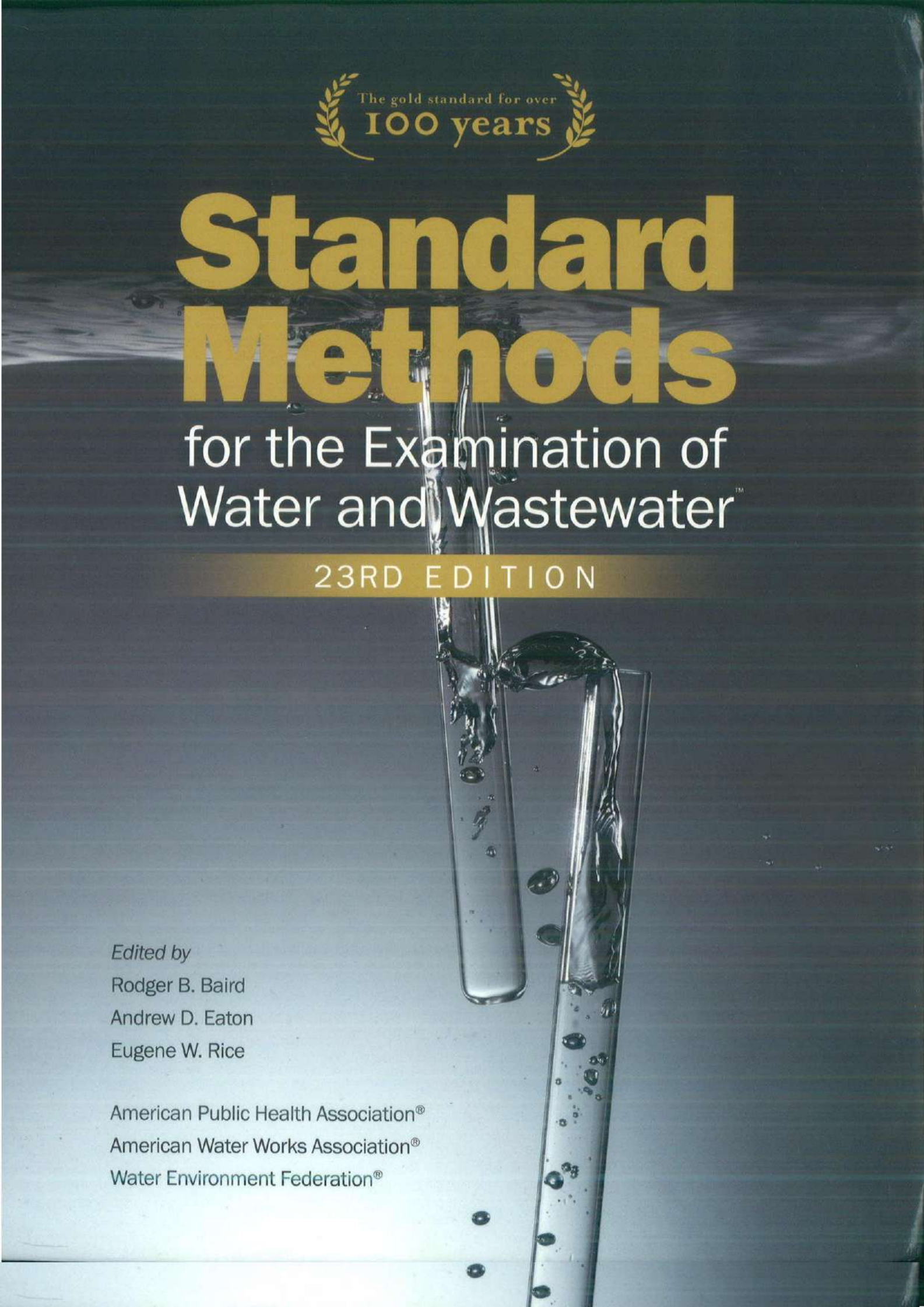
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Standard Methods

for the Examination of Water and Wastewater™

23RD EDITION



Edited by

Rodger B. Baird

Andrew D. Eaton

Eugene W. Rice

American Public Health Association®

American Water Works Association®

Water Environment Federation®

Preparation of Common Types of Desk Reagents Specified in *Standard Methods*

Acid Solutions

Prepare the following reagents by cautiously adding required amount of concentrated acids, with mixing, to designated volume of proper type of distilled water. Dilute to 1000 mL and mix thoroughly.

See Table A for preparation of HCl, H_2SO_4, and HNO_3 solutions.

Alkaline Solutions

a. Stock sodium hydroxide, NaOH, 15*N* (for preparing 6*N*, 1*N*, and 0.1*N* solutions): Cautiously dissolve 625 g solid NaOH in 800 mL distilled water to form 1 L of solution. Remove sodium carbonate precipitate by keeping solution at the boiling point for a few hours in a hot water bath or by letting particles settle for at least 48 h in an alkali-resistant container (wax-lined or polyethylene) protected from atmospheric CO_2 with a soda lime tube. Use the supernate for preparing dilute solutions listed in Table B.

Alternatively prepare dilute solutions by dissolving the weight of solid NaOH indicated in Table B in CO_2-free distilled water and diluting to 1000 mL.

Store NaOH solutions in polyethylene (rigid, heavy-type) bottles with polyethylene screw caps, paraffin-coated bottles with rubber or neoprene stoppers, or borosilicate-glass bottles with rubber or neoprene stoppers. Check solutions periodically. Protect them by attaching a tube of CO_2-absorbing granular material such as soda lime or a commercially available CO_2-removing agent.* Use at least 70 cm of rubber tubing to minimize vapor diffusion from bottle. Replace absorption tube before it becomes exhausted. Withdraw solution by a siphon to avoid opening bottle.

* Ascarite II®, Arthur H. Thomas Co.; or equivalent.

Table B. Preparation of Uniform Sodium Hydroxide Solutions

Normality of NaOH Solution	Required Weight of NaOH to Prepare 1000 mL of Solution *g*	Required Volume of 15*N* NaOH to Prepare 1000 mL of Solution *mL*
6	240	400
1	40	67
0.1	4	6.7

b. Ammonium hydroxide solutions, NH_4OH: Prepare 5*N*, 3*N*, and 0.2*N* NH_4OH solutions by diluting 333 mL, 200 mL, and 13 mL, respectively, of the concentrated reagent (sp gr 0.90, 29.0%, 15*N*) to 1000 mL with distilled water.

Indicator Solutions

a. Phenophthalein indicator solution: Use either the aqueous (1) or alcoholic (2) solution.

1) Dissolve 5 g phenolphthalein disodium salt in distilled water and dilute to 1 L.

2) Dissolve 5 g phenolphthalein in 500 mL 95% ethyl or isopropyl alcohol and add 500 mL distilled water

If necessary, add 0.02*N* NaOH dropwise until a faint pink color appears in solution 1) or 2).

b. Methyl orange indicator solution: Dissolve 500 mg methyl orange powder in distilled water and dilute to 1 L.

Table A: Preparation of Uniform Acid Solutions*

Desired Component	Hydrochloric Acid (HCl)	Sulfuric Acid (H_2SO_4)	Nitric Acid (HNO_3)
Specific gravity (20/4°C) of ACS-grade conc acid	1.174–1.189	1.834–1.836	1.409–1.418
Percent of active ingredient in conc reagent	36–37	96–98	69–70
Normality of conc reagent	11–12	36	15–16
Volume (mL) of conc reagent to prepare 1 L of:			
18*N* solution	—	500 (1 + 1)†	—
6*N* solution	500 (1 + 1)†	167 (1 + 5)†	380
1*N* solution	83 (1 + 11)†	28	64
0.1*N* solution	8.3	2.8	6.4
Volume (mL) of 6*N* reagent to prepare 1 L of			
0.1*N* solution	17	17	17
Volume (mL) of 1*N* reagent to prepare 1 L of			
0.02*N* solution	20	20	20

*All values approximate.

†The *a* + *b* system of specifying preparatory volumes appears frequently throughout *Standard Methods* and means that *a* volumes of the concentrated reagent are diluted with *b* volumes of distilled water to form the required solution.

https://doi.org/10.2105/SMWW.2882.216

Standard Atomic Weights 2015

[Scaled to $A_r(^{12}C) = 12$]

The atomic weights of many elements are not invariant but depend on the origin and treatment of the material. The standard values of $A_r(E)$ and the uncertainties (in parentheses, following the last significant figure to which they are attributed) apply to elements of natural terrestrial origin. The footnotes to this table elaborate the types of variation which may occur for individual elements and that may be larger than the listed uncertainties of values of $A_r(E)$. Names of elements with atomic number 113 to 118 are provisional.

Name	Symbol	Atomic Number	Atomic Weight	Footnotes
Actinium*	Ac	89		
Aluminum	Al	13	26.981 5386(7)	
Americium*	Am	95		
Antimony	Sb	51	121.760(1)	g
Argon	Ar	18	39.948(1)	g, r
Arsenic	As	33	74.921 595(6)	
Astatine*	At	85		
Barium	Ba	56	137.327(7)	
Berkelium*	Bk	97		
Beryllium	Be	4	9.012 182(5)	
Bismuth	Bi	83	208.980 40(1)	
Bohrium*	Bh	107		
Boron	B	5	10.81	m
Bromine	Br	35	79.904	
Cadmium	Cd	48	112.411(4)	g
Calcium	Ca	20	40.078(4)	g
Californium*	Cf	98		
Carbon	C	6	12.011	
Cerium	Ce	58	140.116(1)	g
Cesium	Cs	55	132.905 45196(6)	
Chlorine	Cl	17	35.45	m
Chromium	Cr	24	51.9961(6)	
Cobalt	Co	27	58.933 194(4)	
Copernicium*	Cn	112		
Copper	Cu	29	63.546(3)	r
Curium*	Cm	96		
Darmstadtium	Ds	110		
Dubnium*	Db	105		
Dysprosium	Dy	66	162.500(1)	g
Einsteinium*	Es	99		
Erbium	Er	68	167.259(3)	g
Europium	Eu	63	151.964(1)	g
Fermium*	Fm	100		
Flerovium*	Fl	114		
Fluorine	F	9	18.998 403 163(6)	
Francium*	Fr	87		
Gadolinium	Gd	64	157.25(3)	g
Gallium	Ga	31	69.723(1)	
Germanium	Ge	32	72.630(8)	
Gold	Au	79	196.966 569(5)	
Hafnium	Hf	72	178.49(2)	
Hassium*	Hs	108		
Helium	He	2	4.002 602(2)	g, r
Holmium	Ho	67	164.930 33(2)	
Hydrogen	H	1	1.008	m
Indium	In	49	114.818(1)	
Iodine	I	53	126.904 47(3)	
Iridium	Ir	77	192.217(3)	
Iron	Fe	26	55.845(2)	
Krypton	Kr	36	83.798(2)	g, m
Lanthanum	La	57	138.905 47(7)	g
Lawrencium*	Lr	103		
Lead	Pb	82	207.2(1)	g, r
Lithium	Li	3	[6.938; 6.997]	m
Livermorium*	Lv	116		
Lutetium	Lu	71	174.9668(1)	g
Magnesium	Mg	12	24.3050(6)	
Manganese	Mn	25	54.938 044(3)	
Meitnerium*	Mt	109		
Mendelevium*	Md	101		
Mercury	Hg	80	200.592(3)	
Molybdenum	Mo	42	95.95(1)	g
Moscovium*	Mc	115		
Neodymium	Nd	60	144.242(3)	g
Neon	Ne	10	20.1797(6)	g, m
Neptunium*	Np	93		
Nickel	Ni	28	58.6934(4)	
Nihonium*	Nh	113		
Niobium	Nb	41	92.906 37(2)	
Nitrogen	N	7	14.007	
Nobelium*	No	102		
Oganesson*	Og	118		
Osmium	Os	76	190.23(3)	g
Oxygen	O	8	15.999	
Palladium	Pd	46	106.42(1)	g
Phosphorus	P	15	30.973 761 998(5)	
Platinum	Pt	78	195.084(9)	
Plutonium*	Pu	94		
Polonium*	Po	84		
Potassium	K	19	39.0983(1)	
Praseodymium	Pr	59	140.907 66(2)	
Promethium*	Pm	61		
Protactinium*	Pa	91	231.035 88(2)	
Radium*	Ra	88		
Radon*	Rn	86		
Roentgenium*	Rg	111		
Rhenium	Re	75	186.207(1)	
Rhodium	Rh	45	102.905 50(2)	
Rubidium	Rb	37	85.4678(3)	g
Ruthenium	Ru	44	101.07(2)	g
Rutherfordium*	Rf	104		
Samarium	Sm	62	150.36(2)	g
Scandium	Sc	21	44.955 908(5)	
Seaborgium*	Sg	106		
Selenium	Se	34	78.971(8)	r
Silicon	Si	14	28.085	
Silver	Ag	47	107.8682(2)	g
Sodium	Na	11	22.989 769 28(2)	
Strontium	Sr	38	87.62(1)	g, r
Sulfur	S	16	32.06	
Tantalum	Ta	73	180.947 88(2)	
Technetium*	Tc	43		
Tellurium	Te	52	127.60(3)	g
Terbium	Tb	65	158.925 35(2)	
Thallium	Tl	81	204.38	
Thorium*	Th	90	232.0377(4)	g
Thulium	Tm	69	168.934 22(2)	
Tin	Sn	50	118.710(7)	g
Titanium	Ti	22	47.867(1)	
Tungsten	W	74	183.84(1)	
Uranium*	U	92	238.028 91(3)	g, m
Vanadium	V	23	50.9415(1)	
Xenon	Xe	54	131.293(6)	g, m
Ytterbium	Yb	70	173.045(10)	g
Yttrium	Y	39	88.905 84(2)	
Zinc	Zn	30	65.38(2)	r
Zirconium	Zr	40	91.224(2)	g

* Element has no stable nuclides.

g Geological specimens are known in which the element has an isotopic composition outside the limits for normal material. The difference between the atomic weight of the element in such specimens and that given in the Table may exceed the stated uncertainty.

m Modified isotopic compositions may be found in commercially available material because it has been subjected to an undisclosed or inadvertent isotopic fractionation. Substantial deviations in atomic weight of the element from that given in the table can occur.

r Range in isotopic composition of normal terrestrial material prevents a more precise $A_r(E)$ being given; the tabulated $A_r(E)$ value should be applicable to any normal material.

Source:International Union of Pure and Applied Chemistry. 2016. Atomic weights of the elements, 2013. *Pure Appl. Chem.* 88:265. www.chem.ac.uk/iupac/AtWt/

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PREFACE TO THE TWENTY-THIRD EDITION

The Twenty-Second and Earlier Editions

The first edition of *Standard Methods* was published in 1905. Each subsequent edition has presented significant methodology improvements and enlarged the manual's scope to include techniques suitable for examining many types of samples encountered in the assessment and control of water quality and water pollution.

Standard Methods began as the result of an 1880s movement for "securing the adoption of more uniform and efficient methods of water analysis," which led to the organization of a special committee of the Chemical Section of the American Association for the Advancement of Science. An 1889 report of this committee, "A Method, in Part, for the Sanitary Examination of Water, and for the Statement of Results, Offered for General Adoption," covered five topics:

- "free" and "albuminoid" ammonia;
- oxygen-consuming capacity;
- total nitrogen as nitrates and nitrites;
- nitrogen as nitrites; and
- statement of results.*

Recognizing the need for standard methods in the bacteriological examination of water, members of the American Public Health Association (APHA) sponsored an 1895 convention of bacteriologists to discuss the problem. As a result, an APHA committee was appointed "to draw up procedures for the study of bacteria in a uniform manner and with special references to the differentiation of species." The procedures, which were submitted in 1897,† found wide acceptance.

In 1899, APHA appointed a Committee on Standard Methods of Water Analysis, charged with extending standard procedures to all methods involved in the analysis of water. The committee report, published in 1905, constituted the first edition of *Standard Methods* (then entitled *Standard Methods of Water Analysis*); it included physical, chemical, microscopic, and bacteriological methods of water examination. In its letter of transmittal, the Committee stated:

> The methods of analysis presented in this report as "Standard Methods" are believed to represent the best current practice of American water analysts, and to be generally applicable in connection with the ordinary problems of water purification, sewage disposal and sanitary investigations. Analysts working on widely different problems manifestly cannot use methods which are identical, and special problems obviously require the methods best adapted to them; but, while recognizing these facts, it yet remains true that sound progress in analytical work will advance in proportion to the general adoption of methods which are reliable, uniform and adequate.
>
> It is said by some that standard methods within the field of applied science tend to stifle investigations and that they retard true progress. If such standards are used in the proper spirit, this ought not to be so. The Committee strongly desires that every effort shall be continued to improve the techniques of water analysis and especially to compare current methods with those herein recommended, where different, so that the results obtained may be still more accurate and reliable than they are at present.

APHA published revised and enlarged editions under the title *Standard Methods of Water Analysis* in 1912 (Second Edition), 1917 (Third), 1920 (Fourth), and 1923 (Fifth). In 1925, the American Water Works Association (AWWA) joined APHA in publishing the Sixth Edition, which had the broader title: *Standard Methods of the Examination of Water and Sewage*. Joint publication was continued in the Seventh Edition (1933).

In 1935, the Federation of Sewage Works Associations [now the Water Environment Federation (WEF)] issued a committee report, "Standard Methods of Sewage Analysis."‡ With minor modifications, these methods were incorporated into the Eighth Edition (1936) of *Standard Methods*, which was thus the first to provide methods for examining "sewages, effluents, industrial wastes, grossly polluted waters, sludges, and muds." The Ninth Edition (1946) also contained these methods, and the Federation became a full-fledged publishing partner in 1947. Since then, the work of the *Standard Methods* committees of the three associations—APHA, AWWA, and WEF—has been coordinated by a Joint Editorial Board, on which all three are represented.

The Tenth Edition (1955) included methods specifically for examining industrial wastewaters; this was reflected by a new title: *Standard Methods for the Examination of Water, Sewage and Industrial Wastes.* In the Eleventh Edition (1960), the title was shortened to *Standard Methods for the Examination of Water and Wastewater* in order to describe the contents more accurately and concisely. The title has remained unchanged ever since.

In the Fourteenth Edition (1975), test methods for water were no longer separated from those for wastewater. All methods for analyzing a given component or characteristic appeared in a single section. With minor differences, the organization of the Fourteenth Edition was retained for the Fifteenth (1980) and Sixteenth (1985) Editions.

The Joint Editorial Board made two major policy decisions that were implemented in the Sixteenth Edition. First, the International System of Units (SI) was adopted, except where prevailing field systems or practices require English units. Second, the use of trade names or proprietary materials was eliminated as much as possible, to avoid potential claims regarding restraint of trade or commercial favoritism.

The organization of the Seventeenth Edition (1989) reflected a commitment to develop and retain a permanent numbering system. New numbers were assigned to all sections, and unused numbers were reserved for future use. All Part numbers were expanded to multiples of 1000 instead of 100. The Parts retained their identity from the previous edition, except Part 6000, which was reallocated from automated methods to methods for measuring specific organic compounds. The more general procedures for organics remained in Part 5000.

* *J. Anal. Chem.* 3:398 (1889).
† *Proc. Amer. Pub. Health Assoc.* 23:56 (1897).
‡ *Sewage Works J.* 7:444 (1935).

Also, Part 1000 underwent a major revision in the Seventeenth Edition, and sections dealing with statistical analysis, data quality, and methods development were greatly expanded.

The section on reagent water was updated to include a classification scheme for various types of reagent water. New sections were added at the beginning of Parts 2000 though 10 000 to address quality assurance (QA) and other matters of general application in the specific subject area; the intention was to minimize repetition in each Part.

The Eighteenth Edition (1992) included minor revisions to the new format and new methods in each Part.

In the Nineteenth Edition (1995), sections on laboratory safety and waste management were added to Part 1000. Substantial changes occurred throughout; many sections were revised and/or had new methods added.

Part 1000 was updated in the Twentieth Edition (1998), and substantial changes were made in introductory and quality control (QC) sections in various Parts (notably 3000 and 9000). New methods appeared in Parts 3000, 6000, and 8000. Most other sections were revised.

The Twenty-First Edition (2005) continued the trend to revise methods as issues were identified. The QA requirements in a number of Parts were refined, and new data on precision and bias were added. Several new methods were added to Parts 2000, 4000, 5000, 6000, 7000, 8000, and 9000, and numerous methods were revised.

The Twenty-First Edition methods appeared initially in *Standard Methods* Online (www.standardmethods.org), the Web site inaugurated in April 2004. Since then, all existing, revised, and new methods are available from this source, so *Standard Methods* users will always have access to the most current methods.

The signature undertaking of the Twenty-Second Edition (2012) was clarifying the QC measures necessary to perform the methods in this manual. Sections in Part 1000 were rewritten, and detailed QC sections were added in Parts 2000 through 7000. These changes are a direct and necessary result of the mandate to stay abreast of regulatory requirements and a policy intended to clarify the QC steps considered to be an integral part of each test method. Additional QC steps were added to almost half of the sections.

The Twenty-Third Edition

This edition continues the effort to clarify the QC measures for each method and to create consistency in the QC found in Section 1020 and Parts 2000 through 7000. References and bibliography were updated where necessary and language clarified in certain sections.

The Twenty-Third Edition contains more than 45 sections with significant technical/editorial revisions. Each section may also be found online.

More detailed information on revisions to the sections in the Twenty-Third Edition can be found in the title pages at the beginning of each Part.

Selection and Approval of Methods

For each new edition, both the technical criteria for selecting methods and the formal procedures for approving and including them are reviewed critically. In regard to approval procedures, it is considered particularly important to ensure that the methods presented have been reviewed and are supported by the largest number of qualified people, so they may represent a true consensus of expert opinion.

The system of using Joint Task Groups (initiated with the Fourteenth Edition) was continued for work on each section modified in the Twenty-Third Edition. Individuals generally are appointed to a Joint Task Group based on their expressed interest or recognized expertise in order to assemble a group with maximum available experience with each of the test methods of concern.

Each respective Joint Task Group was charged with review of the methods from the previous edition, review of current methodology in the literature, evaluation of new methods relevant to a Section, and the task of addressing any specific issues of concern that may have come to the attention of the Committee. Once a Joint Task Group was finished with and approved the work on a Section, the manuscript was edited and submitted to Standard Methods Committee members who had asked to review and vote on Sections in a given Part. The Joint Editorial Board reviewed every negative vote and every comment submitted during balloting. Relevant suggestions were referred appropriately for resolution. When negative votes on the first ballot could not be resolved by the Joint Task Group or the Joint Editorial Board, the section was re-balloted among all who voted affirmatively or negatively on the original ballot. Only a few issues could not be resolved in this manner, and the Joint Editorial Board made the final decision.

The general and specific QA/QC sections presented in Part 1000 and Sections 2020, 3020, 4020, 5020, 6020, and 7020 were treated somewhat differently for both the Twenty-Second and Twenty-Third Editions. For the Twenty-Second Edition, Joint Task Groups formed from the Part Coordinators and Joint Editorial Board members were charged with producing consensus drafts, which the Joint Editorial Board reviewed and edited via an iterative process. The draft sections were then sent to the Standard Methods Committee for review, and the resulting comments were used to develop the final drafts. The Twenty-Third Edition work on QC was an attempt by the Joint Editorial Board and Part Coordinators to refine and ensure consistency in these QC sections.

The methods presented here (as in previous editions) are believed to be the best available, generally accepted procedures for analyzing water, wastewaters, and related materials. They represent the recommendations of specialists, ratified by a large number of analysts and others of more general expertise, and as such are truly consensus standards, offering a valid and recognized basis for control and evaluation.

The technical criteria for selecting methods were applied by the Joint Task Groups and the individuals reviewing their recommendations; the Joint Editorial Board provided only general guidelines. In addition to the classical concepts of precision, bias, and minimum detectable concentration, method selection also must consider such issues as the time required to obtain a result, specialized equipment and analyst training needs, and other factors related to the cost of the analysis and the feasibility of its widespread use.

Status of Methods

All of the methods in the Twenty-Third Edition are dated to help users identify the year of approval by the Standard Methods Committee, and determine which ones changed significantly be-

tween editions. The year that a section was approved by the Standard Methods Committee is indicated in a footnote at the beginning of each section. Sections or methods from the Twentieth or Twenty-First Edition that are unchanged, or changed only editorially in the Twenty-Second Edition, show an approval date of 2004 or earlier. Sections or methods that were changed significantly or reaffirmed via general balloting of the Standard Methods Committee during approval of the Twenty-Second Edition, are dated 2005 through 2011. Sections or methods that were changed significantly or reaffirmed via general balloting of the Standard Methods Committee during approval of the Twenty-Third Edition, are dated after 2011. If only an individual method in a section was revised, its approval date is different from that of the rest of the section. Sections with only editorial revisions are noted as such (i.e., Editorial revisions, 2015) to make it easy for users to know whether a prior method is equivalent in protocol (exclusive of the QC issues). All references to individual *Standard Methods* sections should include the approval year in the reference (e.g., 5910-2011 or 5910-11) so users will know which version of the method was used and to facilitate the use of online versions of *Standard Methods*. In the Twenty-Third Edition, the Joint Task Groups that were active since the last full edition are listed at the beginning of each Part, along with a more detailed summary of changes in that Part.

Methods in the Twenty-Third Edition are divided into two fundamental classes: PROPOSED and STANDARD. Regardless of assigned class, all methods must be approved by the Standard Methods Committee. The classes are described as follows:

1. PROPOSED—A PROPOSED method must undergo development and validation that meets the requirements set forth in Section 1040A of *Standard Methods*.

2. STANDARD—A procedure qualifies as a STANDARD method in one of two ways:

a) The procedure has undergone development, validation, and collaborative testing that meet the requirements set forth in Sections 1040 of *Standard Methods*, and it is "WIDELY USED" by the members of the Standard Methods Committee; or

b) The procedure is "WIDELY USED" by the members of the Standard Methods Committee and it has appeared in *Standard Methods* for at least five years.

The Joint Editorial Board assigns method classifications. The Board evaluates the results of the survey on method use by the Standard Methods Committee, which is conducted when the method undergoes general balloting, and considers recommendations offered by Joint Task Groups and the Part Coordinator.

Methods categorized as "PROPOSED" are so designated in their titles; methods with no designation are "STANDARD."

Technical progress makes advisable the establishment of a program to keep *Standard Methods* abreast of advances in research and general practice. The Joint Editorial Board has developed the following procedure for effecting changes in methods:

1. The Joint Editorial Board may elevate any method from "proposed" to "standard" based on adequate published data supporting such a change (as submitted to the Board by the appropriate Joint Task Group). Notices of such a change in status shall be published in the official journals of the three associations sponsoring *Standard Methods* and uploaded to the *Standard Methods* Online Web site.
2. No method may be abandoned or reduced to a lower status without notification via the *Standard Methods* Online Web site.
3. The Joint Editorial Board may adopt a new proposed or standard method at any time, based on the usual consensus procedure. Such methods will be added to *Standard Methods* Online.

Reader comments and questions concerning this manual should be addressed to *Standard Methods* Technical Information Manager at www/standardmethods.org/contact/.

Acknowledgments

For the work in preparing and revising the methods in the Twenty-Third Edition, the Joint Editorial Board gives full credit to the Standard Methods Committees of the American Public Health Association, the American Water Works Association, and the Water Environment Federation. Full credit also is given to those individuals who were not members of the sponsoring societies. A list of all committee members follows these pages. The Joint Editorial Board is indebted to Steve Wendelken [U.S. Environmental Protection Agency (EPA), Office of Groundwater and Drinking Water], and Lemuel Walker (U.S. EPA Office of Science and Technology), who served as Liaisons to the Joint Editorial Board; thanks are due for their interest and help.

The Joint Editorial Board expresses its appreciation to Georges C. Benjamin, M.D., F.A.C.P., Executive Director, American Public Health Association; to David LaFrance, Chief Executive Officer, American Water Works Association; and to Eileen O'Neill, Executive Director, Water Environment Federation; for their cooperation and advice in the development of this publication. Steven J. Posavec, Standard Methods Manager and Joint Editorial Board Secretary, provided a variety of important services that are vital to the preparation of a volume of this type. Ashell Alston, Director of Publications, American Public Health Association, functioned as publisher. Brian Selzer, Assistant Director of Publications, American Public Health Association, served as Production Manager. Special recognition for her valuable services is due to Laura Bridgewater, Managing Editor, who discharged most efficiently the extensive and detailed responsibilities on which this publication depends.

Joint Editorial Board

Rodger B. Baird, Water Environment Federation, Chair
Eugene W. Rice, American Public Health Association
Andrew D. Eaton, American Water Works Association

At several places in this text, a manufacturer's name or trade name of a product, chemical, or chemical compound is referenced. The use of such a name is intended only to be a shorthand reference for the functional characteristics of the manufacturer's item. These references are not intended to be an endorsement of any item by the co-publishers, and materials or reagents with equivalent characteristics may be used.

Rodger B. Baird, Eugene W. Rice, and Steven Posavec

The sponsoring societies, volunteers, and staff of *Standard Methods for the Examination of Water and Wastewater* dedicate the Twenty-Third Edition to Rodger B. Baird, Eugene W. Rice, and Steven Posavec.

Mr. Baird has volunteered his expertise and time for *Standard Methods* for more than 40 years. He began as a member of various Joint Task Groups (JTGs) and chair of the Sulfite JTG for the Fifteenth Edition, became the Part 5000 Coordinator in 1990, and joined the Joint Editorial Board (JEB) in 2007 as the representative for the Water Environment Federation.

Mr. Baird received a B.S. in Chemistry from Claremont Men's College and an M.S. in Chemistry from San Diego State University. He was Manager of Laboratories for the Los Angeles County Sanitation Districts for 32 years, directing 165 scientists and technicians in chemistry, microbiology, biology, and toxicology. He also co-authored *Third Century of Biochemical Oxygen Demand* with former Part 5000 Coordinator Roy-Keith Smith. Mr. Baird retired in 2002.

Dr. Rice has volunteered his expertise and time for *Standard Methods* for nearly 30 years. He began as a JTG member for Sections 9221 and 9222 for the Seventeenth Edition, chaired several JTGs for Eighteenth and Nineteenth Editions, and became Part 9000 Coordinator for the Twentieth Edition. He joined the JEB in 2001 as the representative for the American Public Health Association (APHA).

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The *Standard Methods* community thanks them for their wisdom, expertise, and guidance.

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PART 1000

INTRODUCTION

Part Coordinator
L. Malcolm Baker

Joint Task Group Chairs for the 22nd Edition

1020 Quality Assurance ..... Michael F. Delaney
1050 Expression of Results ..... L. Malcolm Baker

Summary of Major Changes since 2005

The Introduction now includes more information on statistics and dilution/concentration operations, as well as an expanded glossary. Quality Assurance (1020) and Data Quality (1030) were significantly revised to stay abreast of regulatory requirements and clarify the quality control steps considered to be an integral part of each test method. The revisions also are intended to ensure that these sections are consistent with newly revised Sections 2020, 3020, 4020, 5020, 6020, and 7020.

Expression of Results (1050) contains an expanded explanation of units and scientific and engineering notation, analyte nomenclature, and propagation of error. A discussion of equivalents is also new.

The table of sampling and handling requirements in Collection and Preservation of Samples (1060) has been updated.

1010 INTRODUCTION*

1010 A. Scope and Application of Methods

The procedures described in *Standard Methods for the Examination of Water and Wastewater* are intended for use in analyzing a wide range of waters, including surface water, ground water, saline water, domestic and industrial water supplies, cooling or circulating water, boiler water, boiler feed water, and treated and untreated municipal and industrial wastewaters. In recognition of the unity of the water, wastewater, and watershed management fields, the analytical methods are categorized based on constituent, not type of water.

An effort has been made to present methods that apply generally. When alternative methods are necessary for samples of different composition, the basis for selecting the most appropriate method is presented as clearly as possible. In specific instances (e.g., samples with extreme concentrations or otherwise unusual compositions or characteristics), analysts may have to modify a method for it to be suitable. If so, they should plainly state the nature of the modification when reporting the results.

Certain procedures are intended for use with sludges and sediments. Here again, the effort has been made to present methods with the widest possible application. However, these methods may require modification or be inappropriate for chemical sludges or slurries, or other samples with highly unusual composition.

Most of the methods included here have been endorsed by regulators. Regulators may not accept procedures that were modified without formal approval.

Methods for analyzing bulk water-treatment chemicals are not included. American Water Works Association committees prepare and issue standards for water treatment chemicals.

Laboratories that desire to produce analytical results of known quality (i.e., results are demonstrated to be accurate within a specified degree of uncertainty) should use established quality control (QC) procedures consistently. Part 1000 provides a detailed overview of QC procedures used in the individual standard methods as prescribed throughout *Standard Methods*. Other sections of Part 1000 address laboratory safety, sampling procedures, and method development and validation. Material presented in Part 1000 is not necessarily intended to be prescriptive nor to replace or supersede specific QC requirements given in individual sections of this book. Parts 2000 through 9000 contain sections describing QC practices specific to the methods in the respective parts; these practices are considered to be integral to the methods. Most individual methods will contain explicit instructions to be followed for that method (either in general or for certain regulatory applications).

Similarly, the overview of topics covered in Part 1000 is not intended to replace or be the sole basis for technical education and training of analysts. Rather, the discussions are intended as aids to augment and facilitate reliable use of the test procedures herein. Each Section in Part 1000 contains references that can be reviewed to gain more depth or details for topics of interest.

* Reviewed by Standard Methods Committee, 2011.

1010 B. Statistics

1. Normal Distribution

If a measurement is repeated many times under essentially identical conditions, the results of each measurement (x) will be distributed randomly about a mean value (arithmetic average) because of uncontrollable or experimental uncertainty. If an infinite number of such measurements were accumulated, then the individual values would be distributed in a curve similar to those shown in Figure 1010:1. Figure 1010:1A illustrates the Gaussian (normal) distribution, which is described precisely by the mean (μ) and the standard deviation (σ). The *mean* (average) is simply the sum of all values (x_i) divided by the number of values (n).

$$\mu = (\Sigma x_i)/n \text{ for entire population}$$

Because no measurements are repeated infinitely, it is only possible to make an *estimate of the mean* ($\bar{x}$) using the same summation procedure but with n equal to a finite number of repeated measurements (10, 20, 30, etc.):

$$\bar{x} = (\Sigma x_i)/n \text{ for estimated mean}$$

The *standard deviation* of the entire population measured is as follows:

$$\sigma = [\Sigma(x_i - \mu)^2/n]^{1/2}$$

The empirical *estimate of the sample standard deviation* (s) is as follows:

$$s = [\Sigma(x_i - \bar{x})^2/(n-1)]^{1/2}$$

The standard deviation fixes the width (spread) of the normal distribution and consists of a fixed fraction of the measurements that produce the curve. For example, 68.27% of the measure-

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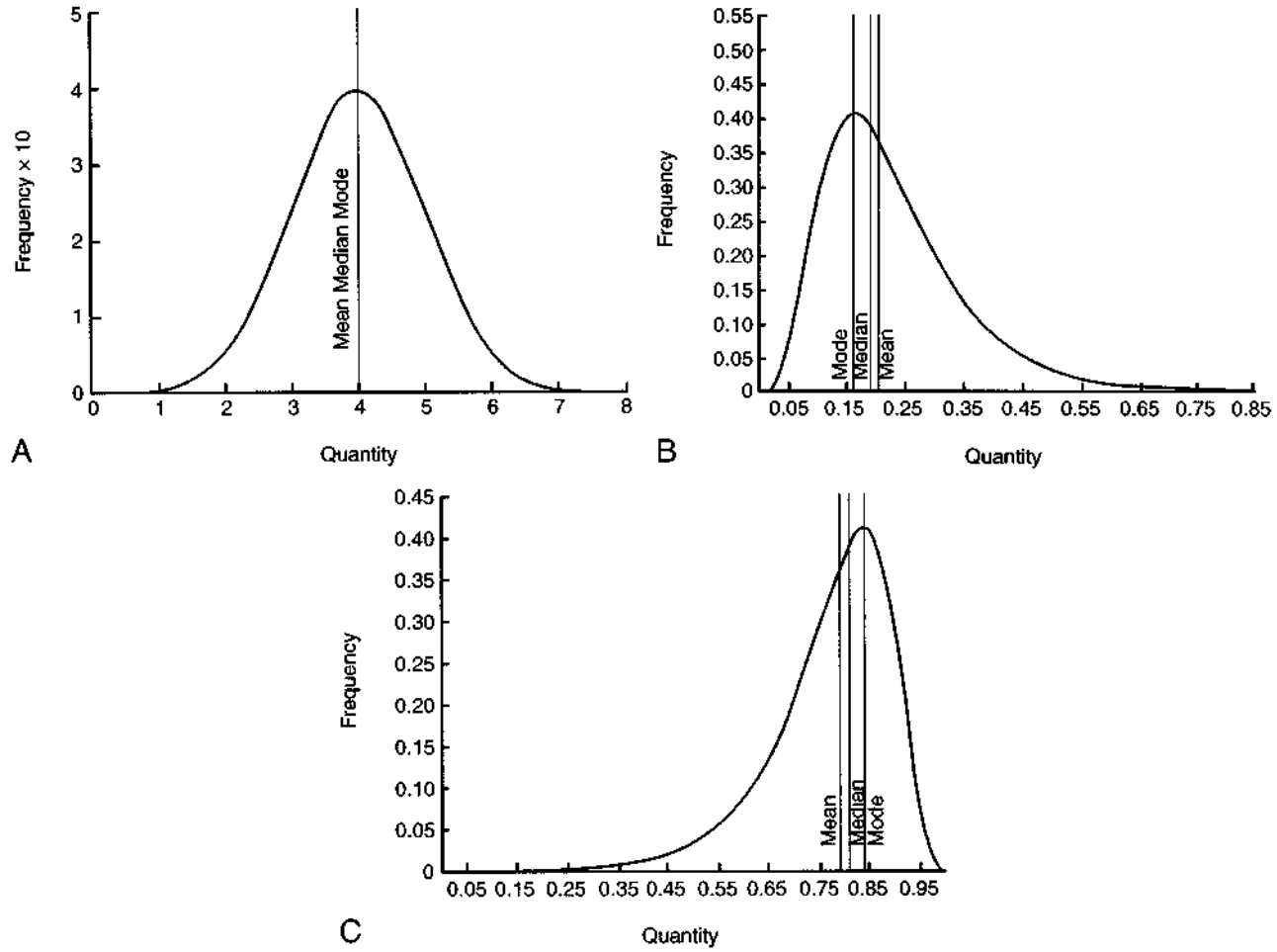


Figure 1010:1. Three types of frequency distribution curves—normal Gaussian (A), positively skewed (B), and negatively skewed (C)—and their measures of central tendency: mean, median, and mode. Courtesy: L. Malcolm Baker.

ments lie within $\mu \pm 1\sigma$, 95.45% between within $\mu \pm 2\sigma$, and 99.73% within $\mu \pm 3\sigma$. (It is sufficiently accurate to state that 95% of the values are within $\mu \pm 2\sigma$ and 99% within $\mu \pm 3\sigma$.) When values are assigned to the $\pm \sigma$ multiples, they are called *confidence limits*, and the range between them is called the *confidence interval*. For example, 10 ± 4 indicates that the confidence limits are 6 and 14, and the confidence interval ranges from 6 to 14.

Another useful statistic is the *standard error of the mean* (σ_μ)—the standard deviation divided by the square root of the number of values ($\sigma/\sqrt{n}$). This is an estimate of sampling accuracy; it implies that the mean of another sample from the same population would have a mean within some multiple of σ_μ. As with σ, 68.27% of the measurements lie within $\mu \pm 1\sigma_\mu$, 95.45% within $\mu \pm 2\sigma_\mu$, and 99.73% within $\mu \pm 3\sigma_\mu$. In practice, a relatively small number of average values is available, so the confidence intervals about the mean are expressed as:

$$\bar{x} \pm ts/\sqrt{n}$$

where t has the following values for 95% confidence intervals:

n	t	n	t
2	12.71	5	2.78
3	4.30	10	2.26
4	3.18	∞	1.96

Using t compensates for the tendency of a small number of values to underestimate uncertainty. For $n > 15$, it is common to use $t = 2$ to estimate the 95% confidence interval.

Still another statistic is the *relative standard deviation* (σ/μ) with its estimate ($s/\bar{x}$), also known as the *coefficient of variation* (*CV*), which commonly is expressed as a percentage. This statistic normalizes σ and sometimes facilitates direct comparisons among analyses involving a wide range of concentrations. For example, if analyses at low concentrations yield a result of 10 ± 1.5 mg/L and at high concentrations yield a result of 100 ± 8 mg/L, the standard deviations do not appear comparable. However, the percent relative standard deviations are 100 (1.5/10) = 15% and 100 (8/100) = 8%, indicating that the variability is not as great as it first appears.

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The mean, median, and mode for each curve in Figure 1010:1 were calculated as follows:

1) *Mean* is the value at the 50th percentile level, or arithmetic average,
2) *Mode* is the value that appears most frequently, and
3) *Median*[1] is estimated as follows:

$$\text{Median} = \tfrac{1}{3}\,(2 \times \text{Mean} + \text{Mode})$$

2. Log-Normal Distribution

In many cases, the results obtained from analyzing environmental samples will not be normally distributed [i.e., a graph of the data distribution will be obviously skewed (see Figure 1010:1B and C)] so the mode, median, and mean will be distinctly different. To obtain a nearly normal distribution, convert the measured variable results to logarithms and then calculate $\bar{x}$ and s. The antilogarithms of $\bar{x}$ and s are the estimates of geometric mean ($\bar{x}_g$) and geometric standard deviation (s_g). The geometric mean is defined as:

$$\bar{x}_g = [\Pi(x_i)]^{1/n} = \text{ antilog } \{1/n[\Sigma \log (x_i)]\}$$

3. Least Square Curve Fitting

Calibration curve data can be fitted to a straight line or quadratic curve by the *least squares method*, which is used to determine the constants of the curve that the data points best fit. To do this, choose the equation that best fits the data points and assume that x is the independent variable and y is the dependent variable (i.e., use x to predict the value of y). The sum of the squares of the differences between each actual data point and its predicted value are minimized.

For a linear least squares fit of

$$y = mx + b$$

the slope (a_1) and the y intercept[1–3] (a_0) are computed as follows:

$$m = \frac{(\Sigma x \Sigma y/n - \Sigma xy)}{[(\Sigma x)^2/n - \Sigma x^2]}$$

$$b = \frac{\Sigma y - m\Sigma x}{n}$$

The correlation coefficient[1–3] (degree of fit) is:

$$r = m\left[\frac{(\Sigma x \Sigma y/n) - \Sigma xy}{\Sigma y^2 - (\Sigma y)^2/n}\right]^{0.5}$$

The best fit is when $r = 1$. There is no fit when $r = 0$.

For a quadratic least squares fit of

$$y = a_2\,x^2 + a_1\,x + a_{0,}$$

the constants ($a_{0,}a_1$, and a_2)[1–3] must be calculated. Typically, these calculations are performed using software provided by instrument manufacturers or independent software vendors. For a more detailed description of the algebraic manipulations, see the cited references.

TABLE 1010:I. CRITICAL VALUES FOR 5% AND 1% TESTS OF DISCORDANCY FOR A SINGLE OUTLIER IN A NORMAL SAMPLE

Number of Measurements n	Critical Value 5%	1%
3	1.15	1.15
4	1.46	1.49
5	1.67	1.75
6	1.82	1.94
7	1.94	2.10
8	2.03	2.22
9	2.11	2.32
10	2.18	2.41
12	2.29	2.55
14	2.37	2.66
15	2.41	2.71
16	2.44	2.75
18	2.50	2.82
20	2.56	2.88
30	2.74	3.10
40	2.87	3.24
50	2.96	3.34
60	3.03	3.41
100	3.21	3.60
120	3.27	3.66

SOURCE: BARNET, V. & T. LEWIS. 1995. Outliers in Statistical Data, 3rd ed. John Wiley & Sons, New York, N.Y.

In this case, the correlation coefficient[1] is:

$$r = \left\{1 - \frac{[\Sigma y^2 - a_0\Sigma y - a_1\Sigma xy - a_2\Sigma x^2 y]}{\left[\Sigma y^2 - \frac{1}{n}(\Sigma y)^2\right]}\right\}^{0.5}$$

4. Rejecting Data

In a series of measurements, one or more results may differ greatly from the others. Theoretically, no result should be arbitrarily rejected because it may indicate either a faulty technique (casting doubt on all results) or a true variant in the distribution. In practice, it is permissible to reject the result of any analysis in which a known error occurred. In environmental studies, extremely high and low concentrations of contaminants may indicate either problematic or uncontaminated areas, so they should not be rejected arbitrarily.

An objective test for outliers has been described.[4] If a set of data is ordered from low to high ($x_L, x_2 \ldots x_H$) and the mean and standard deviation are calculated, then suspected high or low outliers can be tested via the following procedure. First, calculate the statistic T using the discordancy test for outliers:

$$T = (x_H - \bar{x})/s \text{ for a high value, or}$$

$$T = (\bar{x} - x_L)/s \text{ for a low value.}$$

Second, compare T with the value in Table 1010:I for either a 5% or 1% level of significance for the number of measurements (n). If T is larger than that value, then x_H or x_L is an outlier.

Further information on statistical techniques is available elsewhere.[5–7]

5. References

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1010 C. Terminology

This section defines concepts, not regulatory terms. It is not intended to be all-inclusive.

Accuracy—estimate of how close a measured value is to the true value; includes expressions for bias and precision.

Analyte—the element, compound, or component being analyzed.

Bias—consistent deviation of measured values from the true value, caused by systematic errors in a procedure.

Calibration check standard—standard used to determine an instrument's accuracy between recalibrations.

Confidence coefficient—the probability (%) that a measurement will lie within the confidence interval (between the confidence limits).

Confidence interval—set of possible values within which the true value will lie with a specified level of probability.

Confidence limit—one of the boundary values defining the confidence interval.

Detection levels—various levels in use are:

Instrument detection level (IDL)—the constituent concentration that produces a signal greater than five times the instrument's signal:noise ratio. The IDL is similar to the *critical level* and *criterion of detection*, which is 1.645 times the *s* of blank analyses (where *s* is the estimate of standard deviation).

Lower level of detection (LLD) [also called *detection level* and *level of detection* (LOD)]—the constituent concentration in reagent water that produces a signal 2(1.645)*s* above the mean of blank analyses. This establishes both Type I and Type II errors at 5%.

Method detection level (MDL)—the constituent concentration that, when processed through the entire method, produces a signal that has 99% probability of being different from the blank. For seven replicates of the sample, the mean must be 3.14*s* above the blank result (where *s* is the standard deviation of the seven replicates). Compute MDL from replicate measurements of samples spiked with analyte at concentrations more than one to five times the estimated MDL. The MDL will be larger than the LLD because typically 7 or fewer replicates are used. Additionally, the MDL will vary with matrix.

Reporting level (RL)—the lowest quantified level within an analytical method's operational range deemed reliable enough, and therefore appropriate, for reporting by the laboratory. RLs may be established by regulatory mandate or client specifications, or arbitrarily chosen based on a preferred level of acceptable reliability. Examples of RLs typically used (besides the MDL) include:

Level of quantitation (LOQ)/minimum quantifiable level (MQL)—the analyte concentration that produces a signal sufficiently stronger than the blank, such that it can be detected with a specified level of reliability during routine operations. Typically, it is the concentration that produces a signal 10*s* above the reagent water blank signal, and should have a defined precision and bias at that level.

Minimum reporting level (MRL)—the minimum concentration that can be reported as a quantified value for a target analyte in a sample. This defined concentration is no lower than the concentration of the lowest calibration standard for that analyte and can only be used if acceptable QC criteria for this standard are met.

Duplicate—1) the smallest number of replicates (two), or 2) duplicate samples (i.e., two samples taken at the same time from one location) (field duplicate) or replicate of laboratory-analyzed sample.

Fortification—adding a known quantity of analyte to a sample or blank to increase the analyte concentration, usually for the purpose of comparing to test result on the unfortified sample and estimating percent recovery or matrix effects on the test to assess accuracy.

Internal standard—a pure compound added to a sample extract just before instrumental analysis to permit correction for inefficiencies.

Laboratory control standard—a standard usually certified by an outside agency that is used to measure the bias in a procedure. For certain constituents and matrices, use National Institute of Standards and Technology (NIST) or other national or international traceable sources (Standard Reference Materials), when available.

Mean—the arithmetic average (the sum of measurements divided by the number of items being summed) of a data set.

Median—the middle value (odd count) or mean of the two middle values (even count) of a data set.

Mode—the most frequent value in a data set.

Percentile—a value between 1 and 100 that indicates what percentage of the data set is below the expressed value.

Precision (usually expressed as *standard deviation*)—a measure of the degree of agreement among replicate analyses of a sample.

Quality assessment—procedure for determining the quality of laboratory measurements via data from internal and external

quality control measures.

Quality assurance—a definitive plan for laboratory operations that specifies the measures used to produce data with known precision and bias.

Quality control—set of measures used during an analytical method to ensure that the process is within specified control parameters.

Random error—the deviation in any step in an analytical procedure that can be treated by standard statistical techniques. Random error is a major component of measurement error and uncertainty.

Range—the difference of the largest and smallest values in a data set.

Replicate—repeated operation during an analytical procedure. Two or more analyses for the same constituent in an extract of one sample constitute replicate extract analyses.

Spike—see *fortification*.

Surrogate standard—a pure compound added to a sample in the laboratory just before processing so a method's overall efficiency can be determined.

Type I error (also called *alpha error*)—the probability of determining that a constituent is present when it actually is absent.

Type II error (also called *beta error*)—the probability of not detecting a constituent that actually is present.

1010 D. Dilution/Concentration Operations

1. Adjusting Solution Volume

Analysts frequently must dilute or concentrate the amount of analyte in a standard or sample aliquot to within a range suitable for the analytical method so analysis can be performed with specified accuracy. The following equations enable analysts to compute the concentration of a diluted or concentrated aliquot based on the original aliquot concentration and an appropriate factor or fractional constant. (A *factor* in this context is the ratio of final adjusted volume to original volume.) They also can compute the concentration of adjusted aliquot volume based on the original aliquot volume.

$$\text{Concentration of diluted aliquot} = \text{original aliquot concentration} \times \text{dilution fraction}$$

$$\text{Concentration of original aliquot} = \text{diluted aliquot concentration} \times \text{dilution factor}$$

$$\text{Concentration of concentrated aliquot} = \text{original aliquot concentration} \times \text{concentration factor}$$

$$\text{Concentration of original aliquot} = \text{concentrated aliquot concentration} \times \text{concentration fraction}$$

where:

$$\text{Dilution fraction} = \text{original volume/adjusted volume},$$
$$\text{Dilution factor} = \text{adjusted volume/original volume},$$
$$\text{Concentration factor} = \text{original volume/adjusted volume, and}$$
$$\text{Concentration fraction} = \text{adjusted volume/original volume}.$$

2. Types of Dilutions

Several types of dilutions are used in *Standard Methods* procedures. Two of the most common volumetric techniques critical to analytical chemistry results are:

a. Volumetric addition [$a/(a + b)$]: This method typically is used to dilute microbiological samples and prepare reagents from concentrated reagents. It assumes that volumes a and b are additive (i.e., when a is combined with b in one container, the total volume will equal $a + b$, which is not always the case). Most aqueous-solution volumes are additive, but alcoholic solutions or concentrated acid may be only partially volumetrically additive, so be aware of potential problems when combining nonaqueous solutions with aqueous diluents.

b. Volumetric dilution to a measured volume (a/c)*:* This method is used to dilute an aliquot to a given volume via a pipet and volumetric flask. It is the most accurate means of dilution, but when fortifying sample matrices, some error can be introduced if a regular Class A volumetric flask is used. The error will be proportional to the volumes of both spiking solution and flask. For the most accurate work, measure the unfortified sample aliquot in a 100-mL Cassia Class A volumetric flask to the 100-mL mark (0.0 on the flask neck*), and then pipet the volume of fortifying solution. Mix the solution and note the graduated volume on the neck of the flask. The fortified solution's true volume is equal to 100 mL + graduated volume over 100 mL. The true total volume is necessary when computing the dilution factor for the percent recovery of fortified analyte (LFM) in Sections 1020B.12*e* and 4020B.10*a* to obtain the most accurate analytical estimate of recovery.

Dilution factors for multiple volumetric dilutions are calculated as the product of the individual dilutions. Generally, serial dilution is preferred when making dilutions of more than two or three orders of magnitude. Avoid trying to pipet quantities of less than 1.0 mL into large volumes (e.g., <1.0 mL into 100 or 1000 mL) to avoid large relative error propagation.

Some biological test methods (e.g., BOD or toxicity testing) may include dilution techniques that do not strictly conform to the preceding descriptions. For example, such techniques may use continuous-flow dilutors and dilutions prepared directly in test equipment, where volumes are not necessarily prepared via Class A volumetric equipment. Follow the method-specific dilution directions.

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* Pyrex, or equivalent.

1020 QUALITY ASSURANCE*

1020 A. Introduction

This section applies primarily to chemical, some radiochemical, and microbiological analyses. See Sections 7020, 8020, and 9020 for quality assurance and control for specific radiochemical, toxicity, and microbiological analyses.

Quality assurance (QA) is a laboratory operations program that specifies the measures required to produce defensible data with known precision and accuracy. This program is defined in a QA manual, written procedures, work instructions, and records. The manual should include a policy that defines the statistical level of confidence used to express data precision and bias, as well as method detection levels (MDLs) and minimum reporting limits (MRLs). The overall system includes all QA policies and quality control (QC) processes needed to demonstrate the laboratory's competence and to ensure and document the quality of its analytical data. Quality systems are essential for laboratories seeking accreditation under state, federal, or international laboratory certification programs.

QA includes both QC (1020B) and quality assessment (1020C). For information on evaluating data quality, see Section 1030.

1. Quality Assurance Plan

Establish a QA program and prepare a QA manual (or plan). The QA manual and associated documents include the following items[1–5]: cover sheet with approval signatures; quality policy statement; organizational structure; staff responsibilities; document control; analyst training and performance requirements; tests performed by the laboratory; procedures for handling and receiving samples; sample control and documentation procedures; procedures for achieving traceable measurements; major equipment, instrumentation, and reference measurement standards used; standard operating procedures (SOPs) for each analytical method; procedures for generating, approving, and controlling policies and procedures; procedures for procuring reference materials and supplies; procedures for procuring subcontractors' services; internal QC activities; procedures for calibrating, verifying, and maintaining instrumentation and equipment; data-verification practices, including inter-laboratory comparison and proficiency-testing programs; procedures for feedback and corrective actions whenever testing discrepancies are detected; procedures for permitted exceptions to documented policies; procedures for system and performance audits and reviews; procedures for assessing data precision and accuracy and determining MDLs; procedures for data reduction, validation, and reporting; procedures for archiving records; procedures and systems for controlling the testing environment; and procedures for dealing with complaints from data users. Also, the QA manual defines the responsibility for, and frequency of, management review and updates to the QA manual and associated documents.

On the title page, include approval signatures, revision numbers, approval date, and effective date. In the QA manual, include a statement that the manual has been reviewed and determined to be appropriate for the scope, volume, and range of testing activities at the laboratory,[2,3] as well as an indication that management has committed to ensuring that the quality system defined in the QA manual is implemented and followed at all times.

The QA manual also should clearly specify and document the managerial responsibility, authority, quality goals, objectives, and commitment to quality. Write the manual so it is clearly understood and ensures that all laboratory personnel understand their roles and responsibilities.

Implement and follow sample-tracking procedures, including legal chain-of-custody procedures (as required by data users), to ensure that chain of custody is maintained and documented for each sample. Institute procedures to trace a sample and its derivatives through all steps: from collection through analysis, reporting of final results, and sample disposal. Routinely practice adequate and complete documentation, which is critical to ensure that data are defensible, to meet laboratory accreditation/certification requirements, and to ensure that all tests and samples are fully traceable.

Standard operating procedures describe the analytical methods to be used in the laboratory in sufficient detail that a competent analyst unfamiliar with a method can conduct a reliable review and/or obtain acceptable results. SOPs should include, where applicable, the following items[2–4]: title of referenced, consensus test method; sample matrix or matrices; MDL; scope and application; summary of SOP; definitions; interferences; safety considerations; waste management; apparatus, equipment, and supplies; reagents and standards; sample collection, preservation, shipment, and storage requirements; specific QC practices, frequency, acceptance criteria, and required corrective action if acceptance criteria are not met; calibration and standardization; details on the actual test procedure, including sample preparation; calculations; qualifications and performance requirements for analysts (including number and type of analyses); data assessment/data management; references; and any tables, flowcharts, and validation or method-performance data. At a minimum, validate a new SOP before use by first determining the MDL and performing an initial demonstration of capability using relevant regulatory guidelines. (NOTE: MDL does not apply to biological, microbiological, radiological, and some physical and chemical tests.)

Use and document preventive-maintenance procedures for instrumentation and equipment. An effective preventive-maintenance program will reduce instrument malfunctions, maintain more consistent calibration, be cost-effective, and reduce downtime. In the QA manual or appropriate SOP, include measurement traceability to the International System of Units (SI) through a National Metrology Institute, such as the National Institute of Standards and Technology (NIST). Standard refer-

* Reviewed by Standard Methods Committee, 2014.
Joint Task Group: Michael F. Delaney (chair), Clifford G. Annis, Jr., Daniel F. Bender, George T. Bowman, Nilda Cox, Donald G. Davidson, Kenneth E. Osborn, William R. Ray, Kailash C. Srivastava, David W. Tucker.

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ence materials (SRMs) or commercially available reference materials should be certified and traceable to SI standards to establish the integrity of the laboratory calibration and measurement program. Formulate document-control procedures, which are essential to data defensibility, to cover the entire process: document generation, approval, distribution, storage, recall, archiving, and disposal. Maintain logbooks for each test or procedure performed, with complete documentation on preparation and analysis of each sample, including sample identification, associated standards and QC samples, method reference, date/time of preparation/analysis, analyst, weights and volumes used, results obtained, and any problems encountered. Keep logbooks that document maintenance and calibration for each instrument or piece of equipment. Calibration procedures, corrective actions, internal QC activities, performance audits, and data assessments for precision and accuracy (bias) are discussed in 1020B and C.

Data reduction, validation, and reporting are the final steps in the data-generation process. The data obtained from an analytical instrument must first be subjected to the data-reduction processes described in the applicable SOP before the final result can be obtained. In the QA manual or SOP, specify calculations and any correction factors, as well as the steps to be followed when generating the sample result. Also, specify all the data-validation steps to be followed before the final result is made available. Report results in standard units of mass, volume, or concentration, as specified in the method or SOP or as required by regulators or clients. Report results below detection or quantitation levels in accordance with the procedures prescribed in the specific SOP, regulatory requirements, or general laboratory policy.

A statement of uncertainty may be required with each result in specific SOPs, by specific clients, or by a regulatory authority. Uncertainty expression requires statistically relevant data, which may be prescribed within a specific method. Refer to Section 1030B for an overview and references on uncertainty.

See references and bibliography in this section for other useful information and guidance on establishing a QA program and developing an effective QA manual.

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1020 B. Quality Control

Include in each analytical method or SOP the minimum required QC for each analysis. A good QC program consists of at least the following elements, as applicable: initial demonstration of capability (IDC), ongoing demonstration of capability, MDL determination, reagent blank (also referred to as *method blank*), laboratory-fortified blank (LFB) [also referred to as *blank spike* or *laboratory control sample* (LCS)], laboratory-fortified matrix (also referred to as *matrix spike*), laboratory-fortified matrix duplicate (also referred to as *matrix spike duplicate*) or duplicate sample, internal standard, surrogate standard (for organic analysis) or tracer (for radiochemistry), calibration, control charts, and corrective action, frequency of QC indicators, QC acceptance criteria, and definitions of a batch.

Sections 1010 and 1030 describe calculations for evaluating data quality.

1. Initial Demonstration of Capability

Each analyst in the laboratory should conduct an IDC at least once before analyzing any sample to demonstrate proficiency in performing the method and obtaining acceptable results for each analyte. The IDC also is used to demonstrate that the laboratory's modifications to a method will produce results as precise and accurate as those produced by the reference method. As a minimum, include a reagent blank and at least four LFBs at a concentration between 10 times the MDL and the midpoint of the calibration curve (or other level specified in the method). Run the IDC after analyzing all required calibration standards. Ensure that the reagent blank does not contain any analyte of interest at a concentration greater than half the MQL (or other level specified in the method). Ensure that precision (percent relative standard deviation) and accuracy (percent recovery) calculated for LFBs are within the acceptance criteria listed in the method of choice or generated by the laboratory (if there are no established mandatory criteria).

To establish laboratory-generated accuracy and precision limits, calculate the upper and lower control limits from the mean and standard deviation of percent recovery for at least 20 data points:

$$\text{Upper control limit} = \text{Mean} + 3(\text{Standard deviation})$$

$$\text{Lower control limit} = \text{Mean} - 3(\text{Standard deviation})$$

Laboratory-generated acceptance criteria for the IDC (in the absence of established mandatory criteria) generally would meet industry-acceptable guidelines for percent recovery and percent relative standard deviation (%RSD) criteria (e.g., 70 to 130% recovery/20% RSD). Another option is to obtain acceptance criteria from a proficiency testing (PT) sample provider on the inter-laboratory PT studies and translate the data to percent recovery limits per analyte and method of choice.

Also, verify that the method is sensitive enough to meet measurement objectives for detection and quantitation by determining the lower limit of the operational range.

2. Operational Range

Before using a new instrument or instrumental method, determine its operational (calibration) range (upper and lower limits). Use concentrations of standards for each analyte that provide increasing instrument response (linear, weighted, or second-order). Laboratories must define acceptance criteria for the operational range in their QA plans.

3. Ongoing Demonstration of Capability

The ongoing demonstration of capability, sometimes called a *laboratory control sample, laboratory control standard, QC check sample,* or *laboratory-fortified blank,* is used to ensure that the laboratory remains in control while samples are analyzed and separates laboratory performance from method performance on the sample matrix. For initial calibration, the calibration must be verified by comparing it to a second-source calibration standard solution. The laboratory control standard used for ongoing demonstration of capability generally can be either from the same source as the initial calibration standard or from a separate source. Some methods may require that both calibration and spiking solutions be verified with a second (external) source. When verifying the initial calibration control solution, its concentration must be within 10% of the second source's value. See 1020B.6 for further details on the LFB. Analyze QC check samples on at least a quarterly basis.

4. Method Detection Level Determination and Application

Before analyzing samples, determine the MDL for each analyte of interest and method to be used.*

As a starting point for selecting the concentration to use when determining the MDL, use an estimate of five times the estimated true detection level. Start by adding the known amount of constituent to reagent water or sample matrix to achieve the desired concentration. Ideally, prepare and analyze at least seven portions of this solution over a 3-d period to ensure that the MDL determination is more representative of routine measurements in the laboratory. The replicate measurements should be in the range of one to five times the estimated MDL. Calculate the estimated standard deviation, *s*, of the seven replicates, and from a table of one-sided *t* distribution, select *t* for $(7-1) = 6$ degrees of freedom at the 99% confidence level. This value, 3.14, is then multiplied by *s*:

$$\text{MDL} = 3.14s$$

Ideally, estimate *s* using pooled data from several analysts rather than data from one analyst (if the laboratory routinely has multiple analysts running a given test method).

The pooled estimate of σ, which is defined here as S_{pooled}, is a weighted average of the individual analysts' σ. S_{pooled} is calculated from the deviations from the mean of each analyst's data subset squared, which are then summed, divided by the appropriate number of degrees of freedom, and the square root determined. Using S_{pooled} to calculate multiple-analyst standard deviation allows each analyst's error and bias to affect the final result only as much as they have contributed to that result.[1]

$$S_{pooled} = \left[\frac{\sum_{i=1}^{N_1}(X_i - \overline{X}_1)^2 + \sum_{j=1}^{N_2}(X_i - \overline{X}_2)^2 + \sum_{k=1}^{N_3}(X_i - \overline{X_3})^2 + \ldots}{N_1 + N_2 + N_3 \ldots - N_t}\right]^{1/2}$$

where N_t is the number of analysts whose data are being used to compute the pooled standard deviation.

Perform MDL determinations iteratively. If the calculated MDL is not within a factor of l0 of the known addition, repeat determinations at a more suitable concentration. Ideally, conduct MDL determinations or verifications at least annually or on an ongoing basis (or other specified frequency) for each analyte, major matrix category, and method in use at the laboratory. Perform or verify MDL determination for each analyst and instrument, as well as whenever significant modification to the method's instrument or operating conditions also modifies detection or chemistry. Include all sample-preparation steps in the MDL determination.

Generally, apply the MDL to reporting sample results as follows (unless there are regulatory or client constraints to the contrary):

- Report results below the MDL as "not detected" (ND).
- Report results between the MDL and MQL (MRL, LOQ, etc.) with qualification for the quantified value given.
- Report results above the MQL with a value (and its associated uncertainty if required).

5. Reagent Blank

A *reagent blank* (method blank) consists of reagent water (see Section 1080) and all reagents (including preservatives) that normally are in contact with a sample during the entire analytical procedure. The reagent blank is used to determine whether, and how much, reagents and the preparative analytical steps contribute to measurement uncertainty. As a minimum, include one reagent blank with each sample set (batch) or on a 5% basis, whichever is more frequent. Analyze a blank after the daily calibration standard and after highly contaminated samples if carryover is suspected. Evaluate reagent blank results for contamination. If unacceptable contamination is present in the re-

* Some test methods are not amenable to MDL determinations; in such cases, follow the diections in each respective method to determine reporting levels.

agent blank, identify and eliminate the source. Typically, sample results are suspect if analyte(s) in the reagent blank are greater than the MQL. Samples analyzed with a contaminated blank must be re-prepared and re-analyzed. Refer to the method of choice for specific reagent-blank acceptance criteria. General guidelines for qualifying sample results with regard to reagent blank quality are as follows:

- If the reagent blank is less than the MDL and sample results are greater than the MQL, then no qualification is required.
- If the reagent blank is greater than the MDL but less than the MQL and sample results are greater than the MQL, then qualify the results to indicate that analyte was detected in the reagent blank.
- If the reagent blank is greater than the MQL, further corrective action and qualification is required.

6. Laboratory-Fortified Blank/Laboratory Control Standard

A *laboratory-fortified blank* [laboratory control standard (LCS)] is a reagent water sample (with associated preservatives) to which a known concentration of the analyte(s) of interest has been added. An LFB is used to evaluate laboratory performance and analyte recovery in a blank matrix. Its concentration should be high enough to be measured precisely, but not high enough to be irrelevant to measured environmental concentrations. Preferably, rotate LFB concentrations to cover different parts of the calibration range. As a minimum, include one LFB with each sample set (batch) or on a 5% basis, whichever is more frequent. (The definition of a batch is typically method-specific.) Process the LFB through all sample-preparation and analysis steps. Use an added concentration of at least 10 times the MDL/MRL, less than or equal to the midpoint of the calibration curve, or level specified in the method. A low-level LFB fortified at two to five times the detection limit (MDL) can be used as a check for false negatives and for MDL/MRL verification. Control limits for low-level LFB may be variable, depending on the method, but are typically expected to be 50 to 150%. Ideally, the LFB concentration should be less than the MCL (if the contaminant has an MCL). Depending on the method's specific requirements, prepare the addition solution from either the same reference source used for calibration, or from an independent source. Evaluate the LFB for percent recovery of the added analytes by comparing results to method-specified limits, control charts, or other approved criteria. If LFB results are out of control, take corrective action, including re-preparation and re-analysis of associated samples if required. Use LFB results to evaluate batch performance, calculate recovery limits, and plot control charts (see 1020B.13).

7. Laboratory-Fortified Matrix

A *laboratory-fortified matrix* (LFM) is an additional portion of a sample to which a known amount of the analyte(s) of interest is added before sample preparation. Some analytes are not appropriate for LFM analysis; see tables in Sections 2020, 4020, 5020, 6020, 7020, and specific methods for guidance on when an LFM is relevant.

The LFM is used to evaluate analyte recovery in a sample matrix. If an LFM is feasible and the method does not specify LFM frequency requirements, then include at least one LFM with each sample set (batch) or on a 5% basis, whichever is more frequent. Add a concentration that is at least 10 times the MDL/MRL, less than or equal to the midpoint of the calibration curve, or a method-specified level to the selected sample(s). Preferably use the same concentration as for the LFB to allow analysts to separate the matrix's effect from laboratory performance. Prepare the LFM from the same reference source used for the LFB/LCS. Make the addition such that sample background levels do not adversely affect recovery (preferably adjust LFM concentrations if the known sample is more than five times the background level). For example, if the sample contains the analyte of interest, then add approximately as much analyte to the LFM sample as the concentration found in the known sample. Evaluate the results obtained for LFMs for accuracy or percent recovery. If LFM results are out of control, then take corrective action to rectify the matrix effect, use another method, use the method of standard addition, or flag the data if reported. Refer to the method of choice for specific acceptance criteria for LFMs until the laboratory develops statistically valid, laboratory-specific performance criteria. Base sample batch acceptance on results of LFB analyses rather than LFMs alone, because the LFM sample matrix may interfere with method performance.

8. Duplicate Sample/Laboratory-Fortified Matrix Duplicate

Duplicate samples are analyzed randomly to assess precision on an ongoing basis. If an analyte is rarely detected in a matrix type, use an LFM duplicate. An *LFM duplicate* is a second portion of the sample described in 1020B.7 to which a known amount of the analyte(s) of interest is added before sample preparation. If sufficient sample volume is collected, this second portion of sample is added and processed in the same way as the LFM. If there is not enough sample for an LFM duplicate, then use a portion of an alternate sample (duplicate) to gather data on precision. As a minimum, include one duplicate sample or one LFM duplicate with each sample set (batch) or on a 5% basis, whichever is more frequent, and process it independently through the entire sample preparation and analysis. Evaluate LFM duplicate results for precision and accuracy (precision alone for duplicate samples). If LFM duplicate results are out of control, then take corrective action to rectify the matrix effect, use another method, use the method of standard addition, or flag the data if reported. If duplicate results are out of control, then re-prepare and re-analyze the sample and take additional corrective action, as needed. When the value of one or both duplicate samples is less than or equal to five times the MRL, the laboratory may use the MRL as the control limit, and the duplicate results are not used. Refer to the method of choice for specific acceptance criteria for LFM duplicates or duplicate samples until the laboratory develops statistically valid, laboratory-specific performance criteria. If the method of choice does not provide limits, calculate preliminary limits from the IDC. Base sample batch acceptance on results of LFB analyses rather than LFM duplicates alone, because the LFM sample matrix may interfere with method performance.

9. Internal Standard

Internal standards are used for organic analyses by gas chromatography/mass spectrometry (GC/MS), high-performance

liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS), some GC analyses, some ion chromatography (IC) analyses, and some metals analyses by inductively coupled plasma/mass spectrometry (ICP/MS). An *internal standard* is a unique analyte included in each standard and added to each sample or sample extract/digestate just before sample analysis. Internal standards should mimic the analytes of interest and not interfere with the analysis. Choose an internal standard whose retention time or mass spectrum is separate from the analytes of interest and that elutes in a representative area of the chromatogram. Internal standards are used to monitor retention time, calculate relative response, or quantify the analytes of interest in each sample or sample extract/digestate. When quantifying by the internal standard method, measure all analyte responses relative to this internal standard, unless interference is suspected. If internal standard results are out of control, take corrective action, including re-analysis if required. Refer to the method of choice for specific internal standards and their acceptance criteria.

10. Surrogates, Tracers, and Carriers

Surrogates, tracers, and carriers are used to evaluate method performance in each sample. Surrogates are used for organic analyses; tracers and carriers are used for radiochemistry analyses. A *surrogate standard* is a known amount of a unique compound added to each sample before extraction. Surrogates mimic the analytes of interest and are compounds unlikely to be found in environmental samples (e.g., fluorinated compounds or stable, isotopically labeled analogs of the analytes of interest). *Tracers* generally are different isotopes of the analyte or element of interest that are measured based on their characteristic radioactive emissions. Carriers generally are stable isotopes of the element being determined, or analogs thereof, that are measured by chemical or physical means (e.g., gravimetrically or spectroscopically). Surrogates and tracers are introduced to samples before extraction to monitor extraction efficiency and percent recovery in each sample. If surrogate or tracer results are out of control, then take corrective action, including re-preparation and re-analysis if required. Refer to specific SOP for surrogates, tracers, or carriers and their respective acceptance criteria until the laboratory develops statistically valid, laboratory-specific performance criteria.

11. Calibration Curves

For tests that use calibration curves, the following guidance is relevant.

a. Instrument calibration: Perform instrument maintenance and calibration according to method or instrument manual instructions. Conduct instrument performance according to method or SOP instructions.

b. Initial calibration: Perform initial calibration using at least three concentrations of standards for linear curves, at least five concentrations of standards for nonlinear curves, or as specified by the method of choice. Set the lowest concentration at the reporting limit. The highest concentration standard defines the upper end of the calibration range. Ensure that the calibration range encompasses the analytical concentration values expected in samples or required dilutions. Choose calibration standard concentrations with no more than one order of magnitude between concentrations.

A variety of calibration functions may be appropriate: response factor for internal standard calibration, calibration factor for external standard calibration, or calibration curve. Calibration curves may be linear through the origin, linear not through the origin, or nonlinear through or not through the origin. Some nonlinear functions can be linearized via mathematical transformations (e.g., log). The following acceptance criteria are recommended for various calibration functions.

If using response factors or calibration factors, the calculated %RSD for each analyte of interest must be less than the method-specified value. When using response factors (e.g., for GC/MS analysis), evaluate the instrument's performance or sensitivity for the analyte of interest against minimum acceptance values for response factors. Refer to the method of choice for the calibration procedure and acceptance criteria on the response or calibration factors for each analyte.

If linear regression is used, use the minimum correlation coefficient specified in the method. If the minimum correlation coefficient is not specified, then a minimum value of 0.995 is recommended. Compare each calibration point to the curve by recalculating its concentration. If any recalculated concentration is not within the method's acceptance criteria, identify the source of outlier(s) and correct before sample quantitation. Alternatively, a method's calibration can be judged against a reference method by measuring the method's "calibration linearity" or %RSD among the "response factors" at each calibration level or concentration.[2]

Use initial calibration with any of the above functions (response factor, calibration factor, or calibration curve) to quantitate analytes of interest in samples. Use calibration verification (see ¶ *c* below) only for initial-calibration checks, not for sample quantitation, unless otherwise specified by the method of choice. Perform initial calibration when the instrument is set up and whenever calibration-verification criteria are not met.

c. Calibration verification: In *calibration verification,* analysts periodically use a calibration standard to confirm that instrument performance has not changed significantly since initial calibration. Base this verification on time (e.g., every 12 h) or on the number of samples analyzed (e.g., after every 10 samples). Verify calibration by analyzing one standard at a concentration near or at the midpoint of the calibration range. Evaluate the calibration-verification analysis based either on allowable deviations from the values obtained in the initial calibration or from specific points on the calibration curve. If the calibration verification is out of control, then take corrective action, including re-analysis of any affected samples. Refer to the method of choice for the frequency of and acceptance criteria for calibration verification.

12. QC Calculations

The following is a compilation of equations frequently used in QC calculations.

a. Initial calibrations:

Relative response factor (RRF):

$$RRF(x) = \frac{A_x}{A_{is}} \times \frac{C_{is}}{C_x}$$

where:

RRF = relative response factor,
A = peak area or height of characteristic ion measured,
C = concentration,
is = internal standard, and
x = analyte of interest.

Response factor (RF):

$$RF(x) = \frac{A_x}{C_x}$$

where:

RF = response factor,
A = peak area or height,
C = concentration, and
x = analyte of interest.

Calibration factor (CF):

$$\text{CF} = \frac{\text{Peak area (or height) of standards}}{\text{mass injected}}$$

Relative standard deviation (%RSD):

$$\%\ RSD = \frac{s}{(\bar{x})} \times 100$$

$$\text{s} = \sqrt{\sum_{i=1}^{n} \frac{(x_i - \bar{x})^2}{(n-1)}}$$

where:

s = standard deviation,
n = total number of values,
x_i = each individual value used to calculate mean, and
$\bar{x}$ = mean of n values.

b. Calibration verification:
% Difference (%D) for response factor:

$$\%\text{D} = \frac{\overline{RF_i} - RF_c}{RF_i} \times 100$$

where:

$\overline{RF_i}$ = average RF or RRF from initial calibration, and
RF_c = relative RF or RRF from calibration verification standard.

% Difference (%D) for values:

$$\%D = \frac{\text{true value} - \text{found value}}{\text{true value}} \times 100$$

c. Laboratory-fortified blank (laboratory control sample):

$$\%\ \text{Recovery} = \frac{\text{found value}}{\text{true value}} \times 100$$

d. Surrogates:

$$\%\ \text{Recovery} = \frac{\text{quantity measured}}{\text{quantity added}} \times 100$$

e. Laboratory-fortified matrix (LFM) sample (matrix spike sample):

$$\%\ \text{Recovery} = \left[\frac{LFM\ conc \times (spike\ vol + sample\ vol) - (sample\ conc \times sample\ vol)}{spike\ solution\ conc \times spike\ vol}\right] \times 100$$

f. Duplicate sample:
Relative percent difference (RPD):[3]

$$\text{RPD} = \frac{|\text{sample result} - \text{duplicate result}|}{(\text{sample result} + \text{duplicate result})/2} \times 100$$

g. Method of standard additions:

$$\text{Sample concentrations} \times \text{ mg/L} = \frac{S_2 \times V_1 \times C}{(S_1 - S_2) \times V_2}$$

where:

C = concentration of the standard solution, mg/L,
S_1 = signal for fortified portion,
S_2 = signal for unfortified portion,
V_1 = volume of standard addition, L, and
V_2 = volume of sample portion used for method of standard addition, L.

13. Control Charts

Control charts present a graphical record of quality[4] by displaying QC results over time to demonstrate statistical control of an analytical process and to detect apparent changes in the analytical process that may erode such control.[5] These charts are essential QC tools for tests that use accuracy and precision QC measures. Computer-generated and -maintained lists or databases with QC values, limits, and trending may be used as an alternative to manually plotted control charts.

Control charts for batch QC are often based on a single QC result per batch, and decisions on whether to accept or reject that batch may depend on this one result. This special case is referred to as *control charts for individuals* because the rational subgroup size is 1. When the distribution of QC data is markedly asym-

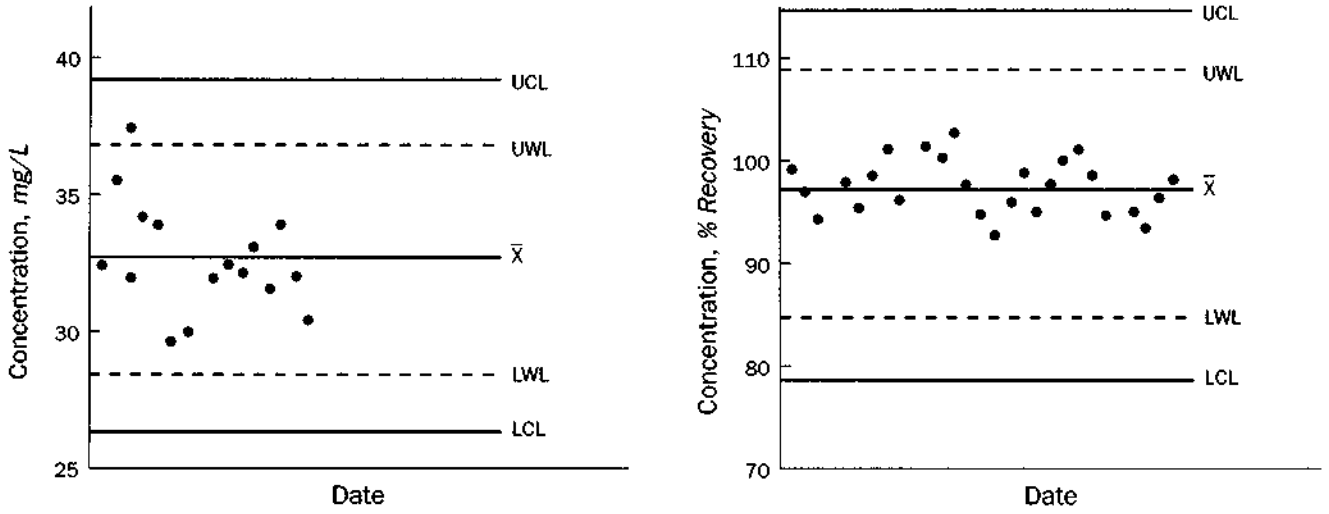


Figure 1020:1. Control charts for means.

metrical (e.g., method blanks), use control charts for individuals with caution.[5]

Two types of control charts commonly used in laboratories are: accuracy (means) charts for QC samples and precision (range) charts for replicate or duplicate analyses.

a. Accuracy (means) chart: The accuracy chart for QC samples (e.g., reagent blanks, LCSs, calibration check standards, LFBs, LFMs, and surrogates) is constructed from the average and standard deviation of a specified number of measurements of the analyte of interest (Figure 1020:1). The accuracy chart includes upper and lower warning levels (WLs) and upper and lower control levels (CLs). Common practice is to use $\pm 2s$ and $\pm 3s$ limits for the WL and CL, respectively, where s represents standard deviation. These calculated limits should not exceed those required in the method. This value, s, should be the average standard deviation derived from a series of trial runs performed before establishing a control chart. Ideally, conduct at least 7 trials using the same number of measurements per trial as anticipated when using the control chart. The standard deviation (s) used in Table 1020:I is the arithmetic average of the individual standard deviations used in the trials. These values are derived from stated or measured values for reference materials. The number of measurements (n) used to determine the estimated standard deviation (s) is specified relative to statistical confidence limits of 95% for WLs and 99% for CLs. Set up an accuracy chart by using either the calculated values for mean and standard deviation or else the percent recovery. (Percent recovery is necessary if the concentration varies.) Construct a chart for each analytical method. Matrix-specific QC may require separate control charts by matrix. Enter results on the chart each time the QC sample is analyzed. It is advisable to re-calculate the initial estimate of s when the number of trials reaches 20 to 50 results.

b. Precision (range) chart: The precision chart also is constructed from the average and standard deviation of a specified number of measurements [e.g., %RSD or relative percent difference (RPD)] for replicate or duplicate analyses of the analyte of interest. If the standard deviation of the method is known, use the factors from Table 1020:I to construct the central line and WLs and CLs as in Figure 1020:2. Perfect agreement between replicates or duplicates results in a difference of zero when the values are subtracted, so the baseline on the chart is zero. Therefore for precision charts, only upper WLs and upper CLs are meaningful. The standard deviation is converted to the range so analysts need

TABLE 1020:I. FACTORS FOR COMPUTING LINES ON RANGE CONTROL CHARTS

Number of Observations n	Factor for Central Line (d_2)	Factor for Control Limits (D_4)
2	1.128	3.267
3	1.693	2.575
4	2.059	2.282
5	2.326	2.114
6	2.534	2.004

SOURCE: Abstracted from Table 6 of AMERICAN SOCIETY FOR TESTING & MATERIALS. 2002. Manual on Presentation of Data and Control Chart Analysis, 7th ed.; 15D, MNL 7A, pp. 67, 112. W. Conshohocken, Pa. Reprinted with permission.

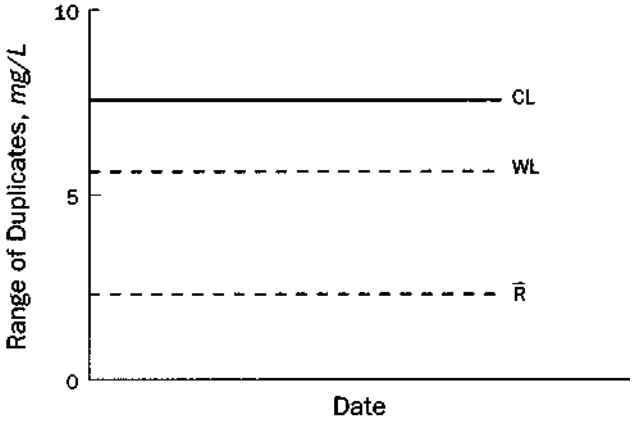


Figure 1020:2. Duplicate analyses of a standard.

only subtract the two results to plot the value on the precision chart. The mean range is computed as:

$$\overline{R} = d_2 s$$

the CL as

$$CL = \overline{R} \pm 3s(R) = D_4\overline{R}$$

and the WL as

$$WL = \overline{R} \pm 2s(R) = \overline{R} \pm 2/3(D_4\overline{R} - \overline{R})$$

where:

$\overline{R}$ = mean range
d_2 = factor to convert s to the mean range (1.128 for duplicates, as given in Table 1020:I),
$s(R)$ = standard deviation of the range, and
D_4 = factor to convert mean range to *CL* (3.267 for duplicates, as given in Table 1020:I). NOTE: When computed lower *CL* or lower *WL* values are negative, record the value as zero because the range value, *R*, is positive by definition.

A precision chart is rather simple when duplicate analyses of a standard are used (Figure 1020:2). For duplicate analyses of samples, the plot will appear different because of variations in sample concentration. If a constant RSD in the concentration range of interest is assumed, then $\overline{R}$, $D_4\overline{R}$, etc., may be computed as above for several concentrations, a smooth curve drawn through the points obtained, and an acceptable range for duplicates determined (Figure 1020:3). A separate table, as suggested below the figure, will be needed to track precision over time.

More commonly, the range can be expressed as a function of RSD (coefficient of variation). The range can be normalized by dividing by the average. Determine the mean range for the pairs analyzed by

$$\overline{R} = (\Sigma R_i)/n$$

Then draw lines on the chart at $\overline{R} + 2s_R$ and $\overline{R} + 3s_R$ and, for each duplicate analysis, calculate normalized range and enter the result on the chart (Figure 1020:4).

c. Chart analyses: If the WLs are at the 95% confidence level, then an average of 1 out of 20 points would exceed that limit, whereas only 1 out of 100 on average would exceed the CLs. There are a number of "rules" (e.g., Western Electric) that may be used to examine control-chart data for trends and other apparent out-of-control changes in method performance.[5] The tradeoff is between missing a change in method performance (false negative) versus investigating and acting on an apparent change in method performance when nothing had actually changed (false positive). The choice of rules to evaluate control charts should balance the risk between false positives and false negatives in method performance; this choice also may be influenced by the rules available in the software or statistical package

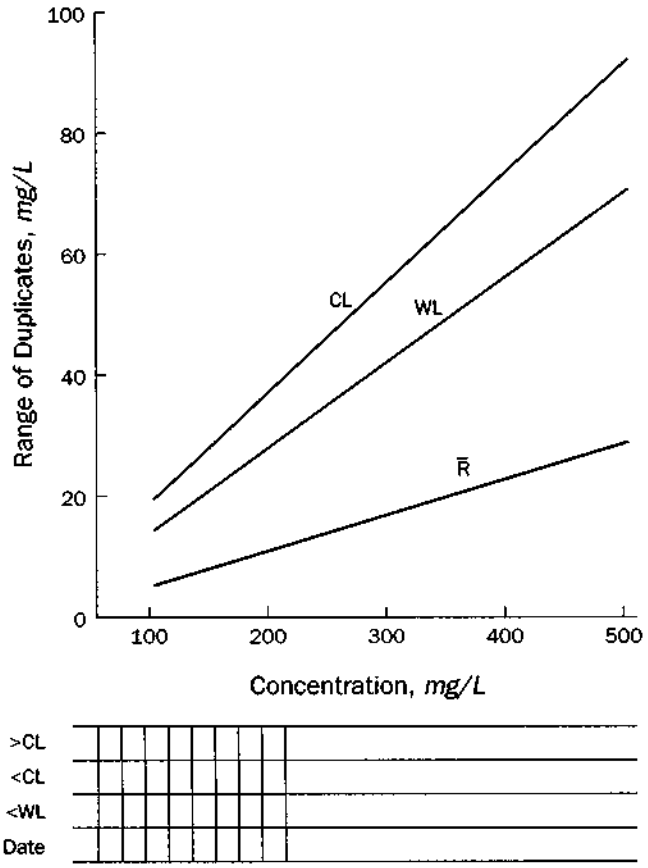


Figure 1020:3. Range chart for variable concentrations.

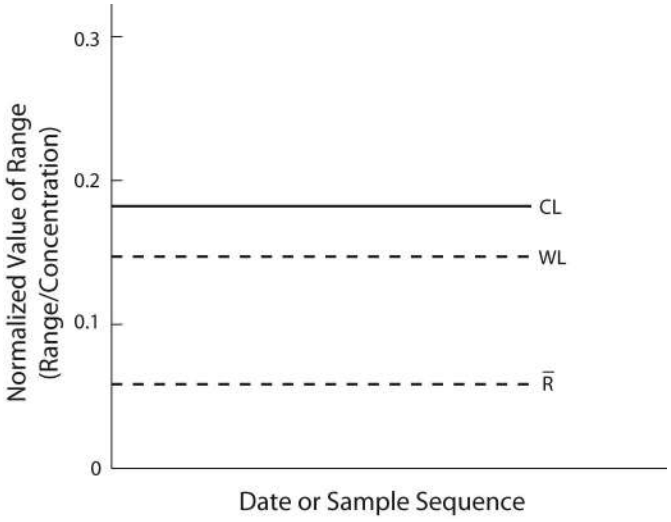


Figure 1020:4. Range chart for variable ranges.

used to analyze control charts. The following are typical guidelines, based on these statistical parameters (Figure 1020:5):

- Control limit—If one measurement exceeds a CL, repeat the analysis immediately. If the repeat measurement is within

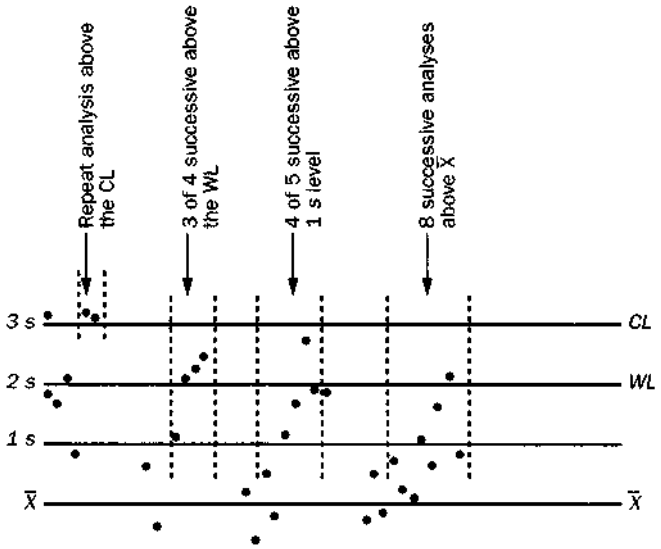


Figure 1020:5. Means control chart with out-of-control data (upper half).

the CL, continue analyses; if it exceeds the CL, discontinue analyses and correct the problem.

- Warning limit—If two out of three successive points exceed a WL, analyze another sample. If the next point is within the WL, continue analyses; if the next point exceeds the WL, evaluate potential bias and correct the problem.
- Standard deviation—If four out of five successive points exceed 1*s*, or are in decreasing or increasing order, analyze another sample. If the next point is less than 1*s*, or changes the order, continue analyses; otherwise, discontinue analyses and correct the problem.
- Trending—If seven successive samples are on the same side of the central line, discontinue analyses and correct the problem.

The above considerations apply when the conditions are either above or below the central line, but not on both sides (e.g., four of five values must exceed either $+1s$ or $-1s$). After correcting the problem, re-analyze the samples analyzed between the last in-control measurement and the out-of-control one.

Another important function of the control chart is assessing improvements in method precision. If measurements never or rarely exceed the WL in the accuracy and precision charts, recalculate the WL and CL using the 10 to 20 most recent data points. Trends in precision can be detected sooner if running averages of 10 to 20 are kept. Trends indicate systematic error; random error is revealed by random exceedance of WLs or CLs.

14. QC Evaluation for Small Sample Sizes

Small sample sizes (e.g., for field blanks and duplicate samples) may not be suitable for QC evaluation with control charts. QC evaluation techniques for small sample sizes are discussed elsewhere.[6]

15. Corrective Action

QC data that are outside the acceptance limits or exhibit a trend are evidence of unacceptable error in the analytical process. Take corrective action promptly to determine and eliminate the source of the error. Do not report data until the cause of the problem is identified and either corrected or qualified (Table 1020:II). Qualifying data does not eliminate the need to take corrective actions, but allows analysts to report data of known quality when it is either impossible or impractical to re-analyze the sample(s). Maintain records of all out-of-control events, determined causes, and corrective action taken. The goal of corrective action is not only to eliminate such events, but also to reduce repetition of the causes.

Table 1020:II. Example Data Qualifiers

Symbol	Explanation
B	Analyte found in reagent blank. Indicates possible reagent or background contamination.
E	Estimated reported value exceeded calibration range.
J	Reported value is an estimate because concentration is less than reporting limit or because certain QC criteria were not met.
N	Organic constituents tentatively identified. Confirmation is needed.
PND	Precision not determined.
R	Sample results rejected because of gross deficiencies in QC or method performance. Re-sampling and/or re-analysis is necessary.
RND	Recovery not determined.
U	Compound was analyzed for, but not detected.

Based on U.S. Environmental Protection Agency guidelines.[1]

Corrective action begins with analysts being responsible for knowing when the analytical process is out of control. Analysts should initiate corrective action when a QC check exceeds acceptance limits or exhibits trending and should report an out-of-control event (e.g., QC outliers, hold-time failures, loss of sample, equipment malfunctions, and evidence of sample contamination) to supervisors. Recommended corrective actions for unacceptable QC data are as follows:

- Check data for calculation or transcription error. Correct results if error occurred.
- Determine whether sample was prepared and analyzed according to the approved method and SOP. If not, prepare and/or analyze again.
- Check calibration standards against an independent standard or reference material. If calibration standards fail, re-prepare calibration standards and/or recalibrate instrument and re-analyze affected sample(s).
- If an LFB fails, analyze another LFB.
- If a second LFB fails, check an independent reference material. If the second source is acceptable, re-prepare and re-analyze affected sample(s).
- If an LFM fails, check LFB. If LFB is acceptable, then qualify the data for the LFM sample, use another method, or use the method of standard addition.
- If an LFM and associated LFB fail, re-prepare and re-analyze affected samples.
- If reagent blank fails, analyze another reagent blank.
- If second reagent blank fails, re-prepare and re-analyze affected sample(s).

- If surrogate or internal standard known addition fails and there are no calculation or reporting errors, re-prepare and re-analyze affected sample(s).

If data qualifiers are used to qualify samples not meeting QC requirements, the data may or may not be usable for the intended purposes. It is the laboratory's responsibility to provide the client or end-user of the data with sufficient information to determine the usability of qualified data.

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1020 C. Quality Assessment

Quality assessment is the process used to ensure that QC measures are being performed as required and to determine the quality of the laboratory's data. It includes proficiency samples, laboratory comparison samples, and performance audits. These are applied to test the precision, accuracy, and detection limits of the methods in use, and to assess adherence to SOP requirements.

1. Laboratory Check Samples (Internal Proficiency)

Evaluate proficiency for each analyte and method in use by periodically analyzing laboratory check samples. To determine each method's percent recovery, use either check samples containing known amounts of the analytes of interest supplied by an outside organization or else blind additions prepared independently in the laboratory.

In general, method performance is established beforehand; acceptable percent recovery consists of values that fall within the established acceptance range. For example, if the acceptable range of recovery for a substance is 85 to 115%, then analysts are expected to achieve a recovery within that range on all laboratory check samples and to take corrective action if results are outside it.

2. Laboratory Comparison Samples

A good QA program requires participation in periodic inter- and intra-laboratory comparison studies. Commercial and some governmental programs supply laboratory comparison samples containing one or more constituents in various matrices. The frequency of participation in comparison studies should depend on the quality of results produced by analysts. For routine procedures, semi-annual analyses are customary. If failures occur, take corrective action and analyze laboratory check samples more frequently until acceptable performance is achieved.

Table 1020:III. Example Audit of a Soil Analysis Procedure

Procedure	Comment	Remarks
1. Sample entered into logbook	Yes	Lab number assigned
2. Sample weighed	Yes	Dry weight
3. Drying procedure followed	No	Maintenance of oven not done
4 a. Balance calibrated	Yes	Once per year
b. Cleaned and zero adjusted	Yes	Weekly
5. Sample ground	Yes	To pass 50 mesh
6. Ball mill cleaned	Yes	Should be after each sample
etc.		
.		
.		
.		

3. Compliance Audits

Compliance audits are conducted to evaluate whether the laboratory meets the applicable SOP or consensus-method requirements that the laboratory claims to follow. Compliance audits can be conducted by internal or external parties. A checklist can be used to document how a sample is treated from time of receipt to final reporting of the result. For example, Table 1020:III provides a partial list of audit items for a hypothetical analytical procedure. The goal of compliance audits is to detect any deviations from the SOP or consensus method so corrective action(s) can be taken.

4. Laboratory Quality Systems Audits

A quality systems audit program is designed and conducted to review all elements of the laboratory quality system and address any issues revealed by different facets of the review. Quality systems audits should be conducted by qualified auditors who

are knowledgeable about the section or analysis being audited. Audit all major elements of the quality system at least annually. Quality system audits may be conducted internally or externally; both types should occur on a regularly scheduled basis and should be handled properly to protect confidentiality. Internal audits are used for self-evaluation and improvement. External audits are used for accreditation, education on client requirements, and approval of the data's end use. Corrective actions should be taken on all audit findings and their effectiveness reviewed at or before the next scheduled audit.

5. Management Review

Review and revision of the quality system is vital to its maintenance and effectiveness. Conducted at least annually by laboratory managers, this review should assess the effectiveness of the quality system and corrective action implementation, and should include internal and external audit results, performance evaluation sample results, input from end user complaints, and corrective actions. This periodic review and revision is vital to the maintenance and implementation of an effective laboratory quality system.

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1030 DATA QUALITY*

1030 A. Introduction

An analytical laboratory's role is to produce measurement-based data that is technically valid, legally defensible, and of known quality. All measurements contain error, which may be systematic (unvarying magnitude) or random (varying magnitude and equal probability of being positive or negative). A method's analytical performance is defined by its unique combination of systematic and random errors.[1] *Quality assurance* is a program designed to make the measurement process as reliable as possible. *Quality control (QC) procedures* are activities designed to identify and determine sources of error.

1. Measures of Quality Control

Two routine indicators of measurement quality that analysts use to assess a method's validity are precision (random error) and bias (systematic error). *Precision* indicates how closely repeated measurements agree. A measurement's precision is acceptable if its random errors are low. *Accuracy* indicates how close a measurement is to the true value. A measurement is acceptably accurate when both systematic and random errors are low. QC results outside acceptance limits (which are set by data quality objectives) are evidence that a method may be out of control due to determinant errors (e.g., contaminated reagents or degraded standards).

2. Measurement Error and Data Use

Both random and systematic measurement errors make laboratory data less reliable. As a measured value decreases, its relative error (e.g., relative standard deviation) may increase, making its validity more uncertain. Reporting tools (e.g., detection or quantitation limits) frequently are used to establish a lower concentration limit for reporting data that incorporate statistical uncertainty.

Laboratory data may be used for such purposes as regulatory monitoring, environmental decision-making, and process control. The procedures used to extract information for different purposes vary and may be diametrically opposed. For example, a regulatory monitoring measurement that is below detection level may be appropriately qualified because the error bar is relatively large and may preclude a statistically sound decision. However, data collected over time may be treated by statistical methods to provide a statistically sound decision even if many of the values are below nominal detection levels.[2]

3. The Analyst's Responsibility

The analyst must understand the QC measures and how to apply them to the data quality objectives (DQOs) of process control, regulatory monitoring, and environmental field studies. It is important that DQOs be clearly defined and detailed before sample analysis begins so the data will be technically correct and legally defensible.

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* Reviewed by Standard Methods Committee, 2011.

Joint Task Group: 20th Edition—Kenneth E. Osborn (chair), Paul W. Britton, Robert D. Gibbons, James M. Gindelberger, Nancy E. Grams, Lawrence H. Keith, Ann E. Rosecrance, Robert K. Wyeth.

1030 B. Measurement Uncertainty

1. Introduction

Even when obtained with the greatest possible care, every measurement has errors that ultimately are unknown and unknowable. These errors collectively result in what is called *measurement uncertainty*. Reporting uncertainty with each measurement—to the extent that it is identified and estimated—is good practice and may spare users from making unwarranted or risky decisions based on the measurement alone.

Measurement error (*E*) is the actual, unknown deviation of the measurement (*M*) from the unknown true value (*T*). *Measurement uncertainty* (*U*) is the state of knowledge about this unknown deviation. *U* may be defined as an uncertainty expression.[1,2] This section concerns the definition of *U*, how to compute it, a recommendation for reporting it, the interpretation and scope of it, and other ways of expressing it.

2. Error

A measurement can be related to the unknown true value and unknown measurement error as follows:

$$M = T + E$$

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This is a simple additive relationship. Other plausible relationships between M and E (e.g., multiplicative or arbitrary functional relationships) are not discussed here.

Because E is unknown, M must be regarded as an uncertain measurement. Sometimes, a true value may be treated as known (e.g., T^* may be a published reference value, a traceable value, or a consensus value) for convenience or because the method that produced T^* has less bias or variation than the one that produced M. For example, based on the average of many measurements, a vessel might be thought to contain $T^* = 50$ μg/L of salt in water. It then may be sampled and routinely measured, resulting in a reported concentration of $M = 51$ μg/L. The actual concentration may be $T = 49.9$ μg/L, resulting in $E = 51 - 49.9 = 1.1$ μg/L.

To generalize the nature of uncertainty, E may be negligible or large in absolute terms (i.e., the original units) or relative terms (i.e., unitless, $E \div T$, or T^*). The acceptability of an absolute error's magnitude depends on its intended use. For example, an absolute error of 1.1 μg/L may be inconsequential for an application in which any concentration over 30 μg/L is sufficient. However, as a precision-measurement standard (e.g., for pharmaceutical ingredients), an absolute error of 1.1 μg/L could be too large.

3. Uncertainty

The reported measurement uncertainty will contain the actual measurement error with a stated level of confidence. For example, if $M \pm U$ is presented as a 95% confidence interval, then approximately 95% of the time, E will fall within the range of $\pm U$.

4. Bias

Bias (systematic error) is the signed (+ or –) deviation between the average measured value and the true value as the number of averaged measurements tends toward infinity and the related uncertainty tends toward zero. For example, the reason a 49.9-μg/L salt solution (T) is thought to be 50 μg/L (T^*) could be a bias ($B = 0.1$ μg/L). The "leftover" error ($1.1 - 0.1 = 1.0$ μg/L) is the random component (*stochastic error*) that changes with each measurement.

The bias is fixed and may be related to the method used to produce T^*. Usually, a recognized method will be used to produce or certify a *traceable standard*—a sample with a certificate stating the accepted true value (T^*). This method may be either the best or most widely accepted method available, chosen because of its minimal bias and stochastic error. Such a traceable standard may be purchased from a standards organization [e.g., National Institute of Standards and Technology (NIST)].

5. Bias and Random Variation

Both E and U can be split into two components:

$$E = Z + B$$

where:

Z = random error, and
B = systematic error.

Random error (Z) is the component that changes from one measurement to the next under certain conditions. It is assumed to be independent and to have a distribution—typically Gaussian (normal distribution). The normal distribution of Z is characterized by a mean (μ) of zero (because any non-zero component is part of bias, by definition) and the traditional standard deviation (σ_E). In other words, about 95% of Z will lie within the interval $\mu \pm 2\sigma_E$. So if there is no bias and E is independent and normally distributed, then $M \pm 2\sigma_E$ would be a suitable way to report a measurement and its uncertainty. (Normal probability tables and statistical software give the proportions of the normal distribution and thus the percent confidence gained that an observation is contained within $\pm k\sigma_E$ for any value of scalar k.)

However, σ_E usually is unknown and must be estimated by the *sample standard deviation* (s_E), which is based on multiple observations and statistical estimation. In this case, scalar k is not chosen based on the normal distribution but rather on the Student's t distribution, taking into account the number of degrees of freedom associated with s_E.

Systematic error (B) is the nonrandom component; it typically is equated with bias and can include outright mistakes (analyst blunders) and lack of control (drifts, fluctuations, etc.).[3] In this manual, the terms *systematic error* and *bias* are intended to be used interchangeably.

B often is more difficult to estimate and make useful than Z is. Knowledge about bias is likely to be hard to obtain; once obtained, it is likely to be exploited to make the measurement less biased or repeated (an appropriate response). If bias is known exactly (or nearly so), the user can subtract it from M to reduce total measurement error.

If bias is unknown (i.e., could be one of a wide but unknown distribution of plausible values), users may adopt a worst-case approach and report an extreme value, re-test, or simply ignore bias altogether. For example, historical data may indicate that interlaboratory biases are significant or that QC measurements of standards shift every time a measurement system is cleaned. Without traceable standards, it is hard for laboratory personnel to do anything other than be ignorant of the potential problem.

The recommended practice for many methods is to conduct routine QA/QC measurements on a suite of internal standards. Plot measurements on control charts, and when an out-of-control condition occurs, recalibrate the system with traceable standards. This permits the laboratory to publish a boundary on bias—assuming that the measurement system's underlying behavior is somewhat predictable and that changes between QA/QC sampling are acceptably small (e.g., slow drifts and small shifts). Many analytical methods are not amenable to use of internal standards in each sample, and external standards and calibration standards must be relied on for an entire set of samples in an analytical run.

6. Repeatability, Reproducibility, and Sources of Bias and Variation

a. Sources and measurement: The sources of bias and variability include sampling error; sample preparation; interference by matrix or other measurement quantities/qualities; variations in calibration error; software errors; counting statistics; an analyst's deviations from the method; instrument differences (e.g.,

chamber volume, voltage level); environmental changes (temperature, humidity, ambient light, etc.); contamination of sample or equipment (e.g., carryover and ambient contamination); variations in purity of solvent, reagent, catalyst, etc.; stability and age of sample, analyte, or matrix; and warm-up or cool-down effects (tendency to drift over time). The simplest strategy for estimating bias is to measure a traceable (known) standard and then compute the difference between M and T^*:

$$M - T^* = B + Z$$

The uncertainty in this case is assumed to be small, although in practice there may be situations in which this assumption is inappropriate. If random uncertainty (Z) is negligible (i.e., $Z \approx 0$), then $M - T^*$ will provide an estimate of bias (B). If Z is not negligible, it can be observed and quantified by repeatedly measuring the same test specimen (if the measurement process is not destructive). This may be part of a QA/QC procedure.

b. Repeatability: Repeatability (also called *intrinsic measurement variability*) is the smallest amount of variation that remains in a measurement system when repeatedly measuring the same specimen while preventing controllable sources of variability from affecting results. It is quantified by the repeatability standard deviation (σ_{RPT}), which can be obtained by pooling sample standard deviations of measurements of J specimens:

$$\sigma_{RPT} = \sqrt{\frac{1}{J} \cdot \sum_{i=1}^{J} {\sigma_{RPT,i}}^2}$$

Repeatability is considered an approximate lower boundary to the standard deviation experienced in practice. The repeatability standard deviation sometimes is used to compute uncertainty intervals ($\pm U$) (referred to as *ultimate instrument variability*) based on the Student's t distribution ($\pm U = \pm k s_{RPT}$).

Common sense and experience demonstrate that repeatability is an overly optimistic estimate of uncertainty for routine measurements, which are subject to many sources of bias and variability that are intentionally eliminated or restrained during a repeatability study. The uncertainty in both B and Z are greater in routine measurements.

c. Reproducibility: Reproducibility is the variation in a measurement system that occurs when repeatedly measuring a sample while allowing (or requiring) selected sources of B or Z to affect results. It is quantified by the reproducibility standard deviation (σ_{RPD}*), accompanied by a list of known applicable sources of* B and Z, and notes on which sources varied.

Barring statistical variation (i.e., variation in estimates of variability, such as the noisiness in sample standard deviations), σ_{RPD} is always greater than σ_{RPT} because it has more components. Typically, one or more of the following varies in a reproducibility study: instrument, analyst, laboratory, or day. Preferably, design a study tailored to the particular measurement system (see 1030B.7). If the sample varies, compute σ_{RPD} separately for each sample, then pool the homogeneous results. Treat factors that vary as random factors and assume they are independent normal random variables with a mean of zero. However, this assumption can often be challenged if the sample and possibly the target populations are small (even identical); there may be a question of "representativeness." Suppose, for example, that out of 20 laboratories (or analysts or instruments) that can do tandem mass spectrometry for a particular analyte and matrix, only six report usable measurements. It is hard to know how representative these six are—especially after a post-study ranking and exclusion process—and whether the Bs of the 20 are normally distributed (probably not discernible from six measurements, even if they are representative).

It may be more appropriate to treat each factor with few known values (e.g., laboratories) as *fixed factors*, which have fixed effects. In other words, each laboratory, analyst, instrument, or day has a different bias, but its distribution is assumed to be unknown (or unknowable), so a small sample cannot be used to estimate distribution parameters, particularly standard deviation. For example, assuming that variables are random, are normal, and have a mean of zero may be inappropriate in an interlaboratory round-robin study. Every laboratory has some B, but it is difficult to characterize because of laboratory anonymity, the small number of laboratories contributing usable data, etc.

Because of these concerns about assumptions and the potential ambiguity of its definition, do not report reproducibility unless it is accompanied with the study design, a list of known sources of B and Z, and notes on which sources varied.

7. Gage Repeatability and Reproducibility, and the Measurement Capability Study

The Gage repeatability and reproducibility (Gage R&R) approach combines repeatability and reproducibility.[4] It treats all factors (including B) as random and is based on the simplest nontrivial model:

$$Z = Z_{RPT} + Z_L$$

where:

Z_{RPT} = normally distributed random variable with mean equal to zero and variance equal to ${\sigma_{RPT}}^2$, and
Z_L = normally distributed random variable with mean equal to zero and with the variance of the factor (e.g., interlaboratory) biases, ${\sigma_L}^2$.

The overall measurement variation then is quantified by

$$\sigma_E = \sigma_{RPD} = \sqrt{{\sigma_{RPT}}^2 + {\sigma_L}^2}$$

Estimates for σ_{RPT} and σ_{RPD} usually are obtained by conducting a nested designed study and analyzing the components of the results' variance. This approach can be generalized to reflect good practice in conducting experiments. The following measurement capability study (MCS) procedure is recommended. The goal is not necessarily to quantify the contribution of every source of B and Z, but rather to study those considered important via systematic error budgeting.

When performing an MCS to assess U via systematic error budgeting, begin by identifying sources of B and Z that affect E. This can be done with a cause-and-effect diagram—perhaps with source categories of equipment, analyst, method (procedure and algorithm), material (aspects of test specimens), and environment.

Select sources to study either empirically or theoretically. Typically, study sources that are influential, that can be varied

during the MCS, and that cannot be eliminated during routine measurement. Select models for the sources. Treat sources of *B* as fixed factors, and sources of *Z* as random factors.

Design and conduct the study, allowing (or requiring) the selected sources to contribute to measurement error. Analyze the data graphically and statistically [e.g., by regression analysis, analysis of variance (ANOVA), or variance components analysis]. Identify and possibly eliminate outliers (observations with responses that are far out of line with the general pattern of the data), and leverage points (observations that exert high, perhaps undue influence).

Refine the models, if necessary (e.g., based on residual analysis), and draw inferences for future measurements. For random effects, this probably will be a confidence interval; for fixed effects, a table of estimated *B*s.

8. Other Assessments of Measurement Uncertainty

The following procedures for assessing measurement uncertainty are discussed below in order of increasing empiricism.

a. Exact theoretical: Some measurement methods are closely tied to exact first-principles models of physics or chemistry. For example, measurement systems that count or track the position and velocity of atomic particles can have exact formulas for uncertainty based on the particles' known theoretical behavior.

b. Delta method (law of propagation of uncertainty): If a result can be expressed as a function of input variables with known error distributions, then sometimes the distribution of such results can be computed exactly.

c. Linearized: The delta method's mathematics may be difficult, so a linearized form of $M = T + E$ may be used instead. It involves a first-order Taylor series expansion about key variables that influence *E*:

$$(M + \delta M) = T + \delta M/\delta G_1 + \delta M/\delta G_2 + \delta M/\delta G_3 + \ldots$$

for sources G_1, G_2, G_3, etc. of *B* and *Z* that are continuous variables (or can be represented by continuous variables). The distribution of this expression may be simpler to determine because it involves the linear combination of scalar multiples of the random variables.

d. Simulation: The delta method also is used to conduct computer simulations. If the distributions of *E*s in input variables are known or can be approximated, then a computer simulation (e.g., Monte Carlo) can empirically obtain the distribution of *E*s in the result. It typically generates 1 to 10 000 sets of random deviates (each set has one random deviate per variable) and computes and archives *M*. The archived distribution is an empirical characterization of *U* in *M*.

e. Sensitivity study (designed experiment): If the identities and distributions of *B* and *Z* sources are known, and the sources are continuous factors but the functional relationship between them and *M* is unknown, then analysts can conduct an empirical sensitivity study (i.e., MCS) to estimate the low-order coefficients ($\delta M/\delta G$) for any factor *G*. This will produce a Taylor series approximation of δM, which can be used to estimate the distribution of δM, as in ¶ *c* above.

f. Random effects study: This is the nested MCS and variance components analysis described in 1030B.7.

g. Passive empirical (QA/QC data): An even more empirical and passive approach is to rely solely on QA/QC or similar data. The estimated standard deviation of sample measurements taken over many days by different analysts using different equipment (perhaps in different laboratories) can provide a useful indication of *U*.

9. Uncertainty Statements

Ideally, measurements should be reported with an uncertainty statement (and its basis). Develop uncertainty statements as follows.[4–8]

With the help of data users, experts on the measurement system's principles and use, and experts on sampling contexts, generate a cause-and-effect diagram for *E* that identifies and prioritizes sources of *B* and *Z* (factors). Consult literature quantifying *B* and *Z*. If needed, conduct one or more MCSs—incorporating the sources considered most important—to provide "snapshot" estimates of *B* and *Z* (sometimes Gage R&R studies may be sufficient).

Institute a QA/QC program in which analysts routinely measure traceable or internal standards and plot the results on *X* and *R* control charts (or equivalent charts). React to out-of-control signals on these charts (e.g., recalibrate using traceable standards when the mean control chart shows a statistically significant change). Use the control charts, relevant literature, and the MCSs to develop uncertainty statements that involve both *B* and *Z*.

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1030 C. Method Detection Level

1. Introduction

Detection levels are controversial, principally because terms are inadequately defined and often confused. For example, the terms *instrument detection level* (IDL) and *method detection level* (MDL) are often incorrectly used interchangeably. That said, most analysts agree that a *detection level* is the smallest amount of a substance that can be detected above the noise in a procedure and within a stated confidence level. Confidence levels are set so the probabilities of both Type I errors (false detection) and Type II errors (false nondetection) are acceptably small. Use of the term "detection limit" has been avoided herein to prevent confusion with regulatory usage of the term.

Currently, there are several types of detection levels—IDL, MDL, lower level of detection (LLD), and level of quantitation (LOQ)—each with a defined purpose (Section 1010C). The relationship among them is approximately IDL:LLD:MDL:LOQ = 1:2:4:10. (Occasionally, analysts use the IDL as a guide for determining the MDL.)

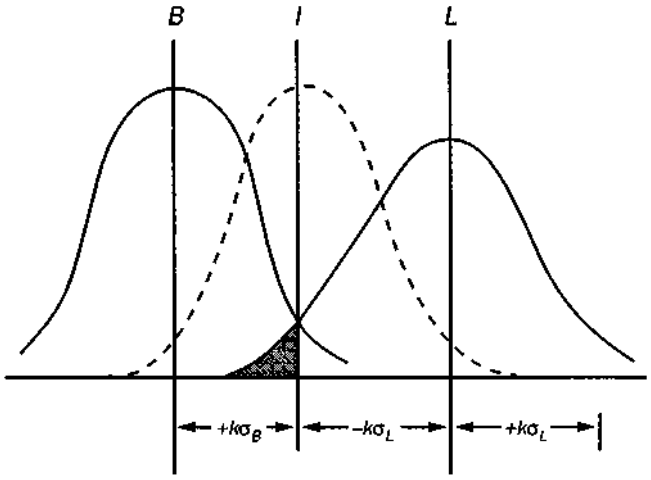


Figure 1030:1. Detection level relationship.

2. Determining Detection Levels

An operating analytical instrument usually produces a signal even when no sample is present (e.g., electronic noise) or when a blank is being analyzed (e.g., molecular noise). Because any QA program requires frequent analysis of blanks, the mean and standard deviation of this background signal become well known; the blank signal can become very precise (i.e., the Gaussian curve of the blank distribution becomes very narrow). The *instrument detection level* is the constituent concentration that produces a signal greater than three standard deviations of the mean noise level or that can be determined by injecting a standard into the instrument to produce a signal that is five times the signal-to-noise ratio. The IDL is useful for estimating the constituent concentration (amount) in an extract needed to produce a signal to permit calculating an estimated MDL.

The *lower level of detection* is the amount of constituent that produces a detectable signal in 99% of trials. Determine the LLD by analyzing multiple samples of a standard at near-zero concentrations (no more than five times the IDL). Determine the standard deviation (s) by the usual method. To reduce the probability of a Type I error to 5%, multiply s by 1.645 (from a cumulative normal probability table). To also reduce the probability of a Type II error to 5%, multiply s by 3.290 instead. For example, if 20 determinations of a low-level standard yield an s of 6 μg/L, then the LLD is $3.29 \times 6 = 20$ μg/L.[1]

The MDL differs from the LLD in that samples containing the constituent of interest are processed through the complete analytical method. MDLs are larger than LLDs because of extraction efficiency and extract concentration factors. The procedure for determining MDLs is outlined in Section 1020B.4.

Although LOQ is useful in a laboratory, the *practical quantitation limit* (PQL) has been proposed as the lowest level achievable among laboratories within specified limits during routine laboratory operations.[2] The PQL is significant because different laboratories will produce different MDLs even when using the same analytical procedures, instruments, and sample matrices. The PQL, which is about three to five times larger than the MDL, is a practical and routinely achievable detection level with a relatively good certainty that any reported value is reliable.

Numerous other definitions of detection and quantitation levels have recently been evaluated and are still under discussion.[3,4] In addition, a few terms are in use as *de facto* specific reporting levels (RLs). These include MDL, PQL, minimum quantifiable level (MQL), and minimum reporting level (MRL). These may be in use in various sections of *Standard Methods* and are included in Section 1010C.

3. Description of Levels

Figure 1030:1 illustrates the detection levels discussed above. For this figure, it is assumed that the signals from an analytical instrument are distributed normally and can be represented by a normal (Gaussian) curve.[5] The curve labeled B is representative of the background or blank signal distribution. As shown, the distribution of blank signals is nearly as broad as for the other distributions (i.e., $\sigma_B = \sigma_I = \sigma_L$). As blank analyses continue, this curve will become narrower because of increased degrees of freedom.

The curve labeled I represents the IDL. Its average value is located $k\sigma_B$ units distant from the blank curve, and k represents the value of t (from the one-sided t distribution) that corresponds to the confidence level chosen to describe instrument performance. For a 95% level and $n = 14$, $k = 1.782$; for a 99% limit, $k = 2.68$. The overlap of the B and I curves indicates the probability of a Type II error.

The curve labeled L represents the LLD. Because only a finite number of determinations is used to calculate IDL and LLD, the curves are similar to the blank, only broader, so it is reasonable

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to choose $\sigma_I = \sigma_L$. Therefore, LLD is $k\sigma_I + k\sigma_L = 2\sigma_L$ from the blank curve.

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1030 D. Data Quality Objectives

1. Introduction

Data quality objectives are systematic planning tools based on the scientific method. They are used to develop data-collection designs and to establish specific criteria for the quality of data to be collected. The process helps planners identify decision-making points for data-collection activities, determine the decisions to be made based on the data collected, and identify the criteria to be used for making each decision. This process documents the criteria for defensible decision-making before an environmental data-collection activity begins.

2. Procedure

The DQO process is composed of the following steps.

a. Stating the issue: Sometimes the reason for performing analyses is straightforward (e.g., to comply with a permit or other regulatory requirement). Sometimes it is far more subjective (e.g., to gather data to support remedial decisions, or to track changes in effluent quality resulting from process changes). A clear statement of the reason for the analyses is integral to establishing appropriate DQOs; this should include a statement of how the data will be used (to determine permit compliance, to support decisions on whether additional process changes will be necessary, etc.).

b. Identifying possible decisions and actions: Initially, express the principal study question. For example: Is the level of contaminant A in environmental medium B higher than regulatory level C? This example is relatively straightforward, but other questions may be more complex. For example: How is aquatic life affected by publicly owned treatment works (POTW) discharges into receiving waters? Break such a question down into several questions that might then be used to develop several decisions. Organize these questions in order of the consensus priority of all participating parties.

Identify alternative actions—including "no action"—that could result from the various possible answers to the principal study questions. In the first example above, if the contaminant level in the environmental medium is higher than the regulatory level, then some cleanup or treatment action may be indicated. If it is lower, the alternative may be "no action," or the study team may wish to evaluate other environmental media and their regulatory levels.

Finally, combine the principal study question with alternative actions to create a decision statement. For the first example, the decision statement might be: "Determine whether the mean level of contaminant A in environmental medium B exceeds regulatory level C and requires remediation." A multi-tiered decision statement might then state: ". . . if not, determine whether the maximum level of contaminant A in environmental medium D exceeds regulatory level E and requires remediation."

c. Identifying inputs: Identify the information needed to make the necessary decision. Inputs may include measurements (e.g., of physical and chemical characteristics), data sources (historical), applicable action levels, or health effects concerns.

Identify and list the sources of information (e.g., previous data, historical records, regulatory guidance, professional judgment, scientific literature, and new data). Evaluate qualitatively whether any existing data are appropriate for the study; such data will be evaluated quantitatively later. Identify the information needed to establish the action level. Define the basis for setting action levels: they may be based on regulatory thresholds or standards, or may be derived from issue-specific considerations (e.g., risk analysis). Determine only the criteria that will be used to set the numerical value; the actual numerical action level will be determined later.

Confirm that the appropriate measurement methods exist to provide the necessary data (i.e., that there are analytical methods for the parameters or contaminants of interest and that they are appropriate for the matrix to be sampled). Consider the potential for matrix interferences, given the samples and analytical methods involved. Ensure that the method's limits (e.g., detection level, quantitation level, reporting level) are appropriate for both the matrix (e.g., drinking water, wastewater, groundwater, leachate, soil, sediment, hazardous waste) and the parameter to be measured. Ensure that a laboratory is available to perform the analyses; determine its capacity, turnaround time, data product, and cost. Include this information as input to the decision-making process.

d. Identifying study limits: Identify both the geographical area and time frame to which the decision will apply. Also, define the scale of decision-making. Identify the smallest, most appropriate subsets of the total population for which decisions will be made. These subsets could be based on spatial or temporal boundaries. For example, while the spatial boundary may be a 300-ac site, samples may be collected from, and decisions made for, each 50-ft square of a grid of such squares drawn on a site map. Also, while the issue's temporal boundary may be the duration of a

storm event, samples may be collected at, and decisions made for, 2-h increments during that event. This type of study might result in a decision to construct a stormwater bypass structure that would carry the first flow, which might contain the highest nutrient load but would not necessarily carry peak flow.

Identify any practical constraints on data collection. Identify any logistical issues that might interfere with data collection, including seasonal conditions, daily variations, meteorological conditions, access conditions, availability of personnel, time, equipment, project budget, regulatory limits, appropriate analytical methods, matrix interferences, detection limits, reporting limits, site access limitations, and expertise.

e. Developing a decision rule: Define the parameter of interest, specify an action level, and integrate outputs from the previous steps into one statement that describes a logical basis for choosing among alternative actions. A decision rule may be worded as follows, substituting case-specific information for the italicized words:

> "If the *factor of interest* within *the scale of decision-making* is greater than *the action level*, then take *alternative action A*; otherwise take *alternative action B*."

The *factor of interest* is a descriptive measure (e.g., an instantaneous value, a mean, a median, or a proportion) that specifies the characteristic (e.g., calcium level in water, PCB level in soil, radon level in air) that the decision-maker would like to know about the statistical population affected by the potential decision (e.g., rivers or streams within a specific watershed, the specified depth of soil within a site boundary, or in basements or crawlspaces within a metropolitan area).

The *scale of decision-making* is the smallest, most appropriate subset for which separate decisions will be made (e.g., each stream segment/river mile, each square of a grid identified on a site map, each section of township X, or range Y of county Z).

The *action level* is the value of the parameter of interest that provides the criterion for choosing among alternative actions (e.g., a stream standard to protect aquatic life, a published regulatory standard, or a health-effects-related level).

Alternative action A is preferred if the action level is exceeded (e.g., initiate nonpoint-source controls, initiate soil cleanup to a specified depth, or distribute technical information to property owners). Noncompliance with the action level is the alternative hypothesis. (Either alternative action can be labeled A without making the decision rule any less valid.)

Alternative action B is preferred if the action level is not exceeded (e.g., continue routine monitoring, leave the soil in place, or provide a summary of the data-collection activity to potential developers). Compliance with the action level is the null hypothesis that is generally the no-action alternative or baseline condition. (Either alternative action can be labeled B without making the decision rule any less valid.)

f. Specifying limits on decision errors: Establish the decision-error limits that the decision-maker will tolerate. Use these limits to establish performance goals for designing the data-collection activity. Base limits on the consequences of making a wrong decision.

Decision-makers are interested in knowing the true state of some feature of the environment. Environmental data can only be an estimate of this true state, so decisions are based on environmental data that are in some degree of error. The goal is to develop a data-collection design that reduces the chances of making a decision error to a level that is acceptable to the decision-maker. Sources of uncertainty include sample design error and measurement error; when combined, they represent the total study error.

Sample design error is the error inherent in using a portion of a population to represent the whole population. It is impractical, for example, to measure and record the concentration of an analyte at every point in a stream continuously; instead, measure analyte concentration at well-defined locations and time intervals to represent this analyte concentration continuum.

Measurement error is the error inherent in the measurement process. A measurement system does not measure, on a molecular level, the amount of an analyte in a sample; it measures an indicator of that amount [e.g., the amount of a specific wavelength of light absorbed by a sample, the change in conductivity of a solution containing the analyte, or the amount of an analyte (in a gaseous or ionized form) that passes through a membrane].

Use data to choose between the one condition of the environment [the null hypothesis (H_0)] and an alternative condition [the alternative hypothesis (H_a)]. A decision error occurs when the decision-maker rejects the null hypothesis when it is true (false-positive decision error) or fails to reject the null hypothesis when it is false (false-negative decision error).*

The null hypothesis usually is treated as the baseline condition presumed to be true in the absence of strong evidence to the contrary. Either condition may be selected as the null hypothesis, but if the null hypothesis is chosen carefully, it can guard against making the decision error that the decision-maker considers to have the more undesirable consequences.

While the possibility of a decision error can never be totally eliminated, it can be controlled by various means, including collecting a large number of samples (to control sampling design error), analyzing individual samples several times, or using more precise laboratory methods (to control measurement error). Better sampling designs also can be developed to collect data that more accurately represent the population of interest. Every study will use a different method to control decision errors, depending on the source of the largest components of total decision error in the data set and the ease of reducing such components.

Reducing the probability of making decision errors generally increases study costs. In many cases, however, it is not necessary to control decision error within very small limits to meet the decision-maker's needs. If the consequences of decision errors are minor, a reasonable decision could be based on relatively crude data. On the other hand, if the consequences of decision errors are severe, the decision-maker will want to control sampling design and measurements within very small limits.

Data quality is judged based on such factors as precision, bias, representativeness, completeness, and comparability. Precision, bias, and completeness can be applied to the measurement system (field and laboratory). Most analytical laboratories have systems to quantify these factors. Laboratory precision can be estimated by analyzing laboratory replicates. Laboratory bias can

* Note that these definitions are not the same as false-positive or false-negative instrument readings, where similar terms commonly are used by laboratory or field personnel to describe a fault in a single result; false-positive and false-negative decision errors are defined in the context of hypothesis testing, where the terms are defined with respect to the null hypothesis.

be estimated by analyzing standards, known additions, and performance evaluation (PE) samples. There is no common system in place to estimate field bias. Field and laboratory completeness can be estimated by comparing the number of analytical results provided by the laboratory with the number of analytical results specified in the sample design. Laboratory representativeness and comparability involve the analytical method used and the lab's performance compared to those of other laboratories (PE studies), which are not commonly quantified.

Precision, bias, representativeness, completeness, and comparability can be applied to the sample design. Precision would indicate how closely this sample design reflects the total population. Bias would indicate how accurately this sample design reflects the total population. Representativeness would indicate to what extent the sample design is representative of the total population. Completeness would indicate how well the sample design reflects the complete population. Comparability would indicate the similarity of the sample design to other sample designs for similar situations. None of these usually is measured.

While data-quality factors provide some insight into sample measurement errors, they do not indicate sample design errors. These errors are additive, so if precision were ±90%, bias were ±90%, and representativeness were ±90%, the combined uncertainty could be up to ±27%.

Because most errors are unquantifiable, a study usually is designed to balance acceptable decision errors with acceptable study cost.

g. Optimizing the design for collection: Identify the most resource-effective design for the study that will achieve DQOs. Use statistical techniques to develop alternative data-collection designs and evaluate their efficiency in meeting DQOs. To develop the optimal study design, it may be necessary to work through this step more than once after revisiting previous steps of the process.

Review DQO outputs and existing environmental data, develop general data-collection design alternatives, and formulate the mathematical expressions needed to solve the design issue for each data-collection design alternative. Develop the following three mathematical expressions:

- a method for testing the statistical hypothesis and a sample size formula that corresponds to the method (e.g., Student's *t* test),
- a statistical model that describes the relationship of the measured value to the "true" value (the model often will describe the components of error or bias believed to exist in the measured value), and
- a cost function that relates the number of samples to the total cost of sampling and analysis.

Select the optimal sample size that satisfies the DQOs for each data-collection design alternative. Using the above mathematical expressions, calculate the optimal sample size that satisfies DQOs. If no design will meet the limits on decision errors within the budget or other constraints, relax one or more constraints by, for example, increasing the sampling and analysis budget, increasing the width of the uncertainty region, increasing the tolerable decision-error rates, relaxing other project constraints (e.g., the schedule), or changing the boundaries. It may be possible to reduce sampling and analysis costs by changing or eliminating subgroups that require separate decisions.

Select the most resource-effective data-collection design that satisfies all DQOs and document the operational details and theoretical assumptions of the selected design in the sampling and analysis plan.

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1030 E. Checking Analyses' Correctness

The following procedures for checking analyses' correctness apply specifically to water samples with relatively complete analyses [e.g., pH, conductivity, total dissolved solids (TDS), and major anionic and cationic constituents that indicate general water quality].[1] These checks do not require additional laboratory analyses. Three involve calculating TDS and conductivity from measured constituents. Sum concentrations of constituents (in milligrams per liter) as follows to calculate TDS:

$$\text{Total dissolved solids} = 0.6(\text{alkalinity*}) + Na^+ + K^+ + Ca^{2+} + Mg^{2+} + Cl^- + SO_4{}^{2-} + SiO_3{}^{2-} + NO_3{}^- + F^-$$

(Note: If pH is <9.0, the hydroxyl ionic conductance is insignificant. If pH is >5.0, the hydrogen ionic conductance is insignificant.)

* As $CaCO_3$.

Calculate electrical conductivity using the procedure in Section 2510B.

1. Anion–Cation Balance[2]

The anion and cation sums (expressed as milliequivalents per liter) must balance because all potable waters are electrically neutral. The test is based on the percentage difference:

$$\%\ \text{difference} = 100 \times \frac{\Sigma\ \text{cations} - \Sigma\ \text{anions}}{\Sigma\ \text{cations} + \Sigma\ \text{anions}}$$

The typical criteria for acceptance are as follows:

Anion Sum *meq/L*	Acceptable Difference
0–3.0	±0.2 meq/L
3.0–10.0	±2%
10.0–800	5%

2. Measured TDS = Calculated TDS[2]

The measured TDS concentration should be larger than the calculated one because the calculation may not include a significant contributor. If the measured value is smaller, then both the higher ion sum and measured value are suspect; the sample should be re-analyzed. If the measured TDS concentration is more than 20% higher than the calculated one, then the low ion sum is suspect; selected constituents should be re-analyzed.

The acceptable ratio is as follows:

$$1.0 < \frac{\text{measured TDS}}{\text{calculated TDS}} < 1.2$$

3. Measured EC = Calculated EC

If the calculated electrical conductivity (EC) is larger than the measured EC value, re-analyze the higher anion or cation analysis sum. If it is smaller, re-analyze the lower anion or cation analysis sum.

The acceptable ratio is as follows:

$$0.9 < \frac{\text{calculated EC}}{\text{measured EC}} < 1.1$$

4. Measured EC and Ion Sums

Both the anion and cation sums should be approximately 1/100 of the measured EC value. If either sum does not meet this criterion, then it is suspect; re-analyze the sample.

The acceptable criteria are as follows:

$$100 \times \text{anion (or cation) sum, meq/L} = (0.9 - 1.1)\ \text{EC}$$

5. Calculated TDS-to-EC Ratio

If the ratio of calculated TDS to conductivity is <0.55, then the lower ion sum is suspect; re-analyze it. If the ratio is >0.7, then the higher anion or cation analysis sum is suspect; re-analyze it. If the lower anion or cation analysis sum does not change after re-analysis, a significant concentration of an unmeasured constituent (e.g., ammonia or nitrite) may be present. If poorly dissociated calcium and sulfate ions are present, then TDS may be as high as 0.8 times the EC.

The acceptable criterion is as follows:

$$\text{calculated TDS/conductivity} = 0.55 - 0.7$$

6. Measured TDS-to-EC Ratio

The measured TDS-to-EC ratio should be between 0.55 and 0.7. If it is outside these limits, then either measured TDS or measured conductivity is suspect; re-analyze.

A more complete exposition of the above QC checks has been published.[3,4]

7. References

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1040 METHOD DEVELOPMENT AND EVALUATION*

1040 A. Introduction

Although standardized test methods are available from many nationally recognized sources, there may be occasions when they cannot be used or when no standard method exists for a particular constituent or characteristic. Therefore, method development may be required. Method development is the set of experimental procedures devised for measuring a known amount of a constituent in various matrices, in the case of chemical analyses; or a known characteristic (e.g., biological or toxicological) of various matrices.

The guidance provided in this section is generalized and slanted towards chemical analyses in most cases. Bioassays, taxonomic, and microbiology testing will frequently demand additional or alternative considerations during development and validation. In some cases, these may be spelled out within other parts of this compendium (e.g. Parts 8000, 9000, 10000) or in other publications or by regulatory authority.

* Reviewed by Standard Methods Committee, 2014.
Joint Task Group: L. Malcolm Baker.

1040 B. Method Validation

Whether an entirely new method is developed by accepted research procedures or an existing method is modified to meet special requirements, validation by a three-step process is required: determination of single-operator precision and bias, analysis of independently prepared unknown samples, and determination of method ruggedness. Universal Acceptance Criteria are difficult to establish and may vary by test type (e.g., chemical, microbiological, toxicological), matrix, intended use, and regulatory authority. It is therefore necessary to establish these criteria during the method-development stage and apply them in the design of validation efforts. The single-operator characteristics may be a good place to begin evaluating and establishing these criteria.

Table 1040:I. Precision and Bias for a Single Concentration in a Single Matrix

Result *mg/L*		Difference (−130)	Squared Difference
1.23		−0.07	0.0049
1.21		−0.09	0.0081
1.30		0.0	0.0
1.59		0.29	0.0841
1.57		0.27	0.0729
1.21		−0.09	0.0081
1.53		0.23	0.0529
1.25		−0.05	0.0025
	Sum	0.49	0.2335

1. Single-Operator Characteristics

This part of the validation procedure requires determining detection and reporting levels as in Section 1020B.4 and 1030C, applicable analytical range, the method's bias (i.e., its systematic error), the precision obtainable by a single operator (i.e., the random error introduced in using the method), matrix effects, and interferences. To make these determinations, analyze at least 7 but preferably 10 or more portions of a standard at each of several concentrations in each matrix that may be used. Use one concentration at, or slightly above, the detection limit and one relatively high so the range of concentrations for which the method is applicable can be specified.

The use of several concentrations to determine bias and precision will reveal the form of the relationship between these method characteristics and the substance's concentration, the substance's characteristic toxicity, or the biological factor of interest. This relationship may be constant, linear, or curvilinear and is a significant characteristic of the method that should be explained clearly. Table 1040:I shows calculation of precision and bias for a single concentration in a single matrix from eight replicate analyses of a standard with a known concentration of 1.30 mg/L.

The bias is 0.49/8 = 0.06 mg/L and the precision is the square root of 0.2335/(8–1) = $\sqrt{0.03336}$, or 0.18 mg/L (Note: This is similar to the calculation for standard deviation).

2. Analysis of Unknown Samples

This step in the method-validation procedure requires analysis of independently prepared standards whose value is unknown to the analyst. Analyze each unknown in replicate by following the standard operating procedure for the method. The mean amount recovered should be within three standard deviations (*s*) of the standard's mean value but preferably within 2 *s*.

Obtain the unknowns from other personnel in the analyst's laboratory using either purchased analytical-grade reagents or standards available from the National Institute of Standards and Technology (NIST). If available for the particular constituent, performance evaluation samples from accredited proficiency test (PT) providers are also recommended.

TABLE 1040:II. VARIATIONS IN FACTORS FOR METHOD RUGGEDNESS DETERMINATION

Factor	Nominal	Variation
Mixing time	10 min	12 min
Portion size	5 g	10 g
Acid concentration	1 *M*	1.1 *M*
Heat to	100°C	95°C
Hold heat for	5 min	10 min
Stirring	yes	no
pH adjust	6.0	6.5

3. Method Ruggedness

A test of the method's ruggedness (i.e., stability of the result produced when steps in the method are varied) is the final validation step. It is especially important to determine this characteristic if the method will be proposed as a standard or reference method. A properly conducted ruggedness test will point out those procedural steps in which rigor is critical and those in which some leeway is permissible.

The Association of Official Analytical Chemists[1] has suggested a method for this test in which eight separate analyses can be used to determine the effect of varying seven different steps in an analytical procedure. To illustrate, suppose the effect of changing the factors in Table 1040:II is to be determined. To make the determination, denote the nominal factors by capital letters A through G and the variations by the corresponding lower-case letters. Then set up a table of the factors (Table 1040:III).

If Combination 1 is analyzed, the result will be *s*. If Combination 2 is analyzed, the result will be *t*, and so on until all eight combinations have been analyzed. To determine the effect of varying a factor, find the four results where the factor was nominal (all caps) and the four where it was varied (all lower case) and compare the averages of the two groups. For example, to compare the effect of changing C to c, use results (s + u + w + y)/4 and (t + v + x + z)/4. Calculate all seven pairs to get seven differences, which can then be ranked to reveal those with a significant effect on the results. If there is no outstanding difference, calculate the average and standard deviation of the eight results s through z. The standard deviation is a realistic estimate of the method's precision. This design tests main effects, not interactions.

TABLE 1040:III. FACTOR MATRIX FOR METHOD RUGGEDNESS DETERMINATION

Factor value	Combinations 1	2	3	4	5	6	7	8
A or a	A	A	A	A	a	a	a	a
B or b	B	B	b	b	B	B	b	b
C or c	C	c	C	c	C	c	C	c
D or d	D	D	d	d	d	d	D	D
E or e	E	e	E	e	e	E	e	E
F or f	F	f	f	F	F	f	f	F
G or g	G	g	g	G	g	G	G	g
Result	s	t	u	v	w	x	y	z

SOURCE: YOUDEN, W.J. & E.H. STEINER. 1987. Statistical Manual of AOAC. Assoc. Official Analytical Chemists, Washington, D.C.

4. Equivalency Testing

After a new method has been validated by the procedures listed above, it may be necessary to test the method for equivalency to standard methods, unless none exist. This requires analyzing at least three concentrations by both the alternate and standard methods. If the range of concentration is very broad, test more concentrations. Once an initial set of analyses (five or more) has been made at each chosen concentration, apply the following statistical steps:[2]

1. Test the distribution of data for normality and transform the data if necessary (Section 1010B).
2. Select an appropriate sample size based on an estimate of the standard deviation.[3]
3. Test the variances of the two methods using the F-ratio statistic.
4. Test the average values of the two methods using a Student's-*t* statistic.

An explanation of each step, with additional techniques and examples, has been published.[4] Because the number of analyses can be very large, the calculations become complex and familiarity with basic statistics is necessary. A listing of standard, reference, and equivalent methods for water analysis is available.[5]

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1040 C. Collaborative Testing

Once a new or modified method has been developed and validated, it is appropriate to determine whether the method should be made a standard method. The procedure to convert a method into a standard method is the *collaborative test*.[1] In this test, different laboratories use the standard operating procedure to analyze a select number of samples to determine the method's bias and precision, as would occur in normal practice.

In planning for a collaborative test, consider the following factors: a precisely written standard operating procedure, the number of variables to be tested, the number of levels to be tested, and the number of replicates required. Because method precision is estimated by the standard deviation, which itself is the result of many sources of variation, the variables that affect it must be tested. These may include the laboratory, operator, apparatus, and concentration range.

1. Variables

Test at least the following variables:

a. Laboratory: Involve at least three different laboratories, although more are desirable to provide a better estimate of the standard deviation. NOTE: Some standards organizations require at least eight laboratories for collaborative testing. Each participating laboratory and analyst should demonstrate proficiency with the new method before inter-laboratory data are generated in a collaborative study.

b. Apparatus: Because model and manufacturer differences can be sources of error, analyze at least two replicates of each concentration per laboratory.

c. Operators: To determine overall precision, involve at least six analysts (no more than two from each laboratory).

d. Levels: If the method development has indicated that the relative standard deviation is constant, test three levels covering the range of the method. If it is not constant, use more levels spread uniformly over the operating range. If developing a new method to compare to an existing standard, use the number of levels equivalent to the existing method's calibration range and requirements.

If matrix effects are suspected, conduct the test evaluation in each medium for which the method was developed. If this is not feasible, use appropriate grades of reagent water as long as this is stipulated in the resulting statement of method characteristics. Results need to be reported as matrix-specific for the types of matrices for which the method is purportedly applicable.

2. Number of Replicates

Calculate the number of replicates after the number of variables to be tested has been determined by using the formula:

$$r > 1\,(30/P)$$

where:

r = number of replicates and
P = the product of several variables.

The minimum number of replicates is three. As an example, if three levels of a substance will be analyzed by single operators in six laboratories on a single apparatus, then P is calculated as follows:

$$P = 3 \times 1 \times 6 \times 1 = 18$$

and the number of replicates is

$$r > 1\,(30/18) > 2.7 \text{ or } r = 3.$$

3. Illustrative Collaborative Test

Send each of five laboratories four concentrations of a compound (4.3, 11.6, 23.4, and 32.7 mg/L) with instructions to analyze in triplicate using the procedure provided. Tabulate results as shown in Table 1040:IV (the results for only one concentration are shown). Because there are no obviously aberrant values (use the method in Section 1010B.4 to reject outliers), use all the data.

Calculate the average and standard deviation for each laboratory; use all 15 results to calculate a grand average and standard deviation. The difference between the average of each laboratory and the grand average reveals any significant bias, such as that shown for Laboratories 1 and 3. The difference between the grand average and the known value is the method bias (e.g., 33.0 – 32.7 = 0.3 mg/L, or 0.9%). The relative standard deviation of the grand average (1.5 mg/L) is 4.5%, which is the

Table 1040:IV. Sample Collaborative Test Results

Laboratory	Result *mg/L*	Experimental $x \pm s$	Deviation From Known	Deviation From Grand Average
1	32.7	34.7 ± 1.8	2.0	1.7
	35.2			
	36.3			
2	32.6	33.3 ± 0.6	0.6	0.3
	33.7			
	33.6			
3	30.6	31.2 ± 1.0	−1.5	−1.8
	30.6			
	32.4			
4	32.6	33.0 ± 0.8	0.3	0
	32.5			
	33.9			
5	32.4	32.9 ± 0.5	0.1	0.2–0.1
	33.4			
	32.9			
	$(\Sigma x)/n = 33$		$\Sigma = 1.5$	$\Sigma = -0.1$
	$s = 1.5$			

method precision, and the s for each laboratory is the single-operator precision.

As noted in Table 1040:IV, the sum of the deviations from the known value for the laboratories was 1.3, so the average deviation (bias) was 1.3/5 = 0.26, rounded to 0.3, which is the same as the difference between the grand average and the known value.

Table 1040:V. Method Precision and Bias

Known Amount *mg/L*	Amount Found *mg/L*	CV (% Standard Deviation)	Bias %
4.3	4.8	12.5	11.5
11.6	12.2	10.2	5.6
23.4	23.8	5.4	1.9
32.7	33	4.5	0.9

For all four unknowns in this test, the percentage results indicated increasing bias and decreasing precision as the concentration decreased. Therefore, to describe the method in a formal statement, the precision would be given by a straight line with the formula $y = mx + b$; where y is the relative standard deviation, m is the slope of the line, x is the concentration, and b is the relative standard deviation at concentration 0. The values found from the collaborative test are shown in Table 1040:V.

These results indicate that the method is acceptable. However, concentrations of less than about 10 mg/L require greater care in analysis.

4. Reference

1. Youden, W.J. & E.H. Steiner. 1975. Statistical Manual of the AOAC. Assoc. Official Analytical Chemists, Washington, D.C.

1050 EXPRESSION OF RESULTS*

1050 A. Units

Standard Methods uses the International System of Units (SI).[1,2] Concentration units for chemical and physical results are generally expressed in mass units/L. The numerical values range from 0.1 to 999.9 mg/L. If the values are less, express them as micrograms per liter (μg/L) and if greater as grams per liter (g/L), with the numerical values adjusted accordingly. Record and report all analytical results to the proper number of significant figures (see 1050B).

1. Radioactivity

For information on reporting radiological results, see Section 7020D.

2. Mass

a. Mass concentrations: Mass concentrations can be expressed in terms of weight/volume (w/v), weight/weight (w/w), and volume/volume (v/v).[3] Those commonly used in *Standard Methods* are listed in Table 1050:I.

Most analysis results are reported in terms of w/v, but some may be expressed in terms of w/w. Since analyses usually are performed for analytes in solution it may be necessary to report results in reference terms other than solution volume, such as milligrams per kilogram, parts per million, or percent by weight. These terms are computed as follows:

$$\text{mg/kg} = \frac{\text{mg/L}}{\rho}$$

$$\text{ppm by weight} = \frac{\text{mg/L}}{\rho}$$

$$\%\text{ by weight} = \frac{\text{mg/L}}{10\,000 \times \rho}$$

where ρ is the density of the sample solution or solid mixture being measured, in kilograms per liter (often reported as the same numeric value as grams per milliliter).

The relative density of water can be computed from Table 1050:II[4] using the densities at t°C and 4°C.

Weight per unit weight (w/w) is defined as mass of analyte per total (wet) mass of solution or mixture. In some cases, the weight fraction results may be relative to total dry mass rather than total wet mass. The units would be multiples of (g analyte/kg dry). This is referred to as "moisture-free basis"; report concentration of total solids at the same time.

TABLE 1050:I. COMMONLY USED EXPRESSIONS OF MASS CONCENTRATION

Proportion	Value	Unit of Expression		
		w/v	w/w	v/v
Parts per hundred (%)	10^{-2}	g/dL	g/100 g	mL/dL
Parts per thousand ($^0/_{00}$)	10^{-3}	g/L	g/kg	mL/L
Parts per million (ppm)	10^{-6}	mg/L	mg/kg	μL/L
Parts per billion (ppb)	10^{-9}	μg/L	μg/kg	nL/L

Adapted from: MILLS, I., T. CVITAS, K. HOMANN, N. KALLAY & K. KUCHITSU. 1993. Quantities, Units and Symbols in Physical Chemistry, 2nd ed. Blackwell Scientific Publications, Oxford, U.K.

b. Other concentration units: To obtain mass concentrations, the analyst may have to compute intermediate results in terms of mass, moles, volume, or equivalent. The most commonly used intermediate forms are defined as follows:

1) *Mass fraction*—analyte mass divided by the total mass of the solution or mixture, expressed as kg/kg;
2) *Volume fraction*—analyte volume divided by total volume of sample where measurements are at equal pressure and temperature, expressed as L/L;
3) *Mole fraction*—number of moles of analyte per total moles in solution;
4) *Molar concentration* (*molarity)*—number of moles of analyte contained in 1 L solution, designated by *M*;
5) *Molal concentration (molality)*—number of moles of analyte dissolved in 1 kg solvent;
6) *Normality*—number of equivalents (see ¶ *c* below) of analyte dissolved and diluted to a 1-L volume, designated by *N*. Also see ¶ *d* below.

c. Equivalency: The unit milligram-equivalents per liter, or millequivalents per liter (me/L), can be valuable for making analytical and water-treatment calculations and for checking analyses by anion–cation balance.

Table 1050:III presents factors for converting concentrations of common ions from milligrams per liter to milliequivalents per liter, and vice versa. The term "milliequivalent" represents 0.001 of an equivalent weight, which is defined as the weight of the ion (sum of the atomic weights of the atoms making up the ion) divided by the number of charges normally associated with the particular ion. The factors for converting results from milligrams per liter to milliequivalents per liter were computed by dividing the ion charge by weight of the ion. Conversely, factors for converting results from milliequivalents per liter to milligrams per liter were calculated by dividing the weight of the ion by the ion charge.

To define equivalents,[5] chemical reactions must be broken down into several types.

1) Neutralization reactions[6]—The equivalent weight of a substance in a neutralization reaction is the weight of acid that will furnish, react with, or be chemically equivalent to, one mole of hydrogen ion in the reaction in which it occurs. For example, the equivalent weight of an acid is its molar mass (molecular weight)

* Reviewed by Standard Methods Committee, 2006.
Joint Task Group: 22nd Edition—L. Malcolm Baker (chair), Stephen W. Johnson, David F. Parkhurst.

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TABLE 1050:II. DENSITY OF WATER FREE FROM DISSOLVED ATMOSPHERIC GASES, AT A PRESSURE OF 101.325 PA

Temperature, t_{68}* °C	Density of Water at Given Temperature kg/m^3									
	.0	.1	.2	.3	.4	.5	.6	.7	.8	.9
0	999.8426	8493	8558	8622	8683	8743	8801	8857	8912	8964
1	999.9015	9065	9112	9158	9202	9244	9284	9323	9360	9395
2	999.9429	9461	9491	9519	9546	9571	9595	9616	9636	9655
3	999.9672	9687	9700	9712	9722	9731	9738	9743	9747	9749
4	999.9750	9748	9746	9742	9736	9728	9719	9709	9696	9683
5	999.9668	9651	9632	9612	9591	9568	9544	9518	9490	9461
6	999.9430	9398	9365	9330	9293	9255	9216	9175	9132	9088
7	999.9043	8996	8948	8898	8847	8794	8740	8684	8627	8569
8	999.8509	8448	8385	8321	8256	8189	8121	8051	7980	7908
9	999.7834	7759	7682	7604	7525	7444	7362	7279	7194	7108
10	999.7021	6932	6842	6751	6658	6564	6468	6372	6274	6174
11	999.6074	5972	5869	5764	5658	5551	5443	5333	5222	5110
12	999.4996	4882	4766	4648	4530	4410	4289	4167	4043	3918
13	999.3792	3665	3536	3407	3276	3143	3010	2875	2740	2602
14	999.2464	2325	2184	2042	1899	1755	1609	1463	1315	1166
15	999.1016	0864	0712	0558	0403	0247	0090	9932†	9772†	9612†
16	998.9450	9287	9123	8957	8791	8623	8455	8285	8114	7942
17	998.7769	7595	7419	7243	7065	6886	6706	6525	6343	6160
18	998.5976	5790	5604	5416	5228	5038	4847	4655	4462	4268
19	998.4073	3877	3680	3481	3282	3081	2880	2677	2474	2269
20	998.2063	1856	1649	1440	1230	1019	0807	0594	0380	0164
21	997.9948	9731	9513	9294	9073	8852	8630	8406	8182	7957
22	997.7730	7503	7275	7045	6815	6584	6351	6118	5883	5648
23	997.5412	5174	4936	4697	4456	4215	3973	3730	3485	3240
24	997.2994	2747	2499	2250	2000	1749	1497	1244	0990	0735
25	997.0480	0223	9965†	9707†	9447†	9186†	8925†	8663†	8399†	8135†
26	996.7870	7604	7337	7069	6800	6530	6259	5987	5714	5441
27	996.5166	4891	4615	4337	4059	3780	3500	3219	2938	2655
28	996.2371	2087	1801	1515	1228	0940	0651	0361	0070	9778*
29	995.9486	9192	8898	8603	8306	8009	7712	7413	7113	6813
30	995.6511	6209	5906	5602	5297	4991	4685	4377	4069	3760
31	995.3450	3139	2827	2514	2201	1887	1572	1255	0939	0621
32	995.0302	9983†	9663†	9342†	9020†	8697†	8373†	8049†	7724†	7397†
33	994.7071	6743	6414	6085	5755	5423	5092	4759	4425	4091
34	994.3756	3420	3083	2745	2407	2068	1728	1387	1045	0703
35	994.0359	0015	9671†	9325†	8978†	8631†	8283†	7934†	7585†	7234†
36	993.6883	6531	6178	5825	5470	5115	4759	4403	4045	3687
37	993.3328	2968	2607	2246	1884	1521	1157	0793	0428	0062
38	992.9695	9328	8960	8591	8221	7850	7479	7107	6735	6361
39	992.5987	5612	5236	4860	4483	4105	3726	3347	2966	2586
40	992.2204									

* t_{68} represents temperature, according to International Practical Temperature Scale 1968.
† The leading figure decreases by 1.0.
Source: MARSH, K.N., ed. 1987. Recommended Reference Materials for the Realization of Physicochemical Properties, Blackwell Scientific Publications, Oxford, U.K.

divided by the number of hydrogen atoms (equivalent number) contained in the acid. Thus, for acids that have reacted completely, the equivalent weight of HCl and HNO_3 would equal the molar mass; the equivalent weight of H_2SO_4 and H_2CO_3 would equal the molar mass divided by 2 and the equivalent weight for H_3PO_4 would equal molar mass divided by 3.

If the chemical formula is written out for the reaction of an acid with a base, the equivalent amounts for each compound in the reaction are obtained by dividing each stoichiometric coefficient in the equation by the equivalent number.[7] For example, in the equation:

$$H_2SO_4 + 2NaOH \rightarrow Na_2SO_4 + 2H_2O$$

dividing by the equivalent number, 2, yields:

$$\frac{H_2SO_4}{2}\ \frac{2NaOH}{2}\ \frac{Na_2SO_4}{2}\ \frac{2H_2O}{2}$$

and reducing to lowest terms yields:

$$\frac{H_2SO_4}{2}\ \frac{NaOH}{1}\ \frac{Na_2SO_4}{2}\ \frac{H_2O}{1}$$

TABLE 1050:III. CONVERSION FACTORS*
(Milligrams per Liter – Milliequivalents per Liter)

Ion (Cation)	me/L = mg/L×	mg/L = me/L×	Ion (Anion)	me/L = mg/L×	mg/L = me/L×
Al^{3+}	0.111 2	8.994	BO_2^-	0.023 36	42.81
B^{3+}	0.277 5	3.604	Br^-	0.012 52	79.90
Ba^{2+}	0.014 56	68.66	Cl^-	0.028 21	35.45
Ca^{2+}	0.049 90	20.04	CO_3^{2-}	0.033 33	30.00
Cr^{3+}	0.057 70	17.33	CrO_4^{2-}	0.017 24	58.00
			F^-	0.052 64	19.00
Cu^{2+}	0.031 47	31.77	HCO_3^-	0.016 39	61.02
Fe^{2+}	0.035 81	27.92	HPO_4^{2-}	0.020 84	47.99
Fe^{3+}	0.053 72	18.62	$H_2PO_4^-$	0.010 31	96.99
H^+	0.992 1	1.008	HS^-	0.030 24	33.07
K^+	0.025 58	39.10	HSO_3^-	0.012 33	81.07
			HSO_4^-	0.010 30	97.07
Li^+	0.144 1	6.941	I^-	0.007 880	126.9
Mg^{2+}	0.082 29	12.15	NO_2^-	0.021 74	46.01
Mn^{2+}	0.036 40	27.47	NO_3^-	0.016 13	62.00
Mn^{4+}	0.072 81	13.73	OH^-	0.058 80	17.01
Na^+	0.043 50	22.99	PO_4^{3-}	0.031 59	31.66
NH_4^+	0.055 44	18.04	S^{2-}	0.062 37	16.03
Pb^{2+}	0.009 653	103.6	SiO_3^{2-}	0.026 29	38.04
Sr^{2+}	0.022 83	43.81	SO_3^{2-}	0.024 98	40.03
Zn^{2+}	0.030 59	32.70	SO_4^{2-}	0.020 82	48.03

* Factors are based on ion charge and not on redox reactions that may be possible for certain of these ions. Cations and anions are listed separately in alphabetical order.

Thus, one-half formula weight of H_2SO_4 and Na_2SO_4, and one formula weight of NaOH and H_2O are equivalent weights in this case.

In some cases, the reaction may not go to completion and the following equivalent entities are obtained.

$$H_2CO_3 + NaOH \rightarrow NaHCO_3 + H_2O$$

Because only one hydrogen was used, the equivalent number is 1.

$$\frac{H_2CO_3}{1} \quad \frac{NaOH}{1} \quad \frac{NaHCO_3}{1} \quad \frac{H_2O}{1}$$

If the cation of the base is multivalent, the number of equivalents is calculated similarly. For example:

$$3HCl + Al(OH)_3 \rightarrow AlCl_3 + 3H_2O$$

$$\frac{HCl}{1} \quad \frac{Al(OH)_3}{3} \quad \frac{AlCl_3}{3} \quad \frac{H_2O}{1}$$

Thus, the equivalent number is equal to the number of hydroxyl groups in a compound.

2) Precipitation and complex reactions—In these reactions the equivalents are equal to the weight of substance that will furnish, react with, or be chemically equivalent to one mole of univalent cation in the precipitate or complex formed. An example of a precipitation reaction is:

$$BaCl_2 + Na_2SO_4 \rightarrow BaSO_4 + 2NaCl$$

$$\frac{BaCl_2}{2} \quad \frac{Na_2SO_4}{2} \quad \frac{BaSO_4}{2} \quad \frac{NaCl}{1}$$

Examples of reactions with complexes are:

$$AgNO_3 + 2KCN \rightarrow Ag(CN)_2^- + KNO_3 + K^+$$

$$\frac{AgNO_3}{1} \quad \frac{2KCN}{1} \quad \frac{Ag(CN)_2^-}{1} \quad \frac{KNO_3}{1} \quad \frac{K^+}{1}$$

$$2AgNO_3 + 2KCN \rightarrow 2AgCN + 2KNO_3$$

$$\frac{2AgNO_3}{2} \quad \frac{2KCN}{2} \quad \frac{2AgCN}{2} \quad \frac{2KNO_3}{2}$$

$$\frac{AgNO_3}{1} \quad \frac{KCN}{1} \quad \frac{AgCN}{1} \quad \frac{KNO_3}{1}$$

$$NiSO_4 + 4KCN \rightarrow Ni(CN)_4^{2-} + K_2SO_4 + 2K^+$$

$$\frac{NiSO_4}{2} \quad \frac{4KCN}{2} \quad \frac{Ni(CN)_4^{2-}}{2} \quad \frac{K_2SO_4}{2} \quad \frac{2K^+}{2}$$

$$\frac{NiSO_4}{2} \quad \frac{2KCN}{1} \quad \frac{Ni(CN)_4^{2-}}{2} \quad \frac{K_2SO_4}{2} \quad \frac{K^+}{1}$$

3) Oxidation–reduction reactions—In these reactions the equivalent weight of a substance is the weight that will furnish, react with, or be chemically equivalent to one electron transferred. First, determine the number of electrons transferred in a redox reaction by writing out a balanced equation and the reduction and oxidation half-reactions. For example:

$$5Na_2C_2O_4 + 2KMnO_4 + 8H_2SO_4 \rightarrow 5Na_2SO_4 + 2MnSO_4 + K_2SO_4 + 10CO_2 + 8H_2O$$

$$5Na_2C_2O_4 + 5H_2SO_4 \rightarrow 5Na_2SO_4 + 10CO_2 + 10H^+ + 10e^-$$

$$2KMnO_4 + 3H_2SO_4 + 10H^+ + 10e^- \rightarrow 2MnSO_4 + K_2SO_4 + 8H_2O$$

Then divide the stoichiometric equation coefficients of the complete balanced equation by the number of electrons transferred in the half-reaction equations (in this case, 10) and reduce the fractions to yield:

$$\frac{Na_2C_2O_4}{2} \quad \frac{KMnO_4}{5} \quad \frac{4H_2SO_4}{5} \quad \frac{Na_2SO_4}{2} \quad \frac{MnSO_4}{5} \quad \frac{K_2SO_4}{10} \quad \frac{CO_2}{1} \quad \frac{4H_2O}{5}$$

With these quantities, only one electron is transferred, and thus the amounts represent the equivalents.

In this example the equivalent weights of these compounds in the reaction can be computed as follows:

$$\text{Equivalent weight} = \frac{\text{molecular weight g/mole}}{\text{equivalent number}}$$

$$\text{Equivalent weight } KMnO_4 = \frac{KMnO_4}{5} = \frac{158.04 \text{ g/mole}}{5}$$

$$= 31.61 \text{ g/equivalent}$$

$$\text{Equivalent weight } Na_2C_2O_4 = \frac{Na_2C_2O_4}{2} = \frac{134.00 \text{ g/mole}}{2}$$

$$= 67.00 \text{ g/equivalent}$$

d. Normality: Normality, as defined in ¶ *b*6) above, may be calculated as follows:

$$\text{Normality} = \frac{\text{weight of substance}}{(\text{equivalent weight of substance})(\text{liters of solution})}$$

The normality concept allows substances to be compared with one another stoichiometrically through the relationship.

$$V_1N_1 = V_2N_2$$

where:

V = volume, mL or L,
N = normality, and
1, 2 = compound 1 or 2.

If one knows the values of three of the variables, then the fourth can be computed.

There is a trend to move from the normality[6] concept because of possible ambiguities in determining normalities. These ambiguities arise because a substance being compared might have more than one computed normality concentration (e.g., potassium cyanide when it reacts with silver ion) and still be in the same concentration.

The above equation relating volume and normality of one substance to those of another can be written using the following equation in terms of molarity:

$$z_1V_1M_1 = z_2V_2M_2$$

where:

z = equivalent number and
M = molarity of substance in solution.

This relationship can be used to compute molarities or volumes of reagents being compared with one another. For example, the above equation could be reduced to the following for potassium permanganate (1) and sodium oxalate (2).

$$5V_1M_1 = 2V_2M_2$$

3. p Functions

Concentrations can be reported in the form of p functions (e.g., pH).

This form is used for expressing values when the analyte concentration varies over orders of magnitude. The pX concept is defined[8,9] in terms of analyte activity, rather than concentration, as follows:

$$pX = -\log (a_x)$$

where:

a_x = activity of analyte X in solution.

Activity coefficients relate activity to concentration:

$$pX = -\log (c_x\gamma_x/c^o)$$

where:

c_x = molar concentration of x, mole/L
γ_x = activity coefficient, dimensionless, and
c° = molar concentration at standard state, mole/L.

With the development of electrode measurement technology, pH and other p functions have become more commonly used, especially for very low concentrations of analytes. However, the electrode measures only activity, a_x, and not concentration, c_x, directly.

The Debye-Hückel equation provides a way to estimate the activity coefficients in low-ionic-strength solutions of 0.001*M* or less. This equation,[4,10–12] which relates concentration to activity coefficient, is:

$$-\log \gamma_i = \frac{Az_i^2I^{1/2}}{1 + B\sigma I^{1/2}}$$

where:

γ_I = analyte ion activity coefficient,
z_i = charge on ion species,
I = ionic strength of all the ions in solution
= $\frac{1}{2}\ \Sigma c_i z_i^2$, mole/L,
σ = radius of ionic atmosphere, picometers (see Table 1050:IV for values), and
A, B = Debye-Hückel equation constants (see Table 1050:V for values given by temperature and molar or molal concentration).

TABLE 1050:IV. EFFECTIVE HYDRATED RADIUS FOR COMMON IONS

Type and Charge of Ion	Ion	Ion Size, σ *pm*
Inorganic, ±1	H^+	900
		800
		700
	Li^+	600
		500
	Na^+, $CdCl^+$, ClO_2^-, IO_3^-, HCO_3^-, $H_2PO_4^-$, HSO_3^-, $H_2AsO_4^-$, $Co(NH_3)_4(NO_2)_2^+$	450
		400
	OH^-, F^-, SCN^-, OCN^-, HS^-, ClO_3^-, ClO_4^-, BrO_3^-, IO_4^-, MnO_4^-	350
	K^+, Cl^-, Br^-, I^-, CN^-, NO_2^-, NO_3^-	300
	Rb^+, Cs^+, NH_4^+, Tl^+, Ag^+	250
Inorganic, ±2	Mg^{2+}, Be^{2+}	800
		700
	Ca^{2+}, Cu^{2+}, Zn^{2+}, Sn^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Co^{2+}	600
	Sr^{2+}, Ba^{2+}, Cd^{2+}, Hg^{2+}, S^{2-}, $S_2O_4^{2-}$, WO_4^{2-}	500
	Pb^{2+}, CO_3^{2-}, SO_3^{2-}, MoO_4^{2-}, $Co(NH_3)_5Cl^{2+}$, $Fe(CN)_5NO^{2+}$	450
	Hg_2^{2+}, SO_4^{2-}, $S_2O_3^{2-}$, $S_2O_6^{2-}$, $S_2O_8^{2-}$, SeO_4^{2-}, CrO_4^{2-}, HPO_4^{2-}	400
Inorganic, ±3	Al^{3+}, Fe^{3+}, Cr^{3+}, Sc^{3+}, Y^{3+}, In^{3+}, lanthanides*	900
		500
	PO_4^{3-}, $Fe(CN)_6^{3-}$, $Cr(NH_3)_6^{3+}$, $Co(NH_3)_6^{3+}$, $Co(NH_3)_5H_2O^{3+}$	400
Inorganic, ±4	Th^{4+}, Zr^{4+}, Ce^{4+}, Sn^{4+}	1100
	$Fe(CN)_6^{4-}$	500
Organic, ±1	$HCOO^-$, $H_2citrate^-$, $CH_3NH_3^+$, $(CH_3)_2NH_2^+$	350
	$NH_3^+CH_2COOH$, $(CH_3)_3NH^+$, $C_2H_5NH_3^+$	400
	CH_3COO^-, CH_2ClCOO^-, $(CH_3)_4N^+$, $(C_2H_5)_2NH_2^+$, $NH_2CH_2COO^-$	450
	$CHCl_2COO^-$, CCl_3COO^-, $(C_2H_5)_3NH^+$, $(C_3H_7)NH^{3+}$	500
	$C_6H_5COO^-$, $C_6H_4OHCOO^-$, $C_6H_4ClCOO^-$, $C_6H_5CH_2COO^-$, $CH_2 = CHCH_2COO^-$, $(CH_3)_2CHCHCOO^-$, $(C_2H_5)_4N^+$, $(C_3H_7)_2NH_2^+$	600
	$[OC_6H_2(NO_3)_3]^-$, $(C_3H_7)_3NH^+$, $CH_3OC_6H_4COO^-$	700
	$(C_6H_5)_2CHCOO^-$, $(C_3H_7)_4N^+$	800
Organic, ±2	$(COO)_2^{2-}$, $Hcitrate^{2-}$	450
	$H_2C(COO)_2^{2-}$, $(CH_2COO)_2^{2-}$, $(CHOHCOO)_2^{2-}$	500
	$C_6H_4(COO)_2^{2-}$, $H_2C(CH_2COO)_2^{2-}$, $CH_2CH_2COO_2^{2-}$	600
	$[OOC(CH_2)_5COO]^{2-}$, $[OOC(CH_2)_6COO]^{2-}$, Congo red $anion^{2-}$	700
Organic, ±3	$Citrate^{3-}$	500

* Elements 57–71 in the periodic table.
SOURCE: Data from KIELLAND, J. 1937. Individual activity coefficients of ions in aqueous solutions. *J. Amer. Chem. Soc.*, 59:1675; table in HARRIS, D.C. 1982. Quantitative Chemical Analysis. W.H. Freeman & Co., New York, N.Y.

Example:

Compute the hydrogen ion concentration of a solution of pure water with sufficient potassium chloride to make it 0.1*M*. The measured pH is 6.98 at 25°C.

The ionic strength of the solution is

$$I = 1/2 \sum c_i z_i^2$$

$$I = 1/2\,[(c_{K^+})(1)^2 + (c_{Cl^-})(1)^2 + (c_{H^+})(1)^2 + (c_{OH^-})(1)^2]$$

The hydrogen ion and the hydroxyl ion concentrations (estimated to be about 10^{-7} mole/L) are insignificant in this example. Thus,

$$I = \tfrac{1}{2}\,[(0.1)(1)^2 + (0.1)(1)^2] = 0.1 \text{ mole/L}$$

Using the computed *I* and the values from Tables 1050:IV and 1050:V for σ (900 pm), A(0.5092), and B(0.003286), the Debye-Hückel equation yields

$$\log \gamma_{H^+} = \frac{-(0.5092)(1)^2\,(0.1)^{0.5}}{1 + (0.003286)(900)(0.1)^{0.5}} = -0.8321$$

$$\gamma_{H^+} = 0.8256$$

Since

TABLE 1050:V. VALUES OF A AND B FROM 0 TO 100°C FOR DEBYE-HÜCKEL EQUATION[12]

Temperature °C	A (Abs. Units) in Terms of Unit Volume of Solution	A′ (Abs. Units) in Terms of Unit Weight of Solvent	B ($cm^{-1} \times 10^8$) in Terms of Unit Volume of Solution	B′ ($cm^{-1} \times 10^8$) in Terms of Unit Weight of Solvent
0	0.4883	0.4883	0.3241	0.3241
5	0.4921	0.4921	0.3249	0.3249
10	0.4961	0.4960	0.3258	0.3258
15	0.5002	0.5000	0.3267	0.3266
18	0.5028	0.5025	0.3273	0.3271
20	0.5046	0.5042	0.3276	0.3273
25	0.5092	0.5085	0.3286	0.3281
30	0.5141	0.5130	0.3297	0.3290
35	0.5190	0.5175	0.3307	0.3297
40	0.5241	0.5221	0.3318	0.3305
45	0.5296	0.5270	0.3330	0.3314
50	0.5351	0.5319	0.3341	0.3321
55	0.5410	0.5371	0.3353	0.3329
60	0.5471	0.5425	0.3366	0.3338
65	0.5534	0.5480	0.3379	0.3346
70	0.5599	0.5537	0.3392	0.3354
75	0.5668	0.5596	0.3406	0.3363
80	0.5739	0.5658	0.3420	0.3372
85	0.5814	0.5722	0.3434	0.3380
90	0.5891	0.5788	0.3450	0.3390
95	0.5972	0.5857	0.3466	0.3399
100	0.6056	0.5929	0.3482	0.3409

SOURCE: MANOV, G.C., R.G. BATES, W.J. HAMER & S.F. ACRES. 1943. Values of the constants in the Debye-Hückel equation for activity coefficients. *J. Amer. Chem. Soc.* 65:1765s.

$$pH = -\log a_{H^+}$$

at the measured pH of 6.98,

$$a_{H^+} = 10^{-6.98} = 1.047 \times 10^{-7} \text{ mole/L}$$

Then,

$$c_{H^+} = (a_{H^+})/(\gamma_{H^+})$$

$$c_{H^+} = 1.047 \times 10^{-7}/0.8256 = 1.268 \times 10^{-7} \text{ mole/L}$$

Note that the hydrogen ion concentration is about 21% greater than was indicated by the hydrogen activity given by the pH electrode.

The same type of computation can be made for determining the ionic concentration from the measured electrode activity for fluoride, silver, and other substances. This is important if the true concentration of the ion is to be determined.

4. Stoichiometric Factors

Some analyses are reported as concentrations of other substances. Conversion factors to accomplish this are called stoichiometric factors. Examples are: alkalinity or acidity reported as $CaCO_3$, total hardness as $CaCO_3$, magnesium hardness as $CaCO_3$, ammonia as nitrogen, nitrate as nitrogen, nitrite as nitrogen, and phosphate as phosphorus.

These stoichiometric factors provide for taking the analysis data in the form analyzed first, dividing the mass concentration by the analyte's formula weight, and multiplying by the formula weight of the reported substance desired. For example,

$$\text{mg/L chloride as NaCl} = \frac{\text{mg } Cl^-}{\text{L}} \times \frac{\text{formula weight of sodium chloride}}{\text{formula weight of chlorine}}$$

Be sure that the formula weights of the substance to be reported and of the analyte include the same number of atoms of the common element.

Some analyses involve multiple reactions. For example, listed below are the reactions involved in the Winkler determination.[13]

$$2MnSO_4 + 4KOH \rightarrow 2Mn(OH)_2 + 2K_2SO_4$$

$$2\,Mn(OH)_2 + O_2 \rightarrow 2MnO(OH)_2$$

$$2\,MnO(OH)_2 + 4KI + 4H_2SO_4 \rightarrow 2I_2 + 2MnSO_4 + 2K_2SO_4 + 6H_2O$$

$$2I_2 + 4Na_2S_2O_3 \rightarrow 4NaI + 2Na_2S_4O_6$$

In the last equation, one mole of sodium thiosulfate is equivalent to one mole of elemental iodine. The number of moles of elemental iodine is equivalent to half the moles of manganese oxide hydroxide [$MnO(OH)_2$] in the second and third equations. Then the number of moles of oxygen is half the number of moles of $MnO(OH)_2$, and thus the equivalent weight of elemental oxygen is one-fourth that of elemental iodine or sodium thiosulfate (32 g/4 = 8g O_2/equivalent weight). This type of reasoning must be used to determine the analytical relationship between the titrant and the analyte being sought.

5. Molality

It is sometimes convenient to relate molality to molarity. That relationship is as follows:[14]

$$M = \frac{1000\rho m}{(1000 + W_B m)}$$

where:

M = molarity, mole/L,
ρ = density of solution, g/cm^3,
m = molality, mole/kg, and
W_B = molecular mass of solute, g.

The molality in terms of molarity is given as

$$m = \frac{1000M}{1000\rho - MW_B}$$

Many electrochemical measurements are made in terms of molality rather than molarity.

6. References

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1050 B. Significant Figures

1. Reporting Requirements

To avoid ambiguity in reporting results or in presenting directions for a procedure, it is customary to use "significant figures." All digits in a reported result are expected to be known definitely, except for the last digit, which may be in doubt. Such a number is said to contain only significant figures. If more than a single doubtful digit is reported, the extra digit or digits are not significant. This is an important distinction. Extra digits should be carried in calculation (see 1050B.2). If an analytical result is reported as "75.6 mg/L," the analyst should be quite certain of the "75," but may be uncertain as to whether the ".6" should be .5 or .7, or even .4 or .8, because of unavoidable uncertainty in the analytical procedure. If the standard deviation were known from previous work to be 2 mg/L , the analyst would have, or should have, rounded off the result to "76 mg/L" before reporting it. On the other hand, if the method was so good that a result of "75.64 mg/L" could have been conscientiously reported, then the analyst should not have rounded it off to 75.6 mg/L.

Report only such figures as are justified by the accuracy of the work. Do not follow the common practice of requiring that quantities listed in a column have the same number of figures to the right of the decimal point.

2. Rounding Off

Round off by dropping digits that are not significant. If the digit 6, 7, 8, or 9 is dropped, increase preceding digit by one unit; if the digit 0, 1, 2, 3, or 4 is dropped, do not alter preceding digit. If the digit 5 is dropped, round off preceding digit to the nearest even number: thus 2.25 becomes 2.2 and 2.35 becomes 2.4.

When making calculations, perform all computations before rounding off results. Repeated rounding off can result in changing the value of a reported result. For example, taking the measured value of 77.46 and rounding off to three significant figures yields 77.5. If the latter number were rounded a second time to two significant figures, the result would be 78. This is clearly a different result from a rounding of the original value of 77.46 to two significant figures (77).

3. Ambiguous Zeros

The digit 0 may record a measured value of zero or it may serve merely as a spacer to locate the decimal point. If the result of a sulfate determination is reported as 420 mg/L, the report recipient may be in doubt whether the zero is significant or not, because the zero cannot be deleted. If an analyst calculates a total residue of 1146 mg/L, but realizes that the

4 is somewhat doubtful and that therefore the 6 has no significance, the answer should be rounded off to 1150 mg/L and so reported but here, too, the report recipient will not know whether the zero is significant. Although the number could be expressed as a power of 10 (e.g., 11.5×10^2 or 1.15×10^3), this form is not used generally because it would not be consistent with the normal expression of results and might be confusing. In most other cases, there will be no doubt as to the sense in which the digit 0 is used. It is obvious that the zeros are significant in such numbers as 104 and 40.08. In a number written as 5.000, it is understood that all the zeros are significant, or else the number could have been rounded off to 5.00, 5.0, or 5, whichever was appropriate. Whenever zero is ambiguous, it is advisable to accompany the result with an estimate of its uncertainty.

Sometimes, significant zeros are dropped without good cause. If a buret is read as "23.60 mL," it should be so recorded, and not as "23.6 mL." The first number indicates that the analyst took the trouble to estimate the second decimal place; "23.6 mL" would indicate an imprecise reading of the buret.

4. Standard Deviation

Suppose that a set of potential results is normally distributed with a standard deviation of 100 mg/L and that a calculated value turns out to be 1450 mg/L. Then that 1450 is the best estimate available of this particular value, and from a Bayesian point of view, there would only be about a 31% chance that the true value was 1400 or lower or that the true value was 1500 or higher. It does not make sense to round the 1450 value to 1400. The arbitrary subtracting of half a standard deviation leaves us with a value that does not represent our best estimate well. The standard deviation should influence the last significant figures of a calculation only by ± 0.5[1] and if more, the number of significant figures cannot be justified.

When reporting numbers in the form $x \pm y$, always state whether y represents standard deviation, standard error, confidence limit, or an estimate of maximum bias. Standard deviations and standard errors often should be reported with extra digits (compared with single measurements) because they are calculated from variances and because they are square roots (see 1050B.5). When interpreting a quantity such as 1480 ± 40, be aware that this notation seldom indicates a belief that the true value lies anywhere in the range from 1440 to 1520 with equal probability; instead, the probability is concentrated near the central value (1480).

5. Calculations

As a starting point, round off the results of any calculation in which several numbers are multiplied and divided to as few significant figures as are present in the factor with the fewest significant figures.[2] However, several potential reasons to modify that guideline are noted below.

Example: Assume that the following calculation must be made to obtain the results of an analysis:

$$\frac{56 \times 0.003\,462 \times 43.22}{1.684}$$

A ten-place calculator yields an answer of "4.975 740 998." If the number 56 is an exact number (a count or a mathematical constant, such as π), it has no error associated with it and is considered to have unlimited significant figures. In that case, round off the result of the calculation to "4.976" because other numbers have only four significant figures. However, if 56 is an approximate measurement with uncertainly associated beyond the second figure, round off the result to "5.0" because 56 has only two significant figures.

When numbers are added or subtracted, the number that has the least precision in its last significant digit limits the number of places that can justifiably be carried in the sum or difference. It is acceptable practice to carry one more digit beyond the least precise significant digit.

Example:

The following numbers are to be added:

```
  0.0072
 12.02
  4.0078
 25.9
4886
```

The number "4886" is the least precise number (decimal place). Round each number in the sum to one or more digits beyond the least precise number and compute the sum.

```
  0.0
 12.0
  4.0
 25.9
4886.
4927.9
```

Round the result to the precision of the least precise number in the sum, 4928.

Some calculators or computers round off numbers by a different rule that tends to bias results toward the larger digit in the last significant figure. Before using such a device, determine which rounding techniques are programmed and if an incorrect rounding method is being used, reprogram to follow the correct rounding method. If it is not possible to reprogram, take unrounded number and manually round off using the correct scientific roundoff method.

Interpret the foregoing guidelines flexibly. For example, consider a series of measurements (u, v, and w) and a derived variable ($y = uv/w$) that is calculated for each case. Suppose measurements give $y = 9.90972$ and $y = 10.11389$ for two cases with similar measured values. According to the guidelines, the first y should be rounded to 9.91; the final digit here is about 0.1% of the overall result. For comparable precision, round the y value for the second case to 10.11 (not 10.1) because the fourth digit is also about 0.1% of the number. To generalize, more significant digits should at times be provided for quantities having 1, 2, or 3 (say) as leading digits than for quantities beginning with 7, 8, or 9.

Flexibility is also needed for the average of several numbers. The standard deviation (standard error) of a mean of N numbers is only $1/\sqrt{N}$ as large as the standard deviation of the individual numbers. Thus a mean of 100 numbers like d.dd is known to a precision of d.ddd. Even if fewer values than 100 are averaged, showing an extra digit may be justifiable. Because variances are averages of squared deviations, they too are more precise than

individual deviations. Therefore, reporting variances with an extra digit or two is often justifiable.

These guidelines refer only to final reported values. When performing any series of mathematical or statistical calculations, do not round measurements or other numbers until the very end of the analysis. Keep two or three extra digits for all intermediate calculations, to reduce round-off errors, which can be substantial.

6. General Arithmetic Functions

In analytical calculations, it may be necessary to use functions other than simple arithmetic, such as logarithmic, exponential, or trigonometric functions. A detailed treatment of significant figures in such cases is available.[3]

7. References

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1050 C. Other Considerations

1. Scientific and Engineering Notation

Scientific notation is defined by placing a number, N, in the format

$$N = Q \times 10^{r}$$

where:

Q = mantissa from 1 to 9.999, and
r = integer exponent.

Engineering notation[1,2] is defined by placing the number, N, in the format

$$N = P \times 10^{3t}$$

where:

P = mantissa from 1 to 999.999, and
$3t$ = exponent in integer multiples of 3.

Engineering notation is a convenient form for reporting results when using SI units. It allows easy selection of the proper SI prefix name.

2. Specifying Analyte Nomenclature

When reporting the concentration of an analyte, include not only the name of the analyte but its equivalent if the concentration units are in terms of the equivalent. A report giving only the analyte name is assumed to include only that chemical entity.

Chemical equivalents, such as nitrate nitrogen, are used to ease accounting of nitrogen chemical forms by allowing them to be added. In the case of organic nitrogen, the analyst may not know the exact organic form of nitrogen analyzed, so for convenience the compound is reported in terms of nitrogen rather than the actual organic compound.

Other concepts, such as alkalinity, are expressed in terms of calcium carbonate because the true form is not always known. Include other information such as temperature of pH measurement, temperature of drying, and temperature of conductance measurement. Other conditions, such as total metals or dissolved metals, are defined by filtration method.

Table 1050:VI gives examples of a number of analytes that may be expressed in other terms. Include complete information on the analyte, because many times the data may be used in other databases. The user may not always be able to assume the chemical form or special physical conditions under which the analyte was analyzed and reported.

3. Propagation of Error

For information on types and sources of error, see Section 1030.

Frequently, an analytical procedure consists of a number of steps in which one measurement is used in computing another measurement. Each measurement has an associated error. The greater the number of measurements used to obtain a computed result, the greater the possible associated error. This process is known as propagation of error.[3] When errors are compounded, they usually increase, but never decrease.

In the propagation of error, we are considering the value of the measurement as well as its associated uncertainty. All measurements have an associated value of uncertainty. The only measurements that have no uncertainty are numbers of events (counts), when properly defined, and mathematical constants.

Further information on propagation of error is available.[4–6]

4. References

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Table 1050:VI. Examples of Alternative Expressions of Analytical Results

Analyte	Units	Possible Reporting Nomenclature
Alkalinity	mg/L	mg/L as $CaCO_3$ or mg/L as HCO_3^-
Ammonia, un-ionized	mg/L	mg/L as N or mg/L as NH_3
Ammonium	mg/L	mg/L as N or mg/L as NH_4^+
Bicarbonate	mg/L	mg/L as HCO_3^- or mg/L as $CaCO_3$
Calcium	mg/L	mg/L as Ca^{2+} or mg/L as $CaCO_3$
Carbonate	mg/L	mg/L as CO_3^{2-} or mg/L as $CaCO_3$
Conductivity	μs/m	μS/m at 25°C or μS/m at t°C
Hydrogen sulfide	mg/L	mg/L as H_2S or mg/L as S^{2-}
Magnesium	mg/L	mg/L as Mg^{2+} or mg/L as $CaCO_3$
Nitrate	mg/L	mg/L as N or mg/L as NO_3^-
Nitrite	mg/L	mg/L as N or mg/L as NO_2^-
PCB	mg/L	mg/L as PCB or mg/L as decachlorobiphenyl or mg/L as Aroclor mixture
pH	—	pH at 25°C or pH at t°C
Silicon	mg/L	mg/L as Si or mg/L as SiO_2
Sulfide	mg/L	mg/L as S or mg/L as H_2S
Total dissolved solids	mg/L	mg/L at 180°C or mg/L at t°C
Uranium	mg/L	mg/L as U or mg/L as U_3O_8
	pCi/L	pCi/L as natural isotopic abundance or pCi/L as specified isotopic abundance
Zinc	mg/L	mg/L Zn as total or mg/L Zn as dissolved
	mg/kg	mg Zn/kg wet or mg Zn/kg dry

1060 COLLECTION AND PRESERVATION OF SAMPLES*

1060 A. Introduction

It is an old axiom that the result of any testing method can be no better than the sample on which it is performed. It is beyond the scope of this publication to specify detailed procedures for the collection of all samples because of varied purposes and analytical procedures. Detailed information is presented in specific methods. This section presents general considerations, applicable primarily to chemical analyses. See appropriate sections for samples to be used in toxicity testing and microbiological, biological, and radiological examinations.

The objective of sampling is to collect a portion of material small enough in volume to be transported conveniently and yet large enough for analytical purposes while still accurately representing the material being sampled. This objective implies that the relative proportions or concentrations of all pertinent components will be the same in the samples as in the material being sampled, and that the sample will be handled in such a way that no significant changes in composition occur before the tests are made.

Frequently, the objective of sampling and testing is to demontrate whether continuing compliance with specific regulatory requirements has been achieved. Samples are presented to the laboratory for specific determinations, with the sampler being responsible for collecting a valid and representative sample. Because of the increasing importance placed on verifying the accuracy and representativeness of data, greater emphasis is placed on proper sample collection, tracking, and preservation techniques. Often, laboratory personnel help plan a sampling program in consultation with the user of the test results. Such consultation is essential to ensure selected samples and analytical methods provide a sound and valid basis for answering the questions that prompted the sampling and that will meet regulatory and/or project-specific requirements.

This section addresses the collection and preservation of water and wastewater samples; the general principles also apply to the sampling of solid or semisolid matrices.

1. General Requirements

Obtain a sample that meets the requirements of the sampling program and handle it so it does not deteriorate or become contaminated or compromised before it is analyzed.

Ensure that all sampling equipment is clean and quality-assured before use. Use sample containers that are clean and free of contaminants. Bake at 450°C all bottles to be used for organic-analysis sampling.

Fill sample containers—without prerinsing—with sample; prerinsing results in loss of any pre-added preservative and sometimes can bias results high when certain components adhere to the sides of the container. Depending on determinations to be performed, fill the container full (most organic compound determinations) or leave space for aeration, mixing, etc. (microbiological and inorganic analyses). If a bottle already contains preservative, take care not to overfill the bottle, as preservative may be lost or diluted. Except when sampling for analysis of volatile organic compounds or radon, leave an air space equivalent to approximately 1% of the container volume to allow for thermal expansion during shipment.

Special precautions (discussed below) are necessary for samples containing organic compounds and trace metals. Because many constituents may be present at low concentrations (micrograms or nanograms per liter), they may be totally or partially lost or easily contaminated when proper sampling and preservation procedures are not followed.

Composite samples can be obtained by collecting over a period of time, depth, or at many different sampling points. The details of collection vary with local conditions, so specific recommendations are not universally applicable. Sometimes it is more informative to analyze numerous separate samples instead of one composite so variability, maxima, and minima can be determined.

Because of the inherent instability of certain properties and compounds, composite sampling for some analytes is not recommended where quantitative values are desired (examples include oil and grease, acidity, alkalinity, carbon dioxide, chlorine residual, iodine, hexavalent chromium, nitrite, volatile organic compounds, radon-222, dissolved oxygen, ozone, temperature, and pH). In certain cases, such as for BOD, phenolics, sulfite, and cyanide, composite samples are routinely required by regulatory agencies. Refrigerate composite samples for BOD, nitrate, ammonia, TKN, TSS, COD, and TOC.

Sample carefully to ensure that analytical results represent actual sample composition. Important factors affecting results are the presence of suspended matter or turbidity, the method chosen for removing a sample from its container, and the physical and chemical changes brought about by storage or aeration. Detailed procedures are essential when processing (blending, sieving, filtering) samples to be analyzed for trace constituents, especially metals and organic compounds. Some determinations can be invalidated by contamination during processing. Treat each sample individually with regard to the substances to be determined, the amount and nature of turbidity present, and other conditions that may influence the results.

Carefully consider the technique for collecting a representative sample and define it in the sampling plan. For metals, it often is appropriate to collect both a filtered and an unfiltered sample to differentiate between total and dissolved metals present in the matrix. Be aware that some metals may partially sorb to filters. Beforehand, determine the acid requirements to bring the pH to <2 on a separate sample. Add the same relative amount of acid to all samples; use ultrapure acid preservative to prevent contamination. Be sure that the dilution caused by acidifying is negligible or sufficiently reproducible for a dilution correction factor. When

* Reviewed by Standard Methods Committee, 2011.
2011 revisions by L. Malcom Baker, Rodger B. Baird, Nilda B. Cox, Andrew D. Eaton.
Joint Task Group: 20th Edition—Lawrence H. Keith (chair), Clifford G. Annis, Gary L. DeKock, Carleton P. Edmunds, Scott J. Mickelson, Mark Wyzalek.

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filtered samples will be collected, filter them in the field, if possible, or at the point of collection before preservation with acid. Filter samples in a laboratory-controlled environment if field conditions could cause error or contamination; in this case, filter as soon as possible. Often, slight turbidity can be tolerated if experience shows that it will cause no interference in gravimetric or volumetric tests and that its influence can be corrected in colorimetric tests, where it has potentially the greatest interfering effect. Sample collector must state whether the sample has been filtered.

Make a record of every sample collected and identify every bottle with a unique sample number, preferably by attaching an appropriately inscribed tag or label. Document sufficient information to provide positive sample identification at a later date, including the unique sample identification number, the name of the sample collector, the date, hour, exact location, and, if possible, sample type (e.g., grab or composite) and any other data that may be needed for correlation, such as water temperature, weather conditions, water level, stream flow, and post-collection conditions. If all pertinent information will not fit on a label or attached tag, record information in a bound sample log book at the sampling site at the time of sample collection. Use waterproof ink to record all information (preferably with black, non-solvent-based ink). Fix sampling points by detailed description in the sampling plan, by maps, or with the aid of stakes, buoys, or landmarks in a manner that will permit their identification by other persons without reliance on memory or personal guidance. Global positioning systems (GPS) also can supply accurate sampling position data. Particularly when sample results are expected to be involved in litigation, use formal "chain-of-custody" procedures (see 1060B.2), which trace sample history from collection to final reporting.

Before collecting samples from distribution systems, flush lines with three to five pipe volumes (or until water is being drawn from the main source) to ensure that the sample is representative of the supply, taking into account the volume of pipe to be flushed and the flow velocity. If the distribution system volume is unavailable, flush with tap fully open for at least 2 to 3 min before sampling. An exception to these guidelines (i.e., collecting a first-draw sample) is when information on areas of reduced or restricted flow is desired or when samples for lead in drinking water are being collected.

Although well-pumping protocols depend on the objectives of an investigation and other factors, such as well characteristics and available equipment, a general rule is to collect samples from wells only after the well has been purged sufficiently (usually with three to ten well volumes) to ensure that the sample represents the groundwater. Purging stagnant water is critical. Sometimes it will be necessary to pump at a specified rate to achieve a characteristic drawdown, if this determines the zones from which the well is supplied; record purging rate and drawdown, if necessary. By using methods with minimal drawdown, purging volumes can be reduced significantly.

When samples are collected from a river or stream, observed results may vary with depth, stream flow, and distance from each shore. Selection of the number and distribution of sites at which samples should be collected depends on study objectives, stream characteristics, available equipment, and other factors. If equipment is available, take an integrated sample from top to bottom in the middle of the main channel of the stream or from side to side at mid-depth. If only grab or catch samples can be collected, preferably take them at various points of equal distance across the stream; if only one sample can be collected, take it in the middle of the main channel of the stream and at mid-depth. Integrated samples are described further in 1060B.1*c*.

Rivers, streams, lakes, and reservoirs are subject to considerable variations from normal causes (e.g., seasonal stratification, diurnal variations, rainfall, runoff, and wind). Choose location, depth, and frequency of sampling depending on local conditions and the purpose of the investigation.

Use the following examples for general guidance. Avoid areas of excessive turbulence because of potential loss of volatile constituents and potential presence of denser-than-air toxic vapors. Avoid sampling at weirs, if possible, because such locations tend to favor retrieval of lighter-than-water immiscible compounds. Generally, collect samples beneath the surface in quiescent areas and open sampling container below surface with the mouth directed toward the current to avoid collecting surface scum unless oil and grease is a constituent of interest; then collect water at the surface. If composite samples are required, ensure that sample constituents are not lost during compositing because of improper handling of portions being composited. If samples will be analyzed for organic constituents, refrigerate composited portions. Do not composite samples for VOC analysis because some of the components will be lost through volatilization.

2. Safety Considerations

Because sample constituents may be toxic, take adequate precautions during sampling and sample handling. Toxic substances can enter through the skin and eyes and, in the case of vapors, also through the lungs. Ingestion can occur via direct contact of toxic materials with foods or by adsorption of vapors onto foods. Precautions may be limited to wearing gloves or may include coveralls, aprons, or other protective apparel. Often, the degree of protection provided by chemical protective clothing (CPC) is specific for different manufacturers and their product models[1]; ensure that the clothing chosen will offer adequate protection. Always wear eye protection (e.g., safety glasses with side shields or goggles). When toxic vapors may be present, sample only in well-ventilated areas, or use an appropriate respirator or self-contained breathing apparatus. In a laboratory, open sample containers in a fume hood. Never have food in the laboratory, near samples, or near sampling locations; always wash hands thoroughly before handling food.[2]

Always prohibit eating, drinking, or smoking near samples, sampling locations, and in the laboratory. Keep sparks, flames, and excessive heat sources away from samples and sampling locations. If flammable compounds are suspected or known to be present and samples will be refrigerated, use only specially designed explosion-proof refrigerators.[2]

Collect samples safely, avoiding situations that may lead to accidents. When in doubt as to the level of safety precautions needed, consult a knowledgeable industrial hygienist or safety professional. Samples with radioactive contaminants may require other safety considerations; consult a health physicist.

Label adequately any sample known or suspected to be hazardous because of flammability, corrosivity, toxicity, oxidizing chemicals, or radioactivity, so appropriate precautions can be taken during sample handling, storage, and disposal.

3. References

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2. Water Pollution Control Federation. 1986. Removal of Hazardous Wastes in Wastewater Facilities—Halogenated Organics; Manual of Practice FD-11. Alexandria, Va.

1060 B. Collection of Samples

1. Types of Samples

a. Grab samples: Grab samples are single samples collected at a specific spot at a site over a short period of time (typically seconds or minutes). Thus, they represent a "snapshot" in both space and time of a sampling area. Discrete grab samples are taken at a selected location, depth, and time. Depth-integrated grab samples are collected over a predetermined part or the entire depth of a water column, at a selected location and time in a given body of water.

A sample can represent only the composition of its source at the time and place of collection. However, when a source is known to be relatively constant in composition over an extended time or over substantial distances in all directions, then the sample may represent a longer time period and/or a larger volume than the specific time and place at which it was collected. In such circumstances, a source may be represented adequately by single grab samples. Examples are protected groundwater supplies, water supplies receiving conventional treatment, some well-mixed surface waters, but rarely wastewater streams, rivers, large lakes, shorelines, estuaries, and groundwater plumes.

When a source is known to vary with time, grab samples collected at suitable intervals and analyzed separately can document the extent, frequency, and duration of these variations. Choose sampling intervals on the basis of the expected frequency of changes, which may vary from 5 min to 1 h or more. Seasonal variations in natural systems may necessitate sampling over months. When the source composition varies in space (i.e., from location to location) rather than time, collect samples from appropriate locations that will meet the objectives of the study (for example, upstream and downstream from a point source).

The same principles apply to sampling wastewater sludges, sludge banks, and muds, although these matrices are not specifically addressed in this section. Take every possible precaution to obtain a representative sample or one conforming to a sampling program.

b. Composite samples: Composite samples should provide a more representative sampling of heterogeneous matrices in which the concentration of the analytes of interest may vary over short periods of time and/or space. Composite samples can be obtained by combining portions of multiple grab samples or by using specially designed automatic sampling devices. Sequential (time) composite samples are collected by using continuous, constant sample pumping or by mixing equal water volumes collected at regular time intervals. Flow-proportional composites are collected by continuous pumping at a rate proportional to the flow, by mixing equal volumes of water collected at time intervals that are inversely proportional to the volume of flow, or by mixing volumes of water proportional to the flow collected during or at regular time intervals.

Advantages of composite samples include reduced costs of analyzing a large number of samples, more representative samples of heterogeneous matrices, and larger sample sizes when amounts of test samples are limited. Disadvantages of composite samples include loss of analyte relationships in individual samples, potential dilution of analytes below detection levels, increased potential analytical interferences, and increased possibility of analyte interactions. In addition, use of composite samples may reduce the number of samples analyzed below the required statistical need for specified data quality objectives or project-specific objectives.

Do not use composite samples with components or characteristics subject to significant and unavoidable changes during storage. Analyze individual samples as soon as possible after collection and preferably at the sampling point. Examples are dissolved gases, residual chlorine, soluble sulfide, temperature, and pH. Changes in components, such as dissolved oxygen or carbon dioxide, pH, or temperature, may produce secondary changes in certain inorganic constituents, such as iron, manganese, alkalinity, or hardness. Some organic analytes also may be changed by changes in the foregoing components. Use time-composite samples only for determining components that can be demonstrated to remain unchanged under the conditions of sample collection, preservation, and storage.

Collect individual portions in a wide-mouth bottle every hour (in some cases, every half hour or even every 5 min) and mix at the end of the sampling period or combine in a single bottle as collected. If preservatives are used, add them to the sample bottle initially so all portions of the composite are preserved as soon as collected.

Automatic sampling devices are available; however, do not use them unless the sample is preserved as described below. Composite samplers running for extended periods (weeks to months) should undergo routine cleaning of containers and sample lines to minimize sample growth and deposits.

c. Integrated (discharge-weighted) samples: For certain purposes, the information needed is best provided by analyzing mixtures of grab samples collected from different points simultaneously, or as nearly so as possible, using discharge-weighted methods [e.g., equal-width increment (EWI) or equal discharge-increment (EDI) procedures and equipment]. An example of the need for integrated sampling occurs in a river or stream that varies in composition across its width and depth. To evaluate average composition or total loading, use a mixture of samples representing various points in the cross-section, in proportion to their relative flows. The need for integrated samples also may exist if combined treatment is proposed for several separate wastewater streams, the interaction of which may have a significant effect on treatability or even on composition. Mathematical prediction of the interactions among chemical components may

be inaccurate or impossible, and testing a suitable integrated sample may provide more useful information.

Both lakes and reservoirs show spatial variations of composition (depth and horizontal location). However, there are conditions under which neither total nor average results are especially useful, but local variations are more important. In such cases, examine samples separately (i.e., do not integrate them).

Preparation of integrated samples usually requires equipment designed to collect a sample water uniformly across the depth profile. Knowledge of the volume, movement, and composition of the various parts of the water being sampled usually is required. Collecting integrated samples is a complicated and specialized process that must be described adequately in a sampling plan.

2. Chain-of-Custody Procedures

Properly designed and executed chain-of-custody forms will ensure sample integrity from collection to data reporting. This includes the ability to trace possession and handling of the sample from the time of collection through analysis and final disposition. This process is referred to as *chain of custody* and is required to demonstrate sample control when the data are to be used for regulation or litigation. Where litigation is not involved, chain-of-custody procedures are useful for routine control of samples.

A sample is considered to be under a person's custody if it is in the individual's physical possession, in the individual's sight, secured and tamper-proofed by that individual, or secured in an area restricted to authorized personnel. The following procedures summarize the major aspects of chain of custody. More detailed discussions are available.[1,2]

a. Sample labels (including bar-code labels): Use labels to prevent sample misidentification. Gummed paper labels or tags generally are adequate. Include at least the following information: a unique sample number, sample type, name of collector, date and time of collection, place of collection, and sample preservative. Also include date and time of preservation for comparison to date and time of collection. Affix tags or self-adhesive labels to sample containers before, or at the time of, sample collection.

b. Sample seals: Use sample seals to detect unauthorized tampering with samples up to the time of analysis. Use self-adhesive paper seals that include at least the following information: sample number (identical with number on sample label), collector's name, and date and time of sampling. Plastic shrink seals also may be used.

Attach seal so that it must be broken to open the sample container or the sample shipping container (e.g., a cooler). Affix seal to container before sample leaves custody of sampling personnel.

c. Field log book: Record all information pertinent to a field survey or sampling in a bound log book. As a minimum, include the following in the log book: purpose of sampling; location of sampling point; name and address of field contact; producer of material being sampled and address (if different from location); type of sample; and method, date, and time of preservation. If the sample is wastewater, identify process producing waste stream. Also provide suspected sample composition, including concentrations; number and volume of sample(s) taken; description of sampling point and sampling method; date and time of collection; collector's sample identification number(s); sample distribution and how transported; references (e.g., maps or photographs of the sampling site); field observations and measurements; and signatures of personnel responsible for observations. Because sampling situations vary widely, it is essential to record sufficient information so one could reconstruct the sampling event without reliance on the collector's memory. Protect log book and keep it in a safe place.

d. Chain-of-custody record: Fill out a chain-of-custody record to accompany each sample or group of samples. The record includes the following information: sample number; signature of collector; date, time, and address of collection; sample type; sample preservation requirements; signatures of persons involved in the chain of possession; and inclusive dates and times of possession.

e. Sample analysis request sheet: The sample analysis request sheet accompanies samples to the laboratory. The collector completes the field portion of the form, which includes most of the pertinent information noted in the log book. The laboratory portion of the form is to be completed by laboratory personnel and includes: name of person receiving the sample, laboratory sample number, date of sample receipt, condition of each sample (if it is cold or warm, whether the container is full or not, color, if more than one phase is present, etc.), and determinations to be performed.

f. Sample delivery to the laboratory: Deliver sample(s) to laboratory as soon as practicable after collection, typically within 2 d. If shorter sample holding times are required, make special arrangements to ensure timely delivery to the laboratory. If samples are shipped by a commercial carrier, include the waybill number in the sample custody documentation. Ensure that samples are accompanied by a completed chain-of-custody record and a sample analysis request sheet. Deliver sample to sample custodian.

g. Receipt and logging of sample: In the laboratory, the sample custodian inspects the condition and seal of the sample and reconciles label information and seal against the chain-of-custody record before the sample is accepted for analysis. After acceptance, the custodian assigns a laboratory number, logs sample in the laboratory log book and/or computerized laboratory information management system, and stores it in a secured storage room or cabinet or refrigerator at the specified temperature until it is assigned to an analyst.

h. Assignment of sample for analysis: The laboratory supervisor usually assigns the sample for analysis. Once the sample is in the laboratory, the supervisor or analyst is responsible for its care and custody.

i. Disposal: Hold samples for the prescribed amount of time for the project or until the data have been reviewed and accepted. Document the disposition of samples. Ensure that disposal is in accordance with local-, state-, and U.S. EPA-approved methods.

3. Sampling Methods

a. Manual sampling: Manual sampling involves minimal equipment but may be unduly costly and time-consuming for routine or large-scale sampling programs. It requires trained field technicians and is often necessary for regulatory and research investigations for which critical appraisal of field conditions and complex sample-collection techniques are essential. Manually collect certain samples, such as waters containing oil and grease.

b. Automatic sampling: Automatic samplers can eliminate human errors in manual sampling, can reduce labor costs, may

provide the means for more frequent sampling,[3] and are increasingly used. Be sure that the automatic sampler does not contaminate the sample. For example, plastic components may be incompatible with certain organic compounds that are soluble in the plastic parts or that can be contaminated (e.g., from phthalate esters) by contact with them. If sample constituents are generally known, contact the manufacturer of an automatic sampler regarding potential incompatibility of plastic components.

Program an automatic sampler in accordance with sampling needs. Carefully match pump speeds and tubing sizes to the type of sample to be taken.

c. Sorbent sampling: Use of solid sorbents, particularly membrane-type disks, is becoming more frequent. These methods offer rapid, inexpensive sampling if the analytes of interest can be adsorbed and desorbed efficiently and the water matrix is free of particulates that plug the sorbent.

4. Sample Containers

The type of sample container used is of utmost importance. Test sample containers and document that they are free of analytes of interest, especially when sampling and analyzing for very low analyte levels. Containers typically are made of plastic or glass, but one material may be preferred over the other. For example, silica, sodium, and boron may be leached from soft glass, but not plastic, and trace levels of some pesticides and metals may sorb onto the walls of glass containers.[4] Thus, hard glass containers* are preferred. For samples containing organic compounds, do not use plastic containers except those made of fluorinated polymers, such as polytetrafluoroethylene (PTFE).[3]

Some sample analytes may dissolve (be absorbed) into the walls of plastic containers; similarly, contaminants from plastic containers may leach into samples. Avoid plastics wherever possible because of potential contamination from phthalate esters. Container failure due to breakdown of the plastic is possible. Therefore, use glass containers for all organics analyses, such as volatile organics, semivolatile organics, pesticides, PCBs, and oil and grease. Some analytes (e.g., bromine-containing compounds and some pesticides, and polynuclear aromatic compounds) are light-sensitive; collect them in amber-colored glass containers to minimize photodegradation. Container caps, typically plastic, also can be a problem. Do not use caps with paper liners. Use foil or PTFE liners but be aware that metal liners can contaminate samples collected for metals analysis and they may also react with the sample if it is acidic or alkaline. Serum vials with PTFE-lined rubber or plastic septa are useful.

In rare situations, it may be necessary to use sample containers not specifically prepared for use, or otherwise unsuitable for the particular situation; thoroughly document these deviations. Documentation should include type and source of container, and the preparation technique (e.g., acid washed with reagent water rinse). For QA purposes, the inclusion of a bottle blank may be necessary.

5. Number of Samples

Because of variability from analytical and sampling procedures (i.e., population variability), a single sample is insufficient to reach any reasonable desired level of confidence. If an overall standard deviation (i.e., the standard deviation of combined sampling and analysis) is known, the required number of samples for a mobile matrix, such as water, may be estimated as follows:[4]

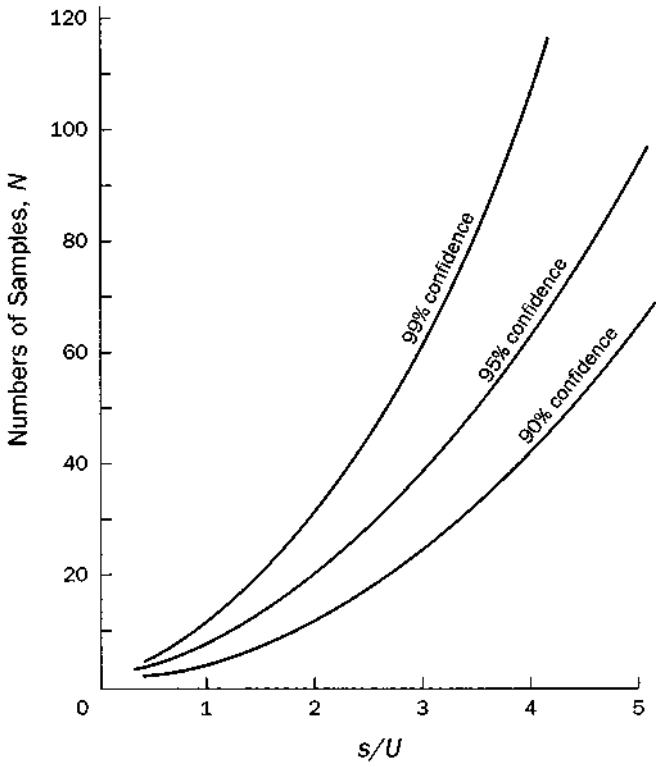


Figure 1060:1. Approximate number of samples required in estimating a mean concentration. SOURCE: Methods for the Examination of Waters and Associated Materials: General Principles of Sampling and Accuracy of Results. 1980. Her Majesty's Stationery Off., London, England.

$$N \geq \left(\frac{ts}{U}\right)^2$$

where:

N = number of samples,
t = Student's t statistic for a given confidence level,
s = overall standard deviation, and
U = acceptable level of uncertainty.

To assist in calculations, use curves such as those in Figure 1060:1. As an example, if s is 0.5 mg/L, U is ±0.2 mg/L, and a 95% confidence level is desired, approximately 25 to 30 samples must be taken.

The above equation assumes that total error (population variability) is known. Total variability consists of all sources of variability, including the distribution of the analytes of interest within the sampling site; collection, preservation, preparation, and analysis of samples; and data handling and reporting. In simpler terms, error (variability) can be divided into sampling and analysis components. Sampling error due to population variability (including heterogeneous distribution of analytes in the environmental matrix) usually is much larger than analytical error components. Unfortunately, sampling error usually is not

* Pyrex, or equivalent.

available and the analyst is left with only the published error of the measurement system (typically obtained by using a reagent water matrix under the best analytical conditions).

More accurate equations are available.[5] These are based on the Z distribution for determining the number of samples needed to estimate a mean concentration when variability is estimated in absolute terms using the standard deviation. The coefficient of variation [relative standard deviation (RSD)] is used when variability is estimated in relative terms.

The number of random samples to be collected at a site can be influenced partly by the method that will be used. The values for standard deviation (SD) or RSD may be obtained from each of the methods or in the literature.[6] However, calculations of estimated numbers of samples needed based only on this information will result in underestimated numbers of samples because only the analytical variances are considered, and the typically larger variances from the sampling operations are not included. Preferably, determine and use SDs or RSDs from overall sampling and analysis operations.

For estimates of numbers of samples needed for systematic sampling (e.g., drilling wells for sampling groundwater or for systematically sampling large water bodies, such as lakes), equations are available[7] that relate number of samples to shape of grid, area covered, and space between nodes of grid. The grid spacing is a complex calculation that depends on the size and shape of any contaminated spot (such as a groundwater plume) to be identified, in addition to the geometric shape of the sampling grid.

See individual methods for types and numbers of quality assurance (QA) and quality control (QC) samples [e.g., for normal-level (procedural) or low-level (contamination) bias or for precision] involving sampling or laboratory analysis (either overall or individually). Estimates of numbers of QC samples needed to achieve specified confidence levels also can be calculated. Rates of false positives (Type I error) and false negatives (Type II error) are useful parameters for estimating required numbers of QC samples. A false positive is the incorrect conclusion that an analyte is present when it is absent. A false negative is the incorrect conclusion that an analyte is absent when it is present. If the frequency of false positives or false negatives desired to be detected is $<10\%$, then

$$n = \frac{\ln \alpha}{\ln (1 - Y)}$$

where:

$\alpha = (1 -$ desired confidence level), and
$Y =$ frequency to detect ($<10\%$).

If the frequency that is desirable to detect is $>10\%$, iterative solution of a binomial equation is necessary.[5,8]

Equations are available as a computer program† for computing sample number by the *Z* distribution, for estimating samples needed in systematic sampling, and for estimating required number of QC samples.

6. Sample Volumes

Collect a 1-L sample for most physical and chemical analyses. For certain determinations, larger samples may be necessary. Table 1060:I lists volumes ordinarily required for analyses, but it is strongly recommended that the laboratory that will conduct the analyses also be consulted to verify the analytical needs of sampling procedures as they pertain to the goals and data quality objective of an investigation.

Do not use samples from the same container for multiple testing requirements (e.g., organic, inorganic, radiological, bacteriological, and microscopic examinations) because methods of collecting and handling are different for each type of test. Always collect enough sample volume in the appropriate container in order to comply with sample handling, storage, and preservation requirements.

7. References

1. U.S. Environmental Protection Agency. 1986. Test Methods for Evaluating Solid Waste: Physical/Chemical Methods, 3rd ed.; Pub. No. SW-846. Off. Solid Waste and Emergency Response, Washington, D.C.
2. U.S. Environmental Protection Agency. 1982. NEIC Policies and Procedures; EPA-330/9/78/001/-R (rev. 1982). Washington, D.C.
3. Water Pollution Control Federation. 1986. Removal of Hazardous Wastes in Wastewater Facilities—Halogenated Organics; Manual of Practice FD-11. Alexandria, Va.
4. Methods for the Examination of Waters and Associated Materials: General Principles of Sampling and Accuracy of Results. 1980. Her Majesty's Stationery Off., London, England.
5. Keith, L.H., G.L. Patton, D.L. Lewis & P.G. Edwards. 1996. Determining numbers and kinds of analytical samples, Chapter 1. *In* Principles of Environmental Sampling, 2nd ed. ACS Professional Reference Book, American Chemical Soc., Washington, D.C.
6. Keith, L.H. 1996. Compilation of EPA's Sampling and Analysis Methods, 2nd ed. Lewis Publ./CRC Press, Boca Raton, Fla.
7. Gilbert, R.O. 1987. Statistical Methods for Environmental Pollution Monitoring. Van Nostrand Reinhold, New York, N.Y.
8. Grant, E.L. & R.S. Leavenworth. 1988. Statistical Quality Control, 6th ed. McGraw-Hill, Inc., New York, N.Y.
9. U.S. Environmental Protection Agency. 2007. 40 CFR Part 136, Table II.
10. U.S. Environmental Protection Agency. 1992. Rules and Regulations. 40 CFR Parts 100–149.

† DQO-PRO, available (free) by downloading from Instant Reference Sources, Inc., http://instantref.com/dqo-form.htm.

TABLE 1060:I. SUMMARY OF SPECIAL SAMPLING AND HANDLING REQUIREMENTS*

Determination	Container†	Minimum Sample Size *mL*	Sample Type‡	Preservation§	Maximum Storage Recommended	Regulatory‖
Acidity	P, G(B), FP	100	g	Cool, ≤6°C	24 h	14 d
Alkalinity	P, G, FP	200	g	Cool, ≤6°C	24 h	14 d
BOD	P, G, FP	1000	g, c	Cool, ≤6°C	6 h	48 h
Boron	F, P (PTFE) or quartz	1000	g, c	HNO_3 to pH<2	28 d	6 months
Bromide	P, G, FP	100	g, c	None required	28 d	28 d
Carbon, organic, total	G(B), P, FP	100	g, c	Analyze immediately, or cool ≤6°C and add HCl, H_3PO_4, or H_2SO_4 to pH	7 d	28 d
Carbon dioxide	P, G	100	g	Analyze immediately	0.25 h	N.S.
COD	P, G, FP	100	g, c	Analyze as soon as possible, or add H_2SO_4 to pH<2; Cool, ≤6°C	7 d	28 d
Chloride	P, G, FP	50	g, c	None required	N.S.	28 d
Chlorine, total, residual	P, G	500	g	Analyze immediately	0.25 h	0.25 h
Chlorine dioxide	P, G	500	g	Analyze immediately	0.25 h	N.S.
Chlorophyll	P, G	500	g	Unfiltered, dark, ≤6°C Filtered, dark, –20°C (Do not store in frost-free freezer)	24–48 h 28 d	N.S.
Color	P, G, FP	500	g, c	Cool, ≤6°C	24 h	48 h
Specific conductance	P, G, FP	500	g, c	Cool, ≤6°C	28 d	28 d
Cyanide						
Total	P, G, FP	1000	g, c	Analyze within 15 min. Add NaOH to pH>12 if sample is to be stored, Cool, ≤6°C, in dark. Add thiosulfate if residual chlorine present	24 h	14 d; 24 h if sulfide present
Amenable to chlorination	P, G, FP	1000	g, c	Remove residual chlorine with thiosulfate and cool ≤6°C	stat	14 d; 24 h if sulfide present
Fluoride	P	100	g, c	None required	28 d	28 d
Hardness	P, G, FP	100	g, c	Add HNO_3 or H_2SO_4 to pH<2	6 months	6 months
Iodine	P, G	500	g	Analyze immediately	0.25 h	N.S.
Metals	P(A), G(A), FP (A)	1000	g, c	For dissolved metals filter immediately, add HNO_3 to pH<2	6 months	6 months
Chromium VI	P(A), G(A), FP (A)	250	g	Cool, ≤6°C, pH 9.3–9.7, ammonium sulfate buffer preservative as specified in method 3500-Cr to extend to 28 d HT	28 d	28 d
Copper by colorimetry	—*	—	g, c	—	—	
Mercury	P(A), G(A), FP(A)	500	g, c	Add HNO_3 to pH<2, Cool ≤6°C	28 d	28 d
Nitrogen						
Ammonia	P, G, FP	500	g, c	Analyze as soon as possible or add H_2SO_4 to pH<2, Cool, ≤6°C	7 d	28 d
Nitrate	P, G, FP	100	g, c	Analyze as soon as possible; Cool, ≤6°C	48 h	48 h (14 d for chlorinated samples)
Nitrate + nitrite	P, G, FP	200	g, c	Add H_2SO_4 to pH<2, Cool, ≤6°C	1–2 d	28 d
Nitrite	P, G, FP	100	g, c	Analyze as soon as possible; Cool, ≤6°C	none	48 h
Organic, Kjeldahl	P, G, FP	500	g, c	Cool, ≤6°C, add H_2SO_4 to pH<2	7 d	28 d
Odor	G	500	g	Analyze as soon as possible; Cool ≤6°C	6 h	24 h (EPA Manual drinking water)
Oil and grease	G, wide-mouth calibrated	1000	g	Add HCl or H_2SO_4 to pH<2, Cool, ≤6°C	28 d	28 d

TABLE 1060:I. CONT.

Determination	Container†	Minimum Sample Size *mL*	Sample Type‡	Preservation§	Maximum Storage Recommended	Regulatory‖
Organic Compounds						
MBAS	P, G, FP	250	g, c	Cool, ≤6°C	48 h	48 h as per CFR 136
Pesticides*	G(S), PTFE-lined cap	1000	g, c	Cool, ≤6°C, add 1000 mg ascorbic acid/L if residual chlorine present (0.008% sodium thiosulfate in CFR 136)	7 d	7 d until extraction; 40 d after extraction
Phenols	P, G, PTFE-lined cap	500	g, c	Cool, ≤6°C, add H_2SO_4 to pH<2	*	28 d until extraction, 2 d after extraction
Purgeables* by purge and trap	G, PTFE-lined cap	2×40	g	Cool, ≤6°C; add HCl to pH<2; add 1000 mg ascorbic acid/L if residual chlorine present (0.008% sodium thiosulfate in CFR 136)	7 d	14 d
Base/neutrals & acids	G(S) amber	1000	g, c	Cool, ≤6°C, 0.008% sodium thiosulfate in CFR 136 if chlorine is present	7 d	7 d until extraction; 40 d after extraction
Oxygen, dissolved	G, BOD bottle	300	g			
Electrode				Analyze immediately	0.25 h	0.25 h
Winkler				Titration may be delayed after acidification	8 h	8 h
Ozone	G	1000	g	Analyze immediately	0.25 h	N.S.
pH	P, G	50	g	Analyze immediately	0.25 h	0.25 h
Phosphate	G(A)	100	g	For dissolved phosphate filter immediately; Cool, ≤6°C	48 h	48 h as per EPA manual for DW
Phosphorus, total	P, G, FP	100	g, c	Add H_2SO_4 to pH<2 and cool, ≤6°C	28 d	28 d
Salinity	G, wax seal	240	g	Analyze immediately or use wax seal	6 months	N.S.
Silica	F, P (PTFE) or quartz	200	g, c	Cool, ≤6°C, do not freeze	28 d	28 d
Sludge digester gas	G, gas bottle	—	g	—	N.S.	
Solids[9]	P, G	200	g, c	Cool, ≤6°C	7 d	2–7 d; see cited reference
Sulfate	P, G, FP	100	g, c	Cool, ≤6°C	28 d	28 d
Sulfide	P, G, FP	100	g, c	Cool, ≤6°C; add 4 drops 2*N* zinc acetate/100 mL; add NaOH to pH >9	28 d	7 d
Temperature	P, G, FP	—	g	Analyze immediately	0.25 h	0.25 h
Turbidity	P, G, FP	100	g, c	Analyze same day; store in dark up to 24 h, Cool, ≤6°C	24 h	48 h

* For determinations not listed, use glass or plastic containers; preferably refrigerate during storage and analyze as soon as possible.

† P = plastic (polyethylene or equivalent); G = glass; G(A) or P(A) = rinsed with 1 + 1 HNO_3; G(B) = glass, borosilicate; G(S) = glass, rinsed with organic solvents or baked; FP = fluoropolymer [polytetrafluoroethylene (PTFE, Teflon) or other fluoropolymer].

‡ g = grab; c = composite.

§ Cool = storage at, >0°C, ≤6°C (above freezing point of water); in the dark; analyze immediately = analyze usually within 15 min of sample collection.

‖ See citation[10] for possible differences regarding container and preservation requirements. N.S. = not stated in cited reference; stat = no storage allowed; analyze immediately (within 15 min).

Some drinking water (DW) and treated wastewater (WW) matrices may be subject to positive interference as a result of preservation. If such interference is demonstrable, samples should be analyzed as soon as possible without preservation. Do not hold for more than 15 min without demonstrating that cyanide (CN) is stable for longer periods in a specific matrix.

NOTE: This table is intended for guidance only. If there is a discrepancy between this table and the method, the information in the current method takes precedence. If performing the method for compliance purposes, be aware that alternative preservation and holding-time requirements may exist. If so, the regulatory requirements should be used.

1060 C. Sample Storage and Preservation

Complete and unequivocal preservation of samples, whether domestic wastewater, industrial wastes, or natural waters, is a practical impossibility because complete stability for every constituent never can be achieved. At best, preservation techniques only retard chemical and biological changes that inevitably continue after sample collection.

1. Sample Storage before Analysis

a. Nature of sample changes: Some determinations are more affected by sample storage than others. Certain cations are subject to loss by adsorption to, or ion exchange with, the walls of glass containers. These include aluminum, cadmium, chromium, copper, iron, lead, manganese, silver, and zinc, which are best collected in a separate clean bottle and acidified with nitric acid to a pH <2.0 to minimize precipitation and adsorption on container walls. Also, some organics may be subject to loss by adsorption to the walls of glass containers.

Temperature changes quickly; pH may change significantly in a matter of minutes; dissolved gases (oxygen, carbon dioxide) may be lost. Because changes in such basic water quality properties may occur so quickly, determine temperature, reduction–oxidation potential, and dissolved gases *in situ* and pH, specific conductance, turbidity, and alkalinity immediately after sample collection. Many organic compounds are sensitive to changes in pH and/or temperature resulting in reduced concentrations during storage.

Changes in the pH–alkalinity–carbon dioxide balance may cause calcium carbonate to precipitate, decreasing the values for calcium and total hardness.

Iron and manganese are readily soluble in their lower oxidation states but relatively insoluble in their higher oxidation states; therefore, these cations may precipitate or they may dissolve from a sediment, depending on the redox potential of the sample. Microbiological activity may affect the nitrate-nitrite-ammonia content, phenol or BOD concentration, or the reduction of sulfate to sulfide. Residual chlorine is reduced to chloride. Sulfide, sulfite, ferrous iron, iodide, and cyanide may be lost through oxidation. Color, odor, and turbidity may increase, decrease, or change in quality. Sodium, silica, and boron may be leached from the glass container. Hexavalent chromium may be reduced to trivalent chromium.

The biological activity in a sample may change the oxidation state of some constituents. Soluble constituents may be converted to organically bound materials in cell structures, or cell lysis may result in release of cellular material into solution. The well-known nitrogen and phosphorus cycles are examples of biological influences on sample composition.

Zero headspace is important in preservation of samples with volatile organic compounds and radon. Avoid loss of volatile materials by collecting sample in a completely filled container. Achieve this by carefully filling the bottle so top of meniscus is above the top of the bottle rim. It is important to avoid spillage or air entrapment if preservatives, such as HCl or ascorbic acid, have already been added to the bottle. After capping or sealing bottle, check for air bubbles by inverting and gently tapping it; if one or more air bubbles are observed then, if practical, discard the sample and repeat refilling bottle with new sample until no air bubbles are observed (this cannot be done if bottle contained preservatives before it was filled).

Serum vials with septum caps are particularly useful in that a sample portion for analysis can be taken through the cap by using a syringe,[1] although the effect of pressure reduction in the headspace must be considered. Pulling a sample into a syringe under vacuum can result in low bias data for volatile compounds and the resulting headspace precludes taking further subsamples.

b. Time interval between collection and analysis: In general, the shorter the time that elapses between collection of a sample and its analysis, the more reliable will be the analytical results. For certain constituents and physical values, immediate analysis in the field is required. For composited samples, it is common practice to use the time at the end of composite collection as the sample-collection time.

Check with the analyzing laboratory to determine how much elapsed time may be allowed between sample collection and analysis; this depends on the character of the sample and the stability of the target analytes under storage conditions. Many regulatory methods limit the elapsed time between sample collection and analysis (see Table 1060:I). Changes caused by growth of microorganisms are greatly retarded by keeping the sample at a low temperature (<6°C but above freezing). When the interval between sample collection and analysis is long enough to produce changes in either the concentration or physical state of the constituent to be measured, follow the preservation practices given in Table 1060:I. Record time elapsed between sampling and analysis, and which preservative, if any, was added.

2. Preservation Techniques

To minimize the potential for volatilization or biodegradation between sampling and analysis, keep samples as cool as possible without freezing. Preferably pack samples in crushed or cubed ice or commercial ice substitutes before shipment. Avoid using dry ice because it will freeze samples and may cause glass containers to break. Dry ice also may effect a pH change in samples. Keep composite samples cool with ice or a refrigeration system set at ≤ 6°C during compositing. Analyze samples as quickly as possible on arrival at the laboratory. If immediate analysis is not possible, preferably store at ≤ 6°C.[1]

No single method of preservation is entirely satisfactory; choose the preservative with due regard to the determinations to be made. Use chemical preservatives only when they do not interfere with the analysis being made. When they are used, add them to the sample bottle initially so all sample portions are preserved as soon as collected. Because a preservation method for one determination may interfere with another one, samples for multiple determinations may need to be split and preserved separately. All preservation methods may be inadequate when applied to suspended matter. Do not use formaldehyde as a preservative for samples collected for chemical analysis because it affects many of the target analytes.

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Preservation methods are relatively limited and are intended generally to retard biological action, retard hydrolysis of chemical compounds and complexes, and reduce volatility of constituents.

Preservation methods are limited to pH control, chemical addition, the use of amber and opaque bottles, refrigeration, filtration, and freezing. Table 1060:I lists preservation methods by constituent. See Section 7010B for sample collection and preservation requirements for radionuclides.

The foregoing discussion is by no means exhaustive and comprehensive. Clearly, it is impossible to prescribe absolute rules for preventing all possible changes. Additional advice will be found in the discussions under individual determinations, but to a large degree, the dependability of an analytical determination rests on the experience and good judgment of the person collecting the sample. Numbers of samples required for confidence levels in data quality objectives, however, rely on statistical equations, such as those discussed earlier.

3. Reference

1. WATER POLLUTION CONTROL FEDERATION. 1986. Removal of Hazardous Wastes in Wastewater Facilities—Halogenated Organics; Manual of Practice FD-11. Alexandria, Va.

4. Bibliography

KEITH, L.H., ed. 1996. Principles of Environmental Sampling, 2nd ed. ACS Professional Reference Book, American Chemical Soc., Washington, D.C.

1080 REAGENT WATER*

1080 A. Introduction

One of the most important aspects of analysis is preparing the reagent water used to prepare and dilute reagents and prepare blanks. *Reagent water* is water with no detectable concentration of the compound or element to be analyzed (i.e., it is below the analytical method's detection level). Reagent water should also be free of substances that interfere with analytical methods. However, its overall quality (concentrations of organic, inorganic, and biological constituents) will depend on the water's intended use(s).

Use any method to prepare reagent water that can meet the applicable quality requirements. Various combinations of reverse osmosis, distillation, and deionization can produce reagent water, as can ultrafiltration and/or ultraviolet irradiation. Keep in mind, however, that improperly operated or maintained water purification systems may add rather than remove contaminants.

This section provides general guidelines for preparing reagent water. Table 1080:I lists commonly available water purification processes and the major classes of contaminants that they remove. For details on preparing water for microbiological tests, see Section 9020B.4*d*. T1

* Reviewed by Standard Methods Committee, 2011.

1080 B. Methods for Preparing Reagent-Grade Water

1. Distillation

Distillation is the process of heating a liquid until it boils, capturing and cooling the resultant hot vapors, and collecting the condensed vapors. Laboratory-grade distilled water should be generated in a still made of all-borosilicate glass, fused quartz, tin, or titanium. To remove ammonia, distill from an acid solution. Remove CO_2 by boiling the water for 15 min and cooling rapidly to room temperature; exclude atmospheric CO_2 by using a tube containing soda lime or a commercially available CO_2-removing agent.*

Impurities may be added to the water during boiling if they leach from the container. Also, freshly replaced filters, cartridges, and resins initially can release impurities. Pretreat feedwater and maintain still periodically to minimize scale formation. Pretreatment may be required if the feedwater contains significant concentrations of calcium, magnesium, and bicarbonate ions; it may involve demineralization via reverse osmosis or ion exchange.

2. Reverse Osmosis

In *reverse osmosis*, water is forced under pressure through a semipermeable membrane, thereby removing some dissolved constituents and suspended impurities. The reagent water quality will depend on both feedwater quality and the type and condition of membranes used.

Reverse osmosis membranes are available in both spiral-wound and hollow-fiber configurations; the choice depends on the feedwater's characteristics and fouling potential. Obtain rejection data for feedwater contaminants (levels of salt and impurities that will pass through the membranes compared to feedwater levels) at the operating pressure that will be used to prepare reagent water. Set the water-production rate to make the most economical use of feedwater without compromising permeate (reagent water) quality.

Pretreatment steps (e.g., filtration) may be needed to minimize membrane fouling (due to colloids or particulates) and/or degradation (due to chlorine, iron, and other oxidizing compounds). Also, the membrane modules need to be backwashed periodically to clean the surface of the membranes. If using a commercially available reverse osmosis system, follow manufacturer's instructions for quality control (QC) and maintenance.

3. Ion Exchange

In an ion exchange process, water passes through a reactor containing negatively charged (anionic) and/or positively charged (cationic) resins. Targeted ions in the water are substituted with specific ions on the resins (ones acceptable in treated water systems), thereby purifying the water. To prepare deionized water, direct feedwater through a mixed-bed ion exchanger, which contains both strong anion and strong cation resins. Proper bed sizing is critical to resin performance. Be sure the bed's length-to-diameter ratio is in accordance with the maximum process flow rate to ensure that optimal face velocities are not exceeded and that residence time is sufficient.

If the system does not generate reagent water continuously, recirculate the water through the ion exchanger. If resin regeneration is economically attractive, use separate anion and cation resin beds, and position the anion exchanger downstream of the cation exchanger to remove leachates from the cation resin. If the feedwater contains significant quantities of organic matter, remove the organics first to minimize the potential for resin fouling. Organics can be removed via prefiltration, distillation,

* Ascarite II, Fisher Scientific Co., or equivalent.

TABLE 1080:I. WATER PURIFICATION PROCESSES

Process	Major Classes of Contaminants*					
	Dissolved Ionized Salts	Dissolved Ionized Gases	Dissolved Organics	Particulates	Bacteria	Pyrogens/ Endotoxins
Distillation	G–E†	P	G	E	E	E
Deionization	E	E	P	P	P	P
Reverse osmosis	G‡	P	G	E	E	E
Carbon adsorption	P	P§	G–E‖	P	P	P
Filtration	P	P	P	E	E	P
Ultrafiltration	P	P	G#	E	E	E
Ultraviolet oxidation	P	P	G–E**	P	G††	P

Permission to use this table from C3–A2, Vol. 11, No. 13, Aug. 1991, "Preparation and Testing of Reagent Water in the Clinical Laboratory—Second Edition" has been granted by the National Committee for Clinical Laboratory Standards. The complete current standard may be obtained from National Committee for Clinical Laboratory Standards. 771 E. Lancaster Ave., Villanova, PA 19085.

* E = Excellent (capable of complete or near total removal), G = Good (capable of removing large percentages), P = Poor (little or no removal).

† The resistivity of water purified via distillation is an order of magnitude less than that in water produced via deionization, mainly due to the presence of CO_2 and sometimes H_2S, NH_3, and other ionized gases (if present in feedwater).

‡ The resistivity of dissolved ionized solids in product water depends on original feedwater resistivity.

§ Activated carbon removes chlorine via adsorption.

‖ When used with other purification processes, special grades of activated carbon and other synthetic adsorbents are excellent at removing organic contaminants. Their use, however, is targeted toward specific compounds and applications.

Ultrafilters reduce specific feedwater organic contaminants based on the membrane's rated molecular weight cut-off.

** 185-nm UV oxidation (batch process) removes trace organic contaminants effectively when used as post-treatment. Feedwater makeup plays a critical role in their performance.

†† While 254-nm UV sterilizers do not physically remove bacteria, they may have bactericidal or bacteriostatic capabilities limited by intensity, contact time, and flow rate.

reverse osmosis, or adsorption. If using commercially prepared resin columns, follow supplier's recommendations for monitoring QC of reagent water from specific equipment.

4. Adsorption

In *adsorption*, water is fed into a reactor filled with an adsorbent material (typically, granular activated carbon, although some resins and other manmade adsorbents are used in specific applications). Chlorine and other organic impurities are drawn from the water to the surface of the adsorbent. How well the process works depends on the organic contaminants involved, the activated carbon's physical characteristics, and the operating conditions. In general, organics-adsorption efficiency is inversely proportional to the solubility of the organics in water and the adsorption process may be inadequate for removing low-molecular-weight, polar compounds. Performance differences among activated carbons are attributable to the raw materials and activation procedures. Even with an optimal activated carbon, proper performance will not be attained unless the column is sized to provide required face velocity and residence time at the maximum process flow rate. If using commercial sorbent systems, follow supplier's recommended flow and QC steps.

Using activated carbon may adversely affect the reagent water's resistivity. This effect may be controlled via reverse osmosis, mixed resins, or special adsorbents. To minimize organic contamination, use mixtures of polishing resins with special carbons and additional treatment steps (e.g., reverse osmosis, natural carbons, ultraviolet oxidation, or ultrafiltration).

1080 C. Reagent Water Quality

1. Quality Guidelines

Guidelines for reagent water vary with the intended use.[1] Table 1080:II lists some characteristics of various qualities of reagent water. In general, low-quality reagent water has a minimum resistivity of 0.1 megohm-cm at 25°C. It may be used to wash glassware, rinse glassware (as a preliminary step), and as a source to produce higher-grade waters.

Medium-quality reagent water typically is produced via distillation or deionization. Resistivity should be >1 megohm-cm at 25°C.

High-quality reagent water has a minimum resistivity of 10 megohms-cm at 25°C. It typically is prepared via distillation, deionization, or reverse osmosis of feedwater followed by mixed-bed deionization and membrane filtration (0.2-μm pore). It also could be prepared via reverse osmosis followed by carbon adsorption and deionization.

Mixed-bed deionizers typically add small amounts of organic matter to water, especially if the beds are fresh, so determine reagent water quality immediately after preparation. Its resistivity (measured in-line) should be >10 megohm-cm at 25°C. However, resistivity measurements do not detect organics or

TABLE 1080:II. REAGENT WATER SPECIFICATIONS

Quality Parameter	High	Medium	Low
Resistivity, megohm-cm at 25°C	>10	>1	>0.1
Conductivity, μmho/cm at 25°C	<0.1	<1	<10
SiO_2, mg/L	<0.05	<0.1	<1

nonionized contaminants, nor accurately assess ionic contaminants at the microgram-per-liter level.

The pH of high- or medium-quality water cannot be measured accurately without contaminating the water, so measure other constituents required for individual tests.

High-quality water cannot be stored without degrading significantly. Medium-quality water may be stored, but keep storage time to a minimum and make sure quality remains consistent with the intended use. Only store it in materials that protect the water from contamination (e.g., TFE and glass for organics analysis or plastics for metals).

2. Reference

1. AMERICAN SOCIETY FOR TESTING & MATERIALS. 2006. Annual Book of ASTM Standards; Vol 11.01, D 1193-06. W. Conshohocken, Pa.

1090 LABORATORY OCCUPATIONAL HEALTH AND SAFETY*

1090 A. Introduction

1. General Discussion

Achievement of a safe and healthful workplace is the responsibility of the organization, the laboratory manager, the supervisory personnel and, finally, the laboratory personnel themselves. All laboratory employees must make every effort to protect themselves and their co-workers by conscientiously adhering to the health and safety program that has been developed and documented specifically for their laboratory.

2. Organizing for Safety

a. Overall program: The responsibility for establishing and enforcing a laboratory health and safety (LH&S) program ultimately rests with the laboratory director. The LH&S program must, at the minimum, address how to protect oneself from the hazards of working with biological (1090H), chemical (1090J), and radiological (1090I) agents. Such a program is a necessary component of an overall laboratory quality system that provides for the health and safety of the entire laboratory staff. As a part of the quality system, all aspects of the LH&S program must be fully documented. Laboratory personnel must be trained. The LH&S program must be fully implemented and its application audited periodically. Appropriate records of all activities must be kept to document performance, meet appropriate regulatory requirements, and document the status of the LH&S program.

In the United States, the minimum standard of practice for health and safety activities is detailed in government documents.[1,2] Each laboratory should appoint as needed a chemical hygiene officer (CHO), a biological hygiene officer (BHO), a radiological hygiene officer (RHO), and, where appropriate or desired, a LH&S committee. The CHO, the committee, and laboratory management must develop, document, and implement a written laboratory hygiene plan (LHP), or chemical hygiene plan (CHP).

b. Specific responsibilities: Specific responsibilities applicable at various levels within the organization are as follows:

1) The chief executive officer (CEO) has ultimate responsibility for LH&S within the organization and must, with other managers and supervisors, provide continuous support for the LH&S program.

2) The supervisor and/or designee has primary responsibility for the LH&S program in his or her work group.

3) The biological hygiene officer (BHO) has the responsibility to work with managers, supervisors, and other employees to develop and implement appropriate biological hygiene policies and practices; monitor procurement, use, and disposal of biological agents used in the laboratory; see that appropriate audits are conducted and that records are maintained; know the current legal requirements concerning working with biological agents; and seek ways to improve the biological hygiene program.

4) The chemical hygiene officer (CHO) has the same responsibilities as the biological hygiene officer, but with respect to chemicals, and also is responsible for helping supervisors (project directors) develop precautions and adequate facilities and for keeping material safety data sheets (MSDSs) available for review.

5) The radiological hygiene officer (RHO), referred to as *radiation safety officer* in most regulatory language, has the same responsibilities as the chemical hygiene officer, but with respect to radiological chemicals and exposure.

6) The laboratory supervisor and/or designee has overall responsibility for chemical hygiene in the laboratory, including ensuring that workers know and follow the chemical hygiene rules, that protective equipment is available and in working order, and that appropriate training has been provided; performing regular, formal chemical hygiene and housekeeping inspections, including routine inspections of emergency equipment, and maintenance of appropriate records; knowing the current legal requirements concerning regulated substances; specifying the required levels of protective apparel and equipment needed to perform the work; and ensuring that facilities and training for use of any material being ordered are adequate.

7) The project director (or a director of a specific operation) has primary responsibility for biological, chemical, and/or radiological hygiene procedures as appropriate for all operations under his or her control.

8) The laboratory worker has the responsibility for planning and conducting each operation in accordance with the institutional chemical hygiene, biological hygiene, and radiological hygiene procedures, and for developing good personal chemical, biological, and radiological hygiene habits.

3. Records

Maintain records of all accidents, including "near-misses," medical care audits, inspections, and training for specified time periods that depend on the nature of the requirement. Keep records on standardized report forms containing sufficient information to enable an investigator to determine who was involved, what happened, when and where it happened, and what injuries or exposures, if any, resulted. Most importantly, these records should enable the formulation of appropriate corrective actions where warranted. The standard of practice for LH&S activities requires that a log (record) be kept of those accidents causing major disability. Record not only all accidents, but also "near-misses," to permit full evaluation of safety program effectiveness. Maintain a file detailing all of the recommendations for the LH&S program.

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* Reviewed by Standard Methods Committee, 2010.
Joint Task Group: 20th Edition—Albert A. Liabastre (chair), Daniel F. Bender, R. Wayne Jackson, Michael C. Nichols, James H. Scott.

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4. Information and Training[2]

The standard of practice for hazard communication or "right-to know" requires that employees be notified about hazards in the workplace.

Laboratory personnel must be under the direct supervision and regular observation of a technically qualified individual who must have knowledge of the hazards present, their health effects, and related emergency procedures. The supervisor must educate laboratory personnel in safe work practices at the time of initial assignment and when a new hazardous substance is introduced into the workplace. Personnel have a right to know what hazardous materials are present, the specific hazards created by those materials, and the required procedures to protect themselves against these hazards. The hazard communication standard[2] requires information and training on material safety data sheets (MSDSs), labeling, chemical inventory of all hazardous substances in the workplace, and informing contractors of hazardous substances.

Training dealing with health and safety techniques and work practices requires a concerted effort by management, and must be conducted on a routine basis by competent and qualified individuals to be effective. Records of training must be maintained.

5. References

1. Occupational Safety and Health Administration. Laboratory Standard. Occupational Exposure to Hazardous Chemicals in Laboratories. 29 CFR 1910.1450.
2. Occupational Safety and Health Administration. 1985. Hazard Communication. Final Rule. *Fed. Reg.* 48–53280. 29 CFR 1910.1200.

6. Bibliography

Dux, J.P. & R.F. Stalzer. 1988. Managing Safety in the Chemical Laboratory. Van Nostrand Reinhold Co., Inc., New York, N.Y.
Furr, A.K., ed. 1990. CRC Handbook of Laboratory Safety, 3rd ed. CRC Press, Inc., Boca Raton, Fla.

1090 B. Safe Laboratory Practices

Use the information, rules, work practices, and/or procedures discussed below for essentially all laboratory work with chemicals.

1. General Rules

a. Accidents and spills:

1) Eye contact—Promptly flush eyes with water for a prolonged period (minimum of 15 min) and seek immediate medical attention.

2) Ingestion—Encourage victim to drink large amounts of water.

3) Skin contact—Promptly flush affected area with water for approximately 15 min and remove any contaminated clothing. If symptoms persist after washing, seek medical attention.

4) Clean-up—Promptly clean up spills, using appropriate protective apparel and equipment and proper disposal procedures.

5) Working alone—Avoid working alone in a building; do not work alone in a laboratory if the procedures to be conducted are hazardous.

b. Vigilance: Be alert to unsafe conditions and see that they are corrected when detected.

2. Work Practices/Rules

a. Work habits: Develop and encourage safe habits, avoid unnecessary exposure to chemicals by any route, and avoid working alone whenever possible.

b. Exhaust ventilation: Do not smell or taste chemicals. Vent any apparatus that may discharge toxic chemicals (vacuum pumps, distillation columns, etc.) into local exhaust devices.

c. Glove boxes: Inspect gloves and test glove boxes before use.

d. Cold and/or warm rooms: Do not allow release of toxic substances in cold rooms and/or warm rooms, because these rooms usually have no provisions for exhausting contaminants.

e. Use/choice of chemicals: Use only those chemicals for which the quality of the available ventilation system is appropriate.

f. Eating, smoking, and related activities: DO NOT eat, drink, smoke, chew gum, or apply cosmetics in areas where laboratory chemicals are present. Always wash hands before conducting these activities.

g. Food storage: DO NOT store, handle, or consume food or beverages in storage areas, refrigerators, or glassware and utensils that also are used for laboratory operations.

h. Equipment and glassware: Handle and store laboratory glassware with care to avoid damage. Do not use damaged glassware. Use extra care with Dewar flasks and other evacuated glass apparatus; shield or wrap them to contain chemicals and fragments should implosion occur. Use equipment for its designed purpose only.

i. Washing: Wash areas of exposed skin well before leaving the laboratory.

j. Horseplay: Do no practical jokes or other behavior that might confuse, startle, or distract another worker.

k. Mouth suction: DO NOT use mouth suction for pipetting or starting a siphon.

l. Personal protective equipment: Do not wear personal protective clothing or equipment in nonlaboratory areas. Remove laboratory coats immediately on significant contamination with hazardous materials.

m. Personal apparel: Confine long hair and loose clothing. Wear shoes at all times in the laboratory but do not wear sandals, open-back, or open-toe shoes.

n. Personal housekeeping: Keep work area clean and uncluttered, with chemicals and equipment properly labeled and stored. Clean up work area on completion of an operation or at the end of each day.

o. Unattended operations: Leave lights on, place an appropriate sign on the door, and provide for containment of toxic substances in the event of failure of a utility service (such as cooling water) to an unattended operation.

3. Personal Protective Equipment

Carefully plan a program addressing the need for, use of, and training with personal protective equipment. Such a program includes seeking information and advice about hazards, developing appropriate protective procedures, and proper positioning of equipment before beginning any new operations.

a. Eye protection: Wear appropriate eye protection (this applies to all persons, including visitors) where chemicals are stored or handled. Avoid use of contact lenses in the laboratory unless necessary; if contact lenses are used, inform supervisor so special precautions can be taken.

b. Skin protection: Wear appropriate gloves when the potential for contact with toxic chemicals exists. Inspect gloves before each use, wash them before removal, and replace periodically. Do not pick up the telephone, touch the door knob, or other common places while wearing gloves.

c. Respiratory protection: Use appropriate respiratory equipment when engineering controls are unable to maintain air contaminant concentrations below the action levels [i.e., one half the permissible exposure limit (PEL)[1] or threshold limit value (TLV)[2] (levels below which no irreversible health affects are expected)]. When work practices are expected to cause routine exposures that exceed the PEL or TLV, respiratory protection is required to prevent overexposure to hazardous chemicals. If respirators are used or provided in the laboratory, then the LH&S standard of practice requires that a complete respiratory protection plan (RPP) be in place. The minimum requirements for an RPP meeting the LH&S standard of practice are published.[1] Periodically inspect respirators before use and check for proper fit.

d. Other protective equipment: Provide and use any other protective equipment and/or apparel as appropriate.

4. Engineering Controls

Fume hoods: Use the hood for operations that might result in the release of toxic chemical vapors or dust. As a rule of thumb, use a hood or other local ventilation device when working with any appreciably volatile substance with a TLV $<$50 ppm. Confirm that hood performance is adequate before use. Open hood minimally during work. Keep hood door closed at all other times except when adjustments within the hood are being made. Keep stored materials in hoods to a minimum, and do not block vents or air flow. Provide at least an 8-cm space around all items used in hoods, and ensure that they are at least 15 cm from the front of the hood.

5. Waste Disposal

Ensure that the plan for each laboratory operation includes plans and training for waste disposal. Deposit chemical waste in appropriately labeled receptacles and follow all other waste disposal procedures of the Chemical Hygiene Plan (see 1090J). Do not discharge any of the following contaminants to the sewer: concentrated acids or bases; highly toxic, malodorous, or lachrymatory substances; substances that might interfere with the biological activity of wastewater treatment plants; and substances that may create fire or explosion hazards, cause structural damage, or obstruct flow. For further information on waste disposal, see Section 1100.

6. Working with Chemicals of Moderate Chronic or High Acute Toxicity

Examples of chemicals in this category include diisopropylfluorophosphate, hydrofluoric acid, hydrogen sulfide, and hydrogen cyanide. The following rules are intended to supplement the rules listed previously for routine laboratory operations. Their purpose is to minimize exposure to these toxic substances by any exposure route using all reasonable precautions. The precautions are appropriate for substances with moderate chronic or high acute toxicity used in significant quantities.

a. Location: Use and store these substances only in areas of restricted access with special warning signs. Always use a hood (previously evaluated to confirm adequate performance with a face velocity of at least 24 m/min) or other containment device for procedures that may result in the generation of aerosols or vapors containing the substance; trap released vapors to prevent their discharge with the hood exhaust.

b. Personal protection: Always avoid skin contact by use of gloves and long sleeves, and other protective apparel as appropriate. Always wash hands and arms immediately after working with these materials.

c. Records: Maintain records of the amounts of these materials on hand, as well as the dates opened and discarded for each container of chemicals.

d. Prevention of spills and accidents: Be prepared for accidents and spills.

Ensure that at least two people are present at all times if a compound in use is highly toxic or of unknown toxicity.

Store breakable containers of these substances in chemically resistant trays; also work and mount apparatus above such trays or cover work and storage surfaces with removable, absorbent, plastic-backed paper. If a major spill occurs outside the hood, evacuate the area; ensure that cleanup personnel wear suitable protective apparel and equipment.

e. Waste: Thoroughly decontaminate or incinerate contaminated clothing or shoes. If possible, chemically decontaminate by chemical conversion. Store contaminated waste in closed, suitably labeled, impervious containers (for liquids, in glass or plastic bottles half-filled with vermiculite).

7. Working with Chemicals of High Chronic Toxicity

Examples of chemicals in this category include (where they are used in quantities above a few milligrams, or a few grams, depending on the substance) dimethyl mercury, nickel carbonyl, benzo(a)pyrene, *N*-nitrosodiethylamine, and other substances with high carcinogenic potency. The following rules are intended to supplement the rules listed previously for routine laboratory operations.

a. Access: Conduct all transfers and work with these substances in a controlled area (i.e., a restricted-access hood, glove box, or portion of a laboratory) designated for use of highly toxic substances, for which all people with access are aware of the substances being used and necessary precautions.

b. Approvals: Prepare a plan for use and disposal of these materials and obtain the approval of the laboratory supervisor.

c. Non-contamination/decontamination: Protect vacuum pumps against contamination by scrubbers or HEPA filters and vent them into the hood. Decontaminate vacuum pumps or other contaminated equipment, including glassware, in the hood before removing them from the controlled area. Decontaminate the controlled area before routine work is resumed.

d. Exiting: On leaving a controlled area, remove any protective apparel (place it in an appropriately labeled container), and thoroughly wash hands, forearms, face, and neck.

e. Housekeeping: Use a wet mop or a vacuum cleaner equipped with a HEPA filter. Do not dry sweep if the toxic substance is a dry powder.

f. Medical surveillance: If using toxicologically significant quantities of such a substance on a regular basis (e.g., three times per week), consult a qualified physician about desirability of regular medical surveillance.

g. Records: Keep accurate records of the amounts of these substances stored and used, the dates of use, and names of users.

h. Signs and labels: Ensure that the controlled area is conspicuously marked with warning and restricted access signs and that all containers of these substances are appropriately labeled with identity and warning labels.

i. Spills: Ensure that contingency plans, equipment, and materials to minimize exposures of people and property are available in case of accident.

j. Storage: Store containers of these chemicals only in a ventilated, limited-access area.

k. Glove boxes: For a negative-pressure glove box, ensure that the ventilation rate is at least 2 volume changes/h and the pressure drop is at least 1.3 cm of water. For a positive-pressure glove box, thoroughly check for leaks before each use. In either case, trap the exit gases or filter them through a HEPA filter and then release them into the hood.

l. Waste: Ensure that containers of contaminated waste (including washings from contaminated flasks) are transferred from the controlled area in a secondary container under the supervision of authorized personnel.

8. Physical Hazards

a. Electrical: Ensure that electrical wiring, connections, and apparatus conform to the requirement of the latest National Electrical Code. Fire, explosion, power outages, and electrical shocks are all serious hazards that may result from incorrect use of electrical devices. Ground all electrical equipment or use double-insulated equipment. Use ground fault interrupter circuit breakers to the maximum extent possible. Do not locate electrical receptacles inside fume hoods, and do not use equipment near volatile flammable solvents. Use approved safety refrigerators. Disconnect electrical equipment from the power supply before service or repair is attempted and never bypass safety interlocks. Attempting to repair equipment using employees not thoroughly acquainted with electrical principles may present particularly dangerous situations.

b. Non-ionizing radiation: Non-ionizing radiation, also called electromagnetic radiation, is generally considered to be the radio frequency region of the radiation spectrum. For the purposes of dealing with personal exposures in laboratories, it also includes the microwave frequency region. Typical laboratory exposures to non-ionizing radiation usually include ultraviolet, visible, infrared, and microwave radiation.

For normal environmental conditions and for incident electromagnetic energy of frequencies from 10 MHz to 100 GHz, the radiation protection guide is 10 mW/cm^2. The radiation protection applies whether the radiation is continuous or intermittent. This means a power density of 10 mW/cm^2 for periods of 0.1 h or more, or an energy density of 1 $mW\text{-}h/cm^2$ during any 0.1-h period. These recommendations apply to both whole-body irradiation and partial body irradiation.

Ultraviolet radiation (UV) and lasers are used frequently. With properly constructed and operated instruments, it is not a significant hazard but can be harmful when used for controlling microorganisms in laboratory rooms or for sterilizing objects.

When using devices that generate or use non-ionizing radiation, observe the following precautions: Wear safety glasses or goggles with solid side pieces whenever there is a possibility of exposure to harmful (UV) radiation. Provide proper shielding (shiny metal surfaces reflect this energy). Shut off all these devices (UV lamps) when not in use. Post warning signs and install indicator lights to serve as a constant reminder when these types of devices are in use (UV lamps).

c. Mechanical: Shield or guard drive belts, pulleys, chain drivers, rotating shafts, and other types of mechanical power-transmission apparatus. Laboratory equipment requiring this guarding includes vacuum pumps, mixers, blenders, and grinders.

Shield portable power tools. Guard equipment such as centrifuges, which have high-speed revolving parts, against "fly-aways." Securely fasten equipment that has a tendency to vibrate (e.g., centrifuges and air compressors) to prevent the tendency to "walk" and locate them away from bottles and other items that may fall from shelves or benches because of the vibration.

d. Compressed gases: Gas cylinders may explode or "rocket" if improperly handled. Leaking cylinders may present an explosion hazard if the contents are flammable; they are an obvious health hazard if the contents are toxic; and they may lead to death by suffocation if the contents are inert gases. The Compressed Gas Association has published procedures governing use and storage of compressed gases. Transfer gas cylinders only with carts, hand trucks, or dollies. Secure gas cylinders properly during storage, transport, and use, and leave valve safety covers on cylinders during storage and transport. Avoid the use of adapters or couplers with compressed gas. Properly identify cylinder contents.

9. Chemical Hazards

a. General precautions: Chemical injuries may be external or internal. External injuries may result from skin exposure to caustic or corrosive substances, such as acids, bases, or reactive salts. Take care to prevent accidents, such as splashes and container spills. Internal injuries may result from the toxic or corrosive effects of substances absorbed by the body. These internal injuries may result from inhalation, skin contact, or ingestion.

Tables 1090:I, II, and III list PELs, TLVs, and/or short-term exposure limits and ceilings for some chemical materials specified

Table 1090:I. Permissible Exposure Limits, Threshold Limit Values, Short-Term Exposure Limits, and/or Ceilings for Some Inorganic Chemicals Specified in *Standard Methods*

Compound	Chemical Abstract No. CAS No.	PEL/TLV STEL(S)* or Ceiling(C) mg/m^3
Chromic acid and chromates†‡ (as CrO_3)	7440-47-3	0.1/0.05
Chromium, soluble chromic, chromous salts (as Cr)	7440-47-3	0.5/0.5
Chromium metal and insoluble salts	7440-47-3	1/0.5
Hydrogen chloride	7647-01-0	7.5(C)/7.5(C)
Hydrogen peroxide	7722-84-1	1.4/1.4
Lead‡	7439-92-1	−/0.15
Mercury†§	7439-97-6	0.1/0.05
Nitric acid	7697-37-2	5/5.2, 10(S)
Phosphoric acid	7664-38-2	1/1, 3(S)
Potassium hydroxide	1310-58-3	−/2(C)
Silver (metal and soluble compounds, as Ag)	7440-22-4	0.01/0.1 metal, 0.01 soluble as Ag
Sodium azide	26628-22-8	−/0.29(C)
Sodium hydroxide	1310-73-2	2(C)/2(C)
Sulfuric acid	7664-93-9	1/1, 3(S)

* Short-term exposure limit. See 29 CFR 1910.1028.
† (Suspect) carcinogen.
‡ Substance has a Biological Exposure Index (BEI).
§ Skin hazard.

in *Standard Methods*, as given in various published sources.[1–8] The PEL values reported in these tables are in some instances higher than the levels that some nations believe to be appropriate. Because the health and safety program should be driven by meeting best industrial hygiene practice, always use the lowest recommended exposure values when protecting human health.

In addition, pay careful attention to equipment corrosion that ultimately may lead to safety hazards from equipment failure.

b. Inorganic acids and bases: Many inorganic acids and bases have PELs and TLVs. Table 1090:I presents PELs (based on U.S. standards) and/or TLVs, as well as short-term exposure limits and ceilings for some inorganic chemicals specified in *Standard Methods*. These PELs and TLVs indicate the maximum air concentration to which workers may be exposed. Fumes of these acids and bases are severe eye and respiratory system irritants. Liquid or solid acids and bases can quickly cause severe burns of the skin and eyes. When acids are heated to increase the rate of digestion of organic materials, they pose a significantly greater hazard because fumes are produced and the hot acid reacts very quickly with the skin.

Store acids and bases separately in well-ventilated areas and away from volatile organic and oxidizable materials. Use containers (rubber or plastic buckets) to transport acids and bases.

Work with strong acids and bases only in a properly functioning chemical fume hood. Slowly add acids and bases to water (with constant stirring) to avoid spattering. If skin contact is made, thoroughly flush the contaminated area with water and seek medical attention if irritation persists. Do not wear contaminated clothing until after it has been cleaned thoroughly. Leather items (e.g., belts and shoes) will retain acids even after rinsing with water and may cause severe burns if worn. If eye contact is made, immediately flush both eyes for at least 15 min with an eye wash and seek medical attention.

c. Perchloric acid and other highly reactive chemicals: Concentrated perchloric acid reacts violently or explosively on contact with organic material and may form explosive heavy metal perchlorates. Do not use laboratory fume hoods used with perchloric acid for organic reagents, particularly volatile solvents. In addition to these hazards, perchloric acid produces severe burns when contact is made with the skin, eyes, or respiratory tract. Preferably provide a dedicated perchloric acid hood. Follow the manufacturer's instructions for proper cleaning, because exhaust ducts become coated and must be washed down regularly.

Use extreme caution when storing and handling highly reactive chemicals, such as strong oxidizers. Improper storage can promote heat evolution and explosion. Do not store strong oxidizers and reducers in close proximity.

d. Organic solvents and reagents: Most solvents specified in *Standard Methods* have PELs and/or TLVs, as well as short-term exposure limits or ceilings for workplace exposures (see Table 1090:II).

Many organic reagents, unlike most organic solvents, do not have PELs/TLVs or short-term exposure limits and ceilings, but this does not mean that they are less hazardous. Table 1090:III contains PELs/TLVs or short-term exposure limits and ceilings for some reagents specified in *Standard Methods*.

Some compounds are suspect carcinogens and should be treated with extreme caution. These compounds include solvents and reagents, such as benzene, carbon tetrachloride, chloroform, dioxane, perchloroethylene, and benzidine. Lists of chemicals with special hazardous characteristics are available from the Occupational Safety and Health Administration and the National Institute for Occupational Safety and Health. In the United States, the lists of "Regulated Carcinogens" and of "Chemicals Having Substantial Evidence of Carcinogenicity" are especially important. Developing and following laboratory handling procedures for compounds on such authoritative lists should significantly reduce the potential for exposures.

Solvents used in the laboratory usually fall into several major categories: alcohols, chlorinated compounds, and hydrocarbons. Exposure to each of these classes of compounds can have a

TABLE 1090:II. PERMISSIBLE EXPOSURE LIMITS, THRESHOLD LIMIT VALUES, SHORT-TERM EXPOSURE LIMITS, AND/OR CEILINGS FOR ORGANIC SOLVENTS SPECIFIED IN *STANDARD METHODS*

Compound	Chemical Abstract No. CAS No.	PEL/TLV STEL(S)* or Ceiling(C) *ppm (v/v)*
Acetic acid	64-19-7	10/10, 15(S)
Acetone	67-64-1	1000/750, 1000(S)
Acetonitrile	75-05-8	40/40, 60(S)
Benzene†‡	71-43-2	10, 25(C), 50 peak 10 min/8 h/10
n-Butyl alcohol§	71-36-3	100/50(C)
tert-Butyl alcohol	75-65-0	100/100, 150(S)
Carbon disulfide‡	75-15-0	20, 30(C), 100 peak 30 min/8 h/10
Carbon tetrachloride†§	56-23-5	10, 25, 200 peak 5 min/4 h/5
Chloroform†	67-66-3	50(C)/10
Cyclohexanone§	108-94-1	50/50
Dioxane§ (diethylene dioxide)	123-91-1	100/25
Ethyl acetate	141-78-6	400/400
Ethyl alcohol	64-17-5	1000/1000
Ethyl ether (diethyl ether)	60-29-7	400/400, 500(S)
Ethylene glycol	107-21-1	−/50(C)
n-Hexane‡	110-54-3	100/50
Isoamyl alcohol (primary and secondary)	123-51-3	100/100, 125(S)
Isobutyl alcohol	78-83-1	100/50
Isopropyl alcohol	67-63-0	400/400, 500(S)
Isopropyl ether	108-20-3	500/250, 310(S)
Methyl alcohol§	67-56-1	200/200, 250(S)
2-Methoxyethanol§ (methyl cellosolve)	109-86-4	25/5
Methylene chloride†	75-09-2	500, 1000(C), 2000 peak 5 min/2 h/50
Pentane	109-66-0	1000/600, 750(S)
Perchloroethylene†‡ (tetrachloroethylene)	127-18-4	100, 200(C), 300 peak 5 min/3 h/50, 200(S)
n-Propyl alcohol§	71-23-8	200/200, 250(S)
Pyridine	110-86-1	5/5
Toluene‡§	108-88-3	200, 300(C), 500 peak 10 min/8 h/50
Xylenes‡ (*o*-, *m*-, *p*-isomers)	1330-20-7 (95-47-6, 108-38-3, 106-42-3)	100/100, 150(S)

* Short-term exposure limit. See 29 CFR 1910.1028.
† (Suspect) carcinogen.
‡ Substance has a Biological Exposure Index (BEI).
§ Skin hazard.

variety of health effects. Alcohols, in general, are intoxicants, capable of causing irritation of the mucous membranes and drowsiness. Chlorinated hydrocarbons cause narcosis and damage to the central nervous system and liver. Hydrocarbons, like the other two groups, are skin irritants and may cause dermatitis after prolonged skin exposure. Because of the volatility of these compounds, hazardous vapor concentrations can occur (fire or explosion hazard). Proper ventilation is essential.

The majority of organic reagents used in this manual fall into four major categories: acids, halogenated compounds, dyes and indicators, and pesticides. Most organic acids have irritant properties. They are predominantly solids from which aerosols may be produced. Dyes and indicators also present an aerosol problem. Handle pesticides with caution because they are poisons, and avoid contact with the skin. Wear gloves and protective clothing. The chlorinated compounds present much the same hazards as the chlorinated solvents (narcosis and damage to the central nervous system and liver). Proper labeling for the compound, including a date for disposal based on the manufacturer's recommendations, permits tracking chemical usage and disposal of outdated chemicals.

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1. OCCUPATIONAL SAFETY AND HEALTH ADMINISTRATION. Respiratory Protection. 29 CFR 1910.134.
2. AMERICAN CONFERENCE OF GOVERNMENTAL INDUSTRIAL HYGIENISTS. 1992. Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices. Cincinnati, Ohio.
3. U.S. PUBLIC HEALTH SERVICE. 1980. Annual Report on Carcinogens. National Toxicology Program, Dept. Health & Human Services, U.S. Government Printing Off., Washington, D.C.
4. INTERNATIONAL AGENCY FOR RESEARCH ON CANCER. (Various dates). IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans. World Health Org. Publications Center, Albany, N.Y.
5. NATIONAL INSTITUTE FOR OCCUPATIONAL SAFETY AND HEALTH. 1985. Occupational Health Guidelines for Chemical Hazards; NIOSH/OSHA-NIOSH Pub. No. 85-123. U.S. Government Printing Off., Washington, D.C.
6. U.S. PUBLIC HEALTH SERVICE, CENTERS FOR DISEASE CONTROL. 1993. Registry of Toxic Effects of Chemical Substances. U.S. Government Printing Off., Washington, D.C.
7. SAX, N.I. 1989. Dangerous Properties of Industrial Materials. Van Nostrand Reinhold, New York, N.Y.

TABLE 1090:III. PERMISSIBLE EXPOSURE LIMITS, THRESHOLD LIMIT VALUES, SHORT-TERM EXPOSURE LIMITS, AND/OR CEILINGS FOR SOME OF THE REAGENTS SPECIFIED IN *STANDARD METHODS*

Compound	Chemical Abstract No. CAS No.	PEL/TLV STEL(S)* or Ceiling(C) *ppm (v/v)*
2-Aminoethanol (ethanolamine)	141-43-5	3/3, 6(S)
Benzidine†‡	92-87-5	Confirmed human carcinogen[1]
Benzyl chloride	100-44-7	1/1
Chlorobenzene	108-90-7	75/10
Diethanolamine	111-42-2	−/3
Naphthalene	91-20-3	10/10, 15(S)
Oxalic acid	144-62-7	1/1 mg/m^3
Phenol‡	108-95-2	5/5
2-Chloro-6-(trichloromethyl) pyridine (nitrapyrin)	1929-82-4	
Total dust		15/10 mg/m^3
Respirable fraction		5/− mg/m^3

* Short-term exposure limit. See 29 CFR 1910.1028.
† (Suspect) carcinogen.
‡ Skin hazard.

8. U.S. PUBLIC HEALTH SERVICE, CENTERS FOR DISEASE CONTROL. 1990. NIOSH Pocket Guide to Chemical Hazards; DHHS (NIOSH) Pub. No. 90-117. U.S. Government Printing Off., Washington, D.C.

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1090 C. Laboratory Facility/Fixed Equipment

1. Facility Design

The laboratory facility must have a general ventilation system with air intakes and exhausts located to avoid intake of contaminated air, well-ventilated stockrooms and/or storerooms, laboratory hoods and sinks, miscellaneous safety equipment including eyewash fountains and safety showers, and arrangements for the disposal of wastes and samples in accordance with applicable federal, state, and local regulations.

2. Facility and Fixed Equipment Maintenance

Maintain facilities and equipment with scheduled maintenance and continual surveillance to ensure proper operation. Give special attention to the adequacy of ventilation system.

a. Facility ventilation systems:

1) General ventilation—The general laboratory ventilation should provide a source of air for breathing and for input to local ventilation devices, such as fume hoods. Do not rely on it for protection from exposure to toxic substances used during the working day. The system should direct air flow into the laboratory from nonlaboratory areas and then exhaust the air directly to the exterior of the building in a manner that will prevent its re-entry.

2) Laboratory fume hoods—As a minimum, provide at least 1 linear m of hood space per worker if workers spend most of their time working with chemicals or if they work with chemical substances with PELs or TLVs less than 100 ppm. Equip each hood with a continuous monitoring device to allow convenient confirmation of adequate hood performance before each use. If this is not possible, avoid work with substances with PELs or TLVs less than 100 ppm or with unknown toxicity, or provide other types of local ventilation devices.

3) Other local ventilation devices—Provide ventilated chemical/biological cabinets, canopy hoods and instrument/work station snorkels as needed. Many local ventilation devices require a separate exhaust duct, as do canopy hoods and snorkels.

4) Special ventilation areas/devices—It may be necessary to pass exhaust air from special ventilation areas or devices such as radiological hoods, glove boxes, and isolation rooms through HEPA filters, scrubbers, or other treatment before release into regular exhaust system. Ensure that cool rooms and warm rooms have provisions for rapid escape and for escape in the event of electrical failure.

5) Ventilation system modifications—Make alteration in the ventilation system only in consultation with an expert qualified in laboratory ventilation system design. Thoroughly test changes in the ventilation system to demonstrate adequate worker protection.

b. Facility ventilation system performance: A ventilation system rate of 4 to 12 room air changes per hour is considered adequate where local exhaust ventilation devices, such as fume hoods, are used as the primary method of control. General ventilation system air flow should not be turbulent and should be relatively uniform throughout the laboratory, with no high-velocity or static areas; air flow into and within laboratory fume hoods should not be excessively turbulent; fume hood face velocity should be adequate for the intended use (for general-purpose fume hoods this is typically 18 to 30 m/min). The effective protection provided by a fume hood depends on a number of factors including hood location and design, and cannot be determined solely on the basis of the face velocity.

c. Facility ventilation system evaluation: Evaluate performance characteristics (quality and quantity) of the ventilation system on installation, re-evaluate whenever a change in local ventilation devices is made, and monitor routinely. Schedule such monitoring with a frequency dictated by the type, age, condition, and any accessories associated with the device, but at least annually; monitor hoods at least quarterly. Document all ventilation system checks or actions, such as flow checks, calibration, alterations, repairs, maintenance, or any other action that may determine or change flow efficiency or characteristics.

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1090 D. Hazard Evaluation

1. Hazard Evaluation

Hazard evaluation refers to the assessment of whether an employee has been overexposed to a hazardous substance or if such an exposure episode is likely to occur and to what extent.

The evaluation does not require monitoring airborne concentrations of the hazardous substances involved. Such an assessment may be informal and simply involve considering, among other factors, the chemical and physical properties of the substance and the quantity of substance used. In addition, the exposure assessment may be sufficient to estimate the probability of an overexposure.

Specify, document, and use hazard assessment criteria. Base such criteria on the toxicity of the substances to be used, the exposure potential of the chemical procedures to be performed, and the capacity of the available engineering control systems.

In cases where continuous monitoring devices are used, include resulting exposure data in the exposure evaluation. Air monitoring *only* provides information for inhalation exposure. Other means are required to determine whether overexposure could have occurred as a result of ingestion, or dermal or eye contact.

2. Spills of Toxic or Hazardous Substances

Spills are usually the result of loss of containment due to equipment failure or breakage (uncontrolled releases). The loss of containment can result in overexposure episodes. Calculations using data from material safety data sheets, the chemical and physical properties of the substance, known laboratory air changes, and work-station air volume will allow assessment of the possibility of an overexposure episode.

3. Work Practice Assignment

Use the information calculated from these exposure assessments to develop the written work practices needed to protect the health of the employee while conducting the procedure.

4. Documentation of Hazard Assessments

Document, validate, and authenticate all hazard assessments, preferably using a standard form.

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Also see references 2 through 8, 1090B.10.

1090 E. Personal Protective Equipment

1. Introduction

The employer must provide and maintain personal protective equipment (PPE) in condition that is sanitary and reliable against hazards in the workplace. All PPE also must be properly designed and constructed for the work to be performed. Several general references on PPE are available.[1–3]

It is essential to select PPE based on an assessment of the hazards[3] or potential hazards to which an employee is exposed, to ensure that the correct PPE will be obtained. Use personal protective equipment only when it is not possible or feasible to provide engineering controls. Such personal protective equipment includes all clothing and other work accessories designed to create a barrier against workplace hazards.

The basic element of any personal protective equipment management program must be an in-depth evaluation of the equipment needed to protect against the hazards at the workplace. Management dedicated to the safety and health of employees must use that evaluation to set a standard operating procedure for personnel, then encourage those employees to use, maintain, and clean the equipment to protect themselves against those hazards.

Using personal protective equipment requires hazard awareness and training on the part of the user. Make employees aware that the equipment does not eliminate the hazard. If the equipment fails, exposure will occur. To reduce the possibility of failure, use equipment that is properly fitted and maintained in a clean and serviceable condition.

Selection of the proper piece of personal protective equipment for the job is important. Employers and employees must understand the equipment's purpose and its limitations. Do not alter or remove equipment even though an employee may find it uncomfortable (equipment may be uncomfortable simply because it does not fit properly).

2. Eye Protection

The LH&S standard of practice requires the use of eye and face protective equipment[3] where there is a reasonable probability of injury prevention through its use. Employers must provide a type of protector suitable for work to be performed, and employees must use the protectors. These requirements also apply to supervisors and management personnel, and to visitors while they are in hazardous areas.

Protectors must provide adequate protection against particular hazards for which they are designed, be reasonably comfortable when worn under the designated conditions, fit snugly without interfering with the movements or vision of the wearer, and be durable, easy to disinfect and clean, and kept in good repair.

In selecting the protector, consider the kind and degree of hazard. Where a choice of protectors is given, and the degree of protection required is not an important issue, worker comfort may be a deciding factor.

Persons using corrective glasses and those who are required to wear eye protection must wear glasses with protective lenses providing optical correction, goggles that can be worn over corrective glasses without disturbing the adjustment of the glasses, or goggles that incorporate corrective lenses mounted behind the protective lenses.

When limitations or precautions are indicated by the manufacturer, transmit them to the user and observe strictly. Safety glasses require special frames. Combinations of normal wire frames with safety lenses are not acceptable.

TABLE 1090:IV. GLOVE SELECTION FOR ORGANIC CHEMICAL HANDLING

Compound	Chemical Abstract No. CAS No.	Chemical Class	Suitable Glove Material						
			Butyl Rubber	Neoprene	Nitrile Rubber	PE	PVC	TFE	Viton
Ethers:		241							
Ethyl ether (diethyl ether)	60-29-7	241						X	
Isopropyl ether	108-20-3	241						X	
2-Methoxyethanol† (methyl cellosolve)	109-86-4	245	X						
Halogen compounds:		261							
Carbon tetrachloride*	56-23-5	261						X	X
Chloroform*	67-66-3	261						X	X
Methylene chloride	75-09-2	261	X					X	X
Perchloroethylene* (tetrachloroethylene)	127-18-4	261							X
Hydrocarbons:		291							
n-Hexane	110-54-3	291			X			X	X
Pentane	109-66-0	291			X				X
Benzene*	71-43-2	292							X
Toluene	108-88-3	292						X	X
Xylenes (*o*-, *m*-, *p*-isomers)	1330-20-7	292						X	X
Hydroxyl compounds:		311							
n-Butyl alcohol	71-36-3	311	X	X	X	X		X	
Ethyl alcohol	64-17-5	311	X		X			X	
Methyl alcohol	67-56-1	311	X					X	
n-Propyl alcohol	71-23-8	311			X			X	
Isoamyl alcohol	123-51-3	311							
Isobutyl alcohol	78-83-1	311	X						
Isopropyl alcohol	67-63-0	312		X	X			X	
tert-Butyl alcohol (2,2-methylpropanol)	75-65-0	313	X						
Ethylene glycol	107-21-1	314	X	X	X	X	X	X	X
Ketones:		391							
Acetone	67-64-1	391	X						X
Cyclohexanone	108-94-1	391	X						
Heterocyclic compounds:		271							
Dioxane† (diethylene dioxide)	123-91-1	278	X					X	
Pyridine	110-86-1	271	X						
Miscellaneous organic compounds:									
Acetic acid	75-07-0	102	X	X		X		X	
Ethyl acetate	141-78-6	222	X					X	
Acetonitrile	75-05-8	431	X					X	
Carbon disulfide	75-15-0	502							X

* (Suspect) carcinogen.
† Skin hazard.

Design, construction, testing, and use of eye and face protection must be in accordance with national standards.[4]

3. Protective Work Gloves

Match glove material to the hazard: such materials as nitrile, neoprene, natural rubber, PVC, latex, and butyl rubber vary widely in chemical resistance. What may be safe with one chemical may prove harmful with another (see Tables 1090:IV and 1090:V). Glove thickness may be as important as glove material in some cases. Many organic reagents, unlike most organic solvents, do not have PELs/TLVs but this does not mean that they are less hazardous.

Evaluate physical properties of the glove material: In addition to chemical resistance, glove materials vary in physical toughness. Select the glove that provides the abrasion, tear, flame, and puncture resistance required for the job.

Maximize comfort and dexterity. Lined gloves absorb perspiration and help insulate the hand. Unlined gloves conform to the hand. Lighter-gauge gloves improve touch sensitivity and flexibility, heavier-gauge gloves add protection and strength.

Ensure a safe grip. Nonslip grips allow for easier and safer handling. Embossed, pebbled, etched, and dotted coatings improve grip in wet or dry working conditions.

TABLE 1090:V. GLOVE SELECTION FOR INORGANIC CHEMICAL HANDLING

Compound	Chemical Abstract No. (CAS No.)	Chemical Class	Suitable Glove Material					
			Butyl Rubber	Natural Rubber	Neoprene	Nitrile Rubber	PE	PVC
Inorganic acids:		370						
Chromic acid,* (Cr^{+6})	7440-47-3	370	X					X
Hydrochloric acid, 30–70% solutions	10035-10-6	370	X	X	X	X		X
Hydrochloric acid, <30% solutions	10035-10-6	370	X	X	X	X		X
Nitric acid, 30–70% solutions	7697-37-2	370	X		X			
Nitric acid, <30% solutions	7697-37-2	370	X	X	X	X		X
Phosphoric acid, >70% solutions	7664-38-2	370		X	X	X	X	X
Phosphoric acid, 30–70% solutions	7664-38-2	370		X	X	X		X
Sulfuric acid, >70% solutions	7664-93-9	370	X				X	
Sulfuric acid, 30–70% solutions	7664-93-9	370	X	X	X		X	X
Sulfuric acid, <30% solutions	7664-93-9	370	X	X	X		X	X
Inorganic bases:		380						
Ammonium hydroxide, 30–70% solutions	7664-41-7	380	X		X	X		
Ammonium hydroxide, <30% solutions	7664-41-7	380	X		X	X		X
Potassium hydroxide, 30–70% solutions	1310-58-3	380	X	X	X	X		X
Sodium hydroxide, >70% solutions	1310-73-2	380			X			X
Sodium hydroxide, 30–70% solutions	1310-73-2	380	X	X	X	X	X	X
Inorganic salt solutions:		340						
Dichromate solutions, <30%,* (Cr^{+6})	7440-47-3	340					X	
Inorganic miscellaneous:								
Hydrogen peroxide, 30–70% solutions	7722-39-3	300		X		X		X
Mercury†	7439-97-6	560			X			X

* (Suspect) carcinogen.
† Skin hazard.

Measure proper size and length. Loose-fitting gloves affect dexterity and can be hazardous. Tight-fitting gloves may cause hand fatigue and tend to wear out faster. Gloves should fit comfortably without restricting motion and they should be long enough to protect the wrist, forearm, elbow, or the entire arm, depending on the application.

4. Head Protection

Water and wastewater laboratories seldom require this kind of personal protection, but field work may require such protection.

Head injuries are caused by falling or flying objects or by bumping the head against a fixed object. Head protection, in the form of protective hats, must both resist penetration and absorb the shock of a blow. Make the shell of the hat of a material hard enough to resist the blow, and utilize a shock-absorbing lining composed of head band and crown straps to keep the shell away from the wearer's skull. Protective materials used in helmets should be water-resistant and slow burning. Helmets consist essentially of a shell and suspension. Ventilation is provided by a space between the headband and the shell. Ensure that each helmet is accompanied by instructions explaining the proper method of adjusting and replacing the suspension and headband.

Visually inspect daily all components, shells, suspensions, headbands, sweatbands, and any accessories for signs of dents, cracks, penetration or any other damage that might reduce the degree of safety originally provided.

Do not store or carry helmets on the rear window deck of an automobile because sunlight and extreme heat may adversely affect the degree of protection.

Further information is available elsewhere.[3,5]

5. Hearing Protection

Exposure to high noise levels can cause hearing loss or impairment, and it can create physical and psychological stress. There is no cure for noise-induced hearing loss, so prevention of excessive noise exposure is the only way to avoid hearing damage. Specifically designed protection is required, depending on the type of noise encountered.

Use preformed or molded ear plugs fitted individually by a professional. Waxed cotton, foam, or fiberglass wool earplugs are self-forming. When properly inserted, they work as well as most molded earplugs. Plain cotton is ineffective as protection against hazardous noise.

Some earplugs are disposable, to be used one time and then thrown away. Clean nondisposable types after each use for proper protection.

Earmuffs need to make a perfect seal around the ear to be effective. Glasses, long sideburns, long hair, and facial movements, such as chewing, can reduce protection. Special equipment is available for use with glasses or beards.

More specific information on hearing conservation is available.[3]

6. Foot and Leg Protection

According to accident reviews most workers who suffered impact injuries to the feet were not wearing protective footwear. Furthermore, most of their employers did not require them to wear safety shoes. The typical foot injury was caused by objects falling less than 1.2 m and the median weight was about 30 kg. Most workers were injured while performing their normal job activities at their worksites.

Safety shoes should be sturdy and have an impact-resistant toe. In some shoes, metal insoles protect against puncture wounds. Additional protection, such as metatarsal guards, may be found in some types of footwear. Safety shoes come in a variety of styles and materials, such as leather and rubber boots and oxfords.

Safety footwear is classified according to its ability to meet minimum requirements for both compression and impact test. Those requirements and testing procedures and further information may be found elsewhere.[3,6]

7. References

1. American Conference of Governmental Industrial Hygienists. 1987. Guidelines for the Selection of Chemical Protective Clothing, 3rd ed. Cincinnati, Ohio.
2. Forsberg, K. & S.Z. Mansdorf. 1993. Quick Selection Guide To Chemical Protective Clothing, 2nd ed. Van Nostrand Reinhold, New York, N.Y.
3. Occupational Safety And Health Administration. General Requirements for Personal Protective Equipment. 29 CFR 1910.132.
4. American National Standards Institute. 1968. Design, Construction, Testing, and Use of Eye and Face Protection; ANSI Z87 1-1968. New York, N.Y.
5. American National Standards Institute. 1986. Safety Requirements for Industrial Head Protection; ANSI Z89.1-1986. New York, N.Y.
6. American National Standards Institute. 1967 & 1983. Men's Safety-Toe Footware; ANSI Z41 1-1967 & Z41-1983. New York, N.Y.

1090 F. Worker Protection Medical Program

1. Preventive Medicine Program

The preventive medicine program should include inoculations to provide protection from tetanus and other diseases that are associated with the types of samples received and analyzed by the laboratory. The scope of this program depends on the diseases prevalent in the area where the samples originate. The program also must comply with the appropriate regulations.

2. Medical Surveillance

Routine surveillance may be indicated for anyone whose work involves routine handling of hazardous chemical or biological substances. Consult a qualified occupational health physician and/or toxicologist to determine whether a regular schedule of medical surveillance is indicated.

3. Environmental Monitoring

a. General: The initiation of environmental monitoring (exposure monitoring) associated with laboratory uses of hazardous chemical substances is triggered by exposures exceeding the action level (usually defined as one-half the PEL or TLV), PEL, or TLV. The employer is responsible for ensuring that employees' exposures to such substances do not exceed the PELs specified in the regulations dealing with air contaminants.[1]

b. Employee exposure determination: Determine a worker's exposure to any hazardous chemical substance if there is reason to believe that exposure levels for that substance routinely exceed the action level. Where there is no action level for a substance the worker exposure must not exceed the PEL or TLV. If the initial monitoring confirms that an employee exposure exceeds the action level, or in the absence of an action level, the PEL, the employer must immediately comply with the exposure monitoring provisions of the relevant national standard. Monitoring may be terminated in accordance with the relevant standard (if one exists) or when the exposures are found to be below the action level (one half the PEL or TLV) or in the absence of an action level, below the PEL or TLV. The workers are to be notified in accordance with national standard; if none exists, they should at least be notified within 15 working days after any monitoring results have become available to the employer, either by contacting the employee individually or by posting the results in an appropriate location accessible to employees.

4. Medical Consultation and Medical Examinations

All employees who work with hazardous chemicals should have an opportunity to receive medical attention (at no personal cost), including any follow-up examinations that the examining physician determines to be necessary, under the following circumstances:

- Whenever an employee develops signs or symptoms associated with an exposure to a hazardous chemical that the employee may have been using.
- Where exposure monitoring reveals an exposure level routinely above the action level (or in the absence of an action level, the PEL or TLV). For a national regulated substance for which there are exposure monitoring and medical surveillance requirements, establish medical surveillance for the affected employee as prescribed by the particular standard.
- Whenever an uncontrolled event, such as a spill, leak, explosion, or other occurrence, takes place in the work area, resulting in the likelihood of a hazardous exposure. Provide the affected employee an opportunity for a medical consultation to determine the need for a medical examination.

All medical examinations and consultations should be performed by, or under the direct supervision of, a licensed physician, without cost to the employee or loss of pay, and at a reasonable time and place. Inform the physician of the identity of the hazardous chemical(s) to which the employee

may have been exposed; the conditions under which the exposure occurred, including quantitative exposure data, if available; and the signs and symptoms of exposure that the employee is experiencing, if any. The employer must obtain from the examining physician a written opinion that includes any recommendation for further medical follow-up, the results of the medical examination and any associated tests, notice of any medical condition revealed during the examination that may place the employee at increased risk as a result of exposure to a hazardous chemical found in the workplace, and a statement that the employee has been informed by the physician of the results of the consultation or medical examination and any medical condition that may require further examination or treatment. The written opinion must not reveal specific findings or diagnoses unrelated to occupational exposure.

5. Reference

1. Occupational Safety and Health Administration. Air Contaminants. 29 CFR 1910.1000.

6. Bibliography

Doull, J., C.D. Klaassen & M.O. Amdur. 1980. Casarett and Doull's Toxicology: The Basic Science of Poisons, 2nd ed. Macmillan Publishing Co., Inc., New York, N.Y.

Williams, P.L. & J.L. Burson. 1985. Industrial Toxicology: Safety and Health Applications in the Workplace. Van Nostrand Reinhold Co., New York, N.Y.

Occupational Safety and Health Administration. Access to Employee Exposure and Medical Records. 29 CFR 1910.20.

Occupational Safety and Health Administration. Occupational Exposure to Hazardous Chemicals in Laboratories. 29 CFR 1910.1450.

1090 G. Provisions for Work with Particularly Hazardous Substances

The information outlined in the following paragraphs meets the LH&S standard of practice[1] and also represents good industrial hygiene practices.

1. Designated Area

Wherever appropriate, the employer must establish a "designated area," that is, an area that may be used for work with select carcinogens, reproductive toxins, or substances having a high degree of acute toxicity. A designated area may be the entire laboratory, an area of a laboratory, or a device such as a laboratory hood.

2. Select Carcinogen

In the United States, a "select carcinogen" means any substance meeting at least one of the following criteria: the substance is regulated by OSHA as a carcinogen; it is listed under the category, "known to be carcinogenic," by the U.S. National Toxicology Program (NTP);[2] it is listed under Group 1 (carcinogenic to humans) by the International Agency for Research on Cancer (IARC);[3] or it is listed in either Group 2A or 2B by IARC[3] or under the category, "reasonably anticipated to be carcinogenic" by NTP,[2] and causes statistically significant tumor incidence in experimental animals after inhalation exposure of 6 to 7 h/d, 5 d/week, for significant portion of a lifetime to dosages of less than 10 mg/m^3, or after repeated skin application of less than 300 mg/kg of body weight/week, or after oral dosages of less than 50 mg/kg of body weight/d.

3. Use of Containment Devices

The work conducted and its scale must be appropriate to the physical facilities available and, especially, to the quality of ventilation.

The general laboratory ventilation system must be capable of providing air for breathing and for input to local ventilation devices. It should not be relied on for protection from toxic substances released into the laboratory, but should ensure that laboratory air is continually replaced, preventing increase of air concentrations of toxic substances during the working day, and that air flows into the laboratory from nonlaboratory areas and out to the exterior of the building.

4. References

1. Occupational Safety and Health Administration. Laboratory Standard. Occupational Exposure to Hazardous Chemicals in Laboratories. 29 CFR 1910.1450.
2. U.S. Public Health Service, National Toxicology Program. 1980. Annual Report on Carcinogens. Dept. Health & Human Services, U.S. Government Printing Off., Washington, D.C.
3. International Agency for Research on Cancer. (Various dates). IARC monographs on risk of chemicals to humans. World Health Org. Publications Center, Albany, N.Y.

5. Bibliography

National Institute's of Health. 1981. Guidelines for the Laboratory Use of Chemical Carcinogens; NIH Pub. No. 81-2385. U.S. Government Printing Off., Washington, D.C.

Also see 1090C.3.

1090 H. Biological Safety

1. Introduction

The analysis of environmental samples involves worker contact with samples that may be contaminated with agents that present microbiological hazards. The majority of these agents involve exposures to pathogenic bacteria, fungi, or viruses that may produce human disease by accidental ingestion, inoculation, injection, or other means of cutaneous penetration. The primary means of exposure to these microbiological hazards involves hand-mouth contact while handling the samples, contaminated laboratory materials and/or aerosols created by incubating, pipetting, centrifuging, or blending of samples or cultures. Use the following rules, work practices and/or procedures to control or minimize exposure to these agents.

2. General Rules

Do not mix dilutions by blowing air through a pipet into a microbiological culture.

When working with grossly polluted samples, such as wastewater or high-density microbial cultures, use a pipetting device attached to a pipetting bulb to prevent accidental ingestion (never pipet by mouth).

Because untreated waters may contain waterborne pathogens, place all used pipets in a jar containing disinfectant solution for decontamination before glassware washing. Do not place used pipets on table tops, on laboratory carts, or in sinks without adequate decontamination.

3. Work Practices

Good personal hygiene practices are essential to control contact exposures. Frequently disinfect hands and working surfaces. Encourage immunization of laboratory staff against tetanus and possibly typhoid and other infectious agents to minimize risk of exposure.

Provide drinking water outside the laboratory, preferably from a foot-operated drinking fountain.

Eliminate flies and other insects to prevent contamination of sterile equipment, media, samples, and bacterial cultures and to prevent spread of infectious organisms to personnel.

Observe appropriate precautions in use of laboratory equipment. Use a leakproof blender tightly covered during operation to minimize contamination. Use a centrifuge tightly covered to minimize exposure if culture-containing tubes should shatter during centrifuging. The tube breakage produces a cleanup problem and microbiological aerosols. Conduct activities, such as inserting a hot loop into a flask of broth culture, in a manner that eliminates or minimizes the hazards due to aerosolized microorganisms. Sterilize contaminated materials (cultures, samples, used glassware, serological discards, etc.) by autoclaving before discarding them or processing for reuse. Preferably use specially marked biohazard bags for disposal. Dispose of contaminated broken glass in a specially marked container.

4. Procedures

Quaternary ammonium compounds that include a compatible detergent, or solutions of sodium hypochlorite are satisfactory disinfectants for pipet discard jars. Use the highest concentrations recommended for these commercial products provided that this concentration does not cause a loss of markings or fogging of pipets.

Sterilize biological waste materials to eliminate all infectious substances, and sterilize all contaminated equipment or apparatus before washing, storage, or disposal, preferably by autoclaving. When decontaminating materials in the autoclave, heat them to at least 121°C under a pressure of 103 kPa for a minimum of 30 min. The contact time is measured from time the contact chamber reaches 121°C. If the waste is contained in bags, add water to the contents to ensure wet heat. Dry heat and chemical treatment also may be used for sterilizing nonplastic items. After sterilization, the wastes can be handled safely and disposed of by conventional disposal systems in accordance with local regulations.

NOTE: It may not be necessary to sterilize domestic waste from leftover wastewater samples, raw sewage, sludges, and inoculated bacteriological media originating from a wastewater treatment process. It could be disposed of at the treatment plant headworks so long as no regulations are violated.

5. Waste Disposal

Sterilize contaminated materials by autoclaving (see 1090H.3) before discarding them.

If combustible materials cannot be decontaminated, burn them with special precautions; permits for burning may be required. Use temporary storage for decay or permanent storage for treating radioactive wastes when alternatives are not available. Collect contaminated combustible wastes and animal carcasses in impermeable containers for disposal by incineration.

6. Bibliography

NATIONAL INSTITUTES OF HEALTH. 1981. Guidelines for the Laboratory Use of Chemical Carcinogens; NIH Pub. No. 81-2385. U.S. Government Printing Off., Washington, D.C.

INSTITUTE OF ENVIRONMENTAL SCIENCES. 1986. Recommended Practice for Laminar Flow Clean Devices; RP-CC-002-86. Mount Prospect, Ill.

NATIONAL SANITATION FOUNDATION. 1987. Class II Biohazard Cabinetry (Laminar Flow); Standard 49-1987. Ann Arbor, Mich.

AMERICAN SOCIETY FOR TESTING & MATERIALS. 1988. Standard Guide for Good Laboratory Practices in Laboratories Engaged in Sampling and Analysis of Water; ASTM D3856. Philadelphia, Pa.

FURR, A.K., ed. 1990. CRC Handbook of Laboratory Safety, 3rd ed. CRC Press, Inc., Boca Raton, Fla.

1090 I. Radiological Safety

1. Introduction

This section discusses ionizing radiation safety related to gas chromatography detectors and specific analytical procedures (see Table 1090:VI). Ionizing radiation includes alpha particles, beta particles, gamma rays, and X-rays. Non-ionizing radiation safety is discussed elsewhere (1090B.8*b*).

All persons are exposed to ionizing radiation. The average annual radiation dose to the whole body from cosmic, terrestrial, and internal sources, medical and dental X-rays, etc., is about 185 mrems/year (1.85 mSieverts/year). It is essential to prevent unnecessary continuous or intermittent occupational exposures, and to take steps to eliminate accidents that may result in dangerous radiation exposure.

Personnel who work in laboratories may be exposed to ionizing radiation sources using both procedures and instruments. Evaluate potential exposures and control the associated equipment and procedures using work practices developed to minimize and/or eliminate exposures.

Users of radioactive materials are responsible for compliance with the requirements of their national nuclear regulatory body (in the United States, the Nuclear Regulatory Commission)[1] and/or related state regulations. In addition, administrative or local requirements may apply at specific facilities. The use of "exempt" quantities is regulated[1] even though the facility may be exempt from specific licensing requirements.

Radionuclides are used in laboratories to develop and evaluate analytical methods, to prepare counting standards, and to calibrate detectors and counting instruments (see Part 7000). Sealed sources, such as the nickel-63 detector cell used in electron capture gas chromatograph units, also are common.

2. Exposures

a. Exposure limits and control: The LH&S standard of practice[2] does not permit the use of personal protective equipment for allowing employee exposures above the limits specified by the NRC.[1] The NRC exposure limits are the maximum permissible exposures for 40 h in any workweek of 7 consecutive days.

The exposure limits may be adjusted proportionately (upward) for a period where the exposure is less than 40 h. However, the limit must be adjusted proportionately (downward) for periods where the exposure period is greater than 40 h.

Limiting exposure to ionizing radiation includes providing engineering (physical safeguards) and administrative (procedural) controls for using radioactive materials. Engineering controls include shields, barriers, and interlocks to limit external exposure, and exhaust ventilation systems and personal protective equipment to limit internal contamination. Administrative controls include conducting periodic surveys and reviews of activities, training in the use of radioactive materials, and documented procedures.

Hazards associated with the use of devices, such as X-ray diffraction apparatus or an electron microscope, can be minimized or eliminated by following the manufacturer's operating instructions and the laboratory safety procedures.

b. Monitoring procedures and equipment: Radiochemical exposure monitoring may be done by collecting and analyzing wipe samples, using portable survey instruments, and/or by collecting and analyzing air samples. More than one technique usually is required.

Survey equipment may either integrate the response over time (e.g., exposure, absorbed dose), or results may be presented as a response rate (e.g., count rate or exposure rate). Typical choices include ion chambers, G-M counters, and scintillation detectors.

Thin-windowed GM-counters are suitable for wipe samples and for monitoring skin and clothing. An alpha scintillation monitor is needed to detect alpha-emitters. An excellent discussion of monitoring techniques for radioisotopes is available.[3]

c. Facility surveys: Conduct periodic surveys to assess the effectiveness of physical and procedural controls. Survey procedures generally use wipe tests for removable contamination

TABLE 1090:VI. PROCEDURES INVOLVING POTENTIAL EXPOSURE TO IONIZING RADIATION

Standard Methods Section	Radionuclide	Type of Radiation	Comment
Part 6000 methods: GC with electron capture detectors	^{63}Ni or ^{3}H	Low-energy beta (^{63}Ni, 17 keV avg ^{3}H, 6 keV avg)	Internal hazard only. Requires survey techniques for low-energy beta radiation.
7110 Gross Alpha and Gross Beta Radioactivity	Alpha: Uranium, ^{230}Th, ^{239}Pu, ^{241}Am	Alpha; beta and gamma; or beta only	Alpha radiation sources primarily an internal hazard. Beta and beta/gamma sources potential external hazards.
	Beta: ^{137}Cs, ^{90}Sr		
7500-Cs Radioactive Cesium	^{134}Cs, ^{137}Cs	Beta, gamma	
7500-I Radioactive Iodine	^{131}I	Beta, gamma	8-d half-life
7500-Ra Radium	^{226}Ra	Alpha, beta, gamma	^{226}Ra and ^{222}Rn daughters must be considered.
7500-Sr Total Radioactive Strontium and Strontium-90	^{89}Sr, ^{90}Sr	Beta-emitters	
7500-^{3}H Tritium	^{3}H	Low-energy beta (6 keV avg)	Internal hazard only
7500-U Uranium	U series	Alpha, beta, gamma	

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and/or portable measurement devices for locating or measuring fixed and removable radioactivity.

1) Sealed sources—Check these sources for integrity by wipe tests at least every 6 months. Electron capture detectors using ^{63}Ni or ^{3}H require counting wipes by liquid scintillation or windowless gas-flow proportional counters to measure low-energy beta radiation effectively.

2) Work and storage areas—Survey these areas periodically to assess possible contamination or external radiation fields using portable survey instruments. The frequency of the surveys is dictated by the documented contamination record for the laboratory. Usually the radioactivities presented using the methods of Part 7000 are not measurable with routine survey instruments. Therefore use blanks in the analytical process in determining the presence of low-level contamination.

3) Documentation and records—Completely document each survey, identifying the personnel involved, the location, the type, model, and serial numbers of survey instruments used, the type and energy of radiations measured, the date and time of the survey, the instrument response to a check source, the instrument background count or exposure rate, and the results of each measurement.[4]

d. Personnel surveys and monitoring: Conduct and document surveys after routine use of unsealed radionuclide sources to confirm that personnel and the work area have not been contaminated by the process. Wear monitoring devices if there is a reasonable probability of exceeding 25% of the occupational exposure dose equivalent limit. Personal monitoring devices include film badges, thermoluminescent dosimeters, and solid state electronic dosimeters. The length of time the personal monitoring badges are worn before evaluation depends on the ability of the device to integrate the exposure over long periods, the probability and magnitude of the exposure, and the need to assure that the device is available and used.

Personnel performing procedures in *Standard Methods* would not be expected to receive exposures approaching 1.25 rem/quarter and may wish to consider 3-month wear periods if dosimetry is required.

Personal (external radiation) exposure is evaluated by using a personal dosimeter, preferably the film dosimeter (badge). The dosimeter badge measures the accumulated radiation over a period of time. Pocket ionization chambers, thermoluminescent dosimeters, and thimble chambers also may be used to supplement the film dosimeter.

Whole body or gamma spectrometry radiation detectors may be used to determine the presence of radioactive substances in the body, but these instruments are expensive and require the operator to be specially trained. Evaluate equipment and supplies that have been, or are suspected to have been, in contact with radioactive substances to determine if contamination is present. Because body waste may contain radioactive contaminants evaluate it also for the presence of contamination where personal exposures have been confirmed.

3. Work Practices

Each individual should be familiar with procedures for dealing with radiation emergencies from small spills to major accidents, depending on facility programs. Emergency procedures should include notifications required, containment methods, clean-up procedures, and survey techniques. Emergency supplies should be readily available for coping with major accidents.

Contamination is typically prevented through proper use of laboratory facilities and procedures. Procedures include the use of gloves, aprons, safety glasses, and other protective clothing to eliminate the possibility of skin contamination and transfer. Learn proper pipetting and weighing techniques before working with radioactive sources. Conduct work with unsealed radioactive sources in unobstructed work areas with adequate means of containing and absorbing potential spillage of liquids.

4. Procedures

Develop and implement a radiation safety plan and provide a copy to all persons working with radioactive materials or radiation-producing machines, and provide both lecture and practical training to all employees.

a. Safety plan elements: The recommended minimum plan should include procedures for obtaining authorization to use, order, handle, and store radionuclides; safe handling of unsealed radioactive material; safe response to radiation accidents; decontamination of personnel and facilities; personnel monitoring; laboratory monitoring; and disposal of radioactive materials.

b. Handling radioactive materials: Become knowledgeable about the hazards associated with the materials to be used. Plan work activities to minimize the time spent handling radioactive materials or in using radioactive sources. Work as far from radioactive sources as possible, use shielding appropriate for the materials to be used, and use radioactive materials only in defined work areas. Wear protective clothing and dosimeters as appropriate. Monitor work areas to ensure maximum contamination control. Minimize the accumulation of waste materials in the work area. Use appropriate personal hygiene and self-monitor after using radioactive materials and after each decontamination procedure.

c. Training of users: Train personnel working with radioactive materials in radiation safety as part of the overall occupational health program. Address at least the following topics: characteristics of ionizing radiation and radioactive contamination; radiation dose limits; environmental radiation background; acute and chronic effects; internal and external modes of exposure; basic protective measures; responsibilities of employer and employees; radiation protection program responsibilities; posting, warning signs, and alarms; radiation monitoring programs; and emergency procedures.

5. Waste Disposal

Generalized disposal criteria for radioactive wastes have been developed by the U.S. National Committee on Radiation Protection and Measurements.[4] Two general philosophies govern the disposal of radioactive wastes: dilution and dispersion to reduce the concentration of radionuclide by carrier dilution or dilution in a receiving medium, and concentration and confinement, usually involving reduction in waste volume with subsequent storage for decay purposes.

Airborne wastes can be treated by either method. Ventilation includes discharge from hooded operations to the atmosphere. Typical radioactive gases include iodine, krypton, and xenon. Iodine can be removed by scrubbing or by reaction with silver

nitrate. Noble gases can be removed by absorption; standard techniques can be used for particulate. Dilution methods are suitable for liquids with low activity. Intermediate levels may be treated by various physical-chemical processes to separate the waste into a nonradioactive portion that can be disposed of by dilution and a high-activity portion to be stored. Solid wastes may consist of equipment, glassware, and other materials. When possible, decontaminate these materials and reuse. Decontamination usually results in a liquid waste.

Dispose of all waste in conformance with the requirements of the regulatory authority having jurisdiction.

Determine the laboratory's status and obtain approval before storing, treating and/or disposing of wastes.

6. References

1. Nuclear Regulatory Commission. Standards for Protection Against Radiation. 10 CFR Part 20.
2. Occupational Safety and Health Administration. Ionizing Radiation. 29 CFR 1910.96.
3. Furr, A.K., ed. 1990. CRC Handbook of Laboratory Safety, 3rd ed. CRC Press, Inc., Boca Raton, Fla.
4. National Council on Radiation Protection and Measurements. 1976. Environmental Radiation Measurements; Rep. No. 50. Washington, D.C.

7. Bibliography

National Council on Radiation Protection and Measurements. 1978. Instrumentation and Monitoring Methods for Radiation Protection; Rep. No. 57. Washington, D.C.

National Council on Radiation Protection and Measurements. 1978. A Handbook of Radioactivity Measurements Procedures; Rep. No. 58. Washington, D.C.

National Council on Radiation Protection and Measurements. 1978. Operational Radiation Safety Program; Rep. No. 59. Washington, D.C.

1090 J. Chemical Hygiene Plan

1. Introduction

The information presented in this section describes the intent and lists the minimum requirements and critical elements of the OSHA laboratory standard.[1] This standard is performance-based and represents good industrial hygiene practice. Any organization that chooses not to follow or use the OSHA standards to meet that standard's requirements must demonstrate that the procedures it uses meet the minimum level of employee protection afforded by the OSHA standard.

Much of the information presented in the preceding subsections of 1090 was provided as guidance and should be used in developing and finalizing the laboratory chemical hygiene plan (CHP).

2. Requirements

Develop and implement a written CHP capable of protecting employees from health hazards associated with the chemicals used in the laboratory. This CHP must be capable of keeping exposures below the permissible exposure limits (PELs/TLVs) and also must be readily available to employees. The CHP must at minimum address the following elements, and must describe specific measures the employer will undertake to ensure laboratory employee protection.

a. Standard operating procedures or work practices: Include procedures and practices relevant to safety and health considerations. These are to be followed when laboratory work involves the use of hazardous chemicals. Include the information contained in MSDSs for hazardous chemicals when conducting a hazard assessment and developing work practices. Some of the guidance presented in an MSDS is intended for use in industrial settings where material is used in large quantities for a full work shift and not for the small-volume, short-duration exposures experienced in laboratories.

b. Exposure hazard criteria and procedures: These will be used to determine and implement control measures for reducing employee exposure to hazardous chemicals while conducting laboratory operations. They include engineering controls, the use of personal protective equipment, and hygiene practices. Pay particular attention to the selection of control measures for work activities that involve chemicals known to be extremely hazardous.

c. Protective equipment performance procedures: These include procedures for evaluating the performance of fume hoods and other protective equipment and specific measures to be taken to ensure proper and adequate performance of such equipment.

d. Employee information and training: This training must be timely, be refreshed periodically, evaluated for effectiveness, and documented.

e. Approval procedures: The plan must state the circumstances under which a specific laboratory operation, procedure, or activity requires prior approval before implementation.

f. Employee medical consultation and medical examinations.

g. Safety personnel: The plan must designate personnel responsible for implementation of the CHP. This should include the formal assignment of a Chemical Hygiene Officer and, where appropriate, the establishment of a Chemical Hygiene Committee.

h. Additional employee protection: The plan should include provisions for working with particularly hazardous substances. These substances include "select carcinogens" (see 1090G.2), reproductive toxins, and substances that have a high degree of acute toxicity. Give specific consideration to establishment of a designated area, use of containment devices such as fume hoods or glove boxes, procedures for safe removal of containment waste, decontamination procedures, emergency plans and procedure (test annually as a minimum), and employer review and evaluation of the effectiveness of the CHP at least annually and updating as necessary.

3. Reference

1. Occupational Safety and Health Administration. Occupational Exposure to Hazardous Chemicals in Laboratories. 29 CFR 1910.1450.

1090 K. Mercury Use Avoidance in Laboratory

Whenever possible, avoid using mercury-containing thermometers, barometers, and manometers in the laboratory because glass breakage may release mercury vapor into the air. Airborne mercury is toxic and can interfere with low-level mercury analysis. Mercury-containing instruments should be replaced with non-mercury alternatives whenever possible. The one exception is a mercury-containing registering thermometer.

Mercury-containing reagents should be avoided when suitable alternatives are available.

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1100 WASTE MINIMIZATION AND DISPOSAL*

1100 A. Introduction

Waste minimization and disposal are part of integrated hazardous materials management. It is important to become familiar with regulations regarding the use and disposal of hazardous materials prior to their purchase, storage, and use for water and wastewater analysis. Proper management of hazardous and radioactive materials will reduce the amount of hazardous waste and associated disposal costs.

* Reviewed by Standard Methods Committee, 2010.

1100 B. Waste Minimization

1. General Considerations

Waste minimization or pollution prevention in the laboratory is the preferred approach in managing laboratory waste. Minimizing waste makes good economic sense: it reduces both costs and liabilities associated with waste disposal. For certain hazardous-waste generators it also is a regulatory requirement.

2. Waste Minimization Methods

Waste minimization methods include source reduction, recycling, and reclamation.[1] Waste treatment, which also may be considered a form of waste minimization, is addressed in 1100C.

Source reduction can be achieved through the purchase and use of smaller quantities of chemicals. While large-volume purchases may seem economical, the costs of disposing of expired-shelf-life materials also must be considered. Date chemical inventory and use oldest stock first, or if possible, use "just in time" material delivery. Commercial laboratories and chemical users in general can return samples or unopened chemicals to sender or supplier for recycling or disposal. Many suppliers will accept unopened containers of chemicals.

Substitute nonhazardous materials for hazardous chemicals where possible. Wherever possible use methods that do not require the use of hazardous chemicals or use micro-scale analytical methods.

Improving laboratory procedures, documentation, and training will increase awareness of waste minimization and proper disposal practices, and may allow different sections within a laboratory to share standards and stock chemicals. Evaluate hazardous materials storage and use areas for potential evaporation, spills, and leaks. Segregate waste streams where possible to keep nonhazardous waste from becoming hazardous waste through contact with hazardous waste. Segregation also facilitates treatment and disposal.

Transfer of unused stock chemicals to other areas of the laboratory or to other institutions where they may be used is a way to minimize waste.[2] Check with laboratory's legal counsel before transferring chemicals.

Recycling/reclamation has limited potential in water and wastewater laboratories. Volumes generated are generally too small for economical reclamation and purity requirements are often too great. However, organic solvents often can be distilled and recovered for reuse and mercury and silver can be recovered.[3]

3. References

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1100 C. Waste Treatment and Disposal

1. General Considerations

Stringent penalties exist for the improper disposal of hazardous wastes. Potential criminal and civil liability exists for both organizations and individuals. Specific requirements vary by state and local jurisdiction and are subject to change. Federal requirements for hazardous waste generators and transporters and for treatment, storage, and disposal facilities

(TSDFs) are found in regulations pursuant to the Resource Conservation and Recovery Act of 1976 (RCRA) as amended by the Hazardous and Solid Waste Amendments of 1984 (HSWA). Many activities, in particular treatment, storage, and disposal of hazardous wastes, require a permit or license.[1,2]

Develop a plan for the safe and legal disposal of chemical and biological substances with the laboratory supervisor and/or safety coordinator. The plan should address the proper transport, storage, treatment, and disposal of hazardous waste. Properly characterize composites and document wastes. Refer to Section 1090 on Safety with regard to protective equipment in the handling of hazardous materials.

2. Waste Treatment and Disposal Methods

Treatment can be used to reduce volume, mobility, and/or toxicity of hazardous waste where expertise and facilities are available. Treatment, even on a small scale, may require a permit. Consult with federal, state, and local regulatory officials.

Waste treatment methods include thermal, chemical, physical, and biological treatment, and combinations of these methods.[1]

a. Thermal treatment: Thermal treatment methods include incineration and sterilization. They involve using high temperatures to change the chemical, physical, or biological character or composition of the waste. Incineration is often used to destroy organic solvents and is preferred for infectious wastes, although sterilization through autoclaving and/or ultraviolet light also may be allowed. Check with local health department officials.

b. Chemical treatment: Methods include chemical reaction (oxidation/reduction, neutralization, ion exchange, chemical fixation, photolysis, coagulation, precipitation) of the waste material. Neutralization of acidic or alkaline wastes is the most common form of chemical treatment. Elementary neutralization of corrosive wastes is exempt from federal RCRA permitting requirements. Before discharge of wastes to a publicly owned treatment works (POTW), ensure that they contain no pollutants (other than corrosivity) exceeding the limits set by the POTW. The oxidation of cyanide to cyanate with a strong chemical oxidant is an example of a toxicity-reducing chemical treatment.

c. Physical treatment: Methods include solidification, compaction, photo-induced reaction, distillation, flocculation, sedimentation, flotation, aeration, filtration, centrifugation, reverse osmosis, ultrafiltration, gravity thickening, and carbon or resin adsorption. Physical treatment generally reduces volume or mobility of waste materials.

d. Biological treatment: Methods include using biosolids to destroy organic compounds, composting organic-rich wastes, and using bioreactors to promote decomposition. Biological treatment usually is economical on a scale larger than is possible in most water and wastewater laboratories.

e. Ultimate disposal: After waste minimization and treatment, remaining waste streams require disposal. Nonhazardous wastes that cannot be treated further can be discharged as wastewater, emitted to the atmosphere, or placed on or in the ground.

With extreme caution, it may be permissible to dispose of limited quantities (at certain concentrations) of laboratory wastes to the sanitary sewer system or to evaporate volatile wastes in chemical ventilation hoods. Obtain written permission of local, state, and federal authorities to dispose of waste in this manner. With increasing regulatory constraints imposed by RCRA, the Clean Air Act, and Clean Water Act, these disposal options are becoming increasingly limited. Wastes disposed of in this manner may contact other substances in the sewer or ventilation systems and produce hazardous reactions.

Most hazardous wastes generated in laboratories must be sent off site for further treatment and disposal. Exercise extreme care in selecting a reputable waste hauler and disposal firm. Many firms will assist laboratories in packaging and manifesting "lab packs," 19- to 208-L (5- to 55-gal) drums containing several smaller containers of wastes.[1] Liability does not disappear when the waste leaves the generator's facility. Ensure that the laboratory receives a copy of the completed manifest and certificate of treatment and/or disposal. If possible, visit the disposal facility in advance to observe how it will manage a waste.

Certain wastes require special handling. As mentioned previously, incinerate infectious waste or sterilize it before disposal. Before reuse, sterilize all nondisposable equipment that has come into contact with infectious waste.

Although most water and wastewater laboratories do not work with radiochemical wastes, some do. Handle radiochemical wastes with extreme care. Generalized disposal criteria for radioactive wastes have been developed by the National Council on Radiation Protection and Measurements.[3] Low-level radioactive waste must be in solid form for final disposal on land. Some firms will process liquid radioactive wastes into solids. Adding absorbent materials to liquid radioactive wastes is not permissible. Certain states allow low-level liquid radioactive waste to be discharged to a permitted POTW.

Other wastes that require special handling include polychlorinated biphenyls (PCBs), dioxin/furans and their precursors, petroleum products, and asbestos. Consult with federal, state, and local officials before disposing of these wastes.

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PART 2000

PHYSICAL AND AGGREGATE PROPERTIES

Part Coordinator
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2170 Flavor Profile Analysis .....Gary A. Burlingame
2570 Asbestos .....Joseph A. Krewer

Summary of Major Changes since 2005

Quality Assurance/Quality Control (2020) was significantly revised to stay abreast of regulatory requirements and clarify the essential quality control practices for test methods in Part 2000.

Flavor Profile Analysis (2170) contains new information on method safety and selecting and training the panel, as well as new procedures for preparing and handling glassware, addressing fatigue, and dechlorinating samples.

The graphical and computer methods that can be used to calculate $CaCO_3$ saturation indices were updated in Calcium Carbonate Saturation (2330). Salinity (2520) now allows the use of the International Temperature Scale of 1990 (ITS-90). The procedure for preparing the filter and its backing was clarified in Asbestos (2570).

Also, many methods now refer users to the quality control practices that are considered to be an integral part of the method.

2010 INTRODUCTION

This part deals primarily with measurement of the physical properties of a sample, as distinguished from the concentrations of chemical or biological components. Many of the determinations included here, such as color, electrical conductivity, and turbidity, fit this category unequivocally. However, physical properties cannot be divorced entirely from chemical composition, and some of the techniques of this part measure aggregate properties resulting from the presence of a number of constituents. Others, for example, calcium carbonate saturation, are related to, or depend on, chemical tests. Also included here are tests for appearance, odor, and taste, which have been classified traditionally among physical properties, although the point could be argued. Finally, Section 2710, Tests on Sludges, includes certain biochemical tests. However, for convenience they are grouped with the other tests used for sludge.

With these minor exceptions, the contents of this part have been kept reasonably faithful to its name. Most of the methods included are either inherently or at least traditionally physical, as distinguished from the explicitly chemical, radiological, biological, or bacteriological methods of other parts.

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2020 QUALITY ASSURANCE/QUALITY CONTROL*

2020 A. Introduction

Quality control (QC) is an important attribute of any laboratory's quality assurance (QA) program. Without QC, there is no confidence in the results of analytical tests. As described in Part 1000, essential QC measures include method calibration, reagent standardization, assessment of each analyst's capabilities, analysis of blind check samples, determination of the method's sensitivity [method detection level (MDL) or quantification limit], and regular evaluation of bias, precision, and the presence of laboratory contamination or other analytical interference. The details of these procedures, their performance frequency, and expected ranges of results should be formalized in a written QA Manual and standard operating procedures. In addition, it is the laboratory's responsibility to qualify and report data values not meeting QC or other method-defined requirements with sufficient information so the client or end user can determine the usability of the qualified data.

While general information on QC procedures is provided in Part 1000 and specific procedures are typically outlined in individual methods, some of the methods in Part 2000 are not amenable to standard QC procedures; they have procedures considered unique to the method that do not necessarily apply to other more conventional analytical methods. For some methods, such as oxygen-consumption rate, bias is not applicable. Several methods in this part do not have acceptance-criteria guidance for either precision or bias of test results. This does not, however, relieve analysts of the responsibility for evaluating the test's accuracy and precision. Laboratories should generate method-specific acceptance criteria for precision or bias (or both) using control-charting techniques.

Evaluate precision by analyzing duplicate samples. However, if these results are "nondetect" or "invalidated," precision cannot be calculated. Laboratory-fortified matrices (LFMs) are not applicable to methods currently in Part 2000, so Table 2020:II has no entry in the LFM column.

Evaluate bias by analyzing standards or samples with known or certifiable parameter values. If a known or certifiable standard analyte cannot be prepared or is otherwise unavailable, then bias cannot be calculated.

To help verify the accuracy of calibration standards and overall method performance, participate in an annual or preferably semi-annual program of analysis of single-blind QC check samples (QCS)—ideally provided by an external entity. Such programs are sometimes called *proficiency testing (PT)/performance evaluation (PE) studies*. An unacceptable result on a PT sample is often a strong indication that a test protocol is not being followed successfully. Investigate circumstances fully to find the cause. In many jurisdictions, participation in PT studies is a required part of laboratory certification/accreditation.

Laboratories may save time and money by purchasing premade standards, titrants, and reagents, but they still must perform the QC checks on these materials required by the analytical methods.

* Reviewed by Standard Methods Committee, 2010. Additional revisions, 2017. Terry E. Baxter (chair), Rodger B. Baird.

2020 B. Quality Control Practices

1. Initial Quality Control

a. Initial demonstration of capability (IDC): Before new analysts run any samples, verify their capability with the method. For methods where bias is applicable (see Table 2020:I), run a laboratory-fortified blank (LFB) (2020B.2*e*), performance evaluation sample, or standard with a known or otherwise certifiable concentration at least four times and compare results to the limits listed in the method or those established by the laboratory. If no limit is specified, use the following procedure to establish limits:

Calculate the standard deviation of the four samples. The LFB's recovery limits are

$$\text{LFB's initial recovery limits} = \text{Mean} \pm (5.84 \times \text{Standard Deviation})$$

where:

5.84 = the two-sided Student's t factor for 99% confidence limits and three degrees of freedom.[1]

Also, verify that the method is sensitive enough to meet measurement objectives for detection and quantitation by determining the lower limit of the operational range. (For basic guidance on demonstrating capability, see Sections 1020B.1 and 2.)

b. Method detection level (MDL): Before analyzing samples, determine the MDL for each analyte or method parameter in accordance with Section 1020B.4. Part 2000 methods considered amenable to MDL determination are indicated in Table 2020:I. Determine MDL at least annually for each analyte or parameter in a method and major matrix category. The laboratory should define all matrix categories in its QA manual.

Ideally, use pooled data from several analysts rather than data from one analyst. (For specific information on MDLs and pooled MDLs, see Section 1020B.4.)

c. Operational range: Before using a new method or instrument, determine its operational range (upper and lower limits), or at least verify that the intended range of use is within the operational range. For each analyte, use standard concentrations that provide increasing instrument or other test response. The minimum reporting level (MRL) is set to a concentration at or above the lowest standard used in the analysis. Quantitation at the MRL must be verified initially and at least quarterly (pref-

Table 2020:I. Methods in Part 2000 Indicating or Amenable to Initial Quality Control

	Section	Bias	Precision	MDL	Operational Range
2120B	Color	–	×	–	–
2120C		–	×	×	–
2120D		–	×	×	–
2120E		–	×	×	–
2120F		–	×	×	–
2130B	Turbidity	–	–	×	–
2170B	Flavor Profile Analysis	–	×	–	–
2310B	Acidity	–	×	–	–
2320B	Alkalinity	×	×	–	–
2340C	Hardness	×	×	–	–
2350B	Oxidant Demand/Requirement	–	–	×	–
2350C		–	–	×	–
2350D		–	–	×	–
2350E		–	–	×	–
2510B	Conductivity	–	×	–	–
2520B	Salinity	–	×	–	×
2520C		–	×	–	–
2530C	Floatables	×	×	×	–
2540B	Solids	–	×	–	–
2540C		–	×	–	–
2540D		–	×	–	–
2540E		–	×	–	–
2560B	Particle Counting and Size Distribution	–	×	×	–
2560C		–	×	×	–
2560D		–	×	×	–
2570B	Asbestos	×	×	–	–
2580B	Oxidation–Reduction Potential	×	×	–	–
2710G	Tests on Sludges	–	×	–	–
2710H		–	×	–	–
2720B	Anaerobic Sludge Digester Gas Analysis	×	×	–	–
2720C		×	×	×	–
2810B	Dissolved Gas Supersaturation	×	×	–	–

erably daily) by analyzing a QC sample (where applicable to the method). Laboratories should define acceptance criteria for the operational range, including MRL, in the QA/QC documentation. In Part 2000, only salinity suggests an initial operating range (see Table 2020:I).

2. Ongoing Quality Control

a. Calibration/standardization: Calibrate the method or standardize titration reagents using the directions in the procedure. Methods in Part 2000 that require calibration or titration reagent standardization are indicated in Table 2020.II. (For basic calibration guidance, see Section 1020B.11.)

b. Calibration/standardization verification: Verify calibration by periodically analyzing a calibration standard and calibration blank during a run—typically, after each batch of ten samples and at the end of the run. The calibration verification standard's analyte or parameter concentration should be varied over the calibration range to determine detector response.

TABLE 2020:II. SUMMARY OF ONGOING QUALITY CONTROL FOR METHODS IN PART 2000

	Section	Calibrate or Standardize	QCS	MB	LFB	Duplicates	LFM
2120B	Color	×	×	–	–	×	–
2120C		×	×	–	–	×	–
2120D		×	×	–	–	×	–
2120E		×	×	–	–	×	–
2120F		×	×	–	–	×	–
2130B	Turbidity	×	×	–	–	–	–
2150B	Odor	–	–	×	–	–	–
2150C		–	–	×	–	×	–
2160B	Taste	–	–	×	–	–	–
2170B	Flavor Profile Analysis	–	–	×	–	×	–
2310B	Acidity	×	×	×	×	×	–
2320B	Alkalinity	×	×	–	×	×	–
2340C	Hardness	×	×	×	×	×	–
2350B	Oxidant Demand/ Requirement	–	–	×	–	–	–
2350C		–	–	×	–	–	–
2350D		–	–	×	–	–	–
2350E		–	–	×	–	–	–
2510B	Conductivity	×	×	–	×	×	–
2520B	Salinity	×	×	–	×	×	–
2520C		×	×	–	–	×	–
2540B	Solids	–	–	–	–	×	–
2540C		–	–	×	×	×	–
2540D		–	–	–	–	×	–
2540E		–	–	×	–	×	–
2540F		–	–	–	–	×	–
2540G		–	–	–	–	×	–
2550B	Temperature	×	–	–	–	–	–
2560B	Particle Counting and Size Distribution	×	×	×	×	×	–
2560C		×	×	×	×	×	–
2560D		×	×	×	×	×	–
2570B	Asbestos	×	–	×	–	×	–
2580B	Oxidation–Reduction Potential	×	–	–	–	×	–
2710B	Tests on Sludges	×	–	–	–	–	–
2710G		–	–	–	–	×	–
2710H		–	–	–	–	×	–
2720B	Anaerobic Sludge Digester Gas Analysis	–	–	–	–	×	–
2720C		×	×	–	–	×	–
2810B	Dissolved Gas Supersaturation	×	–	–	–	×	–

For the calibration verification to be valid, check standard results must not exceed ±10% of its true value, and calibration blank results must not be greater than one-half the reporting level (unless the method specifies otherwise).

If a calibration verification fails, immediately cease analyzing samples and initiate corrective action. The first step may be to re-analyze the calibration verification. If/when the calibration verification passes, continue the analysis. Otherwise, repeat initial calibration and re-analyze samples run since the last acceptable calibration verification.

If the LFB is not prepared from a second source to confirm method accuracy, the laboratory must also verify the accuracy of its standard preparation by analyzing a mid-level second-source calibration standard whenever a new initial calibration curve is prepared. Results must agree within 15% (unless otherwise specified in a method).

Verify standardized titration reagents by periodically re-standardizing. Method parameters in Part 2000 that are determined using standardized titration reagents are acidity, alkalinity, and hardness. Typically, the standardized reagents are stable for several months when sealed to avoid evaporation and stored properly. Re-standardize reagents once a month or when improper storage occurs. If the titration reagent's normality (titer value) has changed, then use the measured value, adjust the normality (titer value) as the procedure describes, or prepare and standardize fresh titration reagent as needed.

c. Quality control sample (QCS): Analyze an externally generated, blind QCS (unknown concentration) at least annually (preferably semi-annually or quarterly). Obtain this sample from a source external to the laboratory, and compare results to that laboratory's acceptance results. If testing results do not pass acceptance criteria, investigate why, take corrective action, and analyze a new QCS. Repeat this process until results meet the acceptance criteria. Methods in Part 2000 considered amenable to QCS determination are indicated in Table 2020.II.

d. Method blank (MB): Include at least one MB daily or with each batch of 20 or fewer samples, whichever is more frequent. Any constituent(s) recovered must generally be less than or equal to one-half the reporting level (unless the method specifies otherwise). If any MB measurements are at or above the reporting level, take immediate corrective action as outlined in Section 1020B.5. This may include re-analyzing the sample batch.

e. Laboratory-fortified blank (LFB): If each initial calibration solution is verified via a second source (2020B.2*b*), the LFB need not be from a second source (unless otherwise specified in a method). Table 2020:II indicates methods in Part 2000 where use of LFB is considered appropriate.

Using stock solutions preferably prepared with the second source, prepare fortified concentrations so they are within the calibration curve. Ideally, vary LFB concentrations to cover the range from the midpoint to the lower part of calibration curve, including the reporting limit.

Calculate percent recovery, plot control charts, and determine control limits (Section 1020B.13) for these measurements to demonstrate ongoing capability. Some methods may have specific limits to use in lieu of plotting control charts. In those cases, control charts may still be useful in identifying potential problems. Ensure that the LFB meets the method's performance criteria when such criteria are specified. Establish corrective actions to be taken if the LFB does not satisfy acceptance criteria.

Include at least one LFB daily or per each batch of 20 or fewer samples. Some regulatory programs require a higher frequency of LFBs. If the sample results are often "nondetect," consider using duplicate LFBs to assess precision.

f. Duplicates: When appropriate (Table 2020:II), randomly select routine samples to be analyzed twice. Independently prepare and analyze duplicate samples. Include at least one duplicate for each matrix type daily or with each batch of 20 or fewer samples. Calculate control limits for duplicates when method-specific limits are not provided. (For basic guidance on duplicates, see Section 1020B.7.) Some regulatory programs require more frequent use of duplicates.

3. Calculations

a. LFB recovery:

$$\text{LFB \% Recovery} = \left[\frac{\textit{measured conc}}{\textit{spiked conc}}\right] \times 100$$

b. Relative percent difference:

$$\%\text{RPD} = \left[\frac{|D_1 - D_2|}{\left(\frac{D_1 + D_2}{2}\right)}\right] \times 100$$

where:

D_1 = concentration determined for first duplicate, and
D_2 = concentration determined for second duplicate.

c. Relative standard deviation (%RSD):

$$\%\text{RSD} = \frac{s}{\bar{x}} \times 100$$

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{(n-1)}}$$

where:

s = standard deviation,
n = total number of values from replicate analyses,
x_i = each individual value used to calculate mean, and
$\bar{x}$ = mean of the total number (n) of values.

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2. U.S. Environmental Protection Agency. 1995. Definition and procedure for the determination of the method detection limit, rev. 1.11. 40 CFR Part 136, Appendix B. *Fed. Reg.* 5:23703.

2110 APPEARANCE

To record the general physical appearance of a sample, use any terms that briefly describe its visible characteristics. These terms may state the presence of color, turbidity, suspended solids, organisms and their immature forms, sediment, floating material, and similar particulate matter detectable by the unaided eye. Use numerical values when they are available, as for color, turbidity, and suspended solids.

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2120 COLOR*

2120 A. Introduction

Color in surface and ground waters results primarily from the presence of natural organic matter, particularly aquatic humic matter. Humic matter consists of humic and fulvic acids; both cause a yellow-brown color. Humic acids give a more intense color, and the presence of iron intensifies the color through the formation of soluble ferric humates. Suspended particles, especially colloidal-size particles such as clays, algae, iron and manganese oxides, give waters an appearance of color; they should be removed before measurement. Industrial wastewaters can contain lignins, tannins, dyes, and other organic and inorganic chemicals that cause color. Humic materials and the color caused by these materials are removed from potable water supplies for aesthetic reasons and for health reasons because they are precursors in the formation of disinfection by-products. Color also is removed to make water suitable for industrial applications. Colored industrial wastewaters may require color removal before discharge into watercourses.

1. Terminology

The term "color" is used here to mean true color, that is, the color of water from which turbidity has been removed. Colloidal and larger suspended particles scatter light interfering with the determination of true color measurements in Method 2120B and in the spectrophotometric procedures of Methods 2120C–F. The term "apparent color" includes not only color due to substances in solution, but also that due to suspended matter. Apparent color is determined on the original sample without filtration. In some waters and wastewaters, apparent color is contributed principally by colloidal or suspended material.

2. Selection of Method

Methods 2120B and C are applicable to measurement of color caused primarily by natural organic matter. The measurements apply to all surface and ground waters; wastewaters, both domestic and industrial; and especially potable waters. While all methods (2120B–F) are suitable for true color measurements, for apparent color measurements use only 2120B; in such cases, determine both true color and apparent color. For comparison among laboratories, calibrate 2120B with 2120C. Methods 2120D–F allow color measurement for any dissolved chemical that gives the appearance of color in the visible-light wavelength range. They are especially applicable to colored waters and wastewaters having color characteristics different from, but not excluding, platinum-cobalt standards.

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* Approved by Standard Methods Committee, 2001. Editorial revisions, 2011. Joint Task Group: 21st Edition—James K. Edzwald (chair), Penny J. Bristol, Brian A. Dempsey, Darren A. Lytle, David J. Pernitsky, Mike J. Sadar, Jeff Throckmorton.

2120 B. Visual Comparison Method

1. General Discussion

a. Principle: Color is determined by visual comparison of the sample with known concentrations of colored solutions. Comparison also may be made with special, properly calibrated glass color disks. The platinum-cobalt method of measuring color is the standard method, the unit of color being that produced by 1 mg platinum/L in the form of the chloroplatinate ion. The ratio of cobalt to platinum given (2120B.4) matches the color of natural waters.

b. Application: The platinum-cobalt method is applicable to natural waters, potable waters, and to wastewaters, both domestic and industrial.

c. Interference: Even a slight turbidity causes the apparent color to be noticeably higher than the true color; therefore remove turbidity by the filtration procedure described in 2120C.

The color value of water is extremely pH-dependent and invariably increases as the pH of the water is raised. When reporting a color value, specify the pH at which color is determined. For research purposes, or when color values are to be compared among laboratories, determine the color response of a given water over a wide range of pH values.[1]

d. Field method: Because the platinum-cobalt standard method is not convenient for field use, compare water color with that of glass disks held at the end of metallic tubes containing glass comparator tubes filled with sample and colorless distilled water. Match sample color with the color of the tube of clear water plus the calibrated colored glass when viewed by looking toward a white surface. Calibrate each disk to correspond with the colors on the platinum-cobalt scale. The glass disks give results in substantial agreement with those obtained by the platinum-cobalt method and their use is recognized as a standard field procedure.

e. Nonstandard laboratory methods: Using glass disks or liquids other than water as standards for laboratory work is permissible only if these have been individually calibrated against platinum-cobalt standards. Waters of highly unusual color, such as those that may occur by mixture with certain

industrial wastes, may have hues so far removed from those of the platinum-cobalt standards that comparison by the standard method is difficult or impossible. For such waters, use the methods in 2120C–F. However, results so obtained are not directly comparable to those obtained with platinum-cobalt standards.

2. Apparatus

a. Nessler tubes, matched, 50-mL, tall form.

b. pH meter, for determining sample pH (see Section 4500-H^+).

c. Filter and filter assembly (for true color measurements): Use a 0.45-μm-pore-diam cellulose membrane filter of 22 or 47 mm diam. Glass fiber filters also can be used. Rinse filters before use and monitor filter blanks. Smaller-pore filters of 0.2 or 0.22 μm or even ultrafiltration may be needed to remove colloidal particles for certain samples such as Mn or Fe oxides or other colloids. Use a glass, TFE, or stainless steel assembly to hold the selected filters.

3. Reagents

a. Organic-free water: Type I reagent water (see Section 1080) or equivalent water. Use for all standard preparation and other procedures.

b. Potassium chloroplatinate (K_2PtCl_6), analytical grade.

c. Cobaltous chloride ($CoCl_2 \cdot 6H_2O$), analytical grade.

d. Hydrochloric acid (HCl), analytical grade.

e. Sodium hydroxide (NaOH), analytical grade.

4. Preparation of Standards

Dissolve 1.246 g potassium chloroplatinate and 1.00 g crystallized cobaltous chloride in water with 100 mL conc HCl and dilute to 1000 mL. This stock solution has a color of 500 color units (CU). Platinum-cobalt standards of 500 CU are available commercially, and are suitable for use as the primary standard.

Prepare standards having CU of 5, 10, 15, 20, 25, 30, 40, 50, and 100 by diluting 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, and 20.0 mL stock color standard with water in 100-mL volumetric flasks. Transfer to nessler tubes for use as standards. Protect standards against evaporation and contamination when not in use. Keep in the dark when not in use, and keep only for 1 month.

5. Procedure

a. Sample collection: Collect samples in acid-washed amber glass bottles or plastic bottles covered to keep out light. Rinse bottles once with sample before filling bottle with sample. Preferably take a sample of at least 100 mL. Analyze sample within 24 h of collection. Keep samples cold until analysis, and warm them up to room temperature before measurement.

b. Sample preparation: Check sample pH. If outside the range of 4 to 10, preferably adjust sample to pH 7 and note the adjustment.

If true color is to be measured, wash membrane filter and filter assembly by passing at least 50 mL water through filter. Filter about 25 mL sample and discard filtrate. Filter a further portion of about 50 mL through the same filter and retain for analysis.

c. Sample measurement: Observe sample color by filling a matched nessler tube to the 50-mL mark with sample and comparing it with standards. Look vertically downward through tubes toward a white or specular surface placed at such an angle that light is reflected upward through the columns of liquid. If turbidity is present and has not been removed, report as "apparent color." If the color exceeds 100 units, dilute sample in known proportions until the color is within the range of the standards.

6. Calculation

a. Calculate color units (CU) by the following equation:

$$\text{Color} = \frac{A \times 50}{B}$$

where:

A = estimated color of a diluted sample, and
B = mL sample taken for dilution.

b. The correct units for true color are CU. One CU is equivalent to one Hazen unit and to one Pt-Co unit. If samples are not filtered, report data as Apparent CU. Report color results in whole numbers and record as follows:

CU	Record to Nearest
1–50	1
51–100	5
101–250	10
251–500	20

c. Report sample pH.

7. Quality Control

The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

a. Replicate measurements: Use at least two portions of filtered sample.

b. Duplicate analyses: Analyze every tenth sample in duplicate (i.e., duplicating the entire procedure) to assess method precision.

c. Pre-programmed spectrophotometers: For spectrophotometers with pre-programmed calibration curves, verify calibration curve regularly with the platinum-cobalt standards prepared under 2120C.4, and adjust pre-programmed curves as needed.

8. Reference

1. Black, A.P. & R.F. Christman. 1963. Characteristics of colored surface waters. *J. Amer. Water Works Assoc.* 55:753.

9. Bibliography

Christman, R.F. & M. Ghassemi. 1966. Chemical nature of organic color in water. *J. Amer. Water Works Assoc.* 58:723.

Sawyer, C.N., P.L. McCarty & G.F. Parkin. 1994. Color. *In* Chemistry for Environmental Engineering, 4th ed., Chap. 14. McGraw Hill, New York, N.Y.

2120 C. Spectrophotometric—Single-Wavelength Method (PROPOSED)

1. General Discussion

a. Principle: Color is determined spectrophotometrically at a wavelength between 450 and 465 nm, with platinum-cobalt solutions as standards.[1–3] True color of real samples and platinum-cobalt standards follows Beer's Law.

b. Application: The spectrophotometric platinum-cobalt method is applicable to natural waters, potable waters, and wastewaters, both domestic and industrial.

c. Interference: The primary interference is from the presence of colloidal and suspended particles that absorb or scatter light at the wavelength of the spectrophotometric method. While in 2120B color measurements can be made without removal of particulate matter as long as they are reported as "Apparent CU", 2120C requires removal of particulate matter before color determination.

Light absorbance of organic matter depends on pH; however, the variation in absorbance is small for the pH range of most waters. Because color measurements are made for aesthetic reasons, preferably do not adjust sample pH as long as it is between 4 and 10. If pH is adjusted, adjust to 7, and note. Further, pH can affect the solubility of substances, which can then interfere with the color measurement if particulate matter is formed.

d. Method detection level: The minimum detectable color depends on the cell path length. Choose a cell size that provides an absorbance within the range that results in good accuracy and linearity of response. This range depends on the quality of the spectrophotometer. If a 50-mm cell is used in the wavelength range of 450 to 465 nm, then an absorbance of 0.005 yields a minimum detectable color of 1 CU. With newer spectrophotometers, a method detection level of 2 CU can be obtained with a path length of 25 mm. Dilute samples with high color to fall within the range of the standard curve. Absorbance readings should fall within the range of 0.005 to 0.8.

2. Apparatus

a. Spectrophotometer: Choose a wavelength between 450 and 465 nm. Use matched glass cells providing a light path of at least 25 mm. Cells with path lengths of 40, 50, or 100 mm may be used. Beer's Law allows flexibility in selecting the cell path length.

b. Filter and filter assembly: See 2120B.2*c*.

3. Reagents

See 2120B.3.

4. Preparation of Standards

Prepare stock color solution of 500 CU according to 2120B.4.

Prepare standards having CU of 5, 10, 15, 20, 30, 40, 50, and 100 by diluting 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, and 20.0 mL stock color standard with water in 100-mL volumetric flasks. Protect standards against evaporation and contamination when not in use. Keep in the dark when not in use, and keep for only 1 month.

5. Spectrophotometric Standard Curve

Let spectrophotometer warm up according to manufacturer's instructions. Choose a wavelength between 450 and 465 nm to develop the standard curve; a good choice is 456 nm. The absorbance of Pt-Co has a broad maximum absorbance within this wavelength range. Use matched spectrophotometer cells. Fill one cell with water to zero the instrument. Read absorbance for each color standard, and prepare a standard curve of CU versus absorbance.

Pre-programmed color curves are available with some spectrophotometers. The curves can be verified by use of the standards prepared in 2120C.4.

6. Procedure

a. Sample collection: See 2120B.5*a*.

b. Sample preparation: See 2120B.5*b*. Always filter sample.

c. Spectrophotometric measurement: Let spectrophotometer warm up according to manufacturer's instructions. Set wavelength at same setting used to develop the standard curve; be sure that the cell path length is the same as that used for the standard curve. Fill one spectrophotometer cell with water and zero the instrument. Rinse the other cell with sample and then refill. Place cell in spectrophotometer and read absorbance. Repeat for remaining samples. Determine sample color using absorbance readings and standard curve relating absorbance and CU. For spectrophotometers with pre-programmed calibration curves for color, zero instrument and take sample measurements according to manufacturer's instructions.

7. Quality Control

See 2120B.7.

8. References

1. Crowther, J. & J. Evans. 1981. Estimating color in Hazen units by spectrophotometry. *J. Amer. Water Works Assoc.* 73:265.
2. Bennett, L. & M. Drikas. 1993. The evaluation of color in natural waters. *Water Res.* 27:1209.
3. Hongve, D. & G. Åkesson. 1996. Spectrophotometric determination of water colour in Hazen units. *Water Res.* 30:2771.

2120 D. Spectrophotometric—Multi-Wavelength Method

1. General Discussion

a. Principle: The color of a filtered sample is expressed in terms that describe the sensation realized when viewing the sample. The hue (red, green, yellow, etc.) is designated by the term "dominant wavelength," the degree of brightness by "luminance," and the saturation (pale, pastel, etc.) by "purity." These values are best determined from the light transmission characteristics of the filtered sample by means of a spectrophotometer.

b. Application: This method is applicable to potable and surface waters and to wastewaters, both domestic and industrial.

c. Interference: The primary interference is from the presence of colloidal and suspended particles that absorb or scatter light.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

a. Spectrophotometer, having absorption cells of a minimum of 10 mm, a narrow (10-nm or less) spectral band, and an effective operating range from 400 to 700 nm.

b. Filter: See 2120B.2*c*.

3. Procedure

a. Sample preparation: Bring two 50-mL samples to room temperature. Use one sample at the original pH; adjust pH of the other to 7.0 by using sulfuric acid (H_2SO_4) and sodium hydroxide (NaOH) of such concentrations that the resulting volume change does not exceed 3%. A standard pH is necessary because of the variation of color with pH. Remove particulate matter from samples before color determination (see 2120B.5*b*).

b. Determination of light transmission characteristics: Thoroughly clean 1-cm absorption cells. Rinse twice with filtered sample, and fill cell with filtered sample.

Determine transmittance values (in percent) at each visible wavelength value presented in Table 2120:I, using the 10 ordinates marked with an asterisk for fairly accurate work and all 30 ordinates for increased accuracy. Set instrument to read 100% transmittance on the distilled water blank and make all determinations with a narrow spectral band.

4. Calculation

a. Tabulate transmittance values corresponding to wavelengths shown in Columns *X*, *Y*, and *Z* in Table 2120:I. Total each transmittance column and multiply totals by the appropriate factors (for 10 or 30 ordinates) shown at the bottom of the table, to obtain tristimulus values *X*, *Y*, and *Z*. The tristimulus value *Y* is percent luminance.

b. Calculate the trichromatic coefficients *x* and *y* from the tristimulus values *X*, *Y*, and *Z* by the following equations:

Table 2120:I. Selected Ordinates for Spectrophotometric Color Determinations*

Ordinate No.	*X*	*Y*	*Z*
	Wavelength *nm*		
1	424.4	465.9	414.1
2	435.5*	489.5*	422.2*
3	443.9	500.4	426.3
4	452.1	508.7	429.4
5*	461.2*	515.2*	432.0*
6	474.0	520.6	434.3
7	531.2	525.4	436.5
8*	544.3*	529.8*	438.6*
9	552.4	533.9	440.6
10	558.7	537.7	442.5
11*	564.1*	541.4*	444.41
12	568.9	544.9	446.3
13	573.2	548.4	448.2
14*	577.4*	551.8*	450.1
15	581.3	555.1	452.1
16	585.0	558.5	454.0
17*	588.7*	561.9*	455.9*
18	592.4	565.3	457.9
19	596.0	568.9	459.9
20*	599.6*	572.5*	462.0*
21	603.3	576.4	464.1
22	607.0	580.4	466.3
23*	610.9*	584.8*	468.7*
24	615.0	589.6	471.4
25	619.4	594.8	474.3
26*	624.2*	600.8*	477.7*
27	629.8	607.7	481.8
28	636.6	616.1	487.2
29*	645.9*	627.3*	495.2*
30	663.0	647.4	511.2
Factors when 30 Ordinates Used			
	0.032 69	0.033 33	0.039 38
Factors when 10 Ordinates Used			
	0.098 06	0.100 00	0.118 14

* Insert in each column the transmittance value (%) corresponding to the wavelength shown. Where limited accuracy is sufficient, use only the ordinates marked with an asterisk.

$$x = \frac{X}{X + Y + Z}$$

$$y = \frac{Y}{X + Y + Z}$$

Locate point *(x, y)* on one of the chromaticity diagrams in Figure 2120:1 and determine the dominant wavelength (in nanometers) and the purity (in percent) directly from the diagram.

Determine hue from the dominant-wavelength value, according to the ranges in Table 2120:II.

5. Expression of Results

Express color characteristics (at pH 7.0 and at the original pH) in terms of *dominant wavelength* (nanometers, to the nearest

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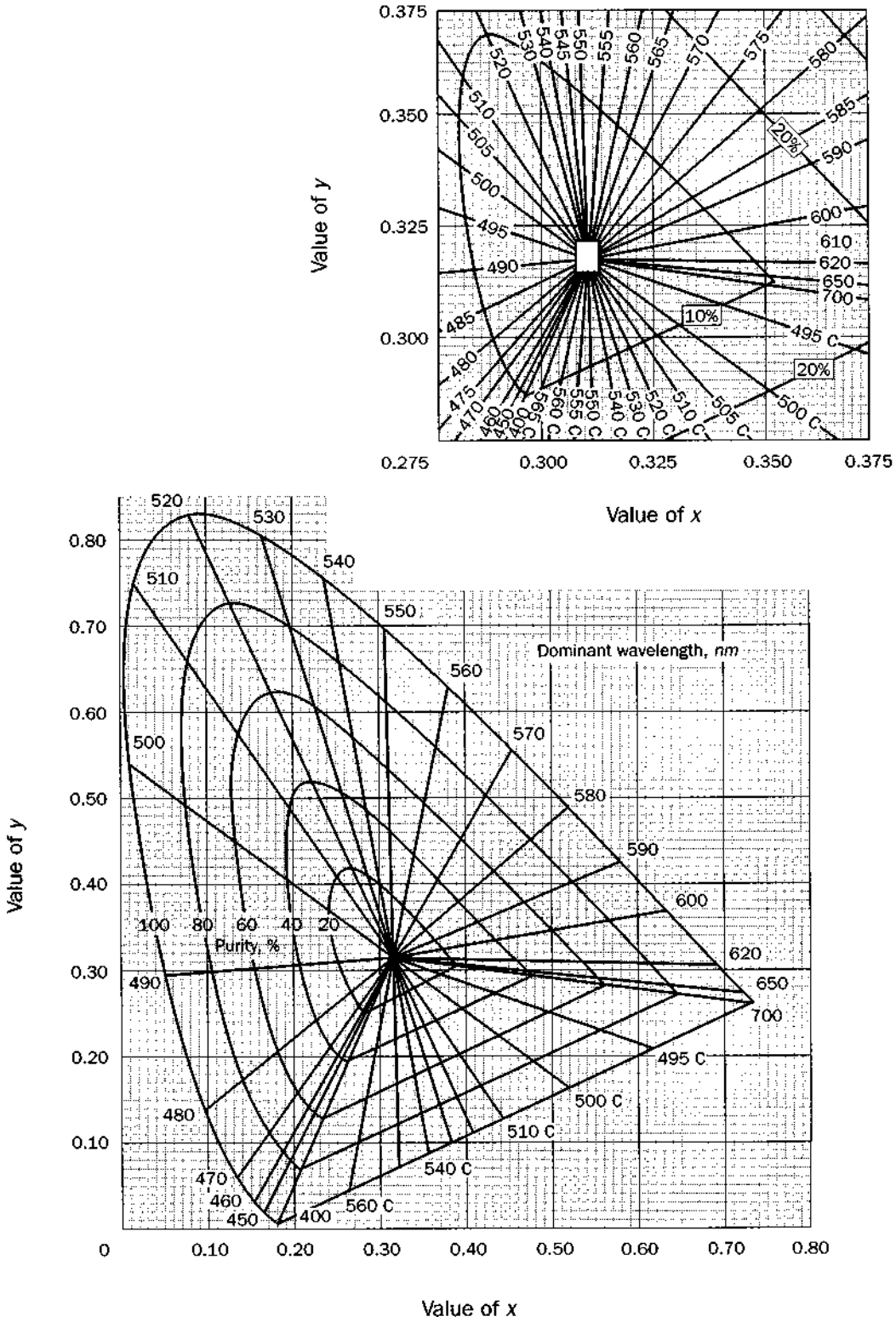


Figure 2120:1. Chromaticity diagrams.

TABLE 2120:II. COLOR HUES FOR DOMINANT WAVELENGTH RANGES

Wavelength Range *nm*	Hue
400–465	Violet
465–482	Blue
482–497	Blue-green
497–530	Green
530–575	Greenish yellow
575–580	Yellow
580–587	Yellowish orange
587–598	Orange
598–620	Orange-red
620–700	Red
400–530c*	Blue-purple
530c–700*	Red-purple

* See Figure 2120:1 for significance of "c".

unit), *hue* (e.g., blue, blue-green, etc.), *luminance* (percent, to the nearest tenth), and *purity* (percent, to the nearest unit). Report type of instrument (i.e., spectrophotometer), number of selected ordinates (10 or 30), and the spectral band width (nanometers) used.

6. Bibliography

HARDY, A.C. 1936. Handbook of Colorimetry. Technology Press, Boston, Mass.

2120 E. Tristimulus Spectrophotometric Method

1. General Discussion

a. Principle: Tristimulus values are a set of three numbers obtained from a spectrophotometer or colorimeter that, when combined in various ways, describe how the human eye perceives a given color. Calculations using the three tristimulus values typically are used to define the color of a specimen for color matching specifications or for routine control purposes.

The percentage of tristimulus light transmitted by the solution is determined and the transmittance values are then converted to trichromatic coefficients and color characteristic values.

b. Application: This method is applicable to potable and surface waters and to domestic and industrial wastewaters.

c. Interference: See 2120C.1*c*.

2. Apparatus

a. Spectrophotometer with narrow spectral band width (10 nm or less), an effective operating range of 380 to 780 nm, and a tungsten lamp light source. Instrument must be able to obtain transmittance values at a multitude of wavelengths and calculate tristimulus values *X*, *Y*, and *Z*[1] to produce a final color result.* Calibrate calculation algorithm software against platinum-cobalt standard reference.

b. Spectrophotometer cells, 1 cm.

c. Filtering apparatus and filter: See 2120 B.2*c*.

* Hach DR/4000 Spectrophotometer, Program No. 1666, or equivalent.

3. Procedure

a. Sample collection: See 2120B.5*a*.

b. Sample preparation: Filter turbid samples according to 2120B.5*b*.

c. Spectrophotometric measurement: Let spectrophotometer warm up in accordance with manufacturer's directions. Set instrument to pre-programmed calibration curve for tristimulus color. Zero instrument and perform measurements according to manufacturer's method directions. Express results as prescribed in 2120D.5.

Alternatively, obtain tristimulus values for color by a published computation method.[1]

4. Quality Control

See 2120B.7.

5. Reference

1. AMERICAN SOCIETY FOR TESTING & MATERIALS. 1995. Standard practice for computing the colors of objects by using the CIE system. E308-95, ASTM Standards on Color and Appearance Measurement, 5th ed. W. Conshohocken, Pa.

6. Bibliography

HACH COMPANY. 1999. Hach DR/4000 Spectrophotometer Procedures Manual, 9th ed. Loveland, Colo.

2120 F. ADMI Weighted-Ordinate Spectrophotometric Method

1. General Discussion

a. Principle: Three properties describe color: hue, chroma, and value. Hue is the "color" (blue or red, for example); chroma is the intensity, brightness, or dullness of color; and value is the amount, or lightness or darkness, of color. In accordance with Adams-Nickerson chromatic value formula,[1] this method calculates single-number color difference values (i.e., uniform color differences). For example, if two colors, A and B, are judged visually to differ from colorless to the same degree, their ADMI color values will be the same.[2] Values are independent of chroma and hue. Spectrophotometrically, transmittance is measured at multiple wavelengths and converted to a set of abstract numbers, which then are converted to a single number that indicates color value. This number is expressed on a scale used by the American Dye Manufacturers Institute (ADMI).[2]

b. Applications: This method is applicable to colored waters and wastewaters having color characteristics significantly different from, but not excluding, platinum-cobalt standards.

c. Interferences: See 2120C.1*c*.

2. Apparatus

a. Spectrophotometer, with narrow spectral band width (10 nm or less), an effective operating range of 400 nm to 700 nm and a tungsten lamp light source. Instrument must be able to obtain transmittance values at a multitude of wavelengths and calculate tristimulus (ordinate) values, preferably by using the weighted-ordinate method.* Calibrate calculation algorithm software against platinum-cobalt standard reference.

b. Spectrophotometer cells, 1 cm.

c. Filtering apparatus and filter: See 2120B.2*c*.

3. Procedure

a. Sample collection: See 2120B.5*a*.

b. Sample preparation: Prepare two 100-mL sample portions, one at the original pH and one at pH 7.0. Filter turbid samples according to 2120B.5*b*.

c. Spectrophotometric measurement: Let spectrophotometer warm up in accordance with the manufacturer's instruction. Set instrument to pre-programmed calibration curve for ADMI Weighted Ordinate Method. Zero instrument and take measurements of original and pH-adjusted samples according to manufacturer's directions. Express results as prescribed in 2120D.5 for both original and pH-adjusted samples.

Alternatively, obtain ADMI weighted-ordinate values for color by a published computation method.[2]

4. Quality Control

See 2120B.7.

5. References

1. McLaren, K. 1970. The Adams-Nickerson colour-difference formula. *J. Soc. Dyers Colorists* 86:354.
2. Allen, W., W.B. Prescott, R.E. Derby, C.E. Garland, J.M. Peret & M. Saltzman. 1973. Determination of color of water and wastewater by means of ADMI color values. *Proc. 28th Ind. Waste Conf.*, Purdue Univ., Eng. Ext. Ser. No. 142:661.

6. Bibliography

Hach Company. 1999. Hach DR/4000 Spectrophotometer Procedures Manual, 9th ed. Loveland, Colo.

* Hach DR/4000 Spectrophotometer, Program No. 1660, or equivalent.

2130 TURBIDITY*

2130 A. Introduction

1. Sources and Significance

Clarity of water is important in producing products destined for human consumption and in many manufacturing operations. Beverage producers, food processors, and potable water treatment plants drawing from a surface water source commonly rely on fluid-particle separation processes such as sedimentation and filtration to increase clarity and ensure an acceptable product. The clarity of a natural body of water is an important determinant of its condition and productivity.

Turbidity in water is caused by suspended and colloidal matter such as clay, silt, finely divided organic and inorganic matter, and plankton and other microscopic organisms. Turbidity is an expression of the optical property that causes light to be scattered and absorbed rather than transmitted with no change in direction or flux level through the sample. Correlation of turbidity with the weight or particle number concentration of suspended matter is difficult because the size, shape, and refractive index of the particles affect the light-scattering properties of the suspension. When present in significant concentrations, particles consisting of light-absorbing materials such as activated carbon cause a negative interference. In low concentrations these particles tend to have a positive influence because they contribute to turbidity. The presence of dissolved, color-causing substances that absorb light may cause a negative interference. Some commercial instruments may have the capability of either correcting for a slight color interference or optically blanking out the color effect.

2. Selection of Method

Historically, the standard method for determination of turbidity has been based on the Jackson candle turbidimeter; however, the lowest turbidity value that can be measured directly on this device is 25 Jackson Turbidity Units (JTU). Because turbidities of water treated by conventional fluid-particle separation processes usually fall within the range of 0 to 1 unit, indirect secondary methods were developed to estimate turbidity. Electronic nephelometers are the preferred instruments for turbidity measurement.

Most commercial turbidimeters designed for measuring low turbidities give comparatively good indications of the intensity of light scattered in one particular direction, predominantly at right angles to the incident light. Turbidimeters with scattered-light detectors located at 90° to the incident beam are called nephelometers. Nephelometers are relatively unaffected by small differences in design parameters and therefore are specified as the standard instrument for measurement of low turbidities. Instruments of different make and model may vary in response. † However, interinstrument variation may be effectively negligible if good measurement techniques are used and the characteristics of the particles in the measured suspensions are similar. Poor measurement technique can have a greater effect on measurement error than small differences in instrument design. Turbidimeters of nonstandard design, such as forward-scattering devices, may be more sensitive than nephelometers to the presence of larger particles. While it may not be appropriate to compare their output with that of instruments of standard design, they still may be useful for process monitoring.

An additional cause of discrepancies in turbidity analysis is the use of suspensions of different types of particulate matter for instrument calibration. Like water samples, prepared suspensions have different optical properties depending on the particle size distributions, shapes, and refractive indices. A standard reference suspension having reproducible light-scattering properties is specified for nephelometer calibration.

Its precision, sensitivity, and applicability over a wide turbidity range make the nephelometric method preferable to visual methods. Report nephelometric measurement results as nephelometric turbidity units (NTU).

3. Storage of Sample

Determine turbidity as soon as possible after the sample is taken. Gently agitate all samples before examination to ensure a representative measurement. Sample preservation is not practical; begin analysis promptly. Refrigerate or cool to 4°C, to minimize microbiological decomposition of solids, if storage is required. For best results, measure turbidity immediately without altering the original sample conditions such as temperature or pH.

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* Approved by Standard Methods Committee, 2001. Editorial revisions, 2011. Joint Task Group: 20th Edition—Raymond D. Letterman (chair), John A. Arrington, Alvin Lieberman, Kemon J. Papacosta, Theodore S. Tanaka, Brannon H. Wilder.

† Nephelometers that instrument manufacturers claim meet the design specifications of this method may not give the same reading for a given suspension, even when each instrument has been calibrated using the manufacturer's manual. This differential performance is especially important when measurements are made for regulatory purposes. Consult regulatory authorities when selecting a nephelometer to be used for making measurements that will be reported for regulatory purposes.

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2130 B. Nephelometric Method

1. General Discussion

a. Principle: This method is based on a comparison of the intensity of light scattered by the sample under defined conditions with the intensity of light scattered by a standard reference suspension under the same conditions. The higher the intensity of scattered light, the higher the turbidity. Formazin polymer is used as the primary standard reference suspension. The turbidity of a specified concentration of formazin suspension is defined as 4000 NTU.

b. Interference: Turbidity can be determined for any water sample that is free of debris and rapidly settling coarse sediment. Dirty glassware and the presence of air bubbles give false results. "True color" (i.e., water color due to dissolved substances that absorb light) causes measured turbidities to be low. This effect usually is not significant in treated water.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

a. Laboratory or process nephelometer consisting of a light source for illuminating the sample and one or more photoelectric detectors with a readout device to indicate intensity of light scattered at 90° to the path of incident light. Use an instrument designed to minimize stray light reaching the detector in the absence of turbidity and to be free from significant drift after a short warmup period. The sensitivity of the instrument should permit detecting turbidity differences of 0.02 NTU or less in the lowest range in waters having a turbidity of less than 1 NTU. Several ranges may be necessary to obtain both adequate coverage and sufficient sensitivity for low turbidities. Differences in instrument design will cause differences in measured values for turbidity even though the same suspension is used for calibration. To minimize such differences, observe the following design criteria:

1) Light source—Tungsten-filament lamp operated at a color temperature between 2200 and 3000K.
2) Distance traversed by incident light and scattered light within the sample tube—Total not to exceed 10 cm.
3) Angle of light acceptance by detector—Centered at 90° to the incident light path and not to exceed ±30° from 90°. The detector and filter system, if used, shall have a spectral peak response between 400 and 600 nm.

b. Sample cells: Use sample cells or tubes of clear, colorless glass or plastic. Keep cells scrupulously clean, both inside and out, and discard if scratched or etched. Never handle them where the instrument's light beam will strike them. Use tubes with sufficient extra length, or with a protective case, so that they may be handled properly. Fill cells with samples and standards that have been agitated thoroughly and allow sufficient time for bubbles to escape.

Clean sample cells by thorough washing with laboratory soap inside and out followed by multiple rinses with distilled or deionized water; let cells air-dry. Handle sample cells only by the top to avoid dirt and fingerprints within the light path.

Cells may be coated on the outside with a thin layer of silicone oil to mask minor imperfections and scratches that may contribute to stray light. Use silicone oil with the same refractive index as glass. Avoid excess oil because it may attract dirt and contaminate the sample compartment of the instrument. Using a soft, lint-free cloth, spread the oil uniformly and wipe off excess. The cell should appear to be nearly dry with little or no visible oil.

Because small differences between sample cells significantly impact measurement, use either matched pairs of cells or the same cell for both standardization and sample measurement.

3. Reagents

a. Dilution water: High-purity water will cause some light scattering, which is detected by nephelometers as turbidity. To obtain low-turbidity water for dilutions, nominal value 0.02 NTU, pass laboratory reagent-grade water through a filter with pore size sufficiently small to remove essentially all particles larger than 0.1 μm;* the usual membrane filter used for bacteriological examinations is not satisfactory. Rinse collecting flask at least twice with filtered water and discard the next 200 mL.

Some commercial bottled demineralized waters have a low turbidity. These may be used when filtration is impractical or a good grade of water is not available to filter in the laboratory. Check turbidity of bottled water to make sure it is lower than the level that can be achieved in the laboratory.

b. Stock primary standard formazin suspension:

1) Solution I—Dissolve 1.000 g hydrazine sulfate, $(NH_2)_2 \cdot H_2SO_4$, in distilled water and dilute to 100 mL in a volumetric flask. **CAUTION: Hydrazine sulfate is a carcinogen; avoid inhalation, ingestion, and skin contact. Formazin suspensions can contain residual hydrazine sulfate.**

2) Solution II—Dissolve 10.00 g hexamethylenetetramine, $(CH_2)_6N_4$, in distilled water and dilute to 100 mL in a volumetric flask.

3) In a flask, mix 5.0 mL Solution I and 5.0 mL Solution II. Let stand for 24 h at 25 ± 3°C. This results in a 4000-NTU suspension. Transfer stock suspension to an amber glass or other UV-light-blocking bottle for storage. Make dilutions from this stock suspension. The stock suspension is stable for up to 1 year when properly stored.

c. Dilute turbidity suspensions: Dilute 4000 NTU primary standard suspension with high-quality dilution water. Prepare immediately before use and discard after use.

d. Secondary standards: Secondary standards are standards that the manufacturer (or an independent testing organization) has certified will give instrument calibration results equivalent (within certain limits) to the results obtained when the instrument is calibrated with the primary standard (i.e., user-prepared formazin). Various secondary standards are available including: commercial stock suspensions of 4000 NTU formazin, commercial suspensions of microspheres of styrene-divinylbenzene co-

* Nuclepore Corp., 7035 Commerce Circle, Pleasanton, CA, or equivalent.

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polymer,† and items supplied by instrument manufacturers, such as sealed sample cells filled with latex suspension or with metal oxide particles in a polymer gel. The U.S. Environmental Protection Agency[1] designates user-prepared formazin, commercial stock formazin suspensions, and commercial styrene-divinylbenzene suspensions as "primary standards," and reserves the term "secondary standard" for the sealed standards mentioned above.

Secondary standards made with suspensions of microspheres of styrene-divinylbenzene copolymer typically are as stable as concentrated formazin and are much more stable than diluted formazin. These suspensions can be instrument-specific; therefore, use only suspensions formulated for the type of nephelometer being used. Secondary standards provided by the instrument manufacturer (sometimes called "permanent" standards) may be necessary to standardize some instruments before each reading and in other instruments only as a calibration check to determine when calibration with the primary standard is necessary.

All secondary standards, even so-called "permanent" standards, change with time. Replace them when their age exceeds the shelf life. Deterioration can be detected by measuring the turbidity of the standard after calibrating the instrument with a fresh formazin or microsphere suspension. If there is any doubt about the integrity or turbidity value of any secondary standard, check instrument calibration first with another secondary standard and then, if necessary, with user-prepared formazin. Most secondary standards have been carefully prepared by their manufacturer and should, if properly used, give good agreement with formazin. Prepare formazin primary standard only as a last resort. Proper application of secondary standards is specific for each make and model of nephelometer. Not all secondary standards have to be discarded when comparison with a primary standard shows that their turbidity value has changed. In some cases, the secondary standard should be simply relabeled with the new turbidity value. Always follow the manufacturer's directions.

4. Procedure

a. General measurement techniques: Proper measurement techniques are important in minimizing the effects of instrument variables as well as stray light and air bubbles. Regardless of the instrument used, the measurement will be more accurate, precise, and repeatable if close attention is paid to proper measurement techniques.

Measure turbidity immediately to prevent temperature changes and particle flocculation and sedimentation from changing sample characteristics. If flocculation is apparent, break up aggregates by agitation. Avoid dilution whenever possible. Particles suspended in the original sample may dissolve or otherwise change characteristics when the temperature changes or when the sample is diluted.

Remove air or other entrained gases in the sample before measurement. Preferably degas even if no bubbles are visible. Degas by applying a partial vacuum, adding a nonfoaming-type surfactant, using an ultrasonic bath, or applying heat. In some cases, two or more of these techniques may be combined for more effective bubble removal. For example, it may be necessary to combine addition of a surfactant with use of an ultrasonic bath for some severe conditions. Any of these techniques, if misapplied, can alter sample turbidity; *use with care*. If degassing cannot be applied, bubble formation will be minimized if the samples are maintained at the temperature and pressure of the water before sampling.

Do not remove air bubbles by letting sample stand for a period of time because during standing, turbidity-causing particulates may settle and sample temperature may change. Both of these conditions alter sample turbidity, resulting in a nonrepresentative measurement.

Condensation may occur on the outside surface of a sample cell when a cold sample is being measured in a warm, humid environment. This interferes with turbidity measurement. Remove all moisture from the outside of the sample cell before placing the cell in the instrument. If fogging recurs, let sample warm slightly by letting it stand at room temperature or by partially immersing it in a warm water bath for a short time. Make sure samples are again well mixed.

b. Nephelometer calibration: Follow the manufacturer's operating instructions. Run at least one standard in each instrument range to be used. Make certain the nephelometer gives stable readings in all sensitivity ranges used. Follow techniques outlined in Sections 2130B.2*b* and 4*a* for care and handling of sample cells, degassing, and dealing with condensation.

c. Measurement of turbidity: Gently agitate sample. Wait until air bubbles disappear and pour sample into cell. When possible, pour well-mixed sample into cell and immerse it in an ultrasonic bath for 1 to 2 s or apply vacuum degassing, causing complete bubble release. Read turbidity directly from instrument display.

d. Calibration of continuous turbidity monitors: Calibrate continuous turbidity monitors for low turbidities by determining turbidity of the water flowing out of them, using a laboratory-model nephelometer, or calibrate the instruments according to manufacturer's instructions with formazin primary standard or appropriate secondary standard.

5. Interpretation of Results

Report turbidity readings as follows:

Turbidity Range *NTU*	Report to the Nearest *NTU*
0–1.0	0.05
1–10	0.1
10–40	1
40–100	5
100–400	10
400–1000	50
>1000	100

When comparing water treatment efficiencies, do not estimate turbidity more closely than specified above. Uncertainties and discrepancies in turbidity measurements make it unlikely that results can be duplicated to greater precision than specified.

† AMCO-AEPA-1 Standard, Advanced Polymer Systems, 3696 Haven Ave., Redwood City, CA, or equivalent.

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2150 ODOR*

2150 A. Introduction

1. Discussion

Odor, like taste, depends on contact of a stimulating substance with the appropriate human receptor cell. The stimuli are chemical in nature and the term "chemical senses" often is applied to odor and taste. Water is a neutral medium, always present on or at the receptors that perceive sensory response. In its pure form, water is odor-free. Man and other animals can avoid many potentially toxic foods and waters because of adverse sensory response. These senses often provide the first warning of potential hazards in the environment.

Odor is recognized[1] as a quality factor affecting acceptability of drinking water (and foods prepared with it), tainting of fish and other aquatic organisms, and esthetics of recreational waters. Most organic and some inorganic chemicals contribute taste or odor. These chemicals may originate from municipal and industrial waste discharges, from natural sources such as decomposition of vegetable matter, or from associated microbial activity, and from disinfectants or their products.

The potential for impairment of the sensory quality of water has increased as a result of expansion in the variety and quantity of waste materials, demand for water disposal of captured air pollutants, and increased reuse of available water supplies by a growing population. Domestic consumers and process industries such as food, beverage, and pharmaceutical manufacturers require water essentially free of tastes and odors.

Some substances, such as certain inorganic salts, produce taste without odor and are evaluated by taste testing (Section 2160). Many other sensations ascribed to the sense of taste actually are odors, even though the sensation is not noticed until the material is taken into the mouth. Because some odorous materials are detectable when present in only a few nanograms per liter, it is usually impractical and often impossible to isolate and identify the odor-producing chemical. The human nose is the practical odor-testing device used in this method. Odor tests are performed to provide qualitative descriptions and approximate quantitative measurements of odor intensity. The method for intensity measurement presented here is the *threshold odor* test, based on a method of limits.[2] This procedure, while not universally preferred,[3] has definite strengths.[4]

Sensory tests are useful as a check on the quality of raw and finished water and for control of odor through the treatment process.[2,3,5] They can assess the effectiveness of different treatments and provide a means of tracing the source of contamination.

Section 6040 provides an analytical procedure for quantifying several organic odor-producing compounds including geosmin and methylisoborneol.

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2150 B. Threshold Odor Test

1. General Discussion

a. Principle: Determine the threshold odor by diluting a sample with odor-free water until the least definitely perceptible odor is achieved. There is no absolute threshold odor concentration, because of inherent variation in individual olfactory capability. A given person varies in sensitivity over time. Day-to-day and within-day differences occur. Furthermore, responses vary as a result of the characteristic, as well as concentration, of odorant. The number of persons selected to measure threshold odor will depend on the objective of the tests, economics, and available personnel. Larger-sized panels are needed for sensory testing when the results must represent the population as a whole or when great precision is desired. Under such circumstances, panels of no fewer than five persons, and preferably ten or more, are recommended.[1] Measurement of threshold levels by one person is often a necessity at water treatment plants. Interpretation of the single tester result requires knowledge of the relative acuity of that person. Some investigators have used specific odorants, such as *m*-cresol or *n*-butanol, to calibrate a tester's response.[2] Others have used the University of Pennsylvania Smell Identification Test to assess an individual's ability to identify odors correctly.[3]

b. Application: This threshold method is applicable to samples ranging from nearly odorless natural waters to industrial wastes with threshold numbers in the thousands. There are no intrinsic difficulties with the highly odorous samples because they are reduced in concentration proportionately before being presented to the test observers.

c. Qualitative descriptions: A fully acceptable system for characterizing odor has not been developed despite efforts over more than a century. Nevertheless, Section 2170 (Flavor Profile Analysis) specifies a set of 23 odor reference standards that may be used if qualitative descriptions are important. These descriptors can be used with the Threshold Odor Test to standardize methods for sensory analysis.

d. Sampling and storage: Collect samples for odor testing in glass bottles with glass or TFE-lined closures. Complete tests as soon as possible after sample collection. If storage is necessary, collect at least 500 mL of sample in a bottle filled to the top; refrigerate, making sure that no extraneous odors can be drawn into the sample as it cools. Do not use plastic containers.

e. Dechlorination: Most tap waters and some wastewaters are chlorinated. Often it is desirable to determine the odor of the chlorinated sample as well as that of the same sample after dechlorination. Dechlorinate with thiosulfate in exact stoichiometric quantity as described under Nitrogen (Ammonia), Section 4500-NH_3.

f. Temperature: Threshold odor values vary with temperature. For most tap waters and raw water sources, a sample temperature of 60°C will permit detection of odors that otherwise might be missed; 60°C is the standard temperature for hot threshold odor tests. For some purposes—because the odor is too fleeting or there is excessive heat sensation—the hot odor test may not be applicable; where experience shows that a lower temperature is needed, use a standard test temperature of 40°C. For special purposes, other temperatures may be used. *Report temperature at which observations are made.*

g. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 2020:II.

2. Apparatus

To assure reliable threshold measurements, use odor-free glassware. Clean glassware shortly before use with nonodorous soap and acid cleaning solution and rinse with odor-free water. Reserve this glassware exclusively for threshold testing. Do not use rubber, cork, or plastic stoppers. Do not use narrow-mouth vessels.

a. Sample bottles, glass-stoppered or with TFE-lined closures.

b. Constant-temperature bath: A water bath or electric hot plate capable of temperature control of ± 1°C for odor tests at elevated temperatures. The bath must not contribute any odor to the odor flasks.

c. Odor flasks: Glass-stoppered, 500-mL (ST 32) erlenmeyer flasks, to hold sample dilutions during testing.

d. Pipets:

1) *Transfer and volumetric pipets or graduated cylinders:* 200-, 100-, 50-, and 25-mL.

2) *Measuring pipets:* 10-mL, graduated in tenths.

e. Thermometer: Zero to 110°C, chemical or metal-stem dial type.

3. Odor-Free Water

a. Sources: Prepare odor-free water by passing distilled, deionized, or tap water through activated carbon. If product water is not odor-free, rebuild or purify the system. In all cases verify quality of product water daily.

b. Odor-free-water generator (Figure 2150:1):* Make the PVC generator from a 2-ft length of 4-in. PVC pipe approved for use for drinking water purposes (e.g., Schedule 80, or National Water Council-approved in U.K.). Thread pipe end to accept threaded caps. Have a small threaded nipple in the cap center for water inlet or outlet. To retain the activated carbon, place coarse glass wool in top and bottom of generator. Regulate water flow to generator by a needle valve and a pressure regulator to provide the minimum pressure for the desired flow. Use activated carbon of approximately 12 to 40 mesh grain size.†

c. Generator operation: Pass tap or purified water through odor-free-water generator at rate of 100 mL/min. When generator is started, flush to remove carbon fines and discard product, or pre-rinse carbon.

Check quality of water obtained from the odor-free-water generator daily at 40 and 60°C before use. The life of the carbon will vary with the condition and amount of water filtered. Subtle odors of biological origin often are found if moist carbon filters stand idle

* For approximate metric dimensions in centimeters multiply dimensions in inches by 2.54.

† Nuchar WV-G, Westvaco, Covington, VA; Filtrasorb 200, Calgon Carbon Corporation, Pittsburgh, PA; or equivalent.

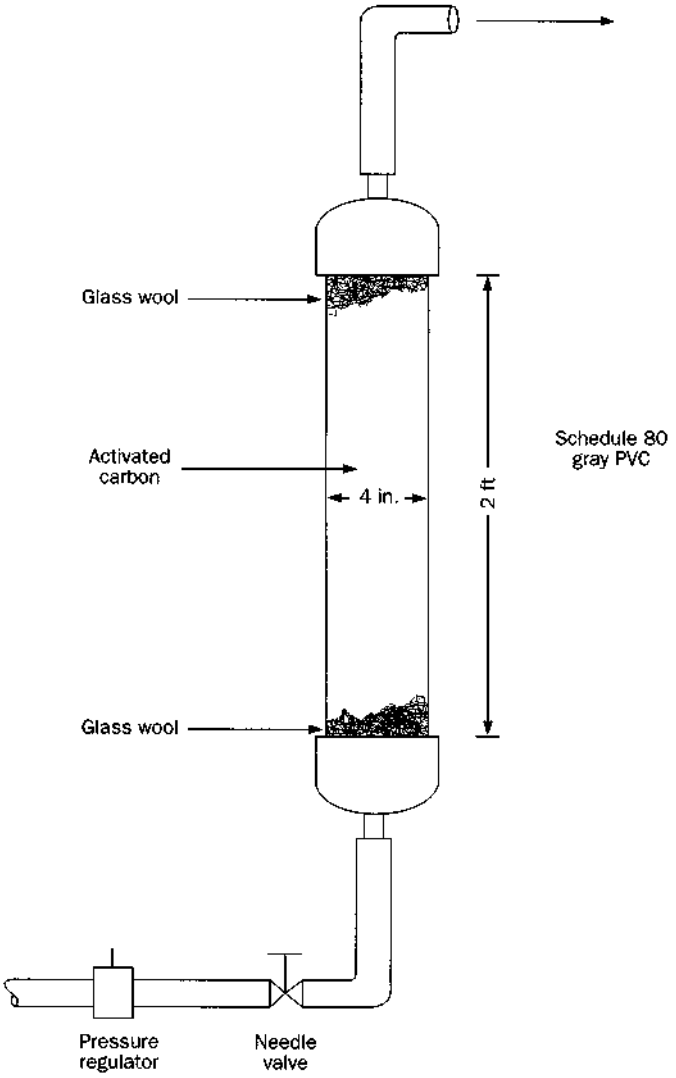


Figure 2150:1. Odor-free-water generator.

between test periods. Detection of odor in the water coming through the carbon indicates that a change of carbon is needed.

4. Procedure

a. Precautions: Carefully select by preliminary tests the persons to make taste or odor tests. Although extreme sensitivity is not required, exclude insensitive persons and concentrate on observers who have a sincere interest in the test. Avoid extraneous odor stimuli such as those caused by smoking and eating before the test or those contributed by scented soaps, perfumes, and shaving lotions. Insure that the tester is free from colds or allergies that affect odor response. Limit frequency of tests to a number below the fatigue level by frequent rests in an odor-free atmosphere. Keep room in which tests are conducted free from distractions, drafts, and odor.[2] If necessary, set aside a special odor-free room ventilated by air that is filtered through activated carbon and maintained at a constant comfortable temperature and humidity.[4]

TABLE 2150:I. THRESHOLD ODOR NUMBERS CORRESPONDING TO VARIOUS DILUTIONS

Sample Volume Diluted to 200 mL *mL*	Threshold Odor No.	Sample Volume Diluted to 200 mL *mL*	Threshold Odor No.
200	1	12.0	17
140	1.4	8.3	24
100	2	5.7	35
70	3	4.0	50
50	4	2.8	70
35	6	2.0	100
25	8	1.4	140
17	12	1.0	200

For precise work use a panel of five or more testers. Do not allow persons making odor measurements to prepare samples or to know dilution concentrations being evaluated. Familiarize testers with the procedure before they participate in a panel test. Present most dilute sample first to avoid tiring the senses with the concentrated sample. Keep temperature of samples during testing within 1°C of the specified temperature.

Because many raw and waste waters are colored or have decided turbidity that may bias results, use opaque or darkly colored odor flasks, such as red actinic erlenmeyer flasks.

b. Characterization: As part of the threshold test or as a separate test, direct each observer to describe the characteristic sample odor using odor reference standards (see Section 2170). Compile the consensus that may appear among testers and that affords a clue to the origin of the odorous component. The value of the characterization test increases as observers become more experienced with a particular category of odor, e.g., earthy, musty, chlorine.

c. Threshold measurement:‡ The "threshold odor number," designated by the abbreviation TON, is the greatest dilution of sample with odor-free water yielding a definitely perceptible odor. Bring total volume of sample and odor-free water to 200 mL in each test. Follow dilutions and record corresponding TON presented in Table 2150:I. These numbers have been computed thus:

$$\text{TON} = \frac{A + B}{A}$$

where:

A = mL sample and
B = mL odor-free water.

1) Place proper volume of odor-free water in the flask first, add sample to water (avoiding contact of pipet or sample with lip or neck of flask), mix by swirling, and proceed as follows:

Determine approximate range of the threshold number by adding 200 mL, 50 mL, 12 mL, and 2.8 mL sample to separate

‡ There are numerous methods of arranging and presenting samples for odor determinations. The methods offered here are practical and economical of time and personnel. If extensive tests are planned and statistical analysis of data is required, become familiar with the more accurate methods that have been used extensively by flavor and allied industries.[5]

TABLE 2150:II. DILUTIONS FOR VARIOUS ODOR INTENSITIES

Sample Volume in Which Odor Is First Noted *mL*	Volumes to Be Diluted to 200 mL *mL*
200	200, 140, 100, 70, 50
50	50, 35, 25, 17, 12
12	12, 8.3, 5.7, 4.0, 2.8
2.8	Intermediate dilution

500-mL glass-stoppered erlenmeyer flasks containing odor-free water to make a total volume of 200 mL. Use a separate flask containing only odor-free water as reference for comparison. Heat dilutions and reference to desired test temperature.

2) Shake flask containing odor-free water, remove stopper, and sniff vapors. Test sample containing least amount of odor-bearing water in the same way. If odor can be detected in this dilution, prepare more dilute samples as described in ¶ 5) below. If odor cannot be detected in first dilution, repeat above procedure using sample containing next higher concentration of odor-bearing water, and continue this process until odor is detected clearly.

3) Based on results obtained in the preliminary test, prepare a set of dilutions using Table 2150:II as a guide. Prepare the five dilutions shown on the appropriate line and the three next most concentrated on the next line in Table 2150:II. For example, if odor was first noted in the flask containing 50 mL sample in the preliminary test, prepare flasks containing 50, 35, 25, 17, 12, 8.3, 5.7, and 4.0 mL sample, each diluted to 200 mL with odor-free water. This array is necessary to challenge the range of sensitivities of the entire panel of testers.

Insert two or more blanks in the series near the expected threshold, but avoid any repeated pattern. Do not let tester know which dilutions are odorous and which are blanks. Instruct tester to smell each flask in sequence, beginning with the least concentrated sample, until odor is detected with certainty.

4) Record observations by indicating whether odor is noted in each test flask. For example:

mL Sample Diluted to 200 mL	12	0	17	25	0	35	50
Response	−	−	−	+	−	+	−

5) If the sample being tested requires more dilution than is provided by Table 2150:II, prepare an intermediate dilution consisting of 20 mL sample diluted to 200 mL with odor-free water. Use this dilution for the threshold determination. Multiply TON obtained by 10 to correct for the intermediate dilution. In rare cases more than one tenfold intermediate dilution step may be required.

5. Calculation

The threshold odor number is the dilution ratio at which odor is just detectable. In the example above, ¶ 4*c*4), the first detectable odor occurred when 25 mL sample was diluted to 200 mL. Thus the threshold is 200 divided by 25, or 8. Table 2150:I lists the threshold numbers corresponding to common dilutions.

The smallest TON that can be observed is 1, as in the case where the odor flask contains 200 mL undiluted sample. If no odor is detected at this concentration, report "No odor observed" instead of a threshold number. (In special applications, fractional threshold numbers have been calculated.[6])

Anomalous responses sometimes occur; a low concentration may be called positive and a higher concentration in the series may be called negative. In such a case, designate the threshold as the point after which no further anomalies occur. For instance:

Increasing Concentration →

Response − − + − + + + +

↓

Threshold

where:

− signifies negative response and
\+ signifies positive response.

Occasionally a flask contains residual odor or is contaminated inadvertently. For precise testing repeat entire threshold odor test to determine if the last flask marked " − " was actually a mislabelled blank of odor-free water or if the previous " + " was a contaminated sample.

Use appropriate statistical methods to calculate the most probable average threshold from large numbers of panel results. For most purposes, express the threshold of a group as the geometric mean of individual thresholds.

6. Interpretation of Results

A threshold number is not a precise value. In the case of the single observer it represents a judgment at the time of testing. Panel results are more meaningful because individual differences have less influence on the result. One or two observers can develop useful data if comparison with larger panels has been made to check their sensitivity. Do not make comparisons of data from time to time or place to place unless all test conditions have been standardized carefully and there is some basis for comparison of observed intensities.

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2160 TASTE*

2160 A. Introduction

1. General Discussion

Taste refers only to gustatory sensations called bitter, salty, sour, and sweet that result from chemical stimulation of sensory nerve endings located in the papillae of the tongue and soft palate. Flavor refers to a complex of gustatory, olfactory, and trigeminal sensations resulting from chemical stimulation of sensory nerve endings located in the tongue, nasal cavity, and oral cavity.[1] Water samples taken into the mouth for sensory analysis always produce a flavor, although taste, odor, or mouthfeel may predominate, depending on the chemical substances present. Methods for sensory analysis presented herein require that the sample be taken into the mouth, that is, be tasted, but technically the sensory analysis requires evaluation of the complex sensation called flavor. As used here, taste refers to a method of sensory analysis in which samples are taken into the mouth but the resultant evaluations pertain to flavor.

Three methods have been developed for the sensory evaluation of water samples taken into the mouth: the flavor threshold test (FTT), the flavor rating assessment (FRA), and the flavor profile analysis (FPA) (Section 2170). The FTT is the oldest. It has been used extensively and is particularly useful for determining if the overall flavor of a sample of finished water is detectably different from a defined standard.[2] The FRA is especially valuable for determining if a sample of finished water is acceptable for daily consumption,[3] and the FPA is most useful for identifying and characterizing individual flavors in a water sample.[4]

Make flavor tests only on samples known to be safe for ingestion. Do not use samples that may be contaminated with bacteria, viruses, parasites, or hazardous chemicals, that contain dechlorinating agents such as sodium arsenite or that are derived from an unesthetic source. Do not make flavor tests on wastewaters or similar untreated effluents. Observe all sanitary and esthetic precautions with regard to apparatus and containers contacting the sample. Properly clean and sterilize containers before using them. Conduct analyses in a laboratory free from interfering background odors and if possible provide non-odorous carbon-filtered air at constant temperature and humidity. Use the procedure described in Section 2150B.3 with respect to taste- and odor-free water to prepare dilution water and reference samples.

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2160 B. Flavor Threshold Test (FTT)

1. General Discussion

Use the FTT to measure detectable flavor quantitatively. More precisely, use the method to compare the sample flavor objectively with that of specified reference water used as diluent.

The flavor threshold number (FTN) is the greatest dilution of sample with reference water yielding a definitely perceptible difference. The FTN is computed as follows:

$$\text{FTN} = \frac{A + B}{A}$$

where:

A = sample volume, mL, and
B = reference water (diluent) volume, mL.

Table 2160:I gives the FTNs corresponding to various dilutions. T1

The QC practices considered to be an integral part of each method are summarized in Table 2020:II.

2. Procedure

a. Panel selection: Carefully select by preliminary trials interested persons to make flavor tests. Exclude insensitive persons and ensure that the testers are free from colds or allergies. Familiarize testers with the procedure before they participate in a panel test, but do not let them prepare samples or know dilution concentrations being evaluated. For precise work use a panel of five or more testers.

b. Taste characterization: Have each observer describe the characteristic sample flavor of the most concentrated sample. Compile the consensus that may appear among testers. The value of characterization increases as observers become more experienced with a particular flavor category such as chlorophenolic, grassy, or musty.

c. Preliminary test: To determine approximate range of the FTN, add 200-, 50-, 12-, and 4-mL sample portions to volumes of reference water (see Section 2150B.3) designated in Table

Table 2160:I. Flavor Threshold Numbers Corresponding to Various Dilutions

Sample Volume *mL*	Diluent Volume *mL*	Flavor Threshold No. *FTN*
200	0	1
100	100	2
70	130	3
50	150	4
35	165	6
25	175	8
17	183	12
12	188	17
8	192	25
6	194	33
4	196	50
3	197	67
2	198	100
1	199	200

2160:I in separate 300-mL glass beakers to make a total of 200 mL in each beaker, and mix gently with clean stirrer. Use separate beaker containing only reference water for comparison. Keep sample temperature during testing within 1°C of specified temperature. Present samples to each taster in a uniform manner, with the reference water presented first, followed by the most dilute sample. If a flavor can be detected in this dilution, prepare an intermediate sample by diluting 20 mL sample to 200 mL with reference water. Use this dilution for threshold determination and multiply FTN obtained by 10 to correct for intermediate dilution. In rare cases a higher intermediate dilution may be required.

If no flavor is detected in the most dilute sample, repeat using the next concentration. Continue this process until flavor is detected clearly.

d. FTN determination: Based on results obtained in the preliminary test, prepare a set of dilutions using Table 2160:II as a guide. Prepare the seven dilutions shown on the appropriate line. This array is necessary to challenge the range of sensitivities of the entire panel of testers. If the sample being tested requires more dilution than is provided by Table 2160:II, make intermediate dilutions as directed in ¶ *c* above.

Use a clean 50-mL beaker filled to the 25-mL level or use an ordinary restaurant-style drinking glass for each dilution and reference sample. Do not use glassware used in sensory testing for other analyses. Between tests, sanitize containers in an automatic dishwasher supplied with water at not less than 60°C.

Table 2160:II. Dilutions for Determining the FTN

Sample Volume in Which Taste Is First Noted *mL*	Volumes to be Diluted to 200 mL *mL*
200	200, 100, 70, 50, 35, 25, 17
50	50, 35, 25, 17, 12, 8, 6
12	12, 8, 6, 4, 3, 2, 1
4	Intermediate dilution

Maintain samples at 15 ± 1°C. However, if temperature of water in the distribution system is higher than 15°C, select an appropriate temperature. Specify temperature in reporting results.

Present series of samples to each tester in order of increasing concentration. Pair each sample with a known reference. Have tester taste sample by taking into the mouth whatever volume is comfortable, moving sample throughout the mouth, holding it for several seconds, and discharging it without swallowing. Have tester compare sample with reference and record whether a flavor or aftertaste is detectable. Insert two or more reference blanks in the series near the expected threshold, but avoid any repeated pattern. Do not let tester know which samples have flavor and which are blanks. Instruct tester to taste each sample in sequence, beginning with the least concentrated sample, until flavor is detected with certainty.

Record observations by indicating whether flavor is noted in each test beaker. For example:

mL Sample Diluted to 200 mL	6	8	12	0	17	25	35	0	50
Response	−	−	−	−	−	+	+	−	+

where:

− signifies negative response and
+ signifies positive response.

3. Calculation

The flavor threshold number is the dilution ratio at which flavor is just detectable. In the example above, the first detectable flavor occurred when 25 mL sample was diluted to 200 mL yielding a threshold number of 8 (Table 2160:I). Reference blanks do not influence calculation of the threshold.

The smallest FTN that can be observed is 1, where the beaker contains 200 mL undiluted sample. If no flavor is detected at this concentration, report "No flavor observed" instead of a threshold number.

Anomalous responses sometimes occur; a low concentration may be called positive and a higher concentration in the series may be called negative. In such cases, designate the threshold as that point after which no further anomalies occur. The following illustrates an approach to an anomalous series (responses to reference blanks are excluded):

	Increasing Concentration →						
Response:	−	+	−	+	+	+	+
			↓				
			Threshold				

Calculate mean and standard deviation of all FTNs if the distribution is reasonably symmetrical; otherwise, express the threshold of a group as the median or geometric mean of individual thresholds.

4. Interpretation of Results

An FTN is not a precise value. In the case of the single observer it represents a judgment at the time of testing. Panel

results are more meaningful because individual differences have less influence on the test result. One or two observers can develop useful data if comparison with larger panels has been made to check their sensitivity. Do not make comparisons of data from time to time or place to place unless all test conditions have been standardized carefully and there is some basis for comparison of observed FTNs.

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2160 C. Flavor Rating Assessment (FRA)

1. General Discussion

When the purpose of the test is to estimate acceptability for daily consumption, use the flavor rating assessment described below. This procedure has been used with samples from public sources in laboratory research and consumer surveys to recommend standards governing mineral content in drinking water. Each tester is presented with a list of nine statements about the water ranging on a scale from very favorable to very unfavorable. The tester's task is to select the statement that best expresses his or her opinion. The individual rating is the scale number of the statement selected. The panel rating for a particular sample is an appropriate measure of central tendency of the scale numbers for all testers for that sample.

2. Samples

Sample finished water ready for human consumption or use experimentally treated water if the sanitary requirements given in 2160A.1 are met fully. Use taste- and odor-free water as described in Section 2150B.3 and a solution of 2000 mg NaCl/L prepared with taste- and odor-free water as criterion samples.

3. Procedure

a. Panel selection and preparation: Give prospective testers thorough instructions and trial or orientation sessions followed by questions and discussion of procedures. In tasting samples, testers work alone. Select panel members on the basis of performance in these trial sessions. Do not let testers know the composition or source of specific samples.

b. Rating test: A single rating session may be used to evaluate up to 10 samples, including the criterion samples mentioned in 2160C.2. Allow at least 30 min rest between repeated rating sessions.

For glassware requirements, see 2160B.2*d*.

Present samples at a temperature that the testers will find pleasant for drinking water; maintain this temperature throughout testing. A temperature of 15°C is recommended, but in any case, do not let the test temperature exceed tap water temperatures customary at the time of the test. Specify test temperature in reporting results.

Independently randomize sample order for each tester. Instruct each to complete the following steps:

1) Taste about half the sample by taking water into the mouth, holding it for several seconds, and discharging it without swallowing;
2) Form an initial judgment on the rating scale;
3) Make a second tasting in a similar manner;
4) Make a final rating and record result on an appropriate data form;
5) Rinse mouth with reference water;
6) Rest 1 min before repeating Steps 1 through 5 on next sample.

c. Characterization: If characterization of flavor also is required, conduct a final rating session wherein each tester is asked to describe the flavor of each sample rated (see 2160B.2*b*).

4. Calculation

Use the following scale for rating. Record ratings as integers ranging from one to nine, with one given the highest quality rating. Calculate mean and standard deviation of all ratings if the distribution is reasonably symmetrical, otherwise express the most typical rating of a group as the median or geometric mean of individual ratings.

Action tendency scale:

1) I would be very happy to accept this water as my everyday drinking water.
2) I would be happy to accept this water as my everyday drinking water.
3) I am sure that I could accept this water as my everyday drinking water.
4) I could accept this water as my everyday drinking water.
5) Maybe I could accept this water as my everyday drinking water.
6) I don't think I could accept this water as my everyday drinking water.
7) I could not accept this water as my everyday drinking water.
8) I could never drink this water.
9) I can't stand this water in my mouth and I could never drink it.

5. Interpretation of Results

Values representing the central tendency and dispersion of quality ratings for a laboratory panel are only estimates of these values for a defined consuming population.

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2170 FLAVOR PROFILE ANALYSIS*

2170 A. Introduction

1. Discussion

Flavor profile analysis (FPA) is one of several techniques for identifying water sample taste(s) and odor(s).[1] (For general information on taste, see Section 2160; for information on odor, see Section 2150.)

FPA is designed to evaluate the tastes and odors important in drinking water. It is not used to judge a sample, to determine preferences between samples, or to determine acceptance of a water sample for public consumption. FPA is used to define a water sample's taste and odor attributes, which then could be related to, or associated with, consumer perceptions (e.g., customer complaints[2]).

One of FPA's strengths is its use of well-trained panelists who use specific, standardized terminology and can discern and express small differences between samples. Each panelist assigns to each sample descriptors that characterize the taste(s) and/or odor(s) detected. Each descriptor is given an intensity (relative to standards) that indicates the strength of each taste and odor. After individual evaluations, panelists share results and attempt to come to a consensus of descriptors and intensities for each sample. The quality of training and data interpretation determine the value of FPA results.

Because dilution may change measured descriptors,[3,4] FPA determines the strength or intensity of each perceived taste or odor without sample dilution.

2. FPA Can Also Be Used for Odor Threshold Measurements

FPA measures two types of thresholds: recognition thresholds and detection thresholds. A *recognition threshold* is the lowest concentration at which a stimulus can be identified and recognized. A *detection threshold* is "a concentration range . . . below which the odor or taste of a substance will not be detectable under any practical circumstances, and above which individuals with a normal sense of smell or taste would readily detect the presence of the substance."[5] Generally, odor detection threshold concentrations are lower than odor recognition threshold concentrations.

Sensory detection thresholds are similar in concept to method detection levels for chemical analyses. As with a method detection level, a sensory threshold is a point where quantification and accuracy are weakest; small changes in testing conditions, or small levels of contamination or background interference, greatly affect results. Moreover, sensory thresholds usually have much greater variability than method detection levels.[6]

Thresholds can be determined for individuals or for a group. Thresholds across individuals are much more variable than group thresholds, although even group thresholds can vary by two orders of magnitude for the same chemical in water. In the sensory threshold region, the effects of human test variability are significant, so reporting a threshold value as a single number can sometimes be misleading.[7,8] Practice and training can produce lower, more consistent thresholds.

Several standardized tests are available[5,9] for detection threshold measurement by individuals and groups. These forced-choice methods use a series of concentrations selected to bracket the expected threshold level. At each concentration, a panelist typically evaluates three samples: one with the odor in it and two without. Each panelist must choose which sample has the odor. The FPA presented here also can be used to estimate individual or group odor thresholds. Typically, an FPA intensity rating of 2 (see 2170B.6) is equated with a recognition threshold. Depending on the concentration range selected, the results occasionally must be extrapolated to achieve the rating of 1. A Weber–Fechner plot[4] of FPA rating versus log concentration can be used for this purpose.

Both analytical and sensory detection thresholds are statistical interpretations of measured data. This may be why a practical method for estimating odor thresholds in drinking water has not yet been widely used by drinking water utilities. Any report of a threshold value must include a description of the panelists, their level of training, the threshold measurement technique, and the statistical analysis used, because these variables can profoundly affect results, and the details are important when evaluating the reported threshold's reliability and context.

3. Safety

See 2170B.2.

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2170 B. Flavor Profile Analysis

1. General Discussion

a. Principle:[1] FPA uses a group of four or five trained panelists to examine the sensory characteristics of samples. Flavor attributes are determined by tasting; odor attributes are determined by sniffing the sample. The method allows more than one flavor, odor, or feeling (e.g., drying, burning) attribute to be determined per sample and each attribute's intensity to be measured. Figure 2170:1 shows

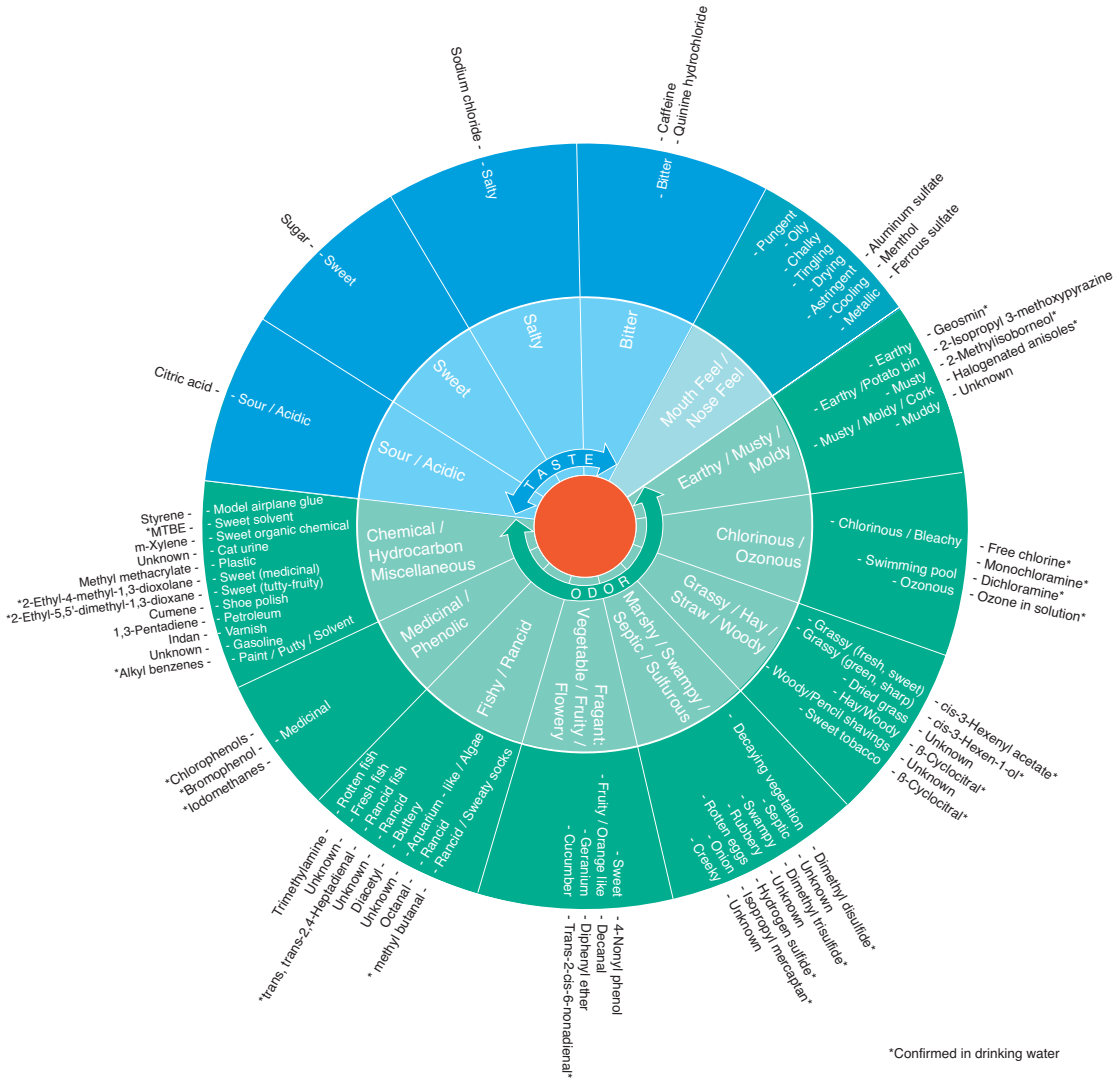


Figure 2170:1. Taste and odor wheel, showing flavor and odor attributes and example compounds. Source: SUFFET, I.H., J. HO, D. CHOU, D. KHIARI & J. MALLEVIALLE 1995. Taste-and-odor problems observed during drinking water treatment. In I.H. Suffet, J. Mallevialle & E. Kawczynski, eds. Advances in Taste and Odor Treatment and Control. American Water Works Assoc. Research Foundation & Lyonnaise des Eaux, Denver, Colo., p.6. (modified 2006). For a color version of this figure, contact *Standard Methods* technical information manager at www.standardmethods.org/contact/.

the relationships among these attributes and factors, as well as specific compounds that exemplify them.

Initially, panelists record their perceptions without discussion. Individual results are considered important, even though the reported result is a consensus. Individual results might alert the panel to a "new" odor or to a change. Once each individual has made an independent assessment of a sample, the panel discusses its findings and reaches a consensus without changing individual results.

b. Interference: Background odors present during analysis affect results. Analyst illness (e.g., cold or allergy) and a recent history of eating, drinking, or smoking can diminish or otherwise alter perception.

c. Application:[1–4] FPA has been applied to drinking water sources, finished drinking water, sampling points in the drinking water treatment train, and bottled waters. It also has been used to investigate customer complaints.

d. Quality control (QC): The QC steps considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Safety

a. Samples: Some substances are health hazards when inhaled or ingested. Do not use this method for industrial wastes or other samples suspected to contain high concentrations of hazardous substances. Chemically analyze suspect samples to determine whether hazardous chemicals are present before making FPA. Do not taste untreated drinking water unless certain that no health threat (biological or chemical) exists.

b. Standards: Avoid human exposure to known or probable human carcinogens. An exposure assessment of chemicals that could be used in FPA[5] revealed that, in most cases, the levels and duration of exposure to chemical standards for training and calibration are well below levels of concern and should pose no known health risks when properly used for FPA.

3. Panel Selection and Training

Carefully select and train a group of panelists according to procedures available from the American Water Works Association.[6] Because four or five panelists are needed to conduct a test, training of additional panelists may be desirable to allow for unavailability of individuals. Because FPA includes discussions during which panelists reach consensus on sample descriptions, avoid including on the panel a person with a dominating personality. The opinions of a senior member or panel leader also may have this undesired effect.

Panelists must be able to detect and recognize various odors present. The training of panelists using odor reference standards is important for developing a consistent terminology within the panel and over time.[7] Trained panelists have been shown to produce more reliable sensory data.[8] FPA requires well-trained panelists and data interpreters. Reproducibility of results depends on the panelists' training and experience.

4. Apparatus[3,4,7,9,10]

Reserve apparatus and glassware exclusively for FPA.

a. Sample bottles: Glass, 1-L, with TFE-lined closure.

b. Containers for odor analysis:[3,4,7,10] Select containers for odor analysis depending on panelists' preference, sample temperature, and container availability.

1) Panel preference—Plastic cups, 7-, 8-, or 16-oz (207-, 237-, or 473-mL), disposable, are convenient. Do not use waxed-lined paper cups. Some panels have found that plastic cups impart a plastic and/or floral odor to samples and therefore prefer wide-mouth, 500-mL Erlenmeyer flasks with ST32 ground-glass stoppers. The panel should determine the acceptable, odor-free test container (glass or plastic) most suitable to its use.

2) Temperature[3,9–11]—If odor samples are tested at 45°C, do not use plastic containers. If odor samples are tested at 25°C, use the same plastic cup and sample for both odor and flavor analysis. Temperature is known to affect the intensity of an odor. For most compounds, the odor will increase as the temperature increases because more molecules of the odor-producing substance (*odorant*) will volatilize from the water sample and enter the nose. Testing at 45°C will produce a greater odor intensity and more likely chance of detection by panelists than testing at 25°C. (NOTE: There are a few exceptions, such as dissolved calcium carbonate, where the compound is less volatile or more soluble as temperature increases).

3) Availability[3]—Containers must be consistently available because panel responses may change when different containers are used.

c. Containers for flavor analysis:[3,7,9,10] Use either disposable odor- and taste-free plastic cups, or glass containers. Do not use wax-lined or paper cups. If using glass containers, verify that they contribute no taste to the sample. Wash glass containers as directed in 2170B.7*a*.

d. Watch glasses to cover the plastic cups.

e. Constant-temperature water bath capable of maintaining a temperature ±1°C. The bath must not contribute any odor to the sample containers or testing room.

f. Thermometer, graduated 0 to 110°C, chemical or metal-stem dial type.

g. Syringes.

h. Ice chest for storing samples.

i. Odor-free testing room:[3,7,9,10] Hold FPA sessions in a clean, well-lit, quiet, odor-free, and temperature-controlled room. The room should have an area with a table and chairs set up so panelists can first perform the tests individually and then hold a group discussion. Place an easel pad in the room so all panelists can see it. Use an easel pad with odor-free markers (e.g., a wax, china marker).

j. Refrigerator capable of maintaining a temperature of 4°C.

5. Reagents

a. Odor-free water: See Section 2150B.3.

b. Crackers: Use salt- and flavor-free crackers* to cleanse the palate during taste testing. Before tasting anything, use the crackers and taste-free water to cleanse the palate. Use crackers between samples to reduce carryover of perceptions.

c. Odor and flavor standards:[3,4,7,9,10,12–14] Odor and flavor reference standards have been developed for use as a guide for qualitative descriptors (names for odors). Odor reference standards help panelists agree on the descriptor for specific odors.

* Carr's Table Water Crackers—plain, or equivalent.

TABLE 2170:I. CONFIRMED ODOR REFERENCES*

Compound	Odor Characteristics	Stock Solution Concentration *mg/L*	Amount Placed into 200 mL Pure Water at 25°C for Presentation
Geosmin	Earthy, red beets	0.2†	300 µL for 300 ng/L
2-Methylisoborneol	Earthy, peat-like, Brazil nut, soil	0.2†	200 µL for 200 ng/L
Free chlorine	Chlorinous	1000 (0.1% solution) @ pH 7	0.1 mL for 0.5 mg/L
Dichloramine	Swimming pool chlorine	1000 (0.1% solution) @ pH 7	0.1 mL for 0.5 mg/L
Monochloramine	Chlorinous	1000 (0.1% solution) @ pH 7	0.1 mL for 0.5 mg/L
trans,2-*cis*,6-Nonadienal	Cucumber, green vegetation	1000	1 µL for 5 µg/L
cis-3-Hexenyl-1-acetate	Grassy	1000	100 µL for 500 µg/L
cis-3-Hexene-1-ol	Grassy, green apple	1000	100 µL for 500 µg/L

NOTE: These compounds have actually been identified as causes of odors in raw and finished drinking water.
* Adapted from AMERICAN WATER WORKS ASSOCIATION. 1993. Flavor Profile Analysis: Screening and Training of Panelists. AWWA Manual. Denver, Colo.
† Compounds are typically dissolved in methanol.

Tables 2170:I, II, and III list some of the odor references for training FPA panelists, with their descriptive names and preparations. Table 2170:I shows Confirmed Odor References, which are odorants that actually occur in water. Table 2170:II shows Representative Odor References, odorants that can occur in the environment in general but have not yet been documented in drinking water or would not be expected to occur in most drinking waters. Table 2170:III shows Substitute Odor References, or ways to reproduce the odor qualities that can occur in water. (Figure 2170:1 is based on these tables.) Unlisted reference standards can be added to suit the panel's needs, provided that safety considerations have been adequately addressed (see 2170B.2). For convenience in odor identification and training, place several drops of stock solutions (producing moderately strong odors) onto sterile cotton moistened with pharmaceutical-grade mineral oil and inserted in 25-mL amber-colored vials with TFE-lined caps.

d. Taste standards:[3,4,7,9,10,13] The chemicals used for taste standards are sucrose (sweet), citric acid (sour), sodium chloride (salt), and caffeine (bitter). Table 2170:IV lists the chemicals used and their concentrations for basic taste standards at "slight," "moderate," and "strong" levels. The taste standards provide reference points for both taste and odor training, as well as intensity ratings. The panelists compare the intensity of what they smell or taste in the samples to the intensity of the standards they have tasted. In general, tastes from all but the sweet standard tend to overpower any subsequent tasting; only the sweet taste standard is recommended for use during actual sample analysis. However, in some instances (due to cultural or lifestyle differences), the use of the salty or another taste standard is preferred for training to recognize the intensities of tastes. If the panel meets as seldom as once a week, make sweet standards available so panelists can "recalibrate" themselves. Panels that meet more than once a week may not need to "recalibrate" as often. Make standards fresh each time they are used.[3]

6. Scale

The strength of a taste or odor is judged according to the following scale:

— (odor- or taste-free)
T (threshold)
2 (very weak)
4 (weak)
6
8 (moderate)
10
12 (strong)

The rating scale has been modified from the terms "slight to strong"[1,15] to use numbers corresponding to the ordinal categories. Points (other than threshold) on the scale can be anchored by the use of taste standards. Thus, a certain concentration of a taste standard is defined as a certain point on the rating scale, thereby anchoring numerical ratings for non-threshold intensities. Threshold ratings are not defined by any standards. Their meaning is different from that of the other ratings, and they are difficult to manipulate mathematically.

7. Procedure

a. Glassware preparation: Prepare sample bottles by washing bottle and cap with detergent. Rinse 10 times with hot water. Optionally rinse with HCl (1:1). In either case, provide a final rinse with odor-free water (see Section 2150B.3) multiple times. If there is residual odor (e.g., chalky), repeat cleaning.

Prepare 500-mL Erlenmeyer flasks by either of the two methods described below, provided that the method imparts no odor to the sample. Make tests with freshly cleaned containers.

1) Without wearing rubber gloves, wash flask with soapy water. Additionally, scrub outside of flask with scouring pad to

Table 2170:II. Representative Odor Reference Standards*

Compound	Odor Characteristics	Stock Solution Concentration *mg/L*	Amount Placed Into 200 mL Pure Water at 25°C for Presentation
2,3,6-Trichloroanisole	Leather, earthy	1000	4 μL for 20 μg/L
2,3-Diethylpyrazine	Mildew, damp basement	1000	2 μL for 10 μg/L
2-Isopropyl-3-methoxypyrazine	Potato bin, musty	1000	40 μL for 200 μg/L
Nonanal	Hay, sweet	1000	40 μL for 200 μg/L
Dimethyl sulfide	Decaying vegetation, canned corn	1000	1 μL for 5 μg/L
Dimethyl disulfide	Septic	1000	2 μL for 10 μg/L
Dimethyltrisulfide	Garlicky, oniony, septic	0.2	50 μL for 50 ng/L
Butyric acid	Putrid, sickening	1000	200 μL for 1 mg/L
trans,2-Nonenal	Cucumber with skin	1000	40 μL for 200 μg/L
Diphenyl ether	Geranium	1000†	20 μL for 100 μg/L
D-Limonene	Citrusy	1000	400 μL for 2 mg/L
Hexanal	Lettuce heart, pumpkin, green pistachio	1000	40 μL for 200 μg/L
2-Isobutyl-3-methoxypyrazine	Green/bell pepper, musty	1000	40 μL for 200 μg/L
trans,2-*trans*,4-Decadienal	Rancid, oily	1000	1000 μL for 5 mg/L
Butanol	Alcohol, solventy	1000	200 μL for 1 mg/L
Pyridine	Sweet, alcohol, organic	1000	400 μL for 2 mg/L

Note: Possible causes of odors or chemical substitutes for causes of odors.
* Adapted from American Water Works Association. 1993. Flavor Profile Analysis: Screening and Training of Panelists. AWWA Manual. Denver, Colo.
† Compounds are typically dissolved in methanol.

Table 2170:III. Substitute Odor Reference Standards*

Compound	Odor Characteristic	Preparation
Cloves	Spicy (like cloves)	Use supermarket brand of dried clove buds (spice). Add 3 clove buds to 200 mL pure water and swirl 1–2 min. Allow to stand overnight at room temperature, then discard the buds.
Dried grass	Hay	Place dried cut grass in Erlenmeyer flask until half full.
Geranium	Trichloramine	Break geranium stems and place in dry stoppered flask
Grass	Decaying vegetation	Weigh 2 g fresh grass and mix into 200 mL pure water and let stand at room temperature. In 1–3 d, the odor will appear.
Grass	Septic	Allow the solution above for decaying vegetation to stand for an additional 1–2 weeks.
Milk of magnesia	Chalky	Place 4 tablets milk of magnesia in 200 mL pure water
Oil of cade	Smokey	Place 1–2 drops in 200 mL pure water in stoppered container
Rubber hose	Rubber hose	Boil a short section of rubber hose in 200 mL pure water for 5 min. Allow to cool and remove the hose.
Soap	Soapy	Place 5 g of chipped, nonscented bar soap in 200 mL pure water.
Pencil shavings	Woody	Instruct panel member to sharpen a wood pencil and sniff the freshly exposed wood.

Note: Standards made from materials rather than chemicals.
* Adapted from American Water Works Association. 1993. Flavor Profile Analysis: Screening and Training of Panelists. AWWA Manual. Denver, Colo.

TABLE 2170:IV. BASIC TASTE STANDARDS

Chemical for Basic Tastes	Food or Beverage Corresponding to Intensity	Concentration %	Intensity Scale (1 to 12)*
Sweet: sugar	Canned fruit or vegetables	5.00	4 W
	Carbonated soda	10.00	8 M
	Syrup, jelly	15.00	12 S
Sour: citric acid	Fresh fruit jelly	0.05	4 W
	Some carbonated sodas	0.10	8 M
	Lemon juice	0.20	12 S
Salt: sodium chloride	Level in bread	0.40	4 W
	Dehydrated soup mix	0.70	8 M
	Soy sauce	1.00	12 S
Bitter: caffeine	Strong coffee	0.05	4 W
		0.10	8 M
		0.20	12 S
or quinine		0.001	4 W
hydrochloride		0.002	8 M
dihydrate		0.004	12 S

* W = weak; M = moderate; S = strong.

remove body oils. Rinse 10 times with hot water and 3 times with odor-free water. To store flask, add 100 to 200 mL odor-free water and ST32 ground-glass stopper. Before use, rinse with 100 mL odor-free water. If there is residual odor, repeat cleaning.

2) Do not handle with rubber gloves. Before use, add 200 mL odor-free water and heat to boiling; lightly lay ST32 ground-glass stopper over flask opening, permitting water vapor to escape. Discard boiled water, and let flask cool to room temperature. If there is residual odor, repeat this procedure or use cleaning alternative 1) above. After analyzing a sample, discard it, rinse flask 10 times with hot tap water, add 200 mL odor-free water and ST32 ground-glass stopper, and store.

For flasks with persistent odor, use commercial glassware cleaning mixture or HCl (1:1).

b. Sample collection: Collect sample in cleaned container. When sampling from a tap, remove all screens and aerators to minimize turbulence. Flush tap at least 5 min, unless a sample indicative of the standing water from the tap is desired. In that case, take an immediate sample from it (i.e., do not flush first). Reduce flow rate during sampling. Rinse bottle with sample, then fill it to the top (no headspace). Chill or refrigerate sample immediately, and analyze as soon after collection as possible—preferably within 24 h but no longer than 48 h.

c. Sample preparation: Pour samples into properly prepared glassware or acceptable disposable containers. Analyze samples at the same temperature. Adjust sample temperatures by placing samples in a water bath 15 min before analysis. Prepare a sample for each panelist. Examine odor samples before flavor samples.

1) Odor analysis—When using 500-mL Erlenmeyer flasks for odor analysis, place 200 mL sample in the flask. Make transfer carefully to avoid loss of volatile components. When 7- to 8-oz (207- to 237-mL) plastic cups are used, place 60-mL sample portions in cups and cover with watch glasses. When 16-oz (473-mL) plastic cups are used, place 200-mL portions in cups and cover with watch glasses.

2) Taste analysis—Bring sample to 25°C before pouring into containers for tasting. Cover each container with a watch glass if samples are not tasted immediately. If samples are tested at 25°C for odor, use the same sample for flavor analysis.

d. Pre-test considerations: Notify panelists (see 2170B.3) well in advance of panel session so a substitute can be found if necessary. The panel consists of at least four or five members. If fewer than four panelists are available, store the sample until a full panel can meet. Panelists who have colds or allergic attacks on the day of the panel are unacceptable; they should ask the panel coordinator to find a substitute. Panelists must not smoke or eat for 30 min before the session. Wearing cologne or perfume or washing hands with scented soap before the session is not permitted.

e. Sample analysis:

1) Odor analysis—Heat samples to proper temperature. If Erlenmeyer flasks are used, sample temperature is 45°C. If plastic cups are used, sample temperature is 25°C. Give each panelist his/her own sample. If a flask is used, hold it with one hand on the bottom and the other on the ST32 ground-glass stopper. Do not touch flask neck. Gently swirl (do not shake) flask circularly to ensure that volatile compounds are released into the headspace. Bring flask close to nose, remove ST32 ground-glass stopper, and sniff at flask opening. Record impressions of odor attributes (in the order perceived) and assessment of each attribute's intensity. If a cup is used, gently swirl the cup for a few seconds without lifting it off the tabletop. Remove watch glass and, keeping hands away from the cup, sniff sample and record perceptions. **CAUTION: Only sniff samples known to be toxicologically safe.** For both the flask and the plastic cup method, smell all samples before going on to the taste test. Do not discuss or interact with other panelists until the time for discussion.

2) Flavor analysis—When tasting, take sample into the mouth and roll it over the entire surface of the tongue. Slurping enhances the odor aspect of flavor. **CAUTION: If samples are of**

unknown, untested water, do not taste. Only taste samples that have been verified by microbiological and organic/inorganic chemical tests to be safe for human consumption. Do not discuss or interact with other panelists until the time for discussion.

3) Intensity—In both taste and odor analysis, each panelist determines intensity ratings by matching the intensity of the flavor or odor perceived with the defined intensities of the basic taste (sweet) standard. This may be difficult initially. Some panels have found it helpful to make a basic taste standard available throughout the analysis so any panelist who wishes to "recalibrate" may do so.

4) Re-examination of samples—First impressions are most important, particularly for intensity. The intensity rating may diminish upon re-examination because of fatigue [see ¶ *e*6) below] and odorant volatility. However, if an odor or flavor is difficult for a panelist to describe, the panelist may go back and re-evaluate the sample before recording results.

5) Individual results—As soon as a sample is tasted or smelled, record individual results. Information recorded includes a description of odors or flavors perceived, their intensity, and the order in which they were perceived.

6) Rest interval—Sniff odor-free water and rest at least 2 min between samples. *Fatigue* (more properly called *adaptation)* is a decrease in a panelist's sensitivity to a given taste- or odor-producing substance (tastant or odorant) because the person has previously been exposed to that substance. Put simply, a panelist is no longer responsive to a taste or odor because that person has had too much sensory stimulation. Geosmin and 2-methylisoborneol are two compounds known to produce fatigue; after exposure to four samples, a person begins to have difficulty smelling these odorants and cannot assess intensity accurately. Other compounds (e.g., sugar) do not seem to produce fatigue. Because odor mixtures are more complex than taste mixtures, olfactory adaptation is more serious.[6]

Because the odorants or tastants in water samples are frequently unknown in advance, it is prudent to give panelists only 4 to 5 samples per FPA testing session and ask them to rest between samples. The rest interval also is important because it prevents carryover of odors between samples. If the samples are known to contain geosmin or 2-methylisoborneol, then definitely limit the session to four samples and ask panelists to wait 3 min between samples and clear their noses or palates with taste- and odor-free water.

Factors that can induce fatigue include odor intensity, type of odorants (some compounds, such as geosmin and chlorine, induce more fatigue than others), number of samples tested during a session, and rest interval between samples.[1,7] The panel leader also may try to order samples so those known to cause fatigue are placed near the end of the sample row.[16] Avoid juxtaposing such samples. Also, use taste- and odor-free blanks between samples.

7) Dechlorination of samples[17]—The odor of chlorine is distinctive and may mask other odors in a sample. If it is desirable to assess odors other than the chlorinous odor, remove the chlorine species via a reducing agent, such as sodium thiosulfate or sodium sulfite (see Section 4500-NH_3). Hydrogen peroxide and ascorbic acid also can be used to reduce chlorine; appropriate quantities are presented in Tables 2170:V and VI. Hydrogen peroxide is not efficient for reducing chloramines, however, because the kinetics are too slow. Take special care not to overdose these chemicals because overdosage can affect the water's flavor. For example, adding too much hydrogen peroxide can impart a sweet odor. Use caution with sodium thiosulfate because adding more than the stoichiometric dose could result in a "sulfur" odor from production of reduced sulfur compounds.

Table 2170:V. Stoichiometric Dosages of Dechlorinating Agents

Dechlorination Agent	Stoichiometric Dose *mg agent/mg Cl as Cl_2*	Effective Dose *mg agent/mg Cl as Cl_2*
Ascorbic acid	2.48	3.0
Hydrogen peroxide	0.48	0.6
Oxalic acid	1.27	45.0
Sodium nitrite	0.97	1.1
Sodium thiosulfate	0.56	2.2

Source: Worley, J.L., A.M. Dietrich & R.C. Hoehn. 2003. Dechlorination techniques to improve sensory odor testing of geosmin and 2–MIB. *J. Amer. Water. Works Assoc.* 95(3):109.

8) Consensus development[7,10,13,16]—When all panelists have had an opportunity to examine the sample, hold a discussion period. Each panelist states his/her impression of the sample, which the panel leader records for everyone to see. On examining the order of appearance, intensity, and descriptors of tastes and odors, the panel leader attempts to group responses together, soliciting comments from panel members as to whether they agree. Sometimes panelists detect an odor that they cannot describe; in the discussion, they may see what another panelist called it and decide to agree with that description. With inexperienced panels, several descriptors may be used for one taste or odor. As the panel gains more experience, these differences tend to be reconciled. It is the panel leader's responsibility to ensure that panelists are provided with standards that duplicate the sample tastes and odors. Descriptions that fewer than 50% of the panelists use are called "other notes" and are listed separately or not included in group results.

9) Recording panel results—Record the following: sample description; time of sampling; identity of each panel member; taste, odor, and feeling descriptors; intensity rating; order of perception; and range and average for each descriptor.

Table 2170:VI. Dechlorinating Agent Dosage Chart for Chlorine

Dechlorinating Agent	Strength of Dechlorinating Agent	Approximate Total Chlorine Concentration *mg/L as Cl_2*	Dosage of Dechlorinating Agent
Ascorbic acid	5 g/L	1	1.2 mL
		2	2.4 mL
		3	3.6 mL
		4	4.8 mL
Hydrogen peroxide	3% (drug-store grade)	1	40 μL
		2	80 μL
		3	120 μL
		4	160 μL

Source: Worley, J.L., A.M. Dietrich & R.C. Hoehn. 2003. Dechlorination techniques to improve sensory odor testing of geosmin and 2–MIB. *J. Amer. Water. Works Assoc.* 95(3):109.

8. Calculation and Interpretation[7,16]

Calculate intensity means (and standard deviations) if at least 50% of panelists agree on a given descriptor or if descriptors can be grouped together (e.g., plastic and vinyl). If a panelist does not give that description, assign an intensity value of zero. For example, FPA of a drinking water sample could produce the following data:

	Intensity				
	Report by Individual Panelist				
Descriptor	I	II	III	IV	Mean
Musty	2	4	0	2	2.0
Chlorinous	2	4	4	4	3.5

Acceptability of the water to consumers should be the underlying guideline for any interpretation of panel results. For example, if plant effluent has an odor or taste intensity average of 5 or higher, and the descriptor is "earthy," this would indicate a potential for consumer complaints. Levels of intensities and descriptors that would constitute a possible problem need to be determined in advance. Panelists should know when results would be atypical for the samples that were analyzed and should notify the proper person(s) immediately for follow-up action. Only experienced and trained panelists should interpret panel results.

9. Quality Control[16]

Expose panelists to samples of known composition, such as odor-free samples (blanks), samples with known additions, and duplicates, as frequently as possible. Analyze blind, coded samples periodically to prevent overfamiliarity with routine samples. The odor-free sample serves to detect guessing. Duplicates check reproducibility. A standard concentration of an odor reference standard in odor-free water or a matrix sample also can be used to check odor recognition and reproducibility of the intensity scale.

Counterbalance samples so each panelist receives them in a different order. This prevents error due to order effects, such as fatigue, carryover, and expectation. When possible, samples should be coded and split within panels from the same laboratory or other laboratory FPA panels, and their results compared (intralaboratory and intrapanel comparsions). This ensures acceptable, reproducible results, as well as confidence in panels' results.

10. Precision and Bias

Initial studies of precision and bias have been completed,[16] including a reproducibility study on the use of reference materials.[15]

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2310 ACIDITY*

2310 A. Introduction

Acidity of a water is its quantitative capacity to react with a strong base to a designated pH. The measured value may vary significantly with the endpoint pH used in the determination. Acidity is a measure of an aggregate property of water and can be interpreted in terms of specific substances only when the chemical composition of the sample is known. Strong mineral acids, weak acids such as carbonic and acetic, and hydrolyzing salts such as iron or aluminum sulfates may contribute to the measured acidity according to the method of determination.

Acids contribute to corrosiveness and influence chemical reaction rates, chemical speciation, and biological processes. The measurement also reflects a change in the quality of the source water.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011.

2310 B. Titration Method

1. General Discussion

a. Principle: Hydrogen ions present in a sample as a result of dissociation or hydrolysis of solutes react with additions of standard alkali. Acidity thus depends on the endpoint pH or indicator used. The construction of a titration curve by recording sample pH after successive small measured additions of titrant permits identification of inflection points and buffering capacity, if any, and allows the acidity to be determined with respect to any pH of interest.

In the titration of a single acidic species, as in the standardization of reagents, the most accurate endpoint is obtained from the inflection point of a titration curve. The inflection point is the pH at which curvature changes from convex to concave or vice versa.

Because accurate identification of inflection points may be difficult or impossible in buffered or complex mixtures, the titration in such cases is carried to an arbitrary endpoint pH based on practical considerations. For routine control titrations or rapid preliminary estimates of acidity, the color change of an indicator may be used for the endpoint. Samples of industrial wastes, acid mine drainage, or other solutions that contain appreciable amounts of hydrolyzable metal ions such as iron, aluminum, or manganese are treated with hydrogen peroxide to ensure oxidation of any reduced forms of polyvalent cations, and boiled to hasten hydrolysis. Acidity results may be highly variable if this procedure is not followed exactly.

b. Endpoints: Ideally the endpoint of the acidity titration should correspond to the stoichiometric equivalence point for neutralization of acids present. The pH at the equivalence point will depend on the sample, the choice among multiple inflection points, and the intended use of the data.

Dissolved carbon dioxide (CO_2) usually is the major acidic component of unpolluted surface waters; handle samples from such sources carefully to minimize the loss of dissolved gases. In a sample containing only carbon dioxide-bicarbonates-carbonates, titration to pH 8.3 at 25°C corresponds to stoichiometric neutralization of carbonic acid to bicarbonate. Because the color change of phenolphthalein indicator is close to pH 8.3, this value generally is accepted as a standard endpoint for titration of total acidity, including CO_2 and most weak acids. Metacresol purple also has an endpoint at pH 8.3 and gives a sharper color change.

For more complex mixtures or buffered solutions selection of an inflection point may be subjective. Consequently, use fixed endpoints of pH 3.7 and pH 8.3 for standard acidity determinations via a potentiometric titration in wastewaters and natural waters where the simple carbonate equilibria discussed above cannot be assumed. Bromphenol blue has a sharp color change at its endpoint of 3.7. The resulting titrations are identified, traditionally, as "methyl orange acidity" (pH 3.7) and "phenolphthalein" or total acidity (pH 8.3) regardless of the actual method of measurement.

c. Interferences: Dissolved gases contributing to acidity or alkalinity, such as CO_2, hydrogen sulfide, or ammonia, may be lost or gained during sampling, storage, or titration. Minimize such effects by titrating to the endpoint promptly after opening sample container, avoiding vigorous shaking or mixing, protecting sample from the atmosphere during titration, and letting sample become no warmer than it was at collection.

In the potentiometric titration, oily matter, suspended solids, precipitates, or other waste matter may coat the glass electrode and cause a sluggish response. Difficulty from this source is likely to be revealed in an erratic titration curve. Do *not* remove interferences from sample because they may contribute to its acidity. Briefly pause between titrant additions to let electrode come to equilibrium or clean the electrodes occasionally.

In samples containing oxidizable or hydrolyzable ions such as ferrous or ferric iron, aluminum, and manganese, the reaction rates at room temperature may be slow enough to cause drifting endpoints.

Do not use indicator titrations with colored or turbid samples that may obscure the color change at the endpoint. Residual free available chlorine in the sample may bleach the indicator. Eliminate this source of interference by adding 1 drop of 0.1*M* sodium thiosulfate ($Na_2S_2O_3$).

d. Selection of procedure: Determine sample acidity from the volume of standard alkali required to titrate a portion to a pH of 8.3 (phenolphthalein acidity) or pH 3.7 (methyl orange acidity of wastewaters and grossly polluted waters). Titrate at room tem-

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perature using a properly calibrated pH meter, electrically operated titrator, or color indicators.

Use the hot peroxide procedure (2310B.4*a*) to pretreat samples known or suspected to contain hydrolyzable metal ions or reduced forms of polyvalent cation, such as iron pickle liquors, acid mine drainage, and other industrial wastes.

Color indicators may be used for routine and control titrations in the absence of interfering color and turbidity and for preliminary titrations to select sample size and strength of titrant (2310B.4*b*).

e. Sample size: The range of acidities found in wastewaters is so large that a single sample size and normality of base used as titrant cannot be specified. Use a sufficiently large volume of titrant (20 mL or more from a 50-mL buret) to obtain relatively good volumetric precision while keeping sample volume sufficiently small to permit sharp endpoints. For samples having acidities less than about 1000 mg as calcium carbonate ($CaCO_3$)/L, select a volume with less than 50 mg $CaCO_3$ equivalent acidity and titrate with 0.02*N* sodium hydroxide (NaOH). For acidities greater than about 1000 mg as $CaCO_3$/L, use a portion containing acidity equivalent to less than 250 mg $CaCO_3$ and titrate with 0.1*N* NaOH. If necessary, make a preliminary titration to determine optimum sample size and/or normality of titrant.

f. Sampling and storage: Collect samples in polyethylene or borosilicate glass bottles and store at a low temperature. Fill bottles completely and cap tightly. Because waste samples may be subject to microbial action and to loss or gain of CO_2 or other gases when exposed to air, analyze samples without delay, preferably within 1 d. If biological activity is suspected analyze within 6 h. Avoid sample agitation and prolonged exposure to air.

g. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

a. Electrometric titrator: Use any commercial pH meter or electrically operated titrator that uses a glass electrode and can be read to 0.05 pH unit. Standardize and calibrate according to the manufacturer's instructions. Pay special attention to temperature compensation and electrode care. If automatic temperature compensation is not provided, titrate at 25 ± 5°C.

b. Titration vessel: The size and form will depend on the electrodes and the sample size. Keep the free space above the sample as small as practicable, but allow room for titrant and full immersion of the indicating portions of electrodes. For conventional-sized electrodes, use a 200-mL, tall-form Berzelius beaker without a spout. Fit beaker with a stopper having three holes, to accommodate the two electrodes and the buret. With a miniature combination glass-reference electrode use a 125-mL or 250-mL Erlenmeyer flask with a two-hole stopper.

c. Magnetic stirrer.

d. Pipets, volumetric.

e. Flasks, volumetric, 1000-, 200-, 100-mL.

f. Burets, borosilicate glass, 50-, 25-, 10-mL.

g. Polyolefin bottle, 1-L.

3. Reagents

a. Carbon dioxide-free water: Prepare all stock and standard solutions and dilution water for the standardization procedure with distilled or deionized water that has been freshly boiled for 15 min and cooled to room temperature. The final pH of the water should be ≥6.0 and its conductivity should be <2 μmhos/cm.

b. Potassium hydrogen phthalate solution, approximately 0.05*N:* Crush 15 to 20 g primary standard $KHC_8H_4O_4$ to about 100 mesh and dry at 120°C for 2 h. Cool in a desiccator. Weigh 10.0 ± 0.5 g (to the nearest mg), transfer to a 1-L volumetric flask, and dilute to 1000 mL.

c. Standard sodium hydroxide titrant, 0.1*N:* Prepare solution approximately 0.1*N* as indicated under Preparation of Desk Reagents. Standardize by titrating 40.00 mL $KHC_8H_4O_4$ solution (3*b*), using a 25-mL buret. Titrate to the inflection point (2310B.1*a*), which should be close to pH 8.7. Calculate normality of NaOH:

$$\text{Normality} = \frac{A \times B}{204.2 \times C}$$

where:

A = g $KHC_8H_4O_4$ weighed into 1-L flask,
B = mL $KHC_8H_4O_4$ solution taken for titration, and
C = mL NaOH solution used.

Use the measured normality in further calculations or adjust to 0.1000*N*; 1 mL = 5.00 mg $CaCO_3$.

d. Standard sodium hydroxide titrant, 0.02*N:* Dilute 200 mL 0.1*N* NaOH to 1000 mL and store in a polyolefin bottle protected from atmospheric CO_2 by a soda lime tube or tight cap. Standardize against $KHC_8H_4O_4$ as directed in ¶ *c* above, using 15.00 mL $KHC_8H_4O_4$ solution and a 50-mL buret. Calculate normality (¶ *c* above); 1 mL = 1.00 mg $CaCO_3$.

e. Hydrogen peroxide (H_2O_2), 30%.

f. Bromphenol blue indicator solution, pH 3.7 indicator: Dissolve 100 mg bromphenol blue, sodium salt, in 100 mL water.

g. Metacresol purple indicator solution, pH 8.3 indicator: Dissolve 100 mg metacresol purple in 100 mL water.

h. Phenolphthalein indicator solution, alcoholic, pH 8.3 indicator.

i. Sodium thiosulfate, 0.1*M:* Dissolve 25 g $Na_2S_2O_3 \cdot 5H_2O$ and dilute to 1000 mL with distilled water.

4. Procedure

If sample is free from hydrolyzable metal ions and reduced forms of polyvalent cations, proceed with analysis according to ¶s *b*, *c*, or *d* below. If sample is known or suspected to contain such substances, below pretreat according to ¶ *a* below.

a. Hot peroxide treatment: Pipet a suitable sample (see 2310B.1*e*) into titration flasks. Measure pH. If pH is above 4.0 add 5-mL increments of 0.02*N* sulfuric acid (H_2SO_4) (Section 2320B.3*c*) to reduce pH to 4 or less. Remove electrodes. Add 5 drops 30% H_2O_2 and boil for 2 to 5 min. Cool to room temperature and titrate with standard alkali to pH 8.3 according to the procedure of ¶ *d* below.

b. Color change: Select sample size and normality of titrant according to criteria of 2310B.1*e*. Adjust sample to room temperature, if necessary, and with a pipet discharge sample into an Erlenmeyer flask, while keeping pipet tip near flask bottom. If free residual chlorine is present add 0.05 mL (1 drop) 0.1*M* $Na_2S_2O_3$ solution, or destroy with ultraviolet radiation.

Add 0.2 mL (5 drops) indicator solution and titrate over a white surface to a persistent color change characteristic of the equivalence point. Commercial indicator solutions or solids designated for the appropriate pH range (3.7 or 8.3) may be used. Check color at endpoint by adding the same concentration of indicator used with sample to a buffer solution at the designated pH.

c. Potentiometric titration curve:

1) Rinse electrodes and titration vessel with distilled water and drain. Select sample size and normality of titrant according to the criteria of 2310B.1*e*. Adjust sample to room temperature, if necessary, and with a pipet discharge sample while keeping pipet tip near the titration vessel bottom.

2) Measure sample pH. Add standard alkali in increments of 0.5 mL or less, such that a change of less than 0.2 pH units occurs per increment. After each addition, mix thoroughly but gently with a magnetic stirrer. Avoid splashing. Record pH when a constant reading is obtained. Continue adding titrant and measure pH until pH 9 is reached. Construct the titration curve by plotting observed pH values versus cumulative milliliters titrant added. A smooth curve showing one or more inflections should be obtained. A ragged or erratic curve may indicate that equilibrium was not reached between successive alkali additions. Determine acidity relative to a particular pH from the curve.

d. Potentiometric titration to pH 3.7 or 8.3: Prepare sample and titration assembly as specified in ¶ *c*1) above. Titrate to preselected endpoint pH (2310B.1*b*) without recording intermediate pH values. As the endpoint is approached make smaller additions of alkali and be sure that pH equilibrium is reached before making the next addition.

5. Calculation

$$\text{Acidity, as mg } CaCO_3\text{/L} = \frac{[(A \times B) - (C \times D)] \times 50\,000}{\text{mL sample}}$$

where:

A = mL NaOH titrant used,
B = normality of NaOH,
C = mL H_2SO_4 used (2310B.4*a*), and
D = normality of H_2SO_4.

Report pH of the endpoint used, as follows: "The acidity to pH _____ = _____ mg $CaCO_3$/L." If a negative value is obtained, report the value as negative. The absolute value of this negative value should be equivalent to the net alkalinity.

6. Precision and Bias

No general statement can be made about precision because of the great variation in sample characteristics. The precision of the titration is likely to be much greater than the uncertainties involved in sampling and sample handling before analysis.

Forty analysts in 17 laboratories analyzed synthetic water samples containing increments of bicarbonate equivalent to 20 mg $CaCO_3$/L. Titration according to the procedure of 2310B.4*d* gave a standard deviation of 1.8 mg $CaCO_3$/L, with negligible bias. Five laboratories analyzed two samples containing sulfuric, acetic, and formic acids and aluminum chloride by the procedures of 2310B.4*b* and 4*d*. The mean acidity of one sample (to pH 3.7) was 487 mg $CaCO_3$/L, with a standard deviation of 11 mg/L. The bromphenol blue titration of the same sample was 90 mg/L greater, with a standard deviation of 110 mg/L. The other sample had a potentiometric titration of 547 mg/L, with a standard deviation of 54 mg/L, while the corresponding indicator result was 85 mg/L greater, with a standard deviation of 56 mg/L. The major difference between the samples was the substitution of ferric ammonium citrate, in the second sample, for part of the aluminum chloride.

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2320 ALKALINITY*

2320 A. Introduction

1. Discussion

Alkalinity of a water is its acid-neutralizing capacity. It is the sum of all the titratable bases. The measured value may vary significantly with the endpoint pH used. Alkalinity is a measure of an aggregate property of water and can be interpreted in terms of specific substances only when the chemical composition of the sample is known.

Alkalinity is significant in many uses and treatments of natural waters and wastewaters. Because the alkalinity of many surface waters is primarily a function of carbonate, bicarbonate, and hydroxide content, it is taken as an indication of the concentration of these constituents. The measured values also may include contributions from borates, phosphates, silicates, or other bases if these are present. Alkalinity in excess of alkaline earth metal concentrations is significant in determining the suitability of a water for irrigation. Alkalinity measurements are used in the interpretation and control of water and wastewater treatment processes. Raw domestic wastewater has an alkalinity less than, or only slightly greater than, that of the water supply. Properly operating anaerobic digesters typically have supernatant alkalinities in the range of 2000 to 4000 mg calcium carbonate ($CaCO_3$)/L.[1]

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* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011.

2320 B. Titration Method

1. General Discussion

a. Principle: Hydroxyl ions present in a sample as a result of dissociation or hydrolysis of solutes react with additions of standard acid. Alkalinity thus depends on the endpoint pH used. For methods of determining inflection points from titration curves and the rationale for titrating to fixed pH endpoints, see Section 2310B.1*a*.

For samples of low alkalinity (less than 20 mg $CaCO_3$/L) use an extrapolation technique based on the near proportionality of concentration of hydrogen ions to excess of titrant beyond the equivalence point. The amount of standard acid required to reduce pH exactly 0.30 pH unit is measured carefully. Because this change in pH corresponds to an exact doubling of the hydrogen ion concentration, a simple extrapolation can be made to the equivalence point.[1,2]

b. Endpoints: When alkalinity is due entirely to carbonate or bicarbonate content, the pH at the equivalence point of the titration is determined by the concentration of carbon dioxide (CO_2) at that stage. CO_2 concentration depends, in turn, on the total carbonate species originally present and any losses that may have occurred during titration. The pH values in Table 2320:I are suggested as the equivalence points for the corresponding alkalinity concentrations as milligrams $CaCO_3$ per liter. "Phenolphthalein alkalinity" is the term traditionally used for the quantity measured by titration to pH 8.3 irrespective of the colored indicator, if any, used in the determination. Phenolphthalein or metacresol purple may be used for alkalinity titration to pH 8.3. Bromcresol green or a mixed bromcresol green-methyl red indicator may be used for pH 4.5.

T1

Table 2320:I Endpoint pH Values

Test Condition	Endpoint pH	
	Total Alkalinity	Phenolphthalein Alkalinity
Alkalinity, mg $CaCO_3$/L:		
30	4.9	8.3
150	4.6	8.3
500	4.3	8.3
Silicates, phosphates known or suspected	4.5	8.3
Routine or automated analyses	4.5	8.3
Industrial waste or complex system	4.5	8.3

c. Interferences: Soaps, oily matter, suspended solids, or precipitates may coat the glass electrode and cause a sluggish response. Allow additional time between titrant additions to let electrode come to equilibrium or clean the electrodes occasionally. Do not filter, dilute, concentrate, or alter sample.

d. Selection of procedure: Determine sample alkalinity from volume of standard acid required to titrate a portion to a designated pH taken from ¶ *b* above. Titrate at room temperature with a properly calibrated pH meter or electrically operated titrator, or use color indicators. If using color indicators, prepare and titrate an indicator blank.

Report alkalinity less than 20 mg $CaCO_3$/L only if it has been determined by the low-alkalinity method of 2320B.4*d*.

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Construct a titration curve for standardization of reagents.

Color indicators may be used for routine and control titrations in the absence of interfering color and turbidity and for preliminary titrations to select sample size and strength of titrant (see ¶ *e* below).

e. Sample size: See Section 2310B.1*e* for selection of size sample to be titrated and normality of titrant, substituting 0.02*N* or 0.1*N* sulfuric (H_2SO_4) or hydrochloric (HCl) acid for the standard alkali of that method. For the low-alkalinity method, titrate a 200-mL sample with 0.02*N* H_2SO_4 from a 10-mL buret.

f. Sampling and storage: See Section 2310B.1*f*.

g. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

See Section 2310B.2.

3. Reagents

a. Sodium carbonate solution, approximately 0.05*N:* Dry 3 to 5 g primary standard Na_2CO_3 at 250°C for 4 h and cool in a desiccator. Weigh 2.5 ± 0.2 g (to the nearest mg), transfer to a 1-L volumetric flask, fill flask to the mark with distilled water, and dissolve and mix reagent. Do not keep longer than 1 week.

b. Standard sulfuric acid or hydrochloric acid, 0.1*N:* Prepare acid solution of approximately 0.1*N* and determine exact normality as follows. Standardize against 40.00 mL 0.05*N* Na_2CO_3 solution, with about 60 mL water, in a beaker by titrating potentiometrically to pH of about 5. Lift out electrodes, rinse into the same beaker, and boil gently for 3 to 5 min under a watch glass cover. Cool to room temperature, rinse cover glass into beaker, and finish titrating to the pH inflection point. Calculate normality:

$$\text{Normality, } N = \frac{A \times B}{53.00 \times C}$$

where:

A = g Na_2CO_3 weighed into 1-L flask,
B = mL Na_2CO_3 solution taken for titration, and
C = mL acid used.

Use measured normality in calculations or adjust to 0.1000*N*; 1 mL 0.1000*N* solution = 5.00 mg $CaCO_3$.

c. Standard sulfuric acid or hydrochloric acid, 0.02*N:* Dilute 200.00 mL 0.1000*N* standard acid to 1000 mL with distilled or deionized water. Standardize by potentiometric titration of 15.00 mL 0.05*N* Na_2CO_3 according to the procedure of ¶ *b* above; 1 mL = 1.00 mg $CaCO_3$.

d. Bromcresol green indicator solution, pH 4.5 indicator: Dissolve 100 mg bromcresol green, sodium salt, in 100 mL distilled water.

e. Mixed bromcresol green-methyl red indicator solution:[3] Use either the aqueous or the alcoholic solution:

1) Dissolve 100 mg bromcresol green sodium salt and 20 mg methyl red sodium salt in 100 mL distilled water.
2) Dissolve 100 mg bromcresol green and 20 mg methyl red in 100 mL 95% ethyl alcohol or isopropyl alcohol.

f. Metacresol purple indicator solution, pH 8.3 indicator: Dissolve 100 mg metacresol purple in 100 mL water.

g. Phenolphthalein solution, alcoholic, pH 8.3 indicator.

4. Procedure

a. Color change: See Section 2310B.4*b*.

b. Potentiometric titration curve: Follow the procedure for determining acidity (Section 2310B.4*c*), substituting the appropriate normality of standard acid solution for standard NaOH, and continue titration to pH 4.5 or lower. Do not filter, dilute, concentrate, or alter the sample.

c. Potentiometric titration to preselected pH: Determine the appropriate endpoint pH according to 2320B.1*b*. Prepare sample and titration assembly (Section 2310B.4*c*). Titrate to the endpoint pH without recording intermediate pH values and without undue delay. As the endpoint is approached make smaller additions of acid and be sure that pH equilibrium is reached before adding more titrant.

d. Potentiometric titration of low alkalinity: For alkalinities less than 20 mg/L titrate 100 to 200 mL according to the procedure of ¶ *c* above, using a 10-mL microburet and 0.02*N* standard acid solution. Stop the titration at a pH in the range 4.3 to 4.7 and record volume and exact pH. Carefully add additional titrant to reduce the pH exactly 0.30 pH unit and again record volume.

5. Calculations

a. Potentiometric titration to endpoint pH:

$$\text{Alkalinity, mg } CaCO_3/L = \frac{A \times N \times 50\,000}{\text{mL sample}}$$

where:

A = mL standard acid used, and
N = normality of standard acid.

or

$$\text{Alkalinity, mg } CaCO_3/L = \frac{A \times t \times 1000}{\text{mL sample}}$$

where:

t = titer of standard acid, mg $CaCO_3$/mL.

Report pH of endpoint used as follows: "The alkalinity to pH _____ = _____ mg $CaCO_3$/L" and indicate clearly if this pH corresponds to an inflection point of the titration curve.

b. Potentiometric titration of low alkalinity:

$$\text{Total alkalinity, mg } CaCO_3/L = \frac{(2\,B - C) \times N \times 50\,000}{\text{mL sample}}$$

where:

B = mL titrant to first recorded pH,

Table 2320:II. Alkalinity Relationships*

Result of Titration	Hydroxide Alkalinity as $CaCO_3$	Carbonate Alkalinity as $CaCO_3$	Bicarbonate Concentration as $CaCO_3$
$P = 0$	0	0	T
$P < \frac{1}{2}T$	0	$2P$	$T - 2P$
$P = \frac{1}{2}T$	0	$2P$	0
$P > \frac{1}{2}T$	$2P - T$	$2(T - P)$	0
$P = T$	T	0	0

* Key: P−phenolphthalein alkalinity; T−total alkalinity.

C = total mL titrant to reach pH 0.3 unit lower, and
N = normality of acid.

c. Calculation of alkalinity relationships: The results obtained from the phenolphthalein and total alkalinity determinations offer a means for stoichiometric classification of the three principal forms of alkalinity present in many waters. The classification ascribes the entire alkalinity to bicarbonate, carbonate, and hydroxide, and assumes the absence of other (weak) inorganic or organic acids, such as silicic, phosphoric, and boric acids. It further presupposes the incompatibility of hydroxide and bicarbonate alkalinities. Because the calculations are made on a stoichiometric basis, ion concentrations in the strictest sense are not represented in the results, which may differ significantly from actual concentrations especially at pH >10. According to this scheme:

1) Carbonate (CO_3^{2-}) alkalinity is present when phenolphthalein alkalinity is not zero but is less than total alkalinity.
2) Hydroxide (OH^-) alkalinity is present if phenolphthalein alkalinity is more than half the total alkalinity.
3) Bicarbonate (HCO_3^-) alkalinity is present if phenolphthalein alkalinity is less than half the total alkalinity. These relationships may be calculated by the following scheme, where P is phenolphthalein alkalinity and T is total alkalinity (2330B.1*b*).

Select the smaller value of P or $(T-P)$. Then, carbonate alkalinity equals twice the smaller value. When the smaller value is P, the balance $(T-2P)$ is bicarbonate. When the smaller value is $(T-P)$, the balance $(2P-T)$ is hydroxide. All results are expressed as $CaCO_3$. The mathematical conversion of the results is shown in Table 2320:II. (A modification of Table 2320:II that is more accurate when $P \simeq {}^1/_2T$ has been proposed.[4])

Alkalinity relationships also may be computed nomographically (see Carbon Dioxide, Section 4500-CO_2). Accurately measure pH, calculate OH^- concentration as milligrams $CaCO_3$ per liter, and calculate concentrations of CO_3^{2-} and HCO_3^- as milligrams $CaCO_3$ per liter from the OH^- concentration, and the phenolphthalein and total alkalinities by the following equations:

$$CO_3^{2-} = 2P - 2[OH^-]$$

$$HCO_3^- = T - 2P + [OH^-]$$

Similarly, if difficulty is experienced with the phenolphthalein endpoint, or if a check on the phenolphthalein titration is desired, calculate phenolphthalein alkalinity as $CaCO_3$ from the results of the nomographic determinations of carbonate and hydroxide ion concentrations:

$$P = 1/2\,[CO_3^{2-}] + [OH^-]$$

6. Precision and Bias

No general statement can be made about precision because of the great variation in sample characteristics. The precision of the titration is likely to be much greater than the uncertainties involved in sampling and sample handling before the analysis.

In the range of 10 to 500 mg/L, when the alkalinity is due entirely to carbonates or bicarbonates, a standard deviation of 1 mg $CaCO_3$/L can be achieved. Forty analysts in 17 laboratories analyzed synthetic samples containing increments of bicarbonate equivalent to 120 mg $CaCO_3$/L. The titration procedure of 2320B.4*b* was used, with an endpoint pH of 4.5. The standard deviation was 5 mg/L and the average bias (lower than the true value) was 9 mg/L.[5]

Sodium carbonate solutions equivalent to 80 and 65 mg $CaCO_3$/L were analyzed by 12 laboratories according to the procedure of 2320B.4*c*.[6] The standard deviations were 8 and 5 mg/L, respectively, with negligible bias.[6] Four laboratories analyzed six samples having total alkalinities of about 1000 mg $CaCO_3$/L and containing various ratios of carbonate/bicarbonate by the procedures of both 2320B.4*a* and *c*. The pooled standard deviation was 40 mg/L, with negligible difference between the procedures.

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2330 CALCIUM CARBONATE SATURATION*

2330 A. Introduction

1. General Discussion

Calcium carbonate ($CaCO_3$) saturation indices are commonly used to evaluate a water's scale-forming and scale-dissolving tendencies to help prevent $CaCO_3$ scaling in piping and equipment (e.g., industrial heat exchangers or domestic water heaters). The indices can be applicable in reducing corrosion in iron, steel, and cement piping, but not in controlling lead and copper release.

Waters oversaturated with $CaCO_3$ tend to precipitate $CaCO_3$; waters undersaturated with $CaCO_3$ tend to dissolve it. Saturated waters (i.e., waters in equilibrium with $CaCO_3$) tend neither to precipitate nor dissolve $CaCO_3$. Saturation is the dividing line between "precipitation being likely" and "precipitation not being likely."

Dissolved total alkalinity (Section 2320), total calcium (Section 3500-Ca), pH (Section 4500-H^+), and temperature (Section 2550) must be measured to calculate the $CaCO_3$ saturation indices described here. Ionic strength also must be calculated or estimated from total dissolved solids (TDS) (Section 2540C) or conductivity (Section 2510) measurements.

Measure pH at the system's water temperature using a temperature-compensated pH meter. If pH is measured at a different temperature in the laboratory, for example, correct the measured pH.[1–5] When measuring pH, minimize CO_2 exchange between sample and atmosphere. Ideally, seal the sample from the atmosphere during measurements;[6] at a minimum, avoid vigorously stirring unsealed samples.

There are two general categories of $CaCO_3$ saturation indices:

- indices that determine whether a water has a *tendency* to precipitate or to dissolve $CaCO_3$, and
- indices that estimate the *quantity* of $CaCO_3$ that can be precipitated or dissolved.

2. Limitations

It is widely assumed that $CaCO_3$ will precipitate from oversaturated waters and cannot be deposited by undersaturated waters, but there are exceptions. For example, phosphates (particularly polyphosphates), certain naturally occurring organics, sulfate, magnesium, and some trace metals (e.g., zinc) can act as sequestering agents or crystal poisons, inhibiting oversaturated waters from depositing $CaCO_3$.[7–10] Conversely, $CaCO_3$ deposits have been found in pipes conveying undersaturated water due to high pH (relative to bulk water pH) next to certain areas (cathodes) of corroding metal surfaces. Even if the bulk water is undersaturated, a locally oversaturated condition can cause a small, but significant, amount of $CaCO_3$ to be deposited.

The calculations referred to here—even the most sophisticated computerized calculations—do not adequately describe these exceptions, so do not consider saturation indices as absolutes. Rather, view them as guides to the behavior of $CaCO_3$ in aqueous systems and supplement them, where possible, with experimentally derived information.

Waters with positive indices are sometimes assumed to be protective, while waters with negative indices are sometimes assumed to be corrosive. In actuality, this relationship is observed with some materials,[11,12] but not with others.[13,14] The indices can relate to corrosion rates through the clogging of reactive areas by $CaCO_3$ precipitation, which can provide a matrix to retain corrosion products, further sealing surfaces. Calcium carbonate scales can reduce corrosion of unlined iron pipe; however, water characteristics not directly involved in calculating the indices [e.g., dissolved oxygen (DO), buffering intensity, chloride, sulfate, and water velocity] can influence corrosion rates appreciably.[10,12,15–20] Calcium carbonate scales can also reduce the release of free lime in cement-mortar-lined and asbestos-cement pipes. Significant $CaCO_3$ films rarely deposit on lead, galvanized, and copper cold-water pipes.[10,20] Calcium carbonate saturation indices are not predictive of corrosion for lead, copper, or leaded brass pipes.[21–23] While the U.S. Environmental Protection Agency required community water-supply systems to determine $CaCO_3$ saturation indices from 1980 to 1994, the requirement was appealed in 1994 due to misuse as corrosion indices.[23]

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2330 B. Indices Indicating a Water's Tendency to Precipitate or Dissolve $CaCO_3$

1. General Discussion

Indices that indicate $CaCO_3$ precipitation or dissolution tendencies define whether a water is oversaturated, saturated, or undersaturated with $CaCO_3$. The most widely used indices are the Saturation Index (SI), Relative Saturation (RS) [also known as the *Driving Force Index* (DFI) and the *Saturation Ratio* (SR)], and the Ryznar Index (RI). The SI is by far the most commonly used and will be described here.

The RS and SI are related (2330D.1, Equation 7). The RI[1] has been used for many years, sometimes with good results. Because it is semi-empirical, however, it may be less reliable than the SI.

2. Saturation Index by Calculation

SI is determined from Equation 1:

$$SI = pH - pH_s \quad (1)$$

where:

pH = measured pH, and
pH_s = pH of the water if it were in equilibrium with $CaCO_3$ at the existing calcium ion $[Ca^{2+}]$ and bicarbonate ion $[HCO_3^-]$ concentrations.

If SI is positive, the water is oversaturated with $CaCO_3$; if negative, the water is undersaturated. If SI is zero, the water is in equilibrium with $CaCO_3$.

a. Analytical solution for pH_s: Determine pH_s as follows:[2]

$$pH_s = pK_2 - pK_s + p[Ca^{2+}] + p[HCO_3^-] + 5pf_m \quad (2)$$

where:

p = when preceding a variable, designates $-\log_{10}$ of that variable,
K_2 = second dissociation constant for carbonic acid at water temperature,
K_s = solubility product constant for $CaCO_3$ at water temperature,
$[Ca^{2+}]$ = calcium ion concentration, moles/L,
$[HCO_3^-]$ = bicarbonate ion concentration, moles/L, and
f_m = activity coefficient for monovalent species at the specified temperature.

Using the equations in Table 2330:I, calculate the values of pK_2, pK_s, and pf_m required to solve Equation 2. To save computation time, values for pK_2 and pK_s have been precalculated for selected temperatures (Table 2330:II). Table 2330:II gives several values for pK_s because different isomorphs of $CaCO_3$ (e.g., calcite, aragonite, and vaterite) can form in aqueous systems, and each has somewhat different solubility properties. To accommodate for such differences when computing pH_s, use the pK_s for the compound most likely to form. For example, calcite is the form of $CaCO_3$ most commonly found in fresh water.

Estimate the calcium ion concentration from total calcium measurements as follows:

$$[Ca^{2+}] = Ca_t - Ca_{ip} \quad (3)$$

where:

Ca_t = total calcium, moles/L, and

TABLE 2330:I. ESTIMATING EQUILIBRIUM CONSTANTS AND ACTIVITY COEFFICIENTS

Equation	Temperature Range *K*	References
When complete mineral analysis is available:		
$I = 1/2 \sum_{i=1}^{i} [X_i] Z_i^2$	–	3
When a complete mineral analysis is not available: $I = TDS/40{,}000$	–	4
When only conductivity is available: $I = 1.6 \times 10^{-5}\ C$	–	5
$pf_m = A \left[\frac{\sqrt{I}}{1 + \sqrt{I}} - 0.3I \right]$ (valid to $I < 0.5$)	–	3
$A = 1.82 \times 10^6\ (ET)^{-1.5}$	–	3
$E = 308.67 e^{-0.0045976(T)}$	273–373	6
$pK_1 = 356.3094 + 0.06091964\ T - 21\ 834.37/T - 126.8339 \log_{10} T + 1\ 684\ 915/T^2$	273–373	7
$pK_2 = 107.8871 + 0.03252849\ T - 5\ 151.79/T - 38.92561 \log_{10} T + 563\ 713.9/T^2$	273–373	7
$pK_w = 4470.99/T + 0.017060\ T - 6.0875$	273–333	8
$pK_{sc} = 171.9065 + 0.077993\ T - 2\ 839.319/T - 71.595 \log_{10} T$	273–363	7
$pK_{sa} = 171.9773 + 0.077993\ T - 2\ 903.293/T - 71.595 \log_{10} T$	273–363	7
$pK_{sv} = 172.1295 + 0.077993\ T - 3\ 074.688/T - 71.595 \log_{10} T$	273–363	7

I = ionic strength
$[X_i]$ = concentration of component i, moles/L
Z_i = charge of species i
TDS = total dissolved solids, mg/L
C = conductivity, μmhos/cm
pY = $-\log_{10}$ of the value of any factor Y
f_m = activity coefficient for monovalent species
E = dielectric constant
T = temperature, K (°C + 273.15)
K_1 = first dissociation constant for carbonic acid, including $CO_{2(aq)}$
K_2 = second dissociation constant for carbonic acid
K_w = dissociation constant for water
K_{sc} = solubility product constant for calcite
K_{sa} = solubility product constant for aragonite
K_{sv} = solubility product constant for vaterite

TABLE 2330:II. PRECALCULATED VALUES FOR *pK* AND *A* AT SELECTED TEMPERATURES

Temperature °C	pK_2	pK_s Calcite	pK_s Aragonite	pK_s Vaterite	pK_w	*A*
0	10.63	8.38	8.22	7.74	14.94	0.489
5	10.55	8.39	8.24	7.77	14.73	0.493
10	10.49	8.41	8.26	7.80	14.53	0.496
15	10.43	8.43	8.28	7.84	14.34	0.501
20	10.38	8.45	8.31	7.87	14.17	0.505
25*	10.33	8.48	8.34	7.91	13.99	0.510
30	10.29	8.51	8.37	7.96	13.83	0.514
35	10.25	8.54	8.41	8.00	13.68	0.520
40	10.22	8.58	8.45	8.05	13.53	0.525
45	10.20	8.62	8.49	8.10	13.39	0.531
50	10.17	8.66	8.54	8.16	13.26	0.537
60	10.14	8.76	8.64	8.28	13.02	0.549
70	10.13	8.87	8.75	8.40	—	0.563
80	10.13	8.99	8.88	8.55	—	0.578
90	10.14	9.12	9.02	8.70	—	0.593

NOTE: All values determined from the equations of Table 2330:I.
A is used to calculate pf_m (see Table 2330:I).
* pf_m estimated from TDS values at 25°C as follows:

TDS	pf_m
100	0.024
200	0.033
400	0.045
800	0.060
1000	0.066

Ca_{ip} = calcium associated with ion pairs (e.g., $CaHCO_3^+$, $CaSO_4^0$, and $CaOH^+$).

Calcium associated with ion pairs is not available to form $CaCO_3$.

Estimate $[HCO_3^-]$, the bicarbonate ion concentration, as follows:[9]

$$[HCO_3^-] = \frac{Alk_t - Alk_0 + 10^{(pf_m - pH)} - 10^{(pH + pf_m - pK_w)}}{1 + 2 \times 10^{(pH + 3pf_m - pK_2)}} \quad (4)$$

where:

Alk_t = total alkalinity, as determined by acid titration to the carbonic acid endpoint, equivalents/L;
Alk_o = alkalinity contributed or consumed by NH_3^0, $H_3SiO_4^-$, $H_2SiO_4^{2-}$, HPO_4^{2-}, PO_4^{3-}, H_3PO_4, $B(OH)_4^-$, CH_3COO^- (acetate), HS^-, S^{2-}, ion pairs (e.g., $CaHCO_3^+$ and $MgOH^+$), and other species that neutralize strong acid when titrating to the carbonic acid endpoint, which can include organic acids (e.g., fulvic and humic acids) with equivalents/L contributions that are usually small compared to those of (HCO_3^-, CO_3^{2-}, OH^-, and H^+); and
K_w = dissociation constant for water, at water temperature.

Calculations can be simplified. In Equation 4 for example, terms containing exponents [e.g., $10^{(pH + pf_m - pk_w)}$] usually can be neglected for waters whose pH is 6.0 to 8.5 and alkalinity is more than about 50 mg/L as $CaCO_3$. The terms Ca_{ip} in Equation 3 and Alk_o in Equation 4 are difficult to calculate without computers, so they usually are neglected in hand calculations. The simplified version of Equation 2 under such conditions is:

$$pH_s = pK_2 - pK_s + p[Ca_t] + p[Alk_t] + 5pf_m \quad (5)$$

1) Sample calculation—The calculation is best illustrated by working through an example. Assume that calcite controls $CaCO_3$ solubility, and determine the SI for a water with the following composition:

Constituent	Calculate Molar Concentration *mg/L ÷ mg/mole = moles/L*		
Calcium	152	40 078	3.79×10^3
Magnesium	39	24 305	1.60×10^3
Sodium	50	22 990	2.17×10^3
Potassium	5	39 098	1.28×10^4
Chloride	53	35 450*	1.50×10^3
Alkalinity (as $CaCO_3$)	130	50 000†	2.60×10^3‡
Sulfate	430	96 060*	4.48×10^{-3}
Silica (as SiO_2)	15	60 083*	2.50×10^{-4}

* Based on conventional atomic weights.[10]

† Although a more accurate value of the equivalent weight of $CaCO_3$ can be calculated from standard atomic weights, a value of 50 000 should be used because that is the value used to convert the results of an alkalinity titration into an alkalinity concentration in units of mg/L as $CaCO_3$ (Section 2320B).

‡ equivalents/L.

Water temperature = 20°C (293.15K); pH = 9.00.

Before evaluating pf_m in Equation 2, determine the ionic strength (I) and another constant (A). Using the first equation in Table 2330:I, estimate ionic strength assuming all the alkalinity is due to bicarbonate ion (an assumption that introduces only a small error at pH 9.00). Use the alkalinity concentration (2.60×10^{-3}) and the bicarbonate charge (–1) to calculate alkalinity's contribution to ionic strength. Likewise, assume silica is mostly H_4SiO_4 at pH 9.00, as the other species typically can be neglected when pH is ≤9. Because H_4SiO_4 has zero charge, it does not contribute to ionic strength.

$$I = \tfrac{1}{2}\sum_{i=1}^{i}[X_i]Z_i^2 =$$

$$0.5 \times [(3.79 \times 10^{-3}) \times 2^2 + (1.60 \times 10^{-3}) \times 2^2 +$$

$$(2.17 \times 10^{-3}) \times 1^2 + (1.28 \times 10^{-4}) \times 1^2 +$$

$$(1.50 \times 10^{-3}) \times 1^2 + (2.60 \times 10^{-3}) \times 1^2 + (4.48 \times 10^{-3}) \times 2^2]$$

$$= 2.29 \times 10^{-2} \text{ moles/L}$$

In the absence of a complete water analysis, estimate ionic strength from TDS measurements or, as a last resort, from conductivity (see alternative equations in Table 2330:I).

Determine the dielectric constant E and then estimate A (see equations in Table 2330:I). Alternatively, use precalculated values of A in Table 2330:II (e.g., $A = 0.505$ at 20°C).

Next, estimate pf_m using the equation in Table 2330:I:

$$pf_m = 0.505 \times \left[\frac{\sqrt{2.29 \times 10^{-2}}}{1 + \sqrt{2.29 \times 10^{-2}}} - 0.3(2.29 \times 10^{-2})\right] = 0.063$$

Determine $[HCO_3^-]$ from Equation 4. Neglect Alk_o, but because the pH is >8.5, calculate the other terms. Table 2330:II indicates that $pK_2 = 10.38$ and $pK_w = 14.16$.

$$[HCO_3^-] = \frac{2.60 \times 10^{-3} + 10^{(0.063-9.00)} - 10^{(9.00+0.063-14.16)}}{1 + 2 \times 10^{[9.00+3(0.063)-10.38]}}$$

$$= 2.30 \times 10^{-3} \text{ moles/L}$$

Therefore, $p[HCO_3^-] = 2.64$.

Determine $[Ca^{2+}]$ from Equation 3; neglect Ca_{ip}:

$$[Ca^{2+}] = Ca_t = 3.80 \times 10^{-3} \text{ moles/L}$$

Therefore, $p[Ca^{2+}] = 2.42$.

Table 2330:II indicates that the pK_s for calcite is 8.45. Determine pH_s using Equation 2:

$$pH_s = 10.38 - 8.45 + 2.42 + 2.64 + 5\ (0.063) = 7.31$$

And finally, determine SI using Equation 1:

$$SI = 9.00 - 7.31 = 1.69$$

The positive SI indicates that the water is oversaturated with calcite.

2) Effects of neglecting Ca_{ip} and Alk_o—If Ca_{ip} is neglected, then pH_s is underestimated and SI is overestimated by $p(1 - Y_{ca_{ip}})$, where $Y_{ca_{ip}}$ is the fraction of total calcium in ion pairs. For example, if $Y_{ca_{ip}} = 0.30$ then the estimate for SI is 0.15 units too high. If Alk_o is neglected, then SI is overestimated by $p(1 - Y_{Alk_o})$, where Y_{Alk_o} is the fraction of total alkalinity contributed by species other than HCO_3^-, CO_3^{2-}, OH^-, and H^+. The effects of neglecting Ca_{ip} and Alk_o are additive.

That said, Ca_{ip} and Alk_o may be neglected if $Y_{ca_{ip}}$ and Y_{Alk_o} are small and do not interfere with the interpretation of SI. These factors are usually small for natural waters with low TDS concentrations and neutral pH values, but may increase in some waters as pH values approach and exceed 9. At high pH values, however, SI is typically much larger than its overestimated index value, in which case neglecting Ca_{ip} and Alk_o is no problem. For instance, if the sample calculation in the example above was done using water-chemistry software (MINTEQA2) that considers Ca_{ip} and Alk_o (see Table 2330:IV), SI would be 1.51 (0.18 units lower than the result obtained by hand calculations). In other words, neglecting Ca_{ip} and Alk_o did not interfere with interpreting the result; both calculations showed the water to be strongly oversaturated.

The potential for misinterpretation is most acute in nearly saturated waters with high sulfate concentration (e.g., recirculating cooling water). The robust $CaSO_4^0$ ion pair sequesters calcium, which can result in an SI overestimated by as much as 0.3 to 0.5 units, even at neutral pH. So SI may be thought to be zero (neither scale-forming nor corrosive) when in fact it is negative. To resolve this problem, determine pH_s using water-chemistry software that considers ion pairs and other forms of alkalinity (2330D). Such calculations are most accurate when a complete mineral analysis is used.

Calcium complexed by organic matter and organic acid contributions to Alk_o can be difficult to characterize and integrate into water chemistry software. The fraction of calcium com-

plexed by organic matter typically is expected to be small in distribution systems.[11] The contribution of organic acids to Alk_0 can be significant in some waters[12] but will be small in natural waters with low concentrations of natural organic matter (NOM).

Another somewhat less rigorous procedure involves direct measurement of calcium ion activity $\{Ca^{2+}\}$ with a calcium-specific ion electrode.[11,13] Use Equation 6 to determine $p[Ca^{2+}]$; then use $p[Ca^{2+}]$ in Equation 2.

$$p[Ca^{2+}] = p\{Ca^{2+}\} - 4pf_m \quad (6)$$

This approach eliminates the need to determine Ca_{ip}.

Alk_0 can be determined in organic-free waters if a complete mineral analysis is used with suitable chemical-equilibrium software. Alternatively, Alk_0 can be determined either in organic-laden or organic-free waters as described below if carbonate alkalinity (Alk_c) can be obtained, where Alk_c is the alkalinity contributed by carbonate species (HCO_3^- and CO_3^{2-}). Alk_c can be calculated if the total inorganic carbon (C_t) concentration is known, where C_t is the sum of the carbonate species [$CO_{2(aq)}$, H_2CO_3, HCO_3^-, and CO_3^{2-}]. C_t is also known as *dissolved inorganic carbon* (DIC). Some total organic carbon (TOC) analyzers can measure C_t via acidification, purging, and $CO_{2(g)}$ detection.

$CO_{2(g)}$ detection with nondispersive infrared (NDIR) sensors virtually eliminates interference from other volatile compounds at low pH (e.g., purgeable organic carbon, hydrogen sulfide) because the sensors use wavelengths that select for $CO_{2(g)}$ absorbance. Conductivity-based $CO_{2(g)}$ detection with a gas-permeable membrane will register interference from volatile weak acids (e.g., hydrogen sulfide), but this interference is negligible for typical waters. If C_t is obtained, calculate Alk_c as follows:

$$Alk_c = C_t\left(\frac{10^{(pf_m-pH-pK_1)} + 2 \times 10^{(4pf_m-pK_1-pK_2)}}{10^{-2pH} + 10^{(pf_m-pH-pK_1)} + 10^{(4pf_m-pK_1-pK_2)}}\right)$$

where:

K_1 = first dissociation constant for carbonic acid, including $CO_{2(aq)}$, at water temperature.

Estimate Alk_0 by subtracting Alk_c from Alk_t (i.e., $Alk_0 = Alk_t - Alk_c$).

Alternatively, bypass the determination of Alk_0 and substitute Alk_c for the quantity "$Alk_t - Alk_0$" in Equation 4 when estimating $[HCO_3^-]$.

b. Graphical solutions for saturation pH: Caldwell–Lawrence diagrams can be used to determine a saturation pH[14–16]; however, graphical methods do not follow the definition of pH_s provided in Equation 2. In graphical methods, the bicarbonate term is a hypothetical quantity calculated as a function of saturation pH; in Equation 2, the calculated bicarbonate concentration is a function of measured pH (see 2330D). The diagrams can be particularly useful for estimating the chemical dosages needed to achieve desired water conditions. See 2330D for more information about the diagrams; consult the references for descriptions of how to use them.

3. Saturation Index by Experimental Determination

a. Saturometry: Saturometers were developed to measure the degree of $CaCO_3$ saturation in seawater. Analysts equilibrate $CaCO_3$ with a water of known calcium concentration and pH in a sealed flask containing a pH electrode. The water temperature is controlled by a constant-temperature bath. During equilibration, pH decreases if $CaCO_3$ precipitates and increases if it dissolves. Equilibrium is said to have been achieved when the pH stops changing. The initial pH and calcium concentration values and the final pH value are used to estimate the carbonate concentration, which can be used to estimate RS.[17] Equation 7 (2330D.1) may then be used to determine *SI*.

A major advantage of this method is that the approach to equilibrium can be tracked by measuring pH, thus minimizing uncertainty about achieving equilibrium. The method is most sensitive in the range of minimum buffering intensity (pH 7.5 to 8.5). Although the calculations do not consider ion pairs or Alk_0, these can be included so long as their pH dependence is known. The technique has been used for *in situ* oceanographic measurements,[18] as well as in the laboratory.

The saturometry calculations discussed above use the K_s of the $CaCO_3$ phase assumed to control solubility. Uncertainties occur if the controlling solid is unknown. To resolve such uncertainties, measure K_s of the controlling solid; it is equal to the $CaCO_3$ activity product ($Ca^{2+} \times CO_3^{2-}$) at equilibrium. Calculate the latter from the equilibrium pH and initial calcium concentration, alkalinity, and pH measurements.[19]

b. Alkalinity difference technique:[20] *SI* also can be determined by equilibrating water of known pH, calcium concentration, and alkalinity with $CaCO_3$ in a sealed, constant-temperature system. The $CaCO_3$ activity product before equilibration is determined from initial calcium, pH, and alkalinity (or total carbonate) values. The $CaCO_3$ solubility product constant (K_s) equals the $CaCO_3$ activity product after equilibration, which is determined by using the alkalinity change that occurred during equilibration. *RS* is found by dividing the initial activity product by K_s. Calculate *SI* using Equation 7 (2330D.1). The advantage of this method is that it makes no assumptions about the identity of the $CaCO_3$ phase. However, it is more difficult to determine when equilibrium is achieved with this method than with the saturometry method.

Whatever the method used, use temperatures that are the same as that of the water of interest. Alternatively, correct test results to the temperature of the water of interest.[20]

4. Quality Assurance/Quality Control

If the calculations in the "Saturation Index by Calculation" section are automated (e.g., in a spreadsheet), check that the equations were properly programmed with the data in Table 2330:III.

5. References

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TABLE 2330:III. QUALITY ASSURANCE/QUALITY CONTROL EXAMPLES FOR SATURATION INDEX BY CALCULATION

Parameter (units)	Values				
Example	A*	B	C	D	E
			Input		
Calcium (mg/L)	152	10	90	90	152
Magnesium (mg/L)	39	2	—	—	39
Sodium (mg/L)	50	10	—	—	50
Potassium (mg/L)	5	1	—	—	5
Chloride (mg/L)	53	5	—	—	53
Alkalinity (mg/L as $CaCO_3$)	130	30	200	200	130
Sulfate (mg/L)	430	10	—	—	430
Silica (mg/L as SiO_2)	15	0	—	—	15
pH	9.00	9.20	7.20	7.20	9.00
Temperature (°C)	20	15	25	25	20
Alk_0 (mg/L as $CaCO_3$)	Neglected	Neglected	Neglected	Neglected	6
Ca_{ip} (mg/L as Ca)	Neglected	Neglected	Neglected	Neglected	49
TDS (mg/L)	—	—	750	—	—
EC (μS/cm)	—	—	—	1230	—
			Output		
I (M)†	2.30×10^{-2}	1.47×10^{-3}	1.88×10^{-2}	3.08×10^{-2}	2.30×10^{-2}
$[HCO_3^-]$ (M)	2.29×10^{-3}	5.22×10^{-4}	3.99×10^{-3}	3.99×10^{-3}	2.19×10^{-3}
$[Ca^{2+}]$ (M)	3.79×10^{-3}	2.50×10^{-4}	2.25×10^{-3}	2.25×10^{-3}	2.57×10^{-3}
pH_s	7.30	8.79	7.19	7.25	7.49
SI	1.70	0.23	0.01	−0.05	1.51

* The result differs from the example presented earlier due to rounding in the hand calculations.
† Assuming all the alkalinity is due to bicarbonate ion, a charge of 2 for Ca_{ip}, and a charge of 1 for Alk_0.

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2330 C. Indices Predicting the Quantity of $CaCO_3$ That Can Be Precipitated or Dissolved

The *calcium carbonate precipitation potential* (CCPP) predicts both the water's tendency to precipitate or dissolve $CaCO_3$ and how much may be precipitated or dissolved. The CCPP also is called *calcium carbonate precipitation capacity* (CCPC).

CCPP is the quantity of $CaCO_3$ that theoretically can be precipitated from oversaturated waters or dissolved by undersaturated waters during equilibration.[1] The amount that actually precipitates or dissolves may be more or less for several possible

reasons, including equilibrium may not be achieved; temperature and ionic strength approximations; failure to accurately account for complexation of species involved in the reaction; the presence of threshold inhibitors; inaccurate assumptions about the solid phase that forms; inaccurate solubility constants, dissociation constants, or measurements; and differences in concentrations between the bulk phase and local environments.[2] CCPP is negative for undersaturated waters, zero for saturated waters, and positive for oversaturated waters.

1. Calculating CCPP

The CCPP does not lend itself to hand calculations; preferably calculate CCPP using computerized water chemistry models, spreadsheets,[3,4] or Caldwell–Lawrence diagrams (see 2330D). The most reliable calculations consider ion pairs and the alkalinity contributions of other species besides HCO_3^-, CO_3^{2-}, OH^-, and H^+. Models that do not consider these factors overestimate how much $CaCO_3$ can be precipitated and underestimate how much can be dissolved.

2. Experimental Determination of CCPP

Estimate CCPP by one of several experimental techniques.

a. Saturometry: See 2330B.3*a*. The CCPP is determined as part of the *RS* calculation.

b. Alkalinity-difference technique: See 2330B.3*b*. The CCPP equals the difference between the initial and equilibrated water's alkalinity (or calcium) values, when expressed as $CaCO_3$.

c. Marble test: The marble test[1,5–8] is similar to the alkalinity-difference technique. The CCPP equals the change in alkalinity (or calcium) values during equilibration, when expressed as $CaCO_3$.

d. Enslow test: The Enslow test[8] is a continuous version of the alkalinity-difference or marble tests. Water is fed continuously to a leveling bulb or separatory funnel partly filled with $CaCO_3$. Bulb/funnel effluent then is filtered through crushed marble, so the filtrate is assumed to be in equilibrium with $CaCO_3$. The CCPP equals the change in alkalinity (or calcium) that occurs when water passes through the apparatus.

e. Calcium carbonate deposition test:[9] The calcium carbonate deposition test (CCDT) is an electrochemical method that measures the electric current produced when DO is reduced on a rotating electrode. When an oversaturated water is placed in the apparatus, it deposits $CaCO_3$ on the electrode, interfering with oxygen transfer and diminishing the current. The $CaCO_3$ deposition rate is directly proportional to the rate at which current declines. The CCDT and the CCPP are related but not identical: CCDT is a rate and CCPP is a quantity.

For realistic assessments of the CCPP (or CCDT), keep test temperature the same as water-source temperature or else correct test results to water-source temperature.

3. References

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2330 D. Graphical and Computer Methods for $CaCO_3$ Indices

1. Description

Table 2330:IV lists some of the graphical and computer methods that can be used to determine SI and CCPP. It also provides a brief description of their characteristics.

Many computer methods calculate *RS* instead of calculating *SI* directly. When *RS* data are presented, calculate *SI* from:[1]

$$SI = \log_{10} RS \quad (7)$$

where:

RS = ratio of $CaCO_3$ activity product to $CaCO_3$ solubility product constant.

The graphical methods use a hypothetical bicarbonate concentration calculated at the saturation pH (i.e., they implicitly define *saturation pH* as the pH the water would exhibit if it were in equilibrium with $CaCO_3$ at existing calcium, total alkalinity, and C_t concentrations), while the bicarbonate concentration in Equation 2 is calculated at the measured value of pH.[1,2] Basing the saturation index on a hypothetical bicarbonate concentration calculated at the saturation pH not only yields different values but also causes the sign of the index to reverse when the pH is greater than the $CaCO_3$ system's second saturation point, which can occur at a pH of approximately pK_2. When sign reversal occurs, a positive value of *SI* (not the usual negative value) connotes an undersaturated water.[3] When *SI*, pH_s, or *RS* is calculated according to the equations in 2330B, sign reversal

TABLE 2330:IV. GRAPHICAL AND COMPUTER METHODS THAT CAN BE USED TO CALCULATE $CaCO_3$ SATURATION INDICES*

Item†	$CaCO_3$ Indices: Basis for Calculation of SI	$CaCO_3$ Indices: CCPP	Approximate Temperature Range °C	Approximate Limit of Ionic Strength	Ion Pairs Considered?	Alk_o Considered?	Operating System
1. Caldwell-Lawrence Diagrams	pH_{sb}	P, D	2–25	0.030	No	No	Graphical Diagrams
2. MINTEQA2	RS	P, D	0–100	0.5	Yes	Yes	Windows 98, NT, 2000, XP
3. PHREEQC	RS	P, D	0–100	0.5	Yes	Yes	Windows 95, 98,
Standard For high salinity waters	RS	P, D	0–80	7–8	Yes	Yes	NT 4.0, ME 2000, XP, Mac OS 9 and X, and Linux
4. WATEQ4F	RS	D	0–100	0.5	Yes	Yes	Any PC capable of running DOS or MS DOS
5. MINEQL+	RS	P, D	0–100	0.5	Yes	Yes	Windows 7, 8 and 10
6. LEQUIL	RS	No	5–90	0.5	Yes	Yes	IBM Compatible PC, 256K RAM, Windows
7. Visual MINTEQ	RS	P, D	0–100	0.5	Yes	Yes	Windows 7, 8 and 10

* SI = saturation index
CCPP = $CaCO_3$ precipitation potential
pH_{sb} = pH_s based on a hypothetical bicarbonate concentration calculated using the saturation pH
P = calculates amount of $CaCO_3$ theoretically precipitated
D = calculates amount of $CaCO_3$ theoretically dissolved
RS = relative saturation (recommended)
PC = personal computer
† 1. Loewenthal and Marais[3] provide 10.2- by 11.4-cm diagrams, with documentation; Merrill[8–10] provides 10.2- by 16.5-cm diagrams, with documentation.
2. U.S. Environmental Protection Agency, Ecosystems Research Division, Center for Exposure Assessment Modeling (CEAM), 960 College Station Road, Athens, GA 30605-2700 (software and documentation[11]) http://www2.epa.gov/exposure-assessment-models/minteqa2. Accessed January 2017.
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does not occur, thereby eliminating the confusing sign change. Furthermore, the *SI* and *RS* values thereby obtained are directly related to the Gibbs free energy (driving force) of the reaction[4–6] and fundamentally consistent with the saturation indices used in numerous other applications.[7]

Some computer methods not listed in Table 2330:IV use Equation 5 (the simplified version of Equation 2), in which bicarbonate is assumed to equal alkalinity. As discussed in 2330B.2*a*, when pH is approximately neutral (6.0 to 8.5) and alkalinity is more than about 50 mg/L as $CaCO_3$, Equations 2 and 5 yield virtually equal values of pH_s because total alkalinity is due almost entirely to the bicarbonate ion. When pH is more than about 8.5, avoid using Equation 5 and only use Equation 7 with *SI* values determined using Equation 2.

Using a graphical or computer method that calculates pH_s from Equation 2, or *RS*, is strongly recommended. Table 2330:IV identifies the saturation pH basis used for each graphical and computer method listed.

Some models only calculate the amount of $CaCO_3$ that can be precipitated, not the amount that can be dissolved. Others calculate both.

Graphical and computer methods can be used to determine many more parameters than $CaCO_3$ saturation indices. A fee may be charged for computer software or graphs. Table 2330:IV describes parameters each code uses to calculate *SI*. Refer to the sources in the table footnotes for current information.

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2340 HARDNESS*

2340 A. Introduction

1. Terminology

Originally, water hardness was understood to be a measure of the capacity of water to precipitate soap. Soap is precipitated chiefly by the calcium and magnesium ions present. Other polyvalent cations also may precipitate soap, but they often are in complex forms, frequently with organic constituents, and their role in water hardness may be minimal and difficult to define. In conformity with current practice, total hardness is defined as the sum of the calcium and magnesium concentrations, both expressed as calcium carbonate, in milligrams per liter.

When hardness numerically is greater than the sum of carbonate and bicarbonate alkalinity, that amount of hardness equivalent to the total alkalinity is called "carbonate hardness"; the amount of hardness in excess of this is called "noncarbonate hardness." When the hardness numerically is equal to or less than the sum of carbonate and bicarbonate alkalinity, all hardness is carbonate hardness and noncarbonate hardness is absent. The hardness may range from zero to hundreds of milligrams per liter, depending on the source and treatment to which the water has been subjected.

2. Selection of Method

Two methods are presented. Method B, hardness by calculation, is applicable to all waters and yields the higher accuracy. If a mineral analysis is performed, hardness by calculation can be reported. Method C, the EDTA titration method, measures the calcium and magnesium ions and may be applied with appropriate modification to any kind of water. The procedure described affords a means of rapid analysis.

3. Reporting Results

When reporting hardness, state the method used, for example, "hardness (calc.)" or "hardness (EDTA)."

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011.

2340 B. Hardness by Calculation

1. Discussion

The preferred method for determining hardness is to compute it from the results of separate determinations of calcium and magnesium.

2. Calculation

$$\text{Hardness, mg equivalent } CaCO_3/L = 2.497\,[\text{Ca, mg/L}] + 4.118\,[\text{Mg, mg/L}]$$

2340 C. EDTA Titrimetric Method

1. General Discussion

a. Principle: Ethylenediaminetetraacetic acid and its sodium salts (abbreviated EDTA) form a chelated soluble complex when added to a solution of certain metal cations. If a small amount of a dye such as Eriochrome Black T or Calmagite is added to an aqueous solution containing calcium and magnesium ions at a pH of 10.0 ± 0.1, the solution becomes wine red. If EDTA is added as a titrant, the calcium and magnesium will be complexed, and when all of the magnesium and calcium has been complexed the solution turns from wine red to blue, marking the endpoint of the titration. Magnesium ion must be present to yield a satisfactory endpoint. To ensure this, a small amount of complexometrically neutral magnesium salt of EDTA is added to the buffer; this automatically introduces sufficient magnesium and obviates the need for a blank correction.

The sharpness of the endpoint increases with increasing pH. However, the pH cannot be increased indefinitely because of the danger of precipitating calcium carbonate, $CaCO_3$, or magnesium hydroxide, $Mg(OH)_2$, and because the dye changes color at high pH values. The specified pH of 10.0 ± 0.1 is a satisfactory compromise. A limit of 5 min is set for the duration of the titration to minimize the tendency toward $CaCO_3$ precipitation.

b. Interference: Some metal ions interfere by causing fading or indistinct endpoints or by stoichiometric consumption of EDTA. Reduce this interference by adding certain inhibitors before titration. MgCDTA [see 2340C.2*b*3)], selectively complexes heavy metals, releases magnesium into the sample, and may be used as a substitute for toxic or malodorous inhibitors. It is useful only when the magnesium substituted for heavy metals does not contribute significantly to the total hardness. With heavy metal or polyphosphate concentrations below those indicated in Table 2340:I, use Inhibitor I or II. When higher concentrations of heavy metals are present, determine calcium and magnesium by a non-EDTA method (see Sections 3500-Ca and 3500-Mg) and obtain hardness by calculation. The values in Table 2340:I are T1

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Table 2340:I. Maximum Concentrations of Interferences Permissible with Various Inhibitors*

Interfering Substance	Max. Interference Concentration *mg/L* Inhibitor I	Inhibitor II
Aluminum	20	20
Barium	†	†
Cadmium	†	20
Cobalt	over 20	0.3
Copper	over 30	20
Iron	over 30	5
Lead	†	20
Manganese (Mn^{2+})	†	1
Nickel	over 20	0.3
Strontium	†	†
Zinc	†	200
Polyphosphate		10

* Based on 25-mL sample diluted to 50 mL.
† Titrates as hardness.

intended as a rough guide only and are based on using a 25-mL sample diluted to 50 mL.

Suspended or colloidal organic matter also may interfere with the endpoint. Eliminate this interference by evaporating the sample to dryness on a steam bath and heating in a muffle furnace at 550°C until the organic matter is completely oxidized. Dissolve the residue in 20 mL 1*N* hydrochloric acid (HCl), neutralize to pH 7 with 1*N* sodium hydroxide (NaOH), and make up to 50 mL with distilled water; cool to room temperature and continue according to the general procedure.

c. Titration precautions: Conduct titrations at or near normal room temperature. The color change becomes impractically slow as the sample approaches freezing temperature. Indicator decomposition becomes a problem in hot water.

The specified pH may produce an environment conducive to $CaCO_3$ precipitation. Although the titrant slowly redissolves such precipitates, a drifting endpoint often yields low results. Completion of the titration within 5 min minimizes the tendency for $CaCO_3$ to precipitate. The following three methods also reduce precipitation loss:

1) Dilute sample with distilled water to reduce $CaCO_3$ concentration. This simple expedient has been incorporated in the procedure. If precipitation occurs at this dilution of 1 + 1 use modification 2) or 3). Using too small a sample contributes a systematic error due to the buret-reading error.

2) If the approximate hardness is known or is determined by a preliminary titration, add 90% or more of titrant to sample *before* adjusting pH with buffer.

3) Acidify sample and stir for 2 min to expel CO_2 *before* pH adjustment. Determine alkalinity to indicate amount of acid to be added.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Reagents

a. Buffer solution:

1) Dissolve 16.9 g ammonium chloride (NH_4Cl) in 143 mL conc ammonium hydroxide (NH_4OH). Add 1.25 g magnesium salt of EDTA (available commercially) and dilute to 250 mL with distilled water.

2) If the magnesium salt of EDTA is unavailable, dissolve 1.179 g disodium salt of ethylenediaminetetraacetic acid dihydrate (analytical reagent grade) and 780 mg magnesium sulfate ($MgSO_4 \cdot 7H_2O$) or 644 mg magnesium chloride ($MgCl_2 \cdot 6H_2O$) in 50 mL distilled water. Add this solution to 16.9 g NH_4Cl and 143 mL conc NH_4OH with mixing and dilute to 250 mL with distilled water. To attain the highest accuracy, adjust to exact equivalence through appropriate addition of a small amount of EDTA or $MgSO_4$ or $MgCl_2$.

Store Solution 1) or 2) in a plastic or borosilicate glass container for no longer than 1 month. Stopper tightly to prevent loss of ammonia (NH_3) or pickup of carbon dioxide (CO_2). Dispense buffer solution by means of a bulb-operated pipet. Discard buffer when 1 or 2 mL added to the sample fails to produce a pH of 10.0 ± 0.1 at the titration endpoint.

3) Satisfactory alternate "odorless buffers" also are available commercially. They contain the magnesium salt of EDTA and have the advantage of being relatively odorless and more stable than the NH_4Cl-NH_4OH buffer. They usually do not provide as good an endpoint as NH_4Cl-NH_4OH because of slower reactions and they may be unsuitable when this method is automated. Prepare one of these buffers by mixing 55 mL conc HCl with 400 mL distilled water and then, slowly and with stirring, adding 300 mL 2-aminoethanol (free of aluminum and heavier metals). Add 5.0 g magnesium salt of EDTA and dilute to 1 L with distilled water.

b. Complexing agents: For most waters no complexing agent is needed. Occasionally water containing interfering ions requires adding an appropriate complexing agent to give a clear, sharp change in color at the endpoint. The following are satisfactory:

1) *Inhibitor I*—Adjust acid samples to pH 6 or higher with buffer or 0.1*N* NaOH. Add 250 mg sodium cyanide (NaCN) in powder form. Add sufficient buffer to adjust to pH 10.0 ± 0.1. [**Caution: NaCN is extremely poisonous. Take extra precautions in its use (see Section 1090)**]. Dispose of solutions containing this inhibitor in accordance with all applicable governmental regulations (see Section 1100C), taking special precautions to prevent its contact with acids, which can liberate volatile poisonous hydrogen cyanide.)

2) *Inhibitor II*—Dissolve 5.0 g sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$) or 3.7 g $Na_2S \cdot 5H_2O$ in 100 mL distilled water. Exclude air with a tightly fitting rubber stopper. This inhibitor deteriorates through air oxidation. It produces a sulfide precipitate that obscures the endpoint when appreciable concentrations of heavy metals are present. Use 1 mL in 2340C.3*b* below.

3) *MgCDTA*—Magnesium salt of 1, 2-cyclohexanediaminetetraacetic acid. Add 250 mg per 100 mL sample and dissolve completely before adding buffer solution. Use this complexing agent to avoid using toxic or odorous inhibitors when interfering substances are present in concentrations that affect the endpoint but will not contribute significantly to the hardness value.

Commercial preparations incorporating a buffer and a complexing agent are available. Such mixtures must maintain pH 10.0 ± 0.1 during titration and give a clear, sharp endpoint when the sample is titrated.

c. Indicators: Many types of indicator solutions have been advocated and may be used if the analyst demonstrates that they yield accurate values. The prime difficulty with indicator solutions is deterioration with aging, giving indistinct endpoints. For example,

alkaline solutions of Eriochrome Black T are sensitive to oxidants and aqueous or alcoholic solutions are unstable. In general, use the least amount of indicator providing a sharp endpoint. It is the analyst's responsibility to determine individually the optimal indicator concentration.

1) *Eriochrome Black T*—Sodium salt of 1-(1-hydroxy-2-naphthylazo)-5-nitro-2-naphthol-4-sulfonic acid; No. 203 in the Color Index. Dissolve 0.5 g dye in 100 g 2,2′,2″-nitrilotriethanol (also called triethanolamine) or 2-methoxymethanol (also called ethylene glycol monomethyl ether). Add 2 drops per 50 mL solution to be titrated. Adjust volume if necessary.

2) *Calmagite*—1-(1-hydroxy-4-methyl-2-phenylazo)-2-naphthol-4-sulfonic acid. This is stable in aqueous solution and produces the same color change as Eriochrome Black T, with a sharper endpoint. Dissolve 0.10 g Calmagite in 100 mL distilled water. Use 1 mL per 50 mL solution to be titrated. Adjust volume if necessary.

3) Indicators 1 and 2 can be used in dry powder form if care is taken to avoid excess indicator. Prepared dry mixtures of these indicators and an inert salt are available commercially.

If the endpoint color change of these indicators is not clear and sharp, it usually means that an appropriate complexing agent is required. If NaCN inhibitor does not sharpen the endpoint, the indicator probably is at fault.

d. Standard EDTA titrant, 0.01*M:* Weigh 3.723 g analytical reagent-grade disodium ethylenediaminetetraacetate dihydrate, also called (ethylenedinitrilo)tetraacetic acid disodium salt (EDTA), dissolve in distilled water, and dilute to 1000 mL. Standardize against standard calcium solution (¶ *e* below) as described in 2340C.3*b* below.

Because the titrant extracts hardness-producing cations from soft-glass containers, store in polyethylene (preferable) or borosilicate glass bottles. Compensate for gradual deterioration by periodic restandardization and by using a suitable correction factor.

e. Standard calcium solution: Weigh 1.000 g anhydrous $CaCO_3$ powder (primary standard or special reagent low in heavy metals, alkalis, and magnesium) into a 500-mL Erlenmeyer flask. Place a funnel in the flask neck and add, a little at a time, 1 + 1 HCl until all $CaCO_3$ has dissolved. Add 200 mL distilled water and boil for a few minutes to expel CO_2. Cool, add a few drops of methyl red indicator, and adjust to the intermediate orange color by adding 3*N* NH_4OH or 1 + 1 HCl, as required. Transfer quantitatively and dilute to 1000 mL with distilled water; 1 mL = 1.00 mg $CaCO_3$.

f. Sodium hydroxide (NaOH), 0.1*N*.

3. Procedure

a. Pretreatment of polluted water and wastewater samples: Use nitric acid-sulfuric acid or nitric acid-perchloric acid digestion (Section 3030).

b. Titration of sample: Select a sample volume that requires less than 15 mL EDTA titrant and complete titration within 5 min, measured from time of buffer addition.

Dilute 25.0 mL sample to about 50 mL with distilled water in a porcelain casserole or other suitable vessel. Add 1 to 2 mL buffer solution. Usually 1 mL will be sufficient to give a pH of 10.0 to 10.1. The absence of a sharp endpoint color change in the titration usually means that an inhibitor must be added at this point (2340C.2*b et seq.*) or that the indicator has deteriorated.

Add 1 to 2 drops indicator solution or an appropriate amount of dry-powder indicator formulation [2340C.2*c*3)]. Add standard EDTA titrant slowly, with continuous stirring, until the last reddish tinge disappears. Add the last few drops at 3- to 5-s intervals. At the endpoint the solution normally is blue. Daylight or a daylight fluorescent lamp is recommended highly because ordinary incandescent lights tend to produce a reddish tinge in the blue at the endpoint.

If sufficient sample is available and interference is absent, improve accuracy by increasing sample size, as described in ¶ *c* below.

c. Low-hardness sample: For ion-exchanger effluent or other softened water and for natural waters of low hardness (less than 5 mg/L), take a larger sample, 100 to 1000 mL, for titration and add proportionately larger amounts of buffer, inhibitor, and indicator. Add standard EDTA titrant slowly from a microburet and run a blank, using redistilled, distilled, or deionized water of the same volume as the sample, to which identical amounts of buffer, inhibitor, and indicator have been added. Subtract volume of EDTA used for blank from volume of EDTA used for sample.

4. Calculation

$$\text{Hardness (EDTA) as mg } CaCO_3\text{/L} = \frac{A \times B \times 1000}{\text{mL sample}}$$

where:

A = mL titration for sample, and
B = mg $CaCO_3$ equivalent to 1.00 mL EDTA titrant.

5. Precision and Bias

A synthetic sample containing 610 mg/L total hardness as $CaCO_3$ contributed by 108 mg Ca/L and 82 mg Mg/L, and the following supplementary substances: 3.1 mg K/L, 19.9 mg Na/L, 241 mg Cl^-/L, 0.25 mg NO_2^--N/L, 1.1 mg NO_3^--N/L, 259 mg SO_4^{2-}/L, and 42.5 mg total alkalinity/L (contributed by $NaHCO_3$) in distilled water was analyzed in 56 laboratories by the EDTA titrimetric method with a relative standard deviation of 2.9% and a relative error of 0.8%.

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2350 OXIDANT DEMAND/REQUIREMENT*

2350 A. Introduction

1. Significance and Chemistry

Oxidants are added to water supplies and wastewater primarily for disinfection. Other beneficial uses include slime removal, oxidation of undesirable inorganic species (e.g., ferrous ion, reduced manganese, sulfide, and ammonia) and oxidation of organic constituents (e.g., taste- and odor-producing compounds). *Oxidant demand* is the difference between the added oxidant dose and the residual oxidant concentration measured after a prescribed contact time at a given pH and temperature. *Oxidant requirement* is the oxidant dose required to achieve a given oxidant residual at a prescribed contact time, pH, and temperature.

The fate of oxidants in water and wastewater is complex. For example, chlorine reacts with sample constituents by three general pathways: oxidation, addition, and substitution. First, chlorine can oxidize reduced species, such as Fe^{2+}, Mn^{2+}, and sulfide. In these reactions, chlorine is reduced to inorganic chloride (Cl^-). Second, chlorine can add to olefins and other double-bond-containing organic compounds to produce chlorinated organic compounds. Third, chlorine can substitute onto chemical substrates. The addition and substitution reactions produce organochlorine species (e.g., chlorination of phenol to chlorophenols) or active chlorine species (e.g., chlorination of ammonia to produce monochloramine). Chlorine reacts with naturally occurring organic compounds by a combination of these mechanisms to generate such products as trihalomethanes. For more information, see Sections 4500-Cl (chlorine), 4500-ClO_2 (chlorine dioxide), and 4500-O_3 (ozone).

Oxidant demand and oxidant requirement are significantly affected by the sample's chemical and physical characteristics and the manner in which oxidant consumption is measured. In particular, oxidant reactivity is influenced by temperature, pH, contact time, and oxidant dose. Oxidant demand and oxidant requirement are defined operationally by the analytical method used to determine the residual oxidant concentration. *Report sample temperature, pH, contact time, oxidant dose, and analytical method with oxidant demand or oxidant requirement.* Sample temperature strongly affects reaction kinetics and thus the demand exerted in a given contact time. Sample pH affects the form of the oxidant and the nature and extent of the demand. For example, ozone is unstable at high pH values, and ozone demand is especially sensitive to sample pH. Oxidant demand increases with time; the demand must be defined for a given contact time. Oxidant demand also depends on oxidant dose. Increasing oxidant dose usually will increase demand, but it is incorrect to assume that doubling the oxidant dose will double the oxidant demand. For these reasons, it is difficult to extrapolate oxidant demand data from one set of conditions to another. *Always study oxidant consumption under the range of conditions expected in the field.*

Oxidant consumption is used to evaluate oxidant demand and oxidant requirement. Report consumption values according to the study's objective. For example, report chlorine *demand* as follows: "The sample dosed at 5.0 mg/L consumed 3.9 mg/L after 24 h at 20°C and pH 7.1, as measured by amperometric titration." By contrast, report ozone *requirement* as follows: "The sample required a dose of 2.1 mg/L to achieve an ozone residual of 0.5 mg/L after 20 min at 15°C and pH 6.5, as measured by the indigo method."

2. Method Selection

Select a method to measure oxidant residuals used in the demand calculation that is specific and has adequate sensitivity. Some oxidant residual measurement techniques are subject to interferences from oxidation-produced oxidants. Interferences affect oxidant demand measurements because the interferents' concentrations may change as the oxidant residual changes. Thus, calculate free chlorine demand in municipal wastewater as the difference between free chlorine dose and free chlorine residual measured after a desired contact time at a given temperature, pH, and chlorine dose for a specified analytical method. Chlorination of non-nitrified municipal wastewater probably produces chloramines. If the analytical method for free chlorine is subject to interferences from chloramines, then the free chlorine residual measurement will be too large (see Section 4500-Cl.A.3) and the resulting free chlorine demand value will be incorrectly low. It is sometimes difficult to predict how oxidant-produced oxidants will affect the demand measurement. The best approach is to use the analytical method most specific to the oxidant of interest, but always indicate the method with the result.

Adding reagents may cause loss of oxidant residual or other changes in oxidant demand. The loss of total chlorine upon addition of acid and KI is discussed in Section 4500-Cl.A.3*a*.

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* Approved by Standard Methods Committee, 2006. Editorial revisions, 2011. Joint Task Group: 20th Edition—Roger A. Yorton (chair), James N. Jensen, Maria T. Morsillo.

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2350 B. Chlorine Demand/Requirement

1. General Discussion

a. Principle: Divide the sample into subsamples, and dose each with the standardized oxidant (chlorine) solution to yield a series of increasing doses. After the appropriate contact time, measure oxidant residual, pH, and temperature and determine the demand/requirement via the difference between initial and final concentrations.

b. Method selection: Chlorine consumption may be tested to examine the demand or requirement for total chlorine, free chlorine, combined chlorine, monochloramine, or dichloramine. Specify the chlorine species consumed in the chlorine demand/requirement test. The analytical method should exhibit minimal interferences for the species examined. For demand/requirement studies with free chlorine, use only amperometric titration (Section 4500-Cl.D) or DPD methods (Sections 4500-Cl.F and 4500-Cl.G).

c. Interference: Refer to Section 4500-Cl.D.1*b* (amperometric titration), 4500-Cl.F.1*d* (DPD ferrous titrimetric method), or 4500-Cl.G.1*b* (DPD colorimetric method). Pay special attention to interferences caused by oxidation products, such as MnO_2, NH_2Cl, and $NHCl_2$. If water's ammonia or organic nitrogen content is significant, combined chlorine may form. See Section 4500-Cl for details. Under these conditions, expect interferences in the measurement of free chlorine by combined chlorine.

d. Minimum detectable concentration: Because it is calculated by difference, the minimum detectable chlorine demand/requirement is $\sqrt{2}$ times the minimum chlorine residual detectable by the analytical method. For minimum detectable chlorine residual, see Section 4500-Cl.F.1*e* (DPD ferrous titrimetric method) or 4500-Cl.G.1*c* (DPD colorimetric method). Minimum detectable demand also is influenced by the amount of oxidant consumed relative to oxidant dose (see 2350B.6).

e. Sampling: Most reliable results are obtained on fresh samples that contain low amounts of suspended solids. If samples will be analyzed within 24 h of collection, refrigerate unacidified at 4°C immediately after collection. To preserve for up to 28 d, freeze unacidified samples at −20°C. Warm chilled samples to desired test condition before analysis.

f. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

See Section 4500-Cl.D.2 (amperometric titration) or 4500-Cl.G.2 (DPD colorimetric method).

3. Reagents

a. Chlorine-demand-free water: See Section 4500-Cl.C.3*m.* Alternatively, prepare dilutions, blanks, and dosing solutions from high-quality distilled water (preferably carbon-filtered redistilled water).

b. Acetic acid, conc (glacial).

c. Potassium iodide (KI), crystals.

d. Standard sodium thiosulfate titrant, 0.025*N:* See Section 4500-Cl.B.2*d*.

e. Starch indicator solution: See Section 4500-Cl.B.2*e*.

f. Reagents for determining residual chlorine: See Section 4500-Cl.D.3 (amperometric titration), 4500-Cl.F.2 (DPD ferrous titrimetric method), or 4500-Cl.G.3 (DPD colorimetric method).

g. Standard chlorine solution: Prepare by bubbling chlorine gas through distilled water or by diluting commercially available 5 to 7% (50 000 to 70 000 mg/L) sodium hypochlorite. Store in the dark or in a brown, glass-stoppered bottle. Standardize each day of use. A suitable strength of chlorine solution usually will be between 100 and 1000 mg/L, preferably about 100 times estimated chlorine demand. Use a solution of sufficient concentration, so adding the chlorine solution will not increase the volume of the treated portions by more than 5%.

Standardization—Place 2 mL acetic acid and 10 to 15 mL chlorine-demand-free water in a flask. Add about 1 g KI. Measure into the flask a suitable volume of chlorine solution. In choosing a convenient volume, note that 1 mL 0.025*N* $Na_2S_2O_3$ titrant is equivalent to about 0.9 mg chlorine as Cl_2. Select volumes that will require no more than 20 mL titrant.

Titrate with standardized 0.025*N* $Na_2S_2O_3$ titrant until the yellow iodine color almost disappears. Add 1 to 2 mL starch indicator solution and continue titrating until the blue color disappears.

Determine the blank by adding identical quantities of acid, KI, and starch indicator to a volume of chlorine-demand-free water corresponding to the sample used for titration. Perform whichever blank titration applies, according to Section 4500-Cl.B.3*d*. Calculate the chlorine stock concentration as described in Section 4500-Cl.B.4.

4. Procedure

Measure sample temperature and pH. Keep sample and sample portions at desired temperature and protect from light throughout the procedure. If pH adjustment is desired, prepare a blank in distilled water containing the same amount of buffer as in the sample. Carry the blank throughout the procedure.

Measure 5* equal sample portions of 200 mL† each into glass-stoppered bottles or flasks of ample capacity to permit mixing. Add increasing amounts of standard chlorine solution 2350B.3*g*) to successive portions in the series. Try to bracket the estimated demand/requirement and satisfy criteria of 2350B.5*a*. Increase dosage between portions in increments of 0.1 mg/L for determining low demands/requirements and up to 1.0 mg/L or more for higher demands. Mix while adding. Dose sample portions according to a staggered schedule that will permit determining the residual after predetermined contact times.

Conduct test over desired contact period. Record contact time. At end of contact period, measure sample temperature, sample pH, and residual chlorine. Record residual measurement method used.

* The number of sample portions can be increased when working with samples of unknown demand and may be decreased when working with samples of familiar origin.

† Size of sample portions is not critical, but must be large enough to ensure reproducible results, as well as provide volume sufficient to measure chlorine residual, pH, and temperature.

5. Calculation

a. Chlorine demand: Select sample portion with a residual at the end of the contact period that satisfies the following criteria:

1) $R_s < D_s - 1.4\ R_{min}$,
2) $R_s > R_{min}$, and
3) Dose is most similar to the dosage range expected in the field

where:

R_s = residual after contact time, mg/L,
D_s = dose, mg/L, and
R_{min} = minimum residual measurable by the method, mg/L.

The first two criteria ensure that the chlorine residual and demand are greater than their respective minimum detection limits. If no sample portion satisfies all criteria, repeat the test and adjust doses accordingly. Calculate chlorine demand as follows:

$$\text{Chlorine demand, mg/L} = (D_S - R_S) - (D_B - R_B)$$

where R_s and D_s are defined as above, and:

D_B = blank dose, mg/L, and
R_B = residual of blank after contact time, mg/L.

When reporting chlorine demand, include dose, contact time, sample temperature, sample pH, and analytical method.

b. Chlorine requirement: Report the chlorine dose that produced the target residual after the desired contact time. When reporting chlorine requirement, include the target residual, contact time, sample temperature, sample pH, and analytical method. Report chlorine demand of blank if it is greater than 10% of the difference between the requirement and the target residual.

6. Precision and Bias

For data on precision and bias of concentration measurements, see analytical method used. Because demand is calculated by difference, the uncertainty associated with the demand value will be greater than the uncertainty of the individual residual measurements. If the standard deviations of the dose measurement and residual measurements are the same, then the standard deviation and minimum detection limit of the oxidant demand will be $\sqrt{2}$ (approximately 1.4) times the standard deviation and minimum detection limit of the measurement technique, respectively.

The chlorine dose and amount consumed affect the precision and bias of demand calculation in two ways. First, the amount consumed must be sufficiently large, relative to the dose, to minimize errors associated with a value calculated from the difference of two numbers of approximately equal value. Second, the amount consumed must be small enough, relative to the dose, to prevent the residual concentration from being too small.

7. Bibliography

See Section 4500-Cl.D.7 or F.6, according to analytical method used.

2350 C. Chlorine Dioxide Demand/Requirement

1. General Discussion

a. Principle: See 2350B.1. Chlorine dioxide consumption studies are made by dosing samples from a ClO_2 stock solution.

b. Selection of method: Use the amperometric method II (Section 4500-ClO_2.E) because of its high degree of accuracy and minimal interferences.

c. Interference: See Section 4500-ClO_2.E.1*b*.

d. Minimum detectable concentration: The minimum detectable chlorine dioxide demand/requirement is $\sqrt{2}$ times the minimum chlorine dioxide residual detectable by the analytical method (see 2350B.1*d* and 6).

e. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

See Section 4500-ClO_2.E.2.

3. Reagents

See Section 4500-ClO_2.B.2 to prepare and standardize ClO_2.

See Section 4500-ClO_2.E.3 for reagents required to determine ClO_2 residual.

4. Procedure

Follow procedure of 2350B.4, using ClO_2 solution, rather than chlorine solution, for dosing sample portions.

Follow procedure of Section 4500-ClO_2.E.4 to measure ClO_2 residual.

5. Calculation

a. Chlorine dioxide demand: See 2350B.5*a*.
b. Chlorine dioxide requirement: See 2350B.5*b*.

6. Precision and Bias

See 2350B.6.

7. Bibliography

See Section 4500-ClO_2.E.6 and 7.

2350 D. Ozone Demand/Requirement—Batch Method

1. General Discussion

a. Principle: See 2350B.1*a*. Samples can be ozonated in batch and semi-batch modes. In batch ozone consumption studies, an ozone stock solution is used to add ozone to the samples. In semi-batch ozone consumption studies, a stream of ozone gas is added continuously to the sample.

Ozone decomposes at high pH. Thus, even pH-buffered distilled water has a non-zero ozone demand/requirement. Analyze blanks with all ozone consumption tests. Do not subtract the ozone demand of the blank from the ozone demand of the sample; report it separately.

b. Selection of method: Ozone produces oxidants that interfere with iodometric methods. The indigo method (Section 4500-O_3.B) is recommended for measuring ozone residuals in ozone consumption studies. The indigo method measures only the demand for ozone; it does not measure the demand for ozone-produced oxidants, such as the hydroxyl radical.

c. Interference: See Section 4500-O_3.B.1*b*.

d. Minimum detectable concentration: The minimum detectable ozone demand/requirement is $\sqrt{2}$ times the minimum ozone residual detectable by the analytical method (see 2350B.1*d* and 6).

For minimum detectable ozone residuals, see Section 4500-O_3.B.1*c*.

e. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

a. Ozone generator: Use a laboratory-scale ozonator capable of providing up to about 5% ozone in the gas phase at a gas flow of up to about 1 L/min.

b. Apparatus for measuring residual ozone: See Section 4500-O_3.B.2.

3. Reagents

a. Ozone-demand-free water: Ozonate reagent water (see Section 1080) for at least 1 h and purge with high-purity (99.995% grade or better) nitrogen gas for at least 1 h. (**CAUTION: Conduct all laboratory ozonations under a vented hood.**)

b. Standard ozone solution: Put about 800 mL of ozone-demand-free water in a 1-L flask. Bubble ozone (approximately 1 to 5% O_3 in the gas phase) through the water for about 30 min while stirring. At room temperature, the ozone solution will contain about 10 to 20 mg O_3/L. If the flask is cooled in an ice bath throughout the procedure, the ozone concentration will be about 30 to 40 mg O_3/L. Standardize the ozone solution by the indigo method. Use a small sample volume (typically 1 mL) as directed in Section 4500-O_3.B.4*a*3).

4. Procedure

Follow procedure in 2350B.4, using standard ozone solution, rather than chlorine solution, for dosing sample portions. Carry a reagent blank through the procedure.

Follow procedure of Section 4500-O_3.B.4 to measure O_3 residual.

5. Calculation

a. Ozone demand: See 2350B.5*a* on selecting the proper sample portion. Calculate ozone demand in sample as follows:

$$\text{Ozone demand, mg/L} = D_S - R_S$$

where:

D_s = sample oxidant dose, mg/L, and
R_s = oxidant residual of sample after contact time, mg/L.

Calculate ozone demand in the blank separately.

$$\text{Ozone demand of blank, mg/L} = D_B - R_B$$

where:

D_B = blank oxidant dose, mg/L, and
R_B = oxidant residual of blank after contact time, mg/L.

Report the ozone demand and the ozone demand of the blank, ozone dose, contact time, sample temperature, sample pH, and analytical method.

b. Ozone requirement: See 2350B.5*b*.

6. Precision and Bias

See 2350B.6.

7. Bibliography

See Sections 4500-O_3.B.7 and 8.

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2350 E. Ozone Demand/Requirement—Semi-Batch Method

1. General Discussion

See 2350D.1.

The semi-batch method involves determining ozone demand via the continuous addition of gaseous ozone to a batch reactor. The results obtained in this method depend on the reactor's mass-transfer characteristics. In addition, some compounds that consume ozone may volatilize during the test.

The quality control practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

All apparatus listed in 2350D.2 is required, plus:

a. Gas washing bottles, borosilicate glass, minimum volume 250 mL.

b. Tubing: Use only stainless steel or TFE tubing.

c. Glassware: Buret, 50 mL; beaker, 400 mL; graduated cylinder, 250 mL.

d. Wash bottle, 500 mL.

e. Magnetic stirrer (optional).

3. Reagents

a. Ozone-demand-free water: See 2350D.3*a*.

b. Sulfuric acid (H_2SO_4), 2*N:* Cautiously add 56 mL conc H_2SO_4 to 800 mL ozone-demand-free water in a 1-L volumetric flask. Mix thoroughly, cool, add up to mark with ozone-demand-free water.

c. Potassium iodide (KI): Dissolve 20 g KI in about 800 mL of ozone-demand-free water in a 1-L volumetric flask. Make up to mark with ozone-demand-free water.

d. Standard sodium thiosulfate titrant ($Na_2S_2O_3$), 0.1*N:* See Section 4500-Cl.B.2*c*.

e. Standard sodium thiosulfate titrant ($Na_2S_2O_3$), 0.005*N:* Dilute the proper volume (approximately 50 mL) of standardized 0.1*N* $Na_2S_2O_3$ to 1 L.

f. Starch indicator solution: See Section 4500-Cl.B.2*e*.

4. Procedure

Determine the ozone generator's output by passing the ozone gas through two serial KI traps (Traps A and B) for about 10 min. For best results, keep gas flow below approximately 1 L/min. Each trap is a gas washing bottle containing a known volume (at least 200 mL) of 2% KI. Quantitatively transfer contents of each trap into a beaker, add 10 mL of 2*N* H_2SO_4, and titrate with standardized 0.005*N* $Na_2S_2O_3$ until the yellow iodine color almost disappears. Add 1 to 2 mL starch indicator solution and continue titrating until the blue color disappears.

Put a known volume (at least 200 mL) of sample in a separate gas washing bottle (label gas washing bottles to avoid contaminating the reaction vessel with iodide). Direct ozone gas through this reaction vessel. For ozone demand studies, direct gas stream leaving reaction vessel through a KI trap (Trap C) prepared as above. Ozonate sample for a given contact time. For ozone demand studies, turn ozonator off at end of contact time and pour contents of Trap C into a beaker. Add 10 mL 2*N* H_2SO_4 and titrate with 0.005*N* $Na_2S_2O_3$ as described above. For ozone requirement studies, remove a portion from the reaction vessel at the end of contact time and measure residual ozone concentration by the indigo method.

5. Calculation

a. Ozone dose:

$$\text{Ozone dose, mg/min} = \frac{(A + B) \times N \times 24}{T}$$

where:

A = mL titrant for Trap A,
B = mL titrant for Trap B,
N = normality of $Na_2S_2O_3$, and
T = ozonation time, min.

b. Ozone demand:

$$\text{Ozone demand, mg/min} = \text{ozone dose, mg/min} - \frac{C \times N \times 24}{T}$$

where:

C = mL titrant for Trap C.

Report sample ozone demand and blank ozone demand, ozone dose, ozonation time, sample temperature, sample pH, sample volume, and analytical method. Because the ozone transfer rate is highly dependent on experimental conditions, also report vessel volume, vessel type, gas flow rate, and sample volume.

c. Ozone requirement: The ozone requirement in the semi-batch test is the ozone dose (mg/min) required to obtain the target ozone residual after the desired ozonation time. See ¶ *a* above to calculate dose. When reporting ozone requirement, also include target oxidant residual and other experimental characteristics listed in ¶ *b* above.

6. Precision and Bias

See 2350B.6.

7. Bibliography

See Sections 4500-O_3.B.7 and 8.

2510 CONDUCTIVITY*

2510 A. Introduction

Conductivity, k, is a measure of the ability of an aqueous solution to carry an electric current. This ability depends on the presence of ions; on their total concentration, mobility, and valence; and on the temperature of measurement. Solutions of most inorganic compounds are relatively good conductors. Conversely, molecules of organic compounds that do not dissociate in aqueous solution conduct a current very poorly, if at all.

1. Terminology and Units of Expression

Conductance, G, is defined as the reciprocal of resistance, R:

$$G = \frac{1}{R}$$

where the unit of R is ohm and G is ohm^{-1} (sometimes written mho). Conductance of a solution is measured between two spatially fixed and chemically inert electrodes. To avoid polarization at the electrode surfaces the conductance measurement is made with an alternating current signal.[1] The conductance of a solution, G, is directly proportional to the electrode surface area, A, cm^2, and inversely proportional to the distance between the electrodes, L, cm. The constant of proportionality, k, such that:

$$G = k\left(\frac{A}{L}\right)$$

is called "conductivity" (preferred to "specific conductance"). It is a characteristic property of the solution between the electrodes. The units of k are 1/ohm-cm or mho per centimeter. Conductivity is customarily reported in micromhos per centimeter (μmho/cm).

In the International System of Units (SI) the reciprocal of the ohm is the siemens (S) and conductivity is reported as millisiemens per meter (mS/m); 1 mS/m = 10 μmhos/cm and 1 μS/cm = 1 μmho/cm. To report results in SI units of mS/m divide μmhos/cm by 10.

To compare conductivities, values of k are reported relative to electrodes with A = 1 cm^2 and L = 1 cm. Absolute conductances, G_s, of standard potassium chloride solutions between electrodes of precise geometry have been measured; the corresponding standard conductivities, k_s, are shown in Table 2510:I.

The equivalent conductivity, Λ, of a solution is the conductivity per unit of concentration. As the concentration is decreased toward zero, Λ approaches a constant, designated as $\Lambda°$. With k in units of micromhos per centimeter it is necessary to convert concentration to units of equivalents per cubic centimeter; therefore:

$$\Lambda = 0.001k/\text{concentration}$$

where the units of Λ, k, and concentration are mho-cm^2/equivalent, μmho/cm, and equivalent/L, respectively. Equivalent conductivity, Λ, values for several concentrations of KCl are listed in Table 2510:I. In practice, solutions of KCl more dilute than 0.001M will not maintain stable conductivities because of absorption of atmospheric CO_2. Protect these dilute solutions from the atmosphere.

Table 2510:I. Equivalent Conductivity, Λ, and Conductivity, k, of Potassium Chloride at 25.0°C.*[2–4]

KCl Concentration *M or equivalent/L*	Equivalent Conductivity, Λ *mho-cm^2/equivalent*	Conductivity, k_s *μmho/cm*
0	149.9	
0.0001	148.9	14.9
0.0005	147.7	73.9
0.001	146.9	146.9
0.005	143.6	717.5
0.01	141.2	1 412
0.02	138.2	2 765
0.05	133.3	6 667
0.1	128.9	12 890
0.2	124.0	24 800
0.5	117.3	58 670
1	111.9	111 900

* Based on the absolute ohm, the 1968 temperature standard, and the dm^3 volume standard.[2] Values are accurate to ±0.1% or 0.1 μmho/cm, whichever is greater.

2. Measurement

a. Instrumental measurements: In the laboratory, conductance, G_s, (or resistance) of a standard KCl solution is measured and from the corresponding conductivity, k_s, (Table 2510:I) a cell constant, C, cm^{-1}, is calculated:

$$C = \frac{k_s}{G_s}$$

Most conductivity meters do not display the actual solution conductance, G, or resistance, R; rather, they generally have a dial that permits the user to adjust the internal cell constant to match the conductivity, k_s, of a standard. Once the cell constant has been determined, or set, the conductivity of an unknown solution,

$$k_u = CG_u$$

will be displayed by the meter.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011. Joint Task Group: 20th Edition—Robert M. Bagdigian (chair), Stephen W. Johnson, William F. Koch, Russell W. Lane, Misha Plam.

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Table 2510:II. Sample Analysis Illustrating Calculation of Conductivity, k_{calc}, for Natural Waters.[7]

Ions	mg/L	m*M*	$\lvert z\rvert\ \lambda^{\circ}_{\pm}$m*M*	z^2m*M*
Ca	55	1.38	164.2	5.52
Mg	12	0.49	52.0	1.96
Na	28	1.22	61.1	1.22
K	3.2	0.08	5.9	0.08
HCO_3	170	2.79	124.2	2.79
SO_4	77	0.80	128.0	3.20
Cl	20	0.56	42.8	0.56
			578.2	15.33

Table 2510:III. Equivalent Conductances, λ°_{+} and λ°_{-}, (mho-cm^2/equivalent) for Ions in Water at 25.0°C.[8]

Cation	Λ°_{+}	Anion	Λ°_{-}
H^+	350	OH^-	198.6
$1/2Ca^{2+}$	59.5	HCO_3^-	44.5
$1/2Mg^{2+}$	53.1	$1/2CO_3^{2-}$	72
Na^+	50.1	$1/2SO_4^{2-}$	80.0
K^+	73.5	Cl^-	76.4
NH_4^+	73.5	Ac^-	40.9
$1/2Fe^{2+}$	54	F^-	54.4
$1/3Fe^{3+}$	68	NO_3^-	71.4
		$H_2PO_4^-$	33
		$1/2HPO_4^{2-}$	57

Distilled water produced in a laboratory generally has a conductivity in the range 0.5 to 3 μmhos/cm. The conductivity increases shortly after exposure to both air and the water container.

The conductivity of potable waters in the United States ranges generally from 50 to 1500 μmhos/cm. The conductivity of domestic wastewaters may be near that of the local water supply, although some industrial wastes have conductivities above 10 000 μmhos/cm. Conductivity instruments are used in pipelines, channels, flowing streams, and lakes and can be incorporated in multiple-parameter monitoring stations using recorders.

Most problems in obtaining good data with conductivity monitoring equipment are related to electrode fouling and to inadequate sample circulation. Conductivities greater than 10 000 to 50 000 μmho/cm or less than about 10 μmho/cm may be difficult to measure with usual measurement electronics and cell capacitance. Consult the instrument manufacturer's manual or published references.[1,5,6]

Laboratory conductivity measurements are used to:

- Establish degree of mineralization to assess the effect of the total concentration of ions on chemical equilibria, physiological effect on plants or animals, corrosion rates, etc.
- Assess degree of mineralization of distilled and deionized water.
- Evaluate variations in dissolved mineral concentration of raw water or wastewater. Minor seasonal variations found in reservoir waters contrast sharply with the daily fluctuations in some polluted river waters. Wastewater containing significant trade wastes also may show a considerable daily variation.
- Estimate sample size to be used for common chemical determinations and to check results of a chemical analysis.
- Determine amount of ionic reagent needed in certain precipitation and neutralization reactions, the endpoint being denoted by a change in slope of the curve resulting from plotting conductivity against buret readings.
- Estimate total dissolved solids (mg/L) in a sample by multiplying conductivity (in micromhos per centimeter) by an empirical factor. This factor may vary from 0.55 to 0.9, depending on the soluble components of the water and on the temperature of measurement. Relatively high factors may be required for saline or boiler waters, whereas lower factors may apply where considerable hydroxide or free acid is present. Even though sample evaporation results in the change of bicarbonate to carbonate the empirical factor is derived for a comparatively constant water supply by dividing dissolved solids by conductivity.
- Approximate the milliequivalents per liter of either cations or anions in some waters by multiplying conductivity in units of micromhos per centimeter by 0.01.

b. Calculation of conductivity: For naturally occurring waters that contain mostly Ca^{2+}, Mg^{2+}, Na^+, K^+, HCO_3^-, SO_4^{2-}, and Cl^- and with TDS less than about 2500 mg/L, the following procedure can be used to calculate conductivity from measured ionic concentrations.[7] The abbreviated water analysis in Table 2510:II illustrates the calculation procedure.

At infinite dilution the contribution to conductivity by different kinds of ions is additive. In general, the relative contribution of each cation and anion is calculated by multiplying equivalent conductances, λ°_{+} and λ°_{-}, mho-cm^2/equivalent, by concentration in equivalents per liter and correcting units. Table 2510:III contains a short list of equivalent conductances for ions commonly found in natural waters.[8] Trace concentrations of ions generally make negligible contribution to the overall conductivity. A temperature coefficient of 0.02/deg is applicable to all ions, except H^+ (0.0139/deg) and OH^- (0.018/deg).

At finite concentrations, as opposed to infinite dilution, conductivity per equivalent decreases with increasing concentration (see Table 2510:I). For solutions composed of one anion type and one cation type (e.g., KCl as in Table 2510:I), the decrease in conductivity per equivalent with concentration can be calculated, ±0.1%, using an ionic-strength-based theory of Onsager.[9] When mixed salts are present, as is nearly always the case with natural and wastewaters, the theory is quite complicated.[10] The following semiempirical procedure can be used to calculate conductivity for naturally occurring waters:

First, calculate infinite dilution conductivity (Table 2510:II, Column 4):

$$k^{\circ} = \Sigma|z_i|(\lambda^{\circ}_{+i})(\mathrm{m}M_i) + \Sigma|z_i|(\lambda^{\circ}_{-i})(\mathrm{m}M_i)$$

where:

$|z_i|$ = absolute value of the charge of the *i*-th ion,
mM_i = millimolar concentration of the *i*-th ion, and
λ°_{+i},λ°_{-i} = equivalent conductance of the *i*-th ion.

If m*M* is used to express concentration, the product, $(\lambda^{\circ}_{+})(\mathrm{m}M_i)$ or $(\lambda^{\circ}_{-})(\mathrm{m}M_i)$, corrects the units from liters to cm^3. In this case k° is 578.2 μmho/cm (Table 2510:II, Column 4).

Next, calculate ionic strength, *IS* in molar units:

$$IS = \Sigma z_i^2(mM_i)/2000$$

The ionic strength is 15.33/2000 = 0.00767 *M* (Table 2510:II, Column 5).

Calculate the monovalent ion activity coefficient, *y*, using the Davies equation for IS ≤ 0.5 *M* and for temperatures from 20 to 30°C.[9,11]

$$y = 10^{-0.5[IS^{1/2}/(1 + IS^{1/2}) - 0.3IS]}$$

In the present example *IS* = 0.00767 *M* and *y* = 0.91.

Finally, obtain the calculated value of conductivity, k_{calc}, from:

$$k_{calc} = k°y^2$$

In the example being considered, k_{calc} = 578.2 × 0.91^2 = 478.8 μmho/cm versus the reported value as measured by the USGS of 477 μmho/cm.

For 39 analyses of naturally occurring waters,[7,12] conductivities calculated in this manner agreed with the measured values to within 2%.

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2510 B. Laboratory Method

1. General Discussion

See 2510A.

The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

a. Self-contained conductivity instruments: Use an instrument capable of measuring conductivity with an error not exceeding 1% or 1 μmho/cm, whichever is greater.

b. Thermometer, capable of being read to the nearest 0.1°C and covering the range 23 to 27°C. Many conductivity meters are equipped to read an automatic temperature sensor.

c. Conductivity cell:

1) Platinum-electrode type—Conductivity cells containing platinized electrodes are available in either pipet or immersion form. Cell choice depends on expected range of conductivity. Experimentally check instrument by comparing instrumental results with true conductivities of the KCl solutions listed in Table 2510:I. Clean new cells, not already coated and ready for use, with chromic-sulfuric acid cleaning mixture [see Section 2580B.3*b*2)] and platinize the electrodes before use. Subsequently, clean and replatinize them whenever the readings become erratic, when a sharp endpoint cannot be obtained, or when inspection shows that any platinum black has flaked off. To platinize, prepare a solution of 1 g chloroplatinic acid, $H_2PtCl_6 \cdot 6H_2O$, and 12 mg lead acetate in 100 mL distilled water. A more concentrated solution reduces the time required to platinize electrodes and may be used when time is a factor (e.g., when the cell constant is 1.0/cm or more). Immerse electrodes in this solution and connect both to the negative terminal of a 1.5-V dry cell battery. Connect positive side of battery to a piece of platinum wire and dip wire into the solution. Use a current such that only a small quantity of gas is evolved. Continue electrolysis until both cell electrodes are coated with platinum black. Save platinizing solution for subsequent use. Rinse electrodes thoroughly and when not in use keep immersed in distilled water.

2) Nonplatinum-electrode type—Use conductivity cells containing electrodes constructed from durable common metals (stainless steel among others) for continuous monitoring and field studies. Calibrate such cells by comparing sample conductivity with results obtained with a laboratory instrument. Use properly designed and mated cell and instrument to minimize errors in cell constant. Very long meter leads can affect performance of a conductivity meter. Under such circumstances, consult the manufacturer's manual for appropriate correction factors if necessary.

3. Reagents

a. Conductivity water: Any of several methods can be used to prepare reagent-grade water. The methods discussed in Section 1080 are recommended. The conductivity should be small compared to the value being measured.

b. Standard potassium chloride solution (KCl), 0.0100*M:* Dissolve 745.6 mg anhydrous KCl in conductivity water and dilute to 1000 mL in a class A volumetric flask at 25°C and store in a CO_2-free atmosphere. This is the standard reference solution, which at 25°C has a conductivity of 1412 μmhos/cm. It is satisfactory for most samples when the cell has a constant between 1 and 2 cm^{-1}. For other cell constants, use stronger or weaker KCl solutions listed in Table 2510:I. Care must be taken when using KCl solutions less than 0.001*M*, which can be unstable because of the influence of carbon dioxide on pure water. For low conductivity standards, Standard Reference Material 3190, with a certified conductivity of 25.0 μS/cm ± 0.3 μS/cm, may be obtained from NIST. Store in a glass-stoppered borosilicate glass bottle.

4. Procedure

a. Determination of cell constant: Rinse conductivity cell with at least three portions of 0.01*M* KCl solution. Adjust temperature of a fourth portion to 25.0 ± 0.1°C. If a conductivity meter displays resistance, *R*, ohms, measure resistance of this portion and note temperature. Compute cell constant, *C:*

$$C,\ cm^{-1} = (0.001412)(R_{KCl})[1 + 0.0191(t - 25)]$$

where:

R_{KCl} = measured resistance, ohms, and
t = observed temperature, °C.

Conductivity meters often indicate conductivity directly. Commercial probes commonly contain a temperature sensor. With such instruments, rinse probe three times with 0.0100*M* KCl, as above. Adjust temperature compensation dial to 0.0191 C^{-1}. With probe in standard KCl solution, adjust meter to read 1412 μmho/cm. This procedure automatically adjusts cell constant internal to the meter.

b. Conductivity measurement: Thoroughly rinse cell with one or more portions of sample. Adjust temperature of a final portion to about 25°C. Measure sample resistance or conductivity and note temperature to ±0.1°C.

5. Calculation

The temperature coefficient of most waters is only approximately the same as that of standard KCl solution; the more the temperature of measurement deviates from 25.0°C, the greater the uncertainty in applying the temperature correction. Report temperature-compensated conductivities as "μmho/cm @ 25.0°C."

a. When sample resistance is measured, conductivity at 25°C is:

$$k = \frac{(1\ 000\ 000)(C)}{R_m[1 + 0.0191(t - 25)]}$$

where:

k = conductivity, μmhos/cm,
C = cell constant, cm^{-1},
R_m = measured resistance of sample, ohms, and
t = temperature of measurement.

b. When sample conductivity is measured without internal temperature compensation conductivity at 25°C is:

$$k,\ \mu mho/cm = \frac{(k_m)}{1 + 0.0191(t - 25)}$$

where:

k_m = measured conductivity in units of μmho/cm at t°C, and other units are defined as above.

For instruments with automatic temperature compensation and readout directly in μmho/cm or similar units, the readout automatically is corrected to 25.0°C. Report displayed conductivity in designated units.

6. Precision and Bias

The precision of commercial conductivity meters is commonly between 0.1 and 1.0%. Reproducibility of 1 to 2% is expected after an instrument has been calibrated with such data as is shown in Table 2510:I.

2520 SALINITY*

2520 A. Introduction

1. General Discussion

Salinity is an important unitless property of industrial and natural waters. It was originally conceived as a measure of the mass of dissolved salts in a given mass of solution. The experimental determination of the salt content by drying and weighing presents some difficulties due to the loss of some components. The only reliable way to determine the true or absolute salinity of a natural water is to make a complete chemical analysis. However, this method is time-consuming and cannot yield the precision necessary for accurate work. Thus, to determine salinity, one normally uses indirect methods involving the measurement of a physical property such as conductivity, density, sound speed, or refractive index. From an empirical relationship of salinity and the physical property detemined for a standard solution it is possible to calculate salinity. The resultant salinity is no more accurate than the empirical relationship. The precision of the measurement of a physical property will determine the precision in salinity. Following are the precisions of various physical measurements and the resultant salinity presently attainable with commercial instruments:

Property	Precision of Measurement	Precision of Salinity
Conductivity	± 0.0002 μmho/cm	±0.0002
Density	$\pm 3 \times 10^{-6}$ g/cm^3	±0.004
Sound speed	±0.02 m/s	±0.01

Although conductivity has the greatest precision, it responds only to ionic solutes. Density, although less precise, responds to all dissolved solutes.

2. Selection of Method

In the past, the salinity of seawater was determined by hydrometric and argentometric methods, both of which were included in previous editions of *Standard Methods* (see Sections 210B and C, 16th Edition). In recent years the conductivity (2520B) and density (2520C) methods have been used because of their high sensitivity and precision. These two methods are recommended for precise field and laboratory work.

3. Quality Assurance

Calibrate salinometer or densimeter against standards of KCl or standard seawater. Expected precision is better than ±0.01 salinity units with careful analysis and use of bracketing standards.

* Approved by Standard Methods Committee, 2010. Editorial revisions, 2011.

2520 B. Electrical Conductivity Method

1. Determination

See Conductivity, Section 2510. Because of its high sensitivity and ease of measurement, the conductivity method is most commonly used to determine salinity.[1] For seawater measurements use the Practical Salinity Scale 1978.[2–5] This scale was developed relative to a KCl solution. A seawater with a conductivity, C, at 15°C equal to that of a KCl solution containing a mass of 32.4356 g in a mass of 1 kg of solution is defined as having a practical salinity of 35. This value was determined as an average of three independent laboratory studies. The salinity dependence of the conductivity ratio, R_t, as a function of temperature (t°C, International Practical Temperature Scale 1968) of a given sample to a standard $S = 35$ seawater is used to determine the salinity. Temperature is currently on the ITS-90 scale and temperature values should be corrected to the corresponding ITS-68 scale ($t_{68} = 1.00024 t_{90}$)[6] before being used in the following relationship.

$$S = a_0 + a_1 R_t^{1/2} + a_2 R_t + a_3 R_t^{3/2} + a_4 R_t^2 + a_5 R_t^{5/2} + \Delta S$$

where ΔS is given by

$$\Delta S = \left[\frac{t - 15}{1 + 0.0162\,(t - 15)}\right](b_0 + b_1 R_t^{1/2} + b_2 R_t + b_3 R_t^{3/2} + b_4 R_t^2 + b_5 R_t^{5/2})$$

and:

$a_0 = 0.0080$	$b_0 = 0.0005$
$a_1 = -0.1692$	$b_1 = -0.0056$
$a_2 = 25.3851$	$b_2 = -0.0066$
$a_3 = 14.0941$	$b_3 = -0.0375$
$a_4 = -7.0261$	$b_4 = 0.0636$
$a_5 = 2.7081$	$b_5 = -0.0144$

valid from $S = 2$ to 42, where:

$$R_t = \frac{C\ (\text{sample at } t)}{C\ (\text{KCl solution at } t)}$$

To measure the conductivity, use a conductivity bridge calibrated with standard seawater* with a known conductivity relative to KCl, following manufacturer's instructions and the procedures noted in Section 2510. If the measurements are to be

* Available from OSIL (Ocean Scientific International, Ltd.), Havant, Hampshire, England.

https://doi.org/10.2105/SMWW.2882.028

made in estuarine waters, make secondary calibrations of weight-diluted seawater of known conductivity to ensure that the bridge is measuring true conductivities.

The Practical Salinity Scale was extended to low salinities[7] using an equation which is valid in the calculation range of 0 to 40 salinity. The equation is:

$$S = S_{PSS} - \frac{a_0}{1 + 1.5X + X^2} - \frac{b_0 \mathrm{f}(t)}{1 + Y^{1/2} + Y^{3/2}}$$

where:

S_{PSS} = value determined from the Practical Salinity Scale given earlier,
$a_0 = 0.008$,
$b_0 = 0.0005$,
$\mathrm{f}(t) = (t-15)/[1 + 0.0162\ (t-15)]$,
$X = 400R_t$, and
$Y = 100R_t$.

The practical salinity breaks with the old salinity-chlorinity relationship, $S = 1.806\ 55\ Cl$. Although the scale can be used for estuarine waters[8–11] and brines[12–14], there are limitations.[13,15–23]

2. Quality Control

The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

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2520 C. Density Method

1. Determination

With a precise vibrating flow densimeter, it is possible to make rapid measurements of the density of natural waters. The measurements are made by passing the sample through a vibrating tube encased in a constant-temperature jacket. The solution density (ρ) is proportional to the square of the period of the vibration (τ).

$$\rho = A + B\tau^2$$

where A and B are terms determined by calibration, B being determined by calibration with a densimeter with standard seawater. The difference between the density of the sample and that of pure water is given by:

$$\rho - \rho_0 = B(\tau^2 - \tau_0^2)$$

where τ and τ_0 are, respectively, the periods of the sample and water. The system is calibrated with two solutions of known density. Follow manufacturer's recommendations for calibration. These two solutions can be nitrogen gas and water or standard seawater and water. The salinity of the sample can be determined from the 1 atm international equation of state for

seawater. This equation relates $(\rho - \rho_0)$ to the practical salinity (S) as a function of temperature.[1]

$$\rho\ (\text{kg/m}^3) = \rho_0 + AS + BS^{3/2} + CS^2$$

where:

$$A = 8.244\ 93 \times 10^{-1} - 4.0899 \times 10^{-3} t + 7.6438 \times 10^{-5} t^2 - 8.2467 \times 10^{-7} t^3 + 5.3875 \times 10^{-9} t^4,$$

$$B = -5.724\ 66 \times 10^{-3} + 1.0227 \times 10^{-4} t - 1.6546 \times 10^{-6} t^2,$$

$$C = 4.8314 \times 10^{-4},$$

and the density of water is given by:

$$\rho_0 = 999.842\ 594 + 6.793\ 952 \times 10^{-2} t - 9.095\ 290 \times 10^{-3} t^2 + 1.001\ 685 \times 10^{-4} t^3 - 1.120\ 083 \times 10^{-6} t^4 + 6.536\ 332 \times 10^{-9} t^5$$

Perform simple iteration by adjusting S until it gives the measured $\rho - \rho_0$ at a given temperature. If the measurements are made at 25°C, the salinity can be determined from the following equation:

$$S = 1.3343\ (\rho - \rho_0) + 2.155\ 306 \times 10^{-4}\ (\rho - \rho_0)^2 - 1.171\ 16 \times 10^{-5}\ (\rho - \rho_0)^3$$

which has a $\tau = 0.0012$ in S. Approximate salinities also can be determined from densities or specific gravities obtained with a hydrometer at a given temperature (Section 210B, 16th Edition).

2. Quality Control

The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

3. Reference

1. Millero, F.J. & A. Poisson. 1981. International one-atmosphere equation of state of seawater. *Deep Sea Res.* 28:625.

2520 D. Algorithm of Practical Salinity

Because all practical salinity measurements are carried out in reference to the conductivity of standard seawater (corrected to $S = 35$), it is the quantity R_t that will be available for salinity calculations. R_t normally is obtained directly by laboratory salinometers, but *in situ* measurements usually produce the quantity R, the ratio of the *in situ* conductivity to the standard conductivity at $S = 35$, $t = 15$°C, $p = 0$ (where p is the pressure above one standard atmosphere and the temperature is on the 1968 International Temperature Scale). R is factored into three parts, i.e.,

$$R = R_p r_t R_t$$

where:

R_p = ratio of *in situ* conductivity to conductivity of the same sample at the same temperature, but at $p = 0$ and r_t = ratio of conductivity of reference seawater, having a practical salinity of 35, at temperature t, to its conductivity at $t = 15$°C. From R_p and r_t calculate R_t using the *in situ* results, i.e.,

$$R_t = \frac{R}{R_p r_t}$$

R_p and r_t can be expressed as functions of the numerical values of the *in situ* parameters, R, t, and p, when t is expressed in °C and p in bars (10^5 Pa), as follows:

$$R_p = 1 + \frac{p(e_1 + e_2 p + e_3 p^2)}{1 + d_1 t + d_2 t^2 + (d_3 + d_4 t) R}$$

where:

$e_1 = 2.070 \times 10^{-4}$, $d_1 = 3.426 \times 10^{-2}$,
$e_2 = -6.370 \times 10^{-8}$, $d_2 = 4.464 \times 10^{-4}$,
$e_3 = 3.989 \times 10^{-12}$, $d_3 = 4.215 \times 10^{-1}$,
and $d_4 = -3.107 \times 10^{-3}$,

and

$$r_t = c_0 + c_1 t + c_2 t^2 + c_3 t^3 + c_4 t^4$$

where:

$c_0 = 0.676\ 609\ 7$,
$c_1 = 2.005\ 64 \times 10^{-2}$,
$c_2 = 1.104\ 259 \times 10^{-4}$,
$c_3 = -6.9698 \times 10^{-7}$, and
$c_4 = 1.0031 \times 10^{-9}$.

https://doi.org/10.2105/SMWW.2882.028

2530 FLOATABLES*

2530 A. Introduction

One important criterion for evaluating the possible effect of waste disposal into surface waters is the amount of floatable material in the waste. Two general types of floating matter are found: particulate matter that includes "grease balls," and liquid components capable of spreading as a thin, highly visible film over large areas. Floatable material in wastewaters is important because it accumulates on the surface, is often highly visible, is subject to wind-induced transport, may contain pathogenic bacteria and/or viruses associated with individual particles, and can significantly concentrate metals and chlorinated hydrocarbons such as pesticides and PCBs. Colloidally dispersed oil and grease behave like other dispersed organic matter and are included in the material measured by the COD, BOD, and TOC tests. The floatable oil test indicates the readily separable fraction. The results are useful in designing oil and grease separators, in ascertaining the efficiency of operating separators, and in monitoring raw and treated wastewater streams. Many cities and districts have specified floatable oil and grease limits for wastewater discharged to sewers.

* Approved by Standard Methods Committee, 2010. Editorial revisions, 2011.

2530 B. Particulate Floatables

1. Discussion

a. Principle: This method depends on the gravity separation of particles having densities less than that of the surrounding water. Particles that collect on the surface and can be filtered out and dried at 103 to 105°C are defined by this test as floatable particles.

b. Application: This method is applicable to raw wastewater, treated primary and secondary effluent, and industrial wastewater. Because of the limited sensitivity, it is not applicable to tertiary effluents or receiving waters, whether freshwater or seawater.

c. Precautions: Even slight differences in sampling and handling during and after collection can give large differences in the measured amount of floatable material. Additionally, uniformity of the TFE* coating of the separation funnel is critical to obtaining reliable results. For a reproducible analysis treat all samples uniformly, preferably by mixing them in a standard manner, before flotation and use consistently prepared separation funnels as much as possible. Because the procedure relies on the difference in specific gravity between the liquid and the floating particles, temperature variations may affect the results. Conduct the test at a constant temperature the same as that of the receiving water body, and report temperature with results.

d. Minimum detectable concentration: The minimum reproducible detectable concentration is approximately 1 mg/L. Although the minimum levels that can be measured are below 1 mg/L, the results are not meaningful within the current established accuracy of the test.

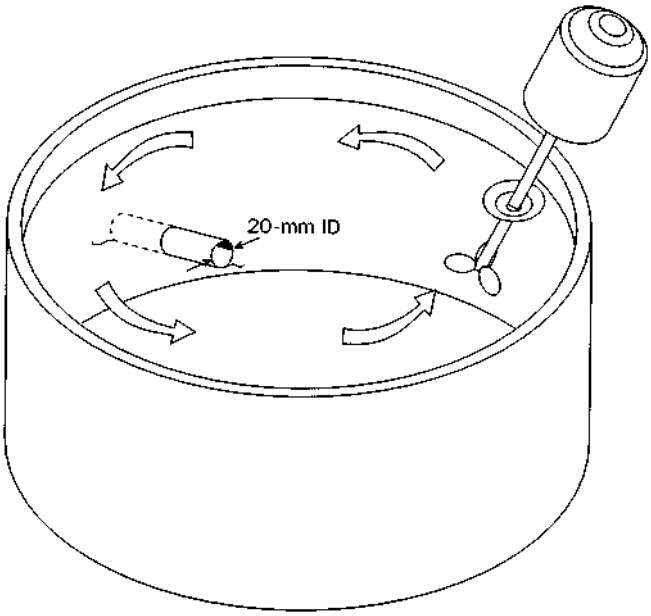


Figure 2530:1. Floatables sampler with mixer.

2. Apparatus

a. Floatables sampler with mixer: Use a metal container of at least 5 L capacity equipped with a propeller mixer on a separate stand (Figure 2530:1), and with a 20-mm-ID bottom outlet cocked at an angle of 45° to the container wall in the direction of fluid movement. The 45° angle assures that even large particles will flow from the container into the flotation funnels where the sample is withdrawn. Fit exterior of bottom outlet with a short piece of tubing and a pinch clamp to allow unrestricted flow through the outlet. Coat inside of container with TFE as uniformly as possible, using a TFE spray to prevent oil and grease from sticking to the surface.

b. Flotation funnel: Use an Imhoff cone provided with a TFE stopcock at the bottom and extended at the top to a total volume

* Teflon, or equivalent.

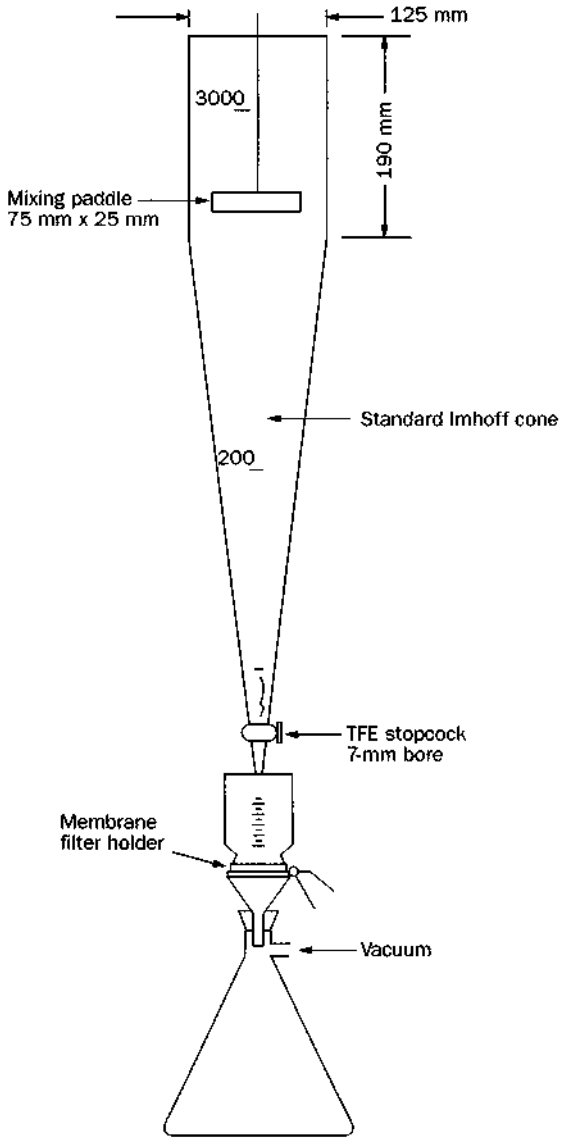


Figure 2530:2. Floatables flotation funnel and filter holder.

of 3.5 L (Figure 2530:2). Coat inside of flotation funnel with TFE as uniformly as possible to prevent floatable grease particles sticking to the sides. Mount flotation funnels as shown in Figure 2530:3 with a light behind the bottom of the funnels to aid in reading levels.

c. Filter holder: Coat inside of top of a standard 500-mL membrane filter holder with TFE, again taking all possible precautions to obtain a uniform TFE coating.

d. Filters, glass fiber, fine porosity.†

e. Vacuum flask, 500 mL.

f. TFE coating: Follow instructions that accompany commercially available coating kits. Alternatively, have necessary glassware coated commercially. Uniform coatings are key to the reliability of the test results, but in practice are difficult to obtain.

† Whatman GF/C, or equivalent.

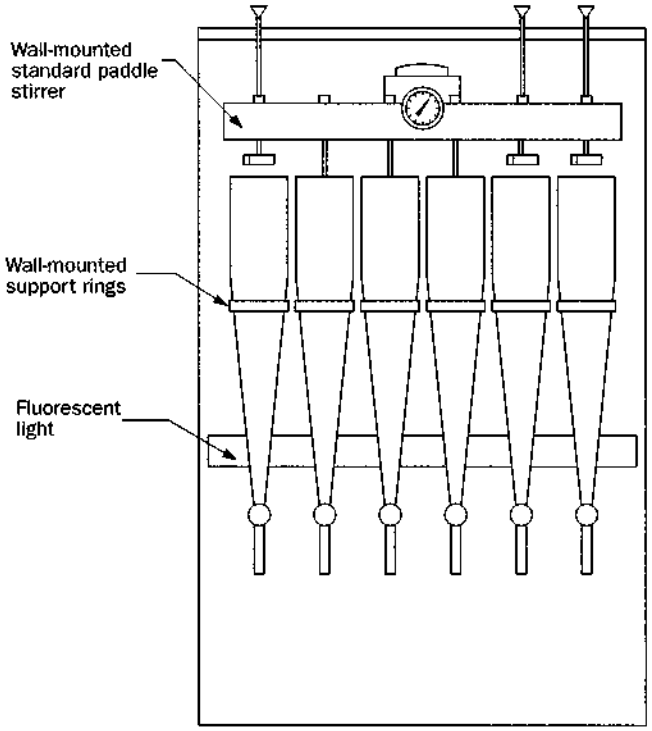


Figure 2530:3. Flotation funnels and mixing unit.

3. Procedure

a. Preparation of glass fiber filters: See Section 2540D.3*a*.

b. Sample collection and treatment: Collect sample in the floatables sampler at a point of complete mixing, transport to the laboratory, and place 3.0 L in the flotation funnel within 2 h after sample collection to minimize changes in the floatable material. While the flotation funnel is being filled, mix sampler contents with a small propeller mixer. Adjust mixing speed to provide uniform distribution of floating particles throughout the liquid but avoid extensive air entrapment through formation of a large vortex.

c. Correction for density and for concentration effects: When a receiving water has a density and ion concentration different from that of the waste, adjust sample density and ion concentration to that of the receiving water. For example, if the receiving water is ocean water, place 1.5 L sample in flotation funnel and add 1.5 L filtered seawater from the receiving area together with mixture of 39.8 g NaCl, 8.0 g $MgCl_2 \cdot 6H_2O$, and 2.3 g $CaCl_2 \cdot 2H_2O$. The final mixture contains the amount of floatables in a 1.5-L sample in a medium of approximately the same density and ion concentration as seawater.

d. Flotation: Mix flotation funnel contents at 40 rpm for 15 min using a paddle mixer (Figure 2530:3). Let settle for 5 min, mix at 100 rpm for 1 min, and let settle for 30 min. Discharge 2.8 L through bottom stopcock at a rate of 500 mL/min. Do not disturb the sample surface in the flotation funnel during discharge. With distilled water from a wash bottle, wash down any floatable material sticking to sides of stirring paddle

and funnel. Let remaining 200 mL settle for 15 min and discharge settled solids and liquid down to the 40-mL mark on the Imhoff cone. Let settle again for 10 min and discharge until only 10 mL liquid and the floating particles remain in funnel. Add 500 mL distilled water and stir by hand to separate entrapped settleable particles from the floatable particles. Let settle for 15 min, then discharge to the 40-mL mark. Let settle for 10 min, then discharge dropwise to the 10-mL mark. Filter remaining 10 mL and floating particles through a preweighed glass fiber filter. Wash sides of flotation funnel with distilled water to transfer all floatable material to filter.

e. Weighing: Dry and weigh glass fiber filter at 103 to 105°C for exactly 2 h (see Section 2540D.3*c*).

4. Calculation

$$\text{mg particulate floatables/L} = \frac{(A - B)}{C}$$

where:

A = weight of filter + floatables, mg,
B = weight of filter, mg, and
C = sample volume, L. (Do not include volume used for density or concentration correction, if used.)

5. Precision and Bias

Precision varies with the concentration of suspended matter in the sample. There is no completely satisfactory procedure for determining the bias of the method for wastewater samples but approximate recovery can be determined by running a second test for floatables on all water discharged throughout the procedure, with the exception of the last 10 mL. Precision and bias are summarized in Table 2530:I. Experience with the method at one municipal treatment plant indicates that the practical lower limit of detection is approximately 1 mg/L.

Table 2530:I. Coefficient of Variation and Recovery for Particulate Floatables Test

Type of Wastewater	Average Floatables Concentration *mg/L*	No. of Samples	Coefficient of Variation %	Recovery %
Raw*	49	5	5.7	96
Raw	1.0	5	20	92
Primary effluent	2.7	5	15	91

* Additional floatable material added from skimmings of a primary sedimentation basin.

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Bracewell, L.W., R.E. Selleck & R. Carter. 1980. Contribution of wastewater discharges to ocean surface particulates. *J. Water Pollut. Control Fed.* 52:2230.

2530 C. Trichlorotrifluoroethane-Soluble Floatable Oil and Grease

1. Discussion

The floatable oil and grease test does not measure a precise class of substances; rather, the results are determined by the conditions of the test. The fraction measured includes oil and grease, both floating and adhering to the sides of the test vessel. The adhering and the floating portions are of similar practical significance because it is assumed that most of the adhering portion would otherwise float under receiving water conditions. The results have been found to represent well the amount of oil removed in separators having overflow rates equivalent to test conditions.

The QC practices considered to be an integral part of each method are summarized in Table 2020:I.

2. Apparatus

a. Floatable oil tube (Figure 2530:4): Before use, carefully clean tube by brushing with a mild scouring powder. Water must form a smooth film on inside of cleaned glass. Do not use lubricant on stopcock.

b. Conical flask, 300 mL.

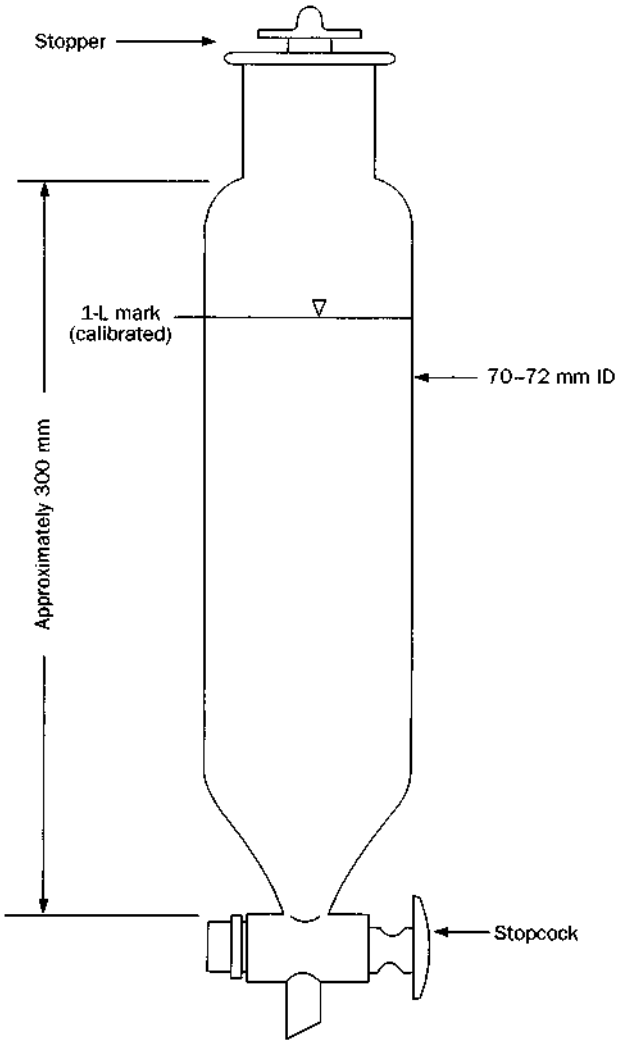


Figure 2530:4. Floatable oil tube, 1-L capacity.

3. Reagents

a. 1,1,2-trichloro-1,2,2-trifluoroethane:* See Section 5520C.3*b*.
b. Hydrochloric acid (HCl), 6*N*.
c. Filter paper.†

4. Procedure

a. Sampling: Collect samples at a place where there is a strong turbulence in the water and where floating material is not trapped at the surface. Fill floatable oil tube to mark by dipping into water. *Do not use samples taken to the laboratory in a bottle, because oil and grease cannot be redispersed to their original condition.*

b. Flotation: Support tube in a vertical position. Start flotation period at sampling site immediately after filling tube. The standard flotation time is 30 min. If a different time is used, state this variation in reporting results. At end of flotation period, discharge the first 900 mL of water carefully through bottom stopcock, stopping before any surface oil or other floating material escapes. Rotate tube slightly back and forth about its vertical axis to dislodge sludge from sides, and let settle for 5 min. Completely discharge sludge that has settled to the bottom or that comes down from the sides with the liquid. Scum on top of the liquid may mix with the water as it moves down the tube. If mixing occurs, stop drawing off water before any floatables have been lost. Let settle for 5 min before withdrawing remainder of water. After removing water, return tube to laboratory to complete test.

c. Extraction: Acidify to pH 2 or lower with a few drops of 6*N* HCl, add 50 to 100 mL trichlorotrifluoroethane, and shake vigorously. Let settle and draw off solvent into a clean dry beaker. Filter solvent through a dry filter paper into a tared 300-mL conical flask, taking care not to get any water on filter paper. Add a second 50-mL portion of trichlorotrifluoroethane and repeat extraction, settling, and filtration into the same 300-mL flask. A third extraction may be needed if the amount of floatables in sample exceeds 4 mg/L. Wash filter paper carefully with fresh solvent discharged from a wash bottle with a fine tip. Evaporate solvent from flask as described in Section 5520B.4. For each solvent batch, determine weight of residue left after evaporation from the same volume as used in the analysis.

5. Calculations

Report results as "soluble floatable oil and grease, 30 min (or other specified) settling time, mg/L."

Trichlorotrifluoroethane-soluble floatable oil and grease, 30 min settling time, mg/L =

$$\frac{(A - B) \times 1000}{\text{mL sample}}$$

where:

A = total gain in weight of tared flask, mg, and
B = calculated residue from solvent blank of the same volume as that used in the test, mg.

6. Precision and Bias

There is no standard against which bias of this test can be determined. Variability of replicates is influenced by sample heterogeneity. If large grease particles are present, the element of chance in sampling may be a major factor. One municipal wastewater discharge and two meat-packing plant discharges, both containing noticeable particles of grease, were analyzed in triplicate. Averages for the three wastewaters were 48, 57, and 25 mg/L; standard deviations averaged 11%. An oil refinery made duplicate determinations of its separator effluent on 15 consecutive days, obtaining results ranging from 5.1 to 11.2 mg/L. The average difference between pairs of samples was 0.37 mg/L.

7. Bibliography

POMEROY, R.D. 1953. Floatability of oil and grease in wastewaters. *Sewage Ind. Wastes* 25:1304.

* Freon, or equivalent.
† Whatman No. 40, or equivalent.

2540 SOLIDS*

2540 A. Introduction

Solids refer to matter suspended or dissolved in potable, surface, and saline waters, as well as domestic and industrial wastewaters. Solids may adversely affect water or effluent quality in a number of ways. Waters with high dissolved solids generally are of inferior palatability and may induce an unfavorable physiological reaction in the transient consumer, so a 500 mg dissolved solids/L limit is desirable for drinking waters. Highly mineralized waters also are unsuitable for many industrial applications. Waters high in suspended solids may be aesthetically unsatisfactory for bathing and other purposes.

Solids analyses are important for controlling water and wastewater treatment processes and assessing compliance with regulatory requirements.

1. Terminology

Fixed solids—the total, suspended, or dissolved solids remaining in a sample after ignition for a specified time at a specified temperature. [Determinations of fixed and volatile solids do not distinguish precisely between inorganic and organic matter because some inorganic compounds can be lost during ignition. Organic matter can be better characterized via total organic carbon (Section 5310), BOD (Section 5210), and COD (Section 5220) methods.]

Settleable solids—the material in a sample that settles out of suspension within a defined period. This may include floating material, depending on the technique used (e.g., 2540F.3*b*).

Total dissolved solids (TDS)—the portion of total solids in a water sample that passes through a filter with a nominal pore size of 2.0 μm (or smaller) under specified conditions.

Total solids—the material left in a sample vessel after evaporation and subsequent oven drying at a defined temperature. *Total solids* includes both total suspended and total dissolved solids, which are physically separated via filtration. Whether a solids particle is filtered into the "suspended" or "dissolved" portion principally depends on a filter's thickness, area, pore size, porosity, and type of holder, as well as the physical nature, particle size, and amount of solids being filtered.

Total suspended solids (TSS)—the portion of total solids in an aqueous sample retained on the filter. NOTE: Some clays and colloids will pass through a 2-μm filter.

Volatile solids—the total, suspended, or dissolved solids lost from a sample after ignition for a specified time at a specified temperature. [Determinations of fixed and volatile solids do not distinguish precisely between inorganic and organic matter because some inorganic compounds can be lost during ignition. Organic matter can be better characterized via total organic carbon (Section 5310), BOD (Section 5210), and COD (Section 5220) methods.]

2. Sources of Error and Variability

Sampling, subsampling, and measuring two- or three-phase samples may introduce significant errors. Maintain sample homogeneity during transfer, and handle carefully to ensure sample integrity. If part of a sample adheres to the container, consider this in evaluating and reporting results. During drying, some samples form a crust that prevents water evaporation; special handling is required to deal with this issue (see below).

Take special care with viscous samples, which might entrain air during mixing and can be difficult to transfer in accurate volumes. Also, take special care with samples with high dissolved solids levels, which are difficult to rinse completely during filtration.

Since total dissolved solids (TDS) and total suspended solids (TSS) are different fractions of the same sample being analyzed, the apparatus and rinsing techniques used may adversely affect TSS and/or TDS results. To avoid this, take care to keep the TDS fraction from migrating into the unrinsed edge of the filter beneath the funnel and being retained as "TSS" weight. If this is suspected, then investigate the filter apparatus seal's effectiveness and/or consider additional rinses.

If using a pipet to measure and transfer sample, place the pipet tip in the center (depth and width) of the well-mixed container. If using a magnetic stir plate and stir bar, set the speed to shear larger particles and blend sample to a more uniform particle size; then pipet sample from mid-depth midway between the container's wall and vortex. Centrifugal force may separate particles of different sizes and densities, resulting in poor precision when sample-withdrawal point varies. Avoid using a magnetic stirrer with samples containing magnetic particles. When using a graduated cylinder, transfer samples to the cylinder immediately after shaking or stirring to avoid any settling of the sample's solids.

Drying temperature, heating duration, and sample matrix can affect weight loss due to volatilization of organic matter, mechanically occluded water, water of crystallization, and gases from heat-induced chemical decomposition. They also can affect weight gains due to oxidation. When liquid samples are put directly into any oven heated above boiling temperature, they may splatter and lose some of the parameter of interest. Therefore, samples can be evaporated to dryness or near dryness at a temperature below boiling using a steam bath, hot plate, or oven before being dried to constant weight per method specifications.

Pay close attention to all samples during post-drying desiccation. Open desiccator as few times as possible to minimize the entry of moist air. If samples are stronger desiccants than the desiccants used in the desiccator, they may take on water. In general, weigh samples as soon as possible after removal from desiccator to minimize water absorption from the atmosphere.

Residues dried at 103–105°C may retain both water of crystallization and some mechanically occluded water. Occluded water, organic matter, and carbonate loss [as carbon dioxide (CO_2)] may slow drying time significantly. Residues dried at 180 ± 2°C should lose mechanically occluded water, but may also lose organic matter and some salts.

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* Approved by Standard Methods Committee, 2015.
Joint Task Group: Michael F. Delaney (chair), Osman M. Aly, David Berwanger, Marianne R. Guzman, Scott A. Jacobs, Keith A. Kibbey, Kim J. Laird, Patty R. Lee, Meaza G. Mariam-Woods, Devon A. Morgan, Lisa M. Ramirez, William R. Ray, Elizabeth J. Robinson, David A. Smith, Zachary B. Smith, J. Mitchell Spears, Mark M. Ultis, Stan K. Van Wagenen, Mark Wyzalek, Meifang Zhou.

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Dry samples to constant weight if possible; this entails multiple cycles of drying, cooling, and weighing for each sample. Results for residues with high oil or grease content may be questionable because such samples are difficult to dry to constant weight in a reasonable timeframe. Any samples that fail to reach a constant weight must be qualified by indicating the number of drying cycles and the final weight change.

When weighing dried samples, be alert to weight changes due to air exposure and/or sample degradation. Make sure samples are cooled to ambient temperature before weighing. Also, remove excess water from glass-fiber filters before placing them on weighing dishes or pans. Excess water will cause filters to adhere to a weighing dish or pan during drying, leading to tearing or loss of material when filter is lifted for weighing, thereby skewing results. This is especially critical for the low-level TSS method. See each method for further discussion of specific interferences.

Use medium-quality reagent water (or better) to rinse filters and filtered solids and to clean labware. Special samples may require a higher-quality water (see Section 1080).

When an analysis deviates from the stated procedures for any reason, record the variations and present them with the results so the reasons for not following the method are documented.

3. Sample Handling and Preservation

Use borosilicate glass, plastic, or fluoropolymer [e.g., polytetrafluoroethylene (PTFE), Teflon®] bottles, so long as suspended solids in sample do not adhere to container walls. Begin analysis as soon as possible, but in no case hold samples >7 d. Settleable solids must be analyzed within 48 h. Between collection and analysis, refrigerate (do NOT freeze) sample at <6°C to minimize microbiological decomposition of solids. Bring samples to room temperature before beginning analysis.

4. Selection of Method

Methods 2540B–F are suitable for determining solids in potable, surface, and saline waters, as well as domestic and industrial wastewaters. The analytical range for 2450B–D is 2.5 to 200 mg/L for a 1000-mL sample, but may be extended by using a small sample volume for analysis. Method 2540G is suitable for determining solids in soils and sediments, as well as solid and semisolid materials produced during water and wastewater treatment.

5. Quality Control (QC)

The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

Analyze ≥5% of all samples in duplicate or at least one duplicate sample with each batch of ≤20 samples. The laboratory may plot duplicate determinations on a control chart for evaluation. Typically, the relative percent difference (RPD) of duplicates should not exceed 10%, but RPDs may vary considerably due to sample matrix and concentration.

Analyze one method blank (MB) per batch of 20 samples for each method except settleable solids (2540F). Blank analysis includes all container- and filter-preparation steps and procedures except sample addition. If any MB measurements are at or above the reporting level, take immediate corrective action (see Section 1020B.5). This may include re-analyzing the sample batch.

Include one laboratory-fortified blank (LFB) per batch of 20 samples for all tests except settleable solids (2540F) and total, fixed, and volatile solids in solid and semisolid samples (2540G). Plot the percent recoveries on a control chart for laboratory evaluation. Laboratories may purchase known standards or prepare in-house working controls for use.

6. Bibliography

THERIAULT, E.J. & H.H. WAGENHALS. 1923. Studies of representative sewage plants. *Pub. Health Bull.* No. 132.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1979. Methods for Chemical Analysis of Water and Wastes; Pub. 600/4-79-020, rev. Mar. 1983. Environmental Monitoring and Support Lab., Cincinnati, Ohio.

2540 B. Total Solids Dried at 103–105°C

1. General Discussion

a. Principle: Evaporate a well-mixed sample in a pre-weighed dish and dry it to constant weight in a 103–105°C oven. The increase compared to the empty pre-weighed dish weight represents total solids. This result may not be the actual weight of dissolved and suspended solids in wastewater samples.

To meet the LFB requirement (2540A.5), a total solids standard can be created as follows: Dry, grind, and sieve a soil for use as a working control. This control may or may not be mixed with other reagents (e.g., Celite 545 or Sigmacell Cellulose Type 20) and may have water added according to the laboratory's procedures.

b. Interferences: Highly mineralized water with a significant concentration of calcium, magnesium, chloride, and/or sulfate may be hygroscopic and require prolonged drying, proper desiccation, and rapid weighing. Exclude large, floating particles or submerged agglomerates of nonhomogeneous materials from sample if they are not desired in the final result.

Optionally, disperse visible floating oil and grease with a blender or homogenizer before withdrawing a sample portion for analysis. If oil and grease sticks to blender sides and blades, thus potentially affecting sample composition, note this in the lab report.

Residues dried at 103–105°C may retain both water of crystallization and some mechanically occluded water. There will be CO_2 loss when bicarbonate converts to carbonate during drying. Usually, very little organic matter will volatilize. It may take a long time to attain constant weight because occluded-water removal is marginal at this temperature.

Because excessive residue in the dish may form a water-trapping crust, limit sample to ≤200 mg residue.

2. Apparatus

a. Sample dishes: Dishes of approximately 90-mm dia and 100-mL capacity made of one of the following materials:

1) Porcelain,
2) Platinum,
3) High-silica glass (may react with highly alkaline samples),* or
4) Other material shown to be resistant to the sample matrix and weight stable at the required evaporation and drying temperatures. Aluminum is NOT appropriate for this purpose.†

*b. Wide-bore pipets,** Class B in glass, mechanical or electronic.

c. Graduated cylinders, Class A.

d. Steam bath (optional) for sample evaporation.

e. Hot plate or block (optional) for sample evaporation. Must be capable of maintaining a temperature <100°C without boiling samples.

f. Pre-drying oven (optional) for sample evaporation that operates at temperatures approximately 2°C below boiling to prevent splattering.

g. Drying oven that operates at 103–105°C.

h. Muffle furnace that operates at 550 ± 50°C.

i. Desiccator, which includes either a desiccant whose color changes in response to moisture concentration or an instrument for measuring moisture (e.g., a hygrometer).

j. Analytical balance, capable of weighing to 0.1 mg.

k. Magnetic stirrer with TFE stirring bar (optional).

l. Blender or homogenizer (optional).

m. Low-form beaker, Class B or better.

3. Procedure

a. Preparation of evaporating dish: If measuring volatile solids, then ignite clean evaporating dish at 550 ± 50°C for ≥15 min in a muffle furnace. If only measuring total solids, then heat clean dish at 103–105°C for ≥1 h. Cool dishes to ambient temperature and weigh. Store weighed dishes in desiccator or oven until needed.

b. Selection of sample size: Choose sample volume to yield between 2.5 and 200 mg dried residue. If necessary, successive sample portions may be added to the same dish after evaporation. Identify any sample that yields residue <2.5 mg or >200 mg, and report the value as described in Sections 1020 and 2020.

c. Sample analysis: Stir or mix sample and quantitatively transfer with a pipet or graduated cylinder to a pre-weighed dish. Evaporate samples to dryness on a steam bath, hot plate, or block, or in a drying oven. Make sure evaporation temperature is ≥2°C below boiling to prevent splattering. Dry evaporated sample for ≥1 h in a 103– 105°C oven. Cool dish in desiccator to ambient temperature, and weigh. Repeat cycle (drying for ≥1 h, cooling, desiccating, and weighing) until weight change is <0.5 mg.

4. Calculation

$$\text{mg total solids/L} = \frac{(A - B) \times 1000}{\text{sample volume, mL}}$$

where:

A = final weight of dried residue + dish, mg, and
B = weight of dish, mg.

5. Precision

Single-laboratory duplicate analyses of 41 samples of water and wastewater were made with a standard deviation of differences of 6.0 mg/L.

6. Bibliography

SYMONS, G.E. & B. MOREY. 1941. The effect of drying time on the determination of solids in sewage and sewage sludges. *Sewage Works J.* 13:936.

* Vycor, product of Corning Glass Works, Corning, NY, or equivalent.
† StableWeigh, Environmental Express, Charleston, SC, or equivalent.

2540 C. Total Dissolved Solids Dried at 180°C

1. General Discussion

a. Principle: Filter a well-mixed sample through a standard glass-fiber filter. Then, transfer the filtrate to a pre-weighed dish, evaporate it to dryness, and dry it to constant weight in an oven at 180 ± 2°C. The increase compared to the empty pre-weighed dish weight represents TDS.

These results may differ from the theoretical value for solids calculated from chemical analysis of sample. Approximation methods for correlating chemical and physical analyses are available.[1] The filtrate collected from the TSS determination (2540D) may be used to determine TDS.

To meet the LFB requirement (2540A.5), analysts can create a TDS standard as follows: Dry NaCl at 103–105°C for ≥1 h, weigh 50 mg, and dilute to 1 L with reagent water. This results in a 50-mg/L TDS standard.

b. Interferences: See 2540A.2. Highly mineralized waters with a considerable calcium, chloride, magnesium, and/or sulfate content may be hygroscopic and require prolonged drying, proper desiccation, and rapid weighing. Samples with high bicarbonate concentrations require careful, possibly prolonged drying at 180 ± 2°C to ensure that bicarbonate completely converts to carbonate.

Residues dried at 180 ± 2°C will lose almost all mechanically occluded water, but some water of crystallization may remain,

especially if sulfates are present. Organic matter may volatilize and be lost, but not completely removed. CO_2 loss occurs when bicarbonates convert to carbonates, and carbonates may be decomposed partially to oxides or basic salts. Some chloride and nitrate salts may be lost. In general, evaporating and drying water samples at 180 ± 2°C yields TDS values closer to those obtained by adding individually determined mineral species than the values obtained when drying at 103–105°C.

Because excessive residue in the dish may form a water-trapping crust, limit sample to ≤200 mg residue.

2. Apparatus

Apparatus listed in 2540B.2*a–l*, and in addition:

a. Glass-fiber filter disks, 22 to 125 mm dia, ≤2-μm nominal pore size without organic binder.*

b. Filtration apparatus: One of the following, suitable for the filter selected:

1) Membrane filter funnel—various capacities, to fit selected filter.
2) Gooch crucible—25- to 40-mL capacity, with Gooch crucible adapter.
3) Filtration apparatus with reservoir and coarse (40- to 60-μm) fritted disk as filter support.†

c. Suction flask with sufficient capacity for sample size selected.

d. Oven that operates at 180 ± 2°C.

3. Procedure

a. Preparation of glass-fiber filter disk: Insert disk with wrinkled side up into filtration apparatus. Apply vacuum and wash disk with three successive volumes of ≥20 mL reagent-grade water. Continue suction to remove all traces of water. If using commercially prepared glass-fiber filter disks, the washing step may be skipped if the manufacturer certifies that the filters meet this method's requirements.

b. Preparation of evaporating dish: If measuring volatile solids, ignite cleaned evaporating dish at 550 ± 50°C for ≥15 min in a muffle furnace. If only measuring TDS, then heat cleaned dish to 180 ± 2°C for ≥1 h in an oven. Cool dishes to ambient temperature and weigh. Store in desiccator or oven until needed.

* Whatman grade 934AH; Gelman type A/E; Millipore type AP40; Ahlstrom grade 161; Environmental Express Pro Weigh; or other products that give demonstrably equivalent results.
† Paul/Gelman No. 4201 magnetic, or equivalent.

c. Selection of filter and sample sizes: Choose sample volume to yield between 2.5 and 200 mg dried residue. If filtration will take >10 min to complete, then increase filter size or decrease sample volume. Identify any sample that yields residue <2.5 mg or >200 mg, and report the value as described in Sections 1020 and 2020.

d. Sample analysis: Stir or mix sample and use a pipet or graduated cylinder to transfer a measured volume onto a glass-fiber filter with applied vacuum. Wash the entire exposed surface of filter with three successive volumes of ≥10 mL reagent-grade water. Allow complete drainage between washings, and continue suction until all traces of water are removed. Transfer total filtrate (with washings) to a pre-weighed evaporating dish and evaporate to dryness on a steam bath, hot plate, or block, or in a drying oven. If necessary, add successive portions to the same dish after evaporation. Dry evaporated sample for ≥1 h in an oven at 180 ± 2°C, cool in a desiccator to ambient temperature, and weigh. Repeat cycle (drying, cooling, desiccating, and weighing) until weight change is <0.5 mg.

If determining volatile solids, follow procedure in 2540E.

4. Calculation

$$\text{mg total dissolved solids/L} = \frac{(A - B) \times 1000}{\text{sample volume, mL}}$$

where:

A = final weight of dried residue + dish, mg, and
B = weight of dish, mg.

5. Precision

Single-laboratory analyses of 77 samples of a known of 293 mg/L were made with a standard deviation of differences of 21.20 mg/L.

6. Reference

1. Sokoloff, V.P. 1933. Water of crystallization in total solids of water analysis. *Ind. Eng. Chem.,* Anal. Ed. 5:336.

7. Bibliography

Howard, C.S. 1933. Determination of total dissolved solids in water analysis. *Ind. Eng. Chem.,* Anal. Ed. 5:4.

U.S. Geological Survey. 1974. Methods for Collection and Analysis of Water Samples for Dissolved Minerals and Gases. Techniques of Water-Resources Investigations; Book 5, Chap. A1. Washington, D.C.

2540 D. Total Suspended Solids Dried at 103–105°C

1. General Discussion

a. Principle: Filter a well-mixed sample through a pre-weighed standard glass-fiber filter, and then dry the filter and the residue retained on it to a constant weight in a 103–105°C oven. The increase in filter weight represents TSS. To estimate an unknown sample matrix TSS concentration, calculate the difference between TDS and total solids.

To meet the LFB requirement (2540A.5), a TSS standard can be created as follows: weigh 100 mg of Sigmacell® Cellulose Type 20

or Celite 545, dilute to 1 L with reagent water, and stir for ≥15 min to mix well. This results in a 100 mg/L TSS standard.

b. Interferences: See 2540A.2 and B.1*b*. Unless representative of source, exclude large floating particles or submerged agglomerates of nonhomogeneous materials from sample. Limit sample size so it yields ≤200 mg residue, because excessive filter residue may form a water-entrapping crust.

If suspended solids clog the filter, prolonging filtration, consider increasing filter diameter or decreasing sample volume.

When samples contain high concentrations of dissolved solids, thoroughly wash all exposed filter surfaces to ensure that dissolved material is removed (see also 2540A.2). Prolonged filtration due to filter clogging may capture more colloidal materials, resulting in artificially high results.

Handle all filters carefully, with the wrinkled side up. Transfer filters using forceps to grip the residue-free edge. Weigh only the filters, not the support pans or dishes, unless a Gooch crucible is used.

2. Apparatus

Apparatus listed in 2540B.2 and C.2, except for evaporating dishes, steam bath, hot plate or block, and 180 ± 2°C drying oven. In addition:

a. Weighing dishes or pans, constructed of aluminum or other inert material, to hold filters.

b. Forceps.

3. Procedure

a. Preparation of glass-fiber filter disk: Insert filter with wrinkled side up in filtration apparatus. Apply vacuum and wash disk with three successive portions of ≥20 mL reagent-grade water. Continue suction to remove all traces of water. Remove filter from filtration apparatus and transfer to an inert weighing dish. If a Gooch crucible is used, remove crucible and filter combination. Dry in a 103–105°C oven for ≥1 h. Cool in desiccator to ambient temperature and weigh. Store filters (on inert dishes or pans) in desiccator or 103–105°C oven until needed. Adequate filter preparation is demonstrated by negligible weight loss or gain for method blanks.

If measuring volatile solids, ignite at 550 ± 50°C for ≥15 min in a muffle furnace. Cool to room temperature before proceeding. (Alternatively, the ignition step may be performed after washing and drying at 103–105°C for ≥1 h, but before weighing.)

If using commercially prepared glass-fiber filters, the ignition, washing, and weighing steps may be eliminated if the manufacturer certifies that the prepared filters meet this method's requirements. Verify filters using method blanks. Filters are verified if the measured weight differs from the manufacturer's weight by less than ±0.5 mg.

b. Selection of filter and sample sizes: Choose sample volumes to yield between 2.5 and 200 mg dried residue. If filtration takes >10 min to complete, increase filter size or decrease sample volume. Identify any sample that yields residue <2.5 mg or >200 mg, and report the value as described in Sections 1020 and 2020.

c. Sample analysis: Stir or mix sample and use a pipet or graduated cylinder to transfer a measured volume onto a glass-fiber filter with applied vacuum. Wash filter with at least three successive volumes of ≥10 mL reagent-grade water. Allow complete drainage between washings, and continue suction until all traces of water are removed. When filtering samples with high dissolved solids concentrations, additional washings may be required to ensure that dissolved material is removed from all exposed filter surfaces.

Using forceps, carefully remove filter from filtration apparatus and transfer to an inert weighing dish or pan as a support. If using a Gooch crucible, remove crucible and filter combination from the crucible adapter. Dry for ≥1 h in a 103–105°C oven, cool in a desiccator to ambient temperature, and weigh. Repeat the cycle (drying, cooling, desiccating, and weighing) until the weight change is <0.5 mg. If determining volatile solids, treat the residue according to 2540E.

4. Calculation

$$\text{mg total suspended solids/L} = \frac{(A - B) \times 1000}{\text{sample volume, mL}}$$

where:

A = final weight of filter + dried residue, mg, and
B = weight of filter, mg.

5. Precision

The standard deviation was 5.2 mg/L (coefficient of variation 33%) at 15 mg/L, 24 mg/L (10%) at 242 mg/L, and 13 mg/L (0.76%) at 1707 mg/L in studies by two analysts of four sets of 10 determinations each.

Single-laboratory duplicate analyses of 50 samples of water and wastewater were made with a standard deviation of differences of 2.8 mg/L.

6. Bibliography

Degen, J. & F.E. Nussberger. 1956. Notes on the determination of suspended solids. *Sewage Ind. Wastes* 28:237.

Chanin, G., E.H. Chow, R.B. Alexander & J. Powers. 1958. Use of glass fiber filter medium in the suspended solids determination. *Sewage Ind. Wastes* 30:1062.

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Trees, C.C. 1978. Analytical analysis of the effect of dissolved solids on suspended solids determination. *J. Water Pollut. Control Fed.* 50:2370.

2540 E. Fixed and Volatile Solids Ignited at 550°C

1. General Discussion

a. Principle: The residue from 2540B, C, or D is ignited to constant weight at 550 ± 50°C. The remaining solids are *fixed* total, dissolved, or suspended solids, while those lost to ignition are *volatile* total, dissolved, or suspended solids. This determination is useful in controlling wastewater treatment plant operations because it offers a rough approximation of the amount of organic matter present in wastewater solids, activated sludge, and industrial wastes.

b. Interferences: There may be negative errors in volatile solids calculations if volatile matter was lost during the drying step of 2540D.3*c*. If a sample contains far more fixed than volatile solids, then volatile solids determined by this procedure may be subject to considerable error; try estimating volatile solids via another test [e.g., total organic carbon (Section 5310)].

Highly alkaline residues may react with any silica in sample or crucibles.

Usually, about 15 min of ignition is required for up to 200 mg residue. However, longer ignition times may be needed if drying heavier residues or more than one sample.

To avoid breakage due to drastic temperature change, dishes and filters may be partially cooled in any oven, steam bath, or hot plate listed in 2540 or else in ambient air until most of the heat dissipates. Transfer to a desiccator for final cooling in a dry atmosphere. Do not overload desiccator. Weigh dish or filter as soon as it has cooled to ambient temperature.

2. Apparatus

See 2540B.2, C.2, and D.2.

3. Procedure

Bring a muffle furnace to 550 ± 50°C. Insert a dish or filter containing residue produced by 2540B, C, or D into furnace. Ignite for at least 15 min at 550 ± 50°C, cool in a desiccator to ambient temperature, and weigh. Repeat cycle (igniting, cooling, desiccating, and weighing) until weight is constant or weight change is <0.5 mg.

4. Calculation

$$\text{mg volatile solids/L} = \frac{(A - B) \times 1000}{\text{sample volume, mL}}$$

$$\text{mg fixed solids/L} = \frac{(B - C) \times 1000}{\text{sample volume, mL}}$$

where:

A = final weight of residue + dish or filter before ignition, mg,
B = final weight of residue + dish or filter after ignition, mg, and
C = weight of dish or filter, mg.

5. Precision

The standard deviation was 11 mg/L at 170 mg/L volatile total solids in studies by three laboratories on four samples and 10 replicates. Bias data on actual samples cannot be obtained.

2540 F. Settleable Solids

1. General Discussion

a. Principle: The settleable solids in surface and saline waters, and in domestic and industrial wastes, may be determined and reported based on either volume (mL/L) or weight (mg/L).

b. Interferences: The volumetric procedure (Imhoff cone) generally has a practical lower measurement limit between 0.1 and 1.0 mL/L, depending on sample composition. The settled-sample-level reading may be affected by sample foaming, sample separation, or pockets of liquid between large settled particles. When measuring heavy sludges whose measured settleable solids may be >100 mL/L, analysts may use a 1000-mL, Class A graduated cylinder instead of a cone. (Imhoff cones typically either lack graduation marks at that level or have marks scaled per 100 mL; a graduated cylinder may offer better resolution.)

If the settled matter contains pockets of liquid between large settled particles, then estimate the liquid volume, subtract it from settled-solids volume, and note in the lab report. If settleable and floating solids separate, do not estimate the floating material as settleable matter. If biological or chemical floc is present, the gravimetric method (2540F.3*b*) is preferred.

2. Apparatus

a. Volumetric:
1) Imhoff cone.
2) Graduated cylinder, Class A.
3) Stir-rod, made of glass or other inert material.

b. Gravimetric:
1) Apparatus listed in 2540D.2.
2) Glass vessel, minimum 9-cm-dia. A standpipe, graduated cylinder, or other vessel may be used as long as it satisfies the 9-cm-dia requirement and can hold the required sample volume.

3. Procedure

a. Volumetric: Fill an Imhoff cone or graduated cylinder to the 1-L mark with a well-mixed sample. Settle for 45 min, then gently agitate sample near the cone sides with a rod or by

spinning. Allow sample to settle for another 15 min, and record volume of settleable solids in the cone as mL/L.

b. Gravimetric:

1) Determine TSS as in 2540D.

2) Stir or mix sample and quantitatively transfer at least 1 L of well-mixed sample into a 9-mm-dia glass vessel to fill vessel to ≥20 cm deep. Use a vessel with a larger diameter and larger sample volume if necessary. Let stand quiescent for 1 h. Then without disturbing the settled or floating material, carefully siphon 250 mL from center of container at a point halfway between the liquid surface and the surface of the settled material. Determine TSS (mg/L) of supernatant (2540D); these are the nonsettleable solids.

4. Calculation

$$\text{mg settleable solids/L} = \text{mg TSS/L} - \text{mg nonsettleable solids/L}$$

5. Precision and Bias

Precision and bias data are currently unavailable.

6. Bibliography

FISCHER, A.J. & G.E. SYMONS. 1944. The determination of settleable sewage solids by weight. *Water Sewage Works* 91:37.

2540 G. Total, Fixed, and Volatile Solids in Solid and Semisolid Samples

1. General Discussion

a. Applicability: This method can be used to determine total solids and its fixed and volatile fractions in such solid and semi-solid samples as river and lake sediments; sludges separated from water and wastewater treatment processes; and sludge cakes from vacuum filtration, centrifugation, or other sludge-dewatering processes.

b. Interferences: The determination of both total and volatile solids in these materials is subject to negative error due to loss of ammonium carbonate and volatile organic matter during drying. Although this is also true for wastewater, the effect tends to be more pronounced with sediments—especially sludges and sludge cakes. The mass of organic matter recovered from sludge and sediment requires a longer ignition time than that specified for wastewaters, effluents, or polluted waters. Carefully observe specified ignition time and temperature to control losses of volatile inorganic salts if these are a problem. Take all weight measurements quickly because wet samples tend to lose weight via evaporation. After drying or ignition, residues often are hygroscopic, rapidly absorbing moisture from the air. Highly alkaline residues may react with silica in samples or silica-containing crucibles.

2. Apparatus

All of the apparatus listed in 2540B.2 except the magnetic stirrer and pipets. A balance capable of weighing to 10 mg may be used.

3. Procedure

a. Total solids:

1) Preparation of evaporating dish—If measuring volatile solids, ignite a clean evaporating dish at 550 ± 50°C for ≥15 min in a muffle furnace. If only measuring total solids, heat dish for ≥1 h in a 103–105°C oven. Cool in desiccator to ambient temperature and weigh. Store in desiccator or 103–105°C oven until needed.

2) Sample analysis

a) Fluid samples—If sample contains enough moisture to flow readily, then stir or shake to homogenize, transfer approximately 25–50 g to a prepared evaporating dish, and weigh (dish plus sample). Evaporate to dryness on a water bath, on a hot plate or block, or in a drying oven, then dry the evaporated sample at 103–105°C for ≥1 h, cool to ambient temperature in a desiccator, and weigh. Repeat cycle (drying, cooling, desiccating, and weighing) until the weight change is <50 mg.

b) Solid samples—If sample consists of discrete pieces of solid material (e.g., dewatered sludge), then take care to obtain a representative sample whose particle size will not impede drying. One of the following manual-processing options may be used:

- take cores from each piece with a No. 7 cork borer and mix crumbled cores together well, or
- pulverize entire sample coarsely on a clean surface by hand (covered with clean gloves) or using a clean mortar and pestle.

Manually process samples as quickly as possible to prevent moisture loss. Processing via mechanical grinding is not recommended because moisture levels could drop during processing.

Transfer approximately 25–50 g to a prepared evaporating dish and weigh. Then, place in a 103–105°C oven for ≥1 h, cool to ambient temperature in a desiccator, and weigh. Repeat cycle (drying, cooling, desiccating, and weighing) until weight change is <50 mg.

b. Fixed and volatile solids: Transfer dried residue from 2540G.3*a*2)a) or b) to a cool muffle furnace, heat furnace to 550 ± 50°C, and then allow ignition to occur for ≥1 h. If residue contains large amounts of organic matter, consider first igniting it over a gas burner under an exhaust hood with enough air to lessen losses due to reducing conditions and to avoid odors in the laboratory. Alternatively, use a muffle furnace in a hood and open the door periodically to ensure air flow.

Cool in desiccator to ambient temperature, and weigh. Repeat cycle (igniting, cooling, desiccating, and weighing) until the weight change is <50 mg.

4. Calculation

$$\%\ \text{total solids} = \frac{(A - B) \times 100}{C - B}$$

$$\%\ \text{volatile solids} = \frac{(A - D) \times 100}{A - B}$$

$$\% \text{ fixed solids} = \frac{(D - B) \times 100}{A - B}$$

where:

A = final weight of dried residue + dish, mg,
B = weight of dish, mg,
C = weight of wet sample + dish, mg, and
D = final weight of residue + dish after ignition, mg.

5. Precision and Bias

Precision and bias data are currently unavailable.

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2550 TEMPERATURE*

2550 A. Introduction

Temperature readings are used in the calculation of various forms of alkalinity, in studies of saturation and stability with respect to calcium carbonate, in the calculation of salinity, in a number of colorimetric tests, and in general laboratory operations. In limnological studies, knowledge of water temperatures as a function of depth often are required. Elevated temperatures resulting from discharges of heated water may have significant ecological impact. The source of water supply, such as deep wells, often can be identified by temperature measurements alone. Industrial plants often require data on water temperature for process use or heat-transmission calculations.

* Approved by Standard Methods Committee, 2010.

2550 B. Laboratory and Field Methods

1. Laboratory and Other Non-Depth Temperature Measurements

Normally, temperature can be measured using any standard liquid-in-glass or electronic thermometer with an analog or digital readout. The device should be able to distinguish temperature changes of 0.1°C or less, and equilibrate rapidly (have a minimal thermal capacity). Abstain from using mercury-filled thermometers whenever possible to avoid the possibility of releasing mercury into the environment if the thermometer breaks. To prevent breakage in field operations, use a thermometer with a metal case.

Periodically check the device's bias (within the temperature range of use) against a reference thermometer certified by the National Institute of Standards and Technology (NIST, formerly National Bureau of Standards) and using tolerances suggested in NIST Handbook 105-6.* The certified thermometer must be used with its certificate and correction chart.

A total immersion thermometer is designed to indicate temperatures correctly when the bulb and the entire liquid column are exposed to the temperature being measured (except for a minimal emergent length for handling). A partial-immersion thermometer has a line around it at the immersion distance from the bottom. It indicates correctly when the bulb and the liquid column to that line are exposed to the temperature being measured and the emergent stem is at ambient temperature.

The quality control (QC) practices considered to be an integral part of each method are summarized in Table 2020:II. T2020

2. Depth Temperature Measurements

Depth temperatures required for limnological studies may be measured with a reversing thermometer, thermophone, or thermistor. The thermistor is most convenient and accurate; however, higher cost may preclude its use. Before field use, verify a temperature measurement device's calibration according to NIST specifications. Make readings with the thermometer or device immersed in water long enough to permit complete equilibration. Report results to the nearest 0.1 or 1.0°C, depending on need.

A reversing type thermometer is commonly used for depth measurements. It often is mounted on the sample-collection apparatus so a water sample can be obtained simultaneously. Correct reversing-thermometer readings for changes due to differences between temperature at reversal and temperature at time of reading. Calculate as follows (all units in degrees):

$$\Delta T = \left[\frac{(T^1 - t)(T^1 + V_0)}{K}\right] \times \left[1 + \frac{(T^1 - t)(T^1 + V_0)}{K}\right] + L$$

where:

ΔT = correction to be added algebraically to uncorrected reading,
T^1 = uncorrected reading at reversal,
t = temperature at which thermometer is read,
V_0 = degree volume of thermometer, the volume of small bulb end of capillary up to 0°C graduation,
K = constant depending on relative thermal expansion of mercury and glass (usual value of $K = 6100$), and
L = calibration correction of thermometer depending on T^1.

If series observations are made, it is convenient to prepare graphs for a thermometer to obtain ΔT from any values of T^1 and t.

* Some commercial thermometers may be as much as 3°C in error.

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2560 PARTICLE COUNTING AND SIZE DISTRIBUTION*

2560 A. Introduction

1. General Discussion

Particles are ubiquitous in natural waters and in water and wastewater treatment streams. Particle counting and size distribution analysis can help to determine the makeup of natural waters, treatment plant influent, process water, and finished water. Similarly, it can aid in designing treatment processes, making decisions about changes in operations, and/or determining process efficiency. The particle size distribution methods included herein depend on electronic measurement devices because manual methods are likely to be too slow for routine analysis. However, when particle size analysis will include size distribution of large (>500-μm) aggregates, use direct microscopic counting and sizing. The principles of various instruments capable of producing both size and number concentration information on particulate dispersions are included. Unless explicitly stated otherwise, the term *size distribution* means an absolute size distribution (i.e., one that includes the number concentration or count).

In most particle-counting instruments, particles pass though a sensing zone where they are measured individually; the only exception included is the static light-scattering instrument. Instruments create an electronic pulse (voltage, current, or resistance) that is proportional to a characteristic particle size. The instrument responses (pulse height, width, or area) are classified by magnitude and counted in each class to yield the particle size distribution.

2. Method Selection

Three types of instruments are included: electrical sensing zone instruments, light-blockage instruments, and light-scattering instruments.

Select instrument consistent with expected use of the particle size analysis. Instruments vary in the particle characteristic being sensed, lower and upper size limits of detection, degree of resolution of the size distribution, particle number concentration range that can be measured accurately, amount of shear to which a sample is subjected before measurement, amount of sample preparation, operator skill required, and the ease with which data can be obtained and manipulated into the desired forms. See 2560B.1, C.1, and D.1, and manufacturers' literature for information on each instrument's characteristics.

Some instruments can be set up for either continuous-flow or batch sampling. Others can only be used for batch analysis. For instruments usable in both modes, check that no systematic differences in particle size distributions occur between continuous-flow measurements and batch samples taken at or near the intake point for continuous-flow samples.

3. Sample Collection and Handling

a. Batch samples: Use extreme care in obtaining, handling, and preparing batch samples to avoid changing total particle count and size distribution.

Choose representative times and locations for sampling. Ensure that particles are not subjected to greater physical forces during collection than in their natural setting. Collect samples from a body of water via submerged vessels to minimize turbulence and bubble entrainment. If sampling from particular depths, use standard samplers designed for that purpose. For flowing systems, make sure that the velocity into the opening of the sampling device is the same as that of the flowing stream (isokinetic sampling) and that the opening diameter is at least 50 times as large as the particles to be measured. For sampling from a tap, let water flow slowly and continuously down the side of the collection vessel.

Minimize particle contamination from the air, dilution water (or, for electrical sensing zone instruments, electrolyte solution) (see 2560A.4), and any vessel or glassware that comes in contact with the sample. Minimize exposure to air by keeping sample in a closed container and by minimizing time between sampling and analysis.

Preferably use glass bottles and other vessels with TFE bottle cap liners.

Clean all glassware scrupulously via automatic dishwashing, vigorous hand brushing, and/or ultrasonication. Rinse glassware with particle-free water immediately before use. Between samples, rinse any part of the instrument that comes in contact with samples with either clean water or the upcoming sample. Alternatively, run multiple replicates and discard the first results.

To avoid breaking up aggregates of particles or flocs, sample and make dilutions very slowly using wide-bore pipets, needles, or other sampling devices; cut off pipet tips to avoid high velocities at the entrance. If sample dilution is required, add sample to dilution water, not vice versa, by submerging the pipet tip in the dilution water and releasing sample slowly. Use the minimum mixing intensity and duration adequate to dilute the suspension into the dilution water. Avoid mechanical stirrers inside the sample or ultrasonication. Simultaneously gently rotate and partially invert entire sample in a closed bottle. Use cylindrical dilution bottles to avoid sharp corners. Leave less than approximately 25% air space during mixing. To avoid sedimentation, make measurements immediately after mixing. Do not mix during measurement unless absolutely necessary to prevent sedimentation.

Most surface and ground waters contain relatively stable particles that aggregate slowly. Particle size distribution in biologically active waters or waters that have been treated with coagulants is more likely to change over short periods. To minimize flocculation, minimize time between sampling and measurement. In highly flocculent systems, maximum holding time should only be a few minutes; for more stable samples, a few hours may be acceptable. Dilution slows flocculation kinet-

* Approved by Standard Methods Committee, 2009. Editorial revisions, 2011.
Joint Task Group: 20th Edition—Desmond F. Lawler (chair), Erika E. Hargesheimer, Carrie M. Lewis, Nancy E. McTigue, Theodore S. Tanaka, John E. Tobiason, Mark R. Wiesner.

ics and, in some cases, makes flocculation less likely. Make dilutions only immediately before measurement.

Samples measured at different temperature or pressure than when collected and those with biological activity may develop entrained air bubbles that interfere with measurement accuracy. If any gas bubbles are visible, let sample stand for a short time to degas naturally or use a mild vacuum to speed degassing. Use ultrasound to aid degassing, but only if floc breakup is not a problem.

Minimize time between sampling and analysis; if at all possible, make measurements immediately after sampling. If storage is unavoidable, refrigerate at 4°C but restore samples to room temperature, preferably in water bath, before measurement.

b. Continuous-flow: Using a particle counter as a continuous-flow monitor may be desirable. Many more samples can be processed by automated particle counting than by batch sample analysis. All the instruments mentioned in 2560B–D can be used in this mode, although instruments and samples not requiring dilution are easier to set up. For some instruments, this type of operation requires custom hardware; for others, there is commercially available hardware.

Batch sample problems are equally relevant to continuous analysis. Other critical considerations include selection of sampling point, velocity at entrance and throughout sample line, maintenance of stable sample flow rate, position of instrument's sensor in the sample line, and absence of flow-modifying devices upstream of sensor.

Choose a sampling point that is representative and away from wall surfaces. Place entrance to sample line facing the flow, with the velocity at the opening nearly the same as the surrounding flow.

Sample preservation within the instrument is difficult because both deposition (or temporary holdup) of particles and floc breakup must be avoided. Deposition occurs by gravity settling of particles onto horizontal surfaces. Floc breakup occurs because of excessive shear forces. Minimize length of transmission lines, especially in horizontal components, and preferably have no horizontal lines. Avoid drastic changes in flow direction or velocity. Do not use flow-modifying devices (pumps, fittings with irregular surfaces, sharp angle changes, flow controllers, etc.) between the sampling point and the sensor. Locate pumps after the sensor. Preferably, provide curved sample lines (e.g., flexible tubing or bent glass tubing) rather than right-angle fittings.

For most instruments, flow rate is specified. Because calibration (see 2560A.5) can change with flow rate within the range, flow control within very narrow limits is essential. Calibrate and use the sensor at the same flow rate. For continuous particle size distribution measurements, flow control (not simply flow monitoring) is necessary. Do not make measurements at a flow rate different from that used for calibration. Provide a flow control system downstream of the sensor, maintain a constant rate, and do not introduce turbulence or pulsations before or through the sensor.

4. Dilution Water

Particle-free water is virtually impossible to obtain, but it is possible to produce water containing very few particles within the size range to be measured. Produce "particle-free" or "clean" water from distilled, deionized water or water taken from the same source as the samples. If all samples are from the same source or have similar chemical characteristics, preferably filter sample water to produce "particle-free" water with no change in chemical environment. Distilled, deionized water produced by ion exchange and cartridge filtration may produce acceptable "particle free" dilution water, but preferably use continuous closed-loop membrane filtration.

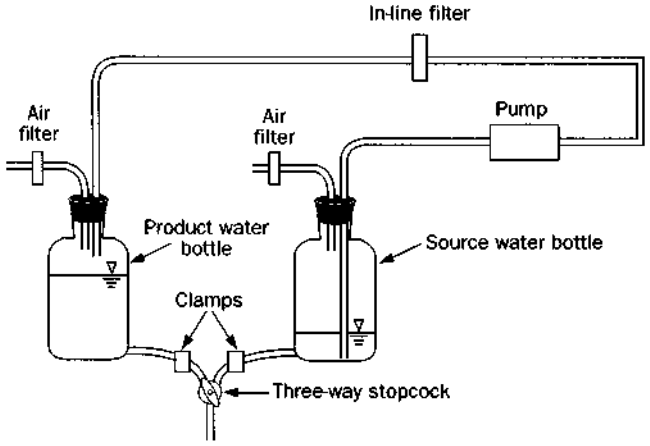


Figure 2560:1. Schematic of filtration apparatus for preparing particle-free dilution water or electrolyte solution.

Dilution water preparation systems are available from particle-counter manufacturers. Alternatively, assemble a system similar to that shown in Figure 2560:1 (or any system that produces a water of sufficient quality). In the system shown, a pump draws the water from a bottle and puts it through the in-line filter. Use membrane filters with nominal pore sizes no more than 10% of the smallest particle size expected; alternatively use cartridge filters. Pass water through the filter several times. The system lets water be passed directly from the product-water bottle to the source-water bottle by opening the clamps and three-way stopcock. Use glass tubing in the bottles, but use flexible tubing to allow product-water bottle to drain into source-water bottle. Attach a glass wool or membrane filter to air inlets. Dilute samples by drawing water directly from the three-way stopcock into the bottle to be used in sample analysis. F1

A simpler system with one-pass filtration directly into the sample bottle may be adequate for many samples, depending on the particle size range to be measured. Such a system would omit the product-water bottle, the connections between the two bottles and associated stopcock and clamps of Figure 2560:1. Dilution water is produced on demand and the effluent is put directly, without additional handling, into the sample container.

Guard against biological growth within filtration systems by frequent disassembly and adequate washing or replacement of components. For many samples, do not use chemical disinfectants because they might change the particle count and size distribution by their oxidizing potential.

5. Calibration

When an instrument's sensing zone detects a particle; an electrical response is generated and sorted into a channel of the instrument based on its magnitude.

Calibrate by determining the channel number into which the instrument sorts particles of known size. Use spherical particles manufactured for this purpose. Calibration particles are available

in suspension or as dry particles. Over time, suspensions are likely to undergo some aggregation; use ultrasound to break up flocs before calibration. Calibration particles are nearly monodisperse but do exhibit a small inherent (true) variance.

The instrument's precision (Section 1030A.1) and resolution influence its ability to sort particles into different channels. *Resolution* is a measure of an instrument's ability to distinguish between particles of similar but different sizes. An instrument with high precision and good resolution will measure monodisperse particles in a very narrow size range; some instruments have sufficient precision and resolution so calibration particles with extremely narrow size distributions (very small variance) are sorted into a few adjacent channels. In such cases, the true variance of the particle size and the measured variance will be nearly equal. An instrument with low precision but high resolution will yield a wide distribution on the same particles (i.e., measured variance $>$ true variance). An instrument with high precision but low resolution (e.g., few channels) may yield a narrow measured distribution (e.g., all the particles in the same channel) even though the true distribution is broader (i.e., measured variance $<$ true variance).

Use at least three sizes of calibration particles in reasonably similar number concentrations to calibrate a sensor. To analyze different-size particles in a mixture, ensure that the different sizes do not interfere with one another. Calibrate under conditions identical with those of sample measurements, (e.g., instrument settings, flow rate, type of sample cell, dilution water or electrolyte solution, and mixing during measurement). Do not exceed the sensor's maximum concentration during calibration.

The calibration curve depends on which particle characteristic the instrument measured (diameter, area, or volume) and whether the pulses are sorted into channels on an arithmetic or logarithmic basis. For example, if an electrical sensing zone instrument (which responds to particle volume) is used in a logarithmic mode, the calibration curve will be the logarithm of particle volume vs. channel number.

Generally, increments between channels are equal on an arithmetic or logarithmic basis. In most light-blockage and light-scattering instruments, the user can set the lower and upper limits for each channel. Because most samples have broad particle size distributions—spanning at least one order of magnitude, and often two or three—preferably use larger increments for larger sizes. This is consistent with equal logarithmic spacing, although other, less systematic intervals that preserve the characteristic of larger increments for larger sizes are permissible. Such intervals also are consistent with the resolution capabilities of most available instruments, which respond to a characteristic particle area or volume (proportional to the square or cube of diameter, respectively), and therefore require greater resolution for larger particles than for smaller ones on a diameter basis.

At a given set of settings for a given sensor, each channel represents a certain average size and size increment. After measuring the channel number associated with several sizes of calibration particles, use a calibration equation to assign average sizes or, in some cases, the lower size limit, to all other channels. Knowledge of the average size and each channel arithmetic and logarithmic increment is necessary for reporting. In contrast to calibration particles, environmental particles are rarely spherical. Generally, report measurements in terms of "equivalent spherical diameter" (i.e., size of any particle taken as that of a sphere that would give the same response in the instrument). Because most sample particles are nonspherical and different instruments respond to different characteristics particle, different instruments produce different measured particle size distributions.

Some light-scattering instruments calculate particle size from first principles and do not require calibration per se. For those instruments, periodically adjust the system optics. Make frequent checks that the instrument response is consistent by following the calibration procedures for other instruments.

6. Data Reporting

a. Particle concentrations: Number, surface, or volume concentration over a specified size range is particularly valuable as a summary factor for particle counting and size distribution. Its variation with some changes in an independent variable in a natural or engineered system may be of interest.

When reporting the number concentration (number per milliliter) of particles, report also the size range measured (both lower and upper size limits). The lower limit is particularly important, because most samples have large number concentrations near the lower detection limit of currently available instruments. Never state or imply a lower size limit of zero.

In some studies, a surface area concentration (μm^2/mL) or volume concentration (μm^3/mL) may be more relevant; convert number concentration to these forms by multiplying by the area or volume, respectively, of a sphere with the mean diameter for each size class. (This is an approximation based on the assumption that particles are spherical.) In such cases, also report the lower and upper limits of size class.

b. Tabulated size distributions: If the distribution itself (i.e., the variation of particle number, surface area, or volume concentration with particle size) will be shown in tabular format, give the number concentration and the associated size range (lower and upper limits) for each instrument channel (or grouping of channels).

c. Graphical size distributions: For graphical reporting, preferably use the count information (on a number, surface area, or volume basis) as the ordinate and particle size as the abscissa.

For the count information, use absolute, rather than relative, scales because they indicate both concentration and size distribution. Also, preferably use differential, rather than cumulative, distributions because they show directly which size range contained the most particles. For ease of plotting, associate the number (or surface area or volume) concentration with a mean size for each channel, rather than with the size range (the lower and upper limit). To account for that change without losing information, normalize the data by dividing the particle concentration in a given size class by the size interval for that class (on either an arithmetic or a logarithmic basis). Normalization prevents creation of artificial (or apparent) peaks or valleys in a distribution and ensures that the same distribution measured by different instruments with different size increments will be the same graphically. If data are not normalized, plot size distribution data (absolute and differential) as a discrete histogram (i.e., as a bar chart with the upper limit of one channel being identical to the lower limit of the next).

Preferably show particle sizes via a logarithmic scale. Most samples have broad distributions and most analyzers use larger increments of diameter with increasing size; these characteristics are consistent with a logarithmic scale, which also intrinsically

Table 2560:I. Example Calculations for Particle Size Distribution Analysis

Channel No. (A)	Lower Limit d_p μm (B)	Upper Limit d_p μm (C)	Mean Diam. d_{pi} μm (D)	Δd_{pi} μm (E)	log d_{pi} d_p in μm (F)	Δ log d_{pi} (G)	Corrected Count (H)	Number Conc. ΔN_i *No./mL* (I)	Volume Conc. ΔV_i $\mu m^3/mL$ (J)	$\Delta N_i/\Delta d_{pi}$ *No./ mL-μm* (K)	$\Delta V_i/\Delta d_{pi}$ $\mu m^3/$ *mL-μm* (L)	$\Delta N_i/\Delta$ log d_{pi} *No./mL* (M)	$\Delta V_i/\Delta$ log d_{pi} $\mu m^3/mL$ (N)	log $\Delta N_i/\Delta d_{pi}$ $\Delta N/\Delta d_p$ in *No./mL-μm* (O)
6	2.95	3.39	3.16	0.44	0.50	0.06	5125	102 500	1.69E+6	2.33E+5	3.85E+6	1.71E+6	2.83E+7	5.37
7	3.39	3.89	3.63	0.50	0.56	0.06	4568	91 360	2.29E+6	1.83E+5	4.58E+6	1.52E+6	2.82E+7	5.26
8	3.89	4.47	4.17	0.58	0.62	0.06	3888	77 760	2.95E+6	1.34E+5	5.09E+6	1.30E+6	4.92E+7	5.13
9	4.47	5.13	4.79	0.66	0.68	0.06	3088	61 760	3.55E+6	9.36E+4	5.38E+6	1.03E+6	5.91E+7	4.97
10	5.13	5.89	5.50	0.76	0.74	0.06	2289	45 780	3.99E+6	6.02E+4	5.25E+6	7.63E+5	6.63E+7	4.78
11	5.89	6.76	6.31	0.87	0.80	0.06	1584	31 680	4.17E+6	3.64E+4	4.86E+6	5.28E+5	6.94E+7	4.56
12	6.76	7.76	7.24	1.00	0.86	0.06	1023	20 460	4.07E+6	2.05E+4	4.07E+6	3.41E+5	6.79E+7	4.31
13	7.76	8.91	8.32	1.15	0.92	0.06	631	12 620	3.81E+6	1.10E+4	3.31E+6	2.10E+5	6.33E+7	4.04
14	8.91	10.23	9.55	1.32	0.98	0.06	363	7 260	3.31E+6	5.50E+3	2.51E+6	1.21E+5	5.52E+7	3.74
15	10.23	11.75	10.96	1.52	1.04	0.06	199	3 980	2.74E+6	2.62E+3	1.80E+6	6.65E+4	4.59E+7	3.42

avoids showing a zero size. Produce the logarithmic scale by showing the log of diameter on an arithmetic scale or the arithmetic values of diameter on a logarithmic scale.

d. Example calculations: A calculation layout (with example data) useful for preparing either tabular or graphical presentation is shown in Table 2560:I. This format may be abbreviated or modified to suit the data presentation being developed.

Columns A–G represent calibration information. Which of these values are set (or determined from calibration by the user) and which are calculated from this primary information depends on the type of instrument. In constructing such a table, preferably place primary information first. In most light-blockage and light-scattering instruments, the lower and upper size limits for each channel are known; calculate mean diameter as the arithmetic or logarithmic (geometric) mean of these limits, depending on whether instrument channels represent arithmetic or logarithmic increments. In electrical sensing zone instruments, the mean size (or log size) is determined directly from the calibration equation and increment width [arithmetic (Δd_{pi}) or logarithmic (Δlog d_{pi})] is preset; calculate lower and upper limits as mean size $\pm$ one-half the arithmetic or logarithmic increment.

Columns H–O represent counting information for a sample. The corrected counts (Column H) are the direct counts from the particle size analyzer minus the background count for each channel. Adjust background count for dilution and differences in sample measured and dilution water measured for background count. Adjust this figure for sample dilution and volume analyzed to obtain the number concentration of particles (Column I). For example, for corrected count n, a 1:10 dilution, and 0.5 mL analyzed, number concentration is calculated as $(n \times 10)/0.5 = 20n$/mL. Volume concentration (Column J) may be approximated as the number of particles multiplied by the volume of a spherical particle with the mean diameter for the channel ($\pi d_p^3/6$). Values in the remaining columns are calculated as shown in the table. The normalized, differential, absolute distribution functions obtained in Columns K (particle size distribution function) through O give the values needed for graphic presentations as follows:

K: particle numbers (arithmetic scale) vs. diameter (arithmetic scale)
L: particle volume (arithmetic scale) vs. diameter (arithmetic scale)
M: particle number (arithmetic scale) vs. diameter (logarithmic scale)
N: particle volume (arithmetic scale) vs. diameter (logarithmic scale)
O: log of particle number (arithmetic scale) vs. diameter (logarithmic scale).

(Alternatively, plot values from Column K directly on a logarithmic scale.)

If distribution K, L, M, or N is plotted, the area under the resulting graph between any two diameter values represents the total number or volume concentration between those size limits. The logarithmic graph (O) shows the entire distribution better than other distributions, but can hide differences between similar distributions. It often is linear over specified size ranges.

7. Quality Control

See Sections 1020 and 1030. Observe precautions for sampling and handling discussed in 2560A.3. Particle counting and size analysis requires experienced analysts capable of judgment in recognizing unusual instrument behavior. Ability to recognize partial blockage of an aperture or sensor, or electronic noise is essential.

Electronic noise can be detected directly in some instruments by making a particle count when no flow is being put through a sensor. Ensure that the noise is not environmental (i.e., due to other nearby instruments, poor grounding, or inconsistent electrical supply). Noise also can be created by excessive cable length between the sensor and instrument or worn cables and connectors. Perform noise checks periodically.

Analyze sample blanks—handled identically to real samples—daily. Discard data from all channels that yield counts in the blanks greater than 5% of the counts for real samples. This procedure accounts for both particle contamination and electronic noise. Develop a maximum acceptable total count for blanks for each sensor (or for each set of standard settings for one sensor); if blanks give more than this maximum, discontinue particle counting until the contamination or noise is eliminated. Set maximum so the 5% rule is met for all channels of interest (i.e., down to some minimum size limit acceptable in the laboratory for the sensor in use and the samples being measured). Recognize that the lower measurement limit for every sensor and every instrument is more likely to be dictated by electronic noise and particle contamination than by electronic settings.

Test cleaning and rinsing for sample bottles by partially filling the container with clean water, swirling the water to contact all sides, and performing a particle count. When the container has been shown to contribute negligible counts, check the cap's cleanliness similarly. Develop and document the standard washing and rinsing procedure, verify its validity as indicated, and follow the procedure without exception.

Also develop standard procedure for mixing samples and dilution water. Test mixing by varying the mixing intensity and/or duration to determine the minimum that gives adequate reproducibility (a sign of uniform concentration) and to determine if greater mixing increases total particle counts (a sign of floc breakup). Follow an established mixing procedure without exception.

To determine precision, make replicate measurements on at least 5% of samples. Report standard deviation (or range) of the total particle number concentration for each sensor or the total of all sensors (if more than one is used). (See Section 1030.)

Particle counting and size-distribution analyzers are calibrated for size, not concentration. Standards for calibrating (or checking) an instrument's ability to measure particle concentration accurately are under development. Currently, only precision, not bias, can be measured well for particle concentration. Obtain an indication of bias by preparing standards of known-density particles at a known suspended solids concentration; multiply total measured particle volume concentration by the known density to estimate the suspended solids concentration from the particle measurements. Compare this estimate with the known suspended solids concentration as an indicator of the combined instrument and laboratory bias.

Periodically calibrate with particles traceable to the National Institute of Standards and Technology (NIST).

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2560 B. Electrical Sensing Zone Method

1. General Discussion

a. Principle: In electrical sensing zone instruments, particles are suspended in an electrolyte solution and pass through a small orifice. A constant current or potential is applied between electrodes on either side of the orifice; the change in resistance caused by the particle taking up volume in the orifice causes a change in potential or current (whichever is not being held constant electronically). The pulse is proportional to the particle volume.

The voltage or current pulses are amplified and sorted into size classes or channels based on their maximum height. Some instruments have fixed channels; others permit selection of number of size classes, channel width and/or lowest size to be measured.

b. Interferences: Interferences are caused by particle contamination, gas bubbles, and electronic noise. See 2560A.3 for sampling precautions, and 2560A.4 for particle-free water preparation. Minimize electronic noise as directed in 2560A.7. Increasing electrolyte strength is another method for minimizing electronic noise.

c. Detectable sizes and concentrations: The lower measurement limit depends on both electronic noise and aperture size. Manufacturers claim that the lower size limit (diameter) is approximately 2% of aperture size, but not lower than approximately 0.4 μm; however, values as low as approximately 0.7 μm have been reported. For the upper size limit, manufacturers state that the maximum size is 40 to 60% of aperture size, but a realistic upper limit for measuring flocs is 20% of aperture size.

The minimum concentration limit depends on the ability to distinguish particles from background counts. For each channel, background count should be less than 5% of total count. In size ranges in which the count is very low (zero or nearly so), counts may be statistically unreliable. Determine if a larger sample volume provides more satisfactory (less scattered) data. Grouping data from several adjacent channels, thereby increasing the size increment, also can give a more accurate size distribution.

The maximum concentration limit depends on the rate at which the instrument can process different pulses and the need to avoid more than one particle in the sensing zone at the same time (coincidence). Follow manufacturers' instructions for upper limits on particle counts for each aperture size. Some instruments can correct for overly concentrated samples, but do not use this feature because each particle is not

measured individually and errors in absolute size distribution may result.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

a. Particle counter and size-distribution analyzer.

b. Glassware: For glassware preparation, see 2560A.3*a* and 7.

3. Reagents

a. Particle-free electrolyte solution: Prepare an electrolyte solution of NaCl, $CaCl_2$, $NaNO_3$, $Ca(NO_3)_2$, or some other simple inorganic salt at a concentration of 1 to 10% by weight. Pass solution through a continuous-flow membrane microfiltration system (see 2560A.4). Alternatively, use a commercially prepared electrolyte solution (filtration also may be required).

b. Calibration particles: See 2560A.5.

4. Procedure

a. Preparation: Let instrument warm up according to manufacturer's instructions. Select proper size aperture for particle sizes of interest; use more than one aperture if size distribution is wider than the range that can be measured with one aperture. Rinse aperture with acid solution, distilled water, and finally clean electrolyte solution. Install aperture. Choose instrument settings for each type of choice, including linear or logarithmic mode (spacing of sizes for each channel; logarithmic is preferable for environmental samples), desired channel width, total number of channels and which channels to include, and method of starting and stopping counting. Counting can be started and stopped manually, by switches built into the manometer representing set sample volumes, by a set maximum count in any one channel, or stopped by time after a manual start. When reporting absolute measurements, choose a method that measures volume sampled.

b. Calibration: See 2560A.5. Calibrate each aperture with at least three particle sizes, determining the channel number at which the maximum count for each size is located. Handle calibration samples identically to those used for measurement. Because calibration can change, check it at each use of the instrument. Plot results as the volume of calibration particles vs. channel number (linear mode) or as the logarithm (base 10 or e) of the volume of calibration particles vs. channel number (logarithmic mode). The results should plot as a straight line. Use an equation for that line to assign an average size to each channel. Convert results from particle volume to particle diameter for reporting.

c. Blank sample: Measure at least one blank sample of electrolyte solution. Run a sample bottle contains only electrolyte solution through procedures identical to those used for samples. Subtract counts from the blank solution (i.e., background counts), channel by channel, from sample counts. (See 2560A.7.)

d. Measurement of samples: Prepare diluted sample, ensuring that sample volume is no greater than the volume of electrolyte solution (dilution water); if a more concentrated suspension is necessary, prepare dilution water with a higher electrolyte concentration. Mix gently (see 2560A.3*a*). Insert sample container into the instrument. Start vacuum to control the mercury column and start particle counting. During counting, continuously watch the instrument monitor to check for aperture blockage by oversized particles. Check that the time for the specified volume (if the count is controlled by the stop and start switches on the manometer) is consistent with that found for particle-free electrolyte solution. When count is complete, check that the concentration did not exceed the manufacturer's recommendations. Repeat with a greater dilution if necessary. When a series of samples with various concentrations is to be measured and sample order is not critical, measure samples in order of expected increasing particle concentration, rinsing with next sample.

5. Calculation

See 2560A.6.

2560 C. Light-Blockage Methods

1. General Discussion

a. Principle: In light-blockage instruments, a focused beam of light shines from one side of the measurement zone toward a photovoltaic cell on the other side. The illuminated volume of liquid is the sensing zone. Particles pass through the sensing zone at a known velocity. The light blocked by the particles creates a change in voltage at the photovoltaic cell. In different instruments, different characteristics of the resulting signal are used to determine size: light-obscuration instruments use pulse height (related to the cross-sectional area of the particle) while time-of-transition instruments use pulse width (proportional to a characteristic length of the particle). In all cases, measurements usually are reported in terms of equivalent spherical diameter.

For light-obscuration instruments, consult manufacturer's literature to determine the relationship between voltage-pulse height and particle size (and increment of particle size).

Time-of-transition devices typically use a laser beam as the light source. The beam may scan a stationary sample at a fixed velocity as it sweeps out an optical sensing zone. Alternatively, a suspension can be passed through a fixed optical sensing zone. In the latter case, the measured particle size is sensitive to flow rate through the sensor.

Devices using light-blockage principles vary in the number of channels (size classes) into which particles are sorted. In some cases, these are preset by the manufacturer; in others, they can be set by the analyst. Different (interchangeable) sensors are available to measure different size ranges and different particle concentrations.

b. Interferences: Interferences are caused by contamination with particles, the presence of gas bubbles, and electronic noise. See 2560A.3*a* for sampling precautions and 2560A.4 for particle-free dilution water preparation. Dilution water is necessary only for samples with particle number concentrations exceeding the sensor's capacity to distinguish different particles. If dilution water will be used, analyze it without sample and subtract particle counts in each channel from sample counts.

For devices in which sample flows through the sensor, some electronic noise can be detected by counting with clean water in the sensor and no flow (that is, with no true particle counts). If noise is present, it is likely to occur as a very large number of counts in the smallest size range (lowest channel). Eliminate this noise by resetting the size range of the smallest channel (increasing the lowest size range to be measured) high enough to avoid significant counts in the lowest channel. For further measures to minimize electronic noise, see 2560A.7.

c. Detectable sizes and concentrations: The lower size limit of measurement depends on both electronic noise and sensor capability. Devices using the principle of light obscuration typically are limited to particles larger than approximately 1 μm. Devices using the time-of-transition principle can detect particles as small as 0.1 μm. The upper particle size limit usually is determined by the size of the orifice through which particles pass. Manufacturers state particle size limits (lower and upper) for each sensor. See 2560B.1*c*.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

a. Particle counter and size distribution analyzer.
b. Glassware: See 2560A.3*a* and 7.

3. Reagents

a. Particle-free dilution water: See 2560A.4.
b. Calibration particles: See 2560A.5.

4. Procedure

a. Preparation: Let instrument warm up according to manufacturer's instructions. Select sensor to measure size and concentration ranges expected; use more than one sensor if size distribution is wider than the range that can be measured with one sensor. Rinse sensor with a volume of particle-free dilution water equivalent to at least three times that used in measuring samples.

Choose instrument settings for each type of choice available (e.g., total number of channels, which channels to include, absolute or relative counts, cumulative or differential counts. Also, choose method of starting and stopping counting based on total count, maximum count in one channel, duration of counting, or analysis of a specified volume of sample. Usually, the known-volume method is preferable because it lends itself to determination of absolute size distribution.

b. Calibration: See 2560A.5. Initial calibration of each sensor on an instrument requires several particle sizes to determine the relationship between particle size and channel number. Either this relationship may be controlled by varying the range of millivolt responses that are sorted into each channel or it is preset by the manufacturer.

After initial calibration, use at least two sizes of calibration particles for each sensor each time the instrument is used. For instruments in which the sample flows through a stationary sensing zone, measure flow rate of sample and adjust it to that used during initial calibration.

c. Blank sample: See 2560B.4*c* but substitute particle-free dilution water for electrolyte solution.

d. Measurement of samples: Dilute sample, if necessary, to keep within manufacturer's guidelines. Mix gently (see 2560A.3*a*). For flow-type instruments with pressure-based samplers, insert sample container into the instrument; for vacuum-based samplers, insert sampler tubing into the sample container. Start counting only after sufficient sample has passed through the tubing connecting the sample and sensor to ensure that the sensor is receiving the sample. For instruments that scan a stationary sample, gently transfer sample into the measurement device and insert.

For all instruments, when the count is complete, check that the concentration did not exceed the manufacturer's recommendations. Repeat with a greater dilution if necessary.

Between samples, rinse with dilution water or the upcoming sample. When a series of samples with various concentrations is to be measured and sample order is not critical, measure samples in order of expected increasing particle concentration; preferably, rinse with the next sample.

5. Calculation

See 2560A.6.

2560 D. Light-Scattering Method

1. General Discussion

a. Principle: Light-scattering instruments may be either flow or static devices. In flow instruments, the direct path of the light beam through the flow cell is blocked by a particle as it flows through the measurement zone with the fluid, and light scattered over a fixed range of angles is collected and measured. Particle size is determined from the angle and intensity of scattering based on the principles of Fraunhofer diffraction and/or Mie scattering. In static instruments, the particles remain quiescent and a laser light beam scans part of the suspension. Scattered light is collected by a photovoltaic cell and the resulting response from all particles scanned is mathematically deconvoluted to generate the size distribution.

Available particle counters vary in the angle or range of angles at which scattered light is measured and in the number of channels (size classes) into which particles are sorted. For flow-type instruments, different (interchangeable) sensors are available to measure different size ranges and different particle concentrations. The particle sizes determined by these instruments are equivalent spherical diameters [i.e., they are determined from the amount of light that would have been scattered at the angle(s) built into the instrument by a calibration sphere of that diameter].

b. Interferences: For both types of instruments, interferences are caused by contamination of the sample with particles, the presence of gas bubbles, and electronic noise. For static instruments, additional sources of interference can be surface contamination (particles, markings, or scratches) on the sample container and sample color. See 2560A.3 for sampling precautions and 2560A.4 for particle-free dilution water preparation. Dilution water is necessary only for samples with particle number concentrations exceeding the sensor's capacity to distinguish different particles. If dilution water will be used, analyze it without sample and subtract particle counts in each channel from sample counts. To correct for color interference, consult the instrument manual.

In flow instruments, some electronic noise can be detected by counting with clean water in the sensor and no flow (i.e., with no true particle counts). If noise is present, it is likely to occur as a very large number of counts in the smallest size range (lowest channel). Eliminate this noise by resetting the size range of the smallest channel (increasing the lowest size range to be measured) high enough to avoid significant counts in the lowest channel. For further measures to minimize electronic noise, see 2560A.7.

For static instruments, use a glass container that is optically clear and has no lettering or markings (at least in the light path). Minimize the effect of specific glassware by using the same sample container for all samples to be compared and ensuring that the orientation of the sample container in the light beam is the same for every sample.

c. Detectable sizes and concentrations: The lower size limit of measurement depends on both electronic noise and sensor capability. For flow instruments, the upper particle size limit usually is determined by the size of the orifice through which particles pass. Manufacturers state particle size limits (lower and upper) for each sensor. See 2560B.1*c*.

Applying Mie theory to the scattering data can extend the range of light-scattering instruments to as low as 0.1 μm. Alternatively, some instruments extend the lower limit of detectable diameters to 0.1 μm by augmenting Fraunhofer diffraction data with scattering intensity measurements at a fixed angle (often 90°) using two or three wavelengths of incident light.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

a. Particle counter and size distribution analyzer.
b. Glassware: See 2560A.3*a* and 7.

3. Reagents

a. Particle-free dilution water: See 2560A.4.
b. Calibration particles: See 2560A.5.

4. Procedure

a. Preparation: Let instrument warm up according to manufacturer's instructions. For flow instruments, select sensor to measure size and concentration ranges expected; use more than one sensor if size distribution is wider than the range that can be measured with one sensor. Preferably use a sensor designed for the concentration range of the undiluted sample rather than diluting samples into the sensor's concentration range. For both types of instruments, place in or pass through the sensor at least three times a volume of particle-free dilution water equivalent to that used in measuring samples, before the first sample.

Choose instrument settings for each type of choice available (e.g., flow rate, total number of channels, which channels to include, absolute or relative counts. Also choose method of starting and stopping counting: total count, maximum count in one channel, duration of counting, or analysis of a specified volume of sample. For flow instruments, the known-volume method is preferable because it lends itself to determination of absolute size distribution. For static analyzers, the analyst does not control sample size directly, but can control duration of measurement. Take care not to exceed maximum particle number concentration limits suggested by manufacturer.

Some flow instruments permit choice between vacuum or pressure systems for transporting the sample through the sensor. If floc preservation is essential, preferably use a vacuum system but ensure that bubbles do not form before the sensor; otherwise, use a pressure system with a pulseless gear pump.

b. Calibration: See 2560A.5. For flow instruments, initial calibration of each sensor on an instrument requires several particle sizes to determine the relationship between particle size and channel number. Either this relationship may be controlled by varying the range of millivolt responses that are sorted into each channel, or it is preset by the manufacturer. Static instruments are preset by the manufacturer because sizing is done by a software conversion of the light sensed. After initial calibration, use at least two sizes of calibration particles for each sensor each time the instrument is used. Although the calculation of particle sizes is based on first principles and not on this calibration, calibrate to ensure that the instrument is functioning properly. For flow instruments, measure flow rate through the sensor and adjust to that used during initial calibration.

c. Blank sample: See 2560B.4*c*, but substitute particle-free dilution water for electrolyte solution.

d. Measurement of samples: If dilution is necessary, dilute sample to keep within manufacturer's guidelines for the maximum counting rate. Mix gently (see 2560A.3*a*).

For flow instruments, initiate the flow from the sample through the sensor. Check that flow rate is the same as that used for sensor calibration. Start counting only after sufficient sample has passed through the tubing connecting the sample and sensor to ensure that the sensor is receiving the sample. When counting is complete, check that the concentration did not exceed the manufacturer's recommendations. Repeat with a greater dilution if necessary. For subsequent samples, rinse with dilution water or the next sample. When a series of samples with varying concentrations will be measured and sample order is not critical, measure samples in order of expected increasing particle concentration; in this case, preferably rinse with next sample.

For static instruments, insert sample beaker or bottle and start particle counting. When counting is complete, check that the concentration did not exceed the manufacturer's recommendations by diluting and measuring again. If the second count is related to the first according to the ratio of the two measurements' dilution factors consider the first measurement acceptable. Repeat with a greater dilution if necessary. For subsequent samples, see directions in preceding paragraph.

5. Calculation

See 2560A.6.

2570 ASBESTOS*

2570 A. Introduction

1. Occurrence and Significance

The term "asbestos" describes a group of naturally occurring, inorganic, highly fibrous silicate minerals that are easily separated into long, thin, flexible fibers when crushed or processed. Included in the definition are the asbestiform (see 2570A.2) varieties of serpentine (chrysotile), riebeckite (crocidolite), grunerite (grunerite asbestos), anthophyllite (anthophyllite asbestos), tremolite (tremolite asbestos), and actinolite (actinolite asbestos).

Asbestos has been used widely as a thermal insulator and in filtration. The tiny, almost indestructible fibers penetrate lung tissue and linings of other body cavities, causing asbestosis and cancer in the lungs and mesothelioma in other cavity linings.[1]

2. Terminology

Asbestiform—having a special type of fibrous habit (form) in which the fibers are separable into thinner fibers and ultimately into fibrils. This habit accounts for greater flexibility and higher tensile strength than other habits of the same mineral. More information on asbestiform mineralogy is available.[2,3]

Aspect ratio—ratio of the length of a fibrous particle to its average width.

Bundle—structure composed of three or more fibers in parallel arrangement with the fibers closer than one fiber diameter to each other.

Cluster—structure with fibers in a random arrangement such that all fibers are intermixed and no single fiber is isolated from the group; groupings of fibers must have more than two points touching.

Fibril—single fiber that cannot be separated into smaller components without losing its fibrous properties or appearance.

Fibrous—composed of parallel, radiating, or interlaced aggregates of fibers, from which the fibers are sometimes separable. The crystalline aggregate of a mineral may be referred to as fibrous even if it is not composed of separable fibers, but has that distinct appearance. "Fibrous" is used in a general mineralogical way to describe aggregates of grains that crystallize in a needle-like habit and appear to be composed of fibers; it has a much more general meaning than "asbestos." While all asbestos minerals are fibrous, not all minerals having fibrous habits are asbestos.

Matrix—fiber or fibers with one end free and the other end embedded in, or hidden by, a particle. The exposed fiber must meet the fiber definition.

Structures—all the types of asbestos particles, including fibers, bundles, clusters, and matrices.

3. References

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* Approved by Standard Methods Committee, 2006. Editorial revisions, 2011. Joint Task Group: 20th Edition—Joseph A. Krewer (chair), Dennis D. Lane, Lilia M. McMillan, James R. Millette, Stephen C. Roesch, George Yamate.

2570 B. Transmission Electron Microscopy Method

1. General Discussion

This method is used to determine the concentration of asbestos structures, expressed as the number of such structures per liter of water. Asbestos identification by transmission electron microscopy (TEM) is based on morphology, selected area electron diffraction (SAED), and energy dispersive X-ray analysis (EDXA). Information about structure size also is generated. Only asbestos structures containing fibers greater than or equal to 10 μm in length are counted. The concentrations of both fibrous asbestos structures greater than 10 μm in length and total asbestos structures per liter of water are determined. The fibrous asbestos structures greater than 10 μm in length are of specific interest for meeting the Federal Maximum Contaminant Level Goal (MCLG) for drinking water, but in many cases the total asbestos concentration provides important additional information.

a. Principle: Sample portions are filtered through a membrane filter. A section of the filter is prepared and transferred to a TEM grid using the direct transfer method. The asbestiform structures are identified, sized, and counted by transmission electron microscopy (TEM), using selected area electron diffraction (SAED) and energy dispersive spectroscopy (EDS or EDXA) at a magnification of 10 000 to 20 000×.

b. Interferences: Certain minerals have properties (i.e., chemical or crystalline structure) that are very similar to those of asbestos minerals and may interfere with the analysis by causing false positives. Maintain references for the following materials in the laboratory for comparison with asbestos minerals, so that they are not misidentified as asbestos minerals: antigorite, at-

tapulgite (palygorskite), halloysite, hornblende, pyroxenes, sepiolite, and vermiculite scrolls.

High concentrations of iron or other minerals in the water may coat asbestos fibers and prevent their full identification.

2. Sampling

a. Containers: Use new, pre-cleaned, capped bottles of glass or low-density (conventional) polyethylene, capable of holding at least 1 L. Do not use polypropylene bottles. Rinse bottles twice by filling approximately one-third full with fiber-free water and shaking vigorously for 30 s. Discard rinse water, fill bottles with fiber-free water, treat in ultrasonic bath (60 to 100 W) for 15 min, and rinse several times with fiber-free water.

Make blank determinations on the bottles before collecting a sample. Use one bottle in each batch or a minimum of one bottle in each 24 to test for background level when using polyethylene bottles. When sampling waters probably containing very low levels of asbestos, or for additional confidence in the bottle blanks, run additional blank determinations.

b. Collection: Follow general principles for water sampling (see Section 1060). Some specific considerations apply to asbestos fibers, which range in length from 0.1 μm to 20 μm or more. In large bodies of water, because of the range of sizes there may be a vertical distribution of particle sizes that may vary with depth. If a representative sample from a water supply tank or impoundment is required, take a carefully designated set of samples representing vertical as well as horizontal distribution and composite for analysis. When sampling from a distribution system, choose a commonly used faucet and remove all hoses or fittings. Let the water run to waste for at least 1 to 3 min or longer to guarantee that the sample collected is representative of the water supply. (Often, the appropriate time to obtain a main's sample can be determined by waiting for a change in water temperature.) Because sediment may build up in valving works, do not adjust faucets or valves until all samples have been collected. Similarly, do not consider samples at hydrants and at dead ends of the distribution systems to be representative of the water in the system. As an additional precaution against contamination, rinse each bottle several times with the source water being sampled. For depth sampling, omit rinsing. Obtain a sample of approximately 800 mL from each sampling site, leaving some air space in each bottle. Using a waterproof marker, label each container with date, time, place, and sampler's initials.

c. Shipment: Ship water samples in a sealed container, but separate from any bulk or air samples intended for asbestos analysis. Preferably, ship in a cooler with ice to retard bacterial or algal growth. Do not freeze the sample. In the laboratory, prepare sample within 48 h of collection.

3. Apparatus

a. High-efficiency particulate air (HEPA) filtered positive-pressure positive-flow hood.

b. Filter funnel assemblies, either 25 mm or 47 mm, of either of the following types:

1) *Disposable plastic units*, or
2) *Glass filtering unit*—With this type of unit, observe the following precautions: Never let unit dry after filtering. Immediately place it in detergent solution, scrub with a test-tube brush, and rinse several times in particle-free water. Periodically treat unit in detergent solution in an ultrasonic bath. Clean unit after each sample is filtered. Run a blank on particle-free water filtered through the glass filtering unit at frequent intervals to ensure absence of residual asbestos contamination.

c. Side-arm filter flask, 1000 mL.

d. Filters: Use either:

1) *Mixed cellulose ester (MCE) membrane filters*, 25- or 47-mm diam, $\leq$ 0.22-μm and 5-μm pore size, or
2) *Polycarbonate (PC) filters*, 25- or 47-mm diam, $\leq$ 0.1-μm pore size.

e. Ultrasonic bath, tabletop model, 60 to 100 W.

f. Graduated pipet, disposable glass, 1, 5, and 10 mL.

g. Cabinet-type desiccator or *low-temperature drying oven.*

h. Cork borer, 7 mm.

i. Glass slides.

j. Petri dishes, glass, approximately 90-mm diam.

k. Mesh screen, stainless steel or aluminum, 30 to 40 mesh.

l. Ashless filter paper filters, 90-mm diam.

m. Exhaust or fume hood.

n. Scalpel blades.

o. Low-temperature plasma asher.

p. High-vacuum carbon evaporator with rotating stage, capable of less than 0.013 Pa pressure. Do not use units that evaporate carbon filaments in a vacuum generated only by oil rotary pump. Use carbon rods sharpened with a carbon rod sharpener to necks about 4 mm long and 1 mm in diam. Install rods in the evaporator so that the points are approximately 100 to 120 mm from surface of microscope slide held in the rotating device.

q. Lens tissue.

r. Copper TEM finder grids, 200 mesh. Use pre-calibrated grids, or determine grid opening area by either of the following methods:

1) Measure at least 20 grid openings in each of 20 random 200-mesh grids (total of 400 grid openings for every 1000 grids used) by placing the 20 grids on a glass slide and examining them under an optical microscope. Use a calibrated reticule to measure average length and width of 20 openings from each of the individual grids. From the accumulated data, calculate the average grid opening area.
2) Measure grid area at the TEM (¶ *s* below) at a calibrated screen magnification low enough to permit the measurement of the sides of the opening. Measure one grid opening for each grid examined. Measure grid openings in both the x and y directions and calculate area.

s. Transmission electron microscope (TEM), 80 to 120 kV, equipped with energy dispersive X-ray system (EDXA), capable of performing electron diffraction with a fluorescent screen inscribed with calibrated gradations. The TEM must have a scanning transmission electron microscopy (STEM) attachment or be capable of producing a spot size of less than 250 nm diam at crossover. Calibrate routinely for magnification, camera constant, and EDXA settings according to procedures of 2570B.5*d*.

4. Reagents and Materials

a. Acetone.

b. Dimethylformamide (DMF). **CAUTION: Toxic; use only in a fume hood.**

c. Glacial acetic acid. **CAUTION: Use in a fume hood.**

d. Chloroform.

e. 1-Methyl-2-pyrrolidone.

f. Particle-free water: Use glass-distilled water or treat by reverse osmosis; filter through a filter with pore diam 0.45 μm or smaller.

g. Non-asbestos standards for minerals listed in 2570B.1*b*.

h. Asbestos standards for minerals listed in 2570A.1.

5. Procedure

a. Sample filtration: Samples with high levels of organic contaminants may require pretreatment. A process using ultraviolet light and ozone bubbling is described elsewhere.[1] Drinking water samples prepared within 48 h of collection do not require pretreatment.

Carefully wet-wipe exterior of sample bottle to remove any possible contamination before taking bottle into a clean preparation area separated from preparation areas for bulk or air-sample handling.

Prepare specimen in a clean HEPA filtered positive-pressure hood. Measure cleanliness of preparation area hoods by cumulative process blank concentrations (see ¶ *b* below).

If using a disposable plastic filter funnel unit, remove funnel assembly and discard top filter supplied with the apparatus, but be sure to retain the coarse polypropylene support pad in place. Assemble unit with the adapter and a properly sized neoprene stopper, and attach funnel to the 1000-mL side-arm vacuum flask. Moisten support pad with a few milliliters distilled water, place a 5.0-μm-pore-size MCE backing filter on support pad, and place an MCE or PC filter (≤0.22 μm or 0.1 μm pore-size) on top of backing filter. After both filters are completely wet, apply vacuum, ensuring that filters are centered and pulled flat without air bubbles. Return flask to atmospheric pressure. If there are any irregularities on the filter surface, discard filters and repeat the process. Alternatively, use glass filtering unit, following the same procedure to set up filters.

Vigorously, by hand, shake capped bottle with sample suspension, then place it in tabletop ultrasonic bath and sonicate for 15 min. The water level in the bath should be approximately the same as that of the sample. After treatment, return sample bottle to work surface of HEPA hood. Carry out all preparation steps, until filters are ready for drying, in this hood.

Shake suspension vigorously by hand for 2 to 3 s. Estimate amount of liquid to be withdrawn to produce an adequate filter preparation. Experience has shown that a light staining of the filter surface will usually yield a suitable preparation. If the sample is relatively clean, use a volumetric cylinder to measure sample. If sample has a high particulate or asbestos content, withdraw a small volume (but at least 1 mL) with disposable glass pipet, inserting pipet halfway into sample.

NOTE: If, after examination in the TEM, the smallest volume measured (1.0 mL) yields an overloaded sample, make additional serial dilutions of the suspension. Shake suspension vigorously by hand for 2 to 3 s before taking serial dilution portion. Do not re-treat in ultrasonic bath either original solution or any dilutions. Mix 10 mL sample with 90 mL particle-free water in a clean sample bottle to obtain a 1:10 serial dilution.

Disassemble filtering unit and carefully remove filter with clean forceps. Place filter, particle side up, in a precleaned, labeled, disposable plastic Petri dish or similar container.

To obtain an optimally loaded filter, make several filtrations with different sample portions. Use new disposable plastic funnel units or carefully cleaned glass units for each filtration. When additional filters are prepared, shake suspension without additional ultrasonic treatment before removing the sample portion. Each new filtration should represent at least a five-fold loading difference.

Dry MCE filters for at least 12 h in an airtight, cabinet-type desiccator, or in a HEPA filtered hood or an asbestos-free oven. Alternatively, to shorten drying time for filters prepared using the acetone collapsing method, plug damp filter and attach to a glass slide as described in ¶ *c*1)a) below. Place the slide with filter plug(s) (up to eight plugs can be attached to one slide) on a bed of desiccant, cover, and place in desiccator for 1 to 2 h.

Place PC filters in a dessicator for at least 30 min before preparation; lengthy drying is not required.

b. Sample blank preparation: Prepare a process blank using ≥50 mL particle-free water for each set of samples analyzed. An acceptable process blank level is ≤0.01 million fibers/L (MFL) >10 μm in length.

Analyze one unused filter from each new box of MCE or PC sample filters. Filter blanks are considered acceptable if they are shown to contain ≤53 asbestos structures/μm^2, which corresponds to ≤3 asbestos structures / 10 grid openings analyzed. Identify source of contamination before making any further analysis. Reject samples that were processed with the contaminated blanks and prepare new samples after source of contamination is found. Take special care with polycarbonate filters, because some have been shown to contain asbestos contamination.

c. Specimen preparation:

1) Mixed cellulose ester (MCE) filters

a) Filter fusing—Use either the acetone or the DMF-acetic acid method.

(1) Acetone fusing method—Remove a section from any quadrant of the sample and blank filters with a 7-mm cork borer. Place filter section (particle side up) on a clean microscope slide. Affix filter section to slide with a gummed page reinforcement or other suitable means. Label slide with a glass scribing tool or permanent marker.

Prepare a fusing dish as follows: Make a pad from five to six ashless paper filters and place in bottom of a glass Petri dish. Place metal screen bridge on top of pad and saturate filter pads with acetone. Place slide on top of bridge and cover the Petri dish. Wait approximately 5 min for sample filter to fuse and clear completely.

(2) DMF-acetic acid fusing method—Place drop of clearing solution (35% dimethylformamide [DMF], 15% glacial acetic acid, and 50% particle-free water by volume) on a clean microscope slide. **CAUTION: DMF is a toxic solvent; use only in a fume hood.** Use an amount of clearing solution that just saturates the filter. Using a clean scalpel blade, cut a wedge-shaped section of filter. A one-eighth filter section is sufficient. Carefully lay filter segment, sample surface upward, on top of solution. Bring filter and solution together at an angle of about 20° to help exclude air bubbles. Remove excess clearing solution with filter paper. Place slide in oven, on a slide-warmer, or on hot

plate, in a fume hood, at 65 to 70°C for 10 to 30 min. The filter section should fuse and clear completely.

b) Plasma etching—Place microscope slide, with attached collapsed filter pieces, in a low-temperature plasma asher. Because plasma ashers vary greatly in their performance, both from unit to unit and between different positions in the asher barrel, it is difficult to specify operating conditions. Insufficient etching will result in a failure to expose embedded fibers; too much etching may result in the loss of particles from the filter surface. Calculate time for ashing on the basis of final sample observations in transmission electron microscope. Additional information on calibration is available.[2,3]

c) Carbon coating—Using high-vacuum carbon evaporator (2570B.3*p*), proceed as follows: Place glass slide holding filters on the rotation device and evacuate evaporator chamber to a pressure of less than 0.013 Pa. Perform evaporation in very short bursts, separated by 3 to 4 s to let electrodes cool. An experienced analyst can judge the thickness of the carbon film. Make initial tests on unused filters. If the carbon film is too thin, large particles will be lost from the TEM specimen, and there will be few complete and undamaged grid openings. A coating that is too thick will lead to a TEM image lacking in contrast and a compromised ability to obtain electron diffraction patterns. The carbon film should be as thin as possible and still remain intact on most of the grid openings of the TEM specimen.

d) Specimen washing—Prepare a Jaffe washer according to any published design.[1,4] One such washer consists of a simple stainless steel bridge contained in a glass Petri dish. Place on the stainless steel bridge several pieces of lens tissue large enough to hang completely over the bridge and into the solvent. In a fume hood, fill Petri dish with either acetone, DMF, 1-methyl-2-pyrrolidone, or chloroform to the level of the stainless steel bridge.

Place TEM grids, shiny side up, on a piece of lens tissue or filter paper so that individual grids can be easily picked up with forceps. Prepare from each sample three grids. Using a curved scalpel blade, excise three square (3-mm × 3-mm) pieces of carbon-coated MCE filter from random areas on the filter. Place each square filter piece, carbon-side up, on top of a TEM specimen grid (2570B.3*r*).

Place all three assemblies (filter/grid) for each sample on the same piece of saturated lens tissue in Jaffe washer. Place lid on the Jaffe washer and let system stand, preferably overnight.

Alternatively, place grids on a low-level (Petri dish is filled only enough to wet paper on screen bridge) DMF Jaffe washer for 60 min. Then add enough solution of equal parts DMF/acetone to fill washer up to screen level. Remove grids after 30 min if they have cleared (i.e., all filter material has been removed from the carbon film) as determined by inspecting in the TEM. Let grids dry before placing in TEM.

2) Polycarbonate (PC) filters—Cover surface of a clean microscope slide with two strips of double-sided cellophane tape. Cut a strip of filter paper slightly narrower than width of slide. Position filter paper strip on center of length of slide. Using a clean, curved scalpel blade, cut a strip of the PC filter approximately 25 × 6 mm. Use a rocking motion of the scalpel blade to avoid tearing filter. Place PC strip, particle side up, on slide perpendicular to long axis of slide, making sure that the ends of the PC strip contact the double-sided cellophane tape. Each slide can hold several PC strips. Label filter paper next to each PC strip with sample number.

Carbon-coat filter strips as directed in ¶ *c*1)c) above. (Etching is not required.) Take special care to avoid overheating filter sections during carbon coating.

Prepare a Jaffe washer as described in ¶ *c*1)d) above, but fill washer with chloroform or 1-methyl-2-pyrrolidone to the level of the screen. Using a clean curved scalpel blade, excise three 3-mm-square filter pieces from each PC strip. Place filter squares, carbon side up, on shiny side of a TEM grid (2570B.3*r*). Pick up grid and filter section together and place them on lens tissue in the Jaffe washer. Place lid on Jaffe washer and leave grids for at least 4 h. Best results are obtained with longer wicking times, up to 12 h. Carefully remove grids from the Jaffe washer and let dry in the grid box before placing them in a clean, marked grid box.

d. Instrument calibration: Calibrate instrumentation regularly, and keep a calibration record for each TEM in the laboratory, in accordance with the laboratory's quality assurance program. Record all calibrations in a log book along with dates of calibration and attached backup documentation.

Check TEM for both alignment and systems operation. Refer to manufacturer's operational manual for detailed instructions.

Calibrate camera length of TEM in electron diffraction (ED) operating mode before observing ED patterns of unknown samples. Measure camera length by using a carbon-coated grid on which a thin film of gold has been sputtered or evaporated. A thin film of gold may be evaporated directly on to a specimen grid containing asbestos fibers. This yields zone-axis ED patterns from the asbestos fibers superimposed on a ring pattern from the polycrystalline gold film. Optimize thickness of gold film so that only one or two sharp rings are obtained. Thicker gold films may mask weaker diffraction spots from the fibrous particles. Because unknown d-spacings of most interest in asbestos analysis are those lying closest to the transmitted beam, multiple gold rings from thicker films are unnecessary. Alternatively, use a gold standard specimen to obtain an average camera constant calculated for that particular instrument, which can then be used for ED patterns of unknowns taken during the corresponding period.

Calibrate magnification at the fluorescent screen at magnification used for structure counting. Use a grating replica (e.g., one containing at least 2160 lines/mm). Define a field of view on the fluorescent screen; the field must be measurable or previously inscribed with a scale or concentric circles (use metric scales). Place grating replica at the same distance from the objective lens as the specimen. For instruments that incorporate a eucentric tilting specimen stage, place all specimens and the grating replica at the eucentric position. Follow the instructions provided with the grating replica to calculate magnification. Frequency of calibration depends on service history of the microscope. Check calibration after any maintenance that involves adjustment of the power supply to the lens, the high-voltage system, or the mechanical disassembly of the electron optical column (apart from filament exchange).

Check smallest spot size of the TEM. At the crossover point, photograph spot size at a magnification of 25 000× (screen magnification 20 000×). An exposure time of 1 s usually is adequate. Measured spot size must be less than or equal to 250 nm.

Verify resolution and calibration of the EDXA as follows: Collect a standard EDXA Cu and Al peak from a Cu grid coated with an aluminum film and compare X-ray energy with channel number for Cu peak to make sure readings are within ±10 eV. Collect a standard EDXA of crocidolite asbestos; elemental analysis of the crocidolite must resolve the Na peak. Collect a standard EDXA spectrum of chrysotile asbestos; elemental analysis of chrysotile must resolve both Si and Mg on a single chrysotile fiber.

e. Sample analysis: Carefully load TEM grid with grid bars oriented parallel/perpendicular to length of specimen holder. Use a hand lens or eye loupe if necessary. This procedure will line up the grid with the x and y translation directions of the microscope. Insert specimen holder into microscope.

Scan entire grid at low magnification (250× to 1000×) to determine its acceptability for high-magnification analysis. Grids are acceptable if the following conditions are met:

- The fraction of grid openings covered by the replica section is at least 50%.
- Relative to that section of the grid covered by the carbon replica, the fraction of intact grid openings is >50%.
- The fractional area of undissolved filter is <10%.
- The fraction of grid squares with overlapping or folded replica film is <50%.
- At least 20 grid squares have no overlapping or folded replica, are <5% covered with holes, and have <5% opaque area due to incomplete filter dissolution.

If the grid meets these criteria, choose grid squares for analysis from various areas of the grid so that the entire grid is represented. To be suitable for analysis, an individual grid square must meet the following criteria:

- It must have <5% holes over its area.
- It must be <25% covered with particulate matter.
- It must be uniformly loaded.

Observe and record orientation of grid at 80 to 150× on a grid map record sheet along with the location of the grid squares examined. If indexed grids are used, a grid map is not needed, but record identifying coordinates of the grid square.

At a screen magnification of 10 000 to 25 000×, evaluate the grids for the most concentrated sample loading. Reject sample if it is estimated to contain more than about 25 asbestos structures per grid opening. Proceed to the next most concentrated sample until a set of grids is obtained that have <25 asbestos structures per grid opening.

Analyze a minimum of four grid squares for each sample.

Analyze approximately one-half of the predetermined sample area on one sample grid preparation and the remainder on a second sample grid preparation.

Use structure definitions given in 2570A.2 to enumerate asbestos structures. Record all data on count sheet. Record asbestos structures >10.0 μm. For fibers and bundles, record greatest length of the structure. For matrices and clusters, record size of visible portion of the longest fiber or bundle involved with the structure, not the greatest overall dimension of the structure. No minimum or maximum width restrictions are applied to the fiber definition, as long as the minimum length and aspect ratio criteria are met. Record "NSD" (no structures detected) when no structures are found in the grid opening.

Record a typical electron diffraction pattern for each type of asbestos observed for each group of samples (or a minimum of every five samples) analyzed. Record micrograph number on count sheet. For chrysotile, record one X-ray spectrum for each tenth structure analyzed. For each type of amphibole, record one X-ray spectrum for each fifth structure analyzed. (More information on identification is available.[1,4]) Attach the printouts to the back of the count sheet. If the X-ray spectrum is stored, record file and disk number on count sheet.

Analytical sensitivity can be improved by increasing amount of liquid filtered, increasing number of grid openings analyzed, or decreasing size of filter used. Occasionally, because of high particle loadings or high asbestos concentration, the desired analytical sensitivity cannot be achieved in practice.

Unless a specific analytical sensitivity is desired, stop analysis on the 10th grid opening or the grid opening that contains the 100th asbestos structure, whichever comes first. If the analysis is stopped at the grid opening that contains the 100th asbestos structure, count entire grid square containing the 100th structure.

After analysis, remove grids from TEM, replace them in grid storage holder, and store for a minimum of one year from the date of the analysis for legal purposes. Sample filters also may be stored in the plastic Petri dishes, if necessary. Prolonged storage of the remaining water sample is not recommended, because microbial growth may cause loss of asbestos structures to the sides of the storage container.

Report the following information for each water sample analyzed: asbestos concentration in structures per liter, for total structures and fibrous asbestos structures greater than 10 μm in length; types of asbestos present; number of asbestos structures counted; effective filtration area; average size of TEM grid openings counted; number of grid openings examined; size category for each structure counted. Include a copy of the TEM count sheet if requested, either hand-written or computer-generated. Also include: upper and lower 95% confidence limits on the mean concentration, volume of sample filtered, and analytical sensitivity.

6. Calculations

Calculate amount of asbestos in a sample as follows:

$$\text{Asbestos concentration, structures /L} = \frac{N \times A_f \times D}{G \times A_G \times V_s}$$

where:

N = number of asbestos structures counted,
A_f = effective filter area of final sampling filter, mm^2,
D = dilution factor (if applicable),
G = number of grid openings counted,
A_G = area of grid openings, mm^2, and
V_s = volume of sample, L.

The same formula may be used to calculate asbestos fibers greater than 10 μm in length per liter based on the total number of fibers and bundles greater than 10 μm in length whether free or associated with matrices and clusters. Express final results as million structures per liter (MSL) and million fibers per liter (MFL).

7. Quality Control/Quality Assurance

The quality control practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

Use the laboratory's quality-control checks to verify that system is performing according to accuracy and consistency specifications. Because of the difficulties in preparing known quantitative asbestos samples, routine quality-control testing focuses on reanalysis of samples (duplicate recounts). Reanalyze 1 out of every 100 samples, not including laboratory blanks.

In addition, set up quality assurance programs according to the criteria developed by Federal agencies.[2,3] These documents cover sample custody, sample preparation, blank checks for contamination, calibration, sample analysis, analyst qualifications, and technical facilities.

8. Precision and Bias

Precision measurements for intralaboratory comparisons have been found to have a relative standard deviation (RSD) of 13 to 22% for standard and environmental water samples, with an RSD of 8.4 to 29% for interlaboratory comparisons.[1] An earlier study found an interlaboratory reproducibility of 25 to 50% in standard samples.[5]

Accuracy measurements from inter- and intralaboratory studies have demonstrated an RSD of 17% for standard chrysotile suspensions, and an RSD of 16% for standard crocidolite suspensions.[1]

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2580 OXIDATION–REDUCTION POTENTIAL (ORP)*

2580 A. Introduction

1. Significance

Oxidation and reduction (redox) reactions mediate the behavior of many chemical constituents in drinking, process, and wastewaters as well as most aquatic compartments of the environment.[1–5] The reactivities and mobilities of important elements in biological systems (e.g., Fe, S, N, and C), as well as those of a number of other metallic elements, depend strongly on redox conditions. Reactions involving both electrons and protons are pH- and Eh-dependent; therefore, chemical reactions in aqueous media often can be characterized by pH and Eh together with the activity of dissolved chemical species. Like pH, Eh represents an intensity factor. It does not characterize the capacity (i.e., poise) of the system for oxidation or reduction.

The potential difference measured in a solution between an inert indicator electrode and the standard hydrogen electrode should not be equated to Eh, a thermodynamic property, of the solution. The assumption of a reversible chemical equilibrium, fast electrode kinetics, and the lack of interfering reactions at the electrode surface are essential for such an interpretation. These conditions rarely, if ever, are met in natural water.

Thus, although measurement of Eh in water is relatively straightforward, many factors limit the interpretation of these values. These factors include irreversible reactions, electrode poisoning, the presence of multiple redox couples, very small exchange currents, and inert redox couples. Eh values measured in the field correlate poorly with Eh values calculated from the redox couples present. Nevertheless, measurement of redox potential, when properly performed and interpreted, is useful in developing a more complete understanding of water chemistry.

* Approved by Standard Methods Committee, 2009. Editorial revisions, 2011. Joint Task Group: 20th Edition—Michael J. Barcelona (chair), William C. Boyle, Thomas R. Holm, Zoltan Kerekes.

2. Sampling and Storage

Do not store samples; analyze on collection. Minimize both atmospheric contact and delay in analysis.

3. References

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2580 B. Oxidation–Reduction Potential Measurement in Clean Water

1. General Discussion

a. Principle: Electrometric measurements are made by potentiometric determination of electron activity (or intensity) with an inert indicator electrode and a suitable reference electrode. Ideally, the indicator electrode will serve as either an electron donor or acceptor with respect to electroactive oxidized or reduced chemical species in solution. At redox equilibrium, the potential difference between the ideal indicator electrode and the reference electrode equals the redox potential of the system. However, no indicator electrodes behave ideally in natural waters. Electrodes made of platinum are most commonly used for Eh measurements. They have limitations,[1] as do alternative materials such as gold and graphite.

The standard hydrogen reference electrode is fragile and impractical for routine laboratory and field use. Therefore, silver:silver-chloride or calomel reference electrodes are used commonly. The redox potential measurement is corrected for the difference between the potential of the reference electrode and that of the standard hydrogen electrode. See Section 4500-H^+, pH Value.

It is not possible to calibrate Eh electrodes over a range of redox potentials (as is done with pH electrodes). Instead, stan-

Table 2580:I. Potential of ZoBell's Solution as Function of Temperature

T °C	E V	T °C	E V
1	0.481	16	0.448
2	0.479	17	0.446
3	0.476	18	0.443
4	0.474	19	0.441
5	0.472	20	0.439
6	0.470	21	0.437
7	0.468	22	0.435
8	0.465	23	0.432
9	0.463	24	0.430
10	0.461	25	0.428
11	0.459	26	0.426
12	0.457	27	0.424
13	0.454	28	0.421
14	0.452	29	0.419
15	0.450	30	0.417

dard solutions that exhibit both chemical stability and known redox potentials for specific indicator electrodes are used to check electrode response at the temperature of measurement.

The potential of the platinum (Pt) Eh electrode versus the Ag/AgCl reference electrode with KCl electrolyte in ZoBell's solution ($3 \times 10^{-3}M$ potassium ferrocyanide and $3 \times 10^{-3}M$ potassium ferricyanide in $0.1M$ KCl)[2] has been measured as a function of temperature.[3] Good agreement was obtained between Eh values measured with this electrode pair in ZoBell's solution and those calculated from the stability constants at 8 to 85°C. The potential of the ZoBell's solution with this electrode configuration as a function of temperature can be calculated:[4]

$$Eh, \text{V} = 0.428 - 0.0022\,(T - 25)$$

where T = solution temperature, °C. Alternatively, select the value from Table 2580:I.

To determine the Eh of a sample relative to the standard hydrogen electrode, measure Eh of both sample and standard solution at the same temperature (within ±0.1°C). Then calculate Eh value of the sample:

$$Eh_{\text{system}} = E_{\text{observed}} + Eh_{\text{ZoBell/reference}} - Eh_{\text{ZoBell observed}}$$

where:

E_{observed} = sample potential relative to reference electrode,
$Eh_{\text{ZoBell/reference}}$ = theoretical Eh of reference electrode and ZoBell's solution, relative to the standard hydrogen electrode (see Table 2580:I), and
$Eh_{\text{ZoBell observed}}$ = observed potential of ZoBell's solution, relative to the reference electrode.

The measurements described above can be applied analogously to other indicator electrode/reference electrode pairs and standard solutions.

b. Interferences: Specific interferences may be due to operation of either indicator or reference electrode, redox capacity or poise of the sample, sample preservation and handling, and temperature equilibration.

1) Sorption and poisoning effects on electrodes—Contamination of the electrode surface, salt bridge, or internal electrolyte in the case of reference electrodes, can lead to excessive drift, poor electrode response, and artifact potentials. Organic matter, sulfide, and bromide may cause these problems, particularly in long-term electrode use.[1,5-7] Abrasive cleaning may improve electrode response times in anoxic systems.[8,9] If excessive drift occurs or erratic performance of paired electrodes is observed in redox standard solutions after appropriate cleaning, refilling, or regeneration procedures, discard the faulty electrode and use a new one.

2) pH variations—Redox potential is sensitive to pH if hydrogen ion or hydroxide ion is involved in the redox half-cells. Cell potentials tend to increase as proton concentration increases (i.e., pH decreases) and Eh values drop as hydroxide concentrations increase (i.e., pH increase).

3) Sample handling and preservation—The sample poise will govern the resistance of the sample to change in redox potential; this phenomenon is analogous to the resistance to pH change afforded by buffer capacity. Except in concentrated process streams, sludges, leachates, and highly reducing or treated waters, the concentrations of oxidized or reduced species may be fairly low (i.e., $<10^{-4}M$). Under these conditions, handle reduced samples very carefully to avoid exposure to atmospheric oxygen. A closed cell sampling configuration may be used.[4,10] Samples cannot be stored or preserved; analyze at sampling.

4) Temperature equilibration—Obtain Eh standard solution reading for the electrode pair at a temperature as close as possible to that of the sample. Temperature determines the Eh reference potential for a particular solution and electrode pair. It also may affect the reversibility of the redox reaction, the magnitude of the exchange current, and the stability of the apparent redox potential reading, particularly in poorly poised solutions. Hold temperature constant for all measurements and report it with Eh results.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

a. pH or millivolt meter: Use a pH meter or other type of high-impedance potentiometer capable of reading either pH or millivolts (mV). For most applications, a meter scale readable to ±1400 mV is sufficient.

b. Reference electrode consisting of a half-cell providing a constant electrode potential. See Section 4500-H^+.B.2*b*.

c. Oxidation-reduction indicator electrode: The platinum electrode is used most commonly. A noble metal or graphite electrode may be useful for specific applications.

1) Noble metal electrode—Noble metal (i.e., gold or platinum) foil, wire, or billet types of electrode are inert and resistant to chemical reaction. Clean and polish electrode surface to ensure reliable performance. Platinum electrodes may be cleaned by strong acid soaking,[11,12] hydrogen peroxide and detergent washing,[13] and anodic activation.[12] Abrasive polishing with crocus cloth, jeweler's rouge, or 400 to 600 grit wet/dry carborundum paper may be best.[5]

2) Graphite electrode—A wax-impregnated graphite (WIG) electrode may be used, especially in aqueous suspensions or

Table 2580:II. Preparation of Redox Standard Solutions

Standard Solution*	Potentials of Pt Electrode vs. Selected Reference Electrodes at 25°C in Standard Solution: Calomel Hg/Hg_2Cl_2 saturated KCl	Silver:Silver Chloride Ag/AgCl: KCl 1.00*M*	Silver:Silver Chloride Ag/AgCl: KCl 4.00*M*	Silver:Silver Chloride Ag/AgCl: KCl saturated	Standard Hydrogen	Weight of Chemicals Needed/1000 mL Aqueous Solution at 25°C
Light's solution[16]	+430	+439	+475	+476	+675	39.21 g ferrous ammonium sulfate, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ 48.22 g ferric ammonium sulfate, $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ 56.2 mL sulfuric acid, H_2SO_4, sp gr 1.84
ZoBell's solution†[2]	+183	+192	+228	+229	+428	1.4080 g potassium ferrocyanide, $K_4Fe(CN)_6 \cdot 3H_2O$ 1.0975 g potassium ferricyanide, $K_3Fe(CN)_6$ 7.4555 g potassium chloride, KCl
Iodide/Tri-iodide solution	+176	+211	+220	+221	+420	Commercially available‡

* Use standards of known or certified (preferred) purity.
† Store in dark plastic bottle in a refrigerator.
‡ Orion Cat. No. 967901, or equivalent.

soils.[14,15] The WIG electrode is more resistant to electrode poisoning than electrodes made of platinum wire.

d. Beakers: Preferably use polyethylene, TFE, or glass beakers.

e. Stirrer: Use a magnetic TFE-coated stirring-bar-type mixer.

f. Flow cell: Use for continuous flow measurements and for poorly buffered solutions.

3. Reagents

a. Standard redox solutions: Standardize the electrode system against redox solutions that provide stable known Eh values over a range of temperatures. Although standard solutions are available, they do not cover the anticipated range of Eh values. Commercially prepared solutions may be used, particularly in field testing. The composition and Eh values of standard solutions are shown in Table 2580:II. With reasonable care, these solutions are stable for several months.

b. Eh electrode cleaners: Use either:

1) Aqua regia—Mix 1 volume conc nitric acid with 3 volumes conc hydrochloric acid. Prepare fresh and dilute by at least 50% with water. Neutralize prior to discarding.
2) Chromic acid—Dissolve 5 g potassium dichromate, $K_2Cr_2O_7$, in 500 mL conc sulfuric acid. Conduct cleaning procedure in a fume hood.

4. Procedure

a. Instrument calibration: Follow manufacturer's instructions for using pH/millivolt meter and in preparing electrodes for use. Use a shorting lead to verify the zero point on the meter's millivolt scale. Equilibrate the standard solution to the temperature of the sample. Immerse electrodes in the gently stirred, standard solution in a beaker (or flow cell). Turn on meter, placing the function switch in the millivolt mode.

After several minutes for electrode equilibration, record reading to nearest millivolt. If the reading is more than ±10 mV from the theoretical redox standard value at that temperature, replace reference electrode fluid and repeat the measurement. If that procedure fails to bring the reading to within ±10 mV of the theoretical value, polish the sensing element of the indicator electrode with carborundum paper, crocus cloth, or jeweler's rouge. Rinse electrode thoroughly and recheck reading with a fresh portion of the standard solution. If the reading is within ±10 mV of the theoretical value, record it and the temperature. If the reading is not within ±10 mV, repeat the cleaning procedure or try another electrode. Then rinse the electrode with distilled water and proceed with the sample measurement. Recalibrate daily and more frequently if turbid, organic-rich, or high-dissolved-solids solutions are being measured.

b. Electrode cleaning procedure: Useful treatments for noble metal electrodes in restoring performance after long periods of use include immersion in warm (70°C) aqua regia for 1 to 2 min or 5 min in 6*N* HNO_3 after bringing to a boil. Alternatively treat with chromic acid solution followed by 6*N* HCl and rinse with water.

c. Sample analysis: Check system for performance with the standard solution, rinse electrodes thoroughly with sample water, then immerse them in the gently stirred sample. Let equilibrate, record Eh value to the nearest millivolt, and temperature to ±0.1°C. Repeat with a second sample portion to confirm successive readings within ±10 mV. Equilibration times vary and may take many minutes in poorly poised solutions. Successive readings that vary less than ±10 mV over 10 min are adequate for most purposes. Make continuous flow or pumped sample measurements, particularly of poorly

TABLE 2580:III. RECOMMENDED COMBINATIONS FOR SELECTED SAMPLE TYPES

Sample Type	Indicator Electrode(s)	Reference Electrode	Type of Sample Cell
Process stream (low Br^-) (S^{2-})	Pt or Au	Calomel or silver: silver chloride	Closed continuous flow (dual indicator electrode)
(high Br^-)	Pt or Au	Calomel or silver: silver chloride with salt bridge (double junction reference electrode)	
Natural waters			
Surface waters	Pt or Au	Calomel or silver: silver chloride	Closed continuous flow (dual indicator electrode) or beaker
Groundwater	Pt or Au	Calomel or silver: silver chloride	Closed continuous flow (dual indicator electrode)
Soils, sludges	WIG, Pt wire	Calomel or silver: silver chloride	Beaker or soil core

poised solutions, in a closed flow cell after external calibration of the electrode system.

See Table 2580:III for recommended combinations of electrodes, standards, and sample handling.

5. Troubleshooting

a. Meter: Use a shorting lead to establish meter reading at zero millivolts whenever possible. If the meter cannot be zeroed, follow the manufacturer's instructions for service.

b. Electrodes: If the potentiometer is in good working order, the fault may be in the electrodes. Frequently, renewal of the filling solution for the salt bridge for the reference electrode is sufficient to restore electrode performance. Another useful check is to oppose the emf of a questionable reference electrode with that of the same type known to be in good order. Using an adapter, plug the good reference electrode into the indicator electrode jack of the potentiometer. Then plug the questionable electrode into the reference electrode jack. With the meter in the millivolt position, immerse electrodes in an electrolyte (e.g., KCl) solution and then into a redox standard solution. The two millivolt readings should be 0 ± 5 mV for both solutions. If different electrodes are used (e.g., silver:silver chloride versus calomel or vice versa), the reading should be 44 ± 5 mV for a good reference electrode.

Unless an indicator electrode has been poisoned, physically damaged, or shorted out, it usually is possible to restore function by proper cleaning.

6. Calculation

$$Eh_{system} = E_{observed} + E_{reference\ standard} - E_{reference\ observed}$$

Report temperature at which readings were made.

7. Precision and Bias

Standard solution measurements made at stable temperatures with a properly functioning electrode system should be accurate to within ±10 mV. Calibration precision as reflected by the agreement of dual platinum electrodes versus an Ag:AgCl reference electrode for over a 2-year period has been estimated at ±15 mV (i.e., one standard deviation) in ZoBell's solution (N = 78) at approximately 12°C. Precision on groundwater samples (N = 234) over the same period has been estimated at ±22 mV (i.e., ± one standard deviation) in a closed flow cell.[17]

8. References

1. WHITFIELD, M. 1974. Thermodynamic limitations on the use of the platinum electrode in Eh measurements. *Limnol. Oceanogr.* 19:857.
2. ZOBELL, C.E. 1946. Studies on redox potential of marine sediments. *Bull. Amer. Assoc. Petroleum Geol.* 30:447.
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2710 TESTS ON SLUDGES*

2710 A. Introduction

This section presents a series of tests uniquely applicable to sludges or slurries. The test data are useful in designing facilities for solids separation and concentration and for assessing operational behavior, especially of the activated sludge process.

* Approved by Standard Methods Committee, 2009. Editorial revisions, 2011. Joint Task Group: 21st Edition—Terry E. Baxter (chair), Billie C. Byrom, Daniel D. Chen, Steven K. Dentel, Stephen R. Gelman, S. Rod Jenkins, Mesut Sezgin.

2710 B. Oxygen-Consumption Rate

1. General Discussion

This test is used to determine the oxygen consumption rate of a sample of a biological suspension such as activated sludge. It is useful in laboratory and pilot-plant studies as well as in the operation of full-scale treatment plants. When used as a routine plant operation test, it often will indicate changes in operating conditions at an early stage. However, because test conditions are not necessarily identical to conditions at the sampling site, the observed measurement may not be identical with actual oxygen consumption rate.

The QC practices considered to be an integral part of each method are summarized in Table 2020:II.

2. Apparatus

a. Oxygen-consumption rate device: Either:

1) *Probe with an oxygen-sensitive electrode* (polarographic or galvanic), or
2) *Manometric or respirometric device* with appropriate readout and sample capacity of at least 300 mL. The device should have an oxygen supply capacity greater than the oxygen consumption rate of the biological suspension, or at least 150 mg/L · h.

b. Stopwatch or other suitable timing device.

c. Thermometer to read to ±0.5°C.

3. Procedure

a. Calibration of oxygen-consumption rate device: Either:

1) Calibrate the oxygen probe and meter according to the method given in Section 4500-O.G, or
2) Calibrate the manometric or respirometric device according to manufacturer's instructions.

b. Volatile suspended solids determination: See Section 2540.

c. Preparation of sample: Adjust temperature of a suitable sample portion to that of the basin from which it was collected or to required evaluation temperature, and maintain constant during analysis. Record temperature. Increase DO concentration of sample by shaking it in a partially filled bottle or by bubbling air or oxygen through it.

d. Measurement of oxygen consumption rate:

1) Fill sample container to overflowing with an appropriate volume of a representative sample of the biological suspension to be tested.

2) If an oxygen-sensing probe is used, immediately insert it into a BOD bottle containing a magnetic stirring bar and the biological suspension. Displace enough suspension with probe to fill flared top of bottle and isolate its contents from the atmosphere. Activate probe stirring mechanism and magnetic stirrer. [NOTE: Adequate mixing is essential. For suspensions with high concentrations of suspended solids (i.e., >5000 mg/L) more vigorous mixing than that provided by the probe stirring mechanism and magnetic stirrer may be required.] If a manometric or respirometric device is used, follow manufacturer's instructions for startup.

3) After meter reading has stabilized, record initial DO and manometric or respirometric reading, and start timing device. Record appropriate DO, manometric, or respirometric data at time intervals of less than 1 min, depending on rate of consumption. Record data over a 15-min period or until DO becomes limiting, whichever occurs first. The oxygen probe may not be accurate below 1 mg DO/L. If a manometric or respirometric device is used, refer to manufacturer's instructions for lower limiting DO value. Low DO (≤2 mg/L at the start of the test) may limit oxygen uptake by the biological suspension and will be indicated by a decreasing rate of oxygen consumption as the test progresses. Reject such data as being unrepresentative of suspension oxygen consumption rate and repeat test beginning with higher initial DO levels.

The results of this determination are quite sensitive to temperature variations and poor precision is obtained unless replicate determinations are made at the same temperature. When oxygen consumption is used as a plant control test, run periodic (at least monthly) replicate determinations to establish the precision of the technique. This determination also is sensitive to the time lag between sample collection and test initiation.

4. Calculations

If an oxygen probe is used, plot observed readings (DO, milligrams per liter) versus time (minutes) on arithmetic graph paper and determine the slope of the line of best fit. The slope is the oxygen consumption rate in milligrams per liter per minute.

If a manometric or respirometric device is used, refer to manufacturer's instructions for calculating the oxygen consumption rate.

https://doi.org/10.2105/SMWW.2882.035

Calculate specific oxygen consumption rate in milligrams per gram per hour as follows:

$$\text{Specific oxygen consumption rate, (mg/g)/h} = \frac{\text{oxygen consumption rate, (mg/L)/min}}{\text{volatile suspended solids, g/L}} \times \frac{60 \text{ min}}{\text{h}}$$

5. Precision and Bias

Bias is not applicable. The precision for this test has not been determined.

6. Bibliography

UMBREIT, W.W., R.H. BURRIS & J.F. STAUFFER. 1964. Manometric Techniques. Burgess Publishing Co., Minneapolis, Minn.

2710 C. Settled Sludge Volume

1. General Discussion

The settled sludge volume of a biological suspension is useful in routine monitoring of biological processes. For activated sludge plant control, a 30-min settled sludge volume or the ratio of the 15-min to the 30-min settled sludge volume has been used to determine the returned-sludge flow rate and when to waste sludge. The 30-min settled sludge volume also is used to determine sludge volume index[1] (2710D).

This method is inappropriate for dilute sludges because of the small volume of settled material. In such cases, use the volumetric test for settleable solids using an Imhoff cone (Section 2540F). Results from Section 2540F are not comparable with those obtained with the procedure herein.

2. Apparatus

a. Settling column: Use 1-L graduated cylinder equipped with a stirring mechanism consisting of one or more thin rods extending the length of the column and positioned within two rod diameters of the cylinder wall. Provide a stirrer able to rotate the stirring rods at approximately, but no more than, 4 rpm (peripheral tip speed of approximately 1.3 cm/s). See Figure 2710:1.

b. Stopwatch.

c. Thermometer.

3. Procedure

Place 1.0 L sample in settling column and distribute solids by covering the top and inverting cylinder three times. Insert stirring rods, activate stirring mechanism, start the stopwatch, and let suspension settle. Continue stirring throughout test. Maintain suspension temperature during test at that in the basin from which the sample was taken.

Determine volume occupied by suspension at measured time intervals (e.g., 5, 10, 15, 20, 30, 45, and 60 min).

Report settled sludge volume of the suspension in milliliters for an indicated time interval.

Test results are applicable to a particular test site and are significantly affected by variables such as suspension temperature, sampling and agitation methods, dimensions of settling column and stirring rods, stirring speed, and time between sampling and start of the determination.

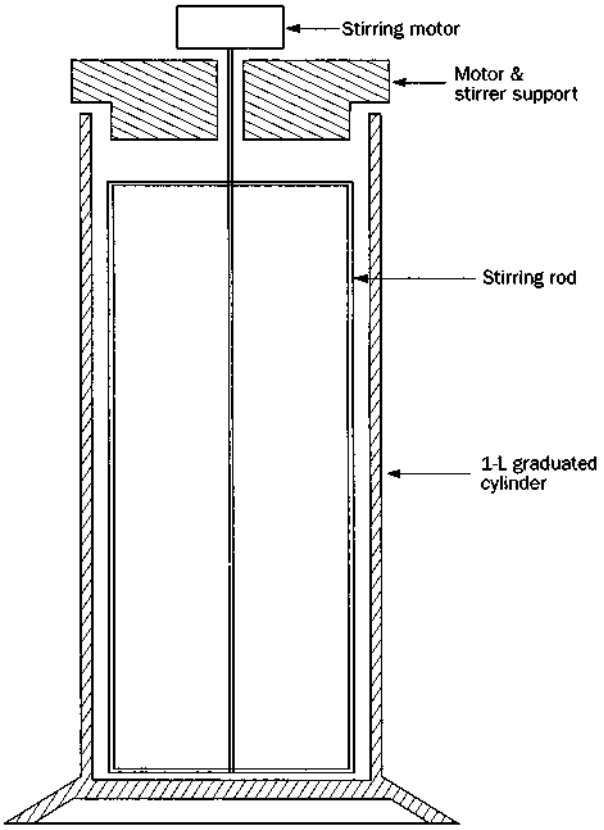


Figure 2710:1. Schematic diagram of settling vessel for settled sludge volume test.

4. Precision and Bias

Bias is not applicable. The precision for this test has not been determined.

5. Reference

1. DICK, R.I. & P.A. VESILIND. 1969. The SVI—What is It? *J. Water Pollut. Control Fed.* 41:1285.

2710 D. Sludge Volume Index

1. General Discussion

The sludge volume index (SVI) is the volume in milliliters occupied by 1 g of a suspension after 30 min settling. SVI typically is used to monitor settling characteristics of activated sludge and other biological suspensions.[1] Although SVI is not supported theoretically,[2] experience has shown it to be useful in routine process control.

2. Procedure

Determine the suspended solids concentration of a well-mixed sample of the suspension (See Section 2540D).

Determine the 30 min settled sludge volume (See 2710C).

3. Calculations

$$\text{SVI} = \frac{\text{settled sludge volume (mL/L)} \times 1000}{\text{suspended solids (mg/L)}}$$

4. Precision and Bias

Precision is determined by the precision achieved in the suspended solids measurement, the settling characteristics of the suspension, and variables associated with the measurement of the settled sludge volume. Bias is not applicable.

5. References

1. Dick, R.I. & P.A. Vesilind. 1969. The SVI—What is it? *J. Water Pollut. Control Fed.* 41:1285.
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2710 E. Zone Settling Rate

1. General Discussion

At high concentrations of suspended solids, suspensions settle in the zone-settling regime. This type of settling takes place under quiescent conditions and is characterized by a distinct interface between the supernatant liquor and the sludge zone. The height of this distinct sludge interface is measured with time. Zone settling data for suspensions that undergo zone settling (e.g., activated sludge and metal hydroxide suspensions) can be used in the design, operation, and evaluation of settling basins.[1–3]

2. Apparatus

a. Settling vessel: Use a transparent cylinder at least 1 m high and 10 cm in diameter. To reduce the discrepancy between laboratory and full-scale thickener results, use larger diameters and taller cylinders.[1,3] Attach a calibrated millimeter tape to outside of cylinder. Equip cylinder with a stirring mechanism (e.g., one or more thin rods positioned within two rod diameters of the internal wall of settling vessel). Stir suspension near vessel wall over the entire depth of suspension at a peripheral speed no greater than 1 cm/s. Greater speeds may interfere with the thickening process and yield inaccurate results.[4] Provide the settling vessel with a port in the bottom plate for filling and draining. See Figure 2710:2.

b. Stopwatch.

c. Thermometer.

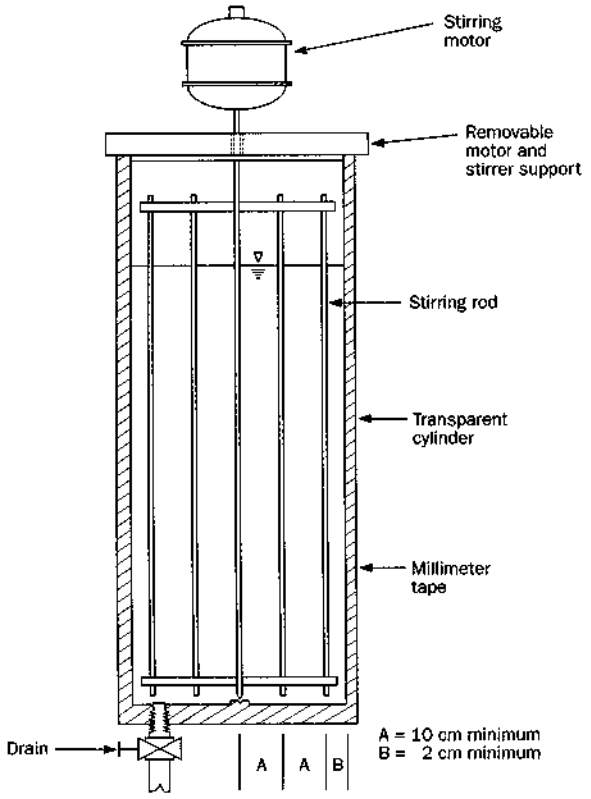


Figure 2710:2. Schematic diagram of settling vessel for zone settling rate test.

3. Procedure

Maintain suspension in a reservoir in a uniformly mixed condition. Adjust temperature of suspension to that of the basin from which it was collected or to required evaluation temperature. Record temperature. Remove a well-mixed sample from reservoir and measure suspended solids concentration (Section 2540D).

Activate stirring mechanism. Fill settling vessel to a fixed height by pumping suspension from reservoir or by gravity flow. Fill at a rate sufficient to maintain a uniform suspended solids concentration throughout settling vessel at end of filling. The suspension should agglomerate (i.e., form a coarse structure with visible fluid channels) within a few minutes. If suspension does not agglomerate, test is invalid and should be repeated.

Record height of solids-liquid interface at intervals of about 1 min. Collect data for sufficient time to assure that suspension is exhibiting a constant zone-settling velocity and that any initial reflocculation period, characterized by an accelerating interfacial settling velocity, has been passed.

Zone settling rate is a function of suspended solids concentration, suspension characteristics, vessel dimensions, and laboratory artifacts. With the filling method described above and a sufficiently large cylinder, these artifacts should be minimized. However, even with careful testing suspensions often may behave erratically. Unpredictable behavior increases for sludges with high solids concentrations and poor settling characteristics, and in small cylinders.

4. Calculations

Plot interface height in centimeters vs. time in minutes.[1,3] Draw straight line through data points, ignoring initial shoulder or reflocculation period and compression shoulder. Calculate interfacial settling rate as slope of line in centimeters per minute.

5. Precision and Bias

Bias is not applicable. The precision for this test has not been determined.

6. References

1. Dick, R.I. 1972. Sludge treatment. *In* W.J. Weber, ed., Physicochemical Processes for Water Quality Control. Wiley-Interscience, New York, N.Y.
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2710 F. Specific Gravity

1. General Discussion

The specific gravity of a sludge is the ratio of the masses of equal volumes of a sludge and distilled water. It is determined by comparing the mass of a known volume of a homogeneous sludge sample at a specific temperature to the mass of the same volume of distilled water at 4°C.

2. Apparatus

Container: A marked flask or bottle to hold a known sludge volume during weighing.

Table 2710:I. Temperature Correction Factor

Temperature °C	Temperature Correction Factor
15	0.9991
20	0.9982
25	0.9975
30	0.9957
35	0.9941
40	0.9922
45	0.9903

3. Procedure

Follow either ¶ *a* or *b* below.

a. Record sample temperature, *T*. Weigh empty container and record weight, *W*. Fill empty container to mark with sample, weigh, and record weight, *S*. Fill empty container to mark with water, weigh, and record weight, *R*. Measure all masses to the nearest 10 mg.

b. If sample does not flow readily, add as much of it to container as possible without exerting pressure, record volume, weigh, and record mass, *P*. Fill container to mark with distilled water, taking care that air bubbles are not trapped in the sludge or container. Weigh and record mass, *Q*. Measure all masses to nearest 10 mg.

4. Calculation

Use *a* or *b*, matching choice of procedure above.

a. Calculate specific gravity, *SG*, from the formula

$$SG_{T/4^\circ C} = \frac{\text{weight of sample}}{\text{weight of equal volume of water at 4°C}} = \frac{S - W}{R - W} \times F$$

The values of the temperature correction factor *F* are given in Table 2710:I.

b. Calculate specific gravity, *SG*, from the formula

$$SG_{T/4^\circ C} = \frac{\text{weight of sample}}{\text{weight of equal volume of water at 4°C}} = \frac{(P - W)}{(R - W) - (Q - P)} \times F$$

For values of *F*, see Table 2710:I.

2710 G. Capillary Suction Time

1. General Discussion

The capillary suction time (CST) test determines rate of water release from sludge. It provides a quantitative measure, reported in seconds, of how readily a sludge releases its water. The results can be used to assist in sludge dewatering processes; to evaluate sludge conditioning aids and dosages; or, when used with a jar test and the settleable solids procedure, to evaluate coagulation effects on the rate of water release from sludges.

The test consists of placing a sludge sample in a small cylinder on a sheet of chromatography paper. The paper extracts liquid from the sludge by capillary action. The time required for the liquid to travel a specified distance is recorded automatically by monitoring the conductivity change occurring at two contact points appropriately spaced and in contact with the chromatography paper. The elapsed time is indicative of the water drainage rate. The CST test has been used as a relative indicator to characterize the performance of most sludge dewatering processes.

The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

a. Test materials and apparatus may be fabricated (see Figure 2710:3) or are commercially available.* The unit includes a paper support block, stainless steel reservoir with 18-mm ID and 25-mm height, and a digital timer.

b. CST paper.†

c. Thermometer to read ±0.5°C.

d. Pipet, 10-mL, plastic with tip trimmed to allow free passage of sludge flocs.

3. Procedure

Turn on and reset CST meter. Dry CST test block and reservoir. Place a new CST paper on lower test block with rough side up and grain parallel to the 9-cm side. Add upper test block, insert sludge reservoir into test block, and seat it using light pressure and a quarter turn to prevent surface leaks. Measure and record temperature of sludge. Pipet 6.4 mL sludge into test cell reservoir; if pipetting is difficult because of sludge consistency, pour a representative sludge sample into the test cell until it is full. The CST device will begin time measurement as liquid being drawn into the paper reaches the inner pair of electrical contacts. Timing ends when the outer contact is reached. Record CST shown on digital display. Empty remaining sludge from reservoir and remove and discard used CST paper. Rinse and dry test block and reservoir. Repeat for a minimum of five determinations

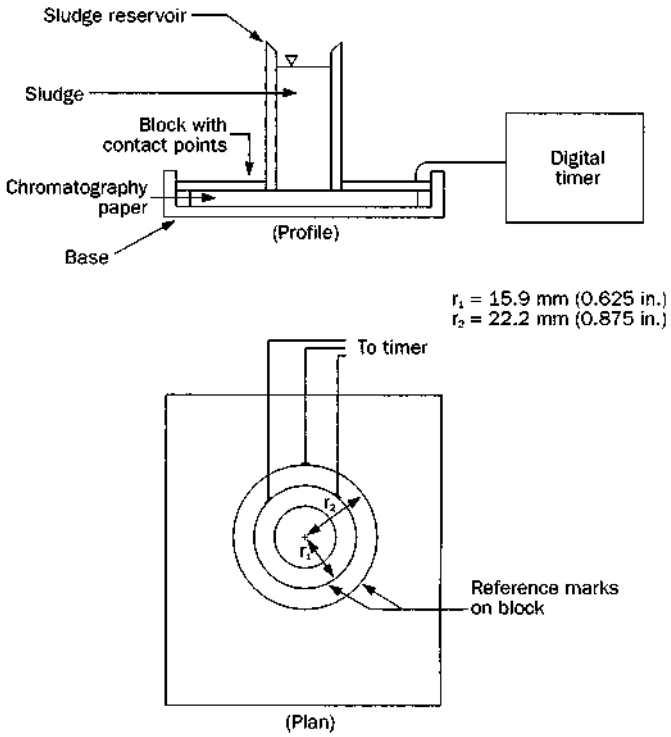


Figure 2710:3. Capillary suction time apparatus.

* Venture Innovations, P.O. Box 53631, Lafayette, LA 70505; or Triton Electronics Ltd., Bigods Hall, Dunmow, Essex, England, CM63BE; or equivalent.

† Available from CST apparatus supplier or use Whatman No. 17 chromatography grade paper cut into 7- × 9-cm sections with grain parallel to long side.

per sample to account for measurement variation and to allow identification of any faulty readings due to leaks or spills.

Variations in sludge temperature and sample volume can affect CST results. Ensure that all analyses are run under similar conditions. Sludge suspended solids concentration has a significant effect on test results. In evaluating sludge conditioners or monitoring operation of a dewatering process, avoid this effect by ensuring homogeneity among sludge samples. Comparison of CST data from different sludge samples from the same source (especially if taken on different days) cannot be made with confidence unless suspended solids concentrations are comparable. Make a rough correction for different solids contents by dividing the sludge's CST value by its corresponding solids concentration.

Characteristics of CST paper may vary between lots. If comparison of CST values for distilled water indicates such variations, subtract times for distilled water blanks from sample times to improve comparisons.

Record CST model used, paper type, sludge type, sludge temperature, and capillary suction time. Measure solids concentration and CST of distilled water using the same paper to provide useful information.

4. Precision and Bias

Ten tests conducted on an anaerobically digested pulp mill sludge resulted in a mean CST of 363.2 s with a standard deviation of 36.2 s. Twenty tests using an anaerobically digested municipal wastewater sludge gave a mean of 85.2 s with a standard deviation of 14.12 s. Triplicate analyses of 30 sample sets of conditioned and unconditioned alum sludge resulted in an average standard deviation of 1.0 s with means between 5 and 80 s. Method bias cannot be determined.

5. Bibliography

BASKERVILLE, R.C. & R.S. GALE. 1968. A simple automatic instrument for determining the filtrability of sewage sludges. *J. Inst. Water Pollut. Control* 67:233.

KAVANAGH, B.A. 1980. The dewatering of activated sludge: measurements of specific resistance to filtration and capillary suction time. *Water Pollut. Control* 79:388.

VESILIND, P.A. 1988. Capillary suction time as a fundamental measure of sludge dewaterability. *J. Water Pollut. Control Fed.* 60:215.

TILLER, F.M., Y.L. SHEN & A. ADIN. 1990. Capillary suction theory for rectangular cells. *Res. J. Water Pollut. Control Fed.* 62:130.

2710 H. Time-to-Filter

1. General Discussion

The time to filter (TTF) correlates with capillary suction time (CST) and is similar to the specific resistance to filtration if sludge solids content and filtrate viscosity do not vary among compared samples. The test requires approximately 200 mL sludge and can be used to assist in the daily operation of sludge dewatering processes or to evaluate sludge-conditioning polymers and dosages.

Testing with a smaller volume is possible in applications to evaluate water drainage rate subsequent to jar tests and settleable solids determination (see Section 2540F). In this case, drain collected sludge from one or more Imhoff cones after decanting as much supernatant as possible; use a small-volume TTF apparatus.

The test consists of placing a sludge sample in a Buchner funnel with a paper support filter, applying vacuum, and measuring the time required for 100 mL filtrate (or, for reduced sample volumes, 50% of original sample) to collect. While similar to the specific resistance to filtration test, the time-to-filter test is superior because of its ease of use and simplicity.

The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

a. Time-to-filter large-volume or small-volume (Figure 2710:4) *assembly.*

*b. Filter paper.**

c. Stopwatch.

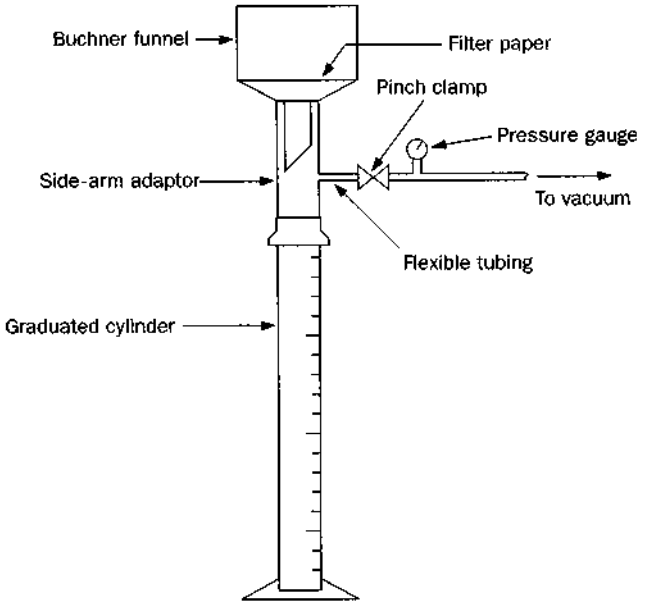


Figure 2710:4. TTF equipment. Large-volume equipment requires a 9-cm-diam Buchner funnel and a 250-mL graduated cylinder. Small-volume equipment requires a 2.5-cm-diam funnel and a 10-mL cylinder.

3. Procedure

Place paper filter in funnel and make a firm seal by prewetting with a small volume of water with vacuum on. If

* Whatman No. 1 or 2, or equivalent.

using large-volume apparatus, take a 200-mL sample of sludge. With vacuum pump providing a constant vacuum of 51 kPa, pour sample into funnel. Start stopwatch or timer and determine time required for 100 mL of sample to collect in graduated cylinder. This is the time to filter. Make a minimum of three replicate determinations.

For the small-volume test, use 7 to 10 mL sludge. Record time required for 50% of sample to collect in graduated cylinder. Compare this time to filter only to other results using the same sample volume.

Sludge suspended solids concentration has a significant effect on test results. In evaluating sludge-conditioning products, compare results for which initial suspended solids concentrations are comparable. Make a rough correction for different solids contents by dividing the time-to-filter value by its corresponding solids concentration. However, variations in solids concentration occur in full-scale applications, and the time-to-filter results may be interpreted as indicating the overall rate of water release from sludges, including the effect of differing solids concentrations.

4. Precision and Bias

Variations in vacuum pressure, support filter type, sludge temperature, and sample volume can affect test results. Triplicate analyses of 18 sample sets of conditioned and unconditioned alum sludge resulted in an average method precision of 19 s (approximately 4% of the average value) for the large-volume TTF test. Triplicate analyses of 9 sample sets of conditioned and unconditioned alum sludge resulted in a method precision of 9 s (approximately 6% of the average value) for the small-volume TTF test. Method bias, which refers to the agreement between the value determined by the test method and the real value, cannot be determined.

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2710 I. Modified Settled Sludge Volume

1. General Discussion

See 2710C.

While 2710C determines settled sludge volume using a 1-L graduated cylinder equipped with a stirring mechanism, the settling column used with the procedure herein is a wide 2-L cylindrical vessel to allow suspension volume to be determined relative to the 1-L graduated test results. Use of a settling column having a diameter wider than the 1-L graduated cylinder has been suggested[1] and the 2-L settling column is frequently used.[2] Results from Section 2710C are not currently considered comparable with those obtained with the procedure herein.

2. Apparatus

a. Settling column: Use a 2-L graduated cylindrical vessel of approximately 13 cm diam, 19 cm height (outer dimensions) equipped with a stirring mechanism consisting of four thin rods extending the length of the column, with the outer rods positioned within two rod-diameters of the cylinder wall. Provide a stirrer able to rotate the stirring rods at approximately, but no more than, 2 rpm (peripheral tip speed of approximately 1.2 cm/s). See Figure 2710:5.

b. Stopwatch.

c. Thermometer.

3. Procedure

Start test as soon as possible after sample collection. Distribute solids in sample without breaking flocculated particles apart by gently inverting or swirling sample container three times. Gently pour 2.0 L sample in settling column and insert stirring rods. Activate stirring mechanism, start stopwatch, and let suspension settle. Continue stirring throughout test. Maintain suspension temperature during test at that in the basin from which the sample was taken.

Determine volume of the suspension occupied at measured time intervals (e.g., 5, 10, 15, 20, 30, 45 and 60 min). Take readings after stirring rod rotates past graduated scale and disturbance at top of settling suspension is minimal.

Report settled sludge volume of the suspension in milliliters for an indicated time interval.

Test results are applicable to a particular test site and are significantly affected by variables such as suspension temperature, sampling and agitation methods, dimensions of settling column and stirring rods, stirring speed, and time between sampling and start of the determination.

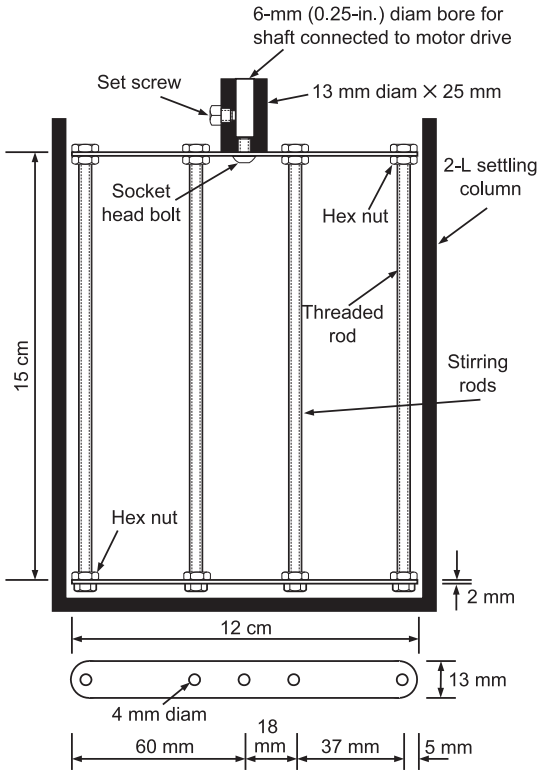


Figure 2710:5. Schematic diagram of settling column and stirring rods for modified sludge volume test. Settling column should be a clear glass or plastic. Stirring mechanism should be stainless steel. Use M4-0.7 (SAE 8-32) nuts, bolts, screws and rods unless noted. All dimensions for stirring mechanism are approximate.

4. Precision and Bias

Bias is not applicable. The precision for this test has not been determined.

5. References

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https://doi.org/10.2105/SMWW.2882.035

2720 ANAEROBIC SLUDGE DIGESTER GAS ANALYSIS*

2720 A. Introduction

Gas produced during the anaerobic decomposition of wastes contains methane (CH_4) and carbon dioxide (CO_2) as the major components with minor quantities of hydrogen (H_2), hydrogen sulfide (H_2S), nitrogen (N_2), and oxygen (O_2). It is saturated with water vapor. Common practice is to analyze the gases produced to estimate their fuel value and to check on the treatment process. The relative proportions of CO_2, CH_4, and N_2 are normally of most concern and the easiest to determine because of the relatively high percentages of these gases.

1. Selection of Method

Two procedures are described for gas analysis, the volumetric method (B), and the gas chromatographic method (C). The volumetric analysis is suitable for the determination of CO_2, H_2, CH_4, and O_2. Nitrogen is estimated indirectly by difference. Although the method is time-consuming, the equipment is relatively simple. Because no calibration is needed before use, the procedure is particularly appropriate when analyses are conducted infrequently.

The principal advantage of gas chromatography is speed. Commercial equipment is designed specifically for isothermal or temperature-programmed gas analysis and permits the routine separation and measurement of CO_2, N_2, O_2, and CH_4 in less than 15 to 20 min. The requirements for a recorder, pressure-regulated bottles of carrier gas, and certified standard gas mixtures for calibration raise costs to the point where infrequent analyses by this method may be uneconomical. The advantages of this system are freedom from the cumulative errors found in sequential volumetric measurements, adaptability to other gas component analyses, adaptability to intermittent on-line sampling and analysis, and the use of samples of 1 mL or less.[1]

2. Sample Collection

When the source of gas is some distance from the apparatus used for analysis, collect samples in sealed containers and bring to the instrument. Displacement collectors are the most suitable containers. Glass sampling bulbs (or tubes) with three-way glass or TFE stopcocks at each end, as indicated in Figure 2720:1, are particularly useful. These also are available with centrally located ports provided with septa for syringe transfer of samples. Replace septa periodically to prevent contamination by atmospheric gases. Connect one end of collector to gas source and vent three-way stopcock to the atmosphere. Clear line of air by passing 10 to 15 volumes of gas through vent and open stopcock to admit sample. If large quantities of gas are available, sweep air away by passing 10 to 15 volumes of gas through tube. If the gas supply is limited, fill the gas sampling bulb (tube) with an acidic salt solution.[2] Then completely displace the acidic salt solution in the gas sampling bulb (tube) with the sample gas. Because the acidic salt solution absorbs gases to some extent, fill the gas sampling bulb completely with the gas and seal off from any contact with displacement fluid during temporary storage. When transferring gas to the gas-analyzing apparatus, do not transfer any fluid.

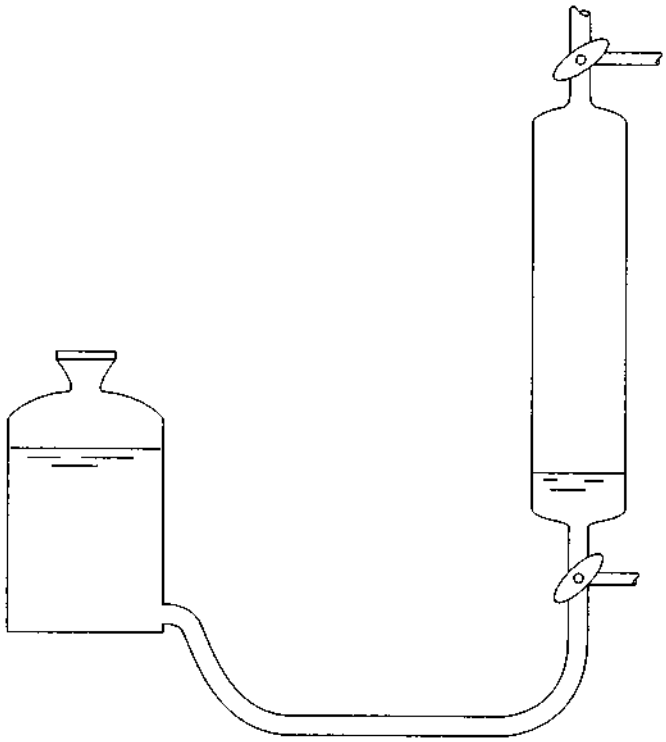


Figure 2720:1. Gas collection apparatus.

3. References

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* Approved by Standard Methods Committee, 2009. Editorial revisions, 2011.

https://doi.org/10.2105/SMWW.2882.036

2720 B. Volumetric Method

1. General Discussion

a. Principle: This method may be used for the analysis either of digester gas or of methane in water (see Section 6211, Methane). A measured volume of gas is passed first through a solution of potassium hydroxide (KOH) to remove CO_2, next through a solution of alkaline pyrogallol to remove O_2, and then over heated cupric oxide, which removes H_2 by oxidation to water. After each of the above steps, the volume of gas remaining is measured; the decrease that results is a measure of the relative percentage of volume of each component in the mixture. Finally, CH_4 is determined by conversion to CO_2 and H_2O in a slow-combustion pipet or a catalytic oxidation assembly. The volume of CO_2 formed during combustion is measured to determine the fraction of methane originally present. Nitrogen is estimated by assuming that it represents the only gas remaining and equals the difference between 100% and the sum of the measured percentages of the other components.

When only CO_2 is measured, report only CO_2. No valid assumptions may be made about the remaining gases present without making a complete analysis.

Follow the equipment manufacturers' recommendations with respect to oxidation procedures.

CAUTION: Do not attempt any slow-combustion procedure on digester gas because of the high probability of exceeding the explosive 5% by volume concentration of CH_4.

b. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

Orsat-type gas-analysis apparatus, consisting of at least:

- a water-jacketed gas buret with leveling bulb;
- a CO_2-absorption pipet;
- an O_2-absorption pipet;
- a cupric oxide-hydrogen oxidation assembly;
- a shielded catalytic CH_4-oxidation assembly or slow-combustion pipet assembly; and
- a leveling bulb. With the slow-combustion pipet use a controlled source of current to heat the platinum filament electrically.

Preferably use mercury as the displacement fluid; alternatively use aqueous Na_2SO_4-H_2SO_4 solution for sample collection. Use any commercially available gas analyzer having these units.

3. Reagents

a. Potassium hydroxide solution: Dissolve 500 g KOH in distilled water and dilute to 1 L.

b. Alkaline pyrogallol reagent: Dissolve 30 g pyrogallol (also called pyrogallic acid) in distilled water and make up to 100 mL. Add 500 mL KOH solution.

c. Oxygen gas: Use approximately 100 mL for each gas sample analyzed.

d. Displacement liquid (acidic salt solution): Dissolve 200 g Na_2SO_4 in 800 mL distilled water; add 30 mL conc H_2SO_4. Add a few drops of methyl orange indicator. When color fades, replace solution.

4. Procedure

a. Sample introduction: Transfer 5 to 10 mL gas sample into gas buret through a capillary-tube connection to the collector. Expel this sample to the atmosphere to purge the system. Transfer up to 100 mL gas sample to buret. Bring sample in buret to atmospheric or reference pressure by adjusting leveling bulb. Measure volume accurately and record as V_1.

b. Carbon dioxide absorption: Remove CO_2 from sample by passing it through the CO_2-absorption pipet charged with the KOH solution. Pass gas back and forth until sample volume remains constant. Before opening stopcocks between buret and any absorption pipet, make sure that the gas in the buret is under a slight positive pressure to prevent reagent in the pipet from contaminating stopcock or manifold. After absorption of CO_2, transfer sample to buret and measure volume. Record as V_2.

c. Oxygen absorption: Remove O_2 by passing sample through O_2-absorption pipet charged with alkaline pyrogallol reagent until sample volume remains constant. Measure volume and record as V_3 . For digester gas samples, continue as directed in ¶ *d* below. For CH_4 in water, store gas in CO_2 pipet and proceed to ¶ *e* below.

d. Hydrogen oxidation: Remove H_2 by passing sample through CuO assembly maintained at a temperature in the range 290 to 300°C. When a constant volume has been obtained, transfer sample back to buret, cool, and measure volume. Record as V_4.

Waste to the atmosphere all but 20 to 25 mL of remaining gas. Measure volume and record as V_5. Store temporarily in CO_2-absorption pipet.

e. Methane oxidation: Purge inlet connections to buret with O_2 by drawing 5 to 10 mL into buret and expelling to the atmosphere. Oxidize CH_4 either by the catalytic oxidation process for digester gas and gas phase of water samples or by the slow-combustion process for gas phase of water samples.

1) Catalytic oxidation process—For catalytic oxidation of digester gas and gas phase of water samples, transfer 65 to 70 mL O_2 to buret and record this volume as V_6 . Pass O_2 into CO_2-absorption pipet so that it will mix with sample stored there. Return this mixture to buret and measure volume. Record as V_7. This volume should closely approximate V_5 plus V_6 . Pass O_2-sample mixture through catalytic oxidation assembly, which should be heated in accordance with the manufacturer's directions. Keep rate of gas passage less than 30 mL/min. After first pass, transfer mixture back and forth through the assembly between buret and reservoir at a rate not faster than 60 mL/min until a constant volume is obtained. Record as V_8.

2) Slow-combustion process—For slow combustion of the gas phase of water samples, transfer 35 to 40 mL O_2 to buret and record volume as V_6 . Transfer O_2 to slow-combustion pipet and then transfer sample from CO_2-absorption pipet to buret. Heat platinum coil in combustion pipet to yellow heat while controlling temperature by adjusting current. Reduce pressure of O_2 in pipet to somewhat less than atmospheric pressure by means of

the leveling bulb attached to the pipet. Pass sample into slow-combustion pipet at rate of approximately 10 mL/min. After the first pass, transfer sample and O_2 mixture back and forth between pipet and buret several times at a faster rate, allowing mercury in pipet to rise to a point just below heated coil. Collect sample in combustion pipet, turn off coil, and cool pipet and sample to room temperature with a jet of compressed air. Transfer sample to buret and measure volume. Record as V_8.

f. Measurement of carbon dioxide produced: Determine amount of CO_2 formed in the reaction by passing sample through CO_2-absorption pipet until volume remains constant. Record volume as V_9 . Check accuracy of determination by absorbing residual O_2 from sample. After this absorption, record final volume as V_{10}.

5. Calculation

a. CH_4 and H_2 usually are the only combustible gases present in sludge digester gas. When this is the case, determine percentage by volume of each gas as follows:

$$\% \, CO_2 = \frac{(V_1 - V_2) \times 100}{V_1}$$

$$\% \, O_2 = \frac{(V_2 - V_3) \times 100}{V_1}$$

$$\% H_2 = \frac{(V_3 - V_4) \times 100}{V_1}$$

$$\% \, CH_4 = \frac{V_4 \times (V_8 - V_9) \times 100}{V_1 \times V_5}$$

$$\% \, N_2 = 100 - (\% \, CO_2 + \% \, O_2 + \% \, H_2 + \% \, CH_4)$$

b. Alternatively, calculate CH_4 by either of the two following equations:

$$\% \, CH_4 = \frac{V_4 \times (V_6 + V_{10} - V_9) \times 100}{2 \times V_1 \times V_5}$$

$$\% \, CH_4 = \frac{V_4 \, (V_7 - V_8) \times 100}{2 \times V_1 \times V_5}$$

Results from the calculations for CH_4 by the three equations should be in reasonable agreement. If not, repeat analysis after checking apparatus for sources of error, such as leaking stopcocks or connections. Other combustible gases, such as ethane, butane, or pentane, will cause a lack of agreement among the calculations; however, the possibility that digester gas contains a significant amount of any of these is remote.

6. Precision and Bias

A gas buret measures gas volume with a precision of 0.05 mL and a probable accuracy of 0.1 mL. With the large fractions of CO_2 and CH_4 normally present in digester gas, the overall error for their determination can be made less than 1%. The error in the determination of O_2 and H_2, however, can be considerable because of the small concentrations normally present. For a concentration as low as 1%, an error as large as 20% can be expected. When N_2 is present in a similar low-volume percentage, the error in its determination would be even greater, because errors in each of the other determinations would be reflected in the calculation for N_2.

7. Bibliography

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2720 C. Gas Chromatographic Method

1. General Discussion

See Section 6010C for discussions of gas chromatography.

The quality control practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

2. Apparatus

a. Gas chromatograph: Use any instrument system equipped with a thermal conductivity detector (TCD), carrier-gas flow controllers, injector and column temperature setting dials, TCD current controller, attenuator, carrier-gas pressure gauge, injection port, signal output, and power switch. Some columns require temperature programming while others are isothermal. Preferably use a unit with a gas sampling loop and valve that allow automatic injection of a constant sample volume.

b. Sample introduction apparatus: An instrument equipped with gas-sampling valves is designed to permit automatic injection of a specific sample volume into the chromatograph. If such an instrument is not available, introduce samples with a 2-mL syringe fitted with a 27-gauge hypodermic needle. Reduce escape of gas by greasing plunger lightly with mineral oil or preferably by using a special gas-tight syringe. One-step separation of oxygen, nitrogen, methane, and carbon dioxide may be accomplished in concentric columns (column within a column) under isothermal conditions at room temperature instead of performing two column analyses. These concentric columns permit the simultaneous use of two different packings for the analysis of gas samples.

*c. Chromatographic column:** Select column on the basis of manufacturer's recommendations.† Report column and packing specifications and conditions of analyses with results.

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* Gas chromatographic methods are extremely sensitive to the materials used. Use of trade names in *Standard Methods* does not preclude the use of other existing or as-yet-undeveloped products that give *demonstrably* equivalent results.

† Commercially available columns (and gases they will separate) include:

Silica gel and activated alumina:
- Silica gel: H_2, air (O_2 + N_2), CO, CH_4, C_2H_6.
- Activated alumina: air (O_2 + N_2), CH_4, C_2H_6, C_3H_8.

Molecular sieves (zeolites):
- Molecular sieve 5A: H_2, (O_2 − Ar), N_2, CH_4, CO.
- Molecular sieve 13X: O_2, N_2, CH_4, CO.

Porous polymers:
- Chromosorb 102: air (H_2, O_2, N_2, CO), CH_4, CO_2.

A two-column system usually is required. A molecular sieve column separates H_2, O_2, N_2, CO, and CH_4 isothermally, but because CO_2 is adsorbed by the molecular sieve, a second column is needed to complete the analysis. A commonly used two-column system utilizes a Chromosorb 102 column and a Molecular Sieve 5A or 13X column to separate H_2, O_2, N_2, CH_4, and CO_2 isothermally.[1,2]

A single-column procedure can be used; however, it requires temperature programming. Columns packed with special porous spherical or granular packing materials effect separation with sharp, well-resolved peaks.[3] With temperature programming, columns packed with Chromosorb 102,[2] Carbosphere,[4] and Carbosieve[3] separate the gases listed.† Commercial equipment specifically designed for such operations is available.[2,4–6]

d. Integrator/recorder: Use a 10-mV full-span strip chart recorder with the gas chromatograph. When minor components such as H_2 and H_2S are to be detected, a 1-mV full-span recorder is preferable.

Integrators that can easily detect very minute quantities of gases are available. Computerized data-processing systems, to record and manipulate the chromatographic signal, chromatographic base line, etc., also are available.

3. Reagents

a. Carrier gases: Preferably use helium for separating digester gases. It is impossible to detect less than 1% hydrogen when helium is used as the carrier gas.[1] Obtain linear TCD responses for molar concentrations of hydrogen between 0 and 60% by using an 8.5% hydrogen–91.5% helium carrier gas mixture.[7] To detect trace quantities of hydrogen use argon or nitrogen as carrier gas.[1]

b. Calibration gases: Use samples of CH_4, CO_2, and N_2 of known purity, or gas mixtures of certified composition, for calibration. Also use samples of O_2, H_2, and H_2S of known purity if these gases are to be measured. Preferably use custom-made gas mixtures to closely approximate digester gas composition.

c. Displacement liquids: See 2720B.3*d.*

4. Procedure

a. Preparation of gas chromatograph: Open main valve of carrier-gas cylinder and adjust carrier-gas flow rate to recommended values. To obtain accurate flow measurements, connect a soap-film flow meter to the TCD vent. Turn power on. Turn on oven heaters, if used, and detector current and adjust to desired values.

Set injection port and column temperatures as specified for the column being used. Set TCD current. Turn on recorder or data processor. Check that the injector/detector temperature has risen to the appropriate level and confirm that column temperature is stabilized. Set range and attenuation at appropriate positions.

The instrument is ready for use when the recorder yields a stable base line. Silica gel and molecular sieve columns gradually lose activity because of adsorbed moisture or materials permanently adsorbed at room temperature. If insufficient separations occur, reactivate by heating or repacking.

b. Calibration: For accurate results, prepare a calibration curve for each gas to be measured because different gas components do not give equivalent detector responses on either a weight or a molar basis. Calibrate with synthetic mixtures or with pure gases.

1) Synthetic mixtures—Use purchased gas mixtures of certified composition or prepare in the laboratory. Inject a standard volume of each mixture into the gas chromatograph and note response for each gas. Compute detector response, either as area under a peak or as height of peak, after correcting for attenuation. Read peak heights accurately and correlate with concentration of component in sample. Reproduce operating parameters exactly from one analysis to the next. If sufficient reproducibility cannot be obtained by this procedure, use peak areas for calibration. Preferably use peak areas when peaks are not symmetrical. Prepare calibration curve by plotting either peak area or peak height against volume or mole percent for each component.

Modern integrators and data-processing systems are able to generate calibration tables for certified gas mixtures.

2) Pure gases—Introduce pure gases into chromatograph individually with a syringe. Inject sample volumes of 0.25, 0.5, 1.0 mL, etc., and plot detector response, corrected for attenuation, against gas volume.

When the analysis system yields a linear detector response with increasing gas component concentration from zero to the range of interest, run standard mixtures along with samples. If the same sample size is used, calculate gas concentration by direct proportions.

c. Sample analysis: If samples are to be injected with a syringe, equip sample collection container with a port closed by a rubber or silicone septum. To take a sample for analysis, expel air from barrel of syringe by depressing plunger and force needle through the septum. Withdraw plunger to take gas volume desired, pull needle from collection container, and inject sample rapidly into chromatograph.

When samples are to be injected through a gas-sampling valve, connect sample collection container to inlet tube. Let gas flow from collection tube through the valve to purge dead air space and fill sample tube. About 15 mL normally are sufficient to clear the lines and to provide a sample of 1 to 2 mL. Transfer sample from loop into carrier gas stream by following manufacturer's instructions. Bring samples to atmospheric pressure before injection.

When calibration curves have been prepared with a synthetic gas mixture of certified composition, use the same sample volume as that used during calibration. When calibration curves are prepared by the procedure using varying volumes of pure gases, inject any convenient gas sample volume up to about 2 mL.

Inject sample and standard gases in sequence to permit calculation of unknown gas concentration in volume (or mole) percent by direct comparison of sample and standard gas peak heights or areas. For more accurate analysis, make duplicate or triplicate injections of sample and standard gases.

5. Calculation

a. When calibration curves have been prepared with synthetic mixtures and the volume of the sample analyzed is the same as that used in calibration, read volume percent of each component

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Porapak Q: (air–CO), CO_2.
HayeSep Q: H_2, air (O_2 + N_2), CH_4, CO_2, C_2H_6, H_2S.
Carbon molecular sieves:
Carbosphere: O_2, N_2, CO, CH_4, CO_2, C_2H_6 (these gases can be eluted isothermally at various temperatures or by temperature programming).
Carbosieve S-II: H_2, air (O_2 + N_2), CO, CH_4, CO_2, C_2H_6 (temperature programming required).

directly from calibration curve after detector response for that component is computed.

b. When calibration curves are prepared with varying volumes of pure gases, calculate the percentage of each gas in the mixture as follows:

$$\text{Volume \%} = \frac{A}{B} \times 100$$

where:

A = partial volume of component (read from calibration curve), and
B = volume sample injected.

c. Where standard mixtures are run with samples and instrument response is linear from zero to the concentration range of interest:

$$\text{Volume \%} = \text{volume \% (std)} \frac{C}{D}$$

where:

C = recorder value of sample, and
D = recorder value of standard.

d. Digester gases usually are saturated with water vapor that is not a digestion product. Therefore, apply corrections to calculate the dry volume percent of each digester gas component as follows:

$$\text{Dry volume \%} = \frac{\text{volume \% as above}}{1 - P_v}$$

where:

P_v = saturated water vapor pressure at room temperature and pressure, decimal %.

The saturation water vapor pressure can be found in common handbooks. The correction factor $\frac{1}{1 - P_v}$ is usually small and is often neglected by analysts.

6. Precision and Bias

Precision and bias depend on the instrument, the column, operating conditions, gas concentrations, and techniques of operation. The upper control limits for replicate analyses of a high-methane-content standard gas mixture (65.01% methane, 29.95% carbon dioxide, 0.99% oxygen, and 4.05% nitrogen) by a single operator were as follows: 65.21% for methane, 30.36% for carbon dioxide, 1.03% for oxygen, and 4.15% for nitrogen. The lower control limits for this same standard gas mixture were: 64.67% for methane, 29.88% for carbon dioxide, 0.85% for oxygen, and 3.85% for nitrogen. The observed relative standard deviations (RSD) were: 0.14% for methane, 0.26% for carbon dioxide, 3.2% for oxygen, and 1.25% for nitrogen.

In a similar analysis of another standard gas mixture (55% methane, 35% carbon dioxide, 10% diatomic gases—2% hydrogen, 6% nitrogen, 2% oxygen), typical upper control limits for precision of duplicate determinations were: 55.21% for methane, 35.39% for carbon dioxide, and 10.26% for the diatomic gases. The lower control limits for this standard gas mixture were: 54.78% for methane, 34.62% for carbon dioxide, and 9.73% for the diatomic gases. The observed RSDs for this analysis were: 0.13% for methane, 0.36% for carbon dioxide, and 0.88% for the diatomic gases.

A low-methane-content standard gas mixture (35.70% methane, 47.70% carbon dioxide, 3.07% oxygen, 8.16% nitrogen, and 5.37% hydrogen) was analyzed as above yielding upper control limits as follows: 34.4% for methane, 49.46% for carbon dioxide, 3.07% for oxygen, and 8.2% for nitrogen. The lower control limits were: 34.18% for methane, 49.11% for carbon dioxide, 2.83% for oxygen, and 7.98% for nitrogen. The observed RSDs were: 0.11% for methane, 0.16% for carbon dioxide, 1.37% for oxygen, and 0.46% for nitrogen.

With digester gas the sum of the percent CH_4, CO_2, and N_2 should approximate 100%. If it does not, suspect errors in collection, handling, storage, and injection of gas, or in instrumental operation or calibration.

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2810 DISSOLVED GAS SUPERSATURATION*

2810 A. Introduction

Water can become supersaturated with atmospheric gases by various means, heating and air entrainment in spilled or pumped water being the most common. The primary sign of gas supersaturation is the formation of bubbles on submerged surfaces or within the vascular systems and tissues of aquatic organisms.

Gas supersaturation can limit aquatic life and interfere with water treatment processes. Levels of supersaturation lethal to aquatic organisms have been found in springs, rivers, wells, lakes, estuaries, and seawater. Gas supersaturation can be produced in pumped or processed water intended for drinking, fish hatchery supply, and laboratory bioassays. Seasonal and other temporal variations in supersaturation may occur in surface waters as a result of solar heating and photosynthesis. Because the rate of equilibration may be slow, supersaturation may persist in flowing water for days and excessive dissolved gas levels thus may persist far from the source of supersaturation.

Gas bubbles form only when the total dissolved gas pressure is greater than the sum of compensating pressures. Compensating pressures include water, barometric and, for organisms, tissue or blood pressure. The total dissolved gas pressure is equal to the sum of the partial pressures of all the dissolved gases, including water vapor. Typically, only nitrogen, oxygen, argon, carbon dioxide, and water vapor pressures need to be considered in most natural waters. Gas bubble disease, of fish or other aquatic organisms, is a result of excessive uncompensated gas pressure. A single supersaturated gas such as oxygen or nitrogen may not necessarily result in gas bubble disease because bubble formation depends largely on total dissolved gas pressure. The degree of gas saturation should be described in terms of pressures rather than concentration or volume units.

* Approved by Standard Methods Committee, 2010. Editorial revisions, 2011. Joint Task Group: 20th Edition—John E. Colt (chair), Larry E. Fidler, John O. Jensen, John W. Sweeney, Barnaby J. Watten.

2810 B. Direct-Sensing Membrane-Diffusion Method

1. General Discussion

a. Principle: This method requires an instrument with a variable length of "gas permeable" tubing, connected to a pressure-measuring device. Dimethyl silicone rubber tubing often is used because it is highly permeable to dissolved gases, including water vapor. At steady state, the gauge pressure inside the tubing is equal to the difference in gas pressure (ΔP) between the total dissolved gas pressure and the ambient barometric pressure. When the water is in equilibrium with the atmosphere, ΔP equals zero. If ΔP is greater than zero, the water is supersaturated. Conversely, if ΔP is negative the water is undersaturated.

b. Working range: The working range of this method depends on the pressure-sensing device used, but typically will range from −150 to +600 mm Hg. Dissolved solids in wastewater will not interfere with this method. The practical depth range for these instruments is 1 to 10 m.

2. Apparatus

Several types of membrane-diffusion instruments are available commercially.* Alternatively, construct a unit from commercially available parts. Several units have been described, including a direct-reading instrument using pressure transducers and a digital readout,[1] an on-line unit that can activate an alarm system,[2] and an early model of the Weiss saturometer.[3] Each of these units has specific advantages and limitations; the instrument of choice will depend on the specific application. All these instruments are portable so that data collection is completed in the field.

Test the instrument for leaks according to the manufacturer's recommendation. Even a very small leak, difficult to detect and locate, will result in useless data. Calibrate the pressure-measuring device with a mercury manometer or certified pressure gauge. If a manometer is used, include fresh mercury that flows freely in the tubing. An alternative method for directly testing membrane-diffusion instruments in a small, closed chamber where induced ΔP levels can be compared against observed ΔP levels is available.[2]

Van Slyke-Neill[4] or gas chromatography methods[1] are inappropriate for calibration but they may be used to verify results. These methods measure individual gas concentrations and require further conversion to ΔP or partial pressure and suffer from sampling and sample handling problems.[5–7]

3. Procedure

At the start of each day, test the instrument for leaks and recalibrate. At a monitoring site, completely submerge the sensing element in the water, preferably below the hydrostatic compensation depth. This is the depth where the hydrostatic and total gas pressures are equal and as a result, bubbles will not form on the tubing. Bubble formation on the silicone rubber tubing seriously reduces accuracy. Compute hydrostatic compensation depth[5] as follows:

$$Z = \frac{\Delta P}{73.42}$$

* Common Sensing, Clark Fork, ID; Eco Enterprises, Seattle, WA; Novatech, Vancouver, BC, Canada; and Sweeney Aquametrics, Stony Creek, CT.

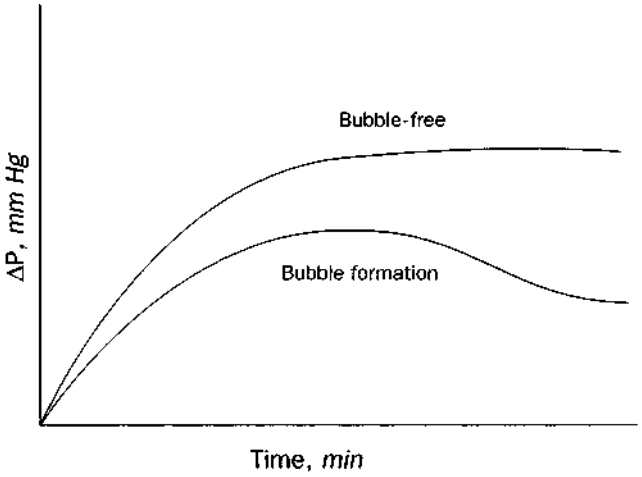


Figure 2810:1 Time response for the membrane-diffusion method.

where:

Z = hydrostatic compensation depth, m, and
ΔP = pressure difference between total dissolved gas pressure and the ambient barometric pressure, mm Hg.

The factor 73.42 is the hydrostatic pressure of fresh water at 20°C expressed in terms of mm Hg/m water depth. Because the variation of hydrostatic pressure with temperature and salinity is small, this equation can be used for all natural waters.

Dislodge formed bubbles on the tubing by gently striking the instrument or moving the instrument rapidly in the water. Movement of water across the silicone rubber tubing also facilitates establishing the equilibrium between gas pressure in the water and in the tubing.

Operate the instrument "bubble free" until a stable ΔP is observed. This may take from 5 to 30 min, depending on the ΔP, water temperature, water flow, and geometry of the system. The time response of the membrane-diffusion method is shown in Figure 2810:1 for "bubble-free" and "bubble" conditions.

If the instrument is used in heavily contaminated water containing oil or other organic compounds, clean the silicone tubing with a mild detergent according to the manufacturer's instructions. Silicone rubber tubing has been used in uncontaminated natural water for at least eight years without being adversely affected by attached algal growth.[2] The tubing can be damaged by abrasive grit, diatoms, biting aquatic organisms, certain organic compounds, and strong acids.[2]

Obtain the barometric pressure with each measurement by using a laboratory mercury barometer, a calibrated portable barometer, or pressure transducer. Barometric pressures reported by weather agencies (or airports) are corrected to sea level and are unusable.

4. Calculation

a. Total gas pressure: Preferably report total gas pressure as ΔP.[2,6,8] Express pressure as millimeters of mercury.

Total gas pressure also has been reported as a percentage of local barometric pressure:

$$\text{TGP } \% = \left[\frac{P_b + \Delta P}{P_b}\right] \times 100$$

where:

P_b = true local barometric pressure, mm Hg.

The reporting of total gas pressure as a percentage is not encouraged.[8]

b. Component gas pressures: When information on component gas supersaturation is needed, express data as partial pressures, differential pressures, or percent saturation.[5,8] This requires additional measurements of dissolved oxygen, temperature, and salinity† at the monitoring site. In a mixture of gases in a given volume, the partial pressure of a gas is the pressure that this gas would exert if it were the only gas present.

1) Oxygen partial pressure—Calculate partial pressure of oxygen as follows:

$$P_{O_2} = \frac{\text{DO}}{\beta_{O_2}} \times 0.5318$$

where:

P_{O_2} = partial pressure of dissolved oxygen, mm Hg,
β_{O_2} = Bunsen coefficient for oxygen (Table 2810:I), L/(L · atm), and
DO = measured concentration of oxygen, mg/L.

Bunsen coefficients for marine waters are available.[5] The factor 0.5318 equals 760/(1000 K), where K is the ratio of molecular weight to molecular volume for oxygen gas.[5]

2) Nitrogen partial pressure—Estimate the partial pressure of nitrogen by subtracting the partial pressures of oxygen and water vapor from the total gas pressure.

$$P_{N_2} = \text{P}_b + \Delta P \; - \; P_{O_2} - P_{H_2O}$$

where:

P_{H_2O} = vapor pressure of water in mm Hg from Table 2810:II.

This term includes a small contribution from argon and any other gases present, including carbon dioxide and methane. The partial pressure of carbon dioxide is negligible in natural waters of pH >7.0.

3) Nitrogen:oxygen partial pressure ratio—The ratio of the partial pressure of nitrogen to the partial pressure of oxygen (N_2:O_2) characterizes the relative contribution of the two gases to the total dissolved gas pressure. In water in equilibrium with air, this ratio is 3.77.

c. Differential pressures: The differential pressure of a gas is the difference between the partial pressures of that gas in water and air. The oxygen differential pressure may be calculated as

$$\Delta P_{O_2} = P_{O_2} \; - \; 0.20946(P_b - P_{H_2O})$$

and the nitrogen differential pressure as

$$\Delta P_{N_2} = \Delta P - \Delta P_{O_2}$$

† Methods for these variables may be found in Sections 4500-O, 2550, and 2520, respectively.

TABLE 2810:I. BUNSEN COEFFICIENT FOR OXYGEN IN FRESH WATER

Temperature °C	Bunsen Coefficient at Given Temperature (to nearest 0.1°C) *L real gas at STP/(L · atmosphere)*									
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0	0.04914	0.04901	0.04887	0.04873	0.04860	0.04847	0.04833	0.04820	0.04807	0.04793
1	0.04780	0.04767	0.04754	0.04741	0.04728	0.04716	0.04703	0.04680	0.04678	0.04665
2	0.04653	0.04640	0.04628	0.04615	0.04603	0.04591	0.04579	0.04567	0.04555	0.04543
3	0.04531	0.04519	0.04507	0.04495	0.04484	0.04472	0.04460	0.04449	0.04437	0.04426
4	0.04414	0.04403	0.04392	0.04381	0.04369	0.04358	0.04347	0.04336	0.04325	0.04314
5	0.04303	0.04292	0.04282	0.04271	0.04260	0.04250	0.04239	0.04229	0.04218	0.04206
6	0.04197	0.04187	0.04177	0.04166	0.04156	0.04146	0.04136	0.04126	0.04116	0.04106
7	0.04096	0.04086	0.04076	0.04066	0.04056	0.04047	0.04037	0.04027	0.04018	0.04008
8	0.03999	0.03989	0.03980	0.03971	0.03961	0.03952	0.03943	0.03933	0.03924	0.03915
9	0.03906	0.03897	0.03888	0.03879	0.03870	0.03861	0.03852	0.03843	0.03835	0.03826
10	0.03817	0.03809	0.03800	0.03791	0.03783	0.03774	0.03766	0.03757	0.03749	0.03741
11	0.03732	0.03724	0.03716	0.03707	0.03699	0.03691	0.03683	0.03675	0.03667	0.03659
12	0.03651	0.03643	0.03635	0.03627	0.03619	0.03611	0.03604	0.03596	0.03588	0.03581
13	0.03573	0.03565	0.03558	0.03550	0.03543	0.03535	0.03528	0.03520	0.03513	0.03505
14	0.03498	0.03491	0.03448	0.03476	0.03469	0.03462	0.03455	0.03448	0.03441	0.03433
15	0.03426	0.03419	0.03412	0.03406	0.03399	0.03392	0.03385	0.03378	0.03371	0.03364
16	0.03358	0.03351	0.03344	0.03338	0.03331	0.03324	0.03318	0.03311	0.03305	0.03298
17	0.03292	0.03285	0.03279	0.03272	0.03266	0.03260	0.03253	0.03247	0.03241	0.03235
18	0.03228	0.03222	0.03216	0.03210	0.03204	0.03198	0.03192	0.03186	0.03180	0.03174
19	0.03168	0.03162	0.03156	0.03150	0.03144	0.03138	0.03132	0.03126	0.03121	0.03115
20	0.03109	0.03103	0.03098	0.03092	0.03086	0.03081	0.03075	0.03070	0.03064	0.03059
21	0.03053	0.03048	0.03042	0.03037	0.03031	0.03026	0.03020	0.03015	0.03010	0.03004
22	0.02999	0.02994	0.02989	0.02983	0.02978	0.02973	0.02968	0.02963	0.02958	0.02952
23	0.02947	0.02942	0.02937	0.02932	0.02927	0.02922	0.02917	0.02912	0.02907	0.02902
24	0.02897	0.02893	0.02888	0.02883	0.02878	0.02873	0.02868	0.02864	0.02859	0.02854
25	0.02850	0.02845	0.02840	0.02835	0.02831	0.02826	0.02822	0.02817	0.02812	0.02808
26	0.02803	0.02799	0.02794	0.02790	0.02785	0.02781	0.02777	0.02772	0.02768	0.02763
27	0.02759	0.02755	0.02750	0.02746	0.02742	0.02737	0.02733	0.02729	0.02725	0.02720
28	0.02716	0.02712	0.02708	0.02704	0.02700	0.02695	0.02691	0.02687	0.02683	0.02679
29	0.02675	0.02671	0.02667	0.02663	0.02659	0.02655	0.02651	0.02647	0.02643	0.02639
30	0.02635	0.02632	0.02628	0.02624	0.02620	0.02616	0.02612	0.02609	0.02605	0.02601
31	0.02597	0.02594	0.02590	0.02586	0.02582	0.02579	0.02575	0.02571	0.02568	0.02564
32	0.02561	0.02557	0.02553	0.02550	0.02546	0.02543	0.02539	0.02536	0.02532	0.02529
33	0.02525	0.02522	0.02518	0.02515	0.02511	0.02508	0.02504	0.02501	0.02498	0.02494
34	0.02491	0.02488	0.02484	0.02481	0.02478	0.02474	0.02471	0.02468	0.02465	0.02461
35	0.02458	0.02455	0.02452	0.02448	0.02445	0.02442	0.02439	0.02436	0.02433	0.02429
36	0.02426	0.02423	0.02420	0.02417	0.02414	0.02411	0.02408	0.02405	0.02402	0.02399
37	0.02396	0.02393	0.02390	0.02387	0.02384	0.02381	0.02378	0.02375	0.02372	0.02369
38	0.02366	0.02363	0.02360	0.02358	0.02355	0.02352	0.02349	0.02346	0.02343	0.02341
39	0.02338	0.02335	0.02332	0.02329	0.02327	0.02324	0.02321	0.02318	0.02316	0.02313
40	0.02310	0.02308	0.02305	0.02302	0.02300	0.02297	0.02294	0.02292	0.02289	0.02286

Based on Benson and Krause.[9,10] $\beta = 9.9902 \times 10^{-4}[\exp(9.7265 - 5.26895 \times 10^3/T + 1.00417 \times 10^6/T^2)]$, where $T = 273.15 + °C$.

d. Percent of saturation: In older literature, supersaturation values have been reported as percent saturation. This method of reporting component gases is discouraged but can be calculated as follows:

$$N_2(\%) = \left[\frac{P_{N_2}}{0.7902\,(P_b - P_{H_2O})}\right] \times 100$$

$$O_2(\%) = \left[\frac{P_{O_2}}{0.20946(P_b - P_{H_2O})}\right] \times 100$$

The following relationships are useful conversions:

$$\mathrm{TGP}(\%) = 0.20946\,\mathrm{O_2}(\%) + 0.7902\,\mathrm{N_2}(\%)$$

$$\Delta P = 0.20946\left[\frac{O_2(\%)}{100} - 1\right][P_b - P_{H_2O}] + 0.7902\left[\frac{N_2(\%)}{100} - 1\right][P_b - P_{H_2O}]$$

$$\Delta P = \frac{DO}{\beta_{O_2}}(0.5318)(1 + N_2{:}O_2) - (P_b - P_{H_2O})$$

Use care with these relationships with older data because both TGP(%) and N_2(%) have been differently defined.[5]

TABLE 2810:II. VAPOR PRESSURE OF FRESH WATER

Temperature °C	Vapor Pressure at Given Temperature (to nearest 0.1°C) mm Hg									
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0	4.58	4.61	4.64	4.68	4.71	4.75	4.78	4.82	4.85	4.89
1	4.92	4.96	4.99	5.03	5.07	5.10	5.14	5.18	5.21	5.25
2	5.29	5.33	5.36	5.40	5.44	5.48	5.52	5.56	5.60	5.64
3	5.68	5.72	5.76	5.80	5.84	5.88	5.92	5.97	6.01	6.05
4	6.09	6.14	6.18	6.22	6.27	6.31	6.36	6.40	6.44	6.49
5	6.54	6.58	6.63	6.67	6.72	6.77	6.81	6.86	6.91	6.96
6	7.01	7.05	7.10	7.15	7.20	7.25	7.30	7.35	7.40	7.45
7	7.51	7.56	7.61	7.66	7.71	7.77	7.82	7.87	7.93	7.98
8	8.04	8.09	8.15	8.20	8.26	8.31	8.37	8.43	8.48	8.54
9	8.60	8.66	8.72	8.87	8.84	8.89	8.95	9.02	9.08	9.14
10	9.20	9.26	9.32	9.39	9.45	9.51	9.58	9.64	9.70	9.77
11	9.83	9.90	9.97	10.03	10.10	10.17	10.23	10.30	10.37	10.44
12	10.51	10.58	10.65	10.72	10.76	10.86	10.93	11.00	11.07	11.15
13	11.22	11.29	11.37	11.44	11.52	11.59	11.67	11.74	11.82	11.90
14	11.98	12.05	12.13	12.21	12.29	12.37	12.45	12.53	12.61	12.69
15	12.78	12.86	12.94	13.05	13.11	13.19	13.28	13.36	13.45	13.54
16	13.62	13.71	13.80	13.89	13.97	14.06	14.15	14.24	14.33	14.43
17	14.52	14.61	14.70	14.80	14.89	14.98	15.08	15.17	15.27	15.37
18	15.46	15.56	15.66	15.76	15.86	15.96	16.06	16.16	16.26	16.36
19	16.46	16.57	16.67	16.77	16.88	16.98	17.09	17.20	17.30	17.41
20	17.52	17.63	17.74	17.85	17.96	18.07	18.18	18.29	18.41	18.52
21	18.64	18.75	18.87	18.98	19.10	19.22	19.33	19.45	19.57	19.69
22	19.81	19.93	20.05	20.48	20.60	20.42	20.55	20.67	20.80	20.93
23	21.05	21.18	21.31	21.44	21.57	21.70	21.83	21.96	22.09	22.23
24	22.36	22.50	22.63	22.77	22.90	23.04	23.18	23.32	23.46	23.60
25	23.74	23.88	24.03	24.17	24.31	24.46	24.60	24.75	24.90	25.04
26	25.19	25.34	25.49	25.64	25.80	25.95	26.10	26.26	26.41	26.57
27	26.72	26.88	27.04	27.20	27.36	27.52	27.68	27.84	28.00	28.17
28	28.33	28.50	28.66	28.83	29.00	29.17	29.34	29.51	29.68	29.85
29	30.03	30.80	30.37	30.55	30.73	30.91	31.08	31.26	31.44	31.62
30	31.81	31.99	32.17	32.36	32.54	32.73	32.92	33.11	33.30	33.49
31	33.68	33.87	34.06	34.26	34.45	34.65	34.85	35.05	35.24	35.44
32	35.65	35.85	36.05	36.25	36.46	36.67	36.87	37.08	37.29	37.50
33	37.71	37.92	38.14	38.35	38.57	38.78	39.00	39.22	39.44	39.66
34	39.88	40.10	40.33	40.55	40.78	41.01	41.23	41.46	41.69	41.92
35	42.16	42.39	42.63	42.86	43.10	43.34	43.58	43.82	44.06	44.30
36	44.55	44.79	45.04	45.28	45.53	45.78	46.03	46.29	46.54	46.79
37	47.05	47.31	47.56	47.82	48.08	48.35	48.61	48.87	49.14	49.41
38	49.67	49.94	50.21	50.49	50.76	51.03	51.31	51.59	51.87	52.14
39	52.43	52.71	52.99	53.28	53.56	53.85	54.14	54.43	54.72	55.01
40	55.31	55.60	55.90	56.20	56.50	56.80	57.10	57.41	57.71	58.02

Based on an equation presented by Green and Carritt.[11] This equation is cumbersome to use. The following equation[9] is adequate for most applications:

$$P_{H_2O} = 760[\exp(11.8571 - 3{,}840.70/T - 216{,}961/T^2)], \text{ where } T = 273.15 + °C.^{5}$$

5. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Tables 2020:I and II.

The precision of the membrane-diffusion method depends primarily on the pressure-sensing instrument. For an experienced operator it is approximately ±1 to 2 mm Hg with an accuracy of ±3 to 5 mm Hg.[3,6] Air leaks, bubble formation, biofilm development, incomplete equilibration, or condensation produce negative errors while direct water leaks can result in positive errors in submersible units.

For accurate work, measure water temperature to the nearest ±0.1°C.

6. Reporting of Results

In reporting results, include the following data:

- Sensor depth, m,
- Barometric pressure, mm Hg,
- Water temperature, °C,
- Dissolved oxygen, mm Hg or mg/L,
- Salinity, g/kg, and
- ΔP, mm Hg.

If component gas information is needed add:

- Partial pressure of oxygen, mm Hg,
- Partial pressure of nitrogen, mm Hg, and

- Nitrogen:oxygen partial pressure ratio

or

- ΔP_{O_2}, mm Hg, and
- ΔP_{N_2}, mm Hg.

7. Interpretation of Results

The biological effects of dissolved gas supersaturation depend on the species, age, depth in water column, length of exposure, temperature, and nitrogen:oxygen partial pressure ratio.[12] Safe limits generally are segregated into wild/natural circumstances, where behavior and hydrostatic pressure can modify the exposure by horizontal and vertical movements away from dangers, and captive environments such as aquaria, hatcheries, or laboratories, where conditions not only preclude escape but also include other significant stresses. Of these two realms, captive circumstances are more likely to cause illness or mortality from gas bubble disease and will do so sooner and at the lower ΔP levels.

In wild/natural circumstances, the limit of safe levels of gas supersaturation depends on the depth available to the species and/or species behavior, but this limit usually occurs at a ΔP between 50 and 150 mm Hg. Under captive conditions, the ΔP should be as close to zero as possible. For sensitive species and life stages, sublethal and lethal effects have been observed at ΔP of 10 to 50 mm Hg.[13]

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PART 3000

METALS

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3020 Quality Assurance/Quality Control .....Cindy A. Bamfield
3125 Metals by Inductively Coupled Plasma/Mass Spectrometry .....Robert Henry
3500-Cr Chromium .....Rock J. Vitale

Summary of Major Changes since 2005

Quality Assurance/Quality Control (3020) was significantly revised to stay abreast of regulatory requirements and clarify the essential quality control practices for test methods in Part 3000.

Metals by Inductively Coupled Plasma/Mass Spectrometry (3125) contains updated QC requirements, including frequency and default limits, to make it consistent with current regulatory requirements. It also incorporates collision cell technology.

Chromium (3500-Cr) contains updated preservation requirements and extends the holding time for hexavalent chromium.

The brief metal sections that do not contain methods in themselves (they merely refer users to appropriate detection methods) have been combined into a new section: Other Metals (3500).

Also, many methods now refer users to the quality control practices that are considered to be an integral part of the method.

3010 INTRODUCTION*

3010 A. General Discussion

1. Significance

The effects of metals in water and wastewater range from beneficial through troublesome to dangerously toxic. Some metals are essential to plant and animal growth while others may adversely affect water consumers, wastewater treatment systems, and receiving waters. The benefits versus toxicity of some metals depend on their concentrations in waters.

2. Types of Methods

Preliminary treatment is often required to present the metals to the analytical methodology in an appropriate form. Alternative methods for pretreatment of samples are presented in Section 3030.

Metals may be determined satisfactorily by a variety of methods, with the choice often depending on the precision and sensitivity required. Part 3000 describes colorimetric methods as well as instrumental methods [i.e., atomic absorption spectrometry, including flame, electrothermal (furnace), hydride, and cold vapor techniques; flame photometry; inductively coupled plasma emission spectrometry; inductively coupled plasma mass spectrometry, and anodic stripping voltammetry]. Flame atomic absorption methods generally are applicable at moderate (0.1- to 10-mg/L) concentrations in clean and complex-matrix samples. Electrothermal methods generally can increase sensitivity if matrix problems do not interfere. Inductively coupled plasma emission techniques are applicable over a broad linear range and are especially sensitive for refractory elements. Inductively coupled plasma mass spectrometry offers significantly increased sensitivity for some elements (as low as 0.01 μg/L) in a variety of environmental matrices. Flame photometry gives good results at higher concentrations for several Group I and II elements. Anodic stripping offers high sensitivity for several elements in relatively clean matrices. Colorimetric methods are applicable to specific metal determinations where interferences are known not to compromise method accuracy; these methods may provide speciation information for some metals. Table 3010:I lists the methods available in Part 3000 for each metal.

3. Terminology

Acid-extractable metals—The concentration of metals in solution after treatment of an unfiltered sample with hot dilute mineral acid. To determine either dissolved or suspended metals, filter sample immediately after collection. Do not preserve with acid until after filtration.

Dissolved metals—Those metals in an unacidified sample that pass through a 0.45-μm membrane filter.

Suspended metals—Those metals in an unacidified sample that are retained by a 0.45-μm membrane filter.

Total metals—The concentration of metals determined in an unfiltered sample after vigorous digestion, or the sum of the concentrations of metals in the dissolved and suspended fractions. Note that total metals are defined operationally by the digestion procedure.

* Joint Task Group: 20th Edition—Brian J. Condike.

3010 B. Sampling and Sample Preservation

Before collecting a sample, decide what fraction is to be analyzed (dissolved, suspended, total, or acid-extractable). This decision will determine in part whether the sample is acidified with or without filtration and the type of digestion required.

Serious errors may be introduced during sampling and storage because of contamination from sampling device, failure to remove residues of previous samples from sample container, and loss of metals by adsorption on and/or precipitation in sample container caused by failure to acidify the sample properly.

1. Sample Containers

The best sample containers are made of quartz or TFE. Because these containers are expensive, the preferred sample container is made of polypropylene or linear polyethylene with a polyethylene cap. Borosilicate glass containers also may be used, but avoid soft glass containers for samples containing metals in the microgram-per-liter range. Store samples for determination of silver in light-absorbing containers. Use only containers and filters that have been acid rinsed.

2. Preservation

Preserve samples immediately after sampling by acidifying with concentrated nitric acid (HNO_3) to pH <2. Filter samples for dissolved metals before preserving (see Section 3030). Usually 1.5 mL conc HNO_3/L sample (or 3 mL 1 + 1 HNO_3/L sample) is sufficient for short-term preservation. For samples with high buffer capacity, increase amount of acid (5 mL may be required for some alkaline or highly buffered samples). Use commercially available high-purity acid* or prepare high-purity acid by sub-boiling distillation of acid.

* Ultrex, J.T. Baker, or equivalent.

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Table 3010:I. Applicable Methods for Elemental Analysis

Element	Flame Atomic Absorption (Direct)	Flame Atomic Absorption (Extracted)	Flame Photometry	Electrothermal Atomic Absorption	Hydride/Cold Vapor Atomic Absorption	Inductively Coupled Plasma (ICP)	ICP/Mass Spectrometry (ICP/MS)	Anodic Stripping Voltammetry	Alternative Methods*
Aluminum	3111D	3111E		3113B		3120B	3125		3500-Al.B
Antimony	3111B			3113B		3120B	3125		
Arsenic				3113B	3114B	3120B	3125		3500-As.B
Barium	3111D	3111E		3113B		3120B	3125		
Beryllium	3111D	3111E		3113B		3120B	3125		
Bismuth	3111B			3113B			3125†		
Boron						3120B	3125†		4500-B.B,C
Cadmium	3111B	3111C		3113B		3120B	3125	3130B	
Calcium	3111B,D	3111E				3120B	3125†		3500-Ca.B
Cesium	3111B						3125†		
Chromium	3111B	3111C		3113B		3120B	3125		3500-Cr.B,C
Cobalt	3111B	3111C		3113B		3120B	3125		
Copper	3111B	3111C		3113B		3120B	3125		3500-Cu.B,C
Gallium				3113B			3125†		
Germanium				3113B			3125†		
Gold	3111B			3113B			3125†		
Indium				3113B			3125†		
Iridium	3111B						3125†		
Iron	3111B	3111C		3113B		3120B	3125†		3500-Fe.B
Lead	3111B	3111C		3113B		3120B	3125	3130B	3500-Pb.B
Lithium	3111B		3500-Li.B			3120B	3125†		
Magnesium	3111B					3120B	3125†		3500-Mg.B,C
Manganese	3111B	3111C		3113B		3120B	3125†		3500-Mn.B
Mercury					3112B		3125†		
Molybdenum	3111D	3111E		3113B		3120B	3125		
Nickel	3111B	3111C		3113B		3120B	3125		
Osmium	3111D	3111E					3125†		
Palladium	3111B						3125†		
Platinum	3111B						3125†		
Potassium	3111B		3500-K.B			3120B	3125†		3500-K.C
Rhenium	3111D	3111E					3125†		
Rhodium	3111B						3125†		
Ruthenium	3111B						3125†		
Selenium				3113B	3114B,C	3120B	3125		3500-Se.C,D,E
Silicon	3111D	3111E				3120B	3125†		
Silver	3111B	3111C		3113B		3120B	3125		
Sodium	3111B		3500-Na.B			3120B	3125†		
Strontium	3111B		3500-Sr.B			3120B	3125		
Tellurium				3113B			3125†		
Thallium	3111B			3113B		3120B	3125		
Thorium	3111D	3111E					3125†		
Tin	3111B			3113B			3125†		
Titanium	3111D	3111E					3125†		
Uranium							3125		
Vanadium	3111D	3111E		3113B		3120B	3125		3500-V.B
Zinc	3111B	3111C		3113B		3120B	3125	3130B	3500-Zn.B

* Additional alternative methods for aluminum, beryllium, cadmium, mercury, selenium, silver, and zinc may be found in the 19th Edition of *Standard Methods*.
† Metal is not specifically mentioned in the method, but 3125 may be used successfully in most cases.

After acidifying sample, preferably store it in a refrigerator at approximately 4°C to prevent change in volume due to evaporation. Under these conditions, samples with metal concentrations of several milligrams per liter are stable for up to 6 months (except mercury, for which the limit is 5 weeks). For microgram-per-liter metal levels, analyze samples as soon as possible after sample collection.

Alternatively, preserve samples for mercury analysis by adding 2 mL/L 20% (w/v) $K_2Cr_2O_7$ solution (prepared in 1 + 1 HNO_3). Store in a refrigerator not contaminated with mercury. (**Caution: Mercury concentrations may increase in samples stored in plastic bottles in mercury-contaminated laboratories.**)

3. Bibliography

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3010 C. General Precautions

1. Sources of Contamination

Avoid introducing contaminating metals from containers, distilled water, or membrane filters. Some plastic caps or cap liners may introduce metal contamination; for example, zinc has been found in black bakelite-type screw caps as well as in many rubber and plastic products, and cadmium has been found in plastic pipet tips. Lead is a ubiquitous contaminant in urban air and dust.

2. Contaminant Removal

Thoroughly clean sample containers with a metal-free nonionic detergent solution, rinse with tap water, soak in acid, and then rinse with metal-free water. For quartz, TFE, or glass materials, use 1 + 1 HNO_3, 1 + 1 HCl, or aqua regia (3 parts conc HCl + 1 part conc HNO_3) for soaking. For plastic material, use 1 + 1 HNO_3 or 1 + 1 HCl. Reliable soaking conditions are 24 h at 70°C. Chromic acid or chromium-free substitutes* may be used to remove organic deposits from containers, but rinse containers thoroughly with water to remove traces of chromium. Do not use chromic acid for plastic containers or if chromium is to be determined. Always use metal-free water in analysis and reagent preparation (see Section 3111B.3*c*). In these methods, the word "water" means metal-free water.

* Nochromix, Godax Laboratories, or equivalent.

3. Airborne Contaminants

For analysis of microgram-per-liter concentrations of metals, airborne contaminants in the form of volatile compounds, dust, soot, and aerosols present in laboratory air may become significant. To avoid contamination use "clean laboratory" facilities such as commercially available laminar-flow clean-air benches or custom-designed work stations and analyze blanks that reflect the complete procedure.

4. Bibliography

MITCHELL, J.W. 1973. Ultrapurity in trace analysis. *Anal. Chem.* 45:492A.

GARDNER, M., D. HUNT & G. TOPPING. 1986. Analytical quality control (AQC) for monitoring trace metals in the coastal and marine environment. *Water Sci. Technol.* 18:35.

3020 QUALITY ASSURANCE/QUALITY CONTROL*

3020 A. Introduction

Quality assurance (QA) is a laboratory operations program that specifies the measures required to produce defensible data with known precision and accuracy. This program is defined in a QA manual, written procedures, work instructions, and records. The manual should include a policy that defines the statistical level of confidence used to express data precision and bias, as well as method detection levels (MDLs) and reporting limits. The overall system includes all QA policies and quality control (QC) processes needed to demonstrate the laboratory's competence and to ensure and document the quality of its analytical data. Quality systems are essential for laboratories seeking accreditation under state or federal laboratory certification programs. Refer to Section 1020 for details on establishing a Quality Assurance Plan.

As described in Part 1000, essential QC measures may include method calibration, reagent preparation and/or standardization, assessment of each analyst's capabilities, analysis of blind check samples, determination of the method's sensitivity [method detection level (MDL, limit of detection (LOD), level of quantification level (LOQ), or minimum reporting level (MRL)], and daily evaluation of bias, precision, and laboratory contamination or other analytical interference.

Some methods in Part 3000 include specific QC procedures, frequencies, and acceptance criteria. These are considered the minimum QCs needed to perform the method successfully. When the words *should* or *preferably* are used, the QC is recommended; when *must* is used, the QC is mandatory. Additional QC procedures should be used when necessary to ensure that results are valid. Some regulatory programs may require additional QC or have alternative acceptance limits. In those cases, the laboratory should follow the more stringent requirements.

The QC program consists of at least the following elements, as applicable to specific methods:

- calibration,
- continuing calibration verification (CCV),
- operational range and MDL determination,
- initial demonstration of capability (IDC),
- ongoing demonstration of capability,
- method blank/reagent blank,
- laboratory-fortified blank (LFB),
- laboratory-fortified matrix (LFM),
- duplicate sample/laboratory-fortified matrix duplicate (LFMD),
- verification of MDL and MRL,
- QC calculations,
- control charts,
- corrective action,
- frequency of QC,
- QC acceptance criteria, and
- definitions of prep and analytical batches.

Sections 1010 and 1030 describe calculations for evaluating data quality.

* Reviewed by Standard Methods Committee, 2005. Additional revisions, 2017. Randy A. Gottler (chair), Rodger B. Baird, Andrew D, Eaton, William C. Lipps.

3020 B. Quality Control Practices

At a minimum, analysts must use the QC practices specified here unless a method specifies alternative practices. Laboratories may save time and money by purchasing premade standards, titrants, and reagents, but they still must perform the QC checks on these materials required by the analytical methods.

1. Calibration

a. Instrument calibration (not applicable to non-instrumental methods): Perform both instrument calibration and maintenance according to the manufacturer's instructions and recommendations. Conduct instrument-performance checks according to method or standard operating procedure (SOP) instructions.

b. Initial calibration: Perform initial calibration using

- at least three concentrations of standards and one blank (for linear calibrations),
- at least five concentrations of standards and one blank (for nonlinear calibrations), or
- as many concentrations as the method specifies.

The lowest concentration must be at or below the MRL, and the highest concentration should be at the upper end of the calibration range. Make sure the calibration range encompasses the concentrations expected in method samples or required dilutions. For the most accurate results, choose calibration standard concentrations no more than one order of magnitude apart [unless calibrating for ion-selective electrode (ISE) methods].

Apply response-factor or linear curve-fitting statistics (depending on what the method allows) to analyze the concentration–instrument response relationship. If the relative standard deviation (%RSD) of the response factors is $\leq$15%, then the average response factor may be used. Otherwise, use a regression equation. The appropriate linear or nonlinear correlation coefficient for standard concentration-to-instrument response should be $\geq$0.995 for linear calibrations. Weighting factors (e.g., $1/x$ or $1/x^2$) may be used to give more weight to the lower

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concentration points of the calibration. Depending on the method, calibration curves may be

- linear through the origin,
- linear not through the origin,
- nonlinear through the origin, or
- nonlinear not through the origin.

Some nonlinear functions can be linearized via mathematical transformations (e.g., log). The following acceptance criteria are recommended for various calibration functions (if the method does not specify acceptance criteria).

Compare each calibration point to the curve and recalculate its concentration. If any recalculated values are not within the method's acceptance criteria—up to twice the MRL ±50%; between 3 and 5 times the MRL ±20%; or greater than 5 times the MRL ±10%—unless otherwise specified in individual methods, identify the source of any outlier(s) and correct before sample quantitation.

NOTE: Do not use the correlation coefficient to verify a calibration's accuracy. That said, many methods still require calculation of the correlation coefficient and comparison to a specific limit.

Verify the initial calibration by analyzing a standard prepared from a different stock standard than that used to create the calibration curve; its concentration should be near the midpoint of the calibration range. The analytical results for this second-source mid-range standard must be within 10% of its true value, except for ICP-AES, which must be within 5% of its true value. If not, determine the cause of the error, take corrective action, and re-verify the calibration. If the re-verification passes, continue the analyses; otherwise, repeat the initial calibration.

See the individual method or manufacturer's instructions for ISE methods.

Use the initial calibration to quantitate analytes of interest in samples. Use CCV (¶ *c* below) only for calibration checks, not for sample quantitation. Perform initial calibration when the instrument is set up and whenever CCV criteria are not met.

c. Continuing calibration verification: In CCV, analysts periodically use a calibration standard to confirm that instrument performance has not changed significantly since initial calibration. Base the CCV interval on the number of samples analyzed (e.g., after every 10 samples and at least once per batch). Verify calibration by analyzing one standard whose concentration is near the midpoint of the calibration range. The results must be within allowable deviations (within 10% of its true value) from either initial-calibration values or specific points on the calibration curve. If the CCV is out of control, then take corrective action—including re-analysis of any samples analyzed since the last acceptable CCV.

Refer to the method for CCV frequency and acceptance criteria; if not specified, use the criteria given here. Other concentrations (e.g., one near the MRL) may be used, but be aware that the acceptance criteria may vary depending on the standard's concentration.

2. Operational Range and MDL Determination

Before using a new method or instrument, you should determine its operational (calibration) range (upper and lower limits). Calibrate according to 3020B.1, or verify the calibration by analyzing prepared standard solutions ranging from low to high concentrations. Determine the maximum concentration that can be measured within 10% of its true value based on the calibration curve: this is the *limit of linearity*. All samples whose concentrations are above the limit of linearity must be diluted.

If reporting results <MRL, initially estimate the MDL as a concentration about 3 to 5 times lower than the minimum calibration standard. This method for determining the MDL is based on the procedure outlined by the U.S. Environmental Protection Agency (EPA).[1]

To determine an MDL, prepare and analyze at least seven portions of a solution spiked at or near the minimum calibration concentration and an equal number of blanks. Analysts should prepare and analyze the spikes and blanks over 3 d rather than doing them all in one batch. If one MDL will be used for multiple instruments, then the MDL analysis must be performed across all of them (however, it is unnecessary to analyze all samples on all instruments). Analysts must prepare and analyze at least two spikes and two blanks on different calendar dates for each instrument. If evaluating more than three instruments, then one set of spikes and blanks can be analyzed on multiple instruments, so long as at least seven sets of spikes and blanks total are used. Alternatively, determine instrument-specific MDLs.

Calculate the estimated sample standard deviation, s_s, of the 7 replicates, and multiply by 3.14 to compute the MDL_s. Calculate MDL_b (MDL based on method blanks) using the following procedure.

If none of the method blanks give a numerical result (positive or negative), then $MDL_b$is not applicable, and MDL = MDL_s. If some give numerical results, then MDL_b equals the highest method blank result. If all of the method blanks give numerical results, calculate MDL_b as

$$MDL_b = X + 3.14S_b$$

where:

X = mean of blank results (set negative values to 0), and
S_b = standard deviation of blank results.

The MDL then equals whichever is greater: MDL_s or MDL_b.

If using more than 7 replicates, adjust the t value from 3.14 using student t tables with n-1 degrees of freedom.

3. Initial Demonstration of Capability

Each analyst in the laboratory should conduct an IDC at least once before analyzing any sample to demonstrate proficiency in performing the method and obtaining acceptable results for each analyte. The IDC also is used to demonstrate that a laboratory's modifications to a method will produce results as precise and accurate as those produced by the reference method. As a minimum, include a reagent blank and at least four LFBs at a concentration between one and four times the MRL (or other level specified in the method). Ensure that the reagent blank does not contain any analyte of interest at a concentration greater than half the lowest calibration point (or other level specified in the method). Ensure that precision and accuracy (percent recovery) calculated for LFBs are within the acceptance criteria listed in the method of choice or generated by the laboratory (if there are no established mandatory criteria).

To establish laboratory-generated accuracy and precision limits, calculate the upper and lower control limits from the mean and standard deviation of percent recovery for ≥20 data points:

$$\text{Upper control limit} = \text{Mean} + 3(\text{Standard deviation})$$

$$\text{Lower control limit} = \text{Mean} - 3(\text{Standard deviation})$$

In the absence of established mandatory criteria, use laboratory-generated acceptance criteria for the IDC or else obtain acceptance criteria from a proficiency testing (PT) provider on PT studies and translate the data to percent recovery limits per analyte and method of choice. Ensure that lab-generated criteria are at least as tight as PT-study criteria, which are typically based on either multiple lab results or PT-provider-fixed limits.

4. Ongoing Demonstration of Capability (Laboratory Control Sample)

The ongoing demonstration of capability, sometimes called a *laboratory control sample* (LCS), *laboratory control standard, QC check sample,* or *laboratory-fortified blank,* is used to ensure that the laboratory analysis remains in control while samples are analyzed and separates laboratory performance from method performance on the sample matrix. This standard should be preserved in accordance with method requirements and carried through the entire procedure, including any digestions, extraction, or filtration. Purchase an external QC standard (if available) from a reputable supplier and use the certified acceptance limits as the laboratory acceptance criteria.

Acceptance criteria will vary depending on the method, matrix, and concentration. The concentration range should be either near the middle of the calibration range or near the maximum contaminant level (MCL), whichever is lower. Alternatively, prepare your own QC standard and calculate acceptance limits as ± 2 standard deviations based on analysis of ≥ 20 replicates, unless the method specifies acceptance limits.

The ongoing demonstration of capability may be one of the following:

- acceptable performance of a blind sample analysis (single blind to the analyst);
- another IDC;
- at least four consecutive LCSs with acceptable levels of precision and accuracy (the laboratory shall determine acceptable precision and accuracy limits before analysis); or
- a documented analyst-review process using QC samples (QC samples can be reviewed to identify individual or group patterns and determine whether corrective action or retraining is necessary).

5. Reagent Blank

A *reagent blank* (method blank) consists of reagent water (see Section 1080) and all reagents (including preservatives) that normally are in contact with a sample during the entire analytical procedure. The reagent blank is used to determine whether and how much reagents and the preparative analytical steps contribute to measurement uncertainty. As a minimum, include one reagent blank with each sample set (batch) or on a 5% basis, whichever is more frequent. Analyze a blank after the CCV standard and before analyzing samples. Evaluate reagent-blank results for contamination; if contamination levels are unacceptable, identify and eliminate the source.

Positive sample results are suspect if analyte(s) in the reagent blank are >1/2MRL, unless the method specifies otherwise. Samples analyzed with a contaminated blank must be re-prepared and re-analyzed unless concentrations are ≥ 10 times those of the blank, concentrations are nondetect, or data user will accept qualified data. See method for specific reagent-blank acceptance criteria. General guidelines for qualifying sample results with regard to reagent-blank quality are as follows:

- If reagent blank is <MDL, then no qualification is required.
- If reagent blank is >1/2MRL but <MRL and sample results are >MRL, then qualify results to indicate that analyte was detected in the reagent blank.
- If reagent blank is >MRL, then further corrective action and qualification is required.

6. Laboratory-Fortified Blank

A *laboratory-fortified blank* (LFB) is a reagent-water sample (with associated preservatives) to which a known concentration of the analyte(s) of interest has been added. The LFB may be used as the LCS (3020B.4) if the method requires a preliminary sample extraction or digestion.

An LFB is used to evaluate laboratory performance and analyte recovery in a blank matrix. Its concentration should be high enough to be measured precisely, but not high enough to be irrelevant to measured environmental concentrations. The analyst should rotate LFB concentrations to cover different parts of the calibration range. As a minimum, include one LFB with each sample set (batch) or on a 5% basis, whichever is more frequent. (The definition of a batch is typically project-specific.)

Process the LFB through all sample-preparation and -analysis steps. Use an added concentration of at least $10 \times$ MDL, or a level specified in a project plan's data quality objectives. Ideally, the LFB concentration should be less than the MCL (if the contaminant has one). Depending on method requirements, prepare the addition solution from either the same reference source used for calibration or an independent source. Evaluate the LFB for percent recovery of the added analytes by comparing results to method-specified limits, control charts, or other approved criteria. If LFB results are out of control, take corrective action, including re-preparation and re-analysis of associated samples if required. Use LFB results to evaluate batch performance, calculate recovery limits, and plot control charts.

7. Laboratory-Fortified Matrix

A *laboratory-fortified matrix* (LFM) is an additional portion of a sample to which a known amount of the analyte(s) of interest is added before sample preparation. Some analytes (e.g., speciation methods) are not appropriate for LFM analysis.

The LFM is used to evaluate analyte recovery in a sample matrix. If an LFM is feasible and the method does not specify LFM frequency requirements, then include at least one LFM with each sample set (batch) or on a 5% basis, whichever is more frequent. Add a concentration that is at least $10 \times$ MRL, less than or equal to the midpoint of the calibration curve, or method-specified level to the selected sample(s). The analyst should use the same concentration as for LFB (3020B.6) to allow analysts to separate the matrix's effect from laboratory performance. Prepare LFM from the same reference source used for LFB. Make

the addition such that sample background levels do not adversely affect recovery (preferably adjust LFM concentrations if the known sample is more than five times the background level). For example, if the sample contains the analyte of interest, then add approximately as much analyte to the LFM sample as the concentration found in the known sample.

Evaluate LFM results for percent recovery; if they are not within control limits, then take corrective action to rectify the matrix effect, use another method, use the method of standard addition, or flag the data if reported. See method for specific LFM-acceptance criteria until the laboratory develops statistically valid, laboratory-specific performance criteria. If the method does not provide limits, use the calculated preliminary limits from the IDC (3020B.3). LFM control limits may be wider than for LFB or LCS, and batch acceptance generally is not contingent upon LFM results.

8. Duplicate Sample/Laboratory-Fortified Matrix Duplicate

Duplicate samples are analyzed to estimate precision. If an analyte is rarely detected in a matrix type, use an LFM duplicate. An *LFM duplicate* is a second portion of the sample described in 3020B.7 to which a known amount of the analyte(s) of interest is added before sample preparation. If sufficient sample volume is collected, this second portion of sample is added and processed in the same way as the LFM. As a minimum, include one duplicate sample or one LFM duplicate with each sample set (batch) or on a 5% basis, whichever is more frequent, and process it independently through the entire sample preparation and analysis.

Evaluate LFM duplicate results for precision and accuracy (precision alone for duplicate samples). If LFM duplicate results are out of control, then take corrective action to rectify the matrix effect, use another method, use the method of standard addition, or flag the data if reported. If duplicate results are out of control, then re-prepare and re-analyze the sample and take additional corrective action, as needed. When the value of one or both duplicate samples is $\leq 5 \times$ MRL, the laboratory may use the MRL as the control limit for percent recovery, and the duplicate results are not used. See method for specific acceptance criteria for LFM duplicates or duplicate samples until the laboratory develops statistically valid, laboratory-specific performance criteria. If the method does not provide limits, use the calculated preliminary limits from the IDC. In general, batch acceptance is not contingent upon LFM duplicate results.

9. Verification of MDL and MRL

With each analytical batch, analyze a reagent-water sample spiked at MRL and ensure that it meets MRL acceptance criteria (generally $\pm 50\%$). If not, re-analyze the entire batch or flag results for all samples in the batch. If the MRL is biased high, nondetect (ND) samples can be reported with flags if the method or regulation allows.

If reporting to the MDL, then verify the MDL at least quarterly by analyzing a sample spiked at the same level used to determine the MDL and ensure that the result is positive. If two consecutive MDL-verification samples do not produce positive results, then recalculate the MDL using the most recent set of at least 7 blanks and MRL level spikes, following the protocols outlined in 3020B.2.

10. QC Calculations

The following is a compilation of equations frequently used in QC calculations.

a. Laboratory-fortified matrix (LFM) sample (matrix spike sample):

$$\text{LFM \% Recovery} = \left[\frac{LFM\ conc \times (spike\ vol + sample\ vol) - (sample\ conc \times sample\ vol)}{spike\ solution\ conc \times spike\ vol}\right] \times 100$$

b. Relative percent difference (RPD):

$$\left[\frac{|LFM - LFMD|}{\left(\frac{LFM + LFMD}{2}\right)}\right] \times 100 = \%RPD$$

or

$$\left[\frac{|D_1 - D_2|}{\left(\frac{D_1 + D_2}{2}\right)}\right] \times 100 = \%RPD$$

where:

LFM = concentration determined for LFM,
$LFMD$ = concentration determined for LFMD,
D_1 = concentration determined for first duplicate, and
D_2 = concentration determined for second duplicate.

c. Initial calibration: See Section 1020B.12*a*.
d. Calibration verification: See Section 1020B.12*b*.
e. Laboratory-fortified blank recovery: See Section 1020B.12*c*.
f. Laboratory-fortified matrix: See Section 1020B.12*e*.
g. Standard additions: See Section 1020B.12*g*.

11. Control Charts

See Section 1020B.13.

12. Reference

1. U.S. Environmental Protection Agency. 2016. III.H. Changes to method detection limit (MDL) procedure. *In* Clean Water Act Methods Update Rule for the Analysis of Effluent. 40 CFR 136.

3030 PRELIMINARY TREATMENT OF SAMPLES*

3030 A. Introduction

Samples containing particulates or organic material generally require pretreatment before spectroscopic analysis. "Total metals" includes all metals, inorganically and organically bound, both dissolved and particulate. Colorless, transparent samples (primarily drinking water) having a turbidity of <1 NTU, no odor, and single phase may be analyzed directly by atomic absorption spectroscopy (flame or electrothermal vaporization) or inductively coupled plasma spectroscopy (atomic emission or mass spectrometry) for total metals without digestion. For further verification or if changes in existing matrices are encountered, compare digested and undigested samples to ensure comparable results. On collection, acidify such samples to pH <2 with conc nitric acid (1.5 mL HNO_3/L is usually adequate for drinking water) and analyze directly. Digest all other samples before determining total metals. Conc HNO_3 preservative must be present in sample bottle for at least 16 h before removal of portion for digestion. To analyze for dissolved metals, filter sample, acidify filtrate, and store until analyses can be performed. To determine suspended metals, filter sample, digest filter and the material on it, and analyze. To determine acid-extractable metals, extract metals as indicated in 3030E–K and analyze extract.

This section describes general pretreatment for samples in which metals are to be determined according to Sections 3110–3500-Zn with several exceptions. The special digestion techniques for mercury are given in Sections 3112B.4*b* and *c*, and those for arsenic and selenium in Sections 3114B.4*c* and *d* and 3500-Se.B.2–5.

Take care not to introduce metals into samples during preliminary treatment. During pretreatment avoid contact with rubber, metal-based paints, cigarette smoke, paper tissues, and all metal products including those made of stainless steel, galvanized metal, and brass. Conventional fume hoods can contribute significantly to sample contamination, particularly during acid digestion in open containers. Keep vessels covered with watch glasses and turn spouts away from incoming air to reduce airborne contamination. Plastic pipet tips often are contaminated with copper, iron, zinc, and cadmium; before use soak in 2*N* HCl or HNO_3 for several days and rinse with deionized water. Avoid using colored plastics, which can contain metals. Use certified metal-free plastic containers and pipet tips when possible. Avoid using glass if analyzing for aluminum or silica.

Use metal-free water (see Section 3111B.3*c*) for all operations. Check reagent-grade acids used for preservation, extraction, and digestion for purity. If excessive metal concentrations are found, purify the acids by distillation or use ultra-pure acids. Inductively coupled plasma mass spectrometry (ICP-MS) may require use of ultra-pure acids and reagents to avoid measurable contamination. Process blanks through all digestion and filtration steps and evaluate blank results relative to corresponding sample results. Either apply corrections to sample results or take other corrective actions as necessary or appropriate.

* Approved by Standard Methods Committee, 2004.
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3030 B. Filtration for Dissolved and Suspended Metals

1. Filtration Procedures

If dissolved or suspended metals (see Section 3010A.3) are to be determined, filter sample at time of collection using a preconditioned plastic filtering device with either vacuum or pressure, containing a filter support of plastic or fluorocarbon, through a prewashed ungridded 0.4- to 0.45-μm-pore-diam membrane filter (polycarbonate or cellulose esters). Before use filter a blank consisting of metal-free (deionized) water to ensure freedom from contamination. Precondition filter and filter device by rinsing with 50 mL deionized water. If the filter blank contains significant metals concentrations, soak membrane filters in approximately 0.5*N* HCl or 1*N* HNO_3 (recommended for electrothermal atomic absorption spectrometry and ICP–MS analyses) and rinse with deionized water before use. NOTE: Take care to avoid potential contamination during filtration of samples.

Before filtering, centrifuge highly turbid samples in acid-washed fluorocarbon or high-density plastic tubes to reduce loading on filters. Stirred, pressure filter units foul less readily than vacuum filters; filter at a pressure of 70 to 130 kPa. After filtration acidify filtrate to pH 2 with conc HNO_3 and store until analyses can be performed. If a precipitate forms on acidification, digest acidified filtrate before analysis as directed (see 3030E). Retain filter and digest it for direct determination of suspended metals.

If it is not possible to field-filter the sample without contaminating it, obtain sample in an "unpreserved" bottle as above and promptly cool to 4°C. Do not acid-preserve the sample. Then, without delay, filter sample under cleaner conditions in the laboratory.

Test pH of a portion of aqueous sample upon receipt in the laboratory to ensure that the sample has been properly filtered and acid-preserved.[1]

NOTE: Different filters display different sorption and filtration characteristics[2]; for trace analysis, test filter and filtration sytem to verify complete recovery of metals.

If suspended metals (see Section 3010A.3) are to be determined, filter sample as above for dissolved metals, but do not centrifuge before filtration. Retain filter and digest it for direct determination of suspended metals. Record sample volume filtered and include a filter in determination of the blank.

CAUTION: Do not use perchloric acid to digest membrane filters. (See 3030H for more information on handling $HClO_4$).

2. References

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1994. Sample Preparation Procedure for Spectrochemical Determination of Total Recoverable Elements; Method 200.2. Environmental Monitoring Systems Lab., Cincinnati, Ohio.
2. HOROWITZ, A.J., K.R. LUM, J.R. GARBARINO, G.E.M. HALL, C. LEMIEUX & C.R. DEMAS. 1996. Problems with using filtration to define dissolved trace element concentrations in natural water samples. *Environ. Sci. Technol.* 30:954.

3030 C. Treatment for Acid-Extractable Metals

Extractable metals (see Section 3010A.3) are lightly adsorbed on particulate material. Because some sample digestion may be unavoidable use rigidly controlled conditions to obtain meaningful and reproducible results. Maintain constant sample volume, acid volume, and contact time. Express results as extractable metals and specify extraction conditions.

At collection, acidify entire sample to pH <2 using 5 mL conc HNO_3/L sample. To prepare sample, mix well, transfer 100 mL to a beaker or flask, and add 5 mL 1 + 1 high-purity HCl. Heat 15 min on a steam bath. Filter through a membrane filter (preconditioned as in 3030B) and carefully transfer filtrate to a tared volumetric flask. Adjust volume to 100 mL with metal-free water, mix, and analyze. If volume is greater than 100 mL, determine volume to nearest 0.1 mL by weight, analyze, and correct final concentration measurement by multiplying by the dilution factor (final volume ÷ 100).

3030 D. Digestion for Metals

To reduce interference by organic matter and to convert metals associated with particulates to a form (usually the free metal ion) that can be determined by electrothermal atomic absorption spectrometry or inductively-coupled plasma spectroscopy, use one of the digestion techniques presented below. Use the least rigorous digestion method required to provide acceptable and consistent recovery compatible with the analytical method and the metal being analyzed.[1–3]

1. Selection of Acid

Nitric acid will digest most samples adequately (3030E). Nitrate is an acceptable matrix for both flame and electrothermal atomic absorption and the preferred matrix for ICP-MS.[4] Some samples may require addition of perchloric, hydrochloric, hydrofluoric, or sulfuric acid for complete digestion. These acids may interfere in the analysis of some metals and all provide a poorer matrix for both electrothermal and ICP-MS analysis. Confirm metal recovery for each digestion and analytical procedure used. Use Table 3030:I as a guide in determining which acids (in addition to HNO_3) to use for complete digestion. As a general rule, HNO_3 alone is adequate for clean samples or easily oxidized materials; HNO_3-H_2SO_4 or HNO_3-HCl digestion is adequate for readily oxidizable organic matter; HNO_3-$HClO_4$ or HNO_3-$HClO_4$-HF digestion is necessary for difficult-to-oxidize organic matter or minerals containing silicates. Although dry ashing is not generally recommended because of the loss of many volatile elements, it may be helpful if large amounts of organic matter are present.

TABLE 3030:I. ACIDS USED WITH HNO_3 FOR SAMPLE PREPARATION

Acid	Recommended for	May Be Helpful for	Not Recommended for
HCl	Ag	Sb, Ru, Sn	Th, Pb
H_2SO_4	Ti	—	Ag, Pb, Ba
$HClO_4$	—	Organic materials	—
HF	—	Siliceous materials	—

2. Digestion Procedures

Dilute samples with Ag concentrations greater than 1 mg/L to contain less than 1 mg Ag/L for flame atomic absorption methods and 25 μg/L or less for electrothermal analysis.[2,5,6] To address problems with silver halide solubility in HNO_3, digest using method 3030F.3*b*.

Report digestion technique used.

Acid digestion techniques (3030E–I) generally yield comparable precision and bias for most sample types that are totally digested by the technique. Because acids used in digestion will add metals to the samples and blanks, minimize the volume of acids used.

Because the acid digestion techniques (3030E and F) normally are not total digestions, the microwave digestion procedure

(3030K) may be used as an alternative. The microwave method is a closed-vessel procedure and thus is expected to provide improved precision when compared with hot-plate techniques. Microwave digestion is recommended for samples being analyzed by ICP-MS. The microwave digestion method is recommended for the analysis of Ag, Al, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Sb, Se, Tl, V, and Zn. Microwave digestion may be acceptable for additional analytes provided its performance for those elements is validated.

Suggested sample volumes are indicated below for flame atomic absorption spectrometry. Lesser volumes, to a minimum of 5 mL, are appropriate for graphite furnace, ICP, and ICP-MS. Do not subsample volumes less than 5 mL, especially when particulates are present. Instead dilute samples with elevated analyte concentrations after digestion. If the recommended volume exceeds digestion vessel capacity, add sample as evaporation proceeds. For samples containing particulates, wide-bore pipets may be useful for volume measurement and transfer.

When samples are concentrated during digestion (e.g., >100 mL sample used) determine metal recovery for each matrix digested, to verify method validity. Using larger samples will require additional acid, which also would increase the concentration of impurities.

Estimated Metal Concentration *mg/L*	Sample Volume* *mL*
<0.1	1000
0.1–10	100
10–100+	10

* For flame atomic absorption spectrometry.

Report results as follows:

$$\text{Metal concentration, mg/L} = A \times \frac{B}{C}$$

where:

A = concentration of metal in digested solution, mg/L,
B = final volume of digested solution, mL, and
C = sample size, mL.

Prepare solid samples or liquid sludges with high solids contents on a weight basis. Mix sample and transfer a suitable amount (typically 1 g of a sludge with 15% total solids) directly into a preweighed digestion vessel. Reweigh and calculate weight of sample. Proceed with one of the digestion techniques presented below. However, as these digestion methods are predominantly for dissolved and extractable metals in aqueous samples, other approaches may be more appropriate for solid samples. For complete mineralization of solid samples, consult methods available elsewhere.[1,4,6,7] Report results on wet- or dry-weight basis as follows:

$$\text{Metal concentrations, mg/kg (wet-weight basis)} = \frac{A \times B}{\text{g sample}}$$

$$\text{Metal concentration, mg/kg (dry-weight basis)} = \frac{A \times B}{\text{g sample}} \times \frac{100}{D}$$

where:

A = concentration of metal in digested solution, mg/L,
B = final volume of digested solution, mL, and
D = total solids, % (see Section 2540G).

Always prepare acid blanks for each type of digestion performed. Although it is always best to eliminate all relevant sources of contamination, a reagent blank prepared with the same acids and subjected to the same digestion procedure as the sample can correct for impurities present in acids and reagent water.

3. References

1. Boumans, P.W.J.M., ed. 1987. Inductively Coupled Plasma Emission Spectroscopy, Part II: Applications and Fundamentals. John Wiley & Sons, New York, N.Y.
2. U.S. Environmental Protection Agency. 1992. Test Methods for Evaluating Solid Waste, Physical/Chemical Methods; SW-846, 3rd ed. Update 1, Methods 3005A, 3010A, 3020A & 3050A. Off. Solid Waste & Emergency Response, Washington, D.C.
3. Hoenig, M. & A.M. de Kersabiec. 1996. Sample preparation steps for analysis by atomic spectroscopy methods: Present status. *Spectrochim. Acta.* B51:1297.
4. Jarvis, K.E., A.L. Gray & R.S. Houk, eds. 1992. Sample preparation for ICP-MS. Chapter 7 *in* Handbook of Inductively Coupled Plasma Mass Spectrometry. Blackie, Glasgow & London, U.K.
5. U.S. Environmental Protection Agency. 1994. Sample Preparation Procedure for Spectrochemical Determination of Total Recoverable Elements; Method 200.2. Environmental Monitoring Systems Lab., Cincinnati, Ohio.
6. Kingston, H.M. & S. Haswell, eds. 1997. Microwave Enhanced Chemistry: Fundamentals, Sample Preparation and Applications. American Chemical Soc., Washington, D.C.
7. Bock, R. 1979. A Handbook of Decomposition Methods in Analytical Chemistry. Blackie, Glasgow, U.K.

3030 E. Nitric Acid Digestion

Because of the wide variation in concentration levels detected by various instrumental techniques and the need to deal adequately with sources of contamination at trace levels, this method presents one approach for high-level analytes (>0.1 mg/L) and another for trace levels (≤0.1 mg/L).

1. Digestion for Flame Atomic Absorption and High-Level Concentrations

a. Apparatus:

1) *Hot plate.*

2) *Conical (Erlenmeyer) flasks,* 125-mL, or *Griffin beakers,* 150-mL, acid-washed and rinsed with water.

3) *Volumetric flasks,* 100-mL.

4) *Watch glasses,* ribbed and unribbed.
5) *Safety shield.*
6) *Safety goggles.*
b. Reagent:
Nitric acid, HNO_3, conc, or trace-metals grade or ultrapure.
c. Procedure: Use protective equipment listed in ¶s *a*5) and 6) above. Transfer a measured volume (100 mL recommended) of well-mixed, acid-preserved sample appropriate for the expected metals concentrations to a flask or beaker (see 3030D for sample volume). In a hood, add 5 mL conc HNO_3. If a beaker is used, cover with a ribbed watch glass to minimize contamination. Boiling chips, glass beads, or Hengar granules may be added to aid boiling and minimize spatter when high concentration levels (>10 mg/L) are being determined. Bring to a reflux temperature of approximately 95°C to achieve a slow boil and evaporate on a hot plate to the lowest volume possible (about 10 to 20 mL) before precipitation occurs. Continue heating and adding conc HNO_3 as necessary until digestion is complete as shown by a light-colored, clear solution. Do not let sample dry during digestion.

Wash down flask or beaker walls and watch glass cover (if used) with metal-free water. Filter if necessary, using TFE or glass fiber filters (see 3030B). Transfer filtrate to a 100-mL volumetric flask with two portions of reagent water, adding these rinsings to the volumetric flask. Cool, dilute to mark, and mix thoroughly. Take portions of this solution for required metal determinations.

2. Digestion for Trace-Level (≤0.1 mg/L) Concentrations for ICP and ICP-MS[1]

a. Apparatus:
1) *Block heater,* dry, with temperature control.
2) *Polypropylene tubes,** graduated, round-bottom tubes with caps, 17 × 100 mm, acid-washed and rinsed with metal-free water. Preferably use tubes that simultaneously match the analysis instrument autosampler and the block digester. A fit with the centrifuge is secondary but also desirable.
3) *Pipettors,* assorted sizes or adjustable.
4) *Pipet tips.*
5) *Centrifuge.*
6) *Safety shield.*
7) *Safety goggles.*
b. Reagent:
Nitric acid (HNO_3), conc, double distilled.†
c. Procedure: Use protective equipment listed in 3030E.1*a*5) and 6). Soak new polypropylene tubes and caps overnight or for several days in 2*N* HNO_3. Triple rinse with metal-free water, and preferably dry in poly rackets or baskets in a low-temperature oven overnight. Store cleaned tubes in plastic bags before use. Pipet tips also may need to be cleaned; evaluate before use.

Pipet 10 mL well-mixed, acid-preserved sample into a precleaned, labeled tube with a macropipet. With a minimum volume change (<0.5 mL), add appropriate amount of analyte for matrix fortified samples. With a pipet, add 0.5 mL conc HNO_3 (or 1.0 mL 1 + 1 HNO_3) to all samples, blanks, standards, and quality control samples.

Place tubes in block heater in a hood and adjust temperature to 105°C. Drape caps over each tube to allow escape of acid vapors while preventing contamination. NOTE: Do not screw on caps at this time. Digest samples for a minimum of 2 h. Do not let samples boil. Add more conc nitric acid as necessary until digestion is complete by observation of a clear solution.

Remove tubes from heat and cool. Dilute back to original 10 mL volume with metal-free water. Adjust over-volume samples to next convenient gradation for calculations and note volume. (Apply concentration correction from 3030D.2.) If tubes contain particulates, centrifuge and decant clear portion into another precleaned tube. Tighten screw caps and store at 4°C until ready for analysis.

3. Reference

1. JARVIS, K.E., A.L. GRAY & R.S. HOUK, eds. 1992. Sample preparation for ICP-MS. Chapter 7 *in* Handbook of Inductively Coupled Plasma Mass Spectrometry. Blackie & Son, Ltd., Glasgow & London, U.K.

* Falcon tubes, or equivalent.

† Ultrex, Optima grade, or equivalent.

3030 F. Nitric Acid-Hydrochloric Acid Digestion

1. Apparatus

See 3030E.1*a*. The following also may be needed:
Steam bath.

2. Reagents

a. Nitric acid, HNO_3, conc, trace-metals grade or ultrapure.
b. Hydrochloric acid, HCl, 1 + 1.
c. Nitric acid, HNO_3, 1 + 1.

3. Procedure

a. Total HNO_3/HCl: Use protective equipment specified in 3030E.1*a*5) and 6). Transfer a measured volume of well-mixed, acid-preserved sample appropriate for the expected metals concentrations to a flask or beaker (see 3030D for sample volume). In a hood add 3 mL conc HNO_3 and cover with a ribbed watch glass. Place flask or beaker on a hot plate and cautiously evaporate to less than 5 mL, making certain that sample does not boil and that no area of the bottom of the container is allowed to go dry. Cool. Rinse down walls of beaker and watch glass with a

minimum of metal-free water and add 5 mL conc HNO_3. Cover container with a nonribbed watch glass and return to hot plate. Increase temperature of hot plate so that a gentle reflux action occurs. Continue heating, adding additional acid as necessary, until digestion is complete (generally indicated when the digestate is light in color or does not change in appearance with continued refluxing). Cool. Add 10 mL 1 + 1 HCl and 15 mL water per 100 mL anticipated final volume. Heat for an additional 15 min to dissolve any precipitate or residue. Cool, wash down beaker walls and watch glass with water, filter to remove insoluble material that could clog the nebulizer (see 3030B), and transfer filtrate to a 100-mL volumetric flask with rinsings. Alternatively centrifuge or let settle overnight. Adjust to volume and mix thoroughly.

b. Recoverable HNO_3/HCl: For this less rigorous digestion procedure, transfer a measured volume of well-mixed, acid-preserved sample to a flask or beaker. Add 2 mL 1 + 1 HNO_3 and 10 mL 1 + 1 HCl and cover with a ribbed watch glass. Heat on a steam bath or hot plate until volume has been reduced to near 25 mL, making certain sample does not boil. Cool and filter to remove insoluble material or alternatively centrifuge or let settle overnight. Quantitatively transfer sample to volumetric flask, adjust volume to 100 mL, and mix.

For trace-level digestion, use precautionary measures similar to those detailed in 3030E.

3030 G. Nitric Acid-Sulfuric Acid Digestion

1. Apparatus

See 3030E.1*a*.

2. Reagents

a. Nitric acid, HNO_3, conc, trace-metals grade or ultrapure.
b. Sulfuric acid, H_2SO_4, conc.

3. Procedure

Use protective equipment specified in 3030E.1*a*5) and 6). Transfer a measured volume of well-mixed, acid-preserved sample appropriate for the expected metals concentrations to a flask or beaker (see 3030D for sample volume). Add 5 mL conc HNO_3 and cover with a ribbed watch glass. Bring to slow boil on hot plate and evaporate to 15 to 20 mL. Add 5 mL conc HNO_3 and 10 mL conc H_2SO_4, cooling flask or beaker between additions. Evaporate on a hot plate until dense white fumes of SO_3 just appear. If solution does not clear, add 10 mL conc HNO_3 and repeat evaporation to fumes of SO_3. Heat to remove all HNO_3 before continuing treatment. All HNO_3 will be removed when the solution is clear and no brownish fumes are evident. Do not let sample dry during digestion.

Cool and dilute to about 50 mL with water. Heat to almost boiling to dissolve slowly soluble salts. Filter if necessary, then complete procedure as directed in 3030E.1*c* beginning with, "Transfer filtrate . . ."

3030 H. Nitric Acid-Perchloric Acid Digestion

1. Apparatus

See 3030E.1*a*.

2. Reagents

a. Nitric acid, HNO_3, conc, trace-metals grade or ultrapure.
b. Perchloric acid, $HClO_4$.
c. Ammonium acetate solution: Dissolve 500 g $NH_4C_2H_3O_2$ in 600 mL water.

3. Procedure

CAUTION: Heated mixtures of $HClO_4$ and organic matter may explode violently. Avoid this hazard by taking the following precautions:

- **do not add $HClO_4$ to a hot solution containing organic matter;**
- **always pretreat samples containing organic matter with HNO_3 before adding $HClO_4$;**
- **avoid repeated fuming with $HClO_4$ in ordinary hoods (For routine operations, use a water pump attached to a glass fume eradicator. Stainless steel fume hoods with adequate water washdown facilities are available commercially and are acceptable for use with $HClO_4$); and**
- **never let samples being digested with $HClO_4$ evaporate to dryness.**

Use protective equipment specified in 3030E.1*a*5) and 6).

Transfer a measured volume of well-mixed, acid-preserved sample appropriate for the expected metals concentrations to a flask or beaker (see 3030D for sample volume). In a hood add 5 mL conc HNO_3 and cover with a ribbed watch glass. Evaporate sample to 15 to 20 mL on a hot plate. Add 10 mL each of conc HNO_3 and $HClO_4$, cooling flask or beaker between additions. Evaporate gently on a hot plate until dense white fumes of $HClO_4$ just appear. If solution is not clear, keep solution just boiling until it clears. If necessary, add 10 mL conc HNO_3 to complete digestion. Cool, dilute to about 50 mL with water, and boil to expel any chlorine or oxides of nitrogen. Filter, then

complete procedure as directed in 3030E.1*c* beginning with, "Transfer filtrate . . ."

If lead is to be determined in the presence of high amounts of sulfate (e.g., determination of Pb in power plant fly ash samples), dissolve $PbSO_4$ precipitate as follows: Add 50 mL ammonium acetate solution to flask or beaker in which digestion was carried out and heat to incipient boiling. Rotate container occasionally to wet all interior surfaces and dissolve any deposited residue. Reconnect filter and slowly draw solution through it. Transfer filtrate to a 100-mL volumetric flask, cool, dilute to mark, mix thoroughly, and set aside for determination of lead.

3030 I. Nitric Acid-Perchloric Acid-Hydrofluoric Acid Digestion

1. Apparatus

a. Hot plate.

b. TFE beakers, 250-mL, acid-washed and rinsed with water.

c. Volumetric flasks, 100-mL, polypropylene or other suitable plastic.

d. Safety shield.

e. Safety goggles.

2. Reagents

a. Nitric acid, HNO_3, conc and 1 + 1, trace-metals grade or ultrapure.

b. Perchloric acid, $HClO_4$.

c. Hydrofluoric acid, HF, 48 to 51%.

3. Procedure

CAUTION: See precautions for using $HClO_4$ in 3030H; handle HF with extreme care and provide adequate ventilation, especially for the heated solution. Avoid all contact with exposed skin. Provide medical attention for HF burns.

Use safety shield and goggles.

Transfer a measured volume of well-mixed, acid-preserved sample appropriate for the expected metals concentrations into a 250-mL TFE beaker (see 3030D for sample volume). Evaporate on a hot plate to 15 to 20 mL. Add 12 mL conc HNO_3 and evaporate to near dryness. Repeat HNO_3 addition and evaporation. Let solution cool, add 20 mL $HClO_4$ and 1 mL HF, and boil until solution is clear and white fumes of $HClO_4$ have appeared. Cool, add about 50 mL water, filter, and proceed as directed in 3030E.1*c* beginning with, "Transfer filtrate . . ."

3030 J. Dry Ashing

The procedure appears in the 18th Edition of *Standard Methods*. It has not been included in subsequent versions of this publication.

3030 K. Microwave-Assisted Digestion

1. Apparatus

a. Microwave unit with programmable power (minimum 545 W) to within ±10 W of required power, having a corrosion-resistant, well-ventilated cavity and having all electronics protected against corrosion for safe operation. Use a unit having a rotating turntable with a minimum speed of 3 rpm to ensure homogeneous distribution of microwave radiation. Use only laboratory-grade microwave equipment and closed digestion containers with pressure relief that are specifically designed for hot acid.[1]

b. Vessels: Construction requires an inner liner of perfluoroalkoxy (PFA) Teflon™,* other TFE, or composite fluorinated polymers,† capable of withstanding pressures of at least 760 ± 70 kPa (110 ± 10 psi), and capable of controlled pressure relief at the manufacturer's maximum pressure rating.

Acid wash all digestion vessels and rinse with water (3030K.2*a*). For new vessels or when changing between high- and low-concentration samples, clean by leaching with hot‡ hydrochloric acid (1:1) for a minimum of 2 h and then with hot nitric acid (1:1) for a minimum of 2 h; rinse with water and dry in a clean environment. Use this procedure whenever the previous use of digestion vessels is unknown or cross-contamination from vessels is suspected.

c. Temperature feedback control system, using shielded thermocouple, fiber-optic probe, or infrared detector.

d. Bottles, polyethylene, 125-mL, with caps.

e. Thermometer, accurate to ±0.1°C.

f. Balance, large-capacity (1500 g), accurate to 0.1 g.

g. Filtration or centrifuge equipment (optional).

h. Plastic container with cover, 1-L, preferably made of PFA Teflon.™§

* Or equivalent.

† Such as TFM™, or equivalent.

‡ At temperatures >80°C, but not boiling.

§ Or equivalent.

2. Reagents

a. Metal-free water: See Section 3111B.3*c*.

b. Nitric acid (HNO_3), conc, sub-boiling distilled: Non-sub-boiling acids can be used if they are shown not to contribute blanks.

3. Calibration of Microwave Unit

NOTE: For microwave units equipped with temperature feedback electronic controls, calibration of the microwave unit is not required provided performance specifications can be duplicated.

For cavity-type microwave equipment, evaluate absolute power (watts) by measuring the temperature rise in 1 kg water exposed to microwave radiation for a fixed time. With this measurement, the relationship between available power (W) and the partial power setting (%) of the unit can be estimated, and any absolute power in watts may be transferred from one unit to another. The calibration format required depends on type of electronic system used by manufacturer to provide partial microwave power. Few units have an accurate and precise linear relationship between percent power settings and absorbed power. Where linear circuits have been used, determine calibration curve by a three-point calibration method; otherwise, use the multiple-point calibration method.

a. Three-point calibration method: Measure power at 100% and 50% power using the procedure described in ¶ *c* below and calculate power setting corresponding to required power in watts as specified in the procedure from the two-point line. Measure absorbed power at the calculated partial power setting. If the measured absorbed power does not correspond to the calculated power within ±10 W, use the multiple-point calibration method, ¶ *b* below. Use this point periodically to verify integrity of calibration.

b. Multiple-point calibration method: For each microwave unit, measure the following power settings: 100, 99, 98, 97, 95, 90, 80, 70, 60, 50, and 40% using the procedure described in ¶ *c* below. These data are clustered about the customary working power ranges. Nonlinearity commonly is encountered at the upper end of the calibration curve. If the unit's electronics are known to have nonlinear deviations in any region of proportional power control, make a set of measurements that bracket the power to be used. The final calibration point should be at the partial power setting that will be used in the test. Check this setting periodically to evaluate the integrity of the calibration. If a significant change (±10 W) is detected, re-evaluate entire calibration.

c. Equilibrate a large volume of water to room temperature (23 ± 2°C). Weigh 1 kg water (1000 g ± 1 g) or measure (1000 mL ± 1 mL) into a plastic, not glass, container, and measure the temperature to ±0.1°C. Condition microwave unit by heating a glass beaker with 500 to 1000 mL tap water at full power for 5 min with the exhaust fan on. Loosely cover plastic container to reduce heat loss and place in normal sample path (at outer edge of rotating turntable); circulate continuously through the microwave field for 120 s at desired power setting with exhaust fan on as it will be during normal operation. Remove plastic container and stir water vigorously. Use a magnetic stirring bar inserted immediately after microwave irradiation; record maximum temperature within the first 30 s to ±0.1°C. Use a new sample for each additional measurement. If the water is reused, return both water and beaker to 23 ± 2°C. Make three measurements at each power setting. When any part of the high-voltage circuit, power source, or control components in the unit have been serviced or replaced, recheck calibration power. If power output has changed by more than ±10 W, re-evaluate entire calibration. Compute absorbed power by the following relationship:

$$P = \frac{(K)(Cp)(m)(\Delta T)}{t}$$

where:

P = apparent power absorbed by sample, W,
K = conversion factor for thermochemical calories sec^{-1} to watts (4.184),
Cp = heat capacity, thermal capacity, or specific heat (cal g^{-1} $°C^{-1}$) of water,
m = mass of water sample, g,
ΔT = final temperature minus initial temperature, °C, and
t = time, s.

For the experimental conditions of 120 s and 1 kg water (*Cp* at 25°C = 0.9997), the calibration equation simplifies to:

$$P = (\Delta T)(34.85)$$

Stable line voltage within the manufacturer's specification is necessary for accurate and reproducible calibration and operation. During measurement and operation it must not vary by more than ±2 V. A constant power supply may be necessary if line voltage is unstable.

4. Procedure

CAUTION: This method is designed for microwave digestion of waters only. It is not intended for the digestion of solids, for which high concentrations of organic compounds may result in high pressures and possibly unsafe conditions.

CAUTION: As a safety measure, never mix different manufacturers' vessels in the same procedure. Vessels constructed differently will retain heat at different rates; control of heating conditions assumes that all vessels have the same heat-transfer characteristics. Inspect casements for cracks and chemical corrosion. Failure to maintain the vessels' integrity may result in catastrophic failure.

Both prescription controls and performance controls are provided for this procedure. Performance controls are the most general and most accurate. When equipment capability permits, use the performance criterion.

a. Performance criterion: The following procedure is based on heating acidified samples in two stages where the first stage is to reach 160 ± 4°C in 10 min and the second stage is to permit a slow rise to 165 to 170°C during the second 10 min. This performance criterion is based on temperature feedback control system capability that is implemented in various ways by different manufacturers. Because the temperature of the acid controls the reaction, this is the essential condition that will reproduce results in this preparation method. Verification of

temperature conditions inside the vessel at these specific times is sufficient to verify the critical procedural requirements.

b. Prescription criterion: For all PFA vessels without liners, a verified program that meets the performance-based temperature-time profile is 545 W for 10 min followed by 344 W for 10 min using five single-wall PFA Teflon™‖ digestion vessels.[2] Any verified program for a given microwave unit depends on unit power and operational power settings, heating times, number, type, and placement of digestion vessels within the unit, and sample and acid volumes. The change in power, time, and temperature profile is not directly proportional to the change in the number of sample vessels. Any deviations from the verified program conditions will require verification of the time-temperature profile to conform to the given two-stage profile. This may be done by laboratory personnel if suitable test equipment is available, or by the manufacturer of the microwave equipment.

c. General conditions: Weigh entire digestion vessel assembly to 0.1 g before use and record (*A*). Accurately transfer 45 mL of well-shaken sample into the digestion vessel. Pipet 5 mL conc HNO_3 into each vessel. Attach all safety equipment required for appropriate and safe vessel operation following manufacturer's specifications. Tighten cap to manufacturer's specifications. Weigh each capped vessel to the nearest 0.1 g (*B*).

Place appropriate number of vessels evenly distributed in the carousel. Treat sample blanks, known additions, and duplicates in the same manner as samples. For prescription control only, when fewer samples than the appropriate number are digested, fill remaining vessels with 45 mL water and 5 mL conc HNO_3 to obtain full complement of vessels for the particular program being used.

Place carousel in unit and seat it carefully on turntable. Program microwave unit to heat samples to 160 ± 4°C in 10 min and then, for the second stage, to permit a slow rise to 165 to 170°C for 10 min. Start microwave generator, making sure that turntable is turning and that exhaust fan is on.

At completion of the microwave program, let vessels cool for at least 5 min in the unit before removal. Cool samples further outside the unit by removing the carousel and letting them cool on a bench or in a water bath. When cooled to room temperature, weigh each vessel (to 0.1 g) and record weight (*C*).

If the net weight of sample plus acid decreased by more than 10%, discard sample.

‖ Or equivalent.

Complete sample preparation by carefully uncapping and venting each vessel in a fume hood. Follow individual manufacturer's specifications for relieving pressure in individual vessel types. Transfer to acid-cleaned noncontaminating plastic bottles. If the digested sample contains particulates, filter, centrifuge, or settle overnight and decant.

5. Calculations

a. Dilution correction: Multiply results by 50/45 or 1.11 to account for the dilution caused by the addition of 5 mL acid to 45 mL sample.

b. Discarding of sample: To determine if the net weight of sample plus acid decreased by more than 10% during the digestion process, use the following calculation

$$\frac{[(B-A)-(C-A)]}{(B-A)} \times 100 > 10\% \text{ (1\% for multilayer vessels)}$$

6. Quality Control

NOTE: When nitric acid digestion is used, recoveries of silver and antimony in some matrices may be unacceptably low. Verify recoveries using appropriate known additions.

Preferably include a quality-control sample in each loaded carousel. Prepare samples in batches including preparation blanks, sample duplicates, and pre-digestion known additions. Determine size of batch and frequency of quality-control samples by method of analysis and laboratory practice. The power of the microwave unit and batch size may prevent including one or more of the quality-control samples in each carousel. Do not group quality-control samples together but distribute them throughout the various carousels to give the best monitoring of digestion.

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3110 METALS BY ATOMIC ABSORPTION SPECTROMETRY

Because requirements for determining metals by atomic absorption spectrometry vary with metal and/or concentration to be determined, the method is presented as follows:

Metals by Flame Atomic Absorption Spectrometry (Section 3111) encompasses:

- Determination of antimony, bismuth, cadmium, calcium, cesium, chromium, cobalt, copper, gold, iridium, iron, lead, lithium, magnesium, manganese, nickel, palladium, platinum, potassium, rhodium, ruthenium, silver, sodium, strontium, thallium, tin, and zinc by direct aspiration into an air-acetylene flame (Section 3111B),
- Determination of low concentrations of cadmium, chromium, cobalt, copper, iron, lead, manganese, nickel, silver, and zinc by chelation with ammonium pyrrolidine dithiocarbamate (APDC), extraction into methyl isobutyl ketone (MIBK), and aspiration into an air-acetylene flame (Section 3111C),
- Determination of aluminum, barium, beryllium, calcium, molybdenum, osmium, rhenium, silicon, thorium, titanium, and vanadium by direct aspiration into a nitrous oxide-acetylene flame (Section 3111D), and
- Determination of low concentrations of aluminum and beryllium by chelation with 8-hydroxyquinoline, extraction into MIBK, and aspiration into a nitrous oxide-acetylene flame (Section 3111E).

Section 3112 covers determination of mercury by the cold vapor technique.

Section 3113 concerns determination of micro quantities of aluminum, antimony, arsenic, barium, beryllium, cadmium, chromium, cobalt, copper, iron, lead, manganese, molybdenum, nickel, selenium, silver, and tin by electrothermal atomic absorption spectrometry.

Section 3114 covers determination of arsenic and selenium by conversion to their hydrides and aspiration into an argon-hydrogen or nitrogen-hydrogen flame.

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3111 METALS BY FLAME ATOMIC ABSORPTION SPECTROMETRY*

3111 A. Introduction

1. Principle

In flame atomic absorption spectrometry, a sample is aspirated into a flame and atomized. A light beam is directed through the flame, into a monochromator, and onto a detector that measures the amount of light absorbed by the atomized element in the flame. For some metals, atomic absorption exhibits superior sensitivity over flame emission. Because each metal has its own characteristic absorption wavelength, a source lamp composed of that element is used; this makes the method relatively free from spectral or radiation interferences. The amount of energy at the characteristic wavelength absorbed in the flame is proportional to the concentration of the element in the sample over a limited concentration range. Most atomic absorption instruments also are equipped for operation in an emission mode, which may provide better linearity for some elements.

2. Selection of Method

See Section 3110.

3. Interferences

a. Chemical interference: Many metals can be determined by direct aspiration of sample into an air-acetylene flame. The most troublesome type of interference is termed "chemical" and results from the lack of absorption by atoms bound in molecular combination in the flame. This can occur when the flame is not hot enough to dissociate the molecules or when the dissociated atom is oxidized immediately to a compound that will not dissociate further at the flame temperature. Such interferences may be reduced or eliminated by adding specific elements or compounds to the sample solution. For example, the interference of phosphate in the magnesium determination can be overcome by adding lanthanum. Similarly, introduction of calcium eliminates silica interference in the determination of manganese. However, silicon and metals such as aluminum, barium, beryllium, and vanadium require the higher-temperature, nitrous oxide-acetylene flame to dissociate their molecules. The nitrous oxide-acetylene flame also can be useful in minimizing certain types of chemical interferences encountered in the air-acetylene flame. For example, the interference caused by high concentrations of phosphate in the determination of calcium in the air-acetylene flame is reduced in the nitrous oxide-acetylene flame.

MIBK extractions with APDC (see 3111C) are particularly useful where a salt matrix interferes, for example, in seawater. This procedure also concentrates the sample so that the detection limits are lowered.

Brines and seawater can be analyzed by direct aspiration but sample dilution is recommended. Aspiration of solutions containing high concentrations of dissolved solids often results in solids buildup on the burner head. This requires frequent shutdown of the flame and cleaning of the burner head. Preferably use background correction when analyzing waters that contain in excess of 1% solids, especially when the primary resonance line of the element of interest is below 240 nm. Make more frequent recovery checks when analyzing brines and seawaters to ensure accurate results in these concentrated and complex matrices.

Barium and other metals ionize in the flame, thereby reducing the ground state (potentially absorbing) population. The addition of an excess of a cation (sodium, potassium, or lithium) having a similar or lower ionization potential will overcome this problem. The wavelength of maximum absorption for arsenic is 193.7 nm and for selenium 196.0 nm—wavelengths at which the air-acetylene flame absorbs intensely. The sensitivity for arsenic and selenium can be improved by conversion to their gaseous hydrides and analyzing them in either a nitrogen-hydrogen or an argon-hydrogen flame with a quartz tube (see Section 3114).

b. Background correction: Molecular absorption and light scattering caused by solid particles in the flame can cause erroneously high absorption values resulting in positive errors. When such phenomena occur, use background correction to obtain accurate values. Use any one of three types of background correction: continuum-source, Zeeman, or Smith-Hieftje correction.

1) Continuum-source background correction—A continuum-source background corrector utilizes either a hydrogen-filled hollow cathode lamp with a metal cathode or a deuterium arc lamp. When both the line source hollow-cathode lamp and the continuum source are placed in the same optical path and are time-shared, the broadband background from the elemental signal is subtracted electronically, and the resultant signal will be background-compensated.

Both the hydrogen-filled hollow-cathode lamp and deuterium arc lamp have lower intensities than either the line source hollow-cathode lamp or electrodeless discharge lamps. To obtain a valid correction, match the intensities of the continuum source with the line source hollow-cathode or electrodeless discharge lamp. The matching may result in lowering the intensity of the line source or increasing the slit width; these measures have the disadvantage of raising the detection limit and possibly causing nonlinearity of the calibration curve. Background correction using a continuum source corrector is susceptible to interference from other absorbing lines in the spectral bandwidth. Miscorrection occurs from significant atomic absorption of the continuum source radiation by elements other than that being determined. When a line source hollow-cathode lamp is used without background correction, the presence of an absorbing line from another element in the spectral bandwidth will not cause an interference unless it overlaps the line of interest.

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Continuum-source background correction will not remove direct absorption spectral overlap, where an element other than that being determined is capable of absorbing the line radiation of the element under study.

2) Zeeman background correction—This correction is based on the principle that a magnetic field splits the spectral line into two linearly polarized light beams parallel and perpendicular to the magnetic field. One is called the pi (π) component and the other the sigma (σ) component. These two light beams have exactly the same wavelength and differ only in the plane of polarization. The π line will be absorbed by both the atoms of the element of interest and by the background caused by broadband absorption and light scattering of the sample matrix. The σ line will be absorbed only by the background.

Zeeman background correction provides accurate background correction at much higher absorption levels than is possible with continuum source background correction systems. It also virtually eliminates the possibility of error from structured background. Because no additional light sources are required, the alignment and intensity limitations encountered using continuum sources are eliminated.

Disadvantages of the Zeeman method include reduced sensitivity for some elements, reduced linear range, and a "rollover" effect whereby the absorbance of some elements begins to decrease at high concentrations, resulting in a two-sided calibration curve.

3) Smith–Hieftje background correction—This correction is based on the principle that absorbance measured for a specific element is reduced as the current to the hollow cathode lamp is increased while absorption of nonspecific absorbing substances remains identical at all current levels. When this method is applied, the absorbance at a high-current mode is subtracted from the absorbance at a low-current mode. Under these conditions, any absorbance due to nonspecific background is subtracted out and corrected for.

Smith–Hieftje background correction provides a number of advantages over continuum-source correction. Accurate correction at higher absorbance levels is possible and error from structured background is virtually eliminated. In some cases, spectral interferences also can be eliminated. The usefulness of Smith-Hieftje background correction with electrodeless discharge lamps has not yet been established.

Table 3111:I. Atomic Absorption Concentration Ranges with Direct Aspiration Atomic Absorption

Element	Wavelength *nm*	Flame Gases*	Instrument Detection Level *mg/L*	Sensitivity *mg/L*	Optimum Concentration Range *mg/L*
Ag	328.1	A–Ac	0.01	0.06	0.1–4
Al	309.3	N–Ac	0.1	1	5–100
Au	242.8	A–Ac	0.01	0.25	0.5–20
Ba	553.6	N–Ac	0.03	0.4	1–20
Be	234.9	N–Ac	0.005	0.03	0.05–2
Bi	223.1	A–Ac	0.06	0.4	1–50
Ca	422.7	A–Ac	0.003	0.08	0.2–20
Cd	228.8	A–Ac	0.002	0.025	0.05–2
Co	240.7	A–Ac	0.03	0.2	0.5–10
Cr	357.9	A–Ac	0.02	0.1	0.2–10
Cs	852.1	A–Ac	0.02	0.3	0.5–15
Cu	324.7	A–Ac	0.01	0.1	0.2–10
Fe	248.3	A–Ac	0.02	0.12	0.3–10
Ir	264.0	A–Ac	0.6	8	—
K	766.5	A–Ac	0.005	0.04	0.1–2
Li	670.8	A–Ac	0.002	0.04	0.1–2
Mg	285.2	A–Ac	0.0005	0.007	0.02–2
Mn	279.5	A–Ac	0.01	0.05	0.1–10
Mo	313.3	N–Ac	0.1	0.5	1–20
Na	589.0	A–Ac	0.002	0.015	0.03–1
Ni	232.0	A–Ac	0.02	0.15	0.3–10
Os	290.9	N–Ac	0.08	1	—
Pb†	283.3	A–Ac	0.05	0.5	1–20
Pt	265.9	A–Ac	0.1	2	5–75
Rh	343.5	A–Ac	0.5	0.3	—
Ru	349.9	A–Ac	0.07	0.5	—
Sb	217.6	A–Ac	0.07	0.5	1–40
Si	251.6	N–Ac	0.3	2	5–150
Sn	224.6	A–Ac	0.8	4	10–200
Sr	460.7	A–Ac	0.03	0.15	0.3–5
Ti	365.3	N–Ac	0.3	2	5–100
V	318.4	N–Ac	0.2	1.5	2–100
Zn	213.9	A–Ac	0.005	0.02	0.05–2

* A–Ac = air-acetylene; N–Ac = nitrous oxide-acetylene.

† The more sensitive 217.0 nm wavelength is recommended for instruments with background correction capabilities.

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4. Sensitivity, Detection Levels, and Optimum Concentration Ranges

The sensitivity of flame atomic absorption spectrometry is defined as the metal concentration that produces an absorption of 1% (an absorbance of approximately 0.0044). The instrument detection level is defined here as the concentration that produces absorption equivalent to twice the magnitude of the background fluctuation. Sensitivity and detection levels vary with the instrument, the element determined, the complexity of the matrix, and the technique selected. The optimum concentration range usually starts from the concentration of several times the detection level and extends to the concentration at which the calibration curve starts to flatten. To achieve best results, use concentrations of samples and standards within the optimum concentration range of the spectrometer. See Table 3111:I for indication of concentration ranges measurable with conventional atomization. In many instances the concentration range shown in Table 3111:I may be extended downward either by scale expansion or by integrating the absorption signal over a long time. The range may be extended upward by dilution, using a less sensitive wavelength, rotating the burner head, or utilizing a microprocessor to linearize the calibration curve at high concentrations.

5. Preparation of Standards

Prepare standard solutions of known metal concentrations in water with a matrix similar to the sample. Use standards that bracket expected sample concentration and are within the method's working range. Very dilute standards should be prepared daily from stock solutions in concentrations greater than 500 mg/L. Stock standard solutions can be obtained from several commercial sources. They also can be prepared from National

Institute of Standards and Technology (NIST) reference materials or by procedures outlined in the following sections.

For samples containing high and variable concentrations of matrix materials, make the major ions in the sample and the dilute standard similar. If the laboratory has reason to believe the sample matrix is complex and the components, surface tension, or viscosity cannot be matched accurately with the standards, use the method of standard additions [Section 3113B.4*d*2)] to correct for matrix effects. If digestion is used, carry standards through the same digestion procedure used for samples.

6. Apparatus

a. Atomic absorption spectrometer, consisting of a light source emitting the line spectrum of an element (hollow-cathode lamp or electrodeless discharge lamp), a device for vaporizing the sample (usually a flame), a means of isolating an absorption line (monochromator or filter and adjustable slit), and a photoelectric detector with its associated electronic amplifying and measuring equipment.

b. Burner: The most common type of burner is a premix, which introduces the spray into a condensing chamber for removal of large droplets. The burner may be fitted with a conventional head containing a single slot; a three-slot Boling head, which may be preferred for direct aspiration with an air-acetylene flame; or a special head for use with nitrous oxide and acetylene.

c. Readout: Most instruments are equipped with either a digital or null meter readout mechanism. Most modern instruments are equipped with microprocessors or stand-alone control computers capable of integrating absorption signals over time and linearizing the calibration curve at high concentrations.

d. Lamps: Use either a hollow-cathode lamp or an electrodeless discharge lamp (EDL). Use one lamp for each element being measured. Multi-element hollow-cathode lamps generally provide lower sensitivity than single-element lamps. EDLs take a longer time to warm up and stabilize.

e. Pressure-reducing valves: Maintain supplies of fuel and oxidant at pressures somewhat higher than the controlled operating pressure of the instrument by using suitable reducing valves. Use a separate reducing valve for each gas.

f. Vent: Place a vent about 15 to 30 cm above the burner to remove fumes and vapors from the flame. This precaution protects laboratory personnel from toxic vapors, protects the instrument from corrosive vapors, and prevents flame stability from being affected by room drafts. A damper or variable-speed blower is desirable for modulating air flow and preventing flame disturbance. Select blower size to provide the air flow recommended by the instrument manufacturer. In laboratory locations with heavy particulate air pollution, use clean laboratory facilities (Section 3010C).

7. Quality Assurance/Quality Control

Some data typical of the precision and bias obtainable with the methods discussed are presented in Tables 3111:II and III.

Analyze a blank between sample or standard readings to verify baseline stability. Rezero when necessary.

Table 3111:II. Interlaboratory Precision and Bias Data for Atomic Absorption Methods—Direct Aspiration and Extracted Metals

Metal	Conc*	SD*	Relative SD %	Relative Error %	No. of Participants
Direct determination:					
Aluminum[1]	4.50	0.19	4.2	8.4	5
Barium[2]	1.00	0.089	8.9	2.7	11
Beryllium[1]	0.46	0.0213	4.6	23.0	11
Cadmium[3]	0.05	0.0108	21.6	8.2	26
Cadmium[1]	1.60	0.11	6.9	5.1	16
Calcium[1]	5.00	0.21	4.2	0.4	8
Chromium[1]	3.00	0.301	10.0	3.7	9
Cobalt[1]	4.00	0.243	6.1	0.5	14
Copper[3]	1.00	0.112	11.2	3.4	53
Copper[1]	4.00	0.331	8.3	2.8	15
Iron[1]	4.40	0.260	5.8	2.3	16
Iron[3]	0.30	0.0495	16.5	0.6	43
Lead[1]	6.00	0.28	4.7	0.2	14
Magnesium[3]	0.20	0.021	10.5	6.3	42
Magnesium[1]	1.10	0.116	10.5	10.0	8
Manganese[1]	4.05	0.317	7.8	1.3	16
Manganese[3]	0.05	0.0068	13.5	6.0	14
Nickel[1]	3.93	0.383	9.8	2.0	14
Silver[3]	0.05	0.0088	17.5	10.6	7
Silver[1]	2.00	0.07	3.5	1.0	10
Sodium[1]	2.70	0.122	4.5	4.1	12
Strontium[1]	1.00	0.05	5.0	0.2	12
Zinc[3]	0.50	0.041	8.2	0.4	48
Extracted determination:					
Aluminum[2]	300	32	10.7	0.7	15
Beryllium[2]	5	1.7	34.0	20.0	9
Cadmium[3]	50	21.9	43.8	13.3	12
Cobalt[1]	300	28.5	9.5	1.0	6
Copper[1]	100	71.7	71.7	12.0	8
Iron[1]	250	19.0	7.6	3.6	4
Manganese[1]	21.5	2.4	11.2	7.4	8
Molybdenum[1]	9.5	1.1	11.6	1.3	5
Nickel[1]	56.8	15.2	26.8	13.6	14
Lead[3]	50	11.8	23.5	19.0	8
Silver[1]	5.2	1.4	26.9	3.0	7

* For direct determinations, mg/L; for extracted determinations, μg/L.
Superscripts refer to reference numbers.
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To one sample out of every ten (or one sample from each group of samples if less than ten are being analyzed) add a known amount of the metal of interest and reanalyze to confirm recovery. The amount of metal added should be approximately equal to the amount found. If little metal is present add an amount close to the middle of the linear range of the test. Recovery of added metal should be between 85 and 115%.

Analyze an additional standard solution after every ten samples or with each batch of samples, whichever is less, to confirm that the test is in control. Recommended concentrations of standards to be run, limits of acceptability, and

TABLE 3111:III. SINGLE-OPERATOR PRECISION AND RECOMMENDED CONTROL RANGES FOR ATOMIC ABSORPTION METHODS—DIRECT ASPIRATION AND EXTRACTED METALS

Metal	Conc*	SD*	Relative SD %	No. of Participants	QC Std.*	Acceptable Range*
Direct determination:						
Aluminum[1]	4.50	0.23	5.1	15	5.00	4.3–5.7
Beryllium[1]	0.46	0.012	2.6	10	0.50	0.46–0.54
Calcium[1]	5.00	0.05	1.0	8	5.00	4.8–5.2
Chromium[1]	7.00	0.69	9.9	9	5.00	3.3–6.7
Cobalt[1]	4.00	0.21	5.3	14	4.00	3.4–4.6
Copper[1]	4.00	0.115	2.9	15	4.00	3.7–4.3
Iron[1]	5.00	0.19	3.8	16	5.00	4.4–5.6
Magnesium[1]	1.00	0.009	0.9	8	1.00	0.97–1.03
Nickel[4]	5.00	0.04	0.8	—	5.00	4.9–5.1
Silver[1]	2.00	0.25	12.5	10	2.00	1.2–2.8
Sodium[4]	8.2	0.1	1.2	—	5.00	4.8–5.2
Strontium[1]	1.00	0.04	4.0	12	1.00	0.87–1.13
Potassium[4]	1.6	0.2	12.5	—	1.6	1.0–2.2
Molybdenum[4]	7.5	0.07	0.9	—	10.0	9.7–10.3
Tin[4]	20.0	0.5	2.5	—	20.0	18.5–21.5
Titanium[4]	50.0	0.4	0.8	—	50.0	48.8–51.2
Vanadium	50.0	0.2	0.4	—	50.0	49.4–50.6
Extracted determination:						
Aluminum[1]	300	12	4.0	15	300	264– 336
Cobalt[1]	300	20	6.7	6	300	220– 380
Copper[1]	100	21	21	8	100	22– 178
Iron[1]	250	12	4.8	4	250	180– 320
Manganese[1]	21.5	202	10.2	8	25	17– 23
Molybdenum[1]	9.5	1.0	10.5	5	10	5.5–14.5
Nickel[1]	56.8	9.2	16.2	14	50	22– 78
Silver[1]	5.2	1.2	23.1	7	5.0	0.5–9.5

* For direct determinations, mg/L; for extracted determinations, μg/L.
Superscripts refer to reference numbers.
SOURCE: AMERICAN SOCIETY FOR TESTING & MATERIALS. 1986. Annual Book of ASTM Standards, Vol. 11.01: Water and Environmental Technology. Philadelphia, Pa.
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reported single-operator precision data are listed in Table 3111:III.

The QC practices considered to be an integral part of each method can be found in Section 3020.

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3111 B. Direct Air-Acetylene Flame Method

1. General Discussion

This method is applicable to the determination of antimony, bismuth, cadmium, calcium, cesium, chromium, cobalt, copper, gold, iridium, iron, lead, lithium, magnesium, manganese, nickel, palladium, platinum, potassium, rhodium, ruthenium, silver, sodium, strontium, thallium, tin, and zinc.

2. Apparatus

Atomic absorption spectrometer and associated equipment: See 3111A.6. Use burner head recommended by the manufacturer.

3. Reagents

a. Air, cleaned and dried through a suitable filter to remove oil, water, and other foreign substances. The source may be a compressor or commercially bottled gas.

b. Acetylene, standard commercial grade. Acetone, which always is present in acetylene cylinders, can be prevented from entering and damaging the burner head by replacing a cylinder when its pressure has fallen to 689 kPa (100 psi) acetylene.

CAUTION: Acetylene gas represents an explosive hazard in the laboratory. Follow instrument manufacturer's directions in plumbing and using this gas. Do not allow gas contact with copper, brass with >65% copper, silver, or liquid mercury; do not use copper or brass tubing, regulators, or fittings with >65% copper content.

c. Metal-free water: Use metal-free water for preparing all reagents and calibration standards and as dilution water. Prepare metal-free water by deionizing tap water and/or by using one of the following processes, depending on the metal concentration in the sample: single distillation, redistillation, or sub-boiling. Always check deionized or distilled water to determine whether the element of interest is present in trace amounts. (NOTE: If the source water contains Hg or other volatile metals, single- or redistilled water may not be suitable for trace analysis because these metals distill over with the distilled water. In such cases, use sub-boiling to prepare metal-free water.)

d. Calcium solution: Dissolve 630 mg calcium carbonate, $CaCO_3$, in 50 mL of 1 + 5 HCl. If necessary, boil gently to obtain complete solution. Cool and dilute to 1000 mL with water.

e. Hydrochloric acid (HCl), 1%, 10%, 20% (all v/v), 1 + 5, 1 + 1, and conc.

f. Lanthanum solution: Dissolve 58.65 g lanthanum oxide, La_2O_3, in 250 mL conc HCl. Add acid slowly until the material is dissolved and dilute to 1000 mL with water.

g. Hydrogen peroxide, 30%.

h. Nitric acid (HNO_3), 2% (v/v), 1 + 1, and conc.

i. Aqua regia: Add 3 volumes conc HCl to 1 volume conc HNO_3.

j. Standard metal solutions: Prepare a series of standard metal solutions in the optimum concentration range by appropriate dilution of the following stock metal solutions with water containing 1.5 mL conc HNO_3/L. Stock standard solutions are available from a number of commercial suppliers. Alternatively, prepare as described below. Thoroughly dry reagents before use. In general, use reagents of the highest purity. For hydrates, use fresh reagents.

1) *Antimony*—Dissolve 0.2669 g $K(SbO)C_4H_4O_6$ in water, add 10 mL 1 + 1 HCl and dilute to 1000 mL with water; 1.00 mL = 100 μg Sb.

2) *Bismuth*—Dissolve 0.100 g bismuth metal in a minimum volume of 1 + 1 HNO_3. Dilute to 1000 mL with 2% (v/v) HNO_3; 1.00 mL = 100 μg Bi.

3) *Cadmium*—Dissolve 0.100 g cadmium metal in 4 mL conc HNO_3. Add 8.0 mL conc HNO_3 and dilute to 1000 mL with water; 1.00 mL = 100 μg Cd.

4) *Calcium*—Suspend 0.2497 g $CaCO_3$ (dried at 180° for 1 h before weighing) in water and dissolve cautiously with a minimum amount of 1 + 1 HNO_3. Add 10.0 mL conc HNO_3 and dilute to 1000 mL with water; 1.00 mL = 100 μg Ca.

5) *Cesium*—Dissolve 0.1267 g cesium chloride, CsCl, in 1000 mL water; 1.00 mL = 100 μg Cs.

6) *Chromium*—Dissolve 0.1923 g CrO_3 in water. When solution is complete, acidify with 10 mL conc HNO_3 and dilute to 1000 mL with water; 1.00 mL = 100 μg Cr.

7) *Cobalt*—Dissolve 0.1000 g cobalt metal in a minimum amount of 1 + 1 HNO_3. Add 10.0 mL 1 + 1 HCl and dilute to 1000 mL with water; 1.00 mL = 100 μg Co.

8) *Copper*—Dissolve 0.100 g copper metal in 2 mL conc HNO_3, add 10.0 mL conc HNO_3 and dilute to 1000 mL with water; 1.00 mL = 100 μg Cu.

9) *Gold*—Dissolve 0.100 g gold metal in a minimum volume of aqua regia. Evaporate to dryness, dissolve residue in 5 mL conc HCl, cool, and dilute to 1000 mL with water; 1.00 mL = 100 μg Au.

10) *Iridium*—Dissolve 0.1147 g ammonium chloroiridate, $(NH_4)_2IrCl_6$, in a minimum volume of 1% (v/v) HCl and dilute to 100 mL with 1% (v/v) HCl; 1.00 mL = 500 μg Ir.

11) *Iron*—Dissolve 0.100 g iron wire in a mixture of 10 mL 1 + 1 HCl and 3 mL conc HNO_3. Add 5 mL conc HNO_3 and dilute to 1000 mL with water; 1.00 mL = 100 μg Fe.

12) *Lead*—Dissolve 0.1598 g lead nitrate, $Pb(NO_3)_2$, in a minimum amount of 1 + 1 HNO_3, add 10 mL conc HNO_3, and dilute to 1000 mL with water; 1.00 mL = 100 μg Pb.

13) *Lithium*—Dissolve 0.5323 g lithium carbonate, Li_2CO_3, in a minimum volume of 1 + 1 HNO_3. Add 10.0 mL conc HNO_3 and dilute to 1000 mL with water; 1.00 mL = 100 μg Li.

14) *Magnesium*—Dissolve 0.1658 g MgO in a minimum amount of 1 + 1 HNO_3. Add 10.0 mL conc HNO_3 and dilute to 1000 mL with water; 1.00 mL = 100 μg Mg.

15) *Manganese*—Dissolve 0.1000 g manganese metal in 10 mL conc HCl mixed with 1 mL conc HNO_3. Dilute to 1000 mL with water; 1.00 mL = 100 μg Mn.

16) *Nickel*—Dissolve 0.1000 g nickel metal in 10 mL hot conc HNO_3, cool, and dilute to 1000 mL with water; 1.00 mL = 100 μg Ni.

17) *Palladium*—Dissolve 0.100 g palladium wire in a minimum volume of aqua regia and evaporate just to dryness. Add 5 mL conc HCl and 25 mL water and warm until dissolution is complete. Dilute to 1000 mL with water; 1.00 mL = 100 μg Pd.

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18) *Platinum*—Dissolve 0.100 g platinum metal in a minimum volume of aqua regia and evaporate just to dryness. Add 5 mL conc HCl and 0.1 g NaCl and again evaporate just to dryness. Dissolve residue in 20 mL of 1 + 1 HCl and dilute to 1000 mL with water; 1.00 mL = 100 μg Pt.

19) *Potassium*—Dissolve 0.1907 g potassium chloride, KCl, (dried at 110°C) in water and make up to 1000 mL; 1.00 mL = 100 μg K.

20) *Rhodium*—Dissolve 0.386 g ammonium hexachlororhodate, $(NH_4)_3RhCl_6 \cdot 1.5H_2O$, in a minimum volume of 10% (v/v) HCl and dilute to 1000 mL with 10% (v/v) HCl; 1.00 mL = 100 μg Rh.

21) *Ruthenium*—Dissolve 0.205 g ruthenium chloride, $RuCl_3$, in a minimum volume of 20% (v/v) HCl and dilute to 1000 mL with 20% (v/v) HCl; 1.00 mL = 100 μg Ru.

22) *Silver*—Dissolve 0.1575 g silver nitrate, $AgNO_3$, in 100 mL water, add 10 mL conc HNO_3, and make up to 1000 mL; 1.00 mL = 100 μg Ag.

23) *Sodium*—Dissolve 0.2542 g sodium chloride, NaCl, dried at 140°C, in water, add 10 mL conc HNO_3 and make up to 1000 mL; 1.00 mL = 100 μg Na.

24) *Strontium*—Suspend 0.1685 g $SrCO_3$ in water and dissolve cautiously with a minimum amount of 1 + 1 HNO_3. Add 10.0 mL conc HNO_3 and dilute to 1000 mL with water: 1 mL = 100 μg Sr.

25) *Thallium*—Dissolve 0.1303 g thallium nitrate, $TlNO_3$, in water. Add 10 mL conc HNO_3 and dilute to 1000 mL with water; 1.00 mL = 100 μg Tl.

26) *Tin*—Dissolve 1.000 g tin metal in 100 mL conc HCl and dilute to 1000 mL with water; 1.00 mL = 1.00 mg Sn.

27) *Zinc*—Dissolve 0.100 g zinc metal in 20 mL 1 + 1 HCl and dilute to 1000 mL with water; 1.00 mL = 100 μg Zn.

4. Procedure

a. Sample preparation: Required sample preparation depends on the metal form being measured.

If dissolved metals are to be determined, see Section 3030B for sample preparation. If total or acid-extractable metals are to be determined, see Sections 3030C–K. For all samples, make certain that the concentrations of acid and matrix modifiers are the same in both samples and standards.

When determining Ca or Mg, dilute and mix 100 mL sample or standard with 10 mL lanthanum solution (3111B.3*f*) before aspirating. When determining Fe or Mn, mix 100 mL with 25 mL of Ca solution (3111B.3*d*) before aspirating. When determining Cr, mix 1 mL 30% H_2O_2 with each 100 mL before aspirating. Alternatively use proportionally smaller volumes.

b. Instrument operation: Because of differences between makes and models of atomic absorption spectrometers, it is not possible to formulate instructions applicable to every instrument. See manufacturer's operating manual. In general, proceed according to the following: Install a hollow-cathode lamp for the desired metal in the instrument and roughly set the wavelength dial according to Table 3111:I. Set slit width according to manufacturer's suggested setting for the element being measured. Turn on instrument, apply to the hollow-cathode lamp the current suggested by the manufacturer, and let instrument warm up until energy source stabilizes, generally about 10 to 20 min. Readjust current as necessary after warmup. Optimize wavelength by adjusting wavelength dial until optimum energy gain is obtained. Align lamp in accordance with manufacturer's instructions.

Install suitable burner head and adjust burner head position. Turn on air and adjust flow rate to that specified by manufacturer to give maximum sensitivity for the metal being measured. Turn on acetylene, adjust flow rate to value specified, and ignite flame. Let flame stabilize for a few minutes. Aspirate a blank consisting of deionized water containing the same concentration of acid in standards and samples. Zero the instrument. Aspirate a standard solution and adjust aspiration rate of the nebulizer to obtain maximum sensitivity. Adjust burner both vertically and horizontally to obtain maximum response. Aspirate blank again and rezero the instrument. Aspirate a standard near the middle of the linear range. Record absorbance of this standard when freshly prepared and with a new hollow-cathode lamp. Refer to these data on subsequent determinations of the same element to check consistency of instrument setup and aging of hollow-cathode lamp and standard.

The instrument now is ready to operate. When analyses are finished, extinguish flame by turning off first acetylene and then air.

c. Standardization: Select at least three concentrations of each standard metal solution (prepared as in 3111B.3*j*) to bracket the expected metal concentration of a sample. Aspirate blank and zero the instrument. Then aspirate each standard in turn into flame and record absorbance.

Prepare a calibration curve by plotting on linear graph paper absorbance of standards versus their concentrations. For instruments equipped with direct concentration readout, this step is unnecessary. With some instruments it may be necessary to convert percent absorption to absorbance by using a table generally provided by the manufacturer. Plot calibration curves for Ca and Mg based on original concentration of standards before dilution with lanthanum solution. Plot calibration curves for Fe and Mn based on original concentration of standards before dilution with Ca solution. Plot calibration curve for Cr based on original concentration of standard before addition of H_2O_2.

d. Analysis of samples: Rinse nebulizer by aspirating water containing 1.5 mL conc HNO_3/L. Aspirate blank and zero instrument. Aspirate sample and determine its absorbance.

5. Calculations

Calculate concentration of each metal ion, in micrograms per liter for trace elements, and in milligrams per liter for more common metals, by referring to the appropriate calibration curve prepared according to 3111B.4*c*. Alternatively, read concentration directly from the instrument readout if the instrument is so equipped. If the sample has been diluted, multiply by the appropriate dilution factor.

6. Bibliography

WILLIS, J.B. 1962. Determination of lead and other heavy metals in urine by atomic absorption spectrophotometry. *Anal. Chem.* 34:614.

Also see 3111A.8 and 9.

3111 C. Extraction/Air-Acetylene Flame Method

1. General Discussion

This method is suitable for the determination of low concentrations of cadmium, chromium, cobalt, copper, iron, lead, manganese, nickel, silver, and zinc. The method consists of chelation with ammonium pyrrolidine dithiocarbamate (APDC) and extraction into methyl isobutyl ketone (MIBK), followed by aspiration into an air-acetylene flame.

2. Apparatus

a. Atomic absorption spectrometer and associated equipment: See 3111A.6.

b. Burner head, conventional. Consult manufacturer's operating manual for suggested burner head.

3. Reagents

a. Air: See 3111B.3*a.*

b. Acetylene: See 3111B.3*b*.

c. Metal-free water: See 3111B.3*c*.

d. Methyl isobutyl ketone (MIBK), reagent grade. For trace analysis, purify MIBK by redistillation or by sub-boiling distillation.

e. Ammonium pyrrolidine dithiocarbamate (APDC) solution: Dissolve 4 g APDC in 100 mL water. If necessary, purify APDC with an equal volume of MIBK. Shake 30 s in a separatory funnel, let separate, and withdraw lower portion. Discard MIBK layer.

f. Nitric acid (HNO_3), conc, ultrapure.

g. Standard metal solutions: See 3111B.3*j*.

h. Potassium permanganate solution ($KMnO_4$), 5% (w/v) aqueous.

i. Sodium sulfate (Na_2SO_4), anhydrous.

j. Water-saturated MIBK: Mix one part purified MIBK with one part water in a separatory funnel. Shake 30 s and let separate. Discard aqueous layer. Save MIBK layer.

k. Hydroxylamine hydrochloride solution, 10% (w/v): This solution can be purchased commercially.

4. Procedure

a. Instrument operation: See 3111B.4*b*. After final adjusting of burner position, aspirate water-saturated MIBK into flame and gradually reduce fuel flow until flame is similar to that before aspiration of solvent.

b. Standardization: Select at least three concentrations of standard metal solutions (prepared as in 3111B.3*j*) to bracket expected sample metal concentration and to be, after extraction, in the optimum concentration range of the instrument. Adjust 100 mL of each standard and 100 mL of a metal-free water blank to pH 3 by adding 1*N* HNO_3 or 1*N* NaOH. For individual element extraction, use the following pH ranges to obtain optimum extraction efficiency:

Element	pH Range for Optimum Extraction
Ag	2–5 (complex unstable)
Cd	1–6
Co	2–10
Cr	3–9
Cu	0.1–8
Fe	2–5
Mn	2–4 (complex unstable)
Ni	2–4
Pb	0.1–6
Zn	2–6

NOTE: For Ag and Pb extraction the optimum pH value is 2.3 ± 0.2. The Mn complex deteriorates rapidly at room temperature, resulting in decreased instrument response. Chilling the extract to 0°C may preserve the complex for a few hours. If this is not possible and Mn cannot be analyzed immediately after extraction, use another analytical procedure.

Transfer each standard solution and blank to individual 200-mL volumetric flasks, add 1 mL APDC solution, and shake to mix. Add 10 mL MIBK and shake vigorously for 30 s. (The maximum volume ratio of sample to MIBK is 40.) Let contents of each flask separate into aqueous and organic layers, then carefully add water (adjusted to the same pH at which the extraction was carried out) down the side of each flask to bring the organic layer into the neck and accessible to the aspirating tube.

Aspirate organic extracts directly into the flame (zeroing instrument on a water-saturated MIBK blank) and record absorbance.

Prepare a calibration curve by plotting on linear graph paper absorbances of extracted standards against their concentrations before extraction.

c. Analysis of samples: Prepare samples in the same manner as the standards. Rinse atomizer by aspirating water-saturated MIBK. Aspirate organic extracts treated as above directly into the flame and record absorbances.

With the above extraction procedure only hexavalent chromium is measured. To determine total chromium, oxidize trivalent chromium to hexavalent chromium by bringing sample to a boil and adding sufficient $KMnO_4$ solution dropwise to give a persistent pink color while the solution is boiled for 10 min. Destroy excess $KMnO_4$ by adding 1 to 2 drops hydroxylamine hydrochloride solution to the boiling solution, allowing 2 min for the reaction to proceed. If pink color persists, add 1 to 2 more drops hydroxylamine hydrochloride solution and wait 2 min. Heat an additional 5 min. Cool, extract with MIBK, and aspirate.

During extraction, if an emulsion forms at the water-MIBK interface, add anhydrous Na_2SO_4 to obtain a homogeneous organic phase. In that case, also add Na_2SO_4 to all standards and blanks.

To avoid problems associated with instability of extracted metal complexes, determine metals immediately after extraction.

5. Calculations

Calculate the concentration of each metal ion in micrograms per liter by referring to the appropriate calibration curve.

6. Bibliography

ALLAN, J.E. 1961. The use of organic solvents in atomic absorption spectrophotometry. *Spectrochim. Acta* 17:467.

SACHDEV, S.L. & P.W. WEST. 1970. Concentration of trace metals by solvent extraction and their determination by atomic absorption spectrophotometry. *Environ. Sci. Technol.* 4:749.

3111 D. Direct Nitrous Oxide-Acetylene Flame Method

1. General Discussion

This method is applicable to the determination of aluminum, barium, beryllium, calcium, molybdenum, osmium, rhenium, silicon, thorium, titanium, and vanadium.

2. Apparatus

a. Atomic absorption spectrometer and associated equipment: See 3111A.6.

b. Nitrous oxide burner head: Use special burner head as suggested in manufacturer's manual. At roughly 20-min intervals of operation it may be necessary to dislodge the carbon crust that forms along the slit surface with a carbon rod or appropriate alternative.

c. T-junction valve or other switching valve for rapidly changing from nitrous oxide to air, so that flame can be turned on or off with air as oxidant to prevent flashbacks.

3. Reagents

a. Air: See 3111B.3*a*.

b. Acetylene: See 3111B.3*b*.

c. Metal-free water: See 3111B.3*c*.

d. Hydrochloric acid (HCl), 1*N,* 1+1, and conc.

e. Nitric acid (HNO_3), conc.

f. Sulfuric acid (H_2SO_4), 1% (v/v).

g. Hydrofluoric acid (HF), 1*N.*

h. Nitrous oxide, commercially available cylinders. Fit nitrous oxide cylinder with a special nonfreezable regulator or wrap a heating coil around an ordinary regulator to prevent flashback at the burner caused by reduction in nitrous oxide flow through a frozen regulator. (Most modern atomic absorption instruments have automatic gas control systems that will shut down a nitrous oxide-acetylene flame safely in the event of a reduction in nitrous oxide flow rate.)

CAUTION: Use nitrous oxide with strict adherence to manufacturer's directions. Improper sequencing of gas flows at startup and shutdown of instrument can produce explosions from flashback.

i. Potassium chloride solution: Dissolve 250 g KCl in water and dilute to 1000 mL.

j. Aluminum nitrate solution: Dissolve 139 g $Al(NO_3)_3 \cdot 9H_2O$ in 150 mL water. Acidify slightly with conc HNO_3 to preclude possible hydrolysis and precipitation. Warm to dissolve completely. Cool and dilute to 200 mL.

k. Standard metal solutions: Prepare a series of standard metal solutions in the optimum concentration ranges by appropriate dilution of stock metal solutions with water containing 1.5 mL conc HNO_3/L. Stock standard solutions are available from a number of commercial suppliers. Alternatively, prepare as described below.

1) *Aluminum*—Dissolve 0.100 g aluminum metal in an acid mixture of 4 mL 1 + 1 HCl and 1 mL conc HNO_3 in a beaker. Warm gently to effect solution. Transfer to a 1-L flask, add 10 mL 1 + 1 HCl, and dilute to 1000 mL with water; 1.00 mL = 100 μg Al.

2) *Barium*—Dissolve 0.1516 g $BaCl_2$ (dried at 250° for 2 h), in about 10 mL water with 1 mL 1+ 1 HCl. Add 10.0 mL 1 + 1 HCl and dilute to 1000 mL with water; 1.00 mL = 100 μg Ba.

3) *Beryllium—Do not dry.* Dissolve 1.966 g $BeSO_4 \cdot 4H_2O$ in water, add 10.0 mL conc HNO_3, and dilute to 1000 mL with water; 1.00 mL = 100 μg Be.

4) *Calcium*—See 3111B.3*j*4).

5) *Molybdenum*—Dissolve 0.2043 g $(NH_4)_2$ MoO_4 in water and dilute to 1000 mL; 1.00 mL = 100 μg Mo.

6) *Osmium*—Obtain standard 0.1*M* osmium tetroxide solution* and store in glass bottle; 1.00 mL = 19.02 mg Os. Make dilutions daily as needed using 1% (v/v) H_2SO_4. **CAUTION: OsO_4 is extremely toxic and highly volatile.**

7) *Rhenium*—Dissolve 0.1554 g potassium perrhenate, $KReO_4$, in 200 mL water. Dilute to 1000 mL with 1% (v/v) H_2SO_4; 1.00 mL = 100 μg Re.

8) *Silica—Do not dry.* Dissolve 0.4730 g $Na_2SiO_3 \cdot 9H_2O$ in water. Add 10.0 mL conc HNO_3 and dilute to 1000 mL with water. 1.00 mL = 100 μg SiO_2. Store in polyethylene.

9) *Thorium*—Dissolve 0.238 g thorium nitrate, $Th(NO_3)_4 \cdot 4H_2O$ in 1000 mL water; 1.00 mL = 100 μg Th.

10) *Titanium*—Dissolve 0.3960 g pure (99.8 or 99.9%) titanium chloride, $TiCl_4$,† in a mixture of equal volumes of 1*N* HCl and 1*N* HF. Make up to 1000 mL with this acid mixture; 1.00 mL = 100 μg Ti.

11) *Vanadium*—Dissolve 0.2297 g ammonium metavanadate, NH_4VO_3, in a minimum amount of conc HNO_3. Heat to dissolve. Add 10 mL conc HNO_3, and dilute to 1000 mL with water; 1.00 mL = 100 μg V.

* GFS Chemicals, Inc., Columbus, OH, Cat. No. 64, or equivalent.

† Alpha Ventron, P.O. Box 299, 152 Andover St., Danvers, MA 01923, or equivalent.

4. Procedure

a. Sample preparation: See 3111B.4*a*.

When determining Al, Ba, or Ti, mix 2 mL KCl solution into 100 mL sample and standards before aspiration. When determining Mo and V, mix 2 mL $Al(NO_3)_3 \cdot 9H_2O$ into 100 mL sample and standards before aspiration.

b. Instrument operation: See 3111B.4*b*. After adjusting wavelength, install a nitrous oxide burner head. Turn on acetylene (without igniting flame) and adjust flow rate to value specified by manufacturer for a nitrous oxide-acetylene flame. Turn off acetylene. With both air and nitrous oxide supplies turned on, set T-junction valve to nitrous oxide and adjust flow rate according to manufacturer's specifications. Turn switching valve to the air position and verify that flow rate is the same. Turn acetylene on and ignite to a bright yellow flame. With a rapid motion, turn switching valve to nitrous oxide. The flame should have a red cone above the burner. If it does not, adjust fuel flow to obtain red cone. After nitrous oxide flame has been ignited, let burner come to thermal equilibrium before beginning analysis.

Aspirate a blank consisting of deionized water containing 1.5 mL conc HNO_3/L and check aspiration rate. Adjust if necessary to a rate between 3 and 5 mL/ min. Zero the instrument. Aspirate a standard of the desired metal with a concentration near the midpoint of the optimum concentration range and adjust burner (both horizontally and vertically) in the light path to obtain maximum response. Aspirate blank again and re-zero the instrument. The instrument now is ready to run standards and samples.

To extinguish flame, turn switching valve from nitrous oxide to air and turn off acetylene. This procedure eliminates the danger of flashback that may occur on direct ignition or shutdown of nitrous oxide and acetylene.

c. Standardization: Select at least three concentrations of standard metal solutions (prepared as in 3111D.3*k*) to bracket the expected metal concentration of a sample. Aspirate each in turn into the flame and record absorbances.

Most modern instruments are equipped with microprocessors and digital readout which permit calibration in direct concentration terms. If instrument is not so equipped, prepare a calibration curve by plotting on linear graph paper absorbance of standards versus concentration. Plot calibration curves for Al, Ba, and Ti based on original concentration of standard before adding KCl solution. Plot calibration curves for Mo and V based on original concentration of standard before adding $Al(NO_3)_3$ solution.

d. Analysis of samples: Rinse atomizer by aspirating water containing 1.5 mL conc HNO_3/L and zero instrument. Aspirate a sample and determine its absorbance.

5. Calculations

Calculate concentration of each metal ion in micrograms per liter by referring to the appropriate calibration curve prepared according to 3111D.4*c*.

Alternatively, read the concentration directly from the instrument readout if the instrument is so equipped. If sample has been diluted, multiply by the appropriate dilution factor.

6. Bibliography

WILLIS, J.B. 1965. Nitrous oxide-acetylene flame in atomic absorption spectroscopy. *Nature* 207:715.

Also see 3111A.8 and 9.

3111 E. Extraction/Nitrous Oxide-Acetylene Flame Method

1. General Discussion

a. Application: This method is suitable for the determination of aluminum at concentrations less than 900 μg/L and beryllium at concentrations less than 30 μg/L. The method consists of chelation with 8-hydroxyquinoline, extraction with methyl isobutyl ketone (MIBK), and aspiration into a nitrous oxide-acetylene flame.

b. Interferences: Concentrations of Fe greater than 10 mg/L interfere by suppressing Al absorption. Iron interference can be masked by addition of hydroxylamine hydrochloride/1,10-phenanthroline. Mn concentrations up to 80 mg/L do not interfere if turbidity in the extract is allowed to settle. Mg forms an insoluble chelate with 8-hydroxyquinoline at pH 8.0 and tends to remove Al complex as a coprecipitate. However, the Mg complex forms slowly over 4 to 6 min; its interference can be avoided if the solution is extracted immediately after adding buffer.

2. Apparatus

Atomic absorption spectrometer and associated equipment: See 3111A.6.

3. Reagents

a. Air: See 3111B.3*a*.

b. Acetylene: See 3111B.3*b*.

c. Ammonium hydroxide (NH_4OH), conc.

d. Buffer: Dissolve 300 g ammonium acetate, $NH_4C_2H_3O_2$, in water, add 105 mL conc NH_4OH, and dilute to 1 L.

e. Metal-free water: See 3111B.3*c*.

f. Hydrochloric acid (HCl), conc.

g. 8-Hydroxyquinoline solution: Dissolve 20 g 8-hydroxyquinoline in about 200 mL water, add 60 mL glacial acetic acid, and dilute to 1 L with water.

h. Methyl isobutyl ketone: See 3111C.3*d*.

i. Nitric acid (HNO_3), conc.

j. Nitrous oxide: See 3111D.3*h*.

k. Standard metal solutions: Prepare a series of standard metal solutions containing 5 to 1000 μg/L by appropriate dilution of the stock metal solutions prepared according to 3111D.3*k*.

l. Iron masking solution: Dissolve 1.3 g hydroxylamine hydrochloride and 6.58 g 1,10-phenanthroline monohydrate in about 500 mL water and dilute to 1 L with water.

4. Procedure

a. Instrument operation: See 3111B.4*b*, C.4*a*, and D.4*b*. After final adjusting of burner position, aspirate MIBK into flame and gradually reduce fuel flow until flame is similar to that before aspiration of solvent. Adjust wavelength setting according to Table 3111:I.

b. Standardization: Select at least three concentrations of standard metal solutions (prepared as in 3111E.3*k*) to bracket the expected metal concentration of a sample and transfer 100 mL of each (and 100 mL water blank) to four different 200-mL volumetric flasks. Add 2 mL 8-hydroxyquinoline solution, 2 mL masking solution (if required), and 10 mL buffer to one flask, immediately add 10 mL MIBK, and shake vigorously. The duration of shaking affects the forms of aluminum complexed. A fast, 10-s shaking time favors monomeric Al, whereas 5 to 10 min of shaking also will complex polymeric species. Adjustment of the 8-hydroxyquinoline to sample ratio can improve recoveries of extremely high or low concentrations of aluminum. Treat each blank, standard, and sample in similar fashion. Continue as in 3111C.4*b*.

c. Analysis of samples: Rinse atomizer by aspirating water-saturated MIBK. Aspirate extracts of samples treated as above, and record absorbances.

5. Calculations

Calculate concentration of each metal in micrograms per liter by referring to the appropriate calibration curve prepared according to 3111E.4*b*.

3112 METALS BY COLD-VAPOR ATOMIC ABSORPTION SPECTROMETRY*

3112 A. Introduction

For general introductory material on atomic absorption spectrometric methods, see Section 3111A.

* Approved by Standard Methods Committee, 2009. Editorial revisions, 2011.

3112 B. Cold-Vapor Atomic Absorption Spectrometric Method

1. General Discussion

This method is applicable to the determination of mercury.

The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

When possible, dedicate glassware for use in Hg analysis. Avoid using glassware previously exposed to high levels of Hg, such as those used in COD, TKN, or Cl^- analysis.

a. Atomic absorption spectrometer and associated equipment: See Section 3111A.6. Instruments and accessories specifically designed for measuring mercury via the cold vapor technique are available commercially and may be substituted.

b. Absorption cell, a glass or plastic tube approximately 2.5 cm in diameter. An 11.4-cm-long tube has been found satisfactory, but a 15-cm-long tube is preferred. Grind tube ends perpendicular to the longitudinal axis, and cement quartz windows in place. Attach gas inlet and outlet ports (6.4 mm diam) 1.3 cm from each end.

c. Cell support: Strap cell to the flat nitrous-oxide burner head or other suitable support and align in light beam to give maximum transmittance.

d. Air pumps: Use any peristaltic pump with electronic speed control capable of delivering an air flow of 2 L/min. Any other regulated compressed air system or air cylinder also is satisfactory.

e. Flowmeter, capable of measuring an air flow of 2 L/min.

f. Aeration tubing, a straight glass frit having a coarse porosity for use in reaction flask.

g. Reaction flask, 250-mL Erlenmeyer flask or a BOD bottle, fitted with a rubber stopper to hold aeration tube.

h. Drying tube, 150-mm × 18-mm-diam, containing 20 g $Mg(ClO_4)_2$. A 60-W light bulb with a suitable shade may be substituted to prevent condensation of moisture inside the absorption cell. Position bulb to maintain cell temperature at 10°C above ambient.

i. Connecting tubing, glass tubing to pass mercury vapor from reaction flask to absorption cell and to interconnect all other components. Clear vinyl plastic tubing* may be substituted for glass.

* Tygon, or equivalent.

3. Reagents†

a. Metal-free water: See Section 3111B.3*c*.

b. Stock mercury solution: Dissolve 0.1354 g mercuric chloride ($HgCl_2$) in about 70 mL water, add 1 mL conc HNO_3, and dilute to 100 mL with water; 1.00 mL = 1.00 mg Hg.

c. Standard mercury solutions: Prepare a series of standard mercury solutions containing 0 to 5 μg/L by appropriate dilution of stock mercury solution with water containing 10 mL conc HNO_3/L. Prepare standards daily.

d. Nitric acid (HNO_3), conc.

e. Potassium permanganate solution: Dissolve 50 g $KMnO_4$ in water and dilute to 1 L.

f. Potassium persulfate solution: Dissolve 50 g $K_2S_2O_8$ in water and dilute to 1 L.

g. Sodium chloride-hydroxylamine sulfate solution: Dissolve 120 g NaCl and 120 g $(NH_2OH)_2 \cdot H_2SO_4$ in water and dilute to 1 L. A 10% hydroxylamine hydrochloride solution may be substituted for the hydroxylamine sulfate.

h. Stannous ion (Sn^{2+}) solution: Use either stannous chloride, ¶ *h*1) below, or stannous sulfate, ¶ *h*2) below, to prepare this solution containing about 7.0 g Sn^{2+}/100 mL.

1) Dissolve 10 g $SnCl_2$ in water containing 20 mL conc HCl and dilute to 100 mL.

2) Dissolve 11 g $SnSO_4$ in water containing 7 mL conc H_2SO_4 and dilute to 100 mL.

Both solutions decompose with aging. If a suspension forms, stir reagent continuously during use. Reagent volume is sufficient to process about 20 samples; adjust volumes prepared to accommodate number of samples processed.

i. Sulfuric acid (H_2SO_4), conc.

4. Procedure

a. Instrument operation: See Section 3111B.4*b*. Set wavelength to 253.7 nm. Install absorption cell and align in light path to give maximum transmission. Connect associated equipment to absorption cell with glass or vinyl plastic tubing, as indicated in Figure 3112:1. Turn on air and adjust flow rate to 2 L/min. Allow air to flow continuously. Alternatively, follow manufacturer's directions for operation. NOTE: Fluorescent lighting may increase baseline noise.

† Use specially prepared reagents low in mercury.

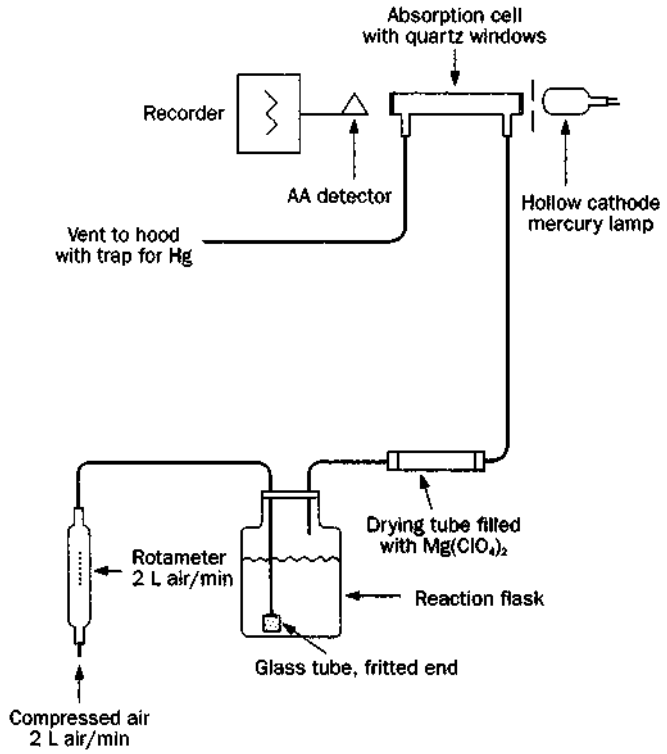


Figure 3112:1. Schematic arrangement of equipment for measuring mercury by cold-vapor atomic absorption technique.

b. Standardization: Transfer 100 mL each of the 1.0, 2.0, and 5.0 μg/L Hg standard solutions and a blank of 100 mL water to 250-mL Erlenmeyer reaction flasks. Add 5 mL conc H_2SO_4 and 2.5 mL conc HNO_3 to each flask. Add 15 mL $KMnO_4$ solution to each flask and let stand at least 15 min. Add 8 mL $K_2S_2O_8$ solution to each flask and heat for 2 h in a water bath at 90 to 95°C. Cool to room temperature.

Treating each flask individually, add enough NaCl-hydroxylamine solution to reduce excess $KMnO_4$, then add 5 mL $SnCl_2$ or $SnSO_4$ solution and immediately attach flask to aeration apparatus. As Hg is volatilized and carried into the absorption cell, absorbance will increase to a maximum within a few seconds. As soon as recorder returns approximately to the baseline, remove stopper holding the frit from reaction flask, and replace with a flask containing water. Flush system for a few seconds, and run the next standard in the same manner. Construct a standard curve by plotting peak height versus micrograms Hg.

c. Analysis of samples: Transfer 100 mL sample or portion diluted to 100 mL containing not more than 5.0 μg Hg/L to a reaction flask. Treat as in 3112B.4*b*. Seawaters, brines, and effluents high in chlorides require as much as another 25 mL $KMnO_4$ solution. During oxidation step, chlorides are converted to free chlorine, which absorbs at 253 nm. Remove all free chlorine before the Hg is reduced and swept into the cell by using an excess (25 mL) of hydroxylamine reagent.

Remove free chlorine by sparging sample gently with air or nitrogen after adding hydroxylamine reducing solution. Use a separate tube and frit to avoid carryover of residual stannous chloride, which could cause reduction and loss of mercury.

Table 3112:I. Interlaboratory Precision and Bias of Cold-Vapor Atomic Absorption Spectrometric Method for Mercury[1]

Form	Conc. μg/L	SD μg/L	Relative SD %	Relative Error %	No. of Participants
Inorganic	0.34	0.077	22.6	21.0	23
Inorganic	4.2	0.56	13.3	14.4	21
Organic	4.2	0.36	8.6	8.4	21

5. Calculation

Determine peak height of sample from recorder chart and read mercury value from standard curve prepared according to 3112B.4*b*.

6. Precision and Bias

Data on interlaboratory precision and bias for this method are given in Table 3112:I.

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3113 METALS BY ELECTROTHERMAL ATOMIC ABSORPTION SPECTROMETRY*

3113 A. Introduction

1. Applications

Electrothermal atomic absorption permits determination of most metallic elements with sensitivities and detection levels from 20 to 1000 times better than those of conventional flame techniques without extraction or sample concentration. This increase in sensitivity results from an increase in atom density within the furnace as compared to flame atomic absorption. Many elements can be determined at concentrations of, or below, 1.0 μg/L. An additional advantage of electrothermal atomic absorption is that only a very small volume of sample is required.

The electrothermal technique is used only at concentration levels below the optimum range of direct flame atomic absorption because it is subject to more interferences than the flame procedure and requires increased analysis time. The method of standard additions may be required to ensure validity of data. Because of the high sensitivity of this technique, it is extremely susceptible to contamination; extra care in sample handling and analysis may be required.

2. Principle

Electrothermal atomic absorption spectroscopy is based on the same principle as direct flame atomization but an electrically heated atomizer or graphite furnace replaces the standard burner head. A discrete sample volume is dispensed into the graphite sample tube (or cup). Typically, determinations are made by heating the sample in three or more stages. First, a low current heats the tube to dry the sample. The second, or charring, stage destroys organic matter and volatilizes other matrix components at an intermediate temperature. Finally, a high current heats the tube to incandescence and, in an inert atmosphere, atomizes the element being determined. Additional stages frequently are added to aid in drying and charring, and to clean and cool the tube between samples. The resultant ground-state atomic vapor absorbs monochromatic radiation from the source. A photoelectric detector measures the intensity of transmitted radiation. The inverse of the transmittance is related logarithmically to the absorbance, which is directly proportional to the number density of vaporized ground-state atoms (the Beer-Lambert law) over a limited concentration range.

3. Interferences

Electrothermal atomization determinations may be subject to significant interferences from molecular absorption as well as chemical and matrix effects. Molecular absorption may occur when components of the sample matrix volatilize during atomization, resulting in broadband absorption. Several background correction techniques are available commercially to compensate for this interference. A continuum source such as a deuterium arc can correct for background up to absorbance levels of about 0.8. Continuum lamp intensity diminishes at long wavelengths and use of continuum background correction is limited to analytical wavelengths below 350 nm. Zeeman effect background correctors can handle background absorbance up to 1.5 to 2.0. The self-reversal (e.g., Smith-Hieftje) correction technique can accommodate background absorbance levels as large as 2.5 to 3.0 (see Section 3111A.3*b*). Both Zeeman and self-reversal background corrections are susceptible to rollover (development of a negative absorbance-concentration relationship) at high absorbances. The rollover absorbance for each element should be available in the manufacturer's literature. Curvature due to rollover should become apparent during calibration; dilution produces a more linear calibration plot. Use background correction when analyzing samples containing high concentrations of acid or dissolved solids and in determining elements for which an absorption line below 350 nm is used.

Matrix modification can be useful in minimizing interference and increasing analytical sensitivity. Determine need for a modifier by evaluating recovery of a sample with a known addition. Recovery near 100% indicates that sample matrix does not affect analysis. Chemical modifiers generally modify relative volatilities of matrix and metal. Some modifiers enhance matrix removal, isolating the metal, while other modifiers inhibit metal volatilization, allowing use of higher ashing/charring temperatures and increasing efficiency of matrix removal. Chemical modifiers are added at high concentration (percent level) and can lead to sample contamination from impurities in the modifier solution. Heavy use of chemical modifiers may reduce the useful life (normally 50 to 100 firings) of the graphite tube. Nonetheless, the use of matrix modifiers is encouraged. Some specific chemical modifiers and approximate concentrations are listed in Table 3113:I.

Addition of a chemical modifier directly to the sample before analysis is restricted to inexpensive additives (e.g., phosphoric acid). Use of palladium salts for matrix modification normally requires methods of co-addition, in which sample and modifier are added consecutively to the furnace either manually or, preferably, with an automatic sampler. Palladium salts (nitrate is preferred, chloride is acceptable) are listed in Table 3113:I as a modifier for many metals. The palladium solution (50 to 2000 mg/L) generally includes citric or ascorbic acid, which aids reduction of palladium in the furnace. Citric acid levels of 1 to 2% are typical. Use of hydrogen (5%) in the purge gas (available commercially as a mixture) also reduces palladium, eliminating need for organic reducing acids. **CAUTION: Do not mix hydrogen and other gases in the laboratory; hydrogen gas is very flammable—handle with caution**. Use low levels of pal-

* Approved by Standard Methods Committee, 2010.
Joint Task Group: 20th Edition—Raymond J. Lovett (chair), David J. Kaptain.

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Table 3113:I. Potential Matrix Modifiers for Electrothermal Atomic Absorption Spectrometry*

Modifier	Analyses for Which Modifier May Be Useful
1500 mg Pd/L + 1000 mg $Mg(NO_3)_2$/L[1]	Ag, As, Au, Bi, Cu, Ge, Mn, Hg, In, Sb, Se, Sn, Te, Tl
500–2000 mg Pd/L + reducing agent[2]†	Ag, As, Bi, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Sb
5000 mg $Mg(NO_3)_2$/L[1]	Be, Co, Cr, Fe, Mn, V
100–500 mg Pd/L[2]	As, Ga, Ge, Sn
50 mg Ni/L[2]	As, Se, Sb
2% PO_4^{3-} + 1000 mg Mg $(NO_3)_2$/L[1]	Cd, Pb

* Assumes 10 µL modifier/10 µL sample.
† Citric acid (1–2%) preferred; ascorbic acid or H_2 acceptable.

Table 3113:II. Detection Levels and Concentration Ranges for Electrothermal Atomization Atomic Absorption Spectrometry

Element	Wavelength *nm*	Estimated Detection Level *µg/L*	Optimum Concentration Range *µg/L*
Al	309.3	3	20–200
Sb	217.6	0.8	20–300
As	193.7	0.5	5–100
Ba	553.6	2	10–200
Be	234.9	0.02	1–30
Cd	228.8	0.05	0.5–10
Cr	357.9	0.1	5–100
Co	240.7	0.7	5–100
Cu	324.7	0.7	5–100
Fe	248.3	1	5–100
Pb*	283.3	0.7	5–100
Mn	279.5	0.2	1–30
Mo	313.3	1	3–60
Ni	232.0	0.6	5–100
Se	196.0	0.6	5–100
Ag	328.1	0.2	1–25
Sn	224.6	1.7	20–300

* The more sensitive 217.0-nm wavelength is recommended for instruments with background correction capabilities.

ladium (50 to 250 mg/L) for normal samples and higher levels for complex samples. Addition of excess palladium modifier may widen atomization peaks; in such cases peak area measurements may provide higher quality results. The recommended mode of modifier use is through co-addition to the furnace of about 10 µL of the palladium (or other) modifier solution. Palladium may not be the best modifier in all cases and cannot be recommended unconditionally. Test samples requiring a modifier first with palladium; test other modifiers only if palladium is unsuccessful or to minimize modifier cost. See 3113B.3*d* for preparation of modifier solution.

Temperature ramping (i.e., gradual heating) can be used to decrease background interferences and permits analysis of samples with complex matrices. Ramping permits a controlled, continuous increase of furnace temperature in any of the various steps of the temperature sequence. Ramp drying is used for samples containing mixtures of solvents or for samples with a high salt content (to avoid spattering). If spattering is suspected, develop drying ramp by visual inspection of the drying stage, using a mirror. Samples that contain a complex mixture of matrix components sometimes require ramp charring to effect controlled, complete thermal decomposition. Ramp atomization may minimize background absorption by permitting volatilization of the element being determined before the matrix. This is especially applicable in the determination of such volatile elements as cadmium and lead. Use of time-resolved absorbance profiles (available on most modern instruments) greatly aids method development. Changes in atomization, notably the element peak appearance time and magnitude of background and metal absorbances, can be monitored directly.

Improve analysis by using a graphite platform, inserted into the graphite tube, as the atomization site. The platform is not heated as directly by the current flowing through the graphite tube; thus the metal atomizes later and under more uniform conditions.

Use standard additions to compensate for matrix interferences. When making standard additions, determine whether the added metal and that in the sample behave similarly under the specified conditions. [See 3113B.4*d*2)]. In the extreme, test every sample for recovery (85 to 115% recovery desired) to determine whether modification of the temperature program or use of the method of standard additions is needed. Test every new sample type for recovery. Recovery of only 40 to 85% generally indicates that standard addition is required. Often, as long as the samples are from sources of consistent properties, a representative recovery can be used to characterize the analysis and determine the necessity of standard addition. Test samples of unknown origin or of complex composition (digestates, for example) individually for metal recovery. Ideally, chemical modifiers and graphite platforms render the sample fit to be analyzed using a standard analytical calibration curve. Always verify this assumption; however, a properly developed method with judicious use of chemical modifiers should eliminate the necessity for standard addition in all but the most extreme samples.

Chemical interaction of the graphite tube with various elements to form refractory carbides occurs at high charring and atomization temperatures. Elements that form carbides are barium, molybdenum, nickel, titanium, vanadium, and silicon. Carbide formation is characterized by broad, tailing atomization peaks and reduced sensitivity. Using pyrolytically coated tubes for these metals minimizes the problem.

4. Sensitivity, Detection Levels, and Optimum Concentration Range

Estimated detection levels and optimum concentration ranges are listed in Table 3113:II. These values may vary with the chemical form of the element being determined, sample composition, or instrumental conditions.

For a given sample, increased sensitivity may be achieved by using a larger sample volume or by reducing flow rate of the purge gas or by using gas interrupt during atomization. Note, however, that these techniques also will increase the effects of any interferences present. Sensitivity can be de-

creased by diluting the sample, reducing sample volume, increasing purge-gas flow, or using a less sensitive wavelength. Use of argon, rather than nitrogen, as the purge gas generally improves sensitivity and reproducibility. Hydrogen mixed with the inert gas may suppress chemical interference and increase sensitivity by acting as a reducing agent, thereby aiding in producing more ground-state atoms. Pyrolytically coated graphite tubes can increase sensitivity for the more refractory elements and are recommended. The optical pyrometer/maximum power accessory available on some instruments also offers increased sensitivity with lower atomization temperatures for many elements.

Using the Stabilized Temperature Platform Furnace (STPF) technique, which is a combination of individual techniques, also offers significant interference reduction with improved sensitivity. Sensitivity changes with sample tube age. Discard graphite tubes when significant variations in sensitivity or poor reproducibility are observed. The use of high acid concentrations, brine samples, and matrix modifiers often drastically reduces tube life. Preferably use the graphite platform in such situations.

5. References

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3113 B. Electrothermal Atomic Absorption Spectrometric Method

1. General Discussion

This method is suitable for determination of micro quantities of aluminum, antimony, arsenic, barium, beryllium, cadmium, chromium, cobalt, copper, iron, lead, manganese, molybdenum, nickel, selenium, silver, and tin.

2. Apparatus

a. Atomic absorption spectrometer: See Section 3111A.6*a*. The instrument must have background correction capability.

b. Source lamps: See Section 3111A.6*d*.

c. Graphite furnace: Use an electrically heated device with electronic control circuitry designed to carry a graphite tube or cup through a heating program that provides sufficient thermal energy to atomize the elements of interest. Furnace heat controllers with only three heating steps are adequate only for fresh waters with low dissolved solids content. For salt waters, brines, and other complex matrices, use a furnace controller with up to seven individually programmed heating steps. Fit the furnace into the sample compartment of the spectrometer in place of the conventional burner assembly. Use argon as a purge gas to minimize oxidation of the furnace tube and to prevent the formation of metallic oxides. Use graphite tubes with platforms to minimize interferences and to improve sensitivity.

d. Readout: See Section 3111A.6*c*.

e. Sample dispensers: Use microliter pipets (5 to 100 μL) or an automatic sampling device designed for the specific instrument.

f. Vent: See Section 3111A.6*f*.

g. Cooling water supply: Cool with tap water flowing at 1 to 4 L/min or use a recirculating cooling device.

h. Membrane filter apparatus: Use an all-glass filtering device and 0.45-μm or smaller-pore-diameter membrane filters. For trace analysis of aluminum, use polypropylene or TFE devices.

3. Reagents

a. Metal-free water: See Section 3111B.3*c*.

b. Hydrochloric acid (HCl), 1 + 1 and conc.

c. Nitric acid (HNO_3), 1 + 1 and conc.

d. Matrix modifier stock solutions:

1) *Magnesium nitrate*, 10 000 mg Mg/L—Dissolve 10.5 g $Mg(NO_3)_2 \cdot 6H_2O$ in water. Dilute to 100 mL.

2) *Nickel nitrate*, 10 000 mg Ni/L—Dissolve 4.96 g $Ni(NO_3)_2 \cdot 6H_2O$ in water. Dilute to 100 mL.

3) *Phosphoric acid*, 10% (v/v)—Add 10 mL conc H_3PO_4 to water. Dilute to 100 mL.

4) *Palladium nitrate*, 4000 mg Pd/L—Dissolve 9.34 g $Pd(NO_3)_2 \cdot H_2O$ in water. Dilute to 1 L.

5) *Citric acid*, 4%—Dissolve 40 g citric acid in water. Dilute to 1 L.

NOTE: All of the modifier solutions recommended in Table 3113:I can be prepared with volumetric combination of the above solutions and water. For preparation of other matrix modifiers, see references or follow manufacturers' instructions.

e. Stock metal solutions: Refer to Sections 3111B.3*j* and 3114B.3.

f. Chelating resin: 100 to 200 mesh* purified by heating at 60°C in 10*N* NaOH for 24 h. Cool resin and rinse 10 times each with alternating portions of 1*N* HCl, metal-free water, 1*N* NaOH, and metal-free water.

g. Metal-free seawater (or brine): Fill a 1.4-cm-ID × 20-cm-long borosilicate glass column to within 2 cm of the top with purified chelating resin. Elute resin with successive 50-mL portions of 1*N* HCl, metal-free water, 1*N* NaOH, and metal-free water at the rate of 5 mL/min just before use. Pass salt water or brine through the column at a rate of 5 mL/min to extract trace metals present. Discard the first 10 bed volumes (300 mL) of eluate.

4. Procedures

a. Sample pretreatment: Before analysis, pretreat all samples as indicated below. Rinse all glassware with 1 + 1 HNO_3 and water. Carry out digestion procedures in a clean, dust-free laboratory area to avoid sample contamination. For digestion of trace aluminum, use polypropylene or TFE utensils to avoid leachable aluminum from glassware.

1) Dissolved metals and metals where sample turbidity is <1 NTU—See Section 3030B. For samples requiring arsenic and/or selenium analysis add 3 mL 30% hydrogen peroxide/100 mL sample and an appropriate volume of nickel nitrate solution (see Table 3113:I) before analysis. Nickel may be deleted if palladium is co-added during analysis. For all other metals no further pretreatment is required except for adding a matrix modifier when necessary.

2) Total recoverable metals (Al, Sb, Ba, Be, Cd, Cr, Co, Cu, Fe, Pb, Mn, Mo, Ni, Ag, and Sn)—NOTE: Sb and Sn are not recovered unless HCl is used in the digestion. See Section 3030D. Quantitatively transfer digested sample to a 100-mL volumetric flask, add an appropriate amount of matrix modifier (see Table 3113:I), and dilute to volume with water.

3) Total recoverable metals (As, Se)—Transfer 100 mL of shaken sample, 1 mL conc HNO_3, and 2 mL 30% H_2O_2 to a clean, acid-washed 250-mL beaker. Heat on a hot plate without allowing solution to boil until volume has been reduced to about 50 mL. Remove from hot plate and let cool to room temperature. Add an appropriate concentration of nickel (see Table 3113:I), and dilute to volume in a 100-mL volumetric flask with water. Substitution of palladium is uneconomical. Nickel may be deleted if palladium is co-added during analysis. Simultaneously prepare a digested blank by substituting water for sample and proceed with digestion as described above.

b. Instrument operation: Mount and align furnace device according to manufacturer's instructions. Turn on instrument and data collection system. Select appropriate light source and adjust to recommended electrical setting. Select proper wavelength and set all conditions according to manufacturer's instructions, including background correction. Background correction is important when elements are determined at short wavelengths or when sample has a high level of dissolved solids. Background correction normally is not necessary at wavelengths longer than 350 nm. If background correction above 350 nm is needed, deuterium arc background correction is not useful and other types must be used.

Select proper inert- or sheath-gas flow. In some cases, it is desirable to interrupt the inert-gas flow during atomization. Such interruption results in increased sensitivity by increasing residence time of the atomic vapor in the optical path. Gas interruption also increases background absorption and intensifies interference effects, but modern background correction methods usually eliminate these problems. Consider advantages and disadvantages of this option for each matrix when optimizing analytical conditions.

To optimize graphite furnace conditions, carefully adjust furnace temperature settings to maximize sensitivity and precision and to minimize interferences. Follow manufacturer's instructions.

Use drying temperatures slightly above the solvent boiling point and provide enough time and temperature for complete evaporation without boiling or spattering.

Select atomization temperature by determining the lowest temperature providing maximum sensitivity without significantly eroding precision. Optimize by a series of successive determinations at various atomization temperatures using a standard solution giving an absorbance of 0.2 to 0.5.

The charring temperature must be high enough to maximize volatilization of interfering matrix components yet too low to volatilize the element of interest. With the drying and atomization temperatures set to their optimum values, analyze a standard solution at a series of charring temperatures in increasing increments of 50 to 100°C. When the optimum charring temperature is exceeded, there will be a significant drop in sensitivity. Plot charring temperature versus sample absorbance: the optimum charring temperature is the highest temperature without reduced sensitivity. Verify optimization with major changes in sample matrix.

c. Instrument calibration: Prepare standard solutions for instrument calibration by diluting metal stock solutions. Prepare standard solutions every 2 weeks, or more frequently as needed if standard response indicates degradation of standard. A decrease in signal of more than 10% requires investigation and indicates the possible need for fresh standards.

Prepare a blank and at least three calibration standards in the appropriate concentration range (see Table 3113:II) for correlating element concentration and instrument response. Match the matrix of the standard solutions to those of the samples as closely as possible. In most cases, this simply requires matching the acid background of the samples. For seawaters or brines, however, use the metal-free matrix (3113B.3*g*) as the standard solution diluent. In addition, add the same concentration of matrix modifier (if required for sample analysis) to the standard solutions.

* Chelex 100, Bio-Rad Laboratories, Richmond, CA, or equivalent.

Inject a suitable portion of each standard solution, in order of increasing concentration. Analyze each standard solution in duplicate to verify method precision.

Construct an analytical curve by plotting the average peak absorbances or peak areas of the standard solution versus concentration on linear graph paper. Alternatively, use electronic instrument calibration if the instrument has this capability.

d. Sample analysis: Analyze all samples except those demonstrated to be free of matrix interferences (based on recoveries of 85 to 115% for known additions) using the method of standard additions. Analyze all samples at least in duplicate or until reproducible results are obtained. A variation of ≤10% is considered acceptable reproducibility. Average replicate values.

1) Direct determination—Inject a measured portion of pretreated sample into the graphite furnace. Use the same volume as was used to prepare the calibration curve. Usually add modifier immediately after the sample, preferably using an automatic sampler or a micropipet. Some methods require modifier to be injected before the sample. Co-addition of the modifier is acceptable. Use the same volume and concentration of modifier for all standards and samples. Dry, char, and atomize according to the preset program. Repeat until reproducible results are obtained.

Compare the average absorbance value or peak area to the calibration curve to determine concentration of the element of interest. Alternatively, read results directly if the instrument is equipped with this capability. If absorbance (or concentration) or peak area of the sample is greater than absorbance (concentration) or peak area of the most concentrated standard solution, dilute sample and reanalyze. If very large dilutions are required, another technique (e.g., flame AA or ICP) may be more suitable for this sample. Large dilution factors magnify small errors on final calculation. Keep acid background and concentration of matrix modifier (if present in the solutions) constant. Dilute the sample in a blank solution of acid and matrix modifiers.

Proceed to 3113B.5*a*.

2) Method of standard additions—Refer to ¶ *c* above. The method of standard additions is valid only when it falls in the linear portion of the calibration curve. Once instrument sensitivity has been optimized for the element of interest and the linear range for the element has been established, proceed with sample analyses.

Inject a measured volume of sample into furnace device. Dry, char or ash, and atomize samples according to preset program. Repeat until reproducible results are obtained. Record instrument response in absorbance or concentration as appropriate. Add a known concentration of the element of interest to a separate portion of sample so as not to change significantly the sample volume. Repeat the determination.

Add a known concentration (preferably twice that used in the first addition) to a separate sample portion. Mix well and repeat the determination. NOTE: These steps must yield results within the linear range of the method.

Using linear graph paper, plot average absorbance or instrument response for the sample and the additions on the vertical axis against the concentrations of the added element on the horizontal axis, using zero as the concentration for the sample. Draw a straight line connecting the three points and extrapolate to zero absorbance. The intercept at the horizontal axis is the negative of the element concentration in the sample. The concentration axis to the left of the origin should be a mirror image of the axis to the right.

Some instruments can perform and calculate methods of standard additions. Use of these capabilities is acceptable.

5. Calculations

a. Direct determination:

$$\mu\text{g metal/L} = C \times F$$

where:

C = metal concentration as read directly from the instrument or from the calibration curve, μg/L, and
F = dilution factor.

b. Method of additions:

$$\mu\text{g metal/L} = C \times F$$

where:

C = metal concentration as read from the method of additions plot, μg/L, and
F = dilution factor.

6. Precision and Bias

Data typical of the precision and bias obtainable are presented in Tables 3113:III, IV, and V.

7. Quality Control

The QC practices considered to be an integral part of each method can be found in Section 3020. Although previous indications were that very low optimum concentration ranges were attainable for most metals (see Table 3113:II), data in Table 3113:III using variations of these protocols show that this may not be so. Exercise extreme care when applying this method to the lower concentration ranges. Verify analyst precision at the beginning of each analytical run by making triplicate analyses. Verify autosampler precision by checking volumes (by weight) delivered by the autosampler at routinely used injection volume settings.

8. Reference

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TABLE 3113:III. INTERLABORATORY SINGLE-ANALYST PRECISION DATA FOR ELECTROTHERMAL ATOMIZATION METHODS[1]

Element	Concentration μg/L	Single-Analyst Precision % RSD: Lab Pure Water	Drinking Water	Surface Water	Effluent 1	Effluent 2	Effluent 3
Al	28	66	108	70	—	—	66
	125	27	35	24	—	—	34
	11 000	11	—	—	22	—	—
	58 300	27	—	—	19	—	—
	460	9	—	—	—	30	—
	2 180	28	—	—	—	4	—
	10.5	20	13	13	13	56	18
	230	10	18	13	21	94	14
As	9.78	40	25	15	74	23	11
	227	10	6	8	11	15	6
Ba	56.5	36	21	29	59	23	27
	418	14	12	20	24	24	18
Be	0.45	18	27	15	30	2	11
	10.9	14	4	9	7	12	12
Cd	0.43	72	49	1	121	35	27
	12	11	17	22	14	11	15
Cr	9.87	24	33	10	23	15	10
	236	16	7	11	13	16	7
Co	29.7	10	17	10	19	24	12
	420	8	11	13	14	9	5
Cu	10.1	49	47	17	17	—	30
	234	8	15	6	21	—	11
	300	6	—	—	—	11	—
	1 670	11	—	—	—	6	—
Fe	26.1	144	52	153	—	—	124
	455	48	37	45	—	—	31
	1 030	17	—	—	30	—	—
	5 590	6	—	—	32	—	—
	370	14	—	—	—	19	—
	2 610	9	—	—	—	18	—
Pb	10.4	6	19	17	21	19	33
	243	17	7	17	18	12	16
Mn	0.44	187	180	—	—	—	275
	14.8	32	19	—	—	—	18
	91.0	15	—	—	48	—	—
	484.0	4	—	—	12	—	—
	111.0	12	—	—	—	21	—
	666.0	6	—	—	—	20	—
Ni	26.2	20	26	25	24	18	9
	461.0	15	11	9	8	11	4
Se	10.0	12	27	16	35	41	13
	235.0	6	6	15	6	13	14
Ag	8.48	10	—	—	15	27	16
	56.5	14	—	—	7	16	23
	0.45	27	166	48	—	—	—
	13.6	15	4	10	—	—	—

Table 3113:IV. Interlaboratory Overall Precision Data for Electrothermal Atomization Methods[1]

Element	Concentration *µg/L*	Overall Precision *% RSD*					
		Lab Pure Water	Drinking Water	Surface Water	Effluent 1	Effluent 2	Effluent 3
Al	28	99	114	124	—	—	131
	125	45	47	49	—	—	40
	11 000	19	—	—	43	—	—
	58 300	31	—	—	32	—	—
	460	20	—	—	—	47	—
	2 180	30	—	—	—	15	—
	10.5	37	19	22	50	103	39
	230	26	16	16	17	180	21
As	9.78	43	26	37	72	50	39
	227	18	12	13	20	15	14
Ba	56.5	68	38	43	116	43	65
	418	35	35	28	38	48	16
Be	0.45	28	31	15	67	50	35
	10.9	33	15	26	20	9	19
Cd	0.43	73	60	5	88	43	65
	12	19	25	41	26	20	27
Cr	9.87	30	53	24	60	41	23
	236	18	14	24	20	14	20
Co	29.7	13	26	17	18	21	17
	420	21	21	17	18	13	13
Cu	10.1	58	82	31	32	—	74
	234	12	33	19	21	—	26
	300	13	—	—	—	14	—
	1 670	12	—	—	—	13	—
Fe	26.1	115	93	306	—	—	204
	455	53	46	53	—	—	44
	1 030	32	—	—	25	—	—
	5 590	10	—	—	43	—	—
	370	28	—	—	—	22	—
	2 610	13	—	—	—	22	—
Pb	10.4	27	42	31	23	28	47
	243	18	19	17	19	19	25
Mn	0.44	299	272	—	—	—	248
	14.8	52	41	—	—	—	29
	91.0	16	—	—	45	—	—
	484.0	5	—	—	17	—	—
	111.0	15	—	—	—	17	—
	666.0	8	—	—	—	24	—
Ni	26.2	35	30	49	35	37	43
	461.0	23	22	15	12	21	17
Se	10.0	17	48	32	30	44	51
	235.0	16	18	18	17	22	34
Ag	8.48	23	—	—	16	35	34
	56.5	15	—	—	24	32	28
	0.45	57	90	368	—	—	—
	13.6	19	19	59	—	—	—

Table 3113:V. Interlaboratory Relative Error Data for Electrothermal Atomization Methods[1]

Element	Concentration μg/L	Relative Error %					
		Lab Pure Water	Drinking Water	Surface Water	Effluent 1	Effluent 2	Effluent 3
Al	28.0	86	150	54	—	—	126
	125.0	4	41	39	—	—	30
	11 000.0	2	—	—	14	—	—
	58 300.0	12	—	—	7	—	—
	460.0	2	—	—	—	11	—
	2 180.0	11	—	—	—	9	—
Sb	10.5	30	32	28	24	28	36
	230.0	35	14	19	13	73	39
As	9.78	36	1	22	106	13	16
	227.0	3	7	10	19	6	13
Ba	56.5	132	54	44	116	59	40
	418.0	4	0	0	13	6	60
Be	0.45	40	16	11	16	10	15
	10.9	13	2	9	7	8	8
Cd	0.43	58	45	37	66	16	19
	12.0	4	6	5	22	18	3
Cr	9.87	10	9	4	2	5	15
	236.0	11	0	9	13	5	8
Co	29.7	7	7	1	6	3	13
	420.0	12	8	8	11	5	18
Cu	10.1	16	48	2	5	—	15
	234.0	8	7	0	4	—	19
	300.0	4	—	—	—	21	—
	1 670.0	6	—	—	—	2	—
Fe	26.1	85	60	379	—	—	158
	455.0	43	22	31	—	—	18
	1 030.0	8	—	—	8	—	—
	5 590.0	2	—	—	12	—	—
	370.0	4	—	—	—	11	—
	2 610.0	35	—	—	—	2	—
Pb	10.4	16	10	17	1	34	14
	243.0	5	15	8	18	15	29
Mn	0.44	332	304	—	—	—	556
	14.8	10	1	—	—	—	36
	91.0	31	—	—	10	—	—
	484.0	42	—	—	4	—	—
	111.0	1	—	—	—	29	—
	666.0	6	—	—	—	23	—
Ni	26.2	9	16	10	7	33	54
	461.0	15	19	18	31	16	18
Se	10.0	12	9	6	36	17	37
	235.0	7	7	0	13	10	17
Ag	8.48	12	—	—	1	51	20
	56.5	16	—	—	8	51	22
	0.45	34	162	534	—	—	—
	13.6	3	12	5	—	—	—

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3114 ARSENIC AND SELENIUM BY HYDRIDE GENERATION/ATOMIC ABSORPTION SPECTROMETRY*

3114 A. Introduction

For general introductory material on atomic absorption spectrometric methods, see Section 3111A.

Two methods are presented in this section: A manual method and a continuous-flow method especially recommended for selenium. Continuous-flow automated systems are preferable to manual hydride generators because the effect of sudden hydrogen generation on light-path transparency is removed and any blank response from contamination of the HCl reagent by the elements being determined is incorporated into the background baseline.

* Approved by Standard Methods Committee, 2009. Editorial revisions, 2011.

3114 B. Manual Hydride Generation/Atomic Absorption Spectrometric Method

1. General Discussion

a. Principle: This method is applicable to the determination of arsenic and selenium by conversion to their hydrides by sodium borohydride reagent and transport into an atomic absorption atomizer.

Arsenous acid and selenous acid, the As(III) and Se(IV) oxidation states of arsenic and selenium, respectively, are instantaneously converted by sodium borohydride reagent in acid solution to their volatile hydrides. The hydrides are purged continuously by argon or nitrogen into a quartz cell heated electrically or by the flame of an atomic absorption spectrometer and converted to the gas-phase atoms. The sodium borohydride reducing agent, by rapid generation of the elemental hydrides in an appropriate reaction cell, minimizes dilution of the hydrides by the carrier gas and provides rapid, sensitive determinations of arsenic and selenium.

CAUTION: Arsenic and selenium and their hydrides are toxic. Handle with care.

At room temperature and solution pH values of 1 or less, arsenic acid, the As(V) oxidation state of arsenic, is reduced relatively slowly by sodium borohydride to As(III), which is then instantaneously converted to arsine. The arsine atomic absorption peaks commonly are decreased by one-fourth to one-third for As(V) when compared to As(III). Determination of total arsenic requires that all inorganic arsenic compounds be in the As(III) state. Organic and inorganic forms of arsenic are first oxidized to As(V) by acid digestion. The As(V) then is quantitatively reduced to As(III) with sodium or potassium iodide before reaction with sodium borohydride.

Selenic acid, the Se(VI) oxidation state of selenium, is not measurably reduced by sodium borohydride. To determine total selenium by atomic absorption and sodium borohydride, first reduce Se(VI) formed during the acid digestion procedure to Se(IV), being careful to prevent reoxidation by chlorine. Efficiency of reduction depends on temperature, reduction time, and HCl concentration. For 4*N* HCl, heat 1 h at 100°C. For 6*N* HCl, boiling for 10 min is sufficient.[1–3] Alternatively, autoclave samples in sealed containers at 121°C for 1 h. NOTE: Autoclaving in sealed containers may result in incomplete reduction, apparently due to the buildup of chlorine gas. To obtain equal instrument responses for reduced Se(VI) and Se (IV) solutions of equal concentrations, manipulate HCl concentration and heating time. For further details, see Section 3500-Se.

b. Equipment selection: Certain atomic absorption atomizers and hydride reaction cells are available commercially for use with the sodium borohydride reagent. A functional manual system that can be constructed in the laboratory is presented in Figure 3114:1. Irrespective of the hydride reaction cell-atomizer system selected, it must meet the following quality-control considerations:

- it must provide a precise and reproducible standard curve between 0 and 20 μg As or Se/L and an instrument detection limit between 0.1 and 0.5 μg As or Se/L;
- when carried through the entire procedure, oxidation state couples [As(III) – As(V) or Se(IV) – Se(VI)] must cause equal instrument response; and
- sample digestion must yield 80% or greater recovery of added cacodylic acid (dimethyl arsinic acid) and 90% or greater recovery of added As(III), As(V), Se(VI), or Se(IV).

Quartz atomization cells provide for the most sensitive arsenic and selenium hydride determinations. The quartz cell can be heated electrically or by an air–acetylene flame in an atomic absorption unit.

c. Digestion techniques: Waters and wastewaters may contain varying amounts of organic arsenic compounds and inorganic compounds of As(III), As(V), Se(IV), and Se(VI). To measure total arsenic and selenium in these samples requires sample digestion to solubilize particulate forms, oxidize reduced forms of arsenic and selenium, and convert any organic compounds to inorganic ones. Organic selenium compounds rarely have been demonstrated in water. It is left to the experienced analyst's judgment whether sample digestion is required.

Various alternative digestion procedures are provided in 3114B.4*c* and *d*. Consider sulfuric–nitric–perchloric acid diges-

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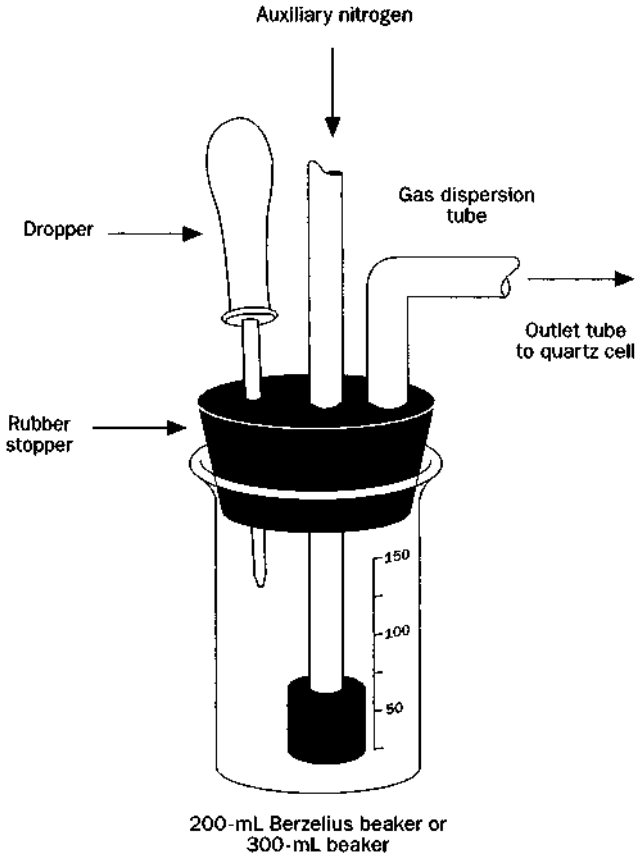


Figure 3114:1. Manual reaction cell for producing As and Se hydrides.

tion (3114B.4*c*) or sulfuric–nitric acid digestion (Section 3030F) as providing a measure of total recoverable arsenic rather than total arsenic because they do not completely convert certain organic arsenic compounds to As(V). Sulfuric–nitric–perchloric acid digestion effectively destroys organics and most particulates in untreated wastewater or solid samples, but does not convert all organic arsenicals to As(V). Potassium persulfate digestion (3114B.4*d*) is effective for converting organic arsenic and selenium compounds to As(V) and Se(VI) in potable and surface waters and in most wastewaters.[4]

The HCl–autoclave reduction of Se(VI) described above and in 3114B.4*f* is an effective digestion procedure for total inorganic selenium; however, it has not been found effective for converting benzene-substituted selenium compounds to inorganic selenium. In all cases, verify the effectiveness of digestion methods by carrying samples with known additions of organic As or Se(IV) through the entire procedure.

d. Interferences: Interferences are minimized because the As and Se hydrides are removed from the solution containing most potentially interfering substances. Slight response variations occur when acid matrices are varied. Control these variations by treating standards and samples in the same manner. Low concentrations of noble metals (approximately 100 μg/L of Ag, Au, Pt, Pd, etc.); concentrations of copper, lead, and nickel at or greater than 1 mg/L; and concentrations between 0.1 and 1 mg/L of hydride-forming elements (Bi, Sb, Sn, and Te) may suppress the response of As and Se hydrides. Interference by transition metals depends strongly on HCl concentration. Interferences are less pronounced at 4 to 6*N* HCl than at lower concentrations.[5] The presence of As or Se in each other's matrices can cause similar suppression. Reduced nitrogen oxides resulting from HNO_3 digestion and nitrite also can suppress instrumental response for both elements. Large concentrations of iodide interfere with the Se determination by reducing Se to its elemental form. When determining Se, do not use any glassware that has been used for iodide reduction of As(V).

When reducing Se(VI) to Se(IV), to prevent chlorine gas produced from reoxidizing Se(IV), generate the hydride within a few hours of the reduction steps or purge the chlorine from the samples by sparging.[6]

Interferences depend on system design and defy quantitative description because of their synergistic effects. Certain waters and wastewaters can contain interferences in sufficient concentration to suppress absorption responses of As and Se. For representative samples in a given laboratory and for initial analyses of unknown wastewaters, add appropriate inorganic forms of As or Se to digested sample portions and measure recovery. If average recoveries are less than 90%, consider using alternative analytical procedures.

e. Detection level and optimum concentration range: For both arsenic and selenium analyzed by aspiration into a nitrogen–hydrogen or argon–hydrogen flame after reduction, the method detection level is 2 μg/L or lower and the optimum concentration range is 2 to 20 μg/L.

For both arsenic and selenium analyzed by atomization into a nitrogen–hydrogen flame after reduction, the method detection limit should be 2 μg/L, with an optimum concentration range of 2 to 20 μg/L. Lower detection limits can be expected with a quartz tube atomizer.

f. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Atomic absorption spectrometer equipped with air–acetylene flame and quartz cell with mounting bracket or an electrically heated quartz cell, As and Se electrodeless discharge lamps with power supply, background correction at measurement wavelengths, and appropriate strip-chart recorder. A good-quality 10-mV recorder with high sensitivity and a fast response time is needed.

b. Atomizer: Use one of the following:

1) *Cylindrical quartz cell,* 10 to 20 cm long, bracket-mountable above air–acetylene burner.

2) *Cylindrical quartz cell*, 10 to 20 cm long, electrically heated by external nichrome wire to 800 to 900°C.[7]

3) *Cylindrical quartz cell* with internal fuel-rich hydrogen–oxygen (air) flame.[8]

The sensitivity of quartz cells deteriorates over several months of use. Sensitivity sometimes may be restored by treatment with 40% HF. **CAUTION: HF is extremely corrosive. Avoid all contact with exposed skin. Handle with care.**

c. Reaction cell for producing As or Se hydrides: See Figure 3114:1 for an example of a manual, laboratory-made system. A commercially available system is acceptable if it uses liquid sodium borohydride reagents; accepts samples digested in accordance with 3114B.4*c–e*; accepts 4 to 6*N* HCl; and is effi-

ciently and precisely stirred by the purging gas and/or a magnetic stirrer.

d. Eye dropper or syringe capable of delivering 0.5 to 3.0 mL sodium borohydride reagent. Exact and reproducible addition is required so production of hydrogen gas does not vary significantly between determinations.

e. Vent: See Section 3111A.6*f*.

3. Reagents

a. Sodium borohydride reagent: Dissolve 8 g $NaBH_4$ in 200 mL 0.1*N* NaOH. Prepare fresh daily.

b. Sodium iodide prereductant solution: Dissolve 50 g NaI in 500 mL water. Prepare fresh daily. Alternatively, use an equivalent KI solution.

c. Sulfuric acid (H_2SO_4), 18*N*.

d. Sulfuric acid, 2.5*N:* Cautiously add 35 mL conc H_2SO_4 to about 400 mL water, let cool, and adjust volume to 500 mL.

e. Potassium persulfate, 5% solution: Dissolve 25 g $K_2S_2O_8$ in water and dilute to 500 mL. Store in glass and refrigerate. Prepare weekly.

f. Nitric acid (HNO_3), conc.

g. Perchloric acid ($HClO_4$), conc.

h. Hydrochloric acid (HCl), conc.

i. Argon (or nitrogen), commercial grade.

j. Arsenic(III) solutions:

1) *Stock As(III) solution*—Dissolve 1.320 g arsenic trioxide, As_2O_3, in water containing 4 g NaOH. Dilute to 1 L; 1.00 mL = 1.00 mg As(III).

2) *Intermediate As(III) solution*—Dilute 10 mL stock As solution to 1000 mL with water containing 5 mL conc HCl; 1.00 mL = 10.0 μg As(III).

3) *Standard As(III) solution*—Dilute 10 mL intermediate As(III) solution to 1000 mL with water containing the same concentration of acid used for sample preservation (2 to 5 mL conc HNO_3); 1.00 mL = 0.100 μg As(III). Prepare diluted solutions daily.

k. Arsenic(V) solutions:

1) *Stock As(V) solution*—Dissolve 1.534 g arsenic pentoxide (As_2O_5) in distilled water containing 4 g NaOH. Dilute to 1 L; 1.00 mL = 1.00 mg As(V).

2) *Intermediate As(V) solution*—Prepare as for As(III) above; 1.00 mL = 10.0 μg As(V).

3) *Standard As(V) solution*—Prepare as for As(III) above; 1.00 mL = 0.100 μg As(V).

l. Organic arsenic solutions:

1) *Stock organic arsenic solution*—Dissolve 1.842 g dimethylarsinic acid (cacodylic acid) [$(CH_3)_2AsOOH$] in water containing 4 g NaOH. Dilute to 1 L; 1.00 mL = 1.00 mg As. [NOTE: Check purity of cacodylic acid reagent against an intermediate arsenic standard (50 to 100 mg As/L) using flame atomic absorption.]

2) *Intermediate organic arsenic solution*—Prepare as for As(III) above; 1.00 mL = 10.0 μg As.

3) *Standard organic arsenic solution*—Prepare as for As(III) above; 1.00 mL = 0.100 μg As.

m. Selenium(IV) solutions:

1) *Stock Se(IV) solution*—Dissolve 2.190 g sodium selenite (Na_2SeO_3) in water containing 10 mL HCl and dilute to 1 L; 1.00 mL = 1.00 mg Se(IV).

2) *Intermediate Se(IV) solution*—Dilute 10 mL stock Se(IV) to 1000 mL with water containing 10 mL conc HCl; 1.00 mL = 10.0 μg Se(IV).

3) *Standard Se(IV) solution*—Dilute 10 mL intermediate Se(IV) solution to 1000 mL with water containing the same concentration of acid used for sample preservation (2 to 5 mL conc HNO_3). Prepare solution daily when checking the equivalency of instrument response for Se(IV) and Se(VI); 1.00 mL = 0.100 μg Se(IV).

n. Selenium(VI) solutions:

1) *Stock Se(VI) solution*—Dissolve 2.393 g sodium selenate (Na_2SeO_4), in water containing 10 mL conc HNO_3. Dilute to 1 L; 1.00 mL = 1.00 mg Se(VI).

2) *Intermediate Se(VI) solution*—Prepare as for Se(IV) above; 1.00 mL = 10.0 μg Se (VI).

3) *Standard Se(VI) solution*—Prepare as for Se(IV) above; 1.00 mL = 0.100 μg Se(VI).

4. Procedure

a. Apparatus setup: Either see Figure 3114:1 or follow manufacturer's instructions. Connect inlet of reaction cell with auxiliary purging gas controlled by flow meter. If a drying cell between the reaction cell and atomizer is necessary, use only anhydrous $CaCl_2$ but not $CaSO_4$ because it may retain SeH_2. Before using the hydride generation/analysis system, optimize operating parameters. Align quartz atomizers for maximum absorbance. Aspirate a blank until memory effects are removed. Establish purging gas flow, concentration and rate of addition of sodium borohydride reagent, solution volume, and stirring rate for optimum instrument response for the chemical species to be analyzed. Optimize quartz cell temperature. Rapid injection of sodium borohydride reagent will increase sensitivities, so injection rates should be both consistent and as rapid as the system will tolerate. Recommended wavelengths are 193.7 and 196.0 nm for As and Se, respectively.

b. Instrument calibration standards: Prepare at least three standards and a blank by transferring appropriate volumes of AS(III) or Se(IV) standard solutions to 100-mL volumetric flasks and bring to volume with water containing the same acid concentration used for sample preservation (typically 2 to 5 mL conc HNO_3/L). Standards should be prepared to cover the linear range of the instrument used (generally from 1 to 20 μg/L). Prepare fresh daily. In all cases, standards must be carried through the same digestion protocol as the samples to monitor digestion effectiveness.

c. Preparation of samples and standards for total recoverable arsenic and selenium: Use digestion procedure described in Section 3030F for samples and standards. Alternatively, add 50 mL sample, As(III), or Se(IV) standard to 200-mL Berzelius beaker or 100-mL micro-Kjeldahl flask. Add 7 mL 18*N* H_2SO_4 and 5 mL conc HNO_3. Add a small boiling chip or glass beads if necessary. Evaporate to SO_3 fumes. Maintain oxidizing conditions at all times by adding small amounts of HNO_3 to prevent solution from darkening. Maintain an excess of HNO_3 until all organic matter is destroyed. Complete digestion usually is indicated by a light-colored solution. Cool slightly, add 25 mL water and 1 mL conc $HClO_4$ and again evaporate to SO_3 fumes to expel oxides of nitrogen. **CAUTION: See Section 3030H for cautions on use of $HClO_4$.** Monitor effectiveness of either

digestion procedure used by adding 5 mL of standard organic arsenic solution or 5 mL of a standard selenium solution to a 50-mL sample and measuring recovery, carrying standards and the sample with known addition through entire procedure. To report total recoverable arsenic as total arsenic, average recoveries of cacodylic acid must exceed 80%. After final evaporation of SO_3 fumes, dilute to 50 mL for arsenic measurements or to 30 mL for selenium measurements. For analysis of both elements in a single sample, increase sample volume to 100 mL and double the volumes of acids used in the digestion. Adjust final digestate volume to 100 mL. Use 50 mL for As and 30 mL for Se determinations, making appropriate volume corrections in calculating results.

d. Preparation of samples and standards for total arsenic and selenium: Add 50 mL undigested sample or standard to a 200-mL Berzelius beaker or 100-mL micro-Kjeldahl flask. Add 1 mL 2.5*N* H_2SO_4 and 5 mL 5% $K_2S_2O_8$. Boil gently on a preheated hot plate for approximately 30 to 40 min or until a final volume of 10 mL is reached. Do not let sample go to dryness. Alternatively, heat in an autoclave at 121°C for 1 h in capped containers. After manual digestion, dilute to 50 mL for subsequent arsenic measurements and to 30 mL for selenium measurements. Monitor digestion effectiveness by measuring recovery of As or Se as above. If poor recovery of arsenic added as cacodylic acid is obtained, re-analyze using double the amount of $K_2S_2O_8$. For analysis of both elements in a single sample, increase sample volume to 100 mL and double the volumes of acids used in the digestion. Adjust final digestate volume to 100 mL. Use 50 mL for As and 30 mL for Se determinations, making appropriate volume corrections in calculating results.

e. Determination of arsenic with sodium borohydride: To 50 mL digested standard or sample in a 200-mL Berzelius beaker (see Figure 3114:1), add 5 mL conc HCl and mix. Add 5 mL NaI prereductant solution, mix, and wait at least 30 min. [NOTE: The NaI reagent has not been found necessary for certain hydride reaction cell designs if a 20 to 30% loss in instrument sensitivity is not important and variables of solution acid conditions, temperatures, and volumes for production of As(V) and arsine can be controlled strictly. Such control requires an automated delivery system (see 3114C.)]

Attach one Berzelius beaker at a time to the rubber stopper containing the gas dispersion tube for the purging gas, the sodium borohydride reagent inlet, and the outlet to the quartz cell. Turn on strip-chart recorder and wait until the baseline is established by the purging gas and all air is expelled from the reaction cell. Add 0.5 mL sodium borohydride reagent. After the instrument absorbance has reached a maximum and returned to the baseline, remove beaker, rinse dispersion tube with water, and proceed to the next sample or standard. Periodically compare standard As(III) and As(V) curves for response consistency. Check for presence of chemical interferences that suppress instrument response for arsine by treating a digested sample with 10 μg/L As(III) or As(V), as appropriate. Average recoveries should be not less than 90%.

f. Determination of selenium with sodium borohydride: To 30 mL digested standard or sample in a 200-mL Berzelius beaker or 100-mL micro-Kjeldahl flask, add 15 mL conc HCl and mix. Heat for a predetermined period at 90 to 100°C. Alternatively, autoclave at 121°C in capped containers for 60 min, or heat for a predetermined time in open test tubes using a 90 to 100°C hot water bath or an aluminum block digester. Check effectiveness of the selected heating time by demonstrating equal instrument responses for calibration curves prepared either from standard Se(IV) or from Se(VI) solutions. Effective heat exposure for converting Se(VI) to Se(IV), with no loss of Se(IV), ranges between 5 and 60 min when open beakers or test tubes are used. Establish a heating time for effective conversion and apply this time to all samples and standards. Do not digest standard Se(IV) and Se(VI) solutions used for this check of equivalency. After prereduction of Se(VI) to Se(IV), attach Berzelius beakers, one at a time, to the purge apparatus. For each, turn on the strip-chart recorder and wait until the baseline is established. Add 0.50 mL sodium borohydride reagent. After the instrument absorbance has reached a maximum and returned to the baseline, remove beaker, rinse dispersion tube with water, and proceed to the next sample or standard. Check for presence of chemical interferences that suppress selenium hydride instrument response by treating a digested sample with 10 μg Se (IV)/L. Average recoveries should be not less than 90%.

5. Calculation

Construct a standard curve by plotting peak heights or areas of standards and blanks versus concentration of standards. Measure peak heights or areas of samples and read concentrations from curve. If sample was diluted (or concentrated) before sample digestion, apply an appropriate factor. On instruments so equipped, read concentrations directly after standard calibration.

6. Precision and Bias

Single-laboratory, single-operator data were collected for As(III) and organic arsenic by both manual and automated methods, and for the manual determination of selenium. Recovery values (%) from seven replicates are given below:

Method	As(III)	Org As	Se(IV)	Se(VI)
Manual with digestion	91.8	87.3	—	—
Manual without digestion	109.4	19.4	100.6	110.8
Automated with digestion	99.8	98.4	—	—
Automated without digestion	92.5	10.4	—	—

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3114 C. Continuous Hydride Generation/Atomic Absorption Spectrometric Method

1. General Discussion

The continuous hydride generator offers the advantages of simplicity in operation, excellent reproducibility, low detection limits, and high sample volume throughput for selenium analysis following preparations as described in Sections 3500-Se.B or 3114B.4*c* and *d*.

a. Principle: See 3114B.1*a*

b. Interferences: Free chlorine in hydrochloric acid is a common but difficult-to-diagnose interference. (The amount of chlorine varies with manufacturer and with each lot from the same manufacturer.) Chlorine oxidizes the hydride and can contaminate the hydride generator to prevent recoveries under any conditions. When interference is encountered, or preferably before using each new bottle of HCl, eliminate chlorine from a 2.3-L bottle of conc HCl by bubbling with helium (commercial grade, 100 mL/min) for 3 h.

Excess oxidant (peroxide, persulfate, or permanganate) from the total selenium digestion can oxidize the hydride. Follow procedures in Section 3500-Se.B.2, 3, or 4 to ensure removal of all oxidizing agents before hydride generation.

Nitrite is a common trace constituent in natural and waste waters, and at levels as low as 10 μg/L, nitrite can reduce the recovery of hydrogen selenide from Se(IV) by more than 50%. Moreover, during the reduction of Se(VI) to Se(IV) by digestion with HCl (Section 3500-Se.B.5), some nitrate is converted to nitrite, which subsequently interferes. When this interference is suspected, add sulfanilamide after sample acidification (or HCl digestion). The diazotization reaction between nitrite and sulfanilamide completely removes the interferent effect (i.e., the standard addition slope is normal).

c. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Continuous hydride generator: The basic unit is composed of two parts: a precision peristaltic pump, which is used to meter and mix reagents and sample solutions, and the gas–liquid separator. At the gas–liquid separator, a constant flow of argon strips out the hydrogen and metal hydride gases formed in the reaction and carries them to the heated quartz absorption cell (3114B.1*b* and 2*b*), which is supported by a metal bracket mounted on top of the regular air acetylene burner head. The spent liquid flows out of the separator via a constant level side drain to a waste bucket. Schematics and operating parameters are shown in Figure 3114:2.

Check flow rates frequently to ensure a steady flow; an uneven flow in any tubing will cause an erratic signal. Remove tubings from pump rollers when not in use. Typical flow rates are: sample, 7 mL/min; acid, 1 mL/min; and borohydride reagent, 1 mL/min. Argon flow usually is pre-fixed, typically at 90 mL/min.

b. Atomic absorption spectrometric equipment: See Section 3111A.6.

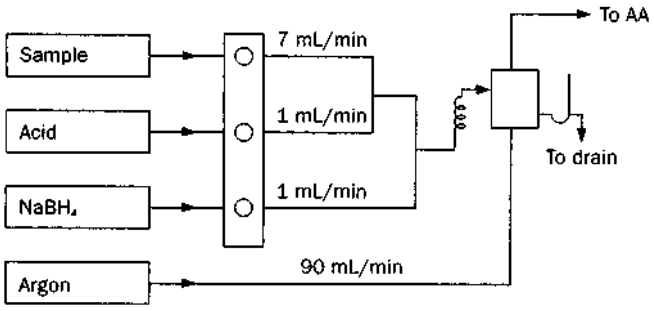


Figure 3114:2. Schematic of a continuous hydride generator.

3. Reagents

a. Hydrochloric acid (HCl), 5 + 1: Handle conc HCl under a fume hood. If necessary, remove free Cl_2 by stripping conc HCl with helium as described in 3114C.1*b*.

b. Borohydride reagent: Dissolve 0.6 g $NaBH_4$ and 0.5 g NaOH in 100 mL water. **CAUTION: Sodium borohydride is toxic, flammable, and corrosive.**

c. Selenium reference standard solution, 1000 mg/L: Use commercially available standard; verify that selenium is Se(IV).

d. Intermediate standard solution, 1 mg/L: Dilute 1 mL reference standard solution to 1 L in a volumetric flask with distilled water.

e. Working standard solutions, 5, 10, 20, 30, and 40 µg/L: Dilute 0.5, 1.0, 2.0, 3.0, and 4.0 mL intermediate standard solution to 100 mL in volumetric flasks.

f. Sulfanilamide solution: Prepare a 2.5% (w/v) solution daily; add several drops conc HCl per 50 mL solution to facilitate dissolution.

4. Procedure

a. Sample preparation: See Section 3500-Se.B or 3114B.4*c* and *d* for preparation steps for various Se fractions or total Se.

b. Preconditioning hydride generator: For newly installed tubing, turn on pump for at least 10 to 15 min before instrument calibration. Sample the highest standard for a few minutes to let volatile hydride react with the reactive sites in the transfer lines and on the quartz absorption cell surfaces.

c. Instrument calibration: Depending on total void volume in sample tubing, sampling time of 15 to 20 s generally is sufficient to obtain a steady signal. Between samples, submerge uptake tube in rinsewater. Calibrate instrument daily after a 45-min lamp warmup time. Use either the hollow cathode or the electrodeless discharge lamp.

d. Antifoaming agents: Certain samples, particularly wastewater samples containing a high concentration of proteinaceous substances, can cause excessive foaming that could carry the liquid directly into the heated quartz absorption cell and cause splattering of salty deposits onto the windows of the spectrometer. Add a drop of antifoaming agent* to eliminate this problem.

* Dow Corning, or equivalent.

e. Nitrite removal: After samples have been acidified, or after acid digestion, add 0.1 mL sulfanilamide solution per 10 mL sample and let react for 2 min.

f. Analysis: Follow manufacturer's instructions for operation of analytical equipment.

5. Calculation

Construct a calibration curve based on absorbance vs. standard concentration. Apply dilution factors on diluted samples.

6. Precision and Bias

Working standards were analyzed together with batches of water samples on a routine production basis. The standards were compounded using chemically pure sodium selenite and sodium selenate. The values of Se(IV) + Se(VI) were determined by converting Se(VI) to Se(IV) by digestion with HCl. Results are tabulated below.

No. Analyses	Mean Se(IV) µg/L	Rel. Dev. %	Se(IV) + Se(VI) µg/L	Rel. Dev. %
21	4.3	12	10.3	7
26	8.5	12	19.7	6
22	17.2	7	39.2	8
20	52.8	5	106.0	6

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3120 METALS BY PLASMA EMISSION SPECTROSCOPY*

3120 A. Introduction

1. General Discussion

Emission spectroscopy using inductively coupled plasma (ICP) was developed in the mid-1960s[1,2] as a rapid, sensitive, and convenient method for the determination of metals in water and wastewater samples.[3–6] Dissolved metals are determined in filtered and acidified samples. Total metals are determined after appropriate digestion. Care must be taken to ensure that potential interferences are dealt with, especially when dissolved solids exceed 1500 mg/L.

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* Approved by Standard Methods Committee, 1999. Editorial revisions, 2011.

3120 B. Inductively Coupled Plasma (ICP) Method

1. General Discussion

a. Principle: An ICP source consists of a flowing stream of argon gas ionized by an applied radio frequency field typically oscillating at 27.1 MHz. This field is inductively coupled to the ionized gas by a water-cooled coil surrounding a quartz "torch" that supports and confines the plasma. A sample aerosol is generated in an appropriate nebulizer and spray chamber and is carried into the plasma through an injector tube located within the torch. The sample aerosol is injected directly into the ICP, subjecting the constituent atoms to temperatures of about 6000 to 8000°K.[1] Because this results in almost complete dissociation of molecules, significant reduction in chemical interferences is achieved. The high temperature of the plasma excites atomic emission efficiently. Ionization of a high percentage of atoms produces ionic emission spectra. The ICP provides an optically "thin" source that is not subject to self-absorption except at very high concentrations. Thus linear dynamic ranges of four to six orders of magnitude are observed for many elements.[2]

The efficient excitation provided by the ICP results in low detection limits for many elements. This, coupled with the extended dynamic range, permits effective multielement determination of metals.[3] The light emitted from the ICP is focused onto the entrance slit of either a monochromator or a polychromator that effects dispersion. A precisely aligned exit slit is used to isolate a portion of the emission spectrum for intensity measurement using a photomultiplier tube. The monochromator uses a single exit slit/photomultiplier and may use a computer-controlled scanning mechanism to examine emission wavelengths sequentially. The polychromator uses multiple fixed exit slits and corresponding photomultiplier tubes; it simultaneously monitors all configured wavelengths using a computer-controlled readout system. The sequential approach provides greater wavelength selection while the simultaneous approach can provide greater sample throughput.

b. Applicable metals and analytical limits: Table 3120:I lists elements for which this method applies, recommended analytical wavelengths, and typical estimated instrument detection levels using conventional pneumatic nebulization. Actual working detection levels are sample-dependent. Typical upper limits for linear calibration also are included in Table 3120:I. T1

c. Interferences: Interferences may be categorized as follows:

1) Spectral interferences—Light emission from spectral sources other than the element of interest may contribute to apparent net signal intensity. Sources of spectral interference include direct spectral line overlaps, broadened wings of intense spectral lines, ion-atom recombination continuum emission, molecular band emission, and stray (scattered) light from the emission of elements at high concentrations.[4] Avoid line overlaps by selecting alternate analytical wavelengths. Avoid or minimize other spectral interference by judicious choice of background correction positions. A wavelength scan of the element line region is useful for detecting potential spectral interferences and for selecting positions for background correction. Make corrections for residual spectral interference using empirically determined correction factors with the computer software supplied by the spectrometer manufacturer or with the calculation detailed below. The empirical correction method cannot be used with scanning spectrometer systems if the analytical and interfering

Table 3120:I. Suggested Wavelengths, Estimated Detection Levels, Alternate Wavelengths, Calibration Concentrations, and Upper Limits

Element	Suggested Wavelength *nm*	Estimated Detection Level *μg/L*	Alternate Wavelength* *nm*	Calibration Concentration *mg/L*	Upper Limit Concentration† *mg/L*
Aluminum	308.22	40	237.32	10.0	100
Antimony	206.83	30	217.58	10.0	100
Arsenic	193.70	50	189.04‡	10.0	100
Barium	455.40	2	493.41	1.0	50
Beryllium	313.04	0.3	234.86	1.0	10
Boron	249.77	5	249.68	1.0	50
Cadmium	226.50	4	214.44	2.0	50
Calcium	317.93	10	315.89	10.0	100
Chromium	267.72	7	206.15	5.0	50
Cobalt	228.62	7	230.79	2.0	50
Copper	324.75	6	219.96	1.0	50
Iron	259.94	7	238.20	10.0	100
Lead	220.35	40	217.00	10.0	100
Lithium	670.78	4§	—	5.0	100
Magnesium	279.08	30	279.55	10.0	100
Manganese	257.61	2	294.92	2.0	50
Molybdenum	202.03	8	203.84	10.0	100
Nickel	231.60	15	221.65	2.0	50
Potassium	766.49	100§	769.90	10.0	100
Selenium	196.03	75	203.99	5.0	100
Silica (SiO_2)	212.41	20	251.61	21.4	100
Silver	328.07	7	338.29	2.0	50
Sodium	589.00	30§	589.59	10.0	100
Strontium	407.77	0.5	421.55	1.0	50
Thallium	190.86‡	40	377.57	10.0	100
Vanadium	292.40	8	—	1.0	50
Zinc	213.86	2	206.20	5.0	100

* Other wavelengths may be substituted if they provide the needed sensitivity and are corrected for spectral interference.
† Defines the top end of the effective calibration range. Do not extrapolate to concentrations beyond highest standard.
‡ Available with vacuum or inert gas purged optical path.
§ Sensitive to operating conditions.

lines cannot be precisely and reproducibly located. In addition, if using a polychromator, verify absence of spectral interference from an element that could occur in a sample but for which there is no channel in the detector array. Do this by analyzing single-element solutions of 100 mg/L concentration and noting for each element channel the apparent concentration from the interfering substance that is greater than the element's instrument detection limit.

2) Nonspectral interferences

a) Physical interferences are effects associated with sample nebulization and transport processes. Changes in the physical properties of samples, such as viscosity and surface tension, can cause significant error. This usually occurs when samples containing more than 10% (by volume) acid or more than 1500 mg dissolved solids/L are analyzed using calibration standards containing ≤5% acid. Whenever a new or unusual sample matrix is encountered, use the test described in 3120B.4*g*. If physical interference is present, compensate for it by sample dilution, by using matrix-matched calibration standards, or by applying the method of standard addition (see 3120B.5*d*).

High dissolved solids content also can contribute to instrumental drift by causing salt buildup at the tip of the nebulizer gas orifice. Using prehumidified argon for sample nebulization lessens this problem. Better control of the argon flow rate to the nebulizer using a mass flow controller improves instrument performance.

b) Chemical interferences are caused by molecular compound formation, ionization effects, and thermochemical effects associated with sample vaporization and atomization in the plasma. Normally these effects are not pronounced and can be minimized by careful selection of operating conditions (incident power, plasma observation position, etc.). Chemical interferences are highly dependent on sample matrix and element of interest. As with physical interferences, compensate for them by using matrix matched standards or by standard addition (3120B.5*d*). To determine the presence of chemical interference, follow instructions in 3120B.4*g*.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. ICP source: The ICP source consists of a radio frequency (RF) generator capable of generating at least 1.1 kW of power, torch, tesla coil, load coil, impedance matching network, nebulizer, spray chamber, and drain. High-quality flow regulators are

required for both the nebulizer argon and the plasma support gas flow. A peristaltic pump is recommended to regulate sample flow to the nebulizer. The type of nebulizer and spray chamber used may depend on the samples to be analyzed as well as on the equipment manufacturer. In general, pneumatic nebulizers of the concentric or cross-flow design are used. Viscous samples and samples containing particulates or high dissolved solids content (>5000 mg/L) may require nebulizers of the Babington type.[5]

b. Spectrometer: The spectrometer may be of the simultaneous (polychromator) or sequential (monochromator) type with air-path, inert gas purged, or vacuum optics. A spectral bandpass of 0.05 nm or less is required. The instrument should permit examination of the spectral background surrounding the emission lines used for metals determination. It is necessary to be able to measure and correct for spectral background at one or more positions on either side of the analytical lines.

3. Reagents and Standards

Use reagents that are of ultra-high-purity grade or equivalent. Redistilled acids are acceptable. Except as noted, dry all salts at 105°C for 1 h and store in a desiccator before weighing. Use deionized water prepared by passing water through at least two stages of deionization with mixed bed cation/anion exchange resins.[6] Use deionized water for preparing all calibration standards, reagents, and for dilution.

a. Hydrochloric acid (HCl), conc and 1+1.

b. Nitric acid (HNO_3), conc.

c. Nitric acid (HNO_3), 1+1: Add 500 mL conc HNO_3 to 400 mL water and dilute to 1 L.

d. Standard stock solutions: See Sections 3111B.3, 3111D.3, and 3114B.3. **CAUTION: Many metal salts are extremely toxic and may be fatal if swallowed. Wash hands thoroughly after handling.**

1) *Aluminum*—See Section 3111D.3*k*1).
2) *Antimony*—See Section 3111B.3*j*1).
3) *Arsenic*—See Section 3114B.3*k*1).
4) *Barium*—See Section 3111D.3*k*2).
5) *Beryllium*—See Section 3111D.3*k*3).
6) *Boron—Do not dry* but keep bottle tightly stoppered and store in a desiccator. Dissolve 0.5716 g anhydrous H_3BO_3 in water and dilute to 1000 mL; 1 mL = 100 μg B.
7) *Cadmium*—See Section 3111B.3*j*3).
8) *Calcium*—See Section 3111B.3*j*4).
9) *Chromium*—See Section 3111B.3*j*6).
10) *Cobalt*—See Section 3111B.3*j*7).
11) *Copper*—See Section 3111B.3*j*8).
12) *Iron*—See Section 3111B.3*j*11).
13) *Lead*—See Section 3111B.3*j*12).
14) *Lithium*—See Section 3111B.3*j*13).
15) *Magnesium*—See Section 3111B.3*j*14).
16) *Manganese*—See Section 3111B.3*j*15).
17) *Molybdenum*—See Section 3111D.3*k*5).
18) *Nickel*—See Section 3111B.3*j*16).
19) *Potassium*—See Section 3111B.3*j*19).
20) *Selenium*—See Section 3114B.3*n*1).
21) *Silica*—See Section 3111D.3*k*8).
22) *Silver*—See Section 3111B.3*j*22).
23) *Sodium*—See Section 3111B.3*j*23).
24) *Strontium*—See Section 3111B.3*j*24).
25) *Thallium*—See Section 3111B.3*j*25).
26) *Vanadium*—See Section 3111D.3*k*11).
27) *Zinc*—See Section 3111B.3*j*27).

e. Calibration standards: Prepare mixed calibration standards containing the concentrations shown in Table 3120:I by combining appropriate volumes of the stock solutions in 100-mL volumetric flasks. Add 2 mL 1+1 HNO_3 and 10 mL 1+1 HCl and dilute to 100 mL with water. Before preparing mixed standards, analyze each stock solution separately to determine possible spectral interference or the presence of impurities. When preparing mixed standards take care that the elements are compatible and stable. Store mixed standard solutions in an FEP fluorocarbon or unused polyethylene bottle. Verify calibration standards initially using the quality control standard; monitor weekly for stability. The following are recommended combinations using the suggested analytical lines in Table 3120:I. Alternative combinations are acceptable.

1) *Mixed standard solution I*—Manganese, beryllium, cadmium, lead, selenium, and zinc.
2) *Mixed standard solution II*—Barium, copper, iron, vanadium, and cobalt.
3) *Mixed standard solution III*—Molybdenum, silica, arsenic, strontium, and lithium.
4) *Mixed standard solution IV*—Calcium, sodium, potassium, aluminum, chromium, and nickel.
5) *Mixed standard solution V*—Antimony, boron, magnesium, silver, and thallium. If addition of silver results in an initial precipitation, add 15 mL water and warm flask until solution clears. Cool and dilute to 100 mL with water. For this acid combination limit the silver concentration to 2 mg/L. Silver under these conditions is stable in a tap water matrix for 30 d. Higher concentrations of silver require additional HCl.

f. Calibration blank: Dilute 2 mL 1+1 HNO_3 and 10 mL 1+1 HCl to 100 mL with water. Prepare a sufficient quantity to be used to flush the system between standards and samples.

g. Method blank: Carry a reagent blank through entire sample preparation procedure. Prepare method blank to contain the same acid types and concentrations as the sample solutions.

h. Instrument check standard: Prepare instrument check standards by combining compatible elements at a concentration of 2 mg/L.

i. Instrument quality control sample: Obtain a certified aqueous reference standard from an outside source and prepare according to instructions provided by the supplier. Use the same acid matrix as the calibration standards.

j. Method quality control sample: Carry the instrument quality control sample (¶ *i* above) through the entire sample preparation procedure.

k. Argon: Use technical or welder's grade. If gas appears to be a source of problems, use prepurified grade.

4. Procedure

a. Sample preparation: See Section 3030F.

b. Operating conditions: Because of differences among makes and models of satisfactory instruments, no detailed operating instructions can be provided. Follow manufacturer's instructions. Establish instrumental detection limit, precision, optimum background correction positions, linear dynamic range,

and interferences for each analytical line. Verify that the instrument configuration and operating conditions satisfy the analytical requirements and that they can be reproduced on a day-to-day basis. An atom-to-ion emission intensity ratio [Cu(I) 324.75 nm/Mn(II) 257.61 nm] can be used to reproduce optimum conditions for multielement analysis precisely. The Cu/Mn intensity ratio may be incorporated into the calibration procedure, including specifications for sensitivity and for precision.[7] Keep daily or weekly records of the Cu and Mn intensities and/or the intensities of critical element lines. Also record settings for optical alignment of the polychromator, sample uptake rate, power readings (incident, reflected), photomultiplier tube attenuation, mass flow controller settings, and system maintenance.

c. Instrument calibration: Set up instrument as directed (¶ *b* above). Warm up for 30 min. For polychromators, perform an optical alignment using the profile lamp or solution. Check alignment of plasma torch and spectrometer entrance slit, particularly if maintenance of the sample introduction system was performed. Make Cu/Mn or similar intensity ratio adjustment.

Calibrate instrument according to manufacturer's recommended procedure using calibration standards and blank. Aspirate each standard or blank for a minimum of 15 s after reaching the plasma before beginning signal integration. Rinse with calibration blank or similar solution for at least 60 s between each standard to eliminate any carryover from the previous standard. Use average intensity of multiple integrations of standards or samples to reduce random error.

Before analyzing samples, analyze instrument check standard. Concentration values obtained should not deviate from the actual values by more than ±5% (or the established control limits, whichever is lower).

d. Analysis of samples: Begin each sample run with an analysis of the calibration blank, then analyze the method blank. This permits a check of the sample preparation reagents and procedures for contamination. Analyze samples, alternating them with analyses of calibration blank. Rinse for at least 60 s with dilute acid between samples and blanks. After introducing each sample or blank let system equilibrate before starting signal integration. Examine each analysis of the calibration blank to verify that no carry-over memory effect has occurred. If carry-over is observed, repeat rinsing until proper blank values are obtained. Make appropriate dilutions and acidifications of the sample to determine concentrations beyond the linear calibration range.

e. Instrumental quality control: Analyze instrument check standard once per 10 samples to determine if significant instrument drift has occurred. If agreement is not within ±5% of the expected values (or within the established control limits, whichever is lower), terminate analysis of samples, correct problem, and recalibrate instrument. If the intensity ratio reference is used, resetting this ratio may restore calibration without the need for reanalyzing calibration standards. Analyze instrument check standard to confirm proper recalibration. Reanalyze one or more samples analyzed just before termination of the analytical run. Results should agree to within ±5%, otherwise all samples analyzed after the last acceptable instrument check standard analysis must be reanalyzed.

Analyze instrument quality control sample within every run. Use this analysis to verify accuracy and stability of the calibration standards. If any result is not within ±5% of the certified value, prepare a new calibration standard and recalibrate the instrument. If this does not correct the problem, prepare a new stock solution and a new calibration standard and repeat calibration.

f. Method quality control: Analyze the method quality control sample within every run. Results should agree to within ±5% of the certified values. Greater discrepancies may reflect losses or contamination during sample preparation.

g. Test for matrix interference: When analyzing a new or unusual sample matrix verify that neither a positive nor negative nonlinear interference effect is operative. If the element is present at a concentration above 1 mg/L, use serial dilution with calibration blank. Results from the analyses of a dilution should be within ±5% of the original result. Alternately, or if the concentration is either below 1 mg/L or not detected, use a post-digestion addition equal to 1 mg/L. Recovery of the addition should be either between 95% and 105% or within established control limits of ±2 standard deviations around the mean. If a matrix effect causes test results to fall outside the critical limits, complete the analysis after either diluting the sample to eliminate the matrix effect while maintaining a detectable concentration of at least twice the detection limit or applying the method of standard additions.

5. Calculations and Corrections

a. Blank correction: Subtract result of an adjacent calibration blank from each sample result to make a baseline drift correction. (Concentrations printed out should include negative and positive values to compensate for positive and negative baseline drift. Make certain that the calibration blank used for blank correction has not been contaminated by carry-over.) Use the result of the method blank analysis to correct for reagent contamination. Alternatively, intersperse method blanks with appropriate samples. Reagent blank and baseline drift correction are accomplished in one subtraction.

b. Dilution correction: If the sample was diluted or concentrated in preparation, multiply results by a dilution factor (*DF*) calculated as follows:

$$DF = \frac{\text{Final weight or volume}}{\text{Initial weight or volume}}$$

c. Correction for spectral interference: Correct for spectral interference by using computer software supplied by the instrument manufacturer or by using the manual method based on interference correction factors. Determine interference correction factors by analyzing single-element stock solutions of appropriate concentrations under conditions matching as closely as possible those used for sample analysis. Unless analysis conditions can be reproduced accurately from day to day, or for longer periods, redetermine interference correction factors found to affect the results significantly each time samples are analyzed.[7, 8] Calculate interference correction factors (K_{ij}) from apparent concentrations observed in the analysis of the high-purity stock solutions:

$$K_{ij} = \frac{\text{Apparent concentration of element } i}{\text{Actual concentration of interfering element } j}$$

where the apparent concentration of element i is the difference between the observed concentration in the stock solution and the observed concentration in the blank. Correct sample concentrations observed for element i (already corrected for baseline drift), for spectral interferences from elements j, k, and l; for example:

$$\text{Concentration of element } i \text{ corrected for spectral interference} =$$

$$\text{Observed concentration of } i - (K_{ij})\ \text{Observed concentration of interfering element } j - (K_{ik})\ \text{Observed concentration of interfering element } k - (K_{il})\ \text{Observed concentration of interfering element } l$$

Interference correction factors may be negative if background correction is used for element i. A negative K_{ij} can result where an interfering line is encountered at the background correction wavelength rather than at the peak wavelength. Determine concentrations of interfering elements j, k, and l within their respective linear ranges. Mutual interferences (i interferes with j and j interferes with i) require iterative or matrix methods for calculation.

d. Correction for nonspectral interference: If nonspectral interference correction is necessary, use the method of standard additions. It is applicable when the chemical and physical form of the element in the standard addition is the same as in the sample, *or* the ICP converts the metal in both sample and addition to the same form; the interference effect is independent of metal concentration over the concentration range of standard additions; and the analytical calibration curve is linear over the concentration range of standard additions.

Use an addition not less than 50% nor more than 100% of the element concentration in the sample so that measurement precision will not be degraded and interferences that depend on element/interferent ratios will not cause erroneous results. Apply the method to all elements in the sample set using background correction at carefully chosen off-line positions. Multielement standard addition can be used if it has been determined that added elements are not interferents.

e. Reporting data: Report analytical data in concentration units of milligrams per liter using up to three significant figures. Report results below the determined detection limit as not detected less than the stated detection limit corrected for sample dilution.

6. Precision and Bias

As a guide to the generally expected precision and bias, see the linear regression equations in Table 3120:II.[9] Additional inter- T2A
laboratory information is available.[10]

7. References

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TABLE 3120:II. ICP PRECISION AND BIAS DATA

Element	Concentration Range $\mu g/L$	Total Digestion* $\mu g/L$	Recoverable Digestion* $\mu g/L$
Aluminum	69–4792	$X = 0.9273C + 3.6$	$X = 0.9380C + 22.1$
		$S = 0.0559X + 18.6$	$S = 0.0873X + 31.7$
		$SR = 0.0507X + 3.5$	$SR = 0.0481X + 18.8$
Antimony	77–1406	$X = 0.7940C - 17.0$	$X = 0.8908C + 0.9$
		$S = 0.1556X - 0.6$	$S = 0.0982X + 8.3$
		$SR = 0.1081X + 3.9$	$SR = 0.0682X + 2.5$
Arsenic	69–1887	$X = 1.0437C - 12.2$	$X = 1.0175C + 3.9$
		$S = 0.1239X + 2.4$	$S = 0.1288X + 6.1$
		$SR = 0.0874X + 6.4$	$SR = 0.0643X + 10.3$
Barium	9–377	$X = 0.7683C + 0.47$	$X = 0.8380C + 1.68$
		$S = 0.1819X + 2.78$	$S = 0.2540X + 0.30$
		$SR = 0.1285X + 2.55$	$SR = 0.0826X + 3.54$
Beryllium	3–1906	$X = 0.9629C + 0.05$	$X = 1.0177C - 0.55$
		$S = 0.0136X + 0.95$	$S = 0.0359X + 0.90$
		$SR = 0.0203X - 0.07$	$SR = 0.0445X - 0.10$
Boron	19–5189	$X = 0.8807C + 9.0$	$X = 0.9676C + 18.7$
		$S = 0.1150X + 14.1$	$S = 0.1320X + 16.0$
		$SR = 0.0742X + 23.2$	$SR = 0.0743X + 21.1$
Cadmium	9–1943	$X = 0.9874C - 0.18$	$X = 1.0137C - 0.65$
		$S = 0.0557X + 2.02$	$S = 0.0585X + 1.15$
		$SR = 0.0300X + 0.94$	$SR = 0.0332X + 0.90$
Calcium	17–47 170	$X = 0.9182C - 2.6$	$X = 0.9658C + 0.8$
		$S = 0.1228X + 10.1$	$S = 0.0917X + 6.9$
		$SR = 0.0189X + 3.7$	$SR = 0.0327X + 10.1$
Chromium	13–1406	$X = 0.9544C + 3.1$	$X = 1.0049C - 1.2$
		$S = 0.0499X + 4.4$	$S = 0.0698X + 2.8$
		$SR = 0.0009X + 7.9$	$SR = 0.0571X + 1.0$
Cobalt	17–2340	$X = 0.9209C - 4.5$	$X = 0.9278C - 1.5$
		$S = 0.0436X + 3.8$	$S = 0.0498X + 2.6$
		$SR = 0.0428X + 0.5$	$SR = 0.0407X + 0.4$
Copper	8–1887	$X = 0.9297C - 0.30$	$X = 0.9647C - 3.64$
		$S = 0.0442X + 2.85$	$S = 0.0497X + 2.28$
		$SR = 0.0128X + 2.53$	$SR = 0.0406X + 0.96$
Iron	13–9359	$X = 0.8829C + 7.0$	$X = 0.9830C + 5.7$
		$S = 0.0683X + 11.5$	$S = 0.1024X + 13.0$
		$SR = -0.0046X + 10.0$	$SR = 0.0790X + 11.5$
Lead	42–4717	$X = 0.9699C - 2.2$	$X = 1.0056C + 4.1$
		$S = 0.0558X + 7.0$	$S = 0.0799X + 4.6$
		$SR = 0.0353X + 3.6$	$SR = 0.0448X + 3.5$
Magnesium	34–13 868	$X = 0.9881C - 1.1$	$X = 0.9879C + 2.2$
		$S = 0.0607X + 11.6$	$S = 0.0564X + 13.2$
		$SR = 0.0298X + 0.6$	$SR = 0.0268X + 8.1$
Manganese	4–1887	$X = 0.9417C + 0.13$	$X = 0.9725C + 0.07$
		$S = 0.0324X + 0.88$	$S = 0.0557X + 0.76$
		$SR = 0.0153X + 0.91$	$SR = 0.0400X + 0.82$
Molybdenum	17–1830	$X = 0.9682C + 0.1$	$X = 0.9707C - 2.3$
		$S = 0.0618X + 1.6$	$S = 0.0811X + 3.8$
		$SR = 0.0371X + 2.2$	$SR = 0.0529X + 2.1$
Nickel	17–47 170	$X = 0.9508C + 0.4$	$X = 0.9869C + 1.5$
		$S = 0.0604X + 4.4$	$S = 0.0526X + 5.5$
		$SR = 0.0425X + 3.6$	$SR = 0.0393X + 2.2$
Potassium	347–14 151	$X = 0.8669C - 36.4$	$X = 0.9355C - 183.1$
		$S = 0.0934X + 77.8$	$S = 0.0481X + 177.2$
		$SR = -0.0099X + 144.2$	$SR = 0.0329X + 60.9$
Selenium	69–1415	$X = 0.9363C - 2.5$	$X = 0.9737C - 1.0$
		$S = 0.0855X + 17.8$	$S = 0.1523X + 7.8$
		$SR = 0.0284X + 9.3$	$SR = 0.0443X + 6.6$

TABLE 3120:II, CONT.

Element	Concentration Range $\mu g/L$	Total Digestion* $\mu g/L$	Recoverable Digestion* $\mu g/L$
Silicon	189–9434	$X = 0.5742C - 35.6$	$X = 0.9737C - 60.8$
		$S = 0.4160X + 37.8$	$S = 0.3288X + 46.0$
		$SR = 0.1987X + 8.4$	$SR = 0.2133X + 22.6$
Silver	8–189	$X = 0.4466C + 5.07$	$X = 0.3987C + 8.25$
		$S = 0.5055X - 3.05$	$S = 0.5478X - 3.93$
		$SR = 0.2086X - 1.74$	$SR = 0.1836X - 0.27$
Sodium	35–47 170	$X = 0.9581C + 39.6$	$X = 1.0526C + 26.7$
		$S = 0.2097X + 33.0$	$S = 0.1473X + 27.4$
		$SR = 0.0280X + 105.8$	$SR = 0.0884X + 50.5$
Thallium	79–1434	$X = 0.9020C - 7.3$	$X = 0.9238C + 5.5$
		$S = 0.1004X + 18.3$	$S = 0.2156X + 5.7$
		$SR = 0.0364X + 11.5$	$SR = -0.0106X + 48.0$
Vanadium	13–4698	$X = 0.9615C - 2.0$	$X = 0.9551C + 0.4$
		$S = 0.0618X + 1.7$	$S = 0.0927X + 1.5$
		$SR = 0.0220X + 0.7$	$SR = 0.0472X + 0.5$
Zinc	7–7076	$X = 0.9356C - 0.30$	$X = 0.9500C + 1.22$
		$S = 0.0914X + 3.75$	$S = 0.0597X + 6.50$
		$SR = -0.0130X + 10.07$	$SR = 0.0153X + 7.78$

* X = mean recovery, μg/L,
C = true value, μg/L,
S = multi-laboratory standard deviation, μg/L, and
SR = single-analyst standard deviation, μg/L.

3125 METALS BY INDUCTIVELY COUPLED PLASMA–MASS SPECTROMETRY*

3125 A. Introduction

1. General Discussion

This method is designed to determine trace metals and metalloids in surface, ground, and drinking waters via inductively coupled plasma–mass spectrometry (ICP–MS). Although best suited for ambient or pristine freshwater matrices, this method can also be used to analyze wastewater, soils, sediments, sludge, and biological samples after appropriate digestion followed by dilution and/or cleanup to reduce matrix effects to a manageable level.[1,2] Various cleanup techniques are available to reduce matrix interferences and/or concentrate analytes of interest.[3–7]

For many analytes, the instrument detection limits (IDLs) are between 1 and 100 ng/L. The quadrupole-based ICP–MS may include collision cell technology (CCT) and/or dynamic reaction cell (DRC), which remove the need for mathematical interference correction for many elements. Additional data (Tables 3125:I and 3125:II) demonstrate the performance for elements seriously affected by polyatomic interferences.[8,9]

The method is intended to be performance-based, so the elemental analyte list can be extended, new "clean" preparation techniques can be implemented, and other appropriate modifications can be made as technology evolves. Any modifications to the base method must be validated via suitable quality control standards. More sources of information on quality assurance and other aspects of ICP–MS analysis of metals are available.[10–12]

Ideally, the analysts who use this method will have experience using ICP–MS, interpreting spectral and matrix interferences, and implementing corrective procedures. Before generating data, analysts should demonstrate their proficiency in this method by successfully analyzing a performance evaluation sample for each matrix type.

Table 3125:I. Method Performance with Calibration Verification Standards*†

Element	Mass	Mean Recovery %	Mean *ng/mL*	SD *ng/mL*	RSD %
V	51	100.00	200.0	9.25	4.62
V CCT	51	100.44	200.9	7.30	3.63
Cr	52	99.56	199.1	9.39	4.72
Cr CCT	52	100.56	201.1	7.16	3.56
Mn	55	101.36	1014	54.09	5.34
Mn CCT	55	98.28	982.8	31.95	3.25
Fe	56	101.84	10184	566.43	5.56
Fe CCT	56	100.25	10025	336.29	3.35
Co	59	101.58	20.32	0.89	4.38
Co CCT	59	101.67	20.33	0.64	3.15
Ni	60	101.08	202.2	9.06	4.48
Ni CCT	60	101.90	203.8	6.61	3.24
Cu	63	101.76	1018	62.46	6.14
Cu CCT	63	105.63	1056	36.79	3.48
Cu	65	102.12	1021	56.06	5.49
Zn	66	100.52	1005	47.63	4.74
Zn CCT	66	99.51	995.1	33.94	3.41
As	75	99.54	19.91	1.02	5.14
As CCT	75	98.24	19.65	0.78	3.99
Se	78	102.77	20.55	1.04	5.07
Se CCT	78	104.78	20.96	1.73	8.26

* Single-laboratory, single-operator data acquired 12-2005 using Thermo Electron X Series in Standard and CCT-KED modes.
† Continuing calibration verification standard ($N = 28$).

2. References

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Table 3125:II. Method Performance with Standard Reference Water*†

Element	Mass	Mean Recovery %	Mean *ng/mL*	SD *ng/mL*	RSD %
V	51	95.49	12.40	0.62	5.01
V CCT	51	101.60	13.20	0.32	2.39
Cr	52	93.34	36.03	0.80	2.21
Cr CCT	52	95.31	36.79	0.89	2.42
Mn	55	99.09	120.40	2.25	1.87
Mn CCT	55	96.99	117.8	2.18	1.85
Fe	56	110.45	37.89	4.32	11.41
Fe CCT	56	102.20	35.06	1.78	5.09
Co	59	98.43	19.96	0.32	1.62
Co CCT	59	99.22	20.12	0.36	1.79
Ni	60	98.78	27.06	0.68	2.51
Ni CCT	60	100.69	27.59	0.50	1.80
Cu†	63	115.36	98.29	3.68	3.74
Cu CCT‡	63	122.91	104.7	2.81	2.68
Cu‡	65	118.83	101.2	2.84	2.80
Zn	66	95.88	51.01	1.36	2.67
Zn CCT	66	97.64	51.95	1.91	3.68
As	75	98.61	26.30	0.48	1.81
As CCT	75	98.38	26.24	0.59	2.26
Se	78	95.83	21.04	0.45	2.15
Se CCT	78	97.58	21.43	1.86	8.66

* Single-laboratory, single-operator data acquired 12-2005 using Thermo Electron X Series in Standard and CCT-KED modes.
† NIST 1640 ($N = 7$).
‡ Copper contamination.

* Approved by Standard Methods Committee, 2009. Editorial revisions, 2011. Joint Task Group: 22nd Edition—Robert Henry (chair), Christopher J. Baggett, Cindy A. Bamfield, Alois F. Clary, William R. Kammin, Gregg Oelker.

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Table 3125:III. Recommended Analyte Masses, Instrument Detection Limits (IDL), and Internal Standards

Element	Analytical Mass	IDL* μg/L	Recommended Internal Standard
Be	9	0.025	Li
Al	27	0.03	Sc
V	51	0.02	Sc
Cr	52	0.04	Sc
Cr	53	0.03	Sc
Mn	55	0.002	Sc
Co	59	0.002	Sc
Ni	60	0.004	Sc
Ni	62	0.025	Sc
Cu	63	0.003	Sc
Cu	65	0.004	Sc
Zn	66	0.017	Ge
Zn	68	0.020	Ge
As	75	0.025	Ge
Se	77	0.093	Ge
Se	82	0.064	Ge
Ag	107	0.003	In
Ag	109	0.002	In
Cd	111	0.006	In
Cd	114	0.003	In
Sb	121	0.07	In
Sb	123	0.07	In
Tl	203	0.03	Th
Tl	205	0.03	Th
Pb	208	0.005	Th
U	235	0.032	Th
U	238	0.001	Th
Mo	98	0.003†	In
Ba	135	0.008†	In
Sr	88	0.001‡	In

* IDLs were determined on a Perkin Elmer Elan 6000 ICP–MS using seven replicate analyses of a 1% nitric acid solution, at Manchester Environmental Laboratory, July 1996.
† From EPA Method 200.8 for the Analysis of Drinking Waters—Application Note, Order No. ENVA-300A, The Perkin Elmer Corporation, 1996.
‡ From Perkin Elmer Technical Summary TSMS-12.

3125 B. Inductively Coupled Plasma–Mass Spectrometry (ICP–MS) Method

1. General Discussion

a. Principle: In this method, analysts introduce sample material to an argon-based, high-temperature radio-frequency plasma, usually via pneumatic nebulization. As energy transfers from the plasma to the sample stream, the target element desolves, atomizes, and ionizes. The resulting ions are extracted from the plasma through a differential vacuum interface and separated based on their mass-to-charge (m/z) ratio by a mass spectrometer. Typically, either a quadrupole (with or without CCT or DRC) or magnetic sector (high-resolution) mass spectrometer is used. An electron multiplier detector counts the separated ions, and a computer-based data-management system processes the resulting information.

b. Applicable elements and analytical limits: This method has been demonstrated to be suitable for aluminum, antimony, arsenic, barium, beryllium, cadmium, chromium, cobalt, copper, lead, manganese, molybdenum, nickel, selenium, silver, strontium, thallium, uranium, vanadium, and zinc (see Table 3125:III). It is also acceptable for other elemental analytes as long as the same quality assurance practices are followed, with documented acceptance limits.

Before implementing the method, determine the instrument detection limit (IDL) and method detection limit (MDL) for all analytes (see Section 1030C). Typical IDLs for method analytes are presented in Table 3125:III.[1,2]

Determining the MDL for each element is critical when analyzing complex matrices, such as seawater, brines, and industrial effluents. In these cases, the MDL typically will be higher than

Table 3125:IV.A. Elemental Abundance Equations and Common Molecular Ion Correction Equations

Elemental and Molecular Equations*†	
Li 6	= C 6
Be 9	= C 9
Al 27	= C 27
Sc 45	= C 45
V 51	= C 51 – (3.127)[(C 53) – (0.113 × C 52)]
Cr 52	= C 52
Cr 53	= C 53
Mn 55	= C 55
Co 59	= C 59
Ni 60	= C 60
Ni 62	= C 62
Cu 63	= C 63
Cu 65	= C 65
Zn 66	= C 66
Zn 68	= C 69
As 75	= C 75 – (3.127)[(C 77) – (0.815 × C 82)]
Se 77	= C 77
Se 82	= C 82 – (1.008696 × C 83)
Sr 88	= C 88
Mo 98	= C 98 – (0.110588 × C 101)
Rh 103	= C 103
Ag 107	= C 107
Ag 109	= C 109
Cd 111	= C 111 – (1.073)[(C 108) – (0.712 × C 106)]
Cd 114	= C 114 – (0.02686 × C 119)
Sb 121	= C 121
Sb 123	= C 123 – (0.127189 × C 125)
Ba 135	= C 135
Ho 165	= C 165
Tl 203	= C 203
Tl 205	= C 205
Pb 208	= C 208 + (1 × C 206) + (1 × C 207)
Th 232	= C 232
U 238	= C 238

* C = calibration blank-corrected counts at indicated masses.
† From EPA Method 200.8 for the Analysis of Drinking Water—Application Note, Order No. ENVA-300A, The Perkin Elmer Corporation, 1996.

the IDL because of background levels of analytes introduced during sample preparation, as well as laboratory-derived contamination and matrix-based interferences. Determine both IDL and MDL when first implementing this method, and then repeat yearly or whenever the instrument configuration changes or major maintenance occurs, whichever happens first.

Also, determine the linear dynamic range (LDR) for all method analytes, including multi-element mixtures (to account for possible inter-element effects). *LDR* is the maximum analyte concentration above the highest calibration point at which the analyte response is within ±10% of its theoretical response. When determining LDRs, avoid using unnecessarily high analyte concentrations because they might damage the detector. Determine LDR when first implementing this method, and then repeat yearly.

c. Interferences: ICP–MS is subject to several types of interferences, including the following:

1) Isobars—*Isobars* are isotopes of different elements that form ions with the same nominal atomic mass units/charge number (m/z) ratio that cannot be resolved by a quadrupole or high-resolution mass spectrometer. Typically, ICP–MS operating software includes all known isobaric interferences and will perform the necessary calculations automatically (see Table 3125:IV). Monitor ^{83}Kr, ^{99}Ru, ^{118}Sn, and ^{125}Te to correct for isobaric interference caused by ^{82}Kr on ^{82}Se, by ^{98}Ru on ^{98}Mo, by ^{114}Sn on ^{114}Cd, by ^{115}Sn on ^{115}In, and by ^{123}Te on ^{123}Sb. Monitor ArCl at mass 77 to estimate chloride interferences. Verify that all elemental and molecular correction equations used in this method are correct and appropriate for the mass spectrometer used and the sample matrix.

2) Abundance sensitivity—*Abundance sensitivity* is the possibility that the low and high "wings" of any abundant mass peak will contribute to or obscure adjacent masses. Adjust the mass spectrometer resolution and quadrupole pole bias to minimize these interferences.

3) Polyatomics—*Polyatomics* are (molecular) ion interferences caused by ions with more than one atom that have the same nominal m/z ratio as the isotope of interest. Most of the common molecular ion interferences have been identified (see Table 3125:V). Because of the severity of chloride ion interference on important analytes, particularly vanadium and arsenic, hydrochloric acid is not recommended for use in ICP–MS sample preparation. Because most environmental samples contain some chloride ion, analysts must use chloride-correction equations for affected masses. Collision cell technology and dynamic reaction cell effectively reduce most polyatomic species to analytically negligible levels in quadrupole-based ICP–MS systems, sometimes removing the need for complex correction equations. A high-resolution ICP–MS resolves many—but not all—interferences caused by polyatomic ions. Polyatomic interferences are strongly influenced by instrument design and plasma operating conditions; they sometimes can be reduced by carefully adjusting nebulizer gas flow and other instrument operating parameters.

4) Doubly-charged—Some elements (e.g., barium and strontium) form significant levels of M^{2+} ions under normal plasma conditions. The M^{2+} ions occur in the mass spectrum at M/2 and, in the case of Ba and Sr, will interfere with some isotopes of zinc and calcium, respectively.

5) Physical interferences—These include differences in viscosity, surface tension, and dissolved solids between samples and calibration standards. To minimize these effects, analytical samples should not contain more than 0.5% of dissolved solids. Dilute water and wastewater samples with higher dissolved solids levels before analyzing them. Use internal standards to correct for physical interferences, provided that their analytical behavior is comparable to the elements being determined.

Table 3125:IV.B includes information on each isotope and cautions on using correction equations. Quadrupole ICP–MS with CCT or DRC is recommended for all analytes, except perhaps beryllium (due to lower sensitivity), because it eliminates many of the equations typically needed in the standard operating mode.

6) Memory interferences—These occur when analytes from a previous sample or standard are measured in the current sample. Use a long enough rinse (flush) between samples to minimize such interferences. Persistent memory interferences may indicate problems in the sample-introduction system. Severe memory interferences may require analysts to disassemble and clean the

TABLE 3125:IV.B. ELEMENTS, MASSES, ABUNDANCES, AND CORRECTION EQUATIONS (UPDATED 2008)

Isotope	Mass	Abundance	Interference Calculation (if required)*	Comments
Li (IS)†	6	7.52	$C\ 6 - 0.08131 \times Li^{7}$	Corrects for natural lithium in samples (I)‡
Be	9	100	*C* 9	
C	13	1.108	*C* 13	For ArC^{52} correction
Al	27	100	C 27	
Ca	43	0.13	$C\ 43 - 0.0004 \times Sr^{88}$	Corrects for Sr^{2+}§
Sc (IS)	45	100	*C* 45	
V	51	99.76	$C\ 51 - 3.1270 \times ClO^{53}$	Corrects for variable chloride matrix (I) [not normally required in CCT Mode]
Cr	52	83.76	$C\ 52 - 0.0900 \times C^{13}$	Corrects for variable carbon content (P)‖ [not normally required in CCT Mode]
ClO (Cr)	53		$C\ 53 - 0.1140 \times Cr^{52}$	CLO interference corrected for chromium (I)
Mn	55	100	*C* 55	
Fe	56	91.52	*C* 56	CCT Mode only (ArO and CaO interferences in Standard Mode)
Co	59	100	*C* 59	
Ni	60	26.16	$C\ 60 - 0.00150 \times Ca^{43}$	Corrects for CaO (P) [not normally required in CCT Mode]
Ni	62	3.66	*C* 62	
Cu	63	69.09	*C* 63	Possibility of ArNa (P) in high sodium matrix (sea or brackish waters)
Cu	65	30.91	*C* 65	
Zn	66	27.81	*C* 66	
Zn	68	18.56	$C\ 68 - 0.0153 \times Ba^{135}$	Ba^{2+} interferes with Zn^{68}
Ge (IS)	72	27.43	*C* 72	Possibility of FeO (P) interference in high iron matrix
As	75	100	$C\ 75 - 3.1270 \times ArCl^{77}$	Corrects for variable chloride matrix (I) Correction not required in CCT Mode (I)
ArCl (Se 77)	77		$C\ 77 - 0.8484 \times Se^{82}$	ArCl interference corrected for Se.
Se	78	23.61	*C* 78	CCT Mode only due to $ArAr^{78}$ in Standard Mode
Se	82	8.84	$C\ 82 - 1.0009 \times Kr^{83}$	Corrects for krypton in argon (I)
Kr	83	11.55	*C* 83	Variable levels in argon
Sr	88	82.56	*C* 88	Forms Sr^{2+} easily
Mo	95	14.78	*C* 95	No isobaric correction required (unlike Mo^{98})
Mo	98	24	$C\ 98 - 0.1307 \times Ru^{101}$	Corrects for variable ruthenium content (I)
Ru	101	16.98	*C* 101	For Mo^{98} correction
Rh (IS)	103	100	*C* 103	
Ag	107	51.35	*C* 107	
Ag	109	48.65	*C* 109	
Cd	111	12.86	$C\ 111 - 0.0017 \times Mo^{95}$	Corrects for MoO (P) [not normally required in CCT Mode]
Cd	114	28.81	$C\ 114 - [0.0271 \times Sn^{118}] - [0.0028 \times Mo^{95}]$	Correction for MoO (P) and Sn (I) [MoO correction not normally required in CCT Mode]
In (IS)	115	95.84	$C\ 115 - 0.0142 \times Sn^{118}$	Corrects for variable tin content (I)
Sn	118	24.01	*C* 118	For Cd^{114} and In^{115} correction
Sb	121	57.25	*C* 121	
Sb	123	42.75	$C\ 123 - 0.0449 \times Te^{125}$	Corrects for variable tellurium content (I)
Te	125	6.99	*C* 125	For Sb^{123} correction
Ba	135	6.59	*C* 135	
Tb (IS)	159	100	*C* 159	
Ho (IS)	165	100	*C* 165	Ho^{2+} may interfere with Se^{82}
Tl	203	29.5	*C* 203	
Tl	205	70.5	*C* 205	
Pb	206	25.15	*C* 206	
Pb	207	21.11	*C* 207	
Pb	208	52.38	$C\ 208 + 1 \times Pb^{206} + 1 \times Pb^{207}$	Correction for variable lead abundance (I)
Bi (IS)	209	100	*C* 209	
U	235	0.715	*C* 235	
U	238	99.28	*C* 238	

* All corrections should be verified and corrected for mass bias.
† IS = internal standard element.
‡ I = isobaric correction.
§ Doubly charged corrections may vary with plasma-tuning conditions and should be verified by the user before use.
‖ P = correction that may vary with plasma-tuning conditions and should be verified by the user before use.

TABLE 3125:V. COMMON MOLECULAR ION INTERFERENCES IN ICP–MS[1]

Molecular Ion	Mass	Element Measurement Affected by Interference
Background molecular ions:		
NH^+	15	—
OH^+	17	—
OH_2^+	18	—
C_2^+	24	Mg
CN^+	26	Mg
CO^+	28	Si
N_2^+	28	Si
N_2H^+	29	Si
NO^+	30	—
NOH^+	31	P
O_2^+	32	S
O_2H^+	33	—
$^{36}ArH^+$	37	Cl
$^{38}ArH^+$	39	K
$^{40}ArH^+$	41	—
CO_2^+	44	Ca
CO_2^+H	45	Sc
ArC^+, ArO^+	52	Cr
ArN^+	54	Cr
$ArNH^+$	55	Mn
ArO^+	56	Fe
ArH^+	57	Fe
$^{40}Ar^{36}Ar^+$	76	Se
$^{40}Ar^{38}Ar$	78	Se
$^{40}Ar_2^+$	80	Se
Matrix molecular ions:		
Bromide:		
$^{81}BrH^+$	82	Se
$^{79}BrO^+$	95	Mo
$^{81}BrO^+$	97	Mo
$^{81}BrOH^+$	98	Mo
$Ar^{81}Br^+$	121	Sb
Chloride:		
$^{35}ClO^+$	51	V
$^{35}ClOH^+$	52	Cr
$^{37}ClO^+$	53	Cr
$^{37}ClOH^+$	54	Cr
$Ar^{35}Cl^+$	75	As
$Ar^{37}Cl^+$	77	Se
Sulfate:		
$^{32}SO^+$	48	Ti
$^{32}SOH^+$	49	—
$^{34}SO^+$	50	V, Cr
$^{34}SOH^+$	51	V
SO_2^+, S_2^+	64	Zn
$Ar^{32}S^+$	72	Ge
$Ar^{34}S^+$	74	Ge
Phosphate:		
PO^+	47	Ti
POH^+	48	Ti
PO_2^+	63	Cu
ArP^+	71	Ga
Group I & II metals:		
$ArNa^+$	63	Cu
ArK^+	79	Br
$ArCa^+$	80	Se
Matrix oxides*		
TiO	62–66	Ni, Cu, Zn
ZrO	106–112	Ag, Cd
MoO	108–116	Cd
NbO	109	Ag

* Oxide interferences normally will be tiny and will affect method elements only when oxide-producing elements are present at relatively high concentrations, or when the instrument is improperly tuned or maintained. Preferably monitor Ti and Zr isotopes in soil, sediment, or solid waste samples, because these samples could contain high levels of such interfering elements.

entire sample-introduction system, including the plasma torch and the sampler and skimmer cones.

7) Ionization interferences—These result when moderate (0.1% to 1%) amounts of a matrix ion change the analyte signal. This effect, which usually reduces the analyte signal, also is known as *suppression*. Correct for suppression by using internal standardization techniques.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Inductively coupled plasma–mass spectrometer: Available from several manufacturers, this instrument includes a mass spectrometer, detector, an ICP source, mass flow controllers for regulating ICP gas flows, a peristaltic pump for introducing samples, and a computerized data acquisition and instrument control system. An x-y autosampler also may be used with appropriate control software.

b. Laboratory ware: Use pre-cleaned plastic laboratory ware for standard and sample preparation. Teflon*—either tetrafluoroethylene hexafluoropropylene-copolymer (FEP), polytetrafluoroethylene (PTFE), or perfluoroalkoxy PTFE (PFA)—is preferred for standard preparation and sample digestion, while high-density polyethylene (HDPE) and other dense, metal-free plastics may be acceptable for internal standards, known-addition solutions, etc. Check each new lot of autosampler tubes for suitability, and pre-clean autosampler tubes and pipet tips if certificate of analysis is unavailable (see Section 3010C.2).

c. Air-displacement pipets, sized 10 to 100 μL, 100 to 1000 μL, and 1 to 10 mL.

d. Analytical balance, accurate to 0.1 mg.

e. Sample-preparation apparatus, such as hot plates, microwave digesters, and heated sand baths. Any sample-preparation device could introduce trace levels of target analytes to the sample.

f. Clean hood (optional), Class 100 (certified to contain less than 100 particles/m^3), for sample preparation and manipulation. If possible, perform all sample manipulations, digestions, dilutions, etc. in a certified Class 100 environment. Alternatively, handle samples in glove boxes, plastic fume hoods, or other environments that minimize random contamination by trace metals.

3. Reagents

a. Acids: Use ultra-high-purity grade (or equivalent) acids to prepare standards and process samples. Redistilled acids are acceptable if each batch is demonstrated to be uncontaminated by target analytes. Use extreme care when handling acids in the laboratory to avoid contaminating them with trace levels of metals.

1) *Nitric acid* (HNO_3), conc (specific gravity 1.41).

2) *Nitric acid,* 1 + 1—Add 500 mL conc HNO_3 to 500 mL reagent water.

3) *Nitric acid,* (v/v) 2%—Add 20 mL conc HNO_3 to 100 mL reagent water; dilute to 1000 mL.

* Or equivalent.

4) *Nitric acid,* (v/v) 1%—Add 10 mL conc HNO_3 to 100 mL reagent water; dilute to 1000 mL.

b. Reagent water: When preparing blanks, standards, and samples, use metal-free water that was prepared using the methods listed in Section 1080, described below, or provided elsewhere (if they have been proven effective). Use only high-purity water to prepare samples and standards. Reagent water containing trace amounts of analyte elements will skew results.

Analysts can produce suitable reagent water using a multistage system that includes a softener/reverse osmosis unit, a dual-column, strong acid/strong base ion exchange unit; an activated carbon filter; and a UV sterilization process. This will produce a polished water free of metals and organics.

c. Stock, standard, and other required solutions: See Sections 3111B.3*j*, 3111D.3*k*, 3114B.3*j–n*, and 3120B.3*d* on preparing standard stock solutions from elemental materials (pure metals, salts). Preferably, purchase high-purity, commercially prepared stock solutions and dilute to required concentrations. Single- or multi-element stock solutions (1000 mg/L) of the following elements are required: aluminum, antimony, arsenic, barium, beryllium, cadmium, cerium, chromium, cobalt, copper, germanium, indium, lead, magnesium, manganese, molybdenum, nickel, rhodium, scandium, selenium, silver, strontium, terbium, thallium, thorium, uranium, vanadium, and zinc. Prepare internal-standard and target-element stock solutions separately; they could be incompatible and cause precipitation or other solution instability.

1) *Internal standard stock solution*—Germanium, indium, lithium, scandium, and thorium are suggested as internal standards. The following masses are monitored: ^{72}Ge, ^{115}In, ^{6}Li, ^{45}Sc, and ^{232}Th. Add enough internal standard to all samples, standards, and quality control (QC) samples to give a suitable counts/second (cps) signal and stability (100 000 to 300 000 cps for most internal standards). Minimize dilution-related errors by using an appropriately high concentration of internal standard mix solution. Maintain volume ratio for all internal standard additions.

Prepare internal standard mix as follows: Prepare a nominal 50-mg/L solution of ^{6}Li by dissolving 0.15 g $^{6}Li_2CO_3$ [isotopically pure (i.e., 95% or greater purity†)] in a minimal amount of 1:1 HNO_3. Pipet 5.0 mL 1000-mg/L germanium, indium, scandium, and thorium standards into the lithium solution, dilute resulting solution to 500.0 mL, and mix thoroughly. The resultant concentration of Ge, In, Sc, and Th will be 10 mg/L. Determine the internal standard concentrations required to achieve acceptable levels of precision, and dilute the internal standard stock accordingly.

Other internal standards, such as bismuth, holmium, rhodium, terbium, and yttrium, may also be used in this method. Ensure that the internal standard mix used is stable and that there are no undesired interactions among elements.

All new sample matrices should be screened for internal standard elements before analysis. Analyzing a few representative samples for internal standards should be sufficient. Analyze samples "as received" or "as digested" (before adding internal standard), then add internal standard mix and re-analyze. Monitor counts at the internal standard masses. If the "as received" or "as digested" samples show appreciable detector counts (10% or higher of samples with added internal standard), dilute sample or use another internal standard. If the response of a sample containing the internal standard is not within 70 to 125% of the response for a calibration blank with the internal standard, either dilute the sample before analysis or use another internal standard. During actual analysis, monitor internal standard masses and note all internal standard recoveries that are more than 125% of the internal standard response in the calibration blank. Interpret results for these samples with caution.

The internal standard mix may be added to blanks, standards, and samples via a "Y" connector after the peristaltic pump to mix it with the sample stream during sample introduction.

2) *Instrument optimization/tuning solution*—containing beryllium, cadmium, cobalt, copper, germanium, indium, rhodium, scandium, terbium, thallium, (for sensitivity and stability evaluation), barium (for doubly-charged evaluation), cerium (for oxide evaluation), magnesium (mass calibration check), and lead (mass calibration check). Prepare this solution in 2% HNO_3. This mix includes all common elements used to optimize and tune various ICP–MS operating parameters. It may be possible to use fewer elements in this solution, depending on the instrument manufacturer's recommendations.

3) *Calibration standards*—A five standard calibration is recommended, from 0 to 100 μg/L.‡ Other calibration regimes are acceptable if the full suite of quality assurance samples and standards is run to validate any method changes. Fewer standards may be used, and a two-point blank/mid-range calibration technique commonly used in ICP optical methods should also produce acceptable results. Calibrate all analytes using the selected concentrations. Prepare all calibration standards and blanks in a matrix of 2% nitric acid. Add internal standard mix to all calibration standards to provide appropriate count rates for interference correction. NOTE: Add the same ratio of internal standard mix to all standards and blanks used in this method.

4) *Method blank (MB)*—A *method blank* (also known as *reagent blank*) is a portion of reagent water (¶ *b* above) treated exactly as a sample, including exposure to all equipment, glassware, procedures, and reagents. It is used to assess whether analytes or interferences are present in the analytical process or system. No MB should contain a warning level of any analyte of interest (based on the end user's requirements). Undertake immediate corrective action for MB measurements above the minimum reporting level (see Section 3020B.5). Include at least one MB with each batch of samples prepared. For dissolved samples, take reagent water through same filtration and preservation processes used for samples. For samples requiring digestion, process reagent water with the same digestion techniques as samples. Add internal standard mix to method blank.

5) *Calibration verification standard*—Prepare a mid-range standard using a different source than that used for the calibration standards, in 2% HNO_3, with equivalent addition of internal standard.

6) *Calibration verification blank*—Use 2% HNO_3, the same solution as the zero calibration standard.

7) *Laboratory-fortified blank (LFB)*—The *laboratory-fortified blank* (also known as a *blank spike*) is a method blank that has

† Cambridge Isotope Laboratories, or equivalent.

‡ Performance data for the method, were obtained with these concentrations.

been fortified with a known concentration of analyte. It is used to evaluate ongoing laboratory performance and analyte recovery in a clean matrix. Prepare fortified concentrations approximating the midpoint of the calibration curve (50 ng/mL) or lower with stock solutions prepared from a different source than that used to develop working standards. Calculate percent recovery, plot control charts, and determine control limits for these measurements. Ensure that the LFB meets the method's performance criteria when such criteria are specified. Establish corrective actions to be taken in case the LFB does not satisfy acceptance criteria. Include at least one LFB with each batch of samples prepared. This standard, sometimes also called a *laboratory control sample* (LCS), is used to validate digestion techniques and known-addition levels.

8) *Reference materials*—Use externally prepared reference material, preferably from National Institute of Standards and Technology§ (NIST) 1643 series or equivalent.

9) *Known-addition solution for samples*—Add stock standard to sample so the volume change is less than 5%. In the absence of information on analyte levels in the sample, prepare known additions at around 50 μg/L or lower. If analyte concentration levels are known, add at 50 to 200% of the sample levels. For samples undergoing digestion, make additions before digestion. For dissolved metals determinations, make additions after filtration, preferably immediately before analysis.

10) *Low-level standards*—Use both a 0.3- and a 1.0-μg/L standard when expected analyte concentration is less than 5 μg/L. Prepare both standards in 2% nitric acid.

Prepare volumetrically a mixed standard containing the method analytes at desired concentration(s) (0.30 μg/L, 1.0 μg/L, or both). Prepare weekly in 100-mL quantities.

d. Argon: Use a prepurified grade of argon unless it can be demonstrated that other grades can be used successfully. Prepurified argon is usually necessary because technical argon often contains significant levels of impurities (e.g., carbon and krypton). ^{82}Kr interferes with the determination of ^{82}Se. Monitor ^{83}Kr at all times.

4. Procedures

a. Sample preparation: See Sections 3010 and 3020 for general guidance and additional specific requirements on sampling and quality control. See Section 3030E for the recommended sample-digestion technique for all analytes except silver and antimony. Use the method in Section 3030F for silver and antimony, paying special attention to chloride ion interferences and using all applicable elemental corrections. Alternative digestion techniques and more guidance on sample preparation are available.[3,4]

Ideally, use a "clean" environment when handling, manipulating, or preparing samples. Preferably perform all sample manipulations in a Class 100 clean hood or room to minimize potential contamination artifacts in digested or filtered samples.

b. Instrument operating conditions: Follow manufacturer's standard operating procedures for initialization, mass calibration, gas flow optimization, and other instrument operating conditions. Maintain complete, detailed information on the instrument's operating status whenever it is used.

§ National Institute of Standards and Technology, www.nist.gov

Table 3125:VI. Suggested Analytical Run Sequence

Sample Type	Comments
Tuning/optimization standard	Check mass calibration and resolution
Tuning/optimization standard	Optimize instrument for maximum rhodium counts while keeping oxides, double-charged ions, and background within instrument specifications
Rinse	—
Reagent blank	Check for contamination
Reagent blank	Calibration standard blank
5-μg/L standard	—
10-μg/L standard	—
20-μg/L standard	—
50-μg/L standard	—
100-μg/L standard	—
Rinse	Check for memory
Initial calibration verification, 50 μg/L	Check for calibration accuracy
Initial calibration blank	Check for memory
0.30-μg/L standard	Low-level calibration verification
1.0-μg/L standard	Low-level calibration verification
External reference material	NIST 1643c or equivalent
Continuing calibration verification	Check for calibration stability
Continuing blank calibration	Check for memory
Project sample method blank	Check for contamination
Project sample laboratory-fortified blank	Check for spike recovery
Project sample 1-4	Check for possible interferences
Project sample 5	—
Project sample 5 with known addition	Check for spike recovery
Project sample 5 duplicate with known addition	Check for reproducibility
Continuing calibration verification (every 10 samples)	Check for calibration stability
Continuing calibration blank (every 10 samples)	Check for memory

c. Analytical run sequence: Table 3125:VI outlines a suggested analytical run sequence, including tuning and optimizing the instrument, checking reagent blanks, calibrating the instrument, verifying the calibration, analyzing samples, and analyzing quality control samples and blanks.

d. Instrument tuning and optimization: Follow manufacturer's instructions for optimizing instrument performance. The most important optimization criteria include nebulizer gas flows, detector and lens voltages, radio-frequency forward power, and mass calibration. Periodically check mass calibration and instrument resolution. Ideally, optimize the instrument to minimize the formation of oxide and doubly charged species. Measure CeO^+:Ce^+ and Ba^{2+}:Ba^+ ratios to monitor the formation of oxide and doubly charged species, respectively. Both ratios should meet the manufacturer's criteria before instrument calibration. Moni-

Table 3125:VII. Summary of Performance Criteria

Performance Characteristic	Criteria
Mass resolution	Manufacturer's specification
Mass calibration	Manufacturer's specification
Ba^{2+}/Ba^{+}	Manufacturer's specification
CeO/Ce	Manufacturer's specification
Background counts at mass 220	Manufacturer's specification
Correlation coefficient	≥0.995
Calibration blanks	< Reporting limit
Calibration verification standards	±10% of true value
Laboratory fortified blank (control sample)	±30% of true value
Precision	±20% relative percent difference for lab duplicates
Known-addition recovery	75–125%
0.3 and 1.0 μg/L standards	Dependent on data quality objectives
Reference materials	Dependent on data quality objectives
Internal standard response	70–125% of response in calibration blank with known addition

tor background counts at mass 220 after optimization, and compare with manufacturer's criteria. (See Table 3125:VII for a summary of method performance criteria related to optimization and tuning, calibration, and analytical performance.)

e. Instrument calibration: After optimization and tuning, calibrate the ICP–MS using an appropriate range of calibration standards. Use appropriate regression techniques to determine calibration responses for each analyte. For acceptable calibrations, the regression curves' correlation coefficients are ideally 0.995 or greater.

Immediately after calibration, run initial calibration verification standard, 3125B.3*c*5); acceptance criteria are ±10% of known analyte concentration. Next, run initial calibration verification blank, 3125B.3*c*6); acceptance criteria are ideally ± the absolute value of the instrument detection limit for each analyte, but in practice, ± the absolute value of the laboratory reporting limit or the laboratory method detection limit for each analyte is acceptable. If analyte concentrations are less than 5 μg/L, verify low-level calibration by using a standard at 40 to 50% of the highest low-level standard.

f. Sample analysis: Ensure that all vessels and reagents are uncontaminated. During the analytical run, include quality-control analyses according to schedule of Table 3125:VIII or follow project-specific QA/QC protocols.

Internal standard recoveries must be between 70 and 125% of internal standard response in the laboratory-fortified blank; otherwise, dilute sample, add internal standard mix, and re-analyze.

Make known-addition analyses for each matrix in a digestion or filtration batch.

5. Calculations and Corrections

Configure instrument software to report internal-standard-corrected results. Water sample results should be reported in micrograms per liter. Report appropriate number of significant figures.

a. Correction for dilutions and solids: Correct all dilution results, and raise the related reporting limits accordingly:

$$R_{Corr} = \frac{R_{Uncorr} \times V}{(V_s/V)}$$

where:

R_{Corr} = diluted corrected results, μg/L,
R_{Uncorr} = uncorrected elemental results, μg/L,
V = volume of digestate (after digestion), L, and
V_s = volume of undiluted sample, L.

Use Method 2540B to determine total solids in solid samples, and report results as micrograms per kilogram, dry weight. Correct all results for solids content of solid samples. Use the following equation to correct solid or sediment sample results for dilution during digestion and moisture content:

Table 3125:VIII. Quality Control Analyses for ICP–MS Method

Analysis	Frequency	Acceptance Criteria
Reference material [3125B.3*c*9)]	Greater of: once per sample batch, or 5%	Dependent on data quality objectives
Preparatory/method blank [3125B.3*c*4)]	Greater of: once per sample batch, or 5%	± Absolute value of instrument detection limit; absolute value of laboratory reporting limit or MDL is acceptable
Laboratory-fortified blank [3125B.3*c*7)]	Greater of: once per sample batch, or 5%	±30% of true value
Duplicate known-addition samples	Greater of: once per sample batch, or 5%	±20% relative percent difference
Continuing calibration verification standards [3125B.3*c*5)]	10%	±10% of known concentration
Continuing calibration verification blank [3125B.3*c*6)]	10%	± Absolute value of instrument detection limit; absolute value of laboratory reporting limit or MDL is acceptable

Table 3125:IX. Method Performance with Calibration Verification Standards*

		Continuing Calibration Verification Standard (N = 44)				Initial Calibration Verification Standard (N = 12)			
Element	Mass	Mean Recovery %	Mean µg/L	Standard Deviation µg/L	Relative Standard Deviation %	Mean Recovery %	Mean µg/L	Standard Deviation µg/L	Relative Standard Deviation %
Be	9	98.71	49.35	3.43	6.94	100.06	50.03	1.90	3.80
Al	27	99.62	49.81	2.99	6.01	98.42	49.21	1.69	3.44
V	51	100.97	50.48	1.36	2.68	99.91	49.96	1.23	2.47
Cr	52	101.39	50.70	1.86	3.66	99.94	49.97	1.47	2.95
Cr	53	100.68	50.34	1.91	3.79	99.13	49.56	1.44	2.90
Mn	55	101.20	50.60	1.98	3.91	99.48	49.74	1.40	2.82
Co	59	101.67	50.83	2.44	4.79	99.44	49.72	1.61	3.24
Ni	60	99.97	49.99	2.14	4.28	97.98	48.99	1.70	3.47
Ni	62	99.79	49.89	2.09	4.18	97.57	48.79	1.32	2.71
Cu	63	100.51	50.25	2.19	4.36	97.87	48.93	1.63	3.33
Cu	65	100.39	50.19	2.26	4.51	98.34	49.17	1.58	3.20
Zn	66	101.07	50.53	1.93	3.82	98.75	49.38	0.87	1.76
Zn	68	100.42	50.21	1.89	3.77	97.75	48.87	0.50	1.02
As	75	100.76	50.38	1.15	2.28	98.83	49.41	0.89	1.80
Se	77	101.71	50.85	1.43	2.81	99.54	49.77	1.01	2.03
Se	82	101.97	50.98	1.50	2.95	99.76	49.88	0.94	1.89
Ag	107	101.50	50.75	1.68	3.30	99.27	49.63	1.17	2.36
Ag	109	101.65	50.83	1.68	3.31	99.66	49.83	1.54	3.08
Cd	111	100.92	50.46	1.94	3.84	98.61	49.30	1.36	2.77
Cd	114	100.90	50.45	2.07	4.10	99.20	49.60	1.41	2.84
Sb	121	100.14	50.07	2.39	4.77	99.38	49.69	1.38	2.78
Sb	123	99.98	49.99	2.48	4.97	99.09	49.54	1.34	2.71
Tl	203	101.36	50.68	1.64	3.23	100.05	50.02	1.01	2.01
Tl	205	102.40	51.20	1.93	3.78	101.23	50.62	1.45	2.87
Pb	208	101.21	50.61	1.65	3.25	99.33	49.67	0.84	1.69
U	238	101.54	50.77	1.93	3.80	99.80	49.90	1.36	2.72

* Single-laboratory, single-operator, single-instrument data, determined using a 50-µg/L standard prepared from sources independent of calibration standard source. Data acquired January–November 1996 during actual sample determinations. Performance of continuing calibration verification standards at different levels may vary. Perkin-Elmer Elan 6000 ICP–MS used for determination.

$$R_{corr} = \frac{R_{uncorr} \times V}{W \times \% TS/100}$$

where:

R_{corr} = corrected result, µg/kg,
R_{uncorr} = uncorrected elemental result, µg/L,
V = volume of digestate (after digestion), L,
W = mass of the wet sample, kg, and
$\% TS$ = percent total solids determined in the sample.

b. Compensation for interferences: Use instrument software to correct for previously listed interferences. See Table 3125:V for a list of the most common molecular ion interferences.

c. Data reporting: Establish appropriate reporting limits for method analytes based on IDLs and the laboratory blank. For regulatory programs, ensure that reporting limits for method analytes are a factor of three below relevant regulatory criteria whenever possible.

If method blank contamination is typically random, sporadic, or otherwise not statistically controllable, do not correct results for the method blank. Consider correcting blank results only if it can be demonstrated that the blank's analyte concentration is within statistical control over a period of months. Report all method blank data explicitly in a manner identical to sample-reporting procedures.

d. Documentation: Maintain documentation for the following (where applicable): instrument tuning, mass calibration, calibration verification, analyses of blanks (method, field, calibration, and equipment blanks), IDL and MDL studies, analyses of samples and duplicates with known additions, laboratory and field duplicate information, serial dilutions, internal standard recoveries, and any relevant quality control charts.

Also, maintain all raw data generated in support of the method, and keep them available for review.[5]

6. Method Performance

Table 3125:III presents IDL data generated by this method; this represents optimal instrument detection capabilities, not recommended method detection or reporting limits. Tables 3125:I, II, and IX through XI contain single-laboratory, single-operator, single-instrument performance data generated by this method for calibration verification standards, low-level standards, and known-addition recoveries for freshwater matrices, as well as data showing standard and CCT performance data for elements usually affected by polyatomic interferences (Tables 3125:I and II). Section 3125 performance data for some analytes are currently unavailable; however, performance data for similar ICP-MS methods are available in the literature.[1,4]

Table 3125:X. Method Performance for Recovery of Known Addition in Natural Waters*

		Total Recoverable Metals†		Dissolved Metals‡	
Element	Mass	Mean Recovery %	Relative Standard Deviation %	Mean Recovery %	Relative Standard Deviation %
Be	9	89.09	5.77	—	—
V	51	87.00	8.82	—	—
Cr	52	87.33	8.42	88.38	6.43
Cr	53	86.93	7.90	88.52	5.95
Mn	55	91.81	10.12	—	—
Co	59	87.67	8.92	—	—
Ni	60	85.07	8.42	89.31	5.70
Ni	62	84.67	8.21	89.00	5.82
Cu	63	84.13	8.46	88.55	8.33
Cu	65	84.37	8.05	88.26	7.80
Zn	66	86.14	23.01	95.59	13.81
Zn	68	81.95	20.31	91.94	13.27
As	75	90.43	4.46	97.30	8.84
Se	77	83.09	4.76	105.36	10.80
Se	82	83.42	4.73	105.36	10.75
Ag	107	—	—	91.98	5.06
Ag	109	—	—	92.25	4.96
Cd	111	91.37	5.47	96.91	6.03
Cd	114	91.47	6.04	97.03	5.42
Sb	121	94.40	5.24	—	—
Sb	123	94.56	5.36	—	—
Tl	203	97.24	5.42	—	—
Tl	205	98.14	6.21	—	—
Pb	208	96.09	7.08	100.69	7.28

* Single-laboratory, single-operator, single-instrument data. Samples were Washington State surface waters from various locations. Data acquired January–November 1996 during actual sample determinations. Performance of known additions at different levels may vary. Perkin-Elmer Elan 6000 ICP–MS used for determination.
† Known-addition level 20 μg/L. Additions made before preparation according to Section 3030E (modified by clean-hood digestion in TFE beakers). $N = 20$.
‡ Known-addition level for Cd and Pb 1 μg/L; for other analytes 10 μg/L. Additions made after filtration through 1:1 HNO_3 precleaned 0.45-μm filters. $N = 28$.

Table 3125:XI. Method Performance with Calibration Verification Standards*

		1.0-μg/L Standard				0.3-μg/L Standard			
Element	Mass	Mean Recovery %	Mean μg/L	Standard Deviation μg/L	Relative Standard Deviation %	Mean Recovery %	Mean μg/L	Standard Deviation μg/L	Relative Standard Deviation %
Be	9	97	0.97	0.06	6.24	95	0.284	0.03	12.11
Al	27	121	1.21	0.32	26.49	196	0.588	0.44	74.30
V	51	104	1.04	0.06	5.83	111	0.332	0.10	28.96
Cr	52	119	1.19	0.34	28.62	163	0.490	0.37	75.90
Cr	53	102	1.02	0.36	35.54	113	0.338	0.32	93.70
Mn	55	103	1.03	0.07	6.55	110	0.329	0.08	25.64
Co	59	103	1.03	0.07	6.42	102	0.307	0.04	12.53
Ni	60	101	1.01	0.05	5.24	107	0.321	0.05	14.14
Ni	62	102	1.02	0.06	5.42	109	0.326	0.05	15.94
Cu	63	107	1.07	0.09	8.78	118	0.355	0.06	18.29
Cu	65	107	1.07	0.10	9.05	117	0.352	0.06	17.69
Zn	66	117	1.17	0.51	43.52	182	0.547	0.68	124.13
Zn	68	116	1.16	0.50	42.90	179	0.537	0.66	122.12
As	75	97	0.97	0.05	5.23	101	0.302	0.06	18.29
Se	77	89	0.89	0.08	8.72	88	0.265	0.08	29.07
Se	82	92	0.92	0.14	15.50	106	0.317	0.14	43.91
Ag	107	101	1.01	0.05	4.53	94	0.282	0.04	15.74
Ag	109	103	1.03	0.07	6.57	92	0.277	0.04	13.68
Cd	111	98	0.98	0.04	3.80	96	0.288	0.03	8.74
Cd	114	100	1.00	0.03	3.39	98	0.293	0.03	8.70
Sb	121	94	0.94	0.05	5.28	93	0.280	0.06	21.89
Sb	123	94	0.94	0.05	5.36	93	0.278	0.06	22.39
Tl	203	101	1.01	0.04	3.57	98	0.294	0.03	11.89
Tl	205	104	1.04	0.05	5.15	100	0.300	0.03	10.43
Pb	208	104	1.04	0.04	3.65	104	0.312	0.03	11.13
U	238	106	1.06	0.05	4.64	102	0.307	0.03	9.92

* Single-laboratory, single-operator, single-instrument data. $N = 24$ for both standards.

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3130 METALS BY ANODIC STRIPPING VOLTAMMETRY*

3130 A. Introduction

Anodic stripping voltammetry (ASV) is one of the most sensitive metal analysis techniques; it is as much as 10 to 100 times as sensitive as electrothermal atomic absorption spectroscopy for some metals. This corresponds to detection levels in the nanogram-per-liter range. The technique requires no sample extraction or preconcentration, it is nondestructive, and it allows simultaneous determination of four to six trace metals, utilizing inexpensive instrumentation. The disadvantages of ASV are that it is restricted to amalgam-forming metals, analysis time is longer than for spectroscopic methods, and interferences and high sensitivity can present severe limitations. The analysis should be performed only by analysts skilled in ASV methodology because of the interferences and potential for trace background contamination.

* Approved by Standard Methods Committee, 2004. Editorial revisions, 2011. Joint Task Group: 20th Edition—Malgorzata Ciszkowska (chair), Margaret M. Goldberg, Janet G. Osteryoung, Robert S. Rodgers, Marek Wojciechowski.

3130 B. Determination of Lead, Cadmium, and Zinc

1. General Discussion

a. Principle: Anodic stripping voltammetry is a two-step electroanalytical technique. In the preconcentration step, metal ions in the sample solution are reduced at negative potential and concentrated into a mercury electrode. The concentration of the metal in the mercury is 100 to 1000 times greater than that of the metal ion in the sample solution. The preconcentration step is followed by a stripping step applying a positive potential scan. The amalgamated metal is oxidized rapidly and the accompanying current is proportional to metal concentration.

b. Detection levels and working range: The level of detection for metal determination using ASV depends on the metal determined, deposition time, stirring rate, solution pH, sample matrix, working electrode (hanging mercury drop electrode, HMDE, or thin mercury film electrode, TMFE), and mode of the stripping potential scan (square wave or differential pulse). Cadmium, lead, and zinc are concentrated efficiently during pre-electrolysis because of their high solubility in mercury and thus have low detection levels (<1 μg/L). Long deposition times and high stirring rates increase the concentration of metal preconcentrated in the mercury phase and reduce detection levels. The effects of solution pH and matrix are more complicated. In general, add a high concentration of inert electrolyte to samples to maintain a high, constant ionic strength. Acidify sample to a low pH or add a pH buffer. If the pH buffer or other component of the sample matrix complexes the metal (*c* below), detection levels often are increased.

The choice of working electrode is determined largely by the working range of concentration required. The HMDE is best suited for analysis from approximately 1 μg/L to 10 mg/L, while the TMFE is superior for detection below 1 μg/L.

c. Interferences: Major interferences include intermetallic compound formation, overlapping stripping peaks, adsorption of organics, and complexation. Intermetallic compounds can form in the mercury phase when high concentrations of certain metals are present simultaneously. Zinc forms intermetallic compounds with cobalt and nickel, and both zinc and cadmium form intermetallic compounds with copper, silver, and gold. As a result, the stripping peak for the constituent metals may be severely depressed or shifted and additional peaks due to intermetallic compound stripping may be observed. Minimize or avoid intermetallic compound formation by use of a hanging mercury drop electrode instead of a thin film mercury electrode when metal concentrations are above 1 μg/L, application of a preconcentration potential sufficiently negative to reduce the desired but not the interfering metal, and use of a relatively short preconcentration period followed by a relatively large pulse modulation (50 mV) during the stripping stage. In general, suspect formation of intermetallic compounds if metals are present in concentrations above 1 mg/L. If metals are present at concentrations above 10 mg/L, do not use anodic stripping voltammetry. Concentrations above 10 mg/L usually can be quantitated by such methods as those given in Sections 3111 and 3120.

Separate overlapping stripping peaks by various methods, including appropriate choice of buffer and electrolyte.[1–3] If only one of the metal peaks is of interest, eliminate interfering peaks by selective complexation with a suitable ligand, such as EDTA. Judicious choice of preconcentration potential can result in the deposition of the selected metal but not the interfering metal in the mercury electrode. Selection of buffer/ligand also may help to distinguish metals during the preconcentration step. Alternatively, use "medium exchange," in which preconcentration is performed with the electrodes in the sample and stripping is performed in a different electrolyte solution. In this procedure, metals are deposited from the sample into the amalgam as usual, but they may be stripped into a medium that provides different stripping peak potentials for the overlapping metals.

Minimize interferences from adsorption of organic compounds and complexation by removal of the organic matter.

Digest samples with high-purity acids as described in Section 3030. Make standard additions to determine if complexation or adsorption remains a problem. Analyze a metal-free solution with a matrix similar to that of the sample both before and after addition of known quantities of standard. Repeat procedure for sample. If the slope of the stripping current versus added metal is significantly different in the sample relative to the metal-free solution, digest sample further. The choice of stripping waveform also is important. While both square-wave and differential-pulse stripping attempt to minimize the contribution of adsorption currents to the total measured stripping current, square-wave stripping does this more effectively. Thus, use square-wave stripping instead of differential-pulse stripping when adsorption occurs.

2. Apparatus

a. Electrochemical analyzer: The basic electrochemical analyzer for ASV applications contains a three-electrode potentiostat, which very precisely controls potential applied to the working electrode relative to the reference electrode, and a sensitive current measuring device. It is capable of delivering potential pulses of various amplitudes and frequencies, and provides several scan rates and current ranges. More advanced ASV instruments offer automated timing, gas purge and stirring, and data processing routines including curve smoothing, baseline correction, and background subtraction.

Two variations of stripping waveforms are commonly used: differential pulse (DPASV) and square wave (SWASV) waveforms. The differential-pulse waveform consists of a series of pulses superimposed on a linear voltage ramp, while the square-wave waveform consists of a series of pulses superimposed on a staircase potential waveform. Square-wave stripping is significantly faster than differential-pulse stripping and is typically ten times more sensitive. Most commercially available ASV instruments perform both differential-pulse and square-wave stripping.

b. Electrodes and cell: Provide working, reference, and auxiliary electrodes. Working electrodes are either hanging mercury drop or thin mercury film electrodes. Hanging mercury drop electrodes must be capable of dispensing mercury in very precisely controlled drop sizes. Three types of electrodes meet this requirement: static mercury drop electrodes, controlled growth mercury drop electrodes, or Kemula-type electrodes. In any case, use a drop knocker to remove an old drop before dispensing a fresh mercury drop.

When the lowest detection levels are required, a thin mercury film electrode is preferred. This electrode consists of a rotating glassy carbon disk plated with mercury *in situ* during preconcentration of the analyte. A high-precision, constant-speed rotator controls the rotation rate of the electrode and provides reproducible mass transport.

Reference electrodes may be either saturated calomel or silver/silver chloride electrodes. Use a platinum wire for the auxiliary electrode.

Use cells constructed of glass, or preferably fused silica or TFE, because they are more resistant to solution adsorption or leaching. Cover cell with a lid that provides reproducible placement of the electrodes and gas purging tubes. Provide an additional hole in the cell lid for addition of standards. Most commercially available mercury drop electrodes include electrolytic cells, reference and auxiliary electrodes, and gas purging tubes.

Use a constant-speed stirring mechanism to provide reproducible mass transport in samples and standards.

Locate the cell in an area where temperature is relatively constant. Alternatively, use a constant-temperature water bath and cell jacket.

c. Oxygen-removal apparatus: Oxygen interferes in electrochemical analyses; remove it from solution before preconcentration by purging with nitrogen or argon. Provide two gas inlet tubes through the cell lid: one extends into the solution and the second purges the space above the solution. A gas outlet hole in the lid provides for removal of oxygen and excess purging gas.

d. Recording device: If the electrochemical analyzer is not equipped with a digital data acquisition system, use an XY plotter to record stripping voltammograms.

e. Timer: If preconcentration and equilibration periods are not controlled by the instrument, use an accurate timing device.

f. Polishing wheel: To obtain the high polish required for a glassy carbon disk electrode, use a motorized polishing wheel.

3. Reagents

Wherever possible, use high-purity reagents that conform to ACS specifications.

CAUTION: Follow proper practices for disposal of any solutions containing mercury.

a. Metal-free water: Use deionized water to prepare buffers, electrolytes, standards, etc. Use water with at least 18 megohm-cm resistivity (see Section 1080).

b. Nitric acid (HNO_3), conc, high-purity.*

c. Nitric acid (HNO_3), 6*N*, 1.6*N* (10%), and 0.01*N*.

d. Purging gas (nitrogen or argon), high-purity: Remove traces of oxygen in nitrogen or argon gas before purging the solution. Pass gas through sequential scrubbing columns containing vanadous chloride in the first, deionized water in the second, and buffer (or electrolyte) solution in the third column.

e. Metal standards: Prepare stock solutions containing 1 mg metal/mL in polyethylene bottles. Purchase these solutions commercially or prepare as in Section 3111. Daily prepare dilutions of stock standards in a matrix similar to that of the samples to cover the concentration range desired.

f. Electrolyte/buffer: Use one of the following:

1) *Acetate buffer,* pH 4.5—Dissolve 16.4 g anhydrous sodium acetate, $NaC_2H_3O_2$, in 800 mL water. Adjust to pH 4.5 with high-purity glacial acetic acid.* Dilute to 1 L with water.

2) *Citrate buffer,* pH 3—Dissolve 42.5 g citric acid (monohydrate) in 700 mL water. Adjust to pH 3 with high-purity NH_4OH.* Dilute to 1 L with water.

3) *Phosphate buffer,* pH 6.8—Dissolve 24 g NaH_2PO_4 in 500 mL water. Adjust to pH 6.8 with 1*N* NaOH. Dilute to 1 L with water.

g. Mercury: Use commercially available triply distilled metallic mercury for hanging mercury drop electrodes. **CAUTION: Mercury vapors are highly toxic. Use only in well-ventilated area.**

h. Mercuric nitrate solution [$Hg(NO_3)_2$], For thin mercury film electrodes, dissolve 0.325 g $Hg(NO_3)_2$ in 100 mL 0.01*N* HNO_3.

* Ultrex, Suprapur, Aristar, or equivalent.

i. Reference electrode filling solution: Available from electrode manufacturer.

j. Amalgamated zinc: Dissolve 2 g $Hg(NO_3)_2$ in 25 mL conc HNO_3; dilute to 250 mL with water. In a separate beaker, clean approximately 50 g mossy zinc by gently oxidizing the surface with 10% HNO_3 and rinse with water. Add $Hg(NO_3)_2$ solution to cleaned zinc and stir with a glass rod. If barely visible bubbles do not appear, add a small amount of 6*N* HNO_3. Zinc should rapidly acquire a shiny, metallic appearance. Decant solution and store for amalgamating future batches of zinc. Rinse amalgamated zinc copiously with water and transfer to a gas scrubbing column.

k. Hydrochloric acid (HCl), conc.

l. Vanadous chloride: Add 2 g ammonium metavanadate, NH_4VO_3, to 25 mL conc HCl and heat to boiling. Solution should turn blue-green. Dilute to 250 mL with water. Pour solution into gas scrubbing column packed with amalgamated zinc and bubble purging gas through it until the solution turns a clear violet color. When the violet color is replaced by a blue, green, or brown color, regenerate vanadous chloride by adding HCl.

m. Siliconizing solution: Preferably use commercially available solutions in sealed ampules for siliconizing capillaries used for hanging mercury drops. **CAUTION: Most commercial siliconizing reagents contain CCl_4, a toxic and cancer-suspect agent. Handle with gloves and avoid breathing vapors.**

n. Alumina suspensions, 1, 0.3, and 0.05 μm: Use commercially available alumina suspensions in water, or make a suspension by adding a small amount of water to the alumina.

o. Hydrofluoric acid (HF), 5%: Dilute 5 mL conc HF to 100 mL with water.

p. Methanol.

q. Sodium hydroxide (NaOH), 1*N*.

4. Procedure

a. Sample preparation and storage: Collect samples in precleaned, acid-soaked polyethylene or TFE bottles. Add 2 mL conc HNO_3/L sample and mix well. Cap tightly and store in refrigerator or freezer until ready for analysis.

b. Cell preparation: Soak clean cell in 6*N* HNO_3 overnight and rinse well with water before use.

c. Electrode preparation:

1) HMDE—Follow manufacturer's guidance for capillary cleaning. If not available, use the following procedure. Remove all mercury from the capillary. Aspirate the following through the capillary in the order listed: 6*N* HNO_3, water, 5% HF, water, methanol, and air. Dry capillary at 100°C for 1 h. Siliconize cooled capillary using a siliconizing solution. Between uses, fill capillary with clean mercury and immerse tip in clean mercury. If the capillary fails to suspend a drop of mercury, repeat cleaning.

2) TMFE—Polish glassy carbon disks used for thin mercury film electrodes to a high metallic sheen with alumina suspensions, progressively decreasing particle size from 1 μm to 0.05 μm. Use a motorized polishing wheel for best results. Completely rinse off all traces of alumina with water. Check disk frequently for etching or pitting; repolish as necessary to maintain reproducible mercury film deposition.

d. Instrumental conditions: Use the following conditions:

Variable	Value
Initial potential	−1.00 V (Pb, Cd); −1.20 V (Zn)
Final potential	0.00 V
Equilibration potential	−1.00 V (Pb, Cd); −1.20 V (Zn)
HMDE drop size	medium
TMFE rotation rate	2000 rpm
DPASV:	
Pulse amplitude	25 mV
Pulse period	0.5 s
Pulse width	50 ms
Sample width	17 ms
Scan rate	5 mV/s
SWASV:	
SW amplitude	25 mV
Step potential	4 mV
Frequency	100 Hz

e. Deoxygenation: Pipet 2 mL sample and 3 mL electrolyte/buffer into cell. If using a TMFE, add 10 μL $Hg(NO_3)_2$ solution. Place electrodes in cell and secure cell lid. Deoxygenate solution with purified purging gas for 10 min while stirring. When solution purge is completed, purge the space above the solution with purified purging gas. Continue head-space purge throughout analysis.

f. Preconcentration: If using a HMDE, dispense a new mercury drop. Start preconcentration, stirring and timing simultaneously. Precisely control and keep constant preconcentration times and stirring rates for solutions and standards. Generally use 120 s and a rotation rate of 2000 rpm for the TMFE. Use longer preconcentration times for very low metal concentrations.

After the metal is sufficiently concentrated in the amalgam, stop stirring or TMFE rotation for an equilibration period of precisely 30 s.

g. Anodic stripping: After equilibration period, begin anodic stripping without stirring and make potential at the working electrode progressively more positive as a function of time. Monitor stripping current and plot as a function of applied potential in stripping voltammograms. Use peak current to quantify metal concentration and peak potential to identify the metal.

h. Add 5 to 50 μL standard solution and repeat analysis, beginning with deoxygenation of sample. Adjust volume of added standard solution to obtain 30 to 70% increase of the stripping peak. If 50 μL addition is not sufficient, use standard solution with a higher concentration of metal. Shorten deoxygenation step to 1 min after initial gas purge.

5. Calculations

Calculate the concentration of metal in the original sample using the following equation:

$$C_o = \frac{C_s \times V_s}{V_o} \times \frac{i_o}{(i_s - i_o)}$$

where:

C_o = concentration of metal in sample, mg/L,
C_s = concentration of metal in standard solution, mg/L,

TABLE 3130:I. PRECISION OF CADMIUM, LEAD, AND ZINC ANALYSIS BY ASV

Sample	Electrode	ASV Mode	Metal Concentration μg/L			RSD %		
			Cd	Pb	Zn	Cd	Pb	Zn
Tap water #1[4]	HMDE	SW	0.068	0.57	—	4.2	4.8	—
Tap water #2[4]	HMDE	SW	—	2.50	—	—	5.1	—
Seawater #1[5]	TFME	DP	0.0121	0.0086	—	10.7	8.1	—
Seawater #2[5]	TFME	DP	0.032	0.032	—	6.3	6.3	—
Soil extract #1[6]	HMDE	SW	189	11.8	—	2.5	5.6	—
Soil extract #1[6]	HMDE	DP	186	11.9	—	2.5	4.0	—
Deionized water[4]	HMDE	SW	0.13	0.79	—	5.5	2.2	—
Wastewater #1[7]	HMDE	DP	—	74	26	—	4.3	4.5
Wastewater #2[7]	HMDE	DP	—	47	86	—	5.2	6.3
Wastewater #3[7]	HMDE	DP	—	46	65	—	4.6	6.2
Wastewater #4[8]	TFME	SW	5.2	60	12	5.2	6.1	7.4

V_s = volume of standard solution added. mL,
V_o = volume of sample, mL,
i_o = stripping peak height in original sample, and
i_s = stripping peak height in sample with standard addition.

6. Quality Control

The QC practices considered to be an integral part of each method can be found in Section 3020. Follow guidelines outlined in Section 3020 with respect to use of additions, duplicates, and blanks for best results. Blanks are critical because of the high sensitivity of the method.

7. Precision and Bias

T1 Table 3130:I gives precision data for analyses of samples with various matrices.

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3500-Al ALUMINUM*

3500-Al A. Introduction

1. Occurrence and Significance

Aluminum (Al) is the second element in Group IIIA of the periodic table; it has an atomic number of 13, an atomic weight of 26.98, and a valence of 3. The average abundance in the earth's crust is 8.1%; in soils it is 0.9 to 6.5%; in streams it is 400 μg/L; in U.S. drinking waters it is 54 μg/L, and in groundwater it is $<$0.1 μg/L. Aluminum occurs in the earth's crust in combination with silicon and oxygen to form feldspars, micas, and clay minerals. The most important minerals are bauxite and corundum, which is used as an abrasive. Aluminum and its alloys are used for heat exchangers, aircraft parts, building materials, containers, etc. Aluminum potassium sulfate (alum) is used in water-treatment processes to flocculate suspended particles, but it may leave a residue of aluminum in the finished water.

Aluminum's occurrence in natural waters is controlled by pH and by very finely suspended mineral particles. The cation Al^{3+} predominates at pH less than 4. Above neutral pH, the predominant dissolved form is $Al(OH)_4^-$. Aluminum is nonessential for plants and animals. Concentrations exceeding 1.5 mg/L constitute a toxicity hazard in the marine environment, and levels below 200 μg/L present a minimal risk. The United Nations Food and Agriculture Organization's recommended maximum level for irrigation waters is 5 mg/L. The possibility of a link between elevated aluminum levels in brain tissues and Alzheimer's disease has been raised. The U.S. EPA secondary drinking water regulations list an optimal secondary maximum contaminant level (SMCL) of 0.05 mg/L and maximum SMCL of 0.2 mg/L.

2. Selection of Method

The atomic absorption spectrometric methods (Sections 3111D and E, and 3113B) and the inductively coupled plasma methods (Sections 3120 and 3125) are free from such common interferences as fluoride and phosphate, and are preferred. The Eriochrome cyanine R colorimetric method (3500-Al.B) provides a means for estimating aluminum with simpler instrumentation.

* Approved by Standard Methods Committee, 2001. Editorial revisions, 2011. Joint Task Group: 20th Edition—Brian J. Condike (chair), Deanna K. Anderson, Anthony Bright, Richard A. Cahill, Alois F. Clary, C. Ellen Gonter, Peter M. Grohse, Daniel C. Hillman, Albert C. Holler, Amy Hughes, J. Charles Jennett, Roger A. Minear, Marlene O. Moore, Gregg L. Oelker, S. Kusum Perera, James G. Poff, Jeffrey G. Skousen, Michael D. Wichman, John L. Wuepper.

3500-Al B. Eriochrome Cyanine R Method

1. General Discussion

a. Principle: With Eriochrome cyanine R dye, dilute aluminum solutions buffered to a pH of 6.0 produce a red to pink complex that exhibits maximum absorption at 535 nm. The intensity of the developed color is influenced by the aluminum concentration, reaction time, temperature, pH, alkalinity, and concentration of other ions in the sample. To compensate for color and turbidity, the aluminum in one portion of sample is complexed with EDTA to provide a blank. The interference of iron and manganese, two elements commonly found in water when aluminum is present, is eliminated by adding ascorbic acid. The optimum aluminum range lies between 20 and 300 μg/L but can be extended upward by sample dilution.

b. Interference: Negative errors are caused by both fluoride and polyphosphates. When the fluoride concentration is constant, the percentage error decreases with increasing amounts of aluminum. Because the fluoride concentration often is known or can be determined readily, fairly accurate results can be obtained by adding the known amount of fluoride to a set of standards. A simpler correction can be determined from the family of curves in Figure 3500-Al:1. A procedure is given for the removal of complex phosphate interference. Orthophosphate in concentrations under 10 mg/L does not interfere. The interference caused by even small amounts of alkalinity is removed by acidifying the sample just beyond the neutralization point of methyl orange. Sulfate does not interfere up to a concentration of 2000 mg/L.

c. Minimum detectable concentration: The minimum aluminum concentration detectable by this method in the absence of fluorides and complex phosphates is approximately 6 μg/L.

d. Sample handling: Collect samples in clean, acid-rinsed bottles, preferably plastic, and examine them as soon as possible after collection. If only soluble aluminum is to be determined, filter a portion of sample through a 0.45-μm membrane filter; discard first 50 mL of filtrate and use succeeding filtrate for the determination. Do not use filter paper, absorbent cotton, or glass wool for filtering any solution that is to be tested for aluminum, because they will remove most of the soluble aluminum.

e. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer,* for use at 535 nm, with a light path of 1 cm or longer.

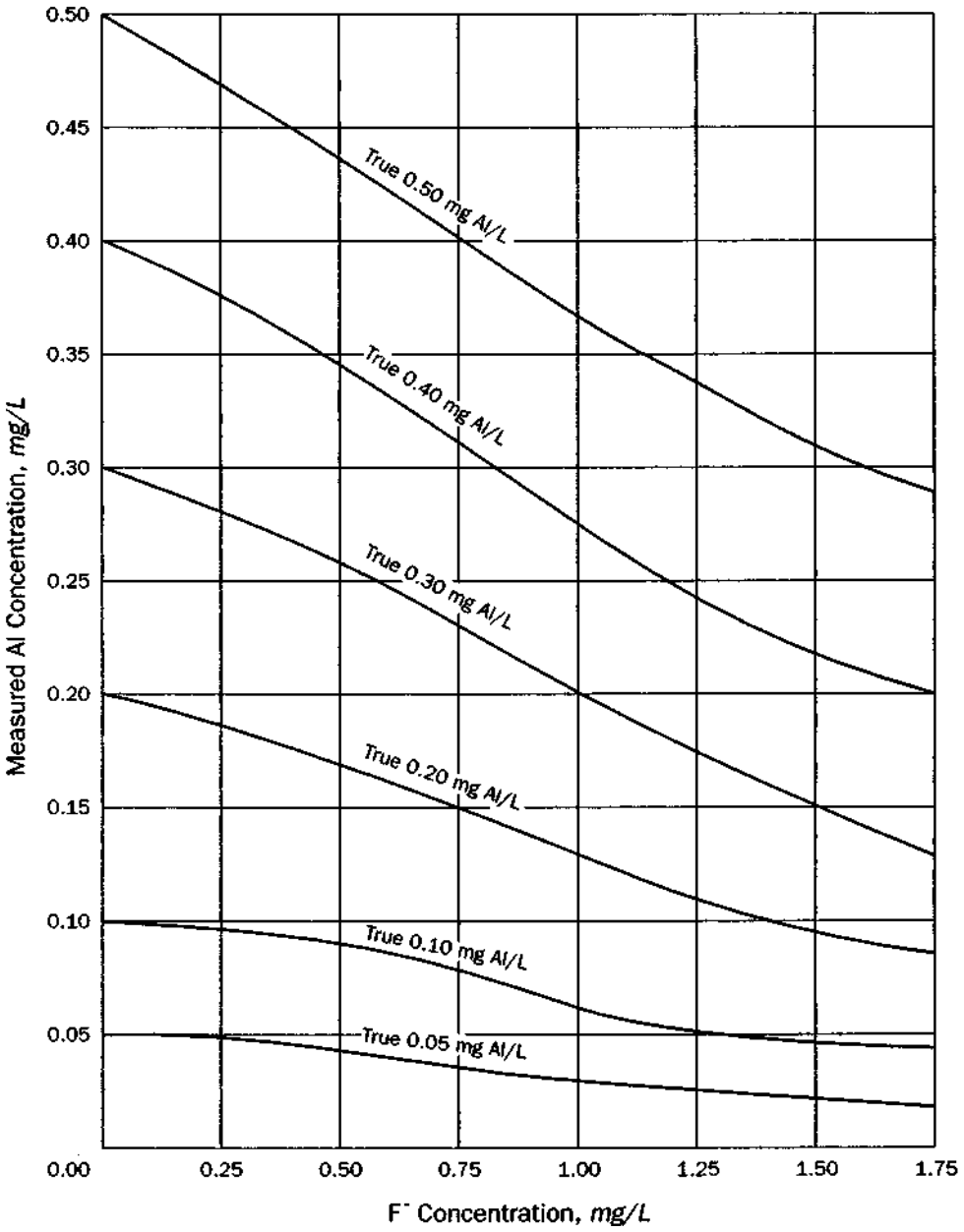


Figure 3500-Al:1. Correction curves for estimation of aluminum in the presence of fluoride. Above the mg F^-/L present, locate the point corresponding to the apparent mg Al/L measured. From this point, interpolate between the curves shown (if the point does not fall directly on one of the curves) to read the true mg Al/L on the ordinate, which corresponds to 0.00 mg F^-/L. For example, an apparent 0.20 mg Al/L in a sample containing 1.00 mg F^-/L would actually be 0.30 mg Al/L if no fluoride were present to interfere.

2) *Filter photometer,* providing a light path of 1 cm or longer and equipped with a green filter with maximum transmittance between 525 and 535 nm.

3) *Nessler tubes,* 50-mL, tall form, matched.

b. Glassware: Treat all glassware with warm 1 + 1 HCl and rinse with aluminum-free distilled water to avoid errors due to materials absorbed on the glass. Rinse sufficiently to remove all acid.

3. Reagents

Use reagents low in aluminum, and aluminum-free distilled water.

a. Stock aluminum solution: Use either the metal (1) or the salt (2) for preparing stock solution; 1.00 mL = 500 μg Al:

1) Dissolve 500.0 mg aluminum metal in 10 mL conc HCl by heating gently. Dilute to 1000 mL with water, or

2) Dissolve 8.791 g aluminum potassium sulfate (also called potassium alum), $AlK(SO_4)_2 \cdot 12H_2O$, in water and dilute to 1000 mL. Correct this weight by dividing by the decimal fraction of assayed $AlK(SO_4)_2 \cdot 12H_2O$ in the reagent used.

b. Standard aluminum solution: Dilute 10.00 mL stock aluminum solution to 1000 mL with water; 1.00 mL = 5.00 μg Al. Prepare daily.

c. Sulfuric acid (H_2SO_4), 0.02*N* and 6*N*.

d. Ascorbic acid solution: Dissolve 0.1 g ascorbic acid in water and make up to 100 mL in a volumetric flask. Prepare fresh daily.

e. Buffer reagent: Dissolve 136 g sodium acetate, $NaC_2H_3O_2 \cdot 3H_2O$, in water, add 40 mL 1*N* acetic acid, and dilute to 1 L.

f. Stock dye solution: Use any of the following products:

1) *Solochrome cyanine R-200** or *Eriochrome cyanine*† —Dissolve 100 mg in water and dilute to 100 mL in a volumetric flask. This solution should have a pH of about 2.9.

2) *Eriochrome cyanine R*‡ —Dissolve 300 mg dye in about 50 mL water. Adjust pH from about 9 to about 2.9 with 1 + 1 acetic acid (approximately 3 mL will be required). Dilute with water to 100 mL.

3) *Eriochrome cyanine R*§ —Dissolve 150 mg in about 50 mL water. Adjust pH from about 9 to about 2.9 with 1 + 1 acetic acid (approximately 2 mL will be required). Dilute with water to 100 mL.

Stock solutions have excellent stability and can be kept for at least a year.

g. Working dye solution: Dilute 10.0 mL of selected stock dye solution to 100 mL in a volumetric flask with water. Working solutions are stable for at least 6 months.

h. Methyl orange indicator solution, or bromcresol green indicator solution specified in the total alkalinity determination (Section 2320B.3*d*).

i. EDTA (sodium salt of ethylenediamine-tetraacetic acid dihydrate), 0.01*M:* Dissolve 3.7 g in water, and dilute to 1 L.

j. Sodium hydroxide (NaOH), 1*N* and 0.1*N*.

4. Procedure

a. Preparation of calibration curve:

1) Prepare a series of aluminum standards from 0 to 7 μg (0 to 280 μg/L based on a 25-mL sample) by accurately measuring the calculated volumes of standard aluminum solution into 50-mL volumetric flasks or nessler tubes. Add water to a total volume of approximately 25 mL.

2) Add 1 mL 0.02*N* H_2SO_4 to each standard and mix. Add 1 mL ascorbic acid solution and mix. Add 10 mL buffer solution and mix. With a volumetric pipet, add 5.00 mL working dye reagent and mix. Immediately make up to 50 mL with distilled water. Mix and let stand for 5 to 10 min. The color begins to fade after 15 min.

3) Read transmittance or absorbance on a spectrophotometer, using a wavelength of 535 nm or a green filter providing maximum transmittance between 525 and 535 nm. Adjust instrument to zero absorbance with the standard containing no aluminum.

Plot concentration of Al (micrograms Al in 50 mL final volume) against absorbance.

b. Sample treatment in absence of fluoride and complex phosphates: Place 25.0 mL sample, or a portion diluted to 25 mL, in a porcelain dish or flask, add a few drops of methyl orange indicator, and titrate with 0.02*N* H_2SO_4 to a faint pink color. Record reading and discard sample. To two similar samples at room temperature add the same amount of 0.02*N* H_2SO_4 used in the titration and 1 mL in excess.

To one sample add 1 mL EDTA solution. This will serve as a blank by complexing any aluminum present and compensating for color and turbidity. To both samples add 1 mL ascorbic acid, 10 mL buffer reagent, and 5.00 mL working dye reagent as prescribed in ¶ *a*2) above.

Set instrument to zero absorbance or 100% transmittance using the EDTA blank. After 5 to 10 min contact time, read transmittance or absorbance and determine aluminum concentration from the calibration curve previously prepared.

c. Visual comparison: If photometric equipment is not available, prepare and treat standards and a sample, as described above, in 50-mL nessler tubes. Make up to mark with water and compare sample color with the standards after 5 to 10 min contact time. A sample treated with EDTA is not needed when nessler tubes are used. If the sample contains turbidity or color, the use of nessler tubes may result in considerable error.

d. Removal of phosphate interference: Add 1.7 mL 6*N* H_2SO_4 to 100 mL sample in a 200-mL Erlenmeyer flask. Heat on a hot plate for at least 90 min, keeping solution temperature just below the boiling point. At the end of the heating period solution volume should be about 25 mL. Add water if necessary to keep it at or above that volume.

After cooling, neutralize to a pH of 4.3 to 4.5 with NaOH, using 1*N* NaOH at the start and 0.1*N* for the final fine adjustment. Monitor with a pH meter. Make up to 100 mL with water, mix, and use a 25-mL portion for the aluminum test.

Run a blank in the same manner, using 100 mL distilled water and 1.7 mL 6*N* H_2SO_4. Subtract blank reading from sample reading or use it to set instrument to zero absorbance before reading the sample.

e. Correction for samples containing fluoride: Measure sample fluoride concentration by the SPADNS or electrode method. Either:

1) Add the same amount of fluoride as in the sample to each aluminum standard, or

2) Determine fluoride correction from the set of curves in Figure 3500-Al:1.

5. Calculation

$$\text{mg Al/L} = \frac{\mu\text{g Al (in 50 mL final volume)}}{\text{mL sample}}$$

6. Precision and Bias

A synthetic sample containing 520 μg Al/L and no interference in distilled water was analyzed by the Eriochrome cyanine

* Arnold Hoffman & Co., Providence, RI.
† K & K Laboratories, K & K Lab. Div., Life Sciences Group, Plainview, NY.
‡ Pfaltz & Bauer, Inc., Stamford, CT.
§ EM Science, Gibbstown, NJ.

R method in 27 laboratories. Relative standard deviation was 34.4% and relative error 1.7%.

A second synthetic sample containing 50 μg Al/L, 500 μg Ba/L, and 5 μg Be/L in distilled water was analyzed in 35 laboratories. Relative standard deviation was 38.5% and relative error 22.0%.

A third synthetic sample containing 500 μg Al/L, 50 μg Cd/L, 110 μg Cr/L, 1000 μg Cu/L, 300 μg Fe/L, 70 μg Pb/L, 50 μg Mn/L, 150 μg Ag/L, and 650 μg Zn/L in distilled water was analyzed in 26 laboratories. Relative standard deviation was 28.8% and relative error 6.2%.

A fourth synthetic sample containing 540 μg Al/L and 2.5 mg polyphosphate/L in distilled water was analyzed in 16 laboratories that hydrolyzed the sample in the prescribed manner. Relative standard deviation was 44.3% and relative error 1.3%. In 12 laboratories that applied no corrective measures, the relative standard deviation was 49.2% and the relative error 8.9%.

A fifth synthetic sample containing 480 μg Al/L and 750 μg F/L in distilled water was analyzed in 16 laboratories that relied on the curve to correct for the fluoride content. Relative standard deviation was 25.5% and relative error 2.3%. The 17 laboratories that added fluoride to the aluminum standards showed a relative standard deviation of 22.5% and a relative error of 7.1%.

7. Bibliography

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3500-As ARSENIC*

3500-As A. Introduction

1. Occurrence and Significance

Arsenic (As) is the third element in Group VA of the periodic table; it has an atomic number of 33, an atomic weight of 74.92, and valences of 3 and 5. The average abundance of As in the earth's crust is 1.8 ppm; in soils it is 5.5 to 13 ppm; in streams it is less than 2 μg/L, and in groundwater it is generally less than 100 μg/L. It occurs naturally in sulfide minerals such as pyrite. Arsenic is used in alloys with lead, in storage batteries, and in ammunition. Arsenic compounds are widely used in pesticides and in wood preservatives.

Arsenic is nonessential for plants but is an essential trace element in several animal species. The predominant form between pH 3 and pH 7 is $H_2AsO_4^-$, between pH 7 and pH 11 it is $HAsO_4^{2-}$, and under reducing conditions it is $HAsO_2$(aq) (or H_3AsO_3). Aqueous arsenic in the form of arsenite, arsenate, and organic arsenicals may result from mineral dissolution, industrial discharges, or the application of pesticides. The chemical form of arsenic depends on its source (inorganic arsenic from minerals, industrial discharges, and pesticides; organic arsenic from industrial discharges, pesticides, and biological action on inorganic arsenic).

Severe poisoning can arise from the ingestion of as little as 100 mg arsenic trioxide; chronic effects may result from the accumulation of arsenic compounds in the body at low intake levels. Carcinogenic properties also have been imputed to arsenic compounds. The toxicity of arsenic depends on its chemical form. Arsenite is many times more toxic than arsenate. For the protection of aquatic life, the average concentration of As^{3+} in water should not exceed 72 μg/L and the maximum should not exceed 140 μg/L. The United Nations Food and Agriculture Organization's recommended maximum level for irrigation waters is 100 μg/L. The U.S. EPA primary drinking water standard MCL is 0.05 mg/L.

2. Selection of Method

Methods are available to identify and determine total arsenic, arsenite, and arsenate. Unpolluted fresh water normally does not contain organic arsenic compounds, but may contain inorganic arsenic compounds in the form of arsenate and arsenite. The electrothermal atomic absorption spectrometric method (Section 3113B) is the method of choice in the absence of overwhelming interferences. The hydride generation/atomic absorption method (Section 3114B) is preferred when interferences are present that cannot be overcome by standard electrothermal techniques (e.g., matrix modifiers, background correction). The silver diethyldithiocarbamate method (3500-As.B), in which arsine is generated by reaction with sodium borohydride in acidic solution, is applicable to the determination of total inorganic arsenic when interferences are absent and when the sample contains no methylarsenic compounds. This method also provides the advantage of being able to identify and quantify arsenate and arsenite separately by generating arsine at different pHs. The inductively coupled plasma (ICP) emission spectroscopy method (Section 3120) is useful at higher concentrations (greater than 50 μg/L) while the ICP-mass spectrometric method (Section 3125) is applicable at lower concentrations if chloride does not interfere. When measuring arsenic species, document that speciation does not change over time. No universal preservative for speciation measurements has been identified.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011. Joint Task Group: 20th Edition—See 3500-Al.

3500-As B. Silver Diethyldithiocarbamate Method

1. General Discussion

a. Principle: Arsenite, containing trivalent arsenic, is reduced selectively by aqueous sodium borohydride solution to arsine, AsH_3, in an aqueous medium of pH 6. Arsenate, methylarsonic acid, and dimethylarsenic acid are not reduced under these conditions. The generated arsine is swept by a stream of oxygen-free nitrogen from the reduction vessel through a scrubber containing glass wool or cotton impregnated with lead acetate solution into an absorber tube containing silver diethyldithiocarbamate and morpholine dissolved in chloroform. The intensity of the red color that develops is measured at 520 nm. To determine total inorganic arsenic in the absence of methylarsenic compounds, a sample portion is reduced at a pH of about l. Alternatively, arsenate is measured in a sample from which arsenite has been removed by reduction to arsine gas at pH 6 as above. The sample is then acidified with hydrochloric acid and another portion of sodium borohydride solution is added. The arsine formed from arsenate is collected in fresh absorber solution.

b. Interferences: Although certain metals—chromium, cobalt, copper, mercury, molybdenum, nickel, platinum, silver, and selenium—influence the generation of arsine, their concentrations in water are seldom high enough to interfere, except in the instance of acid rock drainage. H_2S interferes, but the interference is removed with lead acetate. Antimony is reduced to stibine, which forms a colored complex with an absorption maximum at 510 nm and interferes with the arsenic determination. Methylarsenic compounds are reduced at pH l to methyl-

https://doi.org/10.2105/SMWW.2882.051

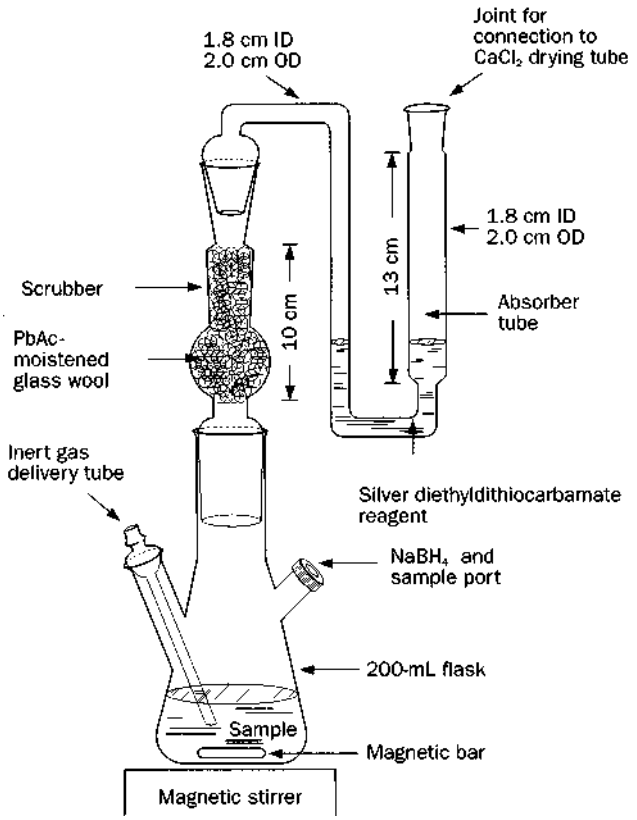


Figure 3500-As:1. Arsine generator and absorber assembly.

arsines, which form colored complexes with the absorber solution. If methylarsenic compounds are present, measurements of total arsenic and arsenate are unreliable. The results for arsenite are not influenced by methylarsenic compounds.

c. Minimum detectable quantity: 1 μg arsenic.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Arsine generator, scrubber, and absorption tube: See Figure 3500-As:1. Use a 200-mL three-necked flask with a sidearm (19/22 or similar size female ground-glass joint) through which the inert gas delivery tube reaching almost to the bottom of the flask is inserted; a 24/40 female ground-glass joint to carry the scrubber; and a second side arm closed with a rubber septum, or preferably by a screw cap with a hole in its top for insertion of a TFE-faced silicone septum. Place a small magnetic stirring bar in the flask. Fit absorber tube (20 mL capacity) to the scrubber and fill with silver diethyldithiocarbamate solution. Do not use rubber or cork stoppers because they may absorb arsine. Clean glass equipment with concentrated nitric acid.

b. Fume hood: Use apparatus in a well-ventilated hood with flask secured on top of a magnetic stirrer.

c. Photometric equipment:

1) *Spectrophotometer,* for use at 520 nm.

2) *Filter photometer,* with green filter having a maximum transmittance in the 500- to 540-nm range.

3) *Cells,* for spectrophotometer or filter photometer, 1-cm, clean, dry, and each equipped with a tightly fitting cover (TFE stopper) to prevent chloroform evaporation.

3. Reagents

a. Reagent water: See Section 1080.

b. Acetate buffer, pH 5.5: Mix 428 mL 0.2*M* sodium acetate, $NaC_2H_3O_2$, and 72 mL 0.2*M* acetic acid, CH_3COOH.

c. Sodium acetate, 0.2*M:* Dissolve 16.46 g anhydrous sodium acetate or 27.36 g sodium acetate trihydrate, $NaC_2H_3O_2 \cdot 3H_2O$, in water. Dilute to 1000 mL with water.

d. Acetic acid (CH_3COOH), 0.2*M:* Dissolve 11.5 mL glacial acetic acid in water. Dilute to 1000 mL.

e. Sodium borohydride solution, 1%: Dissolve 0.4 g sodium hydroxide, NaOH (4 pellets), in 400 mL water. Add 4.0 g sodium borohydride, $NaBH_4$ (check for absence of arsenic). Shake to dissolve and to mix. Prepare fresh every few days.

f. Hydrochloric acid (HCl), 2*M:* Dilute 165 mL conc HCl to 1000 mL with water.

g. Lead acetate solution: Dissolve 10.0 g $Pb(CH_3COO)_2 \cdot 3H_2O$ in 100 mL water.

h. Silver diethyldithiocarbamate solution: Dissolve 1.0 mL morpholine (**CAUTION: Corrosive—avoid contact with skin**) in 70 mL chloroform, $CHCl_3$. Add 0.30 g silver diethyldithiocarbamate, $AgSCSN(C_2H_5)_2$; shake in a stoppered flask until most is dissolved. Dilute to 100 mL with chloroform. Filter and store in a tightly closed brown bottle in a refrigerator.

i. Standard arsenite solution: Dissolve 0.1734 g $NaAsO_2$ in water and dilute to 1000 mL with water. **CAUTION: Toxic—avoid contact with skin and do not ingest.** Dilute 10.0 mL to 100 mL with water; dilute 10.0 mL of this intermediate solution to 100 mL with water; 1.00 mL = 1.00 μg As.

j. Standard arsenate solution: Dissolve 0.416 g $Na_2HAsO_4 \cdot 7H_2O$ in water and dilute to 1000 mL. Dilute 10.0 mL to 100 mL with water; dilute 10 mL of this intermediate solution to 100 mL; 1.00 mL = 1.00 μg As.

4. Procedure

a. Arsenite:

1) Preparation of scrubber and absorber—Dip glass wool into lead acetate solution; remove excess by squeezing glass wool. Press glass wool between pieces of filter paper, then fluff it. Alternatively, if cotton is used treat it similarly but dry in a desiccator and fluff thoroughly when dry. Place a plug of loose glass wool or cotton in scrubber tube. Add 4.00 mL silver diethyldithiocarbamate solution to absorber tube (5.00 mL may be used to provide enough volume to rinse spectrophotometer cell).

2) Loading of arsine generator—Pipet not more than 70 mL sample containing not more than 20.0 μg As (arsenite) into the generator flask. Add 10 mL acetate buffer. If necessary, adjust total volume of liquid to 80 mL. Flush flask with nitrogen at the rate of 60 mL/min.

3) Arsine generation and measurement—While nitrogen is passing through the system, use a 30-mL syringe to inject through the septum 15 mL 1% sodium borohydride solution within 2 min. Stir vigorously with magnetic stirrer. Pass nitrogen through system for an additional 15 min to flush arsine into absorber solution. Pour absorber solution into a clean and dry

spectrophotometric cell and measure absorbance at 520 nm against chloroform. Determine concentration from a calibration curve obtained with arsenite standards. If arsenate also is to be determined for this sample by using the same sample portion, save the liquid in the generator flask.

4) Preparation of standard curves—Treat standard arsenite solution containing 0.0, 1.0, 2.0, 5.0, 10.0, and 20.0 μg As as described in ¶s *a*1)–3) above. Plot absorbance versus micrograms arsenic in the standard.

b. Arsenate: After removal of arsenite as arsine, treat sample to convert arsenate to arsine:

If the lead acetate-impregnated glass wool has become ineffective in removing hydrogen sulfide (if it has become gray to black) replace glass wool [see ¶ *a*1) above]. Pass nitrogen through system at the rate of 60 mL/min. Cautiously add 10 mL 2.0*N* HCl. Generate arsine as directed in ¶ *a*3) above and prepare standard curves with standard solutions of arsenate according to procedure of ¶ *a*4) above.

c. Total inorganic arsenic: Prepare scrubber and absorber as directed in ¶ *a*1) above and load arsine generator as directed in ¶ *a*2) above using 10 mL 2.0*N* HCl instead of acetate buffer. Generate arsine and measure as directed in ¶ *a*3) above. Prepare standard curves according to ¶ *a*4) above. Curves obtained with standard arsenite solution are almost identical to those obtained with arsenate standard solutions. Therefore, use either arsenite or arsenate standards.

5. Calculation

Calculate arsenite, arsenate, and total inorganic arsenic from readings and calibration curves obtained in 3500-As.B.4*a, b,* and *c,* respectively, as follows:

$$\text{mg As/L} = \frac{\mu\text{g As (from calibration curve)}}{\text{mL sample in generator flask}}$$

6. Precision and Bias

Interlaboratory comparisons are not available. The relative standard deviation of results obtained with arsenite/arsenate mixtures containing approximately 10 μg arsenic was less than 10%.

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3500-Ca CALCIUM*

3500-Ca A. Introduction

1. Occurrence and Significance

Calcium (Ca) is the third element in Group IIA of the periodic table; it has an atomic number of 20, an atomic weight of 40.08, and a valence of 2. The average abundance of Ca in the earth's crust is 4.9%; in soils it is 0.07 to 1.7%; in streams it is about 15 mg/L; and in groundwaters it is from 1 to >500 mg/L. The most common forms of calcium are calcium carbonate (calcite) and calcium-magnesium carbonate (dolomite). Calcium compounds are widely used in pharmaceuticals, photography, lime, de-icing salts, pigments, fertilizers, and plasters. Calcium carbonate solubility is controlled by pH and dissolved CO_2. The CO_2, HCO_3^-, and CO_3^{2-} equilibrium is the major buffering mechanism in fresh waters. Hardness is based on the concentration of calcium and magnesium salts, and often is used as a measure of potable water quality.

NOTE: Calcium is necessary in plant and animal nutrition and is an essential component of bones, shells, and plant structures. The presence of calcium in water supplies results from passage over deposits of limestone, dolomite, gypsum, and gypsiferous shale. Small concentrations of calcium carbonate combat corrosion of metal pipes by laying down a protective coating. Because precipitation of calcite in pipes and in heat-exchangers can cause damage, the amount of calcium in domestic and industrial waters is often controlled by water softening (e.g., ion exchange, reverse osmosis). Calcium carbonate saturation and water hardness are discussed in Sections 2330 and 2340, respectively.

Calcium contributes to the total hardness of water. Chemical softening treatment, reverse osmosis, electrodialysis, or ion exchange is used to reduce calcium and the associated hardness.

2. Selection of Method

The atomic absorption methods (Sections 3111B, D, and E) and inductively coupled plasma method (Section 3120) are accurate means of determining calcium. The EDTA titration method ⟨3500-Ca.B) gives good results for control and routine applications, but for samples containing high P levels (>50 mg/L) only the atomic absorption or atomic emission methods are recommended because of interferences often encountered with EDTA indicators.

3. Storage of Samples

The customary precautions are sufficient if care is taken to redissolve any calcium carbonate that may precipitate on standing.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011. Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Ca B. EDTA Titrimetric Method

1. General Discussion

a. Principle: When EDTA (ethylenediaminetetraacetic acid or its salts) is added to water containing both calcium and magnesium, it combines first with the calcium. Calcium can be determined directly, with EDTA, when the pH is made sufficiently high that the magnesium is largely precipitated as the hydroxide and an indicator is used that combines with calcium only. Several indicators give a color change when all of the calcium has been complexed by the EDTA at a pH of 12 to 13.

b. Interference: Under conditions of this test, the following concentrations of ions cause no interference with the calcium hardness determination: Cu^{2+}, 2 mg/L; Fe^{2+}, 20 mg/L; Fe^{3+}, 20 mg/L; Mn^{2+}, 10 mg/L; Zn^{2+}, 5 mg/L; Pb^{2+}, 5 mg/L; Al^{3+}, 5 mg/L; and Sn^{4+}, 5 mg/L. Orthophosphate precipitates calcium at the pH of the test. Strontium and barium give a positive interference and alkalinity in excess of 300 mg/L may cause an indistinct endpoint in hard waters.

c. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Reagents

a. Sodium hydroxide (NaOH), 1*N*.

b. Indicators: Many indicators are available for the calcium titration. Some are described in the literature (3500-Ca.B.6); others are commercial preparations and also may be used. Murexide (ammonium purpurate) was the first indicator available for detecting the calcium endpoint; directions for its use are presented in this procedure. Individuals who have difficulty recognizing the murexide endpoint may find the indicator Eriochrome Blue Black R (color index number 202) or Solochrome Dark Blue an improvement because of the color change from red to pure blue. Eriochrome Blue Black R is sodium-1-(2-hydroxy-1-naphthylazo)-2-naphthol-4-sulfonic acid. Other indicators specifically designed for use as endpoint detectors in EDTA titration of calcium may be used.

1) *Murexide (ammonium purpurate) indicator*—This indicator changes from pink to purple at the endpoint. Prepare by dissolving 150 mg dye in 100 g absolute ethylene glycol. Water solutions of the dye are not stable for longer than 1 d. A ground mixture of dye powder and sodium chloride (NaCl) provides a stable form of the indicator. Prepare by mixing 200 mg murexide

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with 100 g solid NaCl and grinding the mixture to 40 to 50 mesh. Titrate immediately after adding indicator because it is unstable under alkaline conditions. Facilitate endpoint recognition by preparing a color comparison blank containing 2.0 mL NaOH solution, 0.2 g solid indicator mixture (or 1 to 2 drops if a solution is used), and sufficient standard EDTA titrant (0.05 to 0.10 mL) to produce an unchanging color.

2) *Eriochrome Blue Black R indicator*—Prepare a stable form of the indicator by grinding together in a mortar 200 mg powdered dye and 100 g solid NaCl to 40 to 50 mesh. Store in a tightly stoppered bottle. Use 0.2 g of ground mixture for the titration in the same manner as murexide indicator. During titration the color changes from red through purple to bluish purple to a pure blue with no trace of reddish or purple tint. The pH of some (not all) waters must be raised to 14 (rather than 12 to 13) by the use of 8*N* NaOH to get a good color change.

c. Standard EDTA titrant, 0.01*M:* Prepare standard EDTA titrant and standardize against standard calcium solution as described in Section 2340C to obtain EDTA/$CaCO_3$ equivalence. Standard EDTA titrant, 0.0100*M*, is equivalent to 1.000 mg $CaCO_3$/1.00 mL; use titrated equivalent for *B* in the calculations in 4.

3. Procedure

a. Pretreatment of water and wastewater samples: Follow the procedure described in Section 3030E or I if samples require preliminary digestion.

b. Sample preparation: Because of the high pH used in this procedure, titrate immediately after adding alkali and indicator. Use 50.0 mL sample, or a smaller portion diluted to 50 mL so the calcium content is about 5 to 10 mg. Analyze hard waters with alkalinity higher than 300 mg $CaCO_3$/L by taking a smaller portion and diluting to 50 mL. Alternatively, adjust sample pH into the acid range (pH <6), boil for 1 min to dispel CO_2, and cool before beginning titration.

c. Titration: Add 2.0 mL NaOH solution or a volume sufficient to produce a pH of 12 to 13. Stir. Add 0.1 to 0.2 g indicator mixture selected (or 1 to 2 drops if a solution is used). Add EDTA titrant slowly, with continuous stirring to the proper endpoint. When using murexide, check endpoint by adding 1 to 2 drops of titrant in excess to make certain that no further color change occurs.

4. Calculation

$$\text{mg Ca/L} = \frac{A \times B \times 400.8}{\text{mL sample}}$$

$$\text{Calcium hardness as mg } CaCO_3\text{/L} = \frac{A \times B \times 1000}{\text{mL sample}}$$

where:

A = mL titrant for sample and
B = mg $CaCO_3$ equivalent to 1.00 mL EDTA titrant at the calcium indicator endpoint.

5. Precision and Bias

A synthetic sample containing 108 mg Ca/L, 82 mg Mg/L, 3.1 mg K/L, 19.9 mg Na/L, 241 mg Cl^-/L, 1.1 mg NO_3^--N/L, 0.25 mg NO_2^--N/L, 259 mg SO_4^{2-}/L, and 42.5 mg total alkalinity/L (contributed by $NaHCO_3$) in distilled water was analyzed in 44 laboratories by the EDTA titrimetric method, with a relative standard deviation of 9.2% and a relative error of 1.9%.

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3500-Cr CHROMIUM*

3500-Cr A. Introduction

1. Occurrence and Significance

Chromium (Cr) is the first element in Group VIB in the periodic table; it has an atomic number of 24, an atomic weight of 51.99, and valences of 0 and 2 through 6. The average abundance of Cr in the earth's crust is 122 ppm; in soils Cr ranges from 11 to 22 ppm; in streams it averages about 1 μg/L, and in groundwaters it is generally 100 μg/L. Chromium is found chiefly in chrome-iron ore ($FeO \cdot Cr_2O_3$). Chromium is used in alloys, in electroplating, and in pigments. Chromate compounds frequently are added to cooling water for corrosion control.

In natural waters trivalent chromium exists as Cr^{3+}, $Cr(OH)^{2+}$, $Cr(OH)_2^{+}$, and $Cr(OH)_4^{-}$; in the hexavalent form chromium exists as CrO_4^{2-} and as $Cr_2O_7^{2-}$. Cr^{3+} would be expected to form strong complexes with amines, and would be adsorbed by clay minerals.

Chromium is considered nonessential for plants, but an essential trace element for animals. Hexavalent compounds have been shown to be carcinogenic by inhalation and are corrosive to tissue. The chromium guidelines for natural water are linked to the hardness or alkalinity of the water (i.e., the softer the water, the lower the permitted level for chromium). The United Nations Food and Agriculture Organization recommended maximum level for irrigation waters is 100 μg/L. The U.S. EPA primary drinking water standard MCL is 100 μg/L for total chromium.

2. Selection of Method

The colorimetric method (3500-Cr.B) is useful for the determination of hexavalent chromium in a natural or treated water in the range from 100 to 1000 μg/L. This range can be extended by appropriate sample dilution or concentration and/or use of longer cell paths. The ion chromatographic method with photometric detection (3500-Cr.C) is suitable for determining dissolved hexavalent chromium in drinking water, groundwater, and industrial wastewater effluents from 0.5 to 5000 μg/L. The electrothermal atomic absorption spectrometric method (Section 3113B) is suitable for determining low levels of total chromium (<50 μg/L) in water and wastewater, and the flame atomic absorption spectrometric methods (Sections 3111B and C) and the inductively coupled plasma methods (Sections 3120 and 3125) are appropriate for measuring total chromium concentrations up to milligram-per-liter levels.

3. Sample Handling

If dissolved chromium content is desired, then immediately after collection, filter sample through a 0.45-μm membrane filter, and acidify filtrate with conc HNO_3 to pH <2. Analyze within 6 months of collection.

If dissolved hexavalent chromium content is desired, then adjust filtrate pH to between 9.3 and 9.7 with buffer solution plus 600 μL 5*N* NaOH per 100 mL of sample (3500-Cr.B.4*b*). Some samples may require more NaOH (5*N* or 1*N*). Alternatively, use buffer without 5*N* NaOH, and add 1*N* NaOH as necessary within the first 24 h to bring sample pH within range. Never dilute sample volume by more than 10%. Refrigerate to <6°C. Analyze with 28 d of collection. If pH is not within proper range, then either analyze sample or adjust its pH within the 24 h.

If total chromium content is desired, then immediately after collection, acidify unfiltered samples with conc HNO_3 to pH <2. Analyze within 6 months of collection.

If total hexavalent chromium content is desired, then adjust pH of unfiltered samples to 9 with buffer solution plus 600 μL 5*N* NaOH per 100 mL of sample (3500-Cr.B.4*b*). Some samples may require more NaOH (5*N* or 1*N*). Alternatively, use buffer with 5*N* NaOH, and add 1*N* NaOH as necessary within the first 24 h to bring sample pH within range. Never dilute sample volume by more than 10%. Refrigerate to <6°C. Analyze within 28 d of collection. If pH is not within proper range, then either analyze sample or adjust its pH within the first 24 h.

* Approved by Standard Methods Committee, 2009. Editorial revisions, 2011.
Joint Task Group: 22nd Edition—Rock J. Vitale (chair), C. Ellen Gonter, Timothy S. Oostdyk; 20th Edition—See 3500-Al.

3500-Cr B. Colorimetric Method

1. General Discussion

a. Principle: This procedure measures only hexavalent chromium, Cr(VI). For total chromium determination, acid-digest the sample (see Section 3030) and follow with a suitable instrumental analysis technique. The hexavalent chromium is determined colorimetrically by reaction with diphenylcarbazide in acid solution. A red-violet colored complex of unknown composition is produced. The reaction is very sensitive, the molar absorptivity based on chromium being about 40 000 L g^{-1} cm^{-1} at 530 or 540 nm. NOTE: Validation data for 3500-Cr.B were developed at 540 nm. Validation data for 3500-Cr.C were developed at 530 nm.

b. Interferences: The reaction with diphenylcarbazide is nearly specific for chromium. Hexavalent molybdenum and mercury salts will react to form color with the reagent but the intensities are much lower than that for chromium at the specified pH. Concentrations of Mo or Hg as high as 200 mg/L can be tolerated. Vanadium interferes strongly but concentrations up to

10 times that of chromium will not result in significant analytical error. Iron in concentrations greater than 1 mg/L may produce a yellow color but the ferric ion (Fe^{3+}) color is not strong and no difficulty is encountered normally if the absorbance is measured photometrically at the appropriate wavelength.

c. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer*, for use at 530 or 540 nm, with a light path of 1 cm or longer.

2) *Filter photometer*, providing a light path of 1 cm or longer and equipped with a greenish yellow filter having maximum transmittance at 530 or 540 nm.

b. Laboratory ware: Soak all reusable items (glass, plastic, etc.), including sample containers, overnight in laboratory-grade detergent, rinse, and soak for 4 h in a mixture of nitric acid (1 part), hydrochloric acid (2 parts), and reagent water (9 parts). Rinse with tap water and reagent water. NOTE: Never use chromic acid cleaning solution.

c. pH meter: Use any commerical pH meter. Standardize and calibrate according to manufacturer's instructions. pay special attention to temperature compensation and electrode care.

3. Reagents

Use reagent water (see Section 1080) for reagent preparation and analytical procedure.

a. Stock chromium solution: Dissolve 141.4 mg $K_2Cr_2O_7$ in water and dilute to 100 mL; 1.00 mL = 500 μg Cr. **CAUTION: Hexavalent chromium is toxic and a suspected carcinogen. Handle with care.**

5b. Standard chromium solution: Dilute 1.00 mL stock chromium solution to 100 mL; 1.00 mL = 5.00 μg Cr. Prepare calibration standards at time of analysis.

c. Nitric acid (HNO_3), conc.

d. Sulfuric acid (H_2SO_4), conc, 18*N*, and 6*N*.

e. Sulfuric acid (H_2SO_4), 0.2*N:* Dilute 17 mL 6*N* H_2SO_4 to 500 mL with water.

f. Phosphoric acid (H_3PO_4), conc.

g. Diphenylcarbazide solution: Dissolve 250 mg 1,5-diphenylcarbazide (1,5-diphenylcarbohydrazide) in 50 mL acetone. Store in a brown bottle. Prepare weekly. Discard if the solution becomes discolored.

h. Sodium hydroxide, 1*N:* Dissolve 40 g low-level metal impurity NaOH in 1 L water. Store in plastic bottle. Smaller volumes may be prepared.

i. Sodium hydroxide, 5*N:* Dissolve 200 g low-level metal impurity NaOH in 1 L water. Store in plastic bottle. Smaller volumes may be prepared.

j. Buffer solution: Dissolve 33 g ammonium sulfate in 75 mL water. Add 6.5 mL ammonium hydroxide, and dilute to 100 mL with water. Smaller volumes may be prepared.

4. Procedure

a. Preparation of calibration curve: To compensate for possible slight losses of chromium during analytical operations, treat standards and samples with the same procedure. Accordingly, pipet measured volumes of standard chromium solution (5 μg/mL) ranging from 2.00 to 20.0 mL, to give standards for 10 to 100 μg Cr, into 250-mL beakers or conical flasks. Depending on pretreatment used in ¶ *b* below, proceed with subsequent treatment of standards as if they were samples.

Develop color as for samples, transfer a suitable portion of each colored solution to a 1-cm absorption cell, and measure absorbance at 540 nm, using reagent water as reference. Correct absorbance readings of standards by subtracting absorbance of a reagent blank carried through the method.

Construct a calibration curve by plotting corrected absorbance values against micrograms chromium in 102 mL final volume.

b. Treatment of sample: Filter sample through a 0.45-μm filter. Use a portion of sample to rinse syringe filter unit and filter, then collect the required volume of filtrate. Adjust pH to between 9.3 and 9.7 by adding 1 mL buffer plus 600 μL 5*N* NaOH per 100 mL sample. More NaOH (5*N* or 1 *N*) may be required to bring sample pH into range. Alternatively, use the buffer without the 5*N* NaOH, and add 1*N* NaOH as necessary within the first 24 hours to bring sample pH into range. Never dilute sample volume by more than 10%. Ship and store samples at $<6°C$. Bring to room temperature before analysis. Analyze samples within 28 d of collection. If pH is not within the proper range, either analyze the sample or adjust its pH within the first 24 hours. Drinking water samples do not require field filtration.

c. Color development and measurement: Bring samples to room temperature before analysis. Add 0.25 mL (5 drops) H_3PO_4. Use 0.2*N* H_2SO_4 and a pH meter to adjust solution to pH 2.0 $\pm$ 0.5. Transfer solution to a 100-mL volumetric flask, dilute to 100 mL, and mix. Add 2.0 mL diphenylcarbazide solution, mix, and let stand 5 to 10 min for full color development. Transfer an appropriate portion to a 1-cm absorption cell and measure its absorbance at 530 or 540 nm, using reagent water as reference. Correct absorbance reading of sample by subtracting absorbance of a blank carried through the method (see also note below). From the corrected absorbance, determine micrograms chromium present by reference to the calibration curve.

NOTE: If the solution is turbid after dilution to 100 mL in ¶ *c* above, take an absorbance reading before adding carbazide reagent and correct absorbance reading of final colored solution by subtracting the absorbance measured previously.

5. Calculation

$$\text{mg Cr/L} = \frac{\mu\text{g Cr (in 102 mL final volume)}}{A}$$

where:

A = mL original sample.

6. Precision and Bias

Collaborative test data from 16 laboratories were obtained on reagent water, tap water, 10% NaCl solution, treated water from synthetic organic industrial waste, EPA extraction leachate, process water, lake water, and effluent from a steel pickle liquor treatment plant.[1] The test data yielded the following relationships:

Reagent water:

$$S_t = 0.037x + 0.006$$
$$S_o = 0.022x + 0.004$$

Drinking or wastewater:

$$S_t = 0.067x + 0.004$$
$$S_o = 0.037x + 0.002$$

Leachate:

$$S_t = 0.032x + 0.007$$
$$S_o = 0.017x + 0.004$$

where:

S_t = overall precision,
S_o = single-operator precision, and
x = chromium concentration, mg/L.

7. Reference

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3500-Cr C. Ion Chromatographic Method

1. General Discussion

a. Principle: This method is applicable to determination of dissolved hexavalent chromium in drinking water, groundwater, and industrial wastewater effluents. An aqueous sample is filtered and its pH adjusted to 9 to 9.5 with a concentrated buffer. This pH adjustment reduces the solubility of trivalent chromium and preserves the hexavalent chromium oxidation state. The sample is introduced into the instrument's eluent stream of ammonium sulfate and ammonium hydroxide. Trivalent chromium in solution is separated from the hexavalent chromium by the column. After separation, hexavalent chromium reacts with an azide dye to produce a chromogen that is measured at 530 or 540 nm. Note: Validation data for 3500-Cr.C were developed at 530 nm. Hexavalent chromium is identified on the basis of retention time.

Although this method was developed using specific commercial equipment, use of another manufacturer's equipment should be acceptable if appropriate adjustments are made.

b. Interferences: Interferences may come from several sources. Use analytical grade salts for the buffer because trace amounts of chromium may be included.

Several soluble species of trivalent chromium in the sample may be oxidized to the hexavalent form in an alkaline medium in the presence of such oxidants as hydrogen peroxide, ozone, and manganese dioxide. The hexavalent form can be reduced to the trivalent in the presence of reducing species in an acid medium.

High ionic concentration may cause column overload. Samples high in chloride and/or sulfate might show this phenomenon, which is characterized by a change in peak geometry.

Interfering organic compounds are removed by the guard column.

c. Minimum detectable concentrations: The method detection levels obtained in a single laboratory with a 250-μL loop were as follows:

Reagent water	0.4 μg/L
Drinking water	0.3 μg/L
Groundwater	0.3 μg/L
Primary wastewater effluent	0.3 μg/L
Electroplating waste	0.3 μg/L

d. Sample preservation and holding time: Filter sample through a 0.45 μm filter. Use a portion of sample to rinse syringe filter unit and filter, then collect the required volume of filtrate. Adjust pH to between 9.3 and 9.7 by adding 1 mL buffer plus 600 μL 5*N* NaOH per 100 mL of sample. More NaOH (5*N* or 1 *N*) may be required to bring sample pH into range. Alternatively, use the buffer without the 5*N* NaOH, and add 1*N* NaOH as necessary within the first 24 h to bring sample pH into range. Never dilute sample volume by more than 10%. Ship and store samples at <6°C. Bring to room temperature before analysis. Analyze samples within 28 d of collection. If pH is not within the proper range, either analyze the sample or adjust its pH via one of the above methods within the first 24 h. Drinking water samples do not require field filtration.

Ship and store sample at 4°C. Bring to room temperature before analysis. Analyze samples within 24 h of collection.

2. Apparatus

a. Ion chromatograph equipped with a pump capable of precisely delivering a flow of 1 to 5 mL/min. The metallic parts of the pump must not contact sample, eluent, or reagent. Sample loops should be available or the instrument should be capable of delivering from 50 to 250-μL injections of sample. The visible absorption cell should not contain metallic parts that contact the

eluent-sample flow. The cell must be usable at 530 nm. Use plastic pressurized containers to deliver eluent and post-column reagent. Use high-purity helium (99.995%) to pressurize the eluent and post-column reagent vessel.

b. Guard column, to be placed before the separator column, containing an adsorbent capable of adsorbing organic compounds and particulates that would damage or interfere with the analysis or equipment.*

c. Separator column, packed with a high-capacity anion-exchange resin capable of resolving chromate from other sample constituents.†

d. Recorder, integrator, or computer for receiving signals from the detector as a function of time.

e. Laboratory ware: Soak all reusable items (glass, plastic, etc.) including sample containers, overnight in laboratory-grade detergent, rinse, and soak for 4 h in a mixture of nitric acid (1 part), hydrochloric acid (2 parts), and reagent water (9 parts). Rinse with tap water and reagent water. NOTE: Never use chromic acid cleaning solution.

f. Syringe, equipped with male luer-type fitting and a capacity of at least 3 mL.

g. pH meter: Use any commercial pH meter. Standardize and calibrate according to manufacturer's instructions. Pay special attention to temperature compensation and electrode care.

3. Reagents

a. Reagent water: Deionized or distilled water free from interferences at the minimum detection level of each constituent, filtered through a 0.2-μm membrane filter and having a conductance of less than 0.1 μS/cm. Use for preparing all reagents (see Section 1080).

b. Cr(VI) stock solution, 100 mg Cr^{6+}/L: Prepare from primary standard grade potassium dichromate. Dissolve 0.1414 g $K_2Cr_2O_7$ in water and dilute to 500 mL in a volumetric flask. pH adjustment is not required. Store in plastic. **CAUTION: Hexavalent chromium is toxic and a suspected carcinogen; handle with care.**

c. Eluent: Dissolve 33 g ammonium sulfate $(NH_4)_2SO_4$, in 500 mL water and add 6.5 mL conc ammonium hydroxide, NH_4OH. Dilute to 1 L with water.

d. Post-column reagent: Dissolve 0.5 g 1,5-diphenylcarbazide in 100 mL HPLC-grade methanol. Add with stirring to 500 mL water containing 28 mL conc H_2SO_4. Dilute to 1 L with water. Reagent is stable for 4 or 5 d; prepare only as needed.

e. Buffer solution: Dissolve 33 g ammonium sulfate, $(NH_4)_2SO_4$, in 75 mL water and add 6.5 mL conc ammonium hydroxide, NH_4OH. Dilute to 100 mL with water.

f. Sodium hydroxide, 5N: Dissolve 200 g NaOH in 1 L water. Store in plastic bottle. Smaller volumes may be prepared.

4. Procedure

a. Instrument setup: Establish ion chromatograph operating conditions as indicated in Table 3500-Cr:I. Set flow rate of the eluent pump at 1.5 mL/min and adjust pressure of reagent delivery module so that the system flow rate, measured after the detector, is 2.0 mL/min. Measure system flow rate using a graduated cylinder and stopwatch. Allow approximately 30 min after adjustment before measuring flow.

TABLE 3500-Cr:I. ION CHROMATOGRAPHIC CONDITIONS

Variable	Value
Guard column	Dionex IonPac NG1
Separator column	Dionex IonPac AS7
Eluent	250 m*M* $(NH4)_2SO_4$ 100 m*M* NH_4OH
Eluent flow rate	1.5 mL/min
Post-column reagent	2 m*M* diphenylcarbohydrazide 10% v/v CH_3OH 1*N* H_2SO_4
Post-column reagent flow rate	0.5 mL/min
Detector	Visible @ 530 nm
Retention time	3.8 min

Use an injection loop size based on required sensitivity. A 50-μL loop is sufficient, although a 250-μL loop was used to determine the method detection level.

b. Calibration: Before sample analysis, construct a calibration curve using a minimum of a blank and three standards that bracket the expected sample concentration range. Prepare calibration standards from the stock standard (3500-Cr.C.3*b*) by appropriate dilution with reagent water in volumetric flasks. Adjust to pH 9 to 9.5 with buffer solution (3500-Cr.C.3*e*) before final dilution. Injection volumes of standards should be about 10 times the injection loop volume to ensure complete loop flushing.

c. Sample analysis: Bring chilled, pH-adjusted sample to ambient temperature. Fill a clean syringe with sample, attach a 0.45-μm syringe filter, and inject 10 times the sample loop volume into the instrument. Dilute any sample that has a concentration greater than the highest calibration standard.

5. Calculation

Determine area or height of the Cr(VI) peak in the calibration standard chromatograms. Establish a calibration curve by performing a regression analysis of peak height or peak area against standard concentration in mg/L. If correlation coefficient is less than 0.995, do not use these data. Check analytical process.

For samples, measure area (or height) of Cr(VI) peak in sample chromatogram, as determined by retention time. Calculate Cr(VI) concentration by interpolating from the calibration line. Correct data for any dilutions made.

Currently available instrumentation automates the entire measurement process (peak measurement, calibration, and sample measurements and calculations). Ensure that enough quality control samples are analyzed to monitor the instrumental processes.

6. Quality Control

The quality control practices considered to be an integral part of each method can be found in Section 3020.

a. Initial demonstration of performance: Before sample analysis, set up instrument and analyze enough known samples to

* IonPac NG1, Dionex, 4700 Lakeside Drive, Sunnyvale, CA 94086, or equivalent.
† IonPac AS7, Dionex, or equivalent.

determine estimates for the method detection level and linear calibration range. Use the initial demonstration of performance to characterize instrument performance [i.e., method detection levels (MDLs) and linear calibration range].

b. Initial and continuing calibration performance: Initially, after every 10 samples, and after the final sample, analyze an independent check sample and a calibration blank. The concentration of the calibration check sample should be near the mid-calibration range; prepare from a source independent of the calibration standards. Use acceptance criteria for check standard recovery and calibration standard concentration based on project goals for precision and accuracy. Typical values for recovery of the check standard range from 90 to 110%. The acceptance criteria for the calibration blank are typically set at ± the nominal MDL.

c. Reagent blank analysis: Analyze one laboratory reagent blank with each batch of samples. Significant Cr(VI) detected in the reagent blank is a sign of contamination. Identify source and eliminate contamination.

d. Laboratory-fortified matrix (also known as matrix spike) analysis: To a portion of a sample, add a known quantity of Cr(VI). After analysis, calculate percent recovery of the known addition. If the recovery falls outside the control limits (typically 75 to 125%), the matrix may be interfering with the analysis. If matrix interferences are thought to be present, use the method of standard additions, if appropriate, to minimize the effect of interferences. NOTE: There are situations where low recoveries of analyte additions can be overcome, or where the non-detected analyte results can be further validated by the application of three-point method of standard addition. The decision to apply method of standard additions should be a function of achieving desired data quality objectives. Analyze fortified matrix samples as frequently as dictated by project goals and anticipated similarity of matrices in the sample set.

e. Laboratory control sample: Analyze a laboratory control sample (LCS) from an external source with every sample batch. Process LCS and samples identically, including filtering and pH adjustment. Base acceptance criteria for LCS recovery on project goals for precision and bias. Typical values for acceptable recovery range from 90 to 110%.

7. Precision and Bias

The instrument operating conditions and data from a single-laboratory test of the method are shown in Tables 3500-Cr:I and 3500-Cr:II , respectively.

Multilaboratory test data are shown in Table 3500-Cr:III.‡ Fifteen laboratories analyzed samples ranging from 1.2 to 960 μg Cr/L.

For reagent water matrix:

$$S_o = 0.033x + 0.106$$

$$S_t = 0.050x + 0.559$$

$$\text{Mean recovery} = 1.04x + 0.183$$

‡ The multilaboratory precision and bias data cited in this method were the result of a collaborative study carried out jointly between U.S. EPA Environmental Monitoring Systems Laboratory (Cincinnati) and ASTM Committee D-19.

TABLE 3500-Cr:II. SINGLE-LABORATORY PRECISION AND BIAS

Sample Type	Concentration* μg/L	Mean Recovery %	RPD†
Reagent water	100	100	0.8
	1000	100	0.0
Drinking water	100	105	6.7
	1000	98	1.5
Groundwater	100	98	0.0
	1000	96	0.8
Primary wastewater effluent	100	100	0.7
	1000	104	2.7
Electroplating effluent	100	99	0.4
	1000	101	0.4

* Sample fortified at this concentration level.
† RPD = relative percent difference between fortified duplicates.

TABLE 3500-Cr:III. MULTILABORATORY DETERMINATION OF BIAS FOR HEXAVALENT CHROMIUM*

Water	Amount Added μg/L	Amount Found μg/L	S_t	S_o	Bias %
Reagent	6.00	6.68	1.03	0.53	+11.3
	8.00	8.64	1.10		+8.0
	16.0	17.4	2.25	0.77	+8.8
	20.0	21.4	2.31		+7.0
	100	101	1.91	3.76	+1.0
	140	143	5.52		+2.1
	800	819	24.3	12.7	+2.4
	960	966	18.5		+7.3
Waste	6.0	5.63	1.17	0.55	−6.2
	8.0	7.31	1.91		−8.6
	16.0	15.1	2.70	1.85	−5.6
	20.0	19.8	1.01		−1.0
	100	98.9	4.36	3.31	−1.1
	140	138	8.39		−1.4
	800	796	60.6	27.1	−0.5
	960	944	72.1		−1.7

* Each Youden pair was used to calculate one laboratory data point (S_o).

where:

S_o = single-operator precision,
S_t = overall precision, and
x = added amount.

For wastewater matrix:

$$S_o = 0.041x + 0.039$$

$$S_t = 0.059x + 1.05$$

$$\text{Mean recovery} = 0.989x - 0.41$$

The eleven water samples consisted of a reagent water blank and five Youden pairs. The nine wastewater samples consisted of a wastewater blank and four Youden pairs.

8. Bibliography

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3500-Cu COPPER*

3500-Cu A. Introduction

1. Occurrence and Significance

Copper (Cu) is the first element in Group IB in the periodic table; it has an atomic number of 29, an atomic weight of 63.54, and valences of 1 and 2. The average abundance of Cu in the earth's crust is 68 ppm; in soils it is 9 to 33 ppm; in streams it is 4 to 12 μg/L; and in groundwater it is <0.1 mg/L. Copper occurs in its native state, but is also found in many minerals, the most important of which are those containing sulfide compounds (e.g., chalcopyrite), but also those with oxides and carbonates. Copper is widely used in electrical wiring, roofing, various alloys, pigments, cooking utensils, piping, and in the chemical industry. Copper salts are used in water supply systems to control biological growths in reservoirs and distribution pipes and to catalyze the oxidation of manganese. Copper forms a number of complexes in natural waters with inorganic and organic ligands. Among the common aqueous species are Cu^{2+}, $Cu(OH)_2$, and $CuHCO_3^+$. Corrosion of copper-containing alloys in pipe fittings may introduce measurable amounts of copper into the water in a pipe system.

Copper is considered an essential trace element for plants and animals. Some compounds are toxic by ingestion or inhalation. The United Nations Food and Agriculture Organization recommended maximum level for irrigation waters is 200 μg/L. Under the lead-copper rule, the U.S. EPA drinking water 90th percentile action level is 1.3 mg/L.

2. Selection of Method

The atomic absorption spectrometric methods (Sections 3111B and C), the inductively coupled plasma methods (Sections 3120 and 3125), and the neocuproine method (3500-Cu.B) are recommended because of their freedom from interferences. The electrothermal atomic absorption method (Section 3113B) also may be used with success with an appropriate matrix modifier. The bathocuproine method (3500-Cu.C) may be used for potable waters.

3. Sampling and Storage

Copper ion tends to be adsorbed on the surface of sample containers. Therefore, analyze samples as soon as possible after collection. If storage is necessary, use 0.5 mL 1 + 1 HCl/100 mL sample, or acidify to pH <2 with HNO_3, to prevent this adsorption.

* Approved by Standard Methods Committee, 1999. Editorial revisions, 2011. Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Cu B. Neocuproine Method

1. General Discussion

a. Principle: Cuprous ion (Cu^+) in neutral or slightly acidic solution reacts with 2,9-dimethyl-1,10-phenanthroline (neocuproine) to form a complex in which 2 moles of neocuproine are bound by 1 mole of Cu^+ ion. The complex can be extracted by a number of organic solvents, including a chloroform-methanol ($CHCl_3$-CH_3OH) mixture, to give a yellow solution with a molar absorptivity of about 8000 at 457 nm. The reaction is virtually specific for copper; the color follows Beer's law up to a concentration of 0.2 mg Cu/25 mL solvent; full color development is obtained when the pH of the aqueous solution is between 3 and 9; the color is stable in $CHCl_3$-CH_3OH for several days.

The sample is treated with hydroxylamine-hydrochloride to reduce cupric ions to cuprous ions. Sodium citrate is used to complex metallic ions that might precipitate when the pH is raised. The pH is adjusted to 4 to 6 with NH_4OH, a solution of neocuproine in methanol is added, and the resultant complex is extracted into $CHCl_3$. After dilution of the $CHCl_3$ to an exact volume with CH_3OH, the absorbance of the solution is measured at 457 nm.

b. Interference: Large amounts of chromium and tin may interfere. Avoid interference from chromium by adding sulfurous acid to reduce chromate and complex chromic ion. In the presence of much tin or excessive amounts of other oxidizing ions, use up to 20 mL additional hydroxylamine-hydrochloride solution.

Cyanide, sulfide, and organic matter interfere but can be removed by a digestion procedure (see Section 3030).

c. Minimum detectable concentration: The minimum detectable concentration, corresponding to 0.01 absorbance or 98% transmittance, is 3 μg Cu when a 1-cm cell is used and 0.6 μg Cu when a 5-cm cell is used.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer*, for use at 457 nm, providing a light path of 1 cm or longer.

2) *Filter photometer*, providing a light path of 1 cm or longer and equipped with a narrow-band violet filter having maximum transmittance in the range 450 to 460 nm.

b. Separatory funnels, 125-mL, Squibb form, with glass or TFE stopcock and stopper.

3. Reagents

a. Redistilled water, copper-free: Because most ordinary distilled water contains detectable amounts of copper, use redistilled water, prepared by distilling singly distilled water in a resistant-glass still, or distilled water passed through an ion-exchange unit, to prepare all reagents and dilutions.

b. Stock copper solution: To 200.0 mg polished electrolytic copper wire or foil in a 250-mL conical flask, add 10 mL water and 5 mL conc HNO_3. After the reaction has slowed, warm gently to complete dissolution of the copper and boil to expel oxides of nitrogen, using precautions to avoid loss of copper. Cool, add about 50 mL water, transfer quantitatively to a 1-L volumetric flask, and dilute to the mark with water; 1 mL = 200 μg Cu.

c. Standard copper solution: Dilute 50.00 mL stock copper solution to 500 mL with water; 1.00 mL = 20.0 μg Cu.

d. Sulfuric acid (H_2SO_4), conc.

e. Hydroxylamine-hydrochloride solution: Dissolve 50 g $NH_2OH \cdot HCl$ in 450 mL water.

f. Sodium citrate solution: Dissolve 150 g $Na_3C_6H_5O_7 \cdot 2H_2O$ in 400 mL water. Add 5 mL $NH_2OH \cdot HCl$ solution and 10 mL neocuproine reagent. Extract with 50 mL $CHCl_3$ to remove copper impurities and discard $CHCl_3$ layer.

g. Ammonium hydroxide (NH_4OH), 5*N:* Dilute 330 mL conc NH_4OH (28-29%) to 1000 mL with water. Store in a polyethylene bottle.

h. Congo red paper, or other pH test paper showing a color change in the pH range of 4 to 6.

i. Neocuproine reagent: Dissolve 100 mg 2,9-dimethyl-1,10-phenanthroline hemihydrate* in 100 mL methanol. This solution is stable under ordinary storage conditions for a month or more.

j. Chloroform ($CHCl_3$): Avoid or redistill material that comes in containers with metal-lined caps.

k. Methanol (CH_3OH), reagent grade.

l. Nitric acid (HNO_3), conc.

m. Hydrochloric acid (HCl), conc.

4. Procedure

a. Preparation of calibration curve: Pipet 50 mL water into a 125-mL separatory funnel for use as a reagent blank. Prepare standards by pipetting 1.00 to 10.00 mL (20.0 to 200 μg Cu) standard copper solution into a series of 125-mL separatory funnels, and dilute to 50 mL with water. Add 1 mL conc H_2SO_4 and use the extraction procedure given in ¶ *b* below.

Construct a calibration curve by plotting absorbance versus micrograms of copper.

To prepare a calibration curve for smaller amounts of copper, dilute 10.0 mL standard copper solution to 100 mL. Carry 1.00- to 10.00-mL volumes of this diluted standard through the previously described procedure, but use 5-cm cells to measure absorbance.

b. Treatment of sample: Transfer 100 mL sample to a 250-mL beaker, add 1 mL conc H_2SO_4 and 5 mL conc HNO_3. Add a few boiling chips and cautiously evaporate to dense white SO_3 fumes on a hot plate. If solution remains colored, cool, add another 5 mL conc HNO_3, and again evaporate to dense white fumes. Repeat, if necessary, until solution becomes colorless.

Cool, add about 80 mL water, and bring to a boil. Cool and filter into a 100-mL volumetric flask. Make up to 100 mL with water using mostly beaker and filter washings.

Pipet 50.0 mL or other suitable portion containing 4 to 200 μg Cu, from the solution obtained from preliminary treatment, into a 125-mL separatory funnel. Dilute, if necessary, to 50 mL with water. Add 5 mL $NH_2OH \cdot HCl$ solution and 10 mL sodium citrate solution, and mix thoroughly. Adjust pH to approximately 4 by adding 1-mL increments of NH_4OH until Congo red paper is just definitely red (or other suitable pH test paper indicates a value between 4 and 6).

Add 10 mL neocuproine reagent and 10 mL $CHCl_3$. Stopper and shake vigorously for 30 s or more to extract the copper-neocuproine complex into the $CHCl_3$. Let mixture separate into two layers and withdraw lower $CHCl_3$ layer into a 25-mL volumetric flask, taking care not to transfer any of the aqueous layer. Repeat extraction of the water layer with an additional 10 mL $CHCl_3$ and combine extracts. Dilute combined extracts to 25 mL with CH_3OH, stopper, and mix thoroughly.

Transfer an appropriate portion of extract to a suitable absorption cell (1 cm for 40 to 200 μg Cu; 5 cm for lesser amounts) and measure absorbance at 457 nm or with a 450- to 460-nm filter. Use a sample blank prepared by carrying 50 mL water through the complete digestion and analytical procedure.

Determine micrograms copper in final solution by reference to the appropriate calibration curve.

5. Calculation

$$\text{mg Cu/L} = \frac{\mu\text{g Cu (in 25 mL final volume)}}{\text{mL portion taken for extraction}}$$

6. Bibliography

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* GFS Chemicals, Inc., Columbus, OH, or equivalent.

3500-Cu C. Bathocuproine Method

1. General Discussion

a. Principle: Cuprous ion forms a water-soluble orange-colored chelate with bathocuproine disulfonate (2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid, disodium salt). While the color forms over the pH range 3.5 to 11.0, the recommended pH range is between 4 and 5. The sample is buffered at a pH of about 4.3 and reduced with hydroxylamine hydrochloride. The absorbance is measured at 484 nm. The method can be applied to copper concentrations up to at least 5 mg/L with a sensitivity of 20 μg/L.

b. Interference: The following substances can be tolerated with an error of less than ±2%:

Substance	Concentration *mg/L*
Cations	
Aluminum	100
Beryllium	10
Cadmium	100
Calcium	1000
Chromium (III)	10
Cobalt (II)	5
Iron (II)	100
Iron (III)	100
Lithium	500
Magnesium	100
Manganese (II)	500
Nickel (II)	500
Sodium	1000
Strontium	200
Thorium (IV)	100
Zinc	200
Anions	
Chlorate	1000
Chloride	1000
Fluoride	500
Nitrate	200
Nitrite	200
Orthophosphate	1000
Perchlorate	1000
Sulfate	1000
Compounds	
Residual chlorine	1
Linear alkylate sulfonate (LAS)	40

Cyanide, thiocyanate, persulfate, and EDTA also can interfere.

c. Minimum detectable concentration: 20 μg/L with a 5-cm cell.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Colorimetric equipment: One of the following, with a light path of 1 to 5 cm (unless nessler tubes are used):

1) *Spectrophotometer,* for use at 484 nm.

2) *Filter photometer,* equipped with a blue-green filter exhibiting maximum light transmission near 484 nm.

3) *Nessler tubes,* matched, 100-mL, tall form.

b. Acid-washed glassware: Rinse all glassware with conc HCl and then with copper-free water.

3. Reagents

a. Copper-free water: See 3500-Cu.B.3*a*.

b. Stock copper solution: Prepare as directed in 3500-Cu.B.3*b*, but use 20.00 mg copper wire or foil; 1.00 mL = 20.00 μg Cu.

c. Standard copper solution: Dilute 250 mL stock copper solution to 1000 mL with water; 1.00 mL = 5.00 μg Cu. Prepare daily.

d. Hydrochloric acid (HCl), 1 + 1.

e. Hydroxylamine hydrochloride solution: See 3500-Cu.B.3*e*.

f. Sodium citrate solution: Dissolve 300 g $Na_3C_6H_5O_7 \cdot 2H_2O$ in water and make up to 1000 mL.

g. Disodium bathocuproine disulfonate solution: Dissolve 1.000 g $C_{12}H_4N_2(CH_3)_2(C_6H_4)_2$ $(SO_3Na)_2$ in water and make up to 1000 mL.

4. Procedure

Pipet 50.0 mL sample, or a suitable portion diluted to 50.0 mL, into a 250-mL Erlenmeyer flask. In separate 250-mL Erlenmeyer flasks, prepare a 50.0-mL water blank and a series of 50.0-mL copper standards containing 5.0, 10.0, 15.0, 20.0, and 25.0 μg Cu. To sample, blank, and standards add, mixing after each addition, 1.00 mL 1 + 1 HCl, 5.00 mL $NH_2OH \cdot HCl$ solution, 5.00 mL sodium citrate solution, and 5.00 mL disodium bathocuproine disulfonate solution. Transfer to cells and read sample absorbance against the blank at 484 nm. Plot absorbance against micrograms Cu in standards for the calibration curve. Estimate concentration from the calibration curve.

5. Calculation

$$\text{mg Cu/L} = \frac{\mu\text{g Cu (in 66 mL final volume)}}{\text{mL sample}}$$

6. Precision and Bias

A synthetic sample containing 1000 μg Cu/L, 500 μg Al/L, 50 μg Cd/L, 110 μg Cr/L, 300 μg Fe/L, 70 μg Pb/L, 50 μg Mn/L, 150 μg Ag/L, and 650 μg Zn/L was analyzed in 33 laboratories by the bathocuproine method, with a relative standard deviation of 4.1% and a relative error of 0.3%.

7. Bibliography

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3500-Fe IRON*

3500-Fe A. Introduction

1. Occurrence and Significance

Iron (Fe) is the first element in Group VIII of the periodic table; it has an atomic number of 26, an atomic weight of 55.85, and common valences of 2 and 3 (and occasionally valences of 1, 4, and 6). The average abundance of Fe in the earth's crust is 6.22%; in soils Fe ranges from 0.5 to 4.3%; in streams it averages about 0.7 mg/L; and in groundwater it is 0.1 to 10 mg/L. Iron occurs in the minerals hematite, magnetite, taconite, and pyrite. It is widely used in steel and in other alloys.

The solubility of ferrous ion (Fe^{2+}) is controlled by the carbonate concentration. Because groundwater is often anoxic, any soluble iron in groundwater is usually in the ferrous state. On exposure to air or addition of oxidants, ferrous iron is oxidized to the ferric state (Fe^{3+}) and may hydrolyze to form red, insoluble hydrated ferric oxide. In the absence of complex-forming ions, ferric iron is not significantly soluble unless the pH is very low.

Elevated iron levels in water can cause stains in plumbing, laundry, and cooking utensils, and can impart objectionable tastes and colors to foods. The United Nations Food and Agriculture Organization recommended level for irrigation waters is 5 mg/L. The U.S. EPA secondary drinking water standard MCL is 0.3 mg/L.

2. Selection of Method

Sensitivity and detection levels for the atomic absorption spectrometric methods (Sections 3111B and C), the inductively coupled plasma method (Section 3120), and the phenanthroline colorimetric procedure described here (3500-Fe.B) are similar and generally adequate for analysis of natural or treated waters. Lower detection levels can be achieved with electrothermal atomic absorption spectrometry (Section 3113B) when an appropriate matrix modifier is used. The complexing reagents used in the colorimetric procedures are specific for ferrous iron but the atomic absorption procedures are not. However, because of the instability of ferrous iron, which is changed easily to the ferric form in solutions in contact with air, determination of ferrous iron requires special precautions and may need to be done in the field at the time of sample collection.

The procedure for determining ferrous iron using 1,10-phenanthroline (3500-Fe.B.4*c*) has a somewhat limited applicability; avoid long storage time or exposure of samples to light. A rigorous quantitative distinction between ferrous and ferric iron can be obtained with a special procedure using bathophenanthroline. Spectrophotometric methods using bathophenanthroline[1–6] and other organic complexing reagents such as ferrozine[7] or TPTZ[8] are capable of determining iron concentrations as low as 1 μg/L. A chemiluminescence procedure[9] is stated to have a detection limit of 5 ng/L. Additional procedures are described elsewhere.[10–13]

3. Sampling and Storage

Plan in advance the methods of collecting, storing, and pretreating samples. Clean sample container with acid and rinse with reagent water. Equipment for membrane filtration of samples in the field may be required to determine iron in solution (dissolved iron). Dissolved iron, considered to be that passing through a 0.45-μm membrane filter, may include colloidal iron. The value of the determination depends greatly on the care taken to obtain a representative sample. Iron in well or tap water samples may vary in concentration and form with duration and degree of flushing before and during sampling. When taking a sample portion for determining iron in suspension, shake the sample bottle often and vigorously to obtain a uniform suspension of precipitated iron. Use particular care when colloidal iron adheres to the sample bottle. This problem can be acute with plastic bottles.

For a precise determination of total iron, use a separate container for sample collection. Treat with acid at the time of collection to place the iron in solution and prevent adsorption or deposition on the walls of the sample container. Take account of the added acid in measuring portions for analysis. The addition of acid to the sample may eliminate the need for adding acid before digestion (3500-Fe.B.4*a*).

4. References

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3500-Fe B. Phenanthroline Method

1. General Discussion

a. Principle: Iron is brought into solution, reduced to the ferrous state by boiling with acid and hydroxylamine, and treated with 1,10-phenanthroline at pH 3.2 to 3.3. Three molecules of phenanthroline chelate each atom of ferrous iron to form an orange-red complex. The colored solution obeys Beer's law; its intensity is independent of pH from 3 to 9. A pH between 2.9 and 3.5 ensures rapid color development in the presence of an excess of phenanthroline. Color standards are stable for at least 6 months.

b. Interference: Among the interfering substances are strong oxidizing agents, cyanide, nitrite, and phosphates (polyphosphates more so than orthophosphate), chromium, zinc in concentrations exceeding 10 times that of iron, cobalt and copper in excess of 5 mg/L, and nickel in excess of 2 mg/L. Bismuth, cadmium, mercury, molybdate, and silver precipitate phenanthroline. The initial boiling with acid converts polyphosphates to orthophosphate and removes cyanide and nitrite that otherwise would interfere. Adding excess hydroxylamine eliminates errors caused by excessive concentrations of strong oxidizing reagents. In the presence of interfering metal ions, use a larger excess of phenanthroline to replace that complexed by the interfering metals. Where excessive concentrations of interfering metal ions are present, the extraction method may be used.

If noticeable amounts of color or organic matter are present, it may be necessary to evaporate the sample, gently ash the residue, and redissolve in acid. The ashing may be carried out in silica, porcelain, or platinum crucibles that have been boiled for several hours in 6*N* HCl. The presence of excessive amounts of organic matter may necessitate digestion before use of the extraction procedure.

c. Minimum detectable concentration: Dissolved or total concentrations of iron as low as 10 μg/L can be determined with a spectrophotometer using cells with a 5 cm or longer light path. Carry a blank through the entire procedure to allow for correction.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer,* for use at 510 nm, providing a light path of 1 cm or longer.

2) *Filter photometer,* providing a light path of 1 cm or longer and equipped with a green filter having maximum transmittance near 510 nm.

3) *Nessler tubes,* matched, 100-mL, tall form.

b. Acid-washed glassware: Wash all glassware with conc hydrochloric acid (HCl) and rinse with reagent water before use to remove deposits of iron oxide.

c. Separatory funnels: 125-mL, Squibb form, with ground-glass or TFE stopcocks and stoppers.

3. Reagents

Use reagents low in iron. Use reagent water (see Sections 1080 and 3111B.3*c*) in preparing standards and reagent solutions and in procedure. Store reagents in glass-stoppered bottles. The HCl and ammonium acetate solutions are stable indefinitely if tightly stoppered. The hydroxylamine, phenanthroline, and stock iron solutions are stable for several months. The standard iron solutions are not stable; prepare daily as needed by diluting the stock solution. Visual standards in nessler tubes are stable for several months if sealed and protected from light.

a. Hydrochloric acid (HCl), conc, containing less than 0.5 ppm iron.

b. Hydroxylamine solution: Dissolve 10 g $NH_2OH{\cdot}HCl$ in 100 mL water.

c. Ammonium acetate buffer solution: Dissolve 250 g $NH_4C_2H_3O_2$ in 150 mL water. Add 700 mL conc (glacial) acetic acid. Because even a good grade of $NH_4C_2H_3O_2$ contains a significant amount of iron, prepare new reference standards with each buffer preparation.

d. Sodium acetate solution: Dissolve 200 g $NaC_2H_3O_2 \cdot 3H_2O$ in 800 mL water.

e. Phenanthroline solution: Dissolve 100 mg 1,10-phenanthroline monohydrate, $C_{12}H_8N_2 \cdot H_2O$, in 100 mL water by stirring and heating to 80°C. Do not boil. Discard the solution if it darkens. Heating is unnecessary if 2 drops conc HCl are added to the water. (Note: One milliliter of this reagent is sufficient for no more than 100 μg Fe.)

f. Potassium permanganate, 0.02*M:* Dissolve 0.316 g $KMnO_4$ in reagent water and dilute to 100 mL.

g. Stock iron solution: Use metal (1) or salt (2) for preparing the stock solution.

1) Use electrolytic iron wire, or "iron wire for standardizing," to prepare the solution. If necessary, clean wire with fine sandpaper to remove any oxide coating and to produce a bright surface. Weigh 200.0 mg wire and place in a 1000-mL volumetric flask. Dissolve in 20 mL 6*N* sulfuric acid (H_2SO_4) and dilute to mark with water; 1.00 mL = 200 μg Fe.

2) If ferrous ammonium sulfate is preferred, slowly add 20 mL conc H_2SO_4 to 50 mL water and dissolve 1.404 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$. Slowly add potassium permanganate (¶ *f* above) until a faint pink color persists. Add the last few milliliters of the solution dropwise. Approximately 50 mL of the

potassium permanganate will be required. Dilute to 1000 mL with water and mix; 1.00 mL = 200 μg Fe.

h. Standard iron solutions: Prepare daily for use.

1) Pipet 50.00 mL stock solution into a 1000-mL volumetric flask and dilute to mark with water; 1.00 mL = 10.0 μg Fe.

2) Pipet 5.00 mL stock solution into a 1000-mL volumetric flask and dilute to mark with water; 1.00 mL = 1.00 μg Fe.

i. Diisopropyl or isopropyl ether. **CAUTION: Ethers may form explosive peroxides; test before using**.

4. Procedure

a. Total iron: Mix sample thoroughly and measure 50.0 mL into a 125-mL Erlenmeyer flask. If this sample volume contains more than 200 μg iron use a smaller accurately measured portion and dilute to 50.0 mL. Add 2 mL conc HCl and 1 mL $NH_2OH \cdot HCl$ solution. Add a few glass beads and heat to boiling. To ensure dissolution of all the iron, continue boiling until volume is reduced to 15 to 20 mL. (If the sample is ashed, take up residue in 2 mL conc HCl and 5 mL water.) Cool to room temperature and transfer to a 50- or 100-mL volumetric flask or nessler tube. Add 10 mL $NH_4C_2H_3O_2$ buffer solution and 4 mL phenanthroline solution, and dilute to mark with water. Mix thoroughly and allow a minimum of 10 min for maximum color development.

b. Dissolved iron: Immediately after collection filter sample through a 0.45-μm membrane filter into a vacuum flask containing 1 mL conc HCl/100 mL sample. Analyze filtrate for total dissolved iron (¶ *a* above) and/or dissolved ferrous iron (¶ *c* below). (This procedure also can be used in the laboratory if it is understood that normal sample exposure to air during shipment may result in precipitation of iron.)

Calculate suspended iron by subtracting dissolved from total iron.

c. Ferrous iron: Determine ferrous iron at sampling site because of the possibility of change in the ferrous-ferric ratio with time in acid solutions. To determine ferrous iron only, acidify a separate sample with 2 mL conc HCl/100 mL sample at time of collection. Fill bottle directly from sampling source and stopper. Immediately withdraw a 50-mL portion of acidified sample and add 20 mL phenanthroline solution and 10 mL $NH_4C_2H_3O_2$ solution with vigorous stirring. Dilute to 100 mL and measure color intensity within 5 to 10 min. Do not expose to sunlight. (Color development is rapid in the presence of excess phenanthroline. The phenanthroline volume given is suitable for less than 50 μg total iron; if larger amounts are present, use a correspondingly larger volume of phenanthroline or a more concentrated reagent.)

Calculate ferric iron by subtracting ferrous from total iron.

d. Color measurement: Prepare a series of standards by accurately pipetting calculated volumes of standard iron solutions [use solution described in 3500-Fe.B.3*h*2) to measure 1- to 10-μg portions] into 125-mL Erlenmeyer flasks and diluting to 50 mL by adding measured volumes of water. Add 2 mL conc HCl and 1 mL $NH_2OH \cdot HCl$ solution. Carry out the steps in ¶ *a* above beginning with transfer to a 100-mL volumetric flask or nessler tube.

For visual comparison, prepare a set of at least 10 standards, ranging from 1 to 100 μg Fe in the final 100-mL volume. Compare colors in 100-mL tall-form nessler tubes.

TABLE 3500-Fe:I. SELECTION OF LIGHT PATH LENGTH FOR VARIOUS IRON CONCENTRATIONS

Fe μg		
50-mL Final Volume	100-mL Final Volume	Light Path *cm*
50–200	100–400	1
25–100	50–200	2
10–40	20–80	5
5–20	10–40	10

For photometric measurement, use Table 3500-Fe:I as a rough guide for selecting proper light path at 510 nm. Read standards against water set at zero absorbance and plot a calibration curve, including a blank (see Sections 1080 and 3111B.3*c*).

If samples are colored or turbid, carry a second set of samples through all steps of the procedure without adding phenanthroline. Instead of water, use the prepared blanks to set photometer to zero absorbance and read each sample developed with phenanthroline against the corresponding blank without phenanthroline. Translate observed photometer readings into iron values by means of the calibration curve. This procedure does not compensate for interfering ions.

e. Samples containing organic interferences: Digest samples containing substantial amounts of organic substances according to the directions given in Section 3030G or H.

1) If a digested sample has been prepared according to the directions given in Section 3030G or H, pipet 10.0 mL or other suitable portion containing 20 to 500 μg Fe into a 125-mL separatory funnel. If the volume taken is less than 10 mL, add water to make up to 10 mL. To the separatory funnel add 15 mL conc HCl for a 10-mL aqueous volume; or, if the portion taken was greater than 10.0 mL, add 1.5 mL conc HCl/mL sample. Mix, cool, and proceed with ¶ *e*3) below.

2) To prepare a sample solely for determining iron, measure a suitable volume containing 20 to 500 μg Fe and carry it through the digestion procedure described in either Section 3030G or H. However, use only 5 mL H_2SO_4 or $HClO_4$ and omit H_2O_2. When digestion is complete, cool, dilute with 10 mL water, heat almost to boiling to dissolve slowly soluble salts, and, if the sample is still cloudy, filter through a glass-fiber, sintered-glass, or porcelain filter, washing with 2 to 3 mL water. Quantitatively transfer filtrate or clear solution to a 25-mL volumetric flask and make up to 25 mL with water. Empty flask into a 125-mL separatory funnel, rinse flask with 5 mL conc HCl and add to the funnel. Add 25 mL conc HCl measured with the same flask. Mix and cool to room temperature.

3) Extract the iron from the HCl solution in the separatory funnel by shaking for 30 s with 25 mL isopropyl ether (**CAUTION**). Draw off lower acid layer into a second separatory funnel. Extract acid solution again with 25 mL isopropyl ether, drain acid layer into a suitable clean vessel, and add ether layer to the ether in the first funnel. Pour acid layer back into second separatory funnel and re-extract with 25 mL isopropyl ether. Withdraw and discard acid layer and add ether layer to first funnel. Persistence of a yellow color in the HCl solution after three extractions does not signify incomplete separation of iron be-

cause copper, which is not extracted, gives a similar yellow color.

Shake combined ether extracts with 25 mL water to return iron to aqueous phase and transfer lower aqueous layer to a 100-mL volumetric flask. Repeat extraction with a second 25-mL portion of water, adding this to the first aqueous extract. Discard ether layer.

4) Add 1 mL $NH_2OH \cdot HCl$ solution, 10 mL phenanthroline solution, and 10 mL $NaC_2H_3O_2$ solution. Dilute to 100 mL with water, mix thoroughly, and let stand for a minimum of 10 min. Measure absorbance at 510 nm using a 5-cm absorption cell for amounts of iron less than 100 μg or 1-cm cell for quantities from 100 to 500 μg. As reference, use either water or a sample blank prepared by carrying the specified quantities of acids through the entire analytical procedure. If water is used as reference, correct sample absorbance by subtracting absorbance of a sample blank.

Determine micrograms of iron in the sample from the absorbance (corrected, if necessary) by reference to the calibration curve prepared by using a suitable range of iron standards containing the same amounts of phenanthroline, hydroxylamine, and sodium acetate as the sample.

5. Calculation

When the sample has been treated according to 3500-Fe.B.4*a, b, c,* or 4*e*2):

$$\text{mg Fe/L} = \frac{\mu\text{g Fe (in 100 mL final volume)}}{\text{mL sample}}$$

When the sample has been treated according to 4*e*1):

$$\text{mg Fe/L} = \frac{\mu\text{g Fe (in 100 mL final volume)}}{\text{mL sample}} \times \frac{100}{\text{mL portion}}$$

Report details of sample collection, storage, and pretreatment if they are pertinent to interpretation of results.

6. Precision and Bias

Precision and bias depend on the method of sample collection and storage, the method of color measurement, the iron concentration, and the presence of interfering color, turbidity, and foreign ions. In general, optimum reliability of visual comparison in nessler tubes is not better than 5% and often only 10%, whereas, under optimum conditions, photometric measurement may be reliable to 3% or 3 μg, whichever is greater. The sensitivity limit for visual observation in nessler tubes is approximately 1 μg Fe. Sample variability and instability may affect precision and bias of this determination more than will the errors of analysis. Serious divergences have been found in reports of different laboratories because of variations in methods of collecting and treating samples.

A synthetic sample containing 300 μg Fe/L, 500 μg Al/L, 50 μg Cd/L, 110 μg Cr/L, 470 μg Cu/L, 70 μg Pb/L, 120 μg Mn/L, 150 μg Ag/L, and 650 μg Zn/L in distilled water was analyzed in 44 laboratories by the phenanthroline method, with a relative standard deviation of 25.5% and a relative error of 13.3%.

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3500-Pb LEAD*

3500-Pb A. Introduction

1. Occurrence and Significance

Lead (Pb) is the fifth element in Group IVA in the periodic table; it has an atomic number of 82, an atomic weight of 207.19, and valences of 2 and 4. The average abundance of Pb in the earth's crust is 13 ppm; in soils it ranges from 2.6 to 25 ppm; in streams it is 3 μg/L, and in groundwaters it is generally $<$0.1 mg/L. Lead is obtained chiefly from galena (PbS). It is used in batteries, ammunition, solder, piping, pigments, insecticides, and alloys. Lead also was used in gasoline for many years as an anti-knock agent in the form of tetraethyl lead.

The common aqueous species are Pb^{2+} and hydroxide and carbonate complexes. Lead in a water supply may come from industrial, mine, and smelter discharges or from the dissolution of plumbing and plumbing fixtures. Tap waters that are inherently noncorrosive or not suitably treated may contain lead resulting from an attack on lead service pipes, lead interior plumbing, brass fixtures and fittings, or solder pipe joints.

Lead is nonessential for plants and animals. It is toxic by ingestion and is a cumulative poison. The Food and Drug Administration regulates lead content in food and in house paints. Under the lead-copper rule, the U.S. EPA drinking water 90th percentile action level is 15 μg/L.

2. Selection of Method

The atomic absorption spectrometric method (Section 3111B) has a relatively high detection limit in the flame mode and requires an extraction procedure (Section 3111C) for the low concentrations common in potable water. The electrothermal atomic absorption (AA) method (Section 3113B) is more sensitive for low concentrations and does not require extraction. The inductively coupled plasma/mass spectrometric method (Section 3125) is even more sensitive than the electrothermal AA method. The inductively coupled plasma method (Section 3120) has a sensitivity similar to that of the flame atomic absorption method. Anodic stripping voltammetry (Section 3130B) can achieve superior detection levels, but is susceptible to interferences from copper, silver, gold, and organic compounds. The dithizone method (3500-Pb.B) is sensitive and specific as a colorimetric procedure.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011. Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Pb B. Dithizone Method

1. General Discussion

a. Principle: An acidified sample containing microgram quantities of lead is mixed with ammoniacal citrate-cyanide reducing solution and extracted with dithizone in chloroform ($CHCl_3$) to form a cherry-red lead dithizonate. The color of the mixed color solution is measured photometrically.[1,2] Sample volume taken for analysis may be 2 L when digestion is used.

b. Interference: In a weakly ammoniacal cyanide solution (pH 8.5 to 9.5) dithizone forms colored complexes with bismuth, stannous tin, and monovalent thallium. In strongly ammoniacal citrate-cyanide solution (pH 10 to 11.5) the dithizonates of these ions are unstable and are extracted only partially.[3] This method uses a high pH, mixed color, single dithizone extraction. Interference from stannous tin and monovalent thallium is reduced further when these ions are oxidized during preliminary digestion. A modification of the method allows detection and elimination of bismuth interference. Excessive quantities of bismuth, thallium, and tin may be removed.[4]

Dithizone in $CHCl_3$ absorbs at 510 nm; control its interference by using nearly equal concentrations of excess dithizone in samples, standards, and blank.

The method is without interference for the determination of 0.0 to 30.0 μg Pb in the presence of 20 μg Tl^+, 100 μg Sn^{2+}, 200 μg In^{3+}, and 1000 μg each of Ba^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Sr^{2+}, Zn^{2+}, Al^{3+}, Sb^{3+}, As^{3+}, Cr^{3+}, Fe^{3+}, V^{3+}, PO_4^{3-}, and SO_4^{2-}. Gram quantities of alkali metals do not interfere. A modification is provided to avoid interference from excessive quantities of bismuth or tin.

c. Preliminary sample treatment: At time of collection acidify with conc HNO_3 to pH $<$2 but avoid excess HNO_3. Add 5 mL 0.1*N* iodine solution to avoid losses of volatile organo-lead compounds during handling and digesting of samples. Prepare a blank of lead-free water and carry through the procedure.

d. Digestion of samples: Unless digestion is shown to be unnecessary, digest all samples for dissolved or total lead as described in Section 3030H or K.

e. Minimum detectable concentration: 1.0 μg Pb/10 mL dithizone solution.

f. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Spectrophotometer for use at 510 nm, providing a light path of 1 cm or longer.

https://doi.org/10.2105/SMWW.2882.056

b. pH meter.

c. Separatory funnels: 250-mL Squibb type. Clean all glassware, including sample bottles, with 1 + 1 HNO_3. Rinse thoroughly with reagent water.

d. Automatic dispensing burets: Use for all reagents to minimize indeterminate contamination errors.

3. Reagents

Prepare all reagents in lead-free water.

a. Stock lead solution: Dissolve 0.1599 g lead nitrate, $Pb(NO_3)_2$ (minimum purity 99.5%), in approximately 200 mL water. Add 10 mL conc HNO_3 and dilute to 1000 mL with water. Alternatively, dissolve 0.1000 g pure Pb metal in 20 mL 1 + 1 HNO_3 and dilute to 1000 mL with water; 1.00 mL = 100 μg Pb.

b. Working lead solution: Dilute 2.0 mL stock solution to 100 mL with water; 1 mL = 2.00 μg Pb.

c. Nitric acid (HNO_3), 1 + 4: Dilute 200 mL conc HNO_3 to 1 L with water.

d. Ammonium hydroxide (NH_4OH), 1 + 9: Dilute 10 mL conc NH_4OH to 100 mL with water.

e. Citrate-cyanide reducing solution: Dissolve 400 g dibasic ammonium citrate, $(NH_4)_2HC_6H_5O_7$, 20 g anhydrous sodium sulfite, Na_2SO_3, 10 g hydroxylamine hydrochloride, $NH_2OH \cdot HCl$, and 40 g potassium cyanide, KCN (**CAUTION: Poison**) in water and dilute to 1 L. Mix this solution with 2 L conc NH_4OH. **CAUTION: Do not pipet by mouth. Prepare solution in a fume hood.**

f. Stock dithizone solution: The dithizone concentration in the stock dithizone solutions is based on having a 100% pure dithizone reagent. Some commercial grades of dithizone are contaminated with the oxidation product diphenylthiocarbodiazone or with metals. Purify dithizone as directed below. For dithizone solutions not stronger than 0.001% (w/v), calculate the exact concentration by dividing the absorbance of the solution in a 1.00-cm cell at 606 nm by 40.6×10^3, the molar absorptivity.

In a fume hood, dissolve 100 mg dithizone in 50 mL $CHCl_3$ in a 150-mL beaker and filter through a 7-cm-diam paper.* Receive filtrate in a 500-mL separatory funnel or in a 125-mL Erlenmeyer flask under slight vacuum; use a filtering device designed to handle the $CHCl_3$ vapor. Wash beaker with two 5-mL portions $CHCl_3$, and filter. Wash the paper with three 5-mL portions $CHCl_3$, adding final portion dropwise to edge of paper. If filtrate is in flask, transfer with $CHCl_3$ to a 500-mL separatory funnel.

Add 100 mL 1 + 99 NH_4OH to separatory funnel and shake moderately for 1 min; excessive agitation produces slowly breaking emulsions. Let layers separate, swirling funnel gently to submerge $CHCl_3$ droplets held on surface of aqueous layer. Transfer $CHCl_3$ layer to 250-mL separatory funnel, retaining the orange-red aqueous layer in the 500-mL funnel. Repeat extraction, receiving $CHCl_3$ layer in another 250-mL separatory funnel and transferring aqueous layer, using 1 + 99 NH_4OH, to the 500-mL funnel holding the first extract. Repeat extraction, transferring the aqueous layer to 500-mL funnel. Discard $CHCl_3$ layer.

To combined extracts in the 500-mL separatory funnel add 1 + 1 HCl in 2-mL portions, mixing after each addition, until dithizone precipitates and solution is no longer orange-red. Extract precipitated dithizone with three 25-mL portions $CHCl_3$. Dilute combined extracts to 1000 mL with $CHCl_3$; 1.00 mL = 100 μg dithizone.

g. Dithizone working solution: Dilute 100 mL stock dithizone solution to 250 mL with $CHCl_3$; 1 mL = 40 μg dithizone.

h. Special dithizone solution: Dissolve 250 mg dithizone in 250 mL $CHCl_3$. This solution may be prepared without purification because all extracts using it are discarded.

i. Sodium sulfite solution: Dissolve 5 g anhydrous Na_2SO_3 in 100 mL water.

j. Iodine solution: Dissolve 40 g KI in 25 mL water, add 12.7 g resublimed iodine, and dilute to 1000 mL.

4. Procedure

a. With sample digestion: **CAUTION: Perform the following procedure (excluding use of spectrophotometer) in a fume hood.** To a digested sample containing not more than 1 mL conc acid add 20 mL 1 + 4 HNO_3 and filter through lead-free filter paper† and filter funnel directly into a 250-mL separatory funnel. Rinse digestion beaker with 50 mL water and add to filter. Add 50 mL ammoniacal citrate-cyanide solution, mix, and cool to room temperature. Add 10 mL dithizone working solution, shake stoppered funnel vigorously for 30 s, and let layers separate. Insert lead-free cotton in stem of separatory funnel and draw off lower layer. Discard 1 to 2 mL $CHCl_3$ layer, then fill absorption cell. Measure absorbance of extract at 510 nm, using dithizone working solution, 3500-Pb.B.3*g*, to zero spectrophotometer.

b. Without sample digestion: To 100 mL acidified sample (pH 2) in a 250-mL separatory funnel add 20 mL 1 + 4 HNO_3 and 50 mL citrate-cyanide reducing solution; mix. Add 10 mL dithizone working solution and proceed as in ¶ *a* above.

c. Calibration curve: Plot concentration of at least five standards and a blank against absorbance. Determine concentration of lead in extract from curve. All concentrations are μg Pb/10 mL final extract.

d. Removal of excess interferences: The dithizonates of bismuth, tin, and thallium differ from lead dithizonate in maximum absorbance. Detect their presence by measuring sample absorbance at 510 nm and at 465 nm. Calculate corrected absorbance of sample at each wavelength by subtracting absorbance of blank at same wavelength. Calculate ratio of corrected absorbance at 510 nm to corrected absorbance at 465 nm. The ratio of corrected absorbances for lead dithizonate is 2.08 and for bismuth dithizonate is 1.07. If the ratio for the sample indicates interference (i.e., is markedly less than 2.08) proceed as follows with a new 100-mL sample: If the sample has not been digested, add 5 mL Na_2SO_3 solution to reduce iodine preservative. Adjust sample to pH 2.5 using a pH meter and 1 + 4 HNO_3 or 1 + 9 NH_4OH as required. Transfer sample to 250-mL separatory funnel, extract with a minimum of three 10-mL portions special dithizone solution, or until the $CHCl_3$ layer is distinctly green. Extract with 20-mL portions $CHCl_3$ to remove dithizone (absence of green). Add 20 mL 1 + 4 HNO_3, 50 mL citrate-cyanide reducing solution, and 10 mL dithizone working solution. Extract as in ¶ *a* above and measure absorbance.

* Whatman No. 42, or equivalent.

† Whatman No. 541, or equivalent.

5. Calculation

$$\text{mg Pb/L} = \frac{\mu\text{g Pb (in 10 mL, from calibration curve)}}{\text{mL sample}}$$

6. Precision and Bias

Single-operator precision in recovering 0.0104 mg Pb/L from Mississippi River water was 6.8% relative standard deviation and −1.4% relative error. At the level of 0.026 mg Pb/L, recovery was made with 4.8% relative standard deviation and 15% relative error.

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3500-Li LITHIUM*

3500-Li A. Introduction

1. Occurrence and Significance

Lithium (Li) is the second element in Group IA of the periodic table; it has an atomic number of 3, an atomic weight of 6.94, and a valence of 1. The average abundance of Li in the earth's crust is 18 ppm; in soils it is 14 to 32 ppm; in streams it is 3 μg/L, and in groundwaters it is <0.1 mg/L. The more important minerals containing lithium are lepidolite, spodumene, petalite, and amblygonite. Lithium compounds are used in pharmaceuticals, soaps, batteries, welding flux, ceramics, reducing agents (e.g., lithium aluminum hydride), and cosmetics.

Many lithium salts are only slightly soluble, and the metal's concentration in water is controlled by incorporation in clay minerals of soils. Lithium is considered nonessential for plants and animals, but it is essential for some microorganisms. Some lithium salts are toxic by ingestion. The United Nations Food and Agriculture Organization recommended maximum level for lithium in irrigation waters is 2.5 mg/L.

2. Selection of Method

The atomic absorption spectrometric method (Section 3111B) and the inductively coupled plasma method (Section 3120) are preferred. The flame emission photometric method (3500-Li.B) also is available for laboratories not equipped to use preferred methods. The inductively coupled plasma/mass spectrometric method (Section 3125) may be applied successfully in most cases (with lower detection levels), even though lithium is not specifically listed as an analyte in the method.

* Approved by Standard Methods Committee, 2004. Editorial revisions, 2011.
Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Li B. Flame Emission Photometric Method

1. General Discussion

a. Principle: Lithium can be determined in trace amounts by flame photometric methods at a wavelength of 670.8 nm.

b. Interference: A molecular band of strontium hydroxide with an absorption maximum at 671.0 nm interferes in the flame photometric determination of lithium. Ionization of lithium can be significant in both the air-acetylene and nitrous oxide-acetylene flames and can be suppressed by adding potassium. See Section 3500-Na.B.1*b* for additional information on minimizing interferences in flame photometry.

c. Minimum detectable concentration: The minimum lithium concentration detectable is approximately 0.1 μg/L for reagent water analyzed on an atomic absorption spectrophotometer in the emission mode with an air-acetylene flame, or 0.03 μg/L with a nitrous oxide-acetylene flame.

d. Sampling and storage: Preferably collect sample in a polyethylene bottle, although borosilicate glass containers also may be used. At time of collection adjust sample to pH <2 with nitric acid (HNO_3).

2. Apparatus

Flame photometer: A flame photometer or an atomic absorption spectrometer operating in the emission mode using a lean air-acetylene flame is recommended.

3. Reagents

Use reagent water (see Section 3111B.3*c*) in reagent preparation and analysis.

a. Potassium ionization suppressant: Dissolve 95.35 g KCl dried at 110°C and dilute to 1000 mL with water; 1.00 mL = 50 mg K.

b. Stock lithium solution: Dissolve 152.7 mg high-purity anhydrous lithium chloride, LiCl, in water and dilute to 250 mL; 1.00 mL = 100 μg Li. Dry salt overnight in an oven at 105°C. Cool in a desiccator and weigh immediately after removal from desiccator. Alternatively, purchase prepared stock from a reputable supplier.

c. Standard lithium solution: Dilute 10.00 mL stock LiCl solution to 500 mL with water; 1.00 mL = 2.0 μg Li.

4. Procedure

a. Pretreatment of polluted water and wastewater samples: Choose digestion method appropriate to matrix (see Section 3030).

b. Suppressing ionization: If necessary, filter sample through medium-porosity paper, add 1.0 mL potassium ionization suppressant to 50 mL volumetric flask, and dilute with sample for flame photometric determination. Sample solution will be in a 0.1% K matrix.

c. Treatment of standard solutions: Prepare dilutions of the Li standard solution to bracket sample concentration or to establish at least three points on a calibration curve of emission intensity against Li concentration. Prepare standards by adding appropriate volumes of standard lithium solution to 25 mL water + 1.0 mL potassium ionization suppressant reagent in a 50-mL volumetric flask. Dilute to 50.0 mL and mix. Both samples and standards will be in a 0.1% K matrix to suppress ionization of lithium.

d. Flame photometric measurement: Determine lithium concentration by direct intensity measurements at a wavelength of 670.8 nm. The bracketing method (Section 3500-Na.B.4*d*) can be used with some photometric instruments, while the construction of a calibration curve is necessary with others. Run sample, water, and lithium standard as nearly simultaneously as possible. For best results, average several readings on each solution.

Follow the manufacturer's instructions for instrument operation.

5. Calculation

$$\mu g\ Li/L = (\mu g\ Li/L \text{ in portion analyzed}) \times D$$

where:

$$D = \text{dilution ratio} = \frac{\text{mL sample} + \text{mL water}}{\text{mL sample}}$$

6. Quality Control

The quality control practices considered to be an integral part of each method can be found in Section 3020.

Process a QC standard through entire analytical protocol as a way of determining systematic bias. The control limits for precision of duplicate determinations at concentrations (in water) of 4.0 μg/L and 10.0 μg/L were 4.09 $\pm$ 0.056 μg/L and 9.96 $\pm$ 0.094 μg/L, respectively. The single-operator RSD was 1.38% for a lithium solution containing 10 μg/L. See Part 1000 and Section 3020 for specific quality control procedures and acceptance limits to be followed during sample preparation and analysis.

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3500-Mg MAGNESIUM*

3500-Mg A. Introduction

1. Occurrence and Significance

Magnesium (Mg) is the second element in Group IIA of the periodic table; it has an atomic number of 12, an atomic weight of 24.30, and a valence of 2. The average abundance of Mg in the earth's crust is 2.1%; in soils it is 0.03 to 0.84%; in streams it is 4 mg/L, and in groundwaters it is >5 mg/L. Magnesium occurs commonly in the minerals magnesite and dolomite. Magnesium is used in alloys, pyrotechnics, flash photography, drying agents, refractories, fertilizers, pharmaceuticals, and foods.

The common aqueous species is Mg^{2+}. The carbonate equilibrium reactions for magnesium are more complicated than for calcium, and conditions for direct precipitation of dolomite in natural waters are not common. Important contributors to the hardness of a water, magnesium salts break down when heated, forming scale in boilers. Chemical softening, reverse osmosis, or ion exchange reduces magnesium and associated hardness to acceptable levels.

Magnesium is an essential element in chlorophyll and in red blood cells. Some salts of magnesium are toxic by ingestion or inhalation. Concentrations greater than 125 mg/L also can have a cathartic and diuretic effect.

2. Selection of Method

The methods presented are applicable to waters and wastewaters. Direct determinations can be made with the atomic absorption spectrometric method (Section 3111B) and inductively coupled plasma method (Section 3120). The inductively coupled plasma mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though magnesium is not specifically listed as an analyte in the method. These methods can be applied to most concentrations encountered, although sample dilution may be required. Choice of method is largely a matter of personal preference and analyst experience. A calculation method (3500-Mg.B) also is available.

* Approved by Standard Methods Committee, 1997.
Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Mg B. Calculation Method

Magnesium may be estimated as the difference between hardness and calcium as $CaCO_3$ if interfering metals are present in noninterfering concentrations in the calcium titration (Section 3500-Ca.B) and suitable inhibitors are used in the hardness titration (Section 2340C).

$$\text{mg Mg/L} = [\text{total hardness (as mg } CaCO_3\text{/L)} - \text{calcium hardness (as mg } CaCO_3\text{/L)}] \times 0.243$$

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3500-Mn MANGANESE*

3500-Mn A. Introduction

1. Occurrence and Significance

Manganese (Mn) is the first element in Group VIIB in the periodic table; it has an atomic number of 25, an atomic weight of 54.94, and common valences of 2, 4, and 7 (and more rarely, valences of 1, 3, 5, and 6). The average abundance of Mn in the earth's crust is 1060 ppm; in soils it is 61 to 1010 ppm; in streams it is 7 μg/L, and in groundwaters it is $<$0.1 mg/L. Manganese is associated with iron minerals, and occurs in nodules in ocean, fresh waters, and soils. The common ores are pyrolusite (MnO_2) and psilomelane. Manganese is used in steel alloys, batteries, and food additives.

The common aqueous species are the reduced Mn^{2+} and the oxidized Mn^{4+}. The aqueous chemistry of manganese is similar to that of iron. Since groundwater is often anoxic, any soluble manganese in groundwater is usually in the reduced state (Mn^{2+}). Upon exposure to air or other oxidants, groundwater containing manganese usually will precipitate black MnO_2. Elevated manganese levels therefore can cause stains in plumbing/laundry, and cooking utensils. It is considered an essential trace element for plants and animals. The United Nations Food and Agriculture Organization recommended maximum level for manganese in irrigation waters is 0.2 mg/L. The U.S. EPA secondary drinking water standard MCL is 50 μg/L.

2. Selection of Method

The atomic absorption spectrometric methods (Section 3111B and C), the electrothermal atomic absorption method (Section 3113B), and the inductively coupled plasma methods (Sections 3120 and 3125) permit direct determination with acceptable sensitivity and are the methods of choice. Of the various colorimetric methods, the persulfate method (3500-Mn.B) is preferred because the use of mercuric ion can control interference from a limited chloride ion concentration.

3. Sampling and Storage

Manganese may exist in a soluble form in a neutral water when first collected, but it oxidizes to a higher oxidation state and precipitates or becomes adsorbed on the container walls. Determine manganese very soon after sample collection. When delay is unavoidable, total manganese can be determined if the sample is acidified at the time of collection with HNO_3 to pH <2 (Section 3010B.2).

* Approved by Standard Methods Committee, 1999. Editorial revisions, 2011. Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Mn B. Persulfate Method

1. General Discussion

a. Principle: Persulfate oxidation of soluble manganous compounds to form permanganate is carried out in the presence of silver nitrate. The resulting color is stable for at least 24 h if excess persulfate is present and organic matter is absent.

b. Interference: As much as 0.1 g chloride (Cl^-) in a 50-mL sample can be prevented from interfering by adding 1 g mercuric sulfate ($HgSO_4$) to form slightly dissociated complexes. Bromide and iodide still will interfere and only trace amounts may be present. The persulfate procedure can be used for potable water with trace to small amounts of organic matter if the period of heating is increased after more persulfate has been added.

For wastewaters containing organic matter, use preliminary digestion with nitric and sulfuric acids (HNO_3 and H_2SO_4) (see Section 3030G). If large amounts of Cl^- also are present, boiling with HNO_3 helps remove it. Interfering traces of Cl^- are eliminated by $HgSO_4$ in the special reagent.

Colored solutions from other inorganic ions are compensated for in the final colorimetric step.

Samples that have been exposed to air may give low results due to precipitation of manganese dioxide (MnO_2). Add 1 drop 30% hydrogen peroxide (H_2O_2) to the sample, after adding the special reagent, to redissolve precipitated manganese.

c. Minimum detectable concentration: The molar absorptivity of permanganate ion is about 2300 L g^{-1} cm^{-1}. This corresponds to a minimum detectable concentration (98% transmittance) of 210 μg Mn/L when a 1-cm cell is used or 42 μg Mn/L when a 5-cm cell is used.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

Colorimetric equipment: One of the following is required:

a. Spectrophotometer, for use at 525 nm, providing a light path of 1 cm or longer.

b. Filter photometer, providing a light path of 1 cm or longer and equipped with a green filter having maximum transmittance near 525 nm.

c. Nessler tubes, matched, 100-mL, tall form.

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3. Reagents

a. Special reagent: Dissolve 75 g $HgSO_4$ in 400 mL conc HNO_3 and 200 mL distilled water. Add 200 mL 85% phosphoric acid (H_3PO_4), and 35 mg silver nitrate ($AgNO_3$). Dilute the cooled solution to 1 L.

b. Ammonium persulfate [$(NH_4)_2S_2O_8$], solid.

c. Standard manganese solution: Prepare a 0.1*N* potassium permanganate ($KMnO_4$) solution by dissolving 3.2 g $KMnO_4$ in distilled water and making up to 1 L. Age for several weeks in sunlight or heat for several hours near the boiling point, then filter through a fine fritted-glass filter crucible and standardize against sodium oxalate as follows:

Weigh several 100- to 200-mg samples of $Na_2C_2O_4$ to 0.1 mg and transfer to 400-mL beakers. To each beaker, add 100 mL distilled water and stir to dissolve. Add 10 mL 1 + 1 H_2SO_4 and heat rapidly to 90 to 95°C. Titrate rapidly with the $KMnO_4$ solution to be standardized, while stirring, to a slight pink endpoint color that persists for at least 1 min. Do not let temperature fall below 85°C. If necessary, warm beaker contents during titration; 100 mg $Na_2C_2O_4$ will consume about 15 mL permanganate solution. Run a blank on distilled water and H_2SO_4.

$$\text{Normality of } KMnO_4 = \frac{\text{g } Na_2C_2O_4}{(A - B) \times 0.067\,01}$$

where:

A = mL titrant for sample and
B = mL titrant for blank.

Average results of several titrations. Calculate volume of this solution necessary to prepare 1 L of solution so that 1.00 mL = 50.0 μg Mn, as follows:

$$\text{mL } KMnO_4 = \frac{4.55}{\text{normality } KMnO_4}$$

To this volume add 2 to 3 mL conc H_2SO_4 and $NaHSO_3$ solution dropwise, with stirring, until the permanganate color disappears. Boil to remove excess SO_2, cool, and dilute to 1000 mL with distilled water. Dilute this solution further to measure small amounts of manganese.

d. Standard manganese solution (alternate): Dissolve 1.000 g manganese metal (99.8% min.) in 10 mL redistilled HNO_3. Dilute to 1000 mL with 1% (v/v) HCl; 1 mL = 1.000 mg Mn. Dilute 10 mL to 200 mL with distilled water; 1 mL = 0.05 mg Mn. Prepare dilute solution daily.

e. Hydrogen peroxide (H_2O_2), 30%.

f. Nitric acid (HNO_3), conc.

g. Sulfuric acid (H_2SO_4), conc.

h. Sodium nitrite solution: Dissolve 5.0 g $NaNO_2$ in 95 mL distilled water.

i. Sodium oxalate ($Na_2C_2O_4$), primary standard.

j. Sodium bisulfite: Dissolve 10 g $NaHSO_3$ in 100 mL distilled water.

4. Procedure

a. Treatment of sample: If a digested sample has been prepared according to directions for reducing organic matter and/or excessive chlorides in Section 3030G, pipet a portion containing 0.05 to 2.0 mg Mn into a 250-mL conical flask. Add distilled water, if necessary, to 90 mL and proceed as in ¶ *b* below.

b. To a suitable sample portion add 5 mL special reagent and 1 drop H_2O_2. Concentrate to 90 mL by boiling or dilute to 90 mL. Add 1 g $(NH_4)_2S_2O_8$, bring to a boil, and boil for 1 min. Do not heat on a water bath. Remove from heat source, let stand 1 min, then cool under the tap. (Boiling too long results in decomposition of excess persulfate and subsequent loss of permanganate color; cooling too slowly has the same effect.) Dilute to 100 mL with distilled water free from reducing substances and mix. Prepare standards containing 0, 5.00, . . . 1500 μg Mn by treating various amounts of standard Mn solution in the same way.

c. Nessler tube comparison: Use standards prepared as in ¶ *b* above and containing 5 to 100 μg Mn/100 mL final volume. Compare samples and standards visually.

d. Photometric determination: Use a series of standards from 0 to 1500 μg Mn/100 mL final volume. Make photometric measurements against a distilled water blank. The following table shows light path length appropriate for various amounts of manganese in 100 mL final volume:

Mn Range μg	Light Path cm
5–200	15
20–400	5
50–1000	2
100–1500	1

Prepare a calibration curve of manganese concentration vs. absorbance from the standards and determine Mn in the samples from the curve. If turbidity or interfering color is present, make corrections as in ¶ *e* below.

e. Correction for turbidity or interfering color: Avoid filtration because of possible retention of some permanganate on the filter paper. If visual comparison is used, the effect of turbidity only can be estimated and no correction can be made for interfering colored ions. When photometric measurements are made, use the following "bleaching" method, which also corrects for interfering color: As soon as the photometer reading has been made, add 0.05 mL H_2O_2 solution directly to the sample in the optical cell. Mix and, as soon as the permanganate color has faded completely and no bubbles remain, read again. Deduct absorbance of bleached solution from initial absorbance to obtain absorbance due to Mn.

5. Calculation

a. When all of the original sample is taken for analysis:

$$\text{mg Mn/L} = \frac{\text{μg Mn/100 mL}}{\text{mL sample}} \times \frac{100}{\text{mL portion}}$$

b. When a portion of the digested sample (100 mL final volume) is taken for analysis:

$$\text{mg Mn/L} = \frac{\text{μg Mn (in 100 mL final volume)}}{\text{mL sample}}$$

6. Precision and Bias

A synthetic sample containing 120 μg Mn/L, 500 μg Al/L, 50 μg Cd/L, 110 μg Cr/L, 470 μg Cu/L, 300 μg Fe/L, 70 μg Pb/L, 150 μg Ag/L, and 650 μg Zn/L in distilled water was analyzed in 33 laboratories by the persulfate method, with a relative standard deviation of 26.3% and a relative error of 0%.

A second synthetic sample, similar in all respects except for 50 μg Mn/L and 1000 μg Cu/L, was analyzed in 17 laboratories by the persulfate method, with a relative standard deviation of 50.3% and a relative error of 7.2%.

7. Bibliography

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3500-K POTASSIUM*

3500-K A. Introduction

1. Occurrence and Significance

Potassium (K) is the fourth element in Group IA of the periodic table; it has an atomic number of 19, an atomic weight of 39.10, and a valence of 1. The average abundance of K in the earth's crust is 1.84%; in soils it has a range of 0.1 to 2.6%; in streams it is 2.3 mg/L, and in groundwaters it has a range of 0.5 to 10 mg/L. Potassium is commonly associated with aluminosilicate minerals such as feldspars. ^{40}K is a naturally occurring radioactive isotope with a half-life of 1.3×10^9 years. Potassium compounds are used in glass, fertilizers, baking powder, soft drinks, explosives, electroplating, and pigments. Potassium is an essential element in both plant and human nutrition, and occurs in groundwaters as a result of mineral dissolution, from decomposing plant material, and from agricultural runoff.

The common aqueous species is K^+. Unlike sodium, it does not remain in solution, but is assimilated by plants and is incorporated into a number of clay-mineral structures.

2. Selection of Method

Methods for the determination of potassium include flame atomic absorption (Section 3111B), inductively coupled plasma (Section 3120), flame photometry (3500-K.B), and selective ion electrode (3500-K.C). The inductively coupled plasma/mass spectrometric method (Section 3125) usually may be applied successfully (with lower detection levels), even though potassium is not specifically listed as an analyte in the method. The preferred methods are rapid, sensitive, and accurate; selection depends on instrument availability and analyst choice.

3. Storage of Samples

Do not store samples in soft-glass bottles because of the possibility of contamination from leaching of the glass. Use acid-washed polyethylene or borosilicate glass bottles. Adjust sample to pH < 2 with nitric acid. This will dissolve potassium salts and reduce adsorption on vessel walls.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011.
Joint Task Group: 20th Edition—See Section 3500-Al.

3500-K B. Flame Photometric Method

1. General Discussion

a. Principle: Trace amounts of potassium can be determined in either a direct-reading or internal-standard type of flame photometer at a wavelength of 766.5 nm. Because much of the information pertaining to sodium applies equally to the potassium determination, carefully study the entire discussion dealing with the flame photometric determination of sodium (Section 3500-Na.B) before making a potassium determination.

b. Interference: Interference in the internal-standard method may occur at sodium-to-potassium ratios of 5:1 or greater. Calcium may interfere if the calcium-to-potassium ratio is 10:1 or more. Magnesium begins to interfere when the magnesium-to-potassium ratio exceeds 100:1.

c. Minimum detectable concentration: Potassium levels of approximately 0.1 mg/L can be determined.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

See Section 3500-Na.B.2.

3. Reagents

To minimize potassium pickup, store all solutions in plastic bottles. Shake each container thoroughly to dissolve accumulated salts from walls before pouring.

a. Reagent water: See Section 1080. Use this water for preparing all reagents and calibration standards, and as dilution water.

b. Stock potassium solution: Dissolve 1.907 g KCl dried at 110°C and dilute to 1000 mL with water; 1 mL = 1.00 mg K.

c. Intermediate potassium solution: Dilute 10.0 mL stock potassium solution with water to 100 mL; 1.00 mL = 0.100 mg K.

Use this solution to prepare calibration curve in potassium range of 1 to 10 mg/L.

d. Standard potassium solution: Dilute 10.0 mL intermediate potassium solution with water to 100 mL; 1.00 mL = 0.010 mg K. Use this solution to prepare calibration curve in potassium range of 0.1 to 1.0 mg/L.

4. Procedure

Make determination as described in Section 3500-Na.B.4, but measure emission intensity at 766.5 nm.

5. Calculation

See Section 3500-Na.B.5.

6. Precision and Bias

A synthetic sample containing 3.1 mg K^+/L, 108 mg Ca^{2+}/L, 82 mg Mg^{2+}/L, 19.9 mg Na^+/L, 241 mg Cl^-/L, 0.25 mg NO_2^--N/L, 1.1 mg NO_3^--N/L, 259 mg SO_4^{2-}/L, and 42.5 mg total alkalinity/L (contributed by $NaHCO_3$) was analyzed in 33 laboratories by the flame photometric method, with a relative standard deviation of 15.5% and a relative error of 2.3%.

7. Bibliography

MEHLICH, A. & R.J. MONROE. 1952. Report on potassium analyses by means of flame photometer methods. *J. Assoc. Offic. Agr. Chem.* 35:588.
Also see Section 3500-Na.B.7.

3500-K C. Potassium-Selective Electrode Method

1. General Discussion

a. Principle: Potassium ion is measured potentiometrically by using a potassium ion-selective electrode and a double-junction, sleeve-type reference electrode. The analysis is performed with either a pH meter having an expanded millivolt scale capable of being read to the nearest 0.1 mV or a specific ion meter having a direct concentration scale for potassium.

Before measurement, an ionic strength adjustor reagent is added to both standards and samples to maintain a constant ionic strength. The electrode response is measured in standard solutions with potassium concentrations spanning the range of interest using a calibration line derived either by the instrument meter or manually. The electrode response in sample solutions is measured following the same procedure and potassium concentration determined from the calibration line or instrument direct readout.

b. Interferences: Although most sensitive to potassium, the potassium electrode will respond to other cations at high concentrations; this can result in a positive bias. Table 3500-K:I lists the concentration of common cations causing a 10% error at various concentrations of potassium chloride with a background ionic strength of 0.12*N* sodium chloride. Of the cations listed, ammonium ion is most often present in samples at concentrations high enough to result in a significant bias. It can be converted to gaseous ammonia by adjusting to pH >10.

An electrode exposed to interfering cations tends to drift and respond sluggishly. To restore normal performance soak electrode for 1 h in distilled water and then for several hours in a standard potassium solution.

c. Detection limits: Samples containing from 0.1 to 1000 mg K^+/L may be analyzed. To measure higher concentrations dilute the sample.

TABLE 3500-K:I. CONCENTRATION OF CATIONS INTERFERING AT VARIOUS CONCENTRATIONS OF POTASSIUM

	Concentration Causing 10% Error *mg/L*		
Cation	K conc = 1 mg/L	K conc = 10 mg/L	K conc = 100 mg/L
CS^+	1.0	10	100
NH_4^+	2.7	27	270
$T1^+$	31.4	314	3 140
Ag^+	2 765	27 650	276 500
$Tris^+$	3 105	31 050	310 500
Li^+	356	3 560	35 600
Na^+	1 179	11 790	117 900
H^+	3.6*	2.6*	1.6*

* pH.

2. Apparatus

a. Expanded-scale or digital pH meter or ion-selective meter.

b. Potassium ion-selective electrode.

c. Sleeve-type double-junction reference electrode: Fill outer sleeve with reference electrode filling solution (see 3500-K.C.3*b*). Fill inner sleeve with inner filling solution provided with the electrode.

d. pH electrode.

e. Mixer, magnetic, with a TFE-coated stirring bar.

3. Reagents

a. Ionic strength adjustor (ISA): Dissolve 29.22 g NaCl in reagent water and dilute to 100 mL.

b. Reference electrode outer sleeve filling solution: Dilute 2 mL ISA solution to 100 mL with reagent water.

c. Stock potassium solution: See 3500-K.B.3*b*.

d. Sodium hydroxide (NaOH), 6*N*.

e. Reagent water: See Section 1080.

4. Procedure

a. Preparation of standards: Prepare a series of standards containing 100.0, 10.0, 1.0, and 0.1 mg K^+/L by making serial dilutions of the stock potassium solution as in 3500-K.B.3*c* and 3*d*.

b. Instrument calibration: Fill reference electrode according to the manufacturer's instructions using reference electrode filling solution. Transfer 100 mL 0.1 mg K^+/L standard into a 150-mL beaker and add 2 mL ISA. Raise pH to about 11. Stir gently with magnetic mixer. Immerse electrodes, wait approximately 2 min for potential stabilization and record meter reading. Thoroughly rinse electrodes and blot dry. Repeat for each standard solution in order of increasing concentration. Prepare calibration curve on semilogarithmic graph paper by plotting observed potential in millivolts (linear scale) against concentration (log scale). Alternatively, calculate calibration line by regression analysis.

c. Analysis of samples: Transfer 100 mL sample into a 150-mL beaker and follow procedure applied to standards in ¶ *b* above. From the measured response, calculate K^+ concentration from calibration curve.

5. Precision

Reproducibility of potential measured, over the method's range, can be expected to be ± 0.4 mV, corresponding to about ± 2.5% in concentration.

6. Quality Assurance

The slope of the calibration line should be −56 mV/10-fold concentration change. If the slope is outside the range of −56 ±3 mV, the electrode may require maintenance (replace filling solutions). If the proper electrode response cannot be obtained, replace electrode.

The quality control (QC) practices considered to be an integral part of each method can be found in Section 3020.

Analyze an independent check standard with a mid-range potassium concentration throughout analysis of a series, initially, every ten samples, and after final sample. If the value has changed by more than 5%, recalibrate electrode. Analyze a reagent blank at the same frequency. Readings must represent a lower concentration than the lowest concentration standard (0.1 mg/L).

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3500-Se SELENIUM*

3500-Se A. Introduction

1. Occurrence and Significance

Selenium (Se) is the third element in Group VIA in the periodic table; it has an atomic number of 34, an atomic weight of 78.96, and valences of 2, 4, or 6. The average abundance of Se in the earth's crust is 0.2 ppm; in soils it is 0.27 to 0.74 ppm; in streams it is 0.2 μg/L, and in groundwaters it is <0.1 mg/L. Selenium is used in electronics, ceramics, and shampoos.

The inorganic fraction of dissolved selenium consists predominantly of selenium as the selenate ion (SeO_4^{2-}), designated here as Se(VI), and selenium as the selenite ion (SeO_3^{2-}), Se(IV). Other common aqueous species include Se^{2-}, HSe^-, and Se^0. Selenium is considered a nonessential trace element for most plants, but is an essential trace nutrient for most animals, and selenium deficiency diseases are well known in veterinary medicine. Above trace levels, ingested selenium is toxic to animals and may be toxic to humans. While the selenium concentration of most natural waters is low, the pore water in seleniferous soils in semiarid areas may contain up to hundreds or thousands of micrograms dissolved selenium per liter. Certain plants that grow in such areas accumulate large concentrations of selenium and may poison livestock that graze on them. Water drained from such soil may cause severe environmental pollution and wildlife toxicity. Selenopolysulfide ions (SSe^{2-}) may occur in the presence of hydrogen sulfide in waterlogged, anoxic soils. Selenium derived from microbial degradation of seleniferous organic matter includes selenite, selenate, and the volatile organic compounds dimethylselenide and dimethyldiselenide. Nonvolatile organic selenium compounds may be released to water by microbial processes. Soluble selenium may be leached from coal ash and fly ash at electric power plants that burn seleniferous coal.

The United Nations Food and Agriculture Organization recommended maximum level for selenium in irrigation waters is 20 μg/L. The U.S. EPA primary drinking water standard MCL is 50 μg/L.

2. Selection of Method

The selenium methods using hydride generation atomic absorption (Sections 3114B and C), electrothermal atomic absorption (Section 3113B), and derivatization colorimetry (3500-Se.C) are the most sensitive currently available. For determination of selenium at higher concentrations, the inductively coupled plasma methods (Sections 3120 and 3125) may be used.

By using suitable preparatory steps to convert other chemical species to Se(IV), it is possible to distinguish the chemical species in the sample. In drinking water and most surface and ground waters, Se(IV), Se(VI), and particulate selenium frequently are the only significant species. However, when speciation is important, for example, when a new matrix is being analyzed, the general analytical scheme shown in Figure 3500-Se:1 may be carried out as follows: Determine volatile selenium by stripping sample with nitrogen or air and collecting selenium in alkaline hydrogen peroxide (see 3500-Se.D). To obtain an estimate of selenium in suspended particles, determine total selenium, filter sample, and make a second determination of total selenium. In any case, filter sample. Occasionally, a filtered sample may have the odor of hydrogen sulfide and a yellow color; such a sample may contain selenopolysulfides, which may be estimated by comparing results of total selenium analyses before and after acidification, stripping with nitrogen, settling for 10 min, and refiltration. Determine selenite, Se(IV), by analyzing filtered water sample directly by Methods 3114B or C, or by 3500-Se.C or E. In principle, sample digestion with HCl will convert Se(VI) to Se(IV), and the value determined will equal the sum of the two species. In practice, samples frequently contain an unknown masking agent that produces an unduly low result. Test for this effect by analyzing samples with known additions of both species. If recovery is good, the HCl digestion followed by analyses will yield reliable results. If recovery is poor and organic selenium is to be determined subsequently, attempt to remove the interference by sample pretreatment with

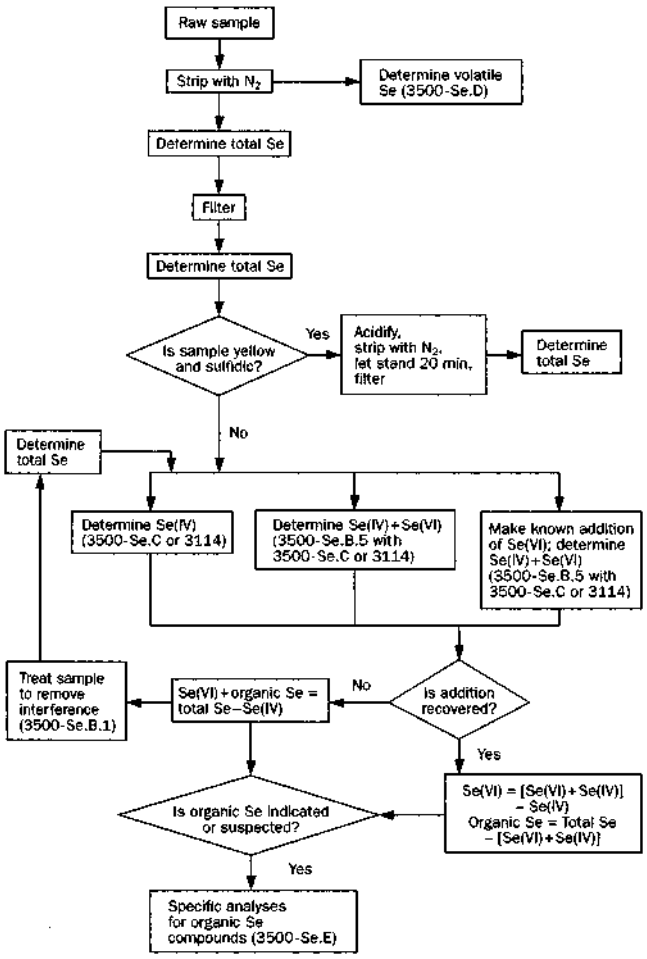


Figure 3500-Se:1. General scheme for speciation of selenium in water.

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resin (3500-Se.B.1). Interference also can be eliminated by digestion with an oxidizing agent (3500-Se.B.2, 3, and 4), but these procedures prevent distinguishing of Se(VI) and organic selenium and also oxidize many organic selenium compounds. To measure nonvolatile organic selenium compounds, use 3500-Se.E.

The choice of digestion method for oxidizing interferences and organic selenium depends on sample matrix. The methods described in 3500-Se.B.2, 3, and 4, in order of increasing complexity and digestive ability, use ammonium (or potassium) persulfate, hydrogen peroxide, and potassium permanganate. Ammonium persulfate digestion is adequate for most filtered ground, drinking, and surface water. Hydrogen peroxide digestion may be required if organic selenium compounds are present, and potassium permanganate digestion may be needed with unfiltered samples or those containing refractory organic selenium compounds. Confirm results obtained with one digestion method by using a more rigorous method when characterizing a new matrix.

3. Interferences

Interferences are found in certain reagents, as well as in samples. Recognition of the presence of an interferant is critical, especially when unknown sample matrices are being analyzed. Routinely add Se(IV) and Se(VI) to test for interference. If present, characterize the interference and correct by the method of standard additions. A slope less than one indicates interference. In cases of mild interference (recoveries reduced by 25% or less), the standard additions method will largely correct determined values.

Because the hydride atomic absorption method is extremely sensitive, samples frequently need to be diluted to bring them within the linear range of the instrument. Diluting a filtered water sample frequently will eliminate sample-related interferences. Include full reagent blanks in each run to ensure absence of contamination from reagents. Hydride generator atomic absorption is susceptible to common interference problems related to nitrite in the sample or free chlorine in the reagent HCl (see Sections 3114B and C).

4. Bibliography

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3500-Se B. Sample Preparation

1. Removal of Organic and Iron Interference by Resin Pretreatment

Interferences are common in selenium analysis, particularly when chemical speciation is attempted. Routine pretreatment of water samples as described here is not necessary. The methods in this section should be tried when poor recovery of standard additions indicates a problem.

Many waters contain iron and/or dissolved organic matter (humic acid) in quantities sufficient to interfere. Reduction of Se(VI) to Se(IV) usually is nonquantitative. When Se(VI) standard additions show poor recovery, treat the sample before analysis. To remove dissolved organic compounds, pass an acidified sample through a resin. Because dissolved organic selenium compounds also may be removed by this treatment, also determine total selenium in the untreated water sample (see 3500-Se.B.2, 3, or 4). To remove iron use a strong base ion exchange resin.* Iron is removed as the anionic chloro complex. In this treatment the acidity and ion exchanger do not alter speciation; complete speciation of selenium is possible.

a. Apparatus:

1) *Chromatography column* for organics removal, glass, about 0.8 cm ID × 30 cm long, with fluorocarbon metering valve.

2) *Chromatography column* for ion exchange, disposable polyethylene.†

3) *pH meter.*

b. Reagents:

1) *Organics-removal resin*—Thoroughly rinse 16 to 50 mesh resin‡ with deionized water and remove resin fines by decanting.

* Bio-Rad AG1-X8, or equivalent.
† Bio-Rad Econo-Columns, or equivalent.
‡ Amberlite XAD-8, Supelco, or equivalent.

Rinse three times with pH 12 solution. Store resin in pH 12 solution and refrigerate to prevent bacterial growth.

2) *Anion exchange resin*—Add 100 to 200 mesh anion exchange resin* to a beaker and thoroughly rinse with deionized water. Cover resin with 4*N* HCl, stir, and let settle. Decant and repeat acid rinse twice more. Store resin in 4*N* HCl.

3) *Hydrochloric acid,* conc—Before use, bubble helium through the acid for 3 h at rate of 100 mL/min. (**CAUTION: Use a fume hood.**)

4) *pH 1.6 solution*—Adjust pH of deionized water to 1.6 with HCl.

5) *pH 12 solution*—Adjust pH of deionized water to 12 with KOH.

c. Procedure:

1) Organic removal—Place 5 cm washed resin in a 0.8-cm-ID column. Precondition column, at 1 mL/min, with 30 mL pH 12 solution and 20 mL pH 1.6 solution. Using HCl and a pH meter adjust sample to pH 1.6 to 1.8. Pass sample through preconditioned column at rate of 1 mL/min. Discard first 10 mL and use next 11 to 50 mL collected for Se(IV) determinations by Methods 3114B or C, or 3500-Se.C preceded, if Se(VI) also will be determined, by 3500-Se.B.5. If more than 50 mL sample are needed, use another column or use a column with twice as much resin.

2) Iron removal—Place 4 cm prepared resin in a small chromatographic column (add resin to column filled with 4*N* HCl to avoid air bubbles). Rinse column with 10 mL 4*N* HCl at flow rate <6 mL/min. Let solution drain to top of resin, but do not let the column run dry. Adjust sample to 4*N* HCl and pour into column. Discard first 10 mL and collect the next 11 to 100 mL for Se(IV) analysis by Methods 3114B or C, or 3500-Se.C preceded, if necessary, by 3500-Se.B.2 and if Se(VI) also is to be determined, by 3500-Se.B.5 below.

2. Removal of Interference by Persulfate Digestion

The combination of this procedure with 3500-Se.B.5 and Methods 3114B or C, or 3500-Se.C is, in most cases, the preferred method for determining total selenium in filtered water. A small amount of ammonium or potassium persulfate is added to the mixture of sample and HCl to remove interference from reducing agents and to oxidize relatively labile organic selenium compounds such as selenoamino acids and methaneseleninic acid.

If the sample contains hydrogen sulfide or a large concentration of organic matter or is otherwise suspect or to confirm method accuracy, reanalyze sample using digestion procedure 3 or 4 below.

Prepare 2% potassium or ammonium persulfate solution by dissolving 2.0 g in 100 mL deionized water (prepare weekly). Add 0.2 mL persulfate solution to the mixed sample and HCl of 3500-Se.B.5*c* before heating and proceeding with pretreatment and analysis.

After completing analysis multiply concentration of selenium determined in the acidified sample by 2.04 to obtain total selenium in original sample.

3. Removal of Interference by Alkaline Hydrogen Peroxide Digestion

Occasionally, digestion with persulfate gives incomplete recovery of total selenium. In this case, digestion with hydrogen peroxide is used to remove all reducing agents that might interfere and to fully oxidize organic selenium to Se(VI). The resulting solution can be analyzed for total selenium after pretreatment according to 3500-Se.B.5 below.

This method is suitable for determining total selenium in unfiltered water samples, where particulate selenium is present. When working with a new matrix, confirm results obtained by reanalyzing the sample using 3500-Se.B.4.

a. Apparatus:

1) *Beakers*, 150-mL.
2) *Watch glasses*.
3) *Hot plate*.
4) *Pipettor,* 1-mL, and tips.
5) *Graduated cylinder,* 25-mL.

b. Reagents:

1) *Hydrogen peroxide* (H_2O_2), 30%—Keep refrigerated.
2) *Sodium hydroxide* (NaOH), 1*N*.
3) *Hydrochloric acid* (HCl), 1.5*N*—Dilute 125 mL conc HCl to 1 L with deionized water.

c. Procedure: Add 2 mL 30% H_2O_2 and 1 mL 1 *N* NaOH to 25 mL sample in a beaker. Cover beaker to control spattering and simmer on hot plate until fine bubbles characteristic of H_2O_2 decomposition subside and are replaced by ordinary boiling. Add 1 mL 1.5*N* HCl to redissolve any precipitate that may have formed, let cool, and pour into graduated cylinder. Rinse beaker with deionized water into graduated cylinder and make volume up to 25 mL. Proceed to 3500-Se.B.5 and chosen analytical method.

4. Removal of Interference by Permanganate Digestion

This digestion method utilizes potassium permanganate to oxidize selenium and remove interfering organic compounds. Excess $KMnO_4$ and MnO_2 are removed by reaction with hydroxylamine. HCl digestion is included here, because it is conveniently performed in the same reaction vial. Selenite may then be determined directly by Methods 3114B or C, or 3500-Se.C.

Verify recovery for the given matrix. This method gives good recovery even with heavily contaminated water samples that contain organic selenium compounds, dissolved organic matter, and visible suspended material.

Permanganate may oxidize chloride ion to free chlorine. Part of the chlorine (which interferes with hydride analysis) is removed by reaction with hydroxylamine, but the best way to eliminate free chlorine is by prolonged heating in an open vial during the digestion step. Excess hydroxylamine may reduce recovery by reducing selenium to Se(0).

a. Apparatus:

1) *Oven with thermostat*, for continuous operation at 110 ± 5°C.
2) *Digestion vials*, 40-mL, with fluorocarbon-lined screw caps.
3) *Metal support rack* to hold 40 digestion vials.

b. Reagents:

1) *Hydrochloric acid* (HCl), conc. [See 3500-Se.B.1*b*3)].
2) *Hydrochloric acid* (HCl), 10*N*—Dilute 1000 mL conc HCl to 1200 mL with deionized water.
3) *Sulfuric acid* (H_2SO_4), conc and 25%—NOTE: Many brands of H_2SO_4 are contaminated with selenium. Run reagent blanks when starting a new bottle. Make 25% v/v solution by adding 250 mL conc H_2SO_4 to 500 mL deionized water, and diluting to 1 L.
4) *Potassium permanganate solution* ($KMnO_4$), 5% (w/v)—Dissolve 50 g $KMnO_4$ in 1000 mL deionized water.

5) *Hydroxylamine hydrochloride solution*—Dissolve 100 g $NH_2OH \cdot HCl$ in 1000 mL deionized water.

c. Procedure: Pipet 5 mL sample into digestion vial, add 5 mL 25% H_2SO_4 and 1 mL $KMnO_4$ solution. Lightly screw cap on and place in preheated oven at 110°C for 1 h. **CAUTION: Excessive pressure may build up in tightly capped vials.** Carefully remove tray with vials from the oven (**CAUTION: potential for acid gas vapor release**) and cool to room temperature. Open vial, carefully add a few drops hydroxylamine hydrochloride solution, mix, and wait until sample is decolorized and residual manganese dioxide is dissolved. Avoid excess hydroxylamine solution, which can cause a low reading. Add 10 mL conc HCl to the sample and heat vial 60 min at 95°C without cap. Let cool to room temperature. Transfer sample to a 25-mL volumetric flask or graduated cylinder, rinse vial into flask, dilute to mark, and mix well. Proceed to analyze by Methods 3114B or C, or 3500-Se.C. If Method 3114 B or C is used, multiply spectrometer readings by the dilution factor as follows:

$$\text{Concentration, } \mu\text{g/L} = \frac{\text{final volume}}{\text{volume of sample}} \times \text{reading}$$

5. Reduction of Se(VI) to Se(IV) by Hydrochloric Acid Digestion

Se(VI) is reduced to Se(IV) by digestion with HCl. Determine Se(IV) + Se(VI) by either hydride generation atomic absorption spectrometer (Method 3114B or C) or as an organic derivative (3500-Se.C).

Test any given sample matrix to ensure recovery of added Se(VI). If recovery is poor, try to remove interference by the procedure of 3500-Se.B.1, or if at least 75% recovery is achieved, use the method of standard additions. The method described here is of limited utility for direct analysis of water samples, but is useful as a step in determining total selenium in a sample where selenium has been oxidized to Se(VI).

NOTE: Available literature does not provide definitive criteria for collection and preservation of samples for which speciation is desired. If speciation is needed, fully evaluate collection and preservation as part of the data quality objectives.

a. Apparatus:

1) *Dispenser,* bottle type, 5-mL, suitable for dispensing concentrated HCl.

2) *Pipettors,* 0.2- and 5-mL.

3) *Screw-cap culture tubes,* borosilicate glass, 25- × 150-mm.

4) *Boiling water bath,* suitable for heating culture tubes; a 1-L beaker on a hot plate is suitable.

b. Reagents:

1) *Sodium selenate additions solution*—Dilute 1000 mg/L stock selenate solution with deionized water to prepare a solution of 1 to 10 mg/L, such that the concentration of the additions solution will be approximately 50 times greater than anticipated total selenium in the sample to be analyzed.

2) *Hydrochloric acid* (HCl), conc—See 3500-Se.B.1*b*3).

c. Procedure: Calibrate acid dispenser using water. Preheat water bath. Pipet 5 mL filtered sample into a culture tube. Add 5 mL conc HCl. Loosely cap tube (do not tighten) and place in boiling water bath for 20 min. Let tube cool and tighten cap. Determine total Se(IV) by Methods 3114B or C, or 3500-Se.C.

Add 0.200 mL additions solution with a microliter pipet to sample and proceed as above. Analyze a deionized water blank and a blank with the addition to ensure absence of contamination and to determine the true value of the addition.

Multiply the concentration of selenium determined in the acidified sample by 2.00 to obtain total concentration of Se(IV) + Se(VI). Multiply reading obtained for sample with addition by 2.04.

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3500-Se C. Colorimetric Method

1. General Discussion

a. Principle: This method is specific to determining selenite ion in aqueous solution. Selenite ion reacts with 2,3-diaminonaphthalene to produce a brightly colored and strongly fluorescent piazselenol compound, which is extracted in cyclohexane and measured colorimetrically.

The optimum pH for formation of the piazselenol complex is approximately 1.5 but should not be above 2.5 because above pH 2, the rate of formation of the colored compound is critically dependent on pH. When indicators are used to adjust pH, results frequently are erratic; results can be improved when pH is monitored electrochemically.

b. Interference: No inorganic compounds are known to give a positive interference. Colored organic compounds extractable by cyclohexane may be encountered, but usually they are absent or can be removed by oxidizing the sample (see 3500-Se.B.2, 3, or 4) or by treating it to remove dissolved organics (see 3500-Se.B.1). Negative interference results from compounds that reduce the concentration of diaminonaphthalene by oxidizing it. Addition of EDTA eliminates negative interference from at least 2.5 mg Fe^{2+}.

c. Minimum detectable quantity: 10 μg Se/L.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Colorimetric equipment: A spectrophotometer, for use at 480 nm, providing a light path of 1 cm or longer.

b. Separatory funnel, 250-mL, preferably with a fluorocarbon stopcock.

c. Thermostatically controlled water bath (50°C) with cover.

d. pH meter.

e. Centrifuge, with rotor for 50-mL tubes (optional).

f. Centrifuge bottles, 60-mL, screw-capped, fluorocarbon.

g. Shaker, suitable for separatory funnel (optional).

3. Reagents

Use reagent water (see Section 1080) in preparing reagents.

a. Selenium standard reference solution: Dissolve 2.190 g sodium selenite, Na_2SeO_3, in water containing 10 mL HCl and dilute to 1 L. 1.00 mL = 1.00 mg Se(IV).

b. Working standard selenium solutions: Dilute selenium reference standard solution with water or suitable background solution to produce a series of working standards spanning the concentration range of interest.

c. Hydrochloric acid (HCl), conc and 0.1*N*.

d. Ammonium hydroxide (NH_4OH), 50% v/v.

e. Cyclohexane (C_6H_{12}).

f. 2,3-Diaminonaphthalene (DAN) solution: Dissolve 200 mg DAN in 200 mL 0.1*N* HCl. Shake 5 min. Extract three times with 25-mL portions of cyclohexane, retaining aqueous phase and discarding organic portions. Filter into opaque container* and store in cool, dark place for no longer than 8 h. **CAUTION: Toxic, handle with extreme care**.

g. Hydroxylamine-EDTA solution (HA-EDTA): Dissolve 4.5 g Na_2EDTA in approximately 450 mL water. Add 12.5 g hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) and adjust volume to 500 mL.

4. Procedure

a. Formation of piazselenol: Add 2 mL HA-EDTA solution to 10 mL sample in 60-mL centrifuge bottle (filtered if Se(IV) is to be determined; oxidized using 3500-Se.B.2, 3, or 4, then reduced using 3500-Se.B.5 for total Se). Adjust to pH 1.5 ± 0.3 with 0.1*N* HCl and 50% NH_4OH, using a pH meter. Add 5 mL DAN solution and heat in a covered water bath at 50°C for 30 min.

b. Extraction of piazselenol: Cool and add 2.0 mL cyclohexane. Cap container securely and shake vigorously for 5 min. Let solution stand for 5 min or until cyclohexane layer becomes well separated. If separation is slow, centrifuge for 5 min at 2000 rpm. Place bottle in a clamp on a ringstand at a 45° angle to the vertical. Remove aqueous phase using a disposable pipet attached to a vacuum line. Transfer organic phase to a small capped container using a clean disposable pipet, or to the spectrophotometer cuvette if absorbance is to be read immediately.

c. Determination of absorbance: Read absorbance at 480 nm using a zero standard. The piazselenol color is very stable but evaporation of the cyclohexane concentrates the color unless the container is capped. **CAUTION: Avoid inhaling cyclohexane vapors**. Beer's Law is obeyed up to 2 mg/L.

* Use Whatman No. 42 filter paper, or equivalent.

5. Calculation

Construct calibration curve using at least a three-point standard curve to bracket the expected sample concentration. Plot absorbance vs. concentration. Correct for digestion blank and any reagent blank.

6. Precision and Bias

Three standard reference materials (wheat flour, water, and a commercial standard) were used to evaluate Se recovery.[1] The wheat flour sample was digested using HNO_3 and $HClO_4$ to convert total selenium to Se(VI), digested with HCl to convert Se(VI) to Se(IV) and finally, the colorimetric method was used. Results were as follows:

	Selenium Concentration *μg Se/L*	
Standard	Expected	Recovered*
NBS, SRM 1567, wheat flour†	1097 ± 197	1113 ± 8
NBS, SRM 1543ib, water	9.7 ± 0.5	8.7 ± 0
Fisher Certified AAS Standard	1002 ± 8	1002 ± 0

* Analyses in triplicate.
† Dry weight basis.

7. Reference

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3500-Se D. Determination of Volatile Selenium

1. General Discussion

Dimethylselenide and dimethyldiselenide are low boiling, extremely malodorous organic compounds sparingly soluble in water. They are produced by microbial processes in seleniferous soil and decaying seleniferous organic matter, and occasionally are present in natural waters. They are readily air stripped from a water sample and can be collected with high efficiency in an alkaline solution of hydrogen peroxide, which oxidizes them quantitatively to Se(VI). Total selenium is determined by digestion with HCl and analysis by the hydride atomic absorption (Section 3114B or C) or colorimetric (3500-Se.C) methods.

Either nitrogen or air may be used to strip the sample. Preferably use nitrogen if air-sensitive compounds (e.g., selenopolysulfides) are suspected.

Because volatile selenium can be lost in the course of sample collection and handling, preferably air-strip the sample in the field immediately after it is collected. After boiling to decompose H_2O_2, return the alkaline peroxide solution to the laboratory for analysis.

The quality control (QC) practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

All apparatus required for selenate reduction (3500-Se.B.5) and Methods 3114B or C, or 3500-Se.C, plus:

a. Gas washing bottles, borosilicate glass, 250-mL, with coarse porous glass gas dispersion frit. Mark 100-mL level on side of bottle.

b. Rotameter, to measure 3 L/min air flow.

c. Gas flow regulator.

d. Hot plate.

e. Graduated cylinder, 100-mL.

f. Beakers, 250-mL.

g. Rubber tubing, to interconnect gas washing bottles and other gas equipment.

h. Rubber gloves.

3. Reagents

All reagents required for selenate reduction (3500-Se.B.5) and Methods 3114B or C, or 3500-Se.C, plus:

a. Hydrogen peroxide (H_2O_2), 30%. Refrigerate.

b. Sodium hydroxide solution (NaOH), 1*N*.

c. Compressed air or nitrogen.

4. Procedure

Set up air-flow train in this order: Regulated air supply → rotameter → gas washing bottle 1 → gas washing bottle 2.

Prepare alkaline peroxide solution immediately before use by pouring 20 mL 30% H_2O_2 into a 100-mL graduated cylinder, adding 50 to 60 mL deionized water and 5 mL 1*N* NaOH, and making up to 100 mL. **CAUTION: Alkaline H_2O_2 is unstable. Do not keep in glass bottle; hold at about 0 °C in oversized plastic bottle. Solution is corrosive; protect eyes and skin.** Pour into gas washing bottle 2. Pour approximately 100 mL freshly collected sample into gas washing bottle 1. Do not attempt to measure sample volume accurately before volatile selenium determination, as unnecessary handling may cause volatile selenium to be lost.

Connect and check all air lines, turn on air and adjust flow to 3 L/min. Strip for 30 min or more. After 30 min, turn off air,disconnect gas washing bottle 2, and place it on the hot plate. Adjust heat to produce a gentle simmering of oxygenbubbles from decomposition of H_2O_2. Continue heating until the characteristic effervescence of oxygen subsides and is replaced by ordinary boiling. Remove from hot plate and let cool. Pour solution into beaker. (Volume will be very near to 100 mL, and correction will usually be unnecessary.) Analyze for total selenium using HCl digestion (3500-Se.B.5) and Methods 3114B or C, or 3500-Se.C. Once boiled, this solution may be safely stored and transported in plastic bottles. Measure volume of sample in gas washing bottle 1.

5. Calculation

The concentration of volatile selenium compounds in the original water sample can be calculated as:

$$C = \frac{100}{\text{volume of original sample}} \times \text{conc of Se in solution}$$

6. Precision and Bias

Approximately 90% of dimethylselenide in samples will be recovered with 30 min air stripping. The recovery of dimethyldiselenide is not known. Loss of gases to the atmosphere during sampling and handling that precede analysis may cause a significant negative error.

3500-Se E. Determination of Nonvolatile Organic Selenium Compounds

1. General Discussion

In principle, the total amount of dissolved organic selenium plus polysulfidic selenium may be estimated by comparing "total Se," determined by oxidation and HCl digestion (3500-Se.B.2 or 3 and B.5, or B.4), followed by Methods 3114B or C, or 3500-Se.C, with Se(IV) + Se(VI) determined by HCl digestion (3500-Se.B.5) and Method 3114C or 3500-Se.C. In practice, this will give a meaningful estimate only if a known addition of Se(VI) is fully recovered. Even if recovery is good, this estimate

may be unreliable, because it is the difference of two (frequently larger) numbers determined by slightly different methods. Comparing total Se before and after treatment with resin [3500-Se.B.1*c*1)] gives a similarly unreliable estimate of nonvolatile organic Se.

It is preferable to separate and directly determine nonvolatile organic selenium. One method involves adsorption of dissolved organic matter onto a C-18 reverse phase HPLC resin, elution with an organic solvent, and determination of selenium in this fraction. While this technique is relatively simple, it is affected by pH and small organic molecules (e.g., individual selenium containing amino acids) are not retained by the resins. Adjust sample pH to 1.5 to 2.0 before using the column, but because the latter problem cannot be solved easily, the use of organic adsorbents provides only an estimate of organic selenium concentration.

Alternatively, isolate specific compounds and determine their selenium content. In some natural waters selenium may be associated with dissolved polypeptides or small proteins, and even small amounts of free selenoamino acids may be present. Because selenoamino acids are the most toxic form of the element, a direct determination is sometimes desirable.

To determine selenium in dissolved peptides, hydrolyze with acid and isolate the free amino acids via ligand exchange chromatography. Elute the selenoamino acids from the column and determine selenium. Selenoamino acids are unstable during acid hydrolysis and even using nonoxidizing methyl sulfonic acid and nitrogen-purged glass ampules, selenoamino acid recoveries are only 50 to 80%. This method is good only for estimating protein-bound selenium. A somewhat more reliable estimate of free selenoamino acids and selenium associated with small oligopeptides is obtained by performing a similar procedure without the hydrolysis step.

While these methods are too intricate for routine use, semiquantitative, and sensitive only to certain classes of organic compounds, at present they are the only ones available with any practical experience.

Imperfect separation of organic selenium compounds from inorganic forms of selenium may cause interference. In parallel with the actual determination, always perform the procedure using a solution compounded to resemble the actual matrix and containing a similar amount of selenium, but in the form of Se(IV) and Se(VI) to determine degree of interference.

The quality control (QC) practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Rotary evaporator, with temperature-control bath and 30-mL pear-shaped flasks.

b. Glass ampule sealing apparatus, or oxygen-gas torch.

c. Heating block, 100°C, or pressure cooker.

d. Glass chromatography columns, 15 cm long, 0.7 cm ID.*

e. Glass syringe, 50-mL.

f. Glass ampules, 10- or 20-mL. Clean by heating in a muffle furnace at 400°C for 24 h.

g. pH meter.

* Bio-Rad glass Econo-Columns, or equivalent.

3. Reagents

a. Hydrochloric acid (HCl), 1*N*.

b. Methyl sulfonic acid, conc.

c. Ammonium hydroxide (NH_4OH), 1.5*N:* Dilute 100 mL conc NH_4OH to 1 L with deionized water.

d. Sodium hydroxide (NaOH), pellets and 1*N* solution.

e. pH 1.6 solution: Adjust pH of deionized water to 1.6 using HCl.

f. pH 9.0 solution: Adjust pH of deionized water to 9.0 using NaOH.

g. Copper sulfate solution ($CuSO_4$), 1*M:* Dissolve 25 g $CuSO_4 \cdot 5H_2O$ in deionized water and dilute to 100 mL.

h. Methanol, purified.

i. C-18 cartridges:† Using a glass syringe, pass the following sequence of reagents through the cartridge to clean the resin (6 mL/min or less): 10 mL deionized water; 20 mL 1*N* HCl; 10 mL deionized water; 20 mL methanol; 10 mL deionized water; and 20 mL pH 1.6 solution. Refrigerate but do not freeze cleaned cartridges.

j. Ligand-exchange chromatographic resin, 100/200 mesh: Rinse resin‡ with deionized water to remove fines. Then rinse resin three times in the following sequence: 1*N* HCl; 1.5*N* NH_4OH; and deionized water. Store resin wet.

k. Copper-treated ligand-exchange chromatographic resin, 100/200 mesh: Rinse resin‡ with deionized water to remove fines. Then rinse three times with 1*N* HCl, followed by deionized water. Using NaOH adjust pH of supernatant above the resin to about 7. Add $CuSO_4$ solution to the resin and stir. After settling, decant supernatant and add more $CuSO_4$ solution. Decant $CuSO_4$ solution and rinse with deionized water until no copper is noticeable in the supernatant. Rinse resin three times with 1.5*N* NH_4OH and three times with deionized water. Store resin wet.

4. Procedures

a. Extractable organic selenium: Adjust sample (5 to 50 mL) to pH 1.5 to 2.0 using HCl and place in a clean glass syringe with an attached cleaned C-18 cartridge. Push sample through the cartridge at a rate of 6 mL/min. After removing the cartridge, draw 2 mL pH 1.6 solution into syringe as a rinse, reattach cartridge, and push the rinse through the cartridge. Repeat two additional times. The cartridge can be refrigerated for storage. To elute organic selenium, push 10 mL methanol through the cartridge at rate of 2 mL/min and collect eluate in a 30-mL pear-shaped flask. Remove methanol by rotary evaporation, with the water bath temperature less than 40°C. Use deionized water to solubilize and transfer the residue into the vessel used for total selenium digestions. Determine total dissolved selenium by digestion with persulfate (3500-Se.B.2) or peroxide (3500-Se.B.3), reduction of Se(VI) (3500-Se.B.5), and analysis by Methods 3114B or C, or 3500-Se.C.

b. Hydrolysis of protein-bound selenium: Place filtered sample in a 10- or 20-mL glass ampule (depending on desired volume), and add conc methyl sulfonic acid to adjust concentration to 4*M*. Purge acidified sample with nitrogen for 10 min and seal top with a torch. Heat sealed vial at 100°C for 24 h in a

† Sep-Pak, Waters Associates, or equivalent.

‡ Chelex 100 (ammonium form) Bio Rad, or equivalent.

heating block or pressure cooker. Transfer cooled hydrolysis solution with deionized water rinses to a 50-mL beaker and place in an ice bath. Using NaOH pellets and 1*N* NaOH, adjust to pH 9.0, taking care not to allow solution to heat to boiling.

c. Determination of selenoamino acids: If sample is not hydrolyzed as in ¶ *b* above, filter, and adjust pH to 9.0 using 1*N* NaOH.

Fill an empty chromatographic column with deionized water and add ammonium-form resin to a depth of 2 cm. Add copper-treated resin to form a 12-cm length of resin. (The ammonium-form resin removes any copper that bleeds from the copper-treated resin above it). Rinse with deionized water until the pH of the effluent is 9.0; maintain flow through the column by gravity. Pass sample through column, rinse sample beaker with 5 mL pH 9 solution, and place the rinse on column (after the last of the sample reaches the top of the resin). Rinse beaker twice more. Discard flow through column.

Place clean beaker under column and add 20 mL 1.5*N* NH_4OH to the column. Neutralize NH_4OH eluate with 2.5 mL conc HCl. Determine total dissolved selenium by digestion with persulfate (3500-Se.B.2) or peroxide (3500-Se.B.3), reduction of Se(VI) (3500-Se.B.5) and analysis by Methods 3114B or C, or 3500-Se.C.

5. Precision and Bias

These procedures are only semiquantitative. Typical relative standard deviation is 12% for the C-18 isolation of dissolved organic selenium, and 15% for protein-bound selenium.

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3500-Na SODIUM*

3500-Na A. Introduction

1. Occurrence and Significance

Sodium (Na) is the third element in Group IA of the periodic table; it has an atomic number of 11, an atomic weight of 22.99, and a valence of 1. The average abundance of Na in the earth's crust is 2.5%; in soils it is 0.02 to 0.62%; in streams it is 6.3 mg/L, and in groundwaters it is generally >5 mg/L. Sodium occurs with silicates and with salt deposits. Sodium compounds are used in many applications, including caustic soda, salt, fertilizers, and water treatment chemicals.

Sodium is very soluble, and its monovalent ion Na^+ can reach concentrations as high as 15 000 mg/L in equilibrium with sodium bicarbonate. The ratio of sodium to total cations is important in agriculture and human physiology. Soil permeability can be harmed by a high sodium ratio. In large concentrations it may affect persons with cardiac difficulties. A limiting concentration of 2 to 3 mg/L is recommended in feedwaters destined for high-pressure boilers. When necessary, sodium can be removed by the hydrogen-exchange process or by distillation. The U.S. EPA advisory limit for sodium in drinking water is 20 mg/L.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011. Joint Task Group: 20th Edition—See Section 3500-Al.

2. Selection of Method

Method 3111B uses an atomic absorption spectrometer in the flame absorption mode. Method 3120B uses inductively coupled plasma; this method is not as sensitive as the other methods, but usually this is not important. Method 3500-Na.B uses either a flame photometer or an atomic absorption spectrometer in the flame emission mode. The inductively coupled plasma/mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection limits), even though sodium is not specifically listed as an analyte in the method. When all of these instruments are available, the choice will depend on factors including relative quality of the instruments, precision and sensitivity required, number of samples and analytes per sample, matrix effects, and relative ease of instrument operation. If an atomic absorption spectrometer is used, operation in the emission mode is preferred.

3. Storage of Sample

Store alkaline samples or samples containing low sodium concentrations in polyethylene bottles to eliminate the possibility of sample contamination due to leaching of the glass container.

3500-Na B. Flame Emission Photometric Method

1. General Discussion

a. Principle: Trace amounts of sodium can be determined by flame emission photometry at 589 nm. Sample is nebulized into a gas flame under carefully controlled, reproducible excitation conditions. The sodium resonant spectral line at 589 nm is isolated by interference filters or by light-dispersing devices such as prisms or gratings. Emission light intensity is measured by a phototube, photomultiplier, or photodiode. The light intensity at 589 nm is approximately proportional to the sodium concentration. Alignment of the wavelength dispersing device and wavelength readout may not be precise. The appropriate wavelength setting, which may be slightly more or less than 589 nm, can be determined from the maximum emission intensity when aspirating a sodium standard solution, and then used for emission measurements. The calibration curve may be linear but has a tendency to level off or even reverse at higher concentrations. Work in the linear to near-linear range.

b. Interferences: Minimize interference by incorporation of one or more of the following:

1) Operate at the lowest practical concentration range.
2) Add releasing agents, such as strontium or lanthanum at 1000 mg/L, to suppress ionization and anion interference. Among common anions capable of causing interference are Cl^-, SO_4^{2-} and HCO_3^- in relatively large amounts.
3) Matrix-match standards and samples by adding identical amounts of interfering substances present in the sample to calibration standards.
4) Apply an experimentally determined correction in those instances where the sample contains a single important interference.
5) Remove interfering ions.
6) Remove burner-clogging particulate matter from the sample by filtration through a filter paper of medium retentiveness.
7) Use the standard addition technique as described in the flame photometric method for strontium (Section 3500-Sr.B). The method involves preparing a calibration curve using the sample matrix as a diluent, and determining the sample concentration either mathematically or graphically.
8) Use the internal standard technique. Potassium and calcium interfere with sodium determination by the internal-standard method if the potassium-to-sodium ratio is ≥5:1 and the calcium-to-sodium ratio is ≥10:1. When these ratios are exceeded, determine calcium and potassium concentrations and matrix-match sodium calibration standards by addition of approximately equivalent concentrations of interfering ions. Interference from magnesium is not significant until the magnesium-to-sodium ratio exceeds 100, a rare occurrence.

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c. Minimum detectable concentration: Better flame photometers or atomic absorption spectrometers operating in the emission mode can be used to determine sodium levels approximating 5 μg/L.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Flame photometer (either direct-reading or internal-standard type) or atomic absorption spectrometer operating in the flame emission mode.

b. Glassware: Rinse all glassware with 1 + 15 HNO_3 followed by several portions of reagent water (3500-Na.B.3*a*).

3. Reagents

To minimize sodium contamination, store all solutions in plastic bottles. Use small containers to reduce the amount of dry element that may be picked up from the bottle walls when the solution is poured. Shake each container vigorously to wash accumulated salts from walls before pouring solution.

a. Reagent water: See Section 1080. Use reagent water to prepare all reagents and calibration standards, and as dilution water.

b. Stock sodium solution: Dissolve 2.542 g NaCl dried at 140°C to constant weight and dilute to 1000 mL with water; 1.00 mL = 1.00 mg Na.

c. Intermediate sodium solution: Dilute 10.00 mL stock sodium solution with water to 100.0 mL; 1.00 mL = 0.10 mg Na (1.00 mL = 100 μg Na). Use this intermediate solution to prepare calibration curve in sodium range of 1 to 10 mg/L.

d. Standard sodium solution: Dilute 10.00 mL intermediate sodium solution with water to 100 mL; 1.00 mL = 10.0 μg Na. Use this solution to prepare calibration curve in sodium range of 0.1 to 1.0 mg/L.

4. Procedure

a. Pretreatment of polluted water and wastewater samples: Follow the procedures described in Section 3030.

b. Instrument operation: Because of differences between makes and models of instruments, it is impossible to formulate detailed operating instructions. Follow manufacturer's recommendation for selecting proper photocell and wavelength, adjusting slit width and sensitivity, appropriate fuel and oxidant gas pressures, and the steps for warm-up, correcting for interferences and flame background, rinsing of burner, igniting flame, and measuring emission intensity.

c. Direct-intensity measurement: Prepare a blank and sodium calibration standards in stepped amounts in any of the following applicable ranges: 0 to 1.0, 0 to 10, or 0 to 100 mg/L. Determine emission intensity at 589 nm. Aspirate calibration standards and samples enough times to secure a reliable average reading for each. Construct a calibration curve from the sodium standards. Determine sodium concentration of sample from the calibration curve. Where a large number of samples must be run routinely, the calibration curve provides sufficient accuracy. If greater precision and less bias are desired and time is available, use the bracketing approach described in ¶ *d* below.

d. Bracketing approach: From the calibration curve, select and prepare sodium standards that immediately bracket the emission intensity of the sample. Determine emission intensities of the bracketing standards (one sodium standard slightly less and the other slightly greater than the sample) and the sample as nearly simultaneously as possible. Repeat the determination on bracketing standards and sample. Calculate the sodium concentration by the equation in 3500-Na.B.5*b* and average the findings.

5. Calculation

a. For direct reference to the calibration curve:

$$\text{mg Na/L} = (\text{mg Na/L in portion}) \times D$$

b. For the bracketing approach:

$$\text{mg Na/L} = \left[\frac{(B - A)\,(s - a)}{(b - a)} + A\right] D$$

where:

B = mg Na/L in upper bracketing standard,
A = mg Na/L in lower bracketing standard,
s = emission intensity of sample,
a = emission intensity of lower bracketing standard,
b = emission intensity of upper bracketing standard, and

$$D = \text{dilution ratio} = \frac{\text{mL sample} + \text{mL water}}{\text{mL sample}}$$

6. Precision and Bias

A synthetic sample containing 19.9 mg Na^+/L, 108 mg Ca^{2+}/L, 82 mg Mg^{2+}/L, 3.1 mg K^+/L, 241 mg Cl^-/L, 0.25 mg NO_2^--N/L, 1.1 mg NO_3^--N/L, 259 mg SO_4^{2-}/L, and 42.5 mg total alkalinity/L (as $CaCO_3$) was analyzed in 35 laboratories by the flame photometric method, with a relative standard deviation of 17.3% and a relative error of 4.0%.

7. Bibliography

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American Society for Testing & Materials. 1988. Method D 1428-82: Standard test methods for sodium and potassium in water and water-formed deposits by flame photometry. *In* Annual Book of ASTM Standards, Vol. 11.01. Philadelphia, Pa.

3500-Sr STRONTIUM*

3500-Sr A. Introduction

1. Occurrence and Significance

Strontium (Sr) is the fourth element in Group IIA of the periodic table; it has an atomic number of 38, an atomic weight of 87.62, and a valence of 2. The average abundance of Sr in the earth's crust is 384 ppm; in soils Sr ranges from 3.6 to 160 ppm; in streams it averages 50 μg/L, and in groundwaters it ranges from 0.01 to 10 mg/L. Strontium is found chiefly in celestite ($SrSO_4$) and in strontianite ($SrCO_3$). Strontium compounds are used in pigments, pyrotechnics, ceramics, and flares. ^{90}Sr is a fission product of nuclear reactor fuels, and was widely distributed on the earth's surface as a result of fallout from nuclear weapons testing.

The common aqueous species is Sr^{2+}. The solubility of strontium is controlled by carbonate and sulfate. Some compounds are toxic by ingestion and inhalation. Although there is no U.S. EPA drinking water standard MCL for concentration of strontium, strontium-90 measurements are required when the gross beta activity of a water sample is greater than 50 pCi/L. The U.S. EPA primary drinking water standard MCL for ^{90}Sr is 8 pCi/L.

A method for determination of ^{90}Sr is found in Section 7500-Sr.

2. Selection of Method

The atomic absorption spectrometric method (Secton 3111B) and inductively coupled plasma methods (Sections 3120 and 3125) are preferred. The flame emission photometric method (3500-Sr.B) also is available for those laboratories that do not have the equipment needed for one of the preferred methods.

3. Sampling and Storage

Polyethylene bottles are preferable for sample storage, although borosilicate glass containers also may be used. At time of collection adjust sample to pH <2 with nitric acid (HNO_3).

* Approved by Standard Methods Committee, 2004. Editorial revisions, 2011. Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Sr B. Flame Emission Photometric Method

1. General Discussion

a. Principle: The flame photometric method can be used for the determination of strontium in the concentration range prevalent in natural waters. The strontium emission is measured at a wavelength of 460.7 nm, while the background intensity is measured at a wavelength of 466 nm. The difference in readings obtained at these two wavelengths measures the light intensity emitted by strontium.

b. Interference: Emission intensity is a linear function of strontium concentration and concentration of other constituents. The standard addition technique distributes the same ions throughout the standards and the sample, thereby equalizing the radiation effect of possible interfering substances. A very low pH (<1) could produce an interference, but sample dilution should eliminate this interference.

c. Minimum detectable concentration: Strontium levels of about 0.2 mg/L can be detected by the flame photometric method without prior sample concentration.

2. Apparatus

Spectrophotometer, equipped with photomultiplier tube and flame accessories; or an atomic absorption spectrophotometer capable of operation in flame emission mode. Use of a fuel-rich nitrous oxide-acetylene flame is recommended.

3. Reagents

a. Stock strontium solution: Dissolve 2.415 g strontium nitrate, $Sr(NO_3)_2$, dried to constant weight at 140°C, in 1000 mL 1% (v/v) HNO_3; 1.00 mL = 1.00 mg Sr.

b. Standard strontium solution: Dilute 25.00 mL stock strontium solution to 1000 mL with water; 1.00 mL = 25.0 μg Sr. Use this solution for preparing Sr standards in the 0.2- to 25-mg/L range.

c. Nitric acid (HNO_3), conc.

4. Procedure

a. Pretreatment of polluted water and wastewater samples: Select an appropriate procedure from Section 3030.

b. Preparation of strontium standards: Dilute samples, if necessary, to contain less than 400 mg Ca or Ba/L and less than 40 mg Sr/L. Add 25.0 mL sample (or a lesser but consistent volume to keep all standards in the linear range of the instrument) to 25.0 mL of each of a series of four or more strontium standards containing from 0 mg/L to a concentration exceeding that of the sample. For most natural waters 0, 2.0, 5.0, and 10.0 mg Sr/L standards are sufficient. A broader range curve might be preferable for brines. Dilute the brine sufficiently to eliminate burner splatter and clogging.

c. Concentration of low-level strontium samples: Concentrate samples containing less than 2 mg Sr/L. Polluted water or

wastewater samples can be concentrated during digestion by starting with a larger volume (see Section 3030D). For other samples, add 3 to 5 drops conc HNO_3 to 250 mL sample and evaporate to about 25 mL. Cool and make up to 50.0 mL with distilled water. Proceed as in ¶ *b* above. The HNO_3 concentration in the sample prepared for atomization can approach 0.4 mL/50 mL without producing interference.

d. Flame photometric measurement: Measure emission intensity of prepared samples (standards plus sample) at wavelengths of 460.7 and 466 nm. Follow manufacturer's instructions for correct instrument operation. Use a fuel-rich nitrous oxide-acetylene flame, if possible.

5. Calculation

a. Using a calculator or computer with linear regression capability, enter the net intensity (reading at 460.7 nm minus reading at 466 nm) versus concentration added to the sample and solve the equation for zero emissions. The negative of this number multiplied by any dilution factor is the sample concentration.

b. Plot net intensity (reading at 460.7 nm minus reading at 466 nm) against strontium concentration added to the sample. Because the plot forms a straight calibration line that intersects the ordinate, strontium concentration can be calculated from the equation:

$$\text{mg Sr/L} = \frac{A - B}{C} \times \frac{D}{E}$$

where:

A = sample emission-intensity reading of sample plus 0 mg/L at 460.7 nm,
B = background radiation reading at 466 nm, and
C = slope of calibration line.

Use the ratio D/E only when E mL of sample are concentrated to a final volume D mL (typically 50 mL).

c. Graphical method: Strontium concentration also can be evaluated by the graphical method illustrated in Figure 3500-Sr:1 . Plot net intensity against strontium concentration added to sample. If the line intersects the ordinate at Y emissions, the strontium concentration is where the abscissa value of the point on the calibration line has an ordinate value of $2Y$ emissions due to the two-fold dilution with standards (if sample and standards are mixed in equal volumes). The calibration line in the example intersects the ordinate at 12. Thus, $Y = 12$ and $2Y = 24$. The strontium concentration of the sample is the abscissa value of the point on the calibration line having an ordinate value of 24. In the example, the strontium concentration is 9.0 mg/L.

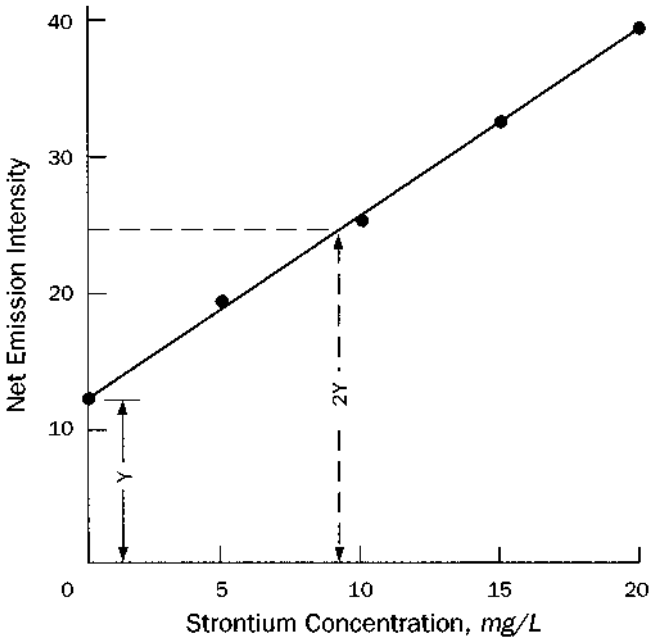


Figure 3500-Sr:1. Graphical method of computing strontium concentration.

d. Report strontium concentrations below 10 mg/L to the nearest 0.1 mg/L and above 10 mg/L to the nearest whole number.

6. Quality Control

The QC practices considered to be an integral part of each method can be found in Section 3020. Also see Section 1020.

7. Precision and Bias

Strontium concentrations in the range 12.0 to 16.0 mg/L can be determined with an accuracy within ±1 to 2 mg/L.

8. Bibliography

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3500-V VANADIUM*

3500-V A. Introduction

1. Occurrence and Significance

Vanadium (V) is the first element in Group VB in the periodic table; it has an atomic number of 23, an atomic weight of 50.94, and valences of 2, 3, 4, and 5. The average abundance of V in the earth's crust is 136 ppm; in soils it ranges from 15 to 110 ppm; in streams it averages about 0.9 μg/L, and in groundwaters it is generally <0.1 mg/L. Though relatively rare, vanadium is found in a variety of minerals; most important among these are vanadinite [$Pb_5(VO_4)_3Cl$], and patronite (possibly VS_4), occurring chiefly in Peru. Vanadium complexes have been noted in coal and petroleum deposits. Vanadium is used in steel alloys and as a catalyst in the production of sulfuric acid and synthetic rubber.

The dominant form in natural waters is V^{5+}. It is associated with organic complexes and is insoluble in reducing environments. It is considered nonessential for most higher plants and animals, although it may be an essential trace element for some algae and microorganisms. Laboratory and epidemiological evidence suggests that vanadium may play a beneficial role in the prevention of heart disease. In water supplies in New Mexico, which has a low incidence of heart disease, vanadium has been found in concentrations of 20 to 150 μg/L. In a state where incidence of heart disease is high, vanadium was not found in water supplies. However, vanadium pentoxide dust causes gastrointestinal and respiratory disturbances. The United Nations Food and Agriculture Organization recommended maximum level for irrigation waters is 0.1 mg/L.

2. Selection of Method

The atomic absorption spectrometric methods (Sections 3111D and E), the electrothermal atomic absorption method (Section 3113B), the inductively coupled plasma methods (Sections 3120 and 3125), and gallic acid method (3500-V.B) are suitable for potable water samples. The atomic absorption spectrometric and inductively coupled plasma methods are preferred for polluted samples. The electrothermal atomic absorption method also may be used successfully with an appropriate matrix modifier.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011.
Joint Task Group: 20th Edition—See Section 3500-Al.

3500-V B. Gallic Acid Method

1. General Discussion

a. Principle: The concentration of trace amounts of vanadium in water is determined by measuring the catalytic effect it exerts on the rate of oxidation of gallic acid by persulfate in acid solution. Under the given conditions of concentrations of reactants, temperature, and reaction time, the extent of oxidation of gallic acid is proportional to the concentration of vanadium. Vanadium is determined by measuring the absorbance of the sample at 415 nm and comparing it with that of standard solutions treated identically.

b. Interference: The substances listed in Table 3500-V:I will interfere in the determination of vanadium if the specified concentrations are exceeded. This is not a serious problem for Cr^{6+}, Co^{2+}, Mo^{6+}, Ni^{2+}, Ag^{+}, and U^{6+} because the tolerable concentration is greater than that commonly encountered in fresh water. However, in some samples the tolerable concentration of Cu^{2+}, Fe^{2+}, and Fe^{3+} may be exceeded. Because of the high sensitivity of the method, interfering substances in concentrations only slightly above tolerance limits can be rendered harmless by dilution.

Traces of Br^- and I^- interfere seriously and dilution alone will not always reduce the concentration below tolerance limits. Mercuric ion may be added to complex these halides and minimize their interference; however, mercuric ion itself interferes if in excess. Adding 350 μg mercuric nitrate, $Hg(NO_3)_2$, per sample permits determination of vanadium in the presence of up to 100 mg Cl^-/L, 250 μg Br^-/L, and 250 μg I^-/L. Dilute samples containing high concentrations of these ions to concentrations below the values given above and add $Hg(NO_3)_2$.

Table 3500-V:I. Concentration at Which Various Ions Interfere in the Determination of Vanadium

Ion	Concentration *mg/L*
Cr^{6+}	1.0
Co^{2+}	1.0
Cu^{2+}	0.05
Fe^{2+}	0.3
Fe^{3+}	0.5
Mo^{6+}	0.1
Ni^{2+}	3.0
Ag^{+}	2.0
U^{6+}	3.0
Br^-	0.1
Cl^-	100.0
I^-	0.001

c. Minimum detectable concentration: 0.025 μg V in approximately 13 mL final volume or approximately 2 μg V/L.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Water bath, capable of being operated at 25 ± 0.5°C.

b. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer,* for measurements at 415 nm, with a light path of 1 to 5 cm.

2) *Filter photometer,* providing a light path of 1 to 5 cm and equipped with a violet filter with maximum transmittance near 415 nm.

3. Reagents

Use reagent water (see Section 1080) in preparation of reagents, for dilutions, and as blanks.

a. Stock vanadium solution: Dissolve 229.6 mg ammonium metavanadate, NH_4VO_3, in a volumetric flask containing approximately 800 mL water and 15 mL 1 + 1 nitric acid (HNO_3). Dilute to 1000 mL; 1.00 mL = 100 μg V.

b. Intermediate vanadium solution: Dilute 1.00 mL stock vanadium solution with water to 100 mL; 1.00 mL = 1.00 μg V.

c. Standard vanadium solution: Dilute 1.00 mL intermediate vanadium solution with water to 100 mL; 1.00 mL = 0.010 μg V.

d. Mercuric nitrate solution: Dissolve 350 mg $Hg(NO_3)_2 \cdot H_2O$ in 1000 mL water.

e. Ammonium persulfate-phosphoric acid reagent: Dissolve 2.5 g $(NH_4)_2S_2O_8$ in 25 mL water. Bring just to a boil, remove from heat, and add 25 mL conc H_3PO_4. Let stand approximately 24 h before use. Discard after 48 h.

f. Gallic acid solution: Dissolve 2 g $H_6C_7O_5$ in 100 mL warm water, heat to a temperature just below boiling, and filter through filter paper.* Prepare a fresh solution for each set of samples.

4. Procedure

a. Preparation of standards and sample: Prepare both blank and sufficient standards by diluting 0- to 8.0-mL portions (0 to 0.08 μg V) of standard vanadium solution to 10 mL with water. Pipet sample (10.00 mL maximum) containing less than 0.08 μg V into a suitable container and adjust volume to 10.0 mL with water. Filter colored or turbid samples. Add 1.0 mL $Hg(NO_3)_2$ solution to each blank, standard, and sample. Place containers in a water bath regulated to 25 ± 0.5°C and allow 30 to 45 min for samples to come to the bath temperature.

b. Color development and measurement: Add 1.0 mL ammonium persulfate-phosphoric acid reagent (temperature equilibrated), swirl to mix thoroughly, and return to water bath. Add 1.0 mL gallic acid solution (temperature equilibrated), swirl to mix thoroughly, and return to water bath. Add gallic acid to successive samples at intervals of 30 s or longer to permit accurate control of reaction time. Exactly 60 min after adding gallic acid, remove sample from water bath and measure its absorbance at 415 nm, using water as a reference. Subtract absorbance of blank from absorbance of each standard and sample. Construct a calibration curve by plotting absorbance values of standards versus micrograms vanadium. Determine amount of vanadium in a sample by referring to the corresponding absorbance on the calibration curve. Prepare a calibration curve with each set of samples.

5. Calculation

$$\text{mg V/L} = \frac{\mu\text{g V (in 13 mL final volume)}}{\text{original sample volume, mL}}$$

6. Precision and Bias

In a synthetic sample containing 6 μg V/L, 40 μg As/L, 250 μg Be/L, 240 μg B/L, and 20 μg Se/L in distilled water, vanadium was measured in 22 laboratories with a relative standard deviation of 20% and no relative error.

7. Bibliography

FISHMAN, M.J. & M.V. SKOUGSTAD. 1964. Catalytic determination of vanadium in water. *Anal. Chem.* 36:1643.

* Whatman No. 42, or equivalent.

3500-Zn ZINC*

3500-Zn A. Introduction

1. Occurrence and Significance

Zinc (Zn) is the first element in Group IIB in the periodic table; it has an atomic number of 30, an atomic weight of 65.38, and a valence of 2. The average abundance of Zn in the earth's crust is 76 ppm; in soils it is 25 to 68 ppm; in streams it is 20 μg/L, and in groundwaters it is <0.1 mg/L. The solubility of zinc is controlled in natural waters by adsorption on mineral surfaces, carbonate equilibrium, and organic complexes. Zinc is used in a number of alloys such as brass and bronze, and in batteries, fungicides, and pigments. Zinc is an essential growth element for plants and animals but at elevated levels it is toxic to some species of aquatic life. The United Nations Food and Agriculture Organization recommended level for zinc in irrigation waters is 2 mg/L. The U.S. EPA secondary drinking water standard MCL is 5 mg/L. Concentrations above 5 mg/L can cause a bitter astringent taste and an opalescence in alkaline waters. Zinc most commonly enters the domestic water supply from deterioration of galvanized iron and dezincification of brass. In such cases lead and cadmium also may be present because they are impurities of the zinc used in galvanizing. Zinc in water also may result from industrial waste pollution.

2. Selection of Method

The atomic absorption spectrometric methods (Sections 3111B and C) and inductively coupled plasma methods (Sections 3120 and 3125) are preferred. The zincon method (3500-Zn.B), suitable for analysis of both potable and polluted waters, may be used if instrumentation for the preferred methods is not available.

3. Sampling and Storage

See Section 3010B.2 for sample handling and storage.

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* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011. Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Zn B. Zincon Method

1. General Discussion

a. Principle: Zinc forms a blue complex with 2-carboxy-2′-hydroxy-5′-sulfoformazyl benzene (zincon) in a solution buffered to pH 9.0. Other heavy metals likewise form colored complexes with zincon. Cyanide is added to complex zinc and heavy metals. Cyclohexanone is added to free zinc selectively from its cyanide complex so that it can be complexed with zincon to form a blue color. Sodium ascorbate reduces manganese interference. The developed color is stable except in the presence of copper (see table below).

b. Interferences: The following ions interfere at concentrations exceeding those listed:

Ion	*mg/L*	Ion	*mg/L*
Cd^{2+}	1	Cr^{3+}	10
Al^{3+}	5	Ni^{2+}	20
Mn^{2+}	5	Cu^{2+}	30
Fe^{3+}	7	Co^{2+}	30
Fe^{2+}	9	CrO_4^{2-}	50

c. Minimum detectable concentration: 0.02 mg Zn/L.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 3020.

2. Apparatus

a. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer,* for measurements at 620 nm, providing a light path of 1 cm or longer.

2) *Filter photometer,* providing a light path of 1 cm or longer and equipped with a red filter having maximum transmittance near 620 nm. Deviation from Beer's Law occurs when the filter band pass exceeds 20 nm.

b. Graduated cylinders, 50-mL, with ground-glass stoppers, Class B or better.

c. Erlenmeyer flasks, 50-mL.

d. Filtration apparatus: 0.45-μm filters and filter holders.

3. Reagents

a. Metal-free water: See Section 3111B.3*c*. Use water for rinsing apparatus and preparing solutions and dilutions.

b. Stock zinc solution: Dissolve 1000 mg (1.000 g) zinc metal in 10 mL 1 + 1 HNO_3. Dilute and boil to expel oxides of nitrogen. Dilute to 1000 mL; 1.00 mL = 1.00 mg Zn.

c. Standard zinc solution: Dilute 10.00 mL stock zinc solution to 1000 mL; 1.00 mL = 10.00 μg Zn.

d. Sodium ascorbate, fine granular powder, USP.

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e. Potassium cyanide solution: Dissolve 1.00 g KCN in approximately 50 mL water and dilute to 100 mL. **CAUTION: Potassium cyanide is a deadly poison. Avoid skin contact or inhalation of vapors. Do not pipet by mouth or bring in contact with acids.**

f. Buffer solution, pH 9.0: Dissolve 8.4 g NaOH pellets in about 500 mL water. Add 31.0 g H_3BO_3 and swirl or stir to dissolve. Dilute to 1000 mL with water and mix thoroughly.

g. Zincon reagent: Dissolve 100 mg zincon (2-carboxy-2′-hydroxy-5′-sulfoformazyl benzene) in 100 mL methanol. Because zincon dissolves slowly, stir and/or let stand overnight.

h. Cyclohexanone, purified.

i. Hydrochloric acid (HCl), conc and 1*N*.

j. Sodium hydroxide (NaOH), 6*N* and 1*N*.

4. Procedure

a. Preparation of colorimetric standards: Accurately deliver 0, 0.5, 1.0, 3.0, 5.0, 10.0, and 14.0 mL standard zinc solution to a series of 50-mL graduated mixing cylinders. Dilute each to 20.0 mL to yield solutions containing 0, 0.25, 0.5, 1.5, 2.5, 5.0, and 7.0 mg Zn/L, respectively. (Lower-range standards may be prepared to extend the quantitation range. Longer optical path cells can be used. Verify linearity of response in this lower concentration range.) Add the following to each solution in sequence, mixing thoroughly after each addition: 0.5 g sodium ascorbate, 5.0 mL buffer solution, 2.0 mL KCN solution, and 3.0 mL zincon solution. Pipet 20.0 mL of the solution into a clean 50-mL Erlenmeyer flask. Reserve remaining solution to zero the instrument. Add 1.0 mL cyclohexanone to the Erlenmeyer flask. Swirl for 10 s and note time. Transfer portions of both solutions to clean sample cells. Use solution without cyclohexanone to zero colorimeter. Read and record absorbance for solution with cyclohexanone after 1 min. The calibration curve does not pass through zero because of the color enhancement effect of cyclohexanone on zincon.

b. Treatment of samples: To determine readily acid-extractable total zinc, add 1 mL conc HCl to 50 mL sample and mix thoroughly. Filter and adjust to pH 7. To determine dissolved zinc, filter sample through a 0.45-μm membrane filter. Adjust to pH 7 with 1*N* NaOH or 1*N* HCl if necessary after filtering.

c. Sample analysis: Cool samples to less than 30°C if necessary. Analyze 20.0 mL of prepared sample as described in ¶ *a* above, beginning with "Add the following to each solution . . ." If the zinc concentration exceeds 7 mg Zn/L prepare a sample dilution and analyze a 20.0-mL portion.

5. Calculation

Read zinc concentration (in milligrams per liter) directly from the calibration curve.

6. Precision and Bias

A synthetic sample containing 650 μg Zn/L, 500 μg Al/L, 50 μg Cd/L, 110 μg Cr/L, 470 μg Cu/L, 300 μg Fe/L, 70 μg Pb/L, 120 μg Mn/L, and 150 μg Ag/L in doubly demineralized water was analyzed in a single laboratory. A series of 10 replicates gave a relative standard deviation of 0.96% and a relative error of 0.15%. A wastewater sample from an industry in Standard Industrial Classification (SIC) No. 3333, primary smelting and refining of zinc, was analyzed by 10 different persons. The mean zinc concentration was 3.36 mg Zn/L and the relative standard deviation was 1.7%. The relative error compared to results from an atomic absorption analysis of the same sample was −1.0%.

7. Bibliography

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3500 OTHER METALS

The following sections contain background information on, and refer the user to analytical methods for, a diverse group of metals that occur in nature and/or have important industrial uses.

3500-Sb Antimony*

Antimony (Sb) is the fourth element in Group VA in the periodic table; it has an atomic number of 51, an atomic weight of 121.75, and valences of 3 and 5. The average abundance of Sb in the earth's crust is 0.2 ppm; in soils it is 1 ppm; in streams it is 1 μg/L, and in groundwaters it is <0.1 mg/L. Antimony is sometimes found native, but more commonly in stibnite (Sb_2S_3). It is used in alloys of lead and in batteries, bullets, solder, pyrotechnics, and semiconductors.

The common aqueous species are SbO_2^-, $HSbO_2$, and complexes with carbonate and sulfate. Soluble salts of antimony are toxic. The U.S. EPA primary drinking water standard MCL is 6 μg/L.

The electrothermal atomic absorption spectrometric method (Section 3113B) or the inductively coupled plasma/mass spectrometric method (Section 3125) are the methods of choice because of their sensitivity. Alternatively use the flame atomic absorption spectrometric method (Section 3111B) or the inductively coupled plasma method (Section 3120) when high sensitivity is not required.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Ba Barium*

Barium (Ba) is the fifth element in Group IIA in the periodic table; it has an atomic number of 56, an atomic weight of 137.33, and a valence of 2. The average abundance of Ba in the earth's crust is 390 ppm; in soils it is 63 to 810 ppm; in streams it is 10 mg/L; in U.S. drinking waters it is 49 μg/L; and in groundwaters it is 0.05 to 1 mg/L. It is found chiefly in barite ($BaSO_4$) or in witherite ($BaCO_3$). Barium's main use is in mud slurries used in drilling oil and exploration wells, but it is also used in pigments, rat poisons, pyrotechnics, and in medicine.

The solubility of barium in natural waters is controlled by the solubility of $BaSO_4$, and somewhat by its adsorption on hydroxides. High concentrations of barium occur in some brines. Concentrations exceeding 1 mg/L constitute a toxicity hazard in the marine environment. The U.S. EPA primary drinking water standard MCL is 1 mg/L.

Perform analyses by the atomic absorption spectrometric methods (Sections 3111D or E), the electrothermal atomic absorption method (Section 3113B), or the inductively coupled plasma methods (Sections 3120 or 3125).

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Be Beryllium*

Beryllium (Be) is the first element in Group IIA of the periodic table; it has an atomic number of 4, an atomic weight of 9.01, and a valence of 2. The average abundance of Be in the earth's crust is 2 ppm; in soils it is 0.8 to 1.3 ppm; in streams it is 0.2 μg/L, in U.S. drinking waters and in groundwaters it is typically <0.1 μg/L. Beryllium occurs in nature in deposits of beryls in granitic rocks. Beryllium is used in high-strength alloys of copper and nickel, windows in X-ray tubes, and as a moderator in nuclear reactors.

Beryllium solubility is controlled in natural waters by the solubility of beryllium hydroxides. The solubility at pH 6.0 is approximately 0.1 μg/L. It is nonessential for plants and animals. Acute toxicity occurs at 130 μg/L, and chronic toxicity at 5 μg/L in freshwater species. The United Nations Food and Agriculture Organization recommended maximum level for irrigation waters is 100 μg/L. The U.S. EPA primary drinking water standard MCL for beryllium is 4 μg/L.

The atomic absorption spectrometric methods (Sections 3111D and E, and 3113B) and the inductively coupled plasma (ICP) methods (Sections 3120 and 3125) are the methods of choice. If atomic absorption or ICP instrumentation is not available, the aluminon colorimetric method detailed in the 19th Edition of *Standard Methods* may be used. This method has poorer precision and bias than the methods of choice.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Bi Bismuth*

Bismuth (Bi) is the fifth element in Group VA in the periodic table; it has an atomic number of 83, an atomic weight of 208.98, and valences of 3 and 5. The average abundance of Bi in the earth's crust in 0.08 ppm; in streams it is <0.02 mg/L, and in groundwaters it is <0.1 mg/L. Bismuth occurs in association with lead and silver ores, and occasionally as the native element. ^{210}Bi, ^{212}Bi, and ^{214}Bi are naturally occuring radioisotopes produced in the decay of uranium and thorium. The metal is used in alloys of lead, tin, and cadmium, and in some pharmaceuticals.

In natural water, Bi^{3+} ion will occur, and complex ions with nitrate and chloride also might be expected. The iodide and telluride compounds are toxic by ingestion or inhalation.

Perform analyses by the atomic absorption spectrometric method (Section 3111B) or by the electrothermal atomic absorption method (Section 3113B). The inductively coupled plasma mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though bismuth is not specifically listed as an analyte in the method.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-B Boron

See Section 4500-B. Cadmium

3500-Cd Cadmium*

Cadmium (Cd) is the second element in Group IIB of the periodic table; it has an atomic number of 48, an atomic weight of 112.41, and a valence of 2. The average abundance of Cd in the earth's crust is 0.16 ppm; in soils it is 0.1 to 0.5 ppm; in streams it is 1 μg/L, and in groundwaters it is from 1 to 10 μg/L. Cadmium occurs in sulfide minerals that also contain zinc, lead, or copper. The metal is used in electroplating, batteries, paint pigments, and in alloys with various other metals. Cadmium is usually associated with zinc at a ratio of about 1 part cadmium to 500 parts zinc in most rocks and soils.

The solubility of cadmium is controlled in natural waters by carbonate equilibria. Guidelines for maximum cadmium concentrations in natural water are linked to the hardness or alkalinity of the water (i.e., the softer the water, the lower the permitted level of cadmium). It is nonessential for plants and animals. Cadmium is extremely toxic and accumulates in the kidneys andliver, with prolonged intake at low levels sometimes leading to dysfunction of the kidneys. The United Nations Food and Agriculture Organization recommended maximum level for cadmium in irrigation waters is 10 μg/L. The U.S. EPA primary drinking water standard MCL is 10 μg/L.

The electrothermal atomic absorption spectrometric method (Section 3113B) is preferred. The flame atomic absorption methods (Sections 3111B and C) and inductively coupled plasma methods (Sections 3120 and 3125) provide acceptable precision and bias, with higher detection limits. Anodic stripping voltammetry (Section 3130B) can achieve superior detection limits, but is susceptible to interferences from copper, silver, gold, and organic compounds. When atomic absorption spectrometric or inductively coupled plasma apparatus is unavailable and the desired precision is not as great, the dithizone method detailed in the 19th Edition of *Standard Methods* is suitable.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Cs Cesium*

Cesium (Cs) is the sixth element in Group IA of the periodic table; it has an atomic number of 55, an atomic weight of 132.90, and a valence of 1. The average abundance of Cs in the earth's crust is 2.6 ppm; in soils it is 1 to 5 ppm; in streams it is 0.02 mg/L; and in groundwaters it is generally <0.1 mg/L. Cesium is found in lepidolite and in the water of certain mineral springs. ^{137}Cs, with a 33-year half-life, is widely dispersed on the earth's surface as a result of the radioactive fallout from the atmospheric testing of nuclear weapons. Cesium compounds are used in photoelectric cells, as a catalyst, and in brewing. Some cesium compounds are fire hazards.

Perform analyses by the flame atomic absorption spectrometric method (Section 3111B). The inductively coupled plasma/mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though cesium is not specifically listed as an analyte in the method.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Co Cobalt*

Cobalt (Co) is the second element in Group VIII in the periodic table; it has an atomic number of 27, an atomic weight of 58.93, and valences of 1, 2, and 3. The average abundance of Co in the earth's crust is 29 ppm; in soils it is 1.0 to 14 ppm; in streams it is 0.2 μg/L; and in groundwaters it is 1 to 10 μg/L. Cobalt occurs only sparingly in ores, usually as the sulfide or the arsenide. It is widely used in alloys of various steels, in electroplating, in fertilizers, and in porcelain and glass.

The solubility of cobalt is controlled by coprecipitation or adsorption by oxides or manganese and iron, by carbonate precipitation, and by the formation of complex ions. Cobalt dust is flammable and is toxic by inhalation. Cobalt is considered essential for algae and some bacteria, nonessential for higher plants, and an essential trace element for animals. The United Nations Food and Agriculture Organization recommended maximum level for irrigation waters is 100 μg/L.

Perform analyses by the flame atomic absorption spectrometric methods (Sections 3111B and C), by the electrothermal atomic absorption method (Section 3113B), or by the inductively coupled plasma methods (Sections 3120 and 3125).

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Ga Gallium*

Gallium (Ga) is the third element in Group IIIA in the periodic table; it has an atomic number of 31, an atomic weight of 67.72, and valences of 1, 2, and 3. The average abundance of Ga in the earth's crust is 19 ppm; in soils it is 1.9 to 29 ppm; in streams it is 0.09 μg/L; and in groundwaters it is $<$0.1 mg/L. Gallium occurs in many zinc ores, and nearly always in bauxite. Gallium compounds are used in semiconducting devices.

The element exists as Ga^{3+} in natural water, and its solubility is controlled by formation of the hydroxide. It is considered nonessential for plants and animals.

Perform analyses by the electrothermal atomic absorption method (Section 3113B). The inductively coupled plasma/mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though gallium is not specifically listed as an analyte in the method.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Ge Germanium*

Germanium (Ge) is the third element in Group IVA in the periodic table; it has an atomic number of 32, an atomic weight of 72.59, and valences of 2 and 4. The average abundance of Ge in the earth's crust is 1.5 ppm; in streams it is 0.03 to 0.1 μg/L; and in groundwaters it is $<$0.1 mg/L. Germanium is found in germanite, in certain zinc ores, and in elevated levels in certain hot spring waters. Germanium alloys are used in transistors, gold alloys, phosphors, and semiconducting devices.

Germanium is present in natural waters in the tetravalent state, and its distribution in natural waters probably is controlled by adsorption on clay mineral surfaces. It is nonessential for plants and animals.

Perform analyses by the electrothermal atomic absorption method (Section 3113B). The inductively coupled plasma/mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though germanium is not specifically listed as an analyte in the method.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Au Gold*

Gold (Au) is the third element in Group IB in the periodic table; it has an atomic number of 79, an atomic weight of 196.97, and valences of 1 and 3. The average abundance of Au in the earth's crust is 0.004 ppm; in streams it is 2 μg/L; and in groundwater it is $<$0.1 mg/L. Gold occurs in the native form, and is associated with quartz or pyrite. The main uses of gold are in jewelry, dentistry, electronics, and the aerospace industry.

Gold solubility is restricted to acidic waters in the presence of oxidizing agents and chloride, or in alkaline solutions in the presence of hydrogen sulfide. Its solubility may be influenced by natural organic acids. Compounds of gold containing thiosulfate and cyanide have some human toxicity.

* Joint Task Group: 20th Edition—See Section 3500-Al.

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Perform analyses by the atomic absorption spectrometric method (Section 3111B) or by the electrothermal atomic absorption method (Section 3113B). The inductively coupled plasma mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though gold is not specifically listed as an analyte in the method.

3500-In Indium*

Indium (In) is the fourth element in Group IIIA in the periodic table; it has an atomic number of 49, an atomic weight of 114.82, and valences of 1, 2, and 3. The average abundance of indium in the earth's crust is 0.19 ppm; in streams it is <0.01 μg/L; and in groundwaters it is <0.1 mg/L. Indium often occurs in combination with zinc ores, and sometimes with pyrites and siderite. Indium is used in alloys for bearings, brazing, solder, and in electrical devices.

Indium exists as In^{3+} and as a number of complex ions. Its solubility is controlled by the formation of the insoluble hydroxide. The metal and its compounds are toxic by inhalation.

Perform analyses by the electrothermal atomic absorption method (Section 3113B). The inductively coupled plasma mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though indium is not specifically listed as an analyte in the method. Iridium

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Ir Iridium*

Iridium (Ir) is the eighth element in Group VIII of the periodic table; it has an atomic number of 77, an atomic weight of 192.2, and valences of 1, 3, and 4. The average abundance of Ir in the earth's crust is probably <0.001 ppm, and in groundwaters it is <0.1 mg/L. Iridium occurs uncombined with platinum and other metals. It is used in alloys with platinum in catalysts, thermocouples, electrodes, and wires.

The aqueous chemistry is controlled by complex compounds, although the solubility in natural waters is relatively unknown.

Perform analyses by the flame atomic absorption spectrometric method (Section 3111B). The inductively coupled plasma/mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though iridium is not specifically listed as an analyte in the method.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Hg Mercury*

Mercury (Hg) is the third element in Group IIB in the periodic table; it has an atomic number of 80, an atomic weight of 200.59, and valences of 1 and 2. The average abundance of Hg in the earth's crust is 0.09 ppm; in soils it is 30 to 160 ppb; in streams it is 0.07 μg/L, and in groundwaters it is 0.5 to 1 μg/L. Mercury occurs free in nature, but the chief source is cinnabar (HgS). Mercury is used in amalgams, mirror coatings, vapor lamps, paints, measuring devices (thermometers, barometers, manometers), pharmaceuticals, pesticides, and fungicides. It is often used in paper mills as a mold retardant for paper.

The common aqueous species are Hg^{2+}, $Hg(OH)_2^0$, Hg^0, and stable complexes with organic ligands. Inorganic mercury can be methylated in sediments when sulfides are present to form dimethyl mercury, $(CH_3)_2Hg$, which is very toxic and can concentrate in the aquatic food chain. Mercury poisoning occurred in Japan in the 1950s as the result of consumption of shellfish that had accumulated mercury. In times past, mercury was used in the haberdashery industry to block hats (the cause of the "mad hatter" syndrome).

Mercury is considered nonessential for plants and animals. The U.S. EPA primary drinking water standard MCL is 2 μg/L.

The cold-vapor atomic absorption method (Section 3112B) is the method of choice for all samples. The inductively coupled plasma mass spectrometric method (Section 3125) also may be applied successfully in some cases, even though mercury is not specifically listed as an analyte in the method. The dithizone method detailed in the 19th edition of *Standard Methods* can be used for determining high levels of mercury (>2 μg/L) in potable waters.

Because mercury can be lost readily from samples, preserve them by treating with HNO_3 to reduce the pH to <2 (see Section 1060). Glass storage containers are preferred to plastic, because they can extend the holding time to 30 d, rather than only the 14 d allowed in plastic containers.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Mo Molybdenum*

Molybdenum (Mo) is the second element in Group VIB in the periodic table; it has an atomic number of 42, and atomic weight of 95.95, and valences of 2, 3, 4, 5, and 6. The average abundance of Mo in the earth's crust is 1.2 ppm; in soils it is 2.5 ppm; in streams it is 1 μg/L, and in groundwaters it is <0.1 mg/L. Molybdenum occurs naturally as molybdenite (MoS_2) and wulfenite ($PbMoO_4$). It is used in alloys, ink pigments, catalysts, and lubricants.

The common aqueous species are $HMoO_4^-$, MoO_4^{2-}, and organic complexes. It is considered an essential trace element for plants and animals. The United Nations Food and Agriculture Organization recommended maximum level for irrigation waters is 0.01 mg/L.

Use one of the flame atomic absorption spectrometric methods (Section 3111D or E), the electrothermal atomic absorption spectrometric method (Section 3113B), or one of the inductively coupled plasma methods (Section 3120 or 3125).

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Ni Nickel*

Nickel (Ni) is the third element in Group VIII in the periodic table; it has an atomic number of 28, an atomic weight of 58.69, and a common valence of 2 and less commonly 1, 3, or 4. The average abundance of Ni in the earth's crust is 1.2 ppm; in soils it is 2.5 ppm; in streams it is 1 μg/L, and in groundwaters it is <0.1 mg/L. Nickel is obtained chiefly from pyrrhotite and garnierite. Nickel is used in alloys, magnets, protective coatings, catalysts, and batteries.

The common aqueous species is Ni^{2+}. In reducing conditions insoluble sulfides can form, while in aerobic conditions nickel complexes with hydroxide, carbonates, and organic ligands can form. It is suspected to be an essential trace element for some plants and animals. The United Nations Food and Agriculture Organization recommended maximum level for irrigation waters is 200 μg/L. The U.S. EPA primary drinking water standard MCL is 0.1 mg/L.

The atomic absorption spectrometric methods (Sections 3111B and C), the inductively coupled plasma methods (Sections 3120 and 3125), and the electrothermal atomic absorption spectrometric method (Section 3113B) are the methods of choice for all samples.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Os Osmium*

Osmium (Os) is the seventh element in Group VIII in the periodic table; it has an atomic number of 76, an atomic weight of 190.2, and valences of 3, 4, and 6, and less commonly 1, 2, 5, 7, and 8. The average abundance of Os in the earth's crust is probably <0.005 ppm, and in groundwaters it is <0.01 mg/L. Osmium occurs in iridosime and in platinum-bearing river sands. Osmium is used as a hardener with iridium and as a catalyst with platinum.

The aqueous chemistry is controlled by complex compounds, although the solubility in natural waters is relatively unknown.

Analyze by flame atomic absorption methods (Sections 3111D and E).

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Pd Palladium*

Palladium (Pd) is the sixth element in Group VIII of the periodic table; it has an atomic number of 46, an atomic weight of 106.42, and valences of 2 and 4. Palladium occurs with platinum in nature. It is used in alloys to make electrical relays, catalysts, in the making f "white gold," and in protective coatings.

Palladium has no known toxic effects. The United Nations Food and Agriculture Organization recommended maximum level for irrigation waters is 5 mg/L.

Preferably analyze by flame atomic absorption method (Section 3111B). The inductively coupled plasma mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though palladium is not specifically listed as an analyte in the method.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Pt Platinum*

Platinum (Pt) is the ninth element in Group VIII of the periodic table; it has an atomic number of 78, an atomic weight of 195.1, and valences of 2 and 4. The average abundance of Pt in the earth's crust is probably <0.01 ppm, and in groundwaters it is <0.1 mg/L. Platinum is usually found in its native state, but also may be found as sperrylite ($PtAs_2$). Platinum is used as a catalyst and in laboratory ware, jewelry, and surgical wire.

The aqueous chemistry in natural waters is relatively unknown, although its solubility is probably controlled by complex compounds. In powder form, platinum can be flammable, and its soluble salts are toxic by inhalation.

Preferably analyze by flame atomic absorption method (Section 3111B). The inductively coupled plasma mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though platinum is not specifically listed as an analyte in the method. Rhenium

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Re Rhenium*

Rhenium (Re) is the third element in Group VIIB in the periodic table; it has an atomic number of 75, an atomic weight of 186.21, and valences of 1 through 7, with 7 being the most stable. The average abundance of Re in the earth's crust is 7 ppm, and in groundwaters it is <0.1 mg/L. Rhenium is found in columbite, tantalite, and wolframite, as well as in molybdenum ore concentrates. It is used in tungsten-molybdenum-based alloys, thermocouples, filaments, and flash bulbs. Rhenium in the powder form can be flammable.

For analysis methods, see flame atomic absorption methods (Sections 3111D and E). The inductively coupled plasma mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though rhenium is not specifically listed as an analyte in the method.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Rh Rhodium*

Rhodium (Rh) is the fifth element in Group VIII in the periodic table; it has an atomic number of 45, an atomic weight of 102.91, and valences of 1 through 6, the most common being 1 and 3. Rhodium is found in its native state in platinum-bearing sands. It is used in platinum alloys for thermocouples, electrical contacts, and jewelry.

The aqueous chemistry in natural waters is relatively unknown. The metal is flammable in the powder form, and its salts are toxic by inhalation.

For analysis see flame atomic absorption method (Section 3111B). The inductively coupled plasma/mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though rhodium is not specifically listed as an analyte in the method.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Ru Ruthenium*

Ruthenium (Ru) is the fourth element in Group VIII in the periodic table; it has an atomic number of 44, an atomic weight of 101.07, and valences of 1 through 7, the most common being 2, 3, and 4. The average abundance of Ru in the earth's crust is probably <0.01 ppm, and in groundwaters it is <0.1 mg/L. It occurs in its native state in platinum-bearing river sands. It is used in jewelry with platinum, in electrical contacts, and as a catalyst.

The aqueous chemistry in natural waters is relatively unknown. Ruthenium has no known toxic effects.

For analysis see flame atomic absorption method (Section 3111B). The inductively coupled plasma mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection levels), even though ruthenium is not specifically listed as an analyte in the method.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Ag Silver*

Silver (Ag) is the second element in Group IB of the periodic table; it has an atomic number of 47, an atomic weight of 107.87, and valences of 1 and 2. The average abundance of Ag in the earth's crust is 0.08 ppm; in soils it is <0.01 to 0.5 ppm; in streams it is 0.3 μg/L; in U.S. drinking waters it is 0.23 μg/L, and in groundwater it is <0.1 μg/L. Silver occurs in its native state and in combination with many nonmetallic elements such as argentite (Ag_2S) and horn silver (AgCl). Lead and copper ores also may yield considerable silver. Silver is widely used in photography, silverware, jewelry, mirrors, and batteries. Silver iodide has been used in the seeding of clouds, and silver oxide to a limited extent is used as a disinfectant for water.

In acidic water Ag^+ would predominate, and in high-chloride water a series of complexes would be expected. Silver is nonessential for plants and animals. Silver can cause argyria, a permanent, blue-gray discoloration of the skin and eyes that imparts a ghostly appearance. Concentrations in the range of 0.4 to 1 mg/L have caused pathological changes in the kidneys, liver, and spleen of rats. Toxic effects on fish in fresh water have been observed at concentrations as low as 0.17 μg/L. For freshwater aquatic life, total recoverable silver should not exceed 1.2 mg/L.

The atomic absorption spectrometric methods (Sections 3111B and C) and the inductively coupled plasma methods (Sections 3120 and 3125) are preferred. The electrothermal atomization method (Section 3113B) is the most sensitive for determining silver in natural waters. The dithizone method detailed in the 19th edition of *Standard Methods* can be used when an atomic absorption spectrometer is unavailable. A method suitable for analysis of silver in industrial or other wastewaters at levels above 1 mg/L is available.[1]

If total silver is to be determined, acidify sample with conc nitric acid (HNO_3) to pH <2 at time of collection. If sample contains particulate matter and only the "dissolved" metal content is to be determined, filter through a 0.45-μm membrane filter at time of collection. After filtration, acidify filtrate with HNO_3 to pH <2. Complete analysis as soon after collection as possible. Some samples may require special storage and digestion; see Section 3030D.

Reference

1. U.S. Environmental Protection Agency. 1994. Approved Inorganic Test Procedures. *Fed. Reg.* 59(20):4504.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Te Tellurium*

Tellurium (Te) is the fourth element in Group VIA in the periodic table; it has an atomic number of 52, an atomic weight of 127.60, and valences of 2, 4, and 6. The average abundance of Te in the earth's crust is 0.002 ppm; in soils it is 0.001 to 0.01 ppm; and in groundwaters it is <0.1 mg/L. Tellurium is found in its native state and as the telluride of gold and other metals. It is used in alloys, catalysts, batteries, and as a coloring agent in glass and ceramics.

The common aqueous species is TeO_3^{2-}. The metal and its compounds are toxic by inhalation.

Perform analyses by the electrothermal atomic absorption method (Section 3113B). The inductively coupled plasma mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection limits), even though tellurium is not specifically listed as an analyte in the method.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Tl Thallium*

Thallium (Tl) is the fifth element in Group IIIA in the periodic table; it has an atomic number of 81, an atomic weight of 204.38, and valences of 1 and 3. The average abundance in the earth's crust is 0.07 ppm, and in groundwaters it is <0.1 mg/L. The metal occurs chiefly in pyrites. Thallium is used in the production of glasses and rodenticides, in photoelectric applications, and in electrodes for dissolved oxygen meters.

The common aqueous species is Tl^+. It is nonessential for plants and animals. Compounds of thallium are toxic on contact with moisture, and by inhalation. The U.S. EPA primary drinking water standard MCL is 2 μg/L.

For analysis, use one of the atomic absorption spectrometric methods (Section 3111B or 3113B), or one of the inductively coupled plasma methods (Section 3120 or 3125), depending upon sensitivity requirements.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Th Thorium*

Thorium (Th) is the first element in the actinium series of the periodic table; it has an atomic number of 90, an atomic weight of 232.04, and a valence of 4. The average abundance in the earth's crust is 8.1 ppm; in soils it is 13 ppm; in streams it is 0.1 μg/L, and in groundwaters it is <0.1 mg/L. Thorium is a radioactive element, with ^{232}Th having a half-life of 1.4×10^{10} years. It is widely distributed in the earth, with the principal mineral being monazite. Thorium is used in sun lamps, photoelectric cells, incandescent lighting, and gas mantles.

The aqueous chemistry of thorium is controlled by the Th^{4+} ion, which forms a set of complex species with hydroxides. Thorium's radioactive decay isotopes are dangerous when inhaled or ingested as thorium dust particles.

Either of the flame atomic absorption spectrometric methods (Section 3111D or E) may be used for analysis. The inductively coupled plasma mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection limits), even though thorium is not specifically listed as an analyte in the method. Tin

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Sn Tin*

Tin (Sn) is the fourth element in Group IVA in the periodic table; it has an atomic number of 50, an atomic weight of 118.69, and valences of 2 and 4. The average abundance in the earth's crust is 2.1 ppm; in soils it is 10 ppm; in streams it is 0.1 μg/L, and in groundwaters it is <0.1 mg/L. Tin is found mostly in the mineral cassiterite (SnO_2), in association with granitic rocks. Tin is used in reducing agents, solder, bronze, pewter, and coatings for various metals.

The common aqueous species are Sn^{4+}, $Sn(OH)_4$, $SnO(OH)_2$, and $SnO(OH)_3^-$. Tin is adsorbed to suspended solids, sulfides, and hydroxides. Tin can be methylated in sediments. Tributyl tin undergoes biodegradation quickly. Organo-tin compounds are toxic. Tin is considered nonessential for plants and animals.

Either the flame atomic absorption method (Section 3111B) or the electrothermal atomic absorption method (Section 3113B) may be used successfully for analyses, depending upon the sensitivity desired. The inductively coupled plasma/mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection limits), even though tin is not specifically listed as an analyte in the method.

* Joint Task Group: 20th Edition—See Section 3500-Al.

3500-Ti Titanium*

Titanium (Ti) is the first element in Group IVB in the periodic table; it has an atomic number of 22, an atomic weight of 47.88, and valences of 2, 3, and 4. The average abundance of Ti in the earth's crust is 0.6%; in soils it is 1700 to 6600 ppm; in streams it is 3 μg/L, and in groundwaters it is <0.1 mg/L. The element is commonly associated with iron minerals. Titanium is used in alloys for aircraft, marine, and food-handling equipment. Compounds of the metal are used in pigments and as a reducing agent.

Titanium species are usually insoluble in natural waters, with the Ti^{4+} species being the most common ion when found. Some compounds are toxic by ingestion and the pure metal is flammable.

Either of the flame atomic absorption spectrometric methods (Section 3111D or E) may be used. The inductively coupled plasma mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection limits), even though titanium is not specifically listed as an analyte in the method.

* Joint Task Group: 20th Edition—See Section 3500-Al.

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3500-U Uranium*

Uranium (U) is the third element in the actinide series of the periodic table; it has an atomic number of 92, an atomic weight of 238.04, and valences of 3, 4, and 6. The average abundance of U in the earth's crust is 2.3 ppm, and in soils it is 1.8 ppm. Concentrations of uranium in drinking waters usually are expressed in terms of picocuries per liter, but that is now being replaced by Becquerel per liter (Bq/L). The approximate conversion factor, assuming equilibrium between ^{234}U and ^{238}U, is 1 μg uranium equals 0.67 pCi. The mean concentration of uranium in drinking water is 1.8 pCi/L. The chief ore is uraninite, or pitchblende, uranous uranate [$U(UO_4)_2$]. Uranium is known mainly for its use in the nuclear industry, but has also been used in glass, ceramics, and photography.

Uranium compounds are radioactive and are thereby toxic by inhalation and ingestion. There are three natural radioisotopes of uranium. Uranium-238 has a half-life of 4.5×10^9years, represents 99% of uranium's natural abundance, and is not fissionable, but can be used to form plutonium-239, which is fissionable. Uranium-235 has a half-life of 7.1×10^8 years, represents 0.75% of uranium's natural abundance, is readily fissionable, and was the energy source in the original atomic bombs. Uranium-234 has a half-life of 2.5×10^5 years and represents only 0.006% of uranium's natural abundance.

The common forms in natural water are U^{4+} and UO_2^{2+}. In natural waters below pH 5, UO_2^{2+} would dominate; in the pH range of 5 to 10, soluble carbonate complexes predominate. Although there is no U.S. EPA drinking water standard MCL for uranium, an analysis for uranium is required if the gross alpha activity of a water sample is greater than 15 pCi/L.

Perform analyses by the inductively coupled plasma/mass spectrometry method (Section 3125) or by one of the methods in Section 7500-U (for regulatory compliance purposes).

* Joint Task Group: 20th Edition—See Section 3500-Al.

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PART 4000

INORGANIC NONMETALLIC CONSTITUENTS

Part Coordinator
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Joint Task Group Chairs for the 23rd Edition

4020 Quality Assurance/Quality Control ..... William C. Lipps
4500-CN^- Cyanide ..... Michael F. Delaney
4500-NO_3^- Nitrate ..... Thomas R. Holm
4500-O Oxygen (Dissolved) ..... Michael A. Michaud

Summary of Major Changes since 2012

Quality Assurance/Quality Control (4020) was revised to clarify the essential quality control (QC) practices for analytical methods in Part 4000 and to be consistent with Sections 1020, 2020, 3020, 5020, and 6020.

Cyanide (4500-CN^-) now includes new information on the preliminary treatment of samples, interferences, and sample preservation. Also, the language was clarified throughout and references were updated. Interferences, reagent preparation, and calibration were updated in Nitrate (4500-NO_3^-), and all of the methods were clarified. An optical-probe method was added to Oxygen (Dissolved) (4500-O).

The language in other methods was edited to clarify procedures.

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4010 INTRODUCTION

The analytical methods included in this part make use of classical wet chemical techniques and their automated variations and such modern instrumental techniques as ion chromatography. Methods that measure various forms of chlorine, nitrogen, and phosphorus are presented. The procedures are intended for use in the assessment and control of receiving water quality, the treatment and supply of potable water, and the measurement of operation and process efficiency in wastewater treatment. The methods also are appropriate and applicable in evaluation of environmental water-quality concerns. The introduction to each procedure contains reference to special field sampling conditions, appropriate sample containers, proper procedures for sampling and storage, and the applicability of the method.

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4020 QUALITY ASSURANCE/QUALITY CONTROL*

4020 A. Introduction

Quality assurance (QA) is a laboratory operations program that specifies the measures required to produce defensible data with known precision and accuracy. This program is defined in a QA manual, written procedures, work instructions, and records. The manual should include a policy that defines the statistical level of confidence used to express data precision and bias, as well as method detection levels (MDLs) and reporting limits. The overall system includes all QA policies and quality control (QC) processes needed to demonstrate the laboratory's competence and to ensure and document the quality of its analytical data. Quality systems are essential for laboratories seeking accreditation under state or federal laboratory certification programs. Refer to Section 1020 for details on establishing a Quality Assurance Plan.

As described in Part 1000, essential QC measures may include method calibration, reagent preparation and/or standardization, assessment of each analyst's capabilities, analysis of blind check samples, determination of the method's sensitivity [method detection level (MDL, limit of detection (LOD), level of quantification level (LOQ), or minimum reporting level (MRL)], and daily evaluation of bias, precision, and laboratory contamination or other analytical interference.

Some methods in Part 4000 include specific QC procedures, frequencies, and acceptance criteria. These are considered the minimum QCs needed to perform the method successfully. When the words *should* or *preferably* are used, the QC is recommended; when *must* is used, the QC is mandatory. Additional QC procedures should be used when necessary to ensure that results are valid. Some regulatory programs may require additional QC or have alternative acceptance limits. In those cases, the laboratory should follow the more stringent requirements.

The QC program consists of at least the following elements, as applicable to specific methods:

- calibration,
- continuing calibration verification (CCV),
- operational range and MDL determination,
- initial demonstration of capability (IDC),
- ongoing demonstration of capability,
- method blank/reagent blank,
- laboratory-fortified blank (LFB),
- laboratory-fortified matrix (LFM),
- duplicate sample/laboratory-fortified matrix duplicate (LFMD),
- verification of MDL and MRL,
- QC calculations,
- control charts,
- corrective action,
- frequency of QC,
- QC acceptance criteria, and
- definitions of prep and analytical batches.

Sections 1010 and 1030 describe calculations for evaluating data quality.

* Approved by Standard Methods Committee, 2014.
William C. Lipps (chair), Rodger B. Baird, L. Malcolm Baker, Terry E. Baxter, Andrew D, Eaton, Randy A. Gottler, John R. Gumpper, Robin S. Parnell.

4020 B. Quality Control Practices

At a minimum, analysts must use the QC practices specified here unless a method specifies alternative practices. Laboratories may save time and money by purchasing premade standards, titrants, and reagents, but they still must perform the QC checks on these materials required by the analytical methods.

1. Calibration

a. Instrument calibration (not applicable to non-instrumental methods): Perform both instrument calibration and maintenance according to the manufacturer's instructions and recommendations. Conduct instrument-performance checks according to method or standard operating procedure (SOP) instructions.

b. Initial calibration: Perform initial calibration using

- at least three concentrations of standards and one blank (for linear calibrations),
- at least five concentrations of standards and one blank (for nonlinear calibrations), or
- as many concentrations as the method specifies.

The lowest concentration must be at or below the MRL, and the highest concentration should be at the upper end of the calibration range. Make sure the calibration range encompasses the concentrations expected in method samples or required dilutions. For the most accurate results, choose calibration standard concentrations no more than one order of magnitude apart [unless calibrating pH and ion-selective electrode (ISE) methods]. Some methods and instruments respond better to more orders of magnitude between concentrations. Refer to the individual method or manufacturer's instructions for calibrating pH and ISE methods.

Apply response-factor, linear, or quadratic-curve-fitting statistics (depending on what the method allows) to analyze the concentration–instrument response relationship. If the relative

standard deviation (%RSD) of the response factors is ≤15%, then the average response factor may be used. Otherwise, use a regression equation. The appropriate linear or nonlinear correlation coefficient for standard concentration-to-instrument response should be ≥0.995 for linear calibrations and ≥0.990 for quadratic calibrations. Weighting factors (e.g., 1/x or $1/x^2$) may be used to give more weight to the lower concentration points of the calibration. Depending on the method, calibration curves may be

- linear through the origin,
- linear not through the origin,
- nonlinear through the origin, or
- nonlinear not through the origin.

Some nonlinear functions can be linearized via mathematical transformations (e.g., log). The following acceptance criteria are recommended for various calibration functions (if the method does not specify acceptance criteria).

Compare each calibration point to the curve and recalculate its concentration. If any recalculated values are not within the method's acceptance criteria—up to twice the MRL ±50%; between 3 and 5 times the MRL ±20%; or greater than 5 times the MRL ±10%—unless otherwise specified in individual methods, identify the source of any outlier(s) and correct before sample quantitation.

NOTE: Do not use the correlation coefficient to verify a calibration's accuracy. That said, many methods still require calculation of the correlation coefficient and comparison to a specific limit.

Verify the initial calibration by analyzing a standard prepared from a different stock standard than that used to create the calibration curve; its concentration should be near the midpoint of the calibration range. The analytical results for this second-source mid-range standard must be within 10% of its true value. If not, determine the cause of the error, take corrective action, and re-verify the calibration. If the re-verification passes, continue the analyses; otherwise, repeat the initial calibration.

See the individual method or manufacturer's instructions for pH or ISE methods.

Use the initial calibration to quantitate analytes of interest in samples. Use CCV (¶ *c* below) only for calibration checks, not for sample quantitation. Perform initial calibration when the instrument is set up and whenever CCV criteria are not met.

c. Continuing calibration verification: In CCV, analysts periodically use a calibration standard to confirm that instrument performance has not changed significantly since initial calibration. Base the CCV interval on the number of samples analyzed (e.g., after every 10 samples and at least once per batch). Verify calibration by analyzing one standard whose concentration is near the midpoint of the calibration range. The results must be within allowable deviations from either initial-calibration values or specific points on the calibration curve. If the CCV is out of control, then take corrective action—including re-analysis of any samples analyzed since the last acceptable CCV.

Refer to the method for CCV frequency and acceptance criteria; if not specified, use the criteria given here. Other concentrations (e.g., one near the MRL) may be used, but be aware that the acceptance criteria may vary depending on the standard's concentration.

2. Operational Range and MDL Determination

Before using a new method or instrument, you should determine its operational (calibration) range (upper and lower limits). Calibrate according to 4020B.1, or verify the calibration by analyzing prepared standard solutions ranging from low to high concentrations. Determine the maximum concentration that can be measured within 10% of its true value based on the calibration curve: this is the *limit of linearity*. All samples whose concentrations are above the limit of linearity or the highest calibration point, whichever is lower, must be diluted.

If reporting results <MRL, initially estimate the MDL as a concentration about 3 to 5 times lower than the minimum calibration standard. The method for determining the MDL is based on the procedure outlined by the U.S. Environmental Protection Agency (EPA).[1]

To determine an MDL, prepare and analyze at least seven portions of a solution spiked at or near the minimum calibration concentration and an equal number of blanks. Analysts should prepare and analyze the spikes and blanks over ≥3 d rather than doing them all in one batch. If one MDL will be used for multiple instruments, then the MDL analysis must be performed across all of them (however, it is unnecessary to analyze all samples on all instruments). Analysts must prepare and analyze at least two spikes and two blanks on different calendar dates for each instrument. If evaluating more than three instruments, then one set of spikes and blanks can be analyzed on multiple instruments, so long as at least seven sets of spikes and blanks total are used. Alternatively, determine instrument-specific MDLs.

Calculate the estimated sample standard deviation, s_s, of the 7 replicates, and multiply by 3.14 to compute the MDL_s. Calculate MDL_b (MDL based on method blanks) using the following procedure.

If none of the method blanks give a numerical result (positive or negative), then MDL_b is not applicable, and MDL = MDL_s. If some give numerical results, then MDL_b equals the highest method blank result. If all of the method blanks give numerical results, calculate MDL_b as

$$MDL_b = X + 3.14S_b$$

where:

X = mean of blank results (set negative values to 0), and
S_b = standard deviation of blank results.

The MDL then equals whichever is greater: MDL_s or MDL_b.

If using more than 7 replicates, adjust the t value from 3.14 using student t tables with n-1 degrees of freedom.

For methods in this section, spike recovery for MDL determinations must be within 50 to 150%, with a %RSD of <20%. If it does not meet these criteria, the MRL spiking level and calculated MDL are too low and must be repeated at a higher concentration.

3. Initial Demonstration of Capability

Each analyst in the laboratory should conduct an IDC at least once before analyzing any sample to demonstrate proficiency in performing the method and obtaining acceptable results for each

analyte. The IDC also is used to demonstrate that a laboratory's modifications to a method will produce results as precise and accurate as those produced by the reference method. As a minimum, include a reagent blank and at least four LFBs at a concentration between one and four times the MRL (or other level specified in the method). Run the IDC after analyzing all required calibration standards. Ensure that the reagent blank does not contain any analyte of interest at a concentration greater than half the lowest calibration point (or other level specified in the method). Ensure that precision and accuracy (percent recovery) calculated for LFBs are within the acceptance criteria listed in the method of choice or generated by the laboratory (if there are no established mandatory criteria).

To establish laboratory-generated accuracy and precision limits, calculate the upper and lower control limits from the mean and standard deviation of percent recovery for ≥20 data points:

$$\text{Upper control limit} = \text{Mean} + 3(\text{Standard deviation})$$

$$\text{Lower control limit} = \text{Mean} - 3(\text{Standard deviation})$$

In the absence of established mandatory criteria, use laboratory-generated acceptance criteria for the IDC or else obtain acceptance criteria from a proficiency testing (PT) provider on PT studies and translate the data to percent recovery limits per analyte and method of choice. Ensure that lab-generated criteria are at least as tight as PT-study criteria, which are typically based on either multiple lab results or PT-provider-fixed limits.

4. Ongoing Demonstration of Capability

The ongoing demonstration of capability, sometimes called a *laboratory control sample* (LCS), *laboratory control standard, QC check sample,* or *laboratory-fortified blank,* is used to ensure that the laboratory analysis remains in control while samples are analyzed and separates laboratory performance from method performance on the sample matrix. This standard should be preserved in accordance with method requirements and carried through the entire procedure, including any digestions, extraction, or filtration. Purchase an external QC standard (if available) from a reputable supplier and use the certified acceptance limits as the laboratory acceptance criteria.

Acceptance criteria will vary depending on the method, matrix, and concentration. The concentration range should be either near the middle of the calibration range or near the maximum contaminant level (MCL), whichever is lower. Alternatively, prepare your own QC standard and calculate acceptance limits as ±2 standard deviations based on analysis of ≥20 replicates, unless the method specifies acceptance limits.

The ongoing demonstration of capability may be one of the following:

- acceptable performance of a blind sample analysis (single blind to the analyst);
- another IDC;
- at least four consecutive LCSs with acceptable levels of precision and accuracy (the laboratory shall determine acceptable precision and accuracy limits before analysis and shall tabulate or be able to readily retrieve four consecutive passing LCSs per method per analyst per year); or
- a documented analyst-review process using QC samples (QC samples can be reviewed to identify individual or group patterns and determine whether corrective action or retraining is necessary).

If none of these options is technically feasible, then review the analyses of real-world samples to ensure that results are within predefined acceptance criteria (established by the laboratory or method, whichever is tighter).

5. Reagent Blank

A *reagent blank* (method blank) consists of reagent water (see Section 1080) and all reagents (including preservatives) that normally are in contact with a sample during the entire analytical procedure. The reagent blank is used to determine whether and how much reagents and the preparative analytical steps contribute to measurement uncertainty. As a minimum, include one reagent blank with each sample set (batch) or on a 5% basis, whichever is more frequent. Analyze a blank after the CCV standard and before analyzing samples. Evaluate reagent-blank results for contamination; if contamination levels are unacceptable, identify and eliminate the source.

Positive sample results are suspect if analyte(s) in the reagent blank are >1/2MRL, unless the method specifies otherwise. Samples analyzed with a contaminated blank must be re-prepared and re-analyzed unless concentrations are ≥10 times those of the blank, concentrations are nondetect, or data user will accept qualified data. See method for specific reagent-blank acceptance criteria. General guidelines for qualifying sample results with regard to reagent-blank quality are as follows:

- If reagent blank is <MDL and sample results are >MRL, then no qualification is required.
- If reagent blank is >1/2MRL but <MRL and sample results are >MRL, then qualify results to indicate that analyte was detected in the reagent blank.
- If reagent blank is >MRL, then further corrective action and qualification is required.

6. Laboratory-Fortified Blank

A *laboratory-fortified blank* (LFB) is a reagent-water sample (with associated preservatives) to which a known concentration of the analyte(s) of interest has been added. The LFB may be used as the LCS (4020B.4) if the method requires a preliminary sample extraction or digestion.

An LFB is used to evaluate laboratory performance and analyte recovery in a blank matrix. Its concentration should be high enough to be measured precisely, but not high enough to be irrelevant to measured environmental concentrations. The analyst should rotate LFB concentrations to cover different parts of the calibration range. As a minimum, include one LFB with each sample set (batch) or on a 5% basis, whichever is more frequent. (The definition of a batch is typically project-specific.)

Process the LFB through all sample-preparation and -analysis steps. Use an added concentration of at least 10 × MDL, at or below the midpoint of the calibration curve, a method-specified level, or a level specified in a project plan's data quality objectives. Ideally, the LFB concentration should be less than the MCL (if the contaminant has one). Depending on method requirements, prepare the addition solution from either the same

TABLE 4020:I. MINIMUM QUALITY CONTROLS FOR METHODS IN PART 4000

SECTION	METHOD BLANK	LFB*	LFM† & LFMD‡	OTHER	SECTION	METHOD BLANK	LFB*	LFM† & LFMD‡	OTHER
4110B	×	×	×	1,3	4500-N B	×	×	×	3
4110C	×	×	×	1,3	4500-N C	×	×	×	3
4110D	×	×	×	1,3	4500-N D	×	×	×	3
4120B	×	×	×	1,3	4500-NH_3 C	×	×	×	7
					4500-NH_3 D	×	×	×	9
4140	×	×	×	1,3	4500-NH_3 E	×	×	×	9
					4500-NH_3 F	×	×	×	3
4500-B.B	×	×	×	3	4500-NH_3 G	×	×	×	3
4500-B.C	×	×	×	3	4500-NH_3 H	×	×	×	3
4500-Br^- B	×	×	×	3	4500-NO_2^-B	×	×	×	3
4500-Br^- D	×	×	×	3					
					4500-NO_3^-B	×	×	×	3
4500-CO_2 B	–	–	–	4	4500-NO_3^-C	×	×	×	3
4500-CO_2 C	×	–	–	2,8	4500-NO_3^-D	×	×	×	9
4500-CO_2 D	×	–	–	2	4500-NO_3^-E	×	×	×	3
					4500-NO_3^-F	×	×	×	3
4500-CN^- C	×	×	×	3	4500-NO_3^-H	×	×	×	3
4500-CN^- D	×	×	×	8	4500-NO_3^- I	×	×	×	3
4500-CN^- E	×	×	×	3					
4500-CN^- F	×	×	×	9	4500-N_{org} B	×	×	×	3
4500-CN^- G	×	×	×	3	4500-N_{org} C	×	×	×	3
4500-CN^- H	×	×	×	3	4500-N_{org} D	×	×	×	3
4500-CN^- I	×	×	×	3					
4500-CN^- J	×	–	–	3	4500-O B	–	–	–	2,7
4500-CN^- L	×	×	×	9	4500-O C	–	–	–	2,7
4500-CN^-M	×	×	×	3	4500-O E	–	–	–	2,7
4500-CN^- N	×	×	×	3	4500-O F	–	–	–	2,7
4500-CN^- O	×	×	×	3	4500-O G	–	–	–	2,6
					4500-O H				2,6
4500-Cl B	×	×	–	2,7					
4500-Cl C	×	×	–	2,7	4500-O_3 B	×	–	–	2
4500-Cl D	×	×	–	2,7					
4500-Cl E	×		–	2,7	4500-P C	×	×	×	3
4500-Cl F	×	×	–	2,7	4500-P D	×	×	×	3
4500-Cl G	×	×	–	2,3	4500-P E	×	×	×	3
4500-Cl H	×	×	–	2,3	4500-P F	×	×	×	3
4500-Cl I	×	×	–	2,9	4500-P G	×	×	×	3
					4500-P H	×	×	×	3
4500-Cl^- B	×	×	×	7	4500-P I	×	×	×	3
4500-Cl^- C	×	×	×	7	4500-P J	×	×	×	3
4500-Cl^- D	×	×	×	7					
4500-Cl^- E	×	×	×	3	4500- $KMnO_4$ B	×	×	×	3
4500-Cl^- G	×	×	×	3					
					4500-SiO_2 C	×	×	×	3
4500-ClO2 B	×	–	–	7	4500-SiO_2 D	×	×	×	3
4500-ClO_2 C	×	–	–	7	4500-SiO_2 E	×	×	×	3
4500-ClO_2 E	×	–	–	7	4500-SiO_2 F	×	×	×	3
4500-F^-C	×	×	×	9	4500-S^{2-} D	×	×	×	3
4500-F^-D	×	×	×	3	4500-S^{2-} E	×	×	×	3
4500-F^-E	×	×	×	3	4500-S^{2-} F	×	×	×	7
4500-F^-G	×	×	×	3	4500-S^{2-} G	×	×	×	9
					4500-S^{2-} I	×	×	×	3
4500-H^+ B	–	–	–	2,5,9	4500-S^{2-} J	×	×	×	3
4500-I B	×	×	×	3	4500-SO_3^{2-} B	×	×	–	7
4500-I C	×	×	×	7	4500-SO_3^{2-} C	×	×	–	3

TABLE 4020:I. CONT.

SECTION	METHOD BLANK	LFB*	LFM† & LFMD‡	OTHER	SECTION	METHOD BLANK	LFB*	LFM† & LFMD‡	OTHER
4500-I^- B	×	×	×	3	4500-SO_4^{2-} C	×	×	×	8
4500-I^- C	×	×	×	3	4500-SO_4^{2-} D	×	×	×	8
4500-I^- D	×	×	×	7	4500-SO_4^{2-} E	×	×	×	3
					4500-SO_4^{2-} F	×	×	×	3
4500-IO_3^- B	×	×	×	7	4500-SO_4^{2-} G	×	×	×	3

* Laboratory-fortified blank.
† Laboratory-fortified matrix.
‡ Laboratory-fortified matrix duplicate.
× indicates that a QC type is mandatory for the method.
1. Additional QC guidelines in method.
2. Duplicates of the sample will be run.
3. Refer to 4020B for further QC requirements.
4. Compare to results from Section 4500-CO_2.D.
5. Additional QC check with pH standard whose value is bracketed by calibration standards.
6. Zero check with zero oxygen sample.
7. Refer to 4020B for other QC requirements, no calibration curve required (use or standardize against a primary standard).
8. Refer to 4020B for other QC requirements, no calibration curve required (verify the accuracy of analytical balances with NIST-traceable weights).
9. Refer to 4020B for other QC requirements (verify slope according to manufacturer's instructions).
This table is not comprehensive; refer to the specific method and 4020B for further details.

reference source used for calibration or an independent source. Evaluate the LFB for percent recovery of the added analytes by comparing results to method-specified limits, control charts, or other approved criteria. If LFB results are out of control, take corrective action, including re-preparation and re-analysis of associated samples if required. Use LFB results to evaluate batch performance, calculate recovery limits, and plot control charts.

7. Laboratory-Fortified Matrix

A *laboratory-fortified matrix* (LFM) is an additional portion of a sample to which a known amount of the analyte(s) of interest is added before sample preparation. Some analytes are not appropriate for LFM analysis; see Table 4020:I and specific methods for guidance on when an LFM is relevant.

The LFM is used to evaluate analyte recovery in a sample matrix. If an LFM is feasible and the method does not specify LFM frequency requirements, then include at least one LFM with each sample set (batch) or on a 5% basis, whichever is more frequent. Add a concentration that is at least 10 × MRL, less than or equal to the midpoint of the calibration curve, or method-specified level to the selected sample(s). The analyst should use the same concentration as for LFB (4020B.6) to allow analysts to separate the matrix's effect from laboratory performance. Prepare LFM from the same reference source used for LFB. Make the addition such that sample background levels do not adversely affect recovery (preferably adjust LFM concentrations if the known sample is more than five times the background level). At a minimum, the spike must at least equal the background concentration, unless the method specifies otherwise. For example, if the sample contains the analyte of interest, then add approximately as much analyte to the LFM sample as the concentration found in the known sample.

Evaluate LFM results for percent recovery; if they are not within control limits, then take corrective action to rectify the matrix effect, use another method, use the method of standard addition, or flag the data if reported. See method for specific LFM-acceptance criteria until the laboratory develops statistically valid, laboratory-specific performance criteria. If the method does not provide limits, use the calculated preliminary limits from the IDC (4020B.3). LFM control limits may be wider than for LFB or LCS, and batch acceptance generally is not contingent upon LFM results.

8. Duplicate Sample/Laboratory-Fortified Matrix Duplicate

Duplicate samples are analyzed to estimate precision. If an analyte is rarely detected in a matrix type, use an LFM duplicate. An *LFM duplicate* is a second portion of the sample described in 4020B.7 to which a known amount of the analyte(s) of interest is added before sample preparation. If sufficient sample volume is collected, this second portion of sample is added and processed in the same way as the LFM. As a minimum, include one duplicate sample or one LFM duplicate with each sample set (batch) or on a 5% basis, whichever is more frequent, and process it independently through the entire sample preparation and analysis.

Evaluate LFM duplicate results for precision and accuracy (precision alone for duplicate samples). If LFM duplicate results are out of control, then take corrective action to rectify the matrix effect, use another method, use the method of standard addition, or flag the data if reported. If duplicate results are out of control, then re-prepare and re-analyze the sample and take additional corrective action, as needed. When the value of one or both duplicate samples is ≤5 × MRL, the laboratory may use the MRL as the control limit for percent recovery, and the duplicate results are not used to measure precision. See method for specific acceptance criteria for LFM duplicates or duplicate samples until the laboratory develops statistically valid, laboratory-specific performance criteria. If the method does not provide limits, use the calculated preliminary limits from the IDC. In general, batch acceptance is not contingent upon LFM duplicate results.

9. Verification of MDL and MRL

With each analytical batch, analyze a reagent-water sample spiked at MRL and ensure that it meets MRL acceptance criteria

(generally ±50%). If not, re-analyze the entire batch or flag results for all samples in the batch. If the MRL is biased high, nondetect (ND) samples can be reported with flags if the method or regulation allows.

If reporting to the MDL, then verify the MDL at least quarterly by analyzing a sample spiked at the same level used to determine the MDL and ensure that the result is positive. If two consecutive MDL-verification samples do not produce positive results, then recalculate the MDL using the most recent set of at least 7 blanks and MRL level spikes, following the protocols outlined in 4020B.2.

10. QC Calculations

The following is a compilation of equations frequently used in QC calculations.

a. Laboratory-fortified matrix (LFM) sample (matrix spike sample):

$$\text{LFM \% Recovery} = \left[\frac{LFM\ conc \times (spike\ vol + sample\ vol) - (sample\ conc \times sample\ vol)}{spike\ solution\ conc \times spike\ vol}\right] \times 100$$

b. Relative percent difference (RPD):

$$\left[\frac{|LFM - LFMD|}{\left(\frac{LFM + LFMD}{2}\right)}\right] \times 100 = \%RPD$$

or

$$\left[\frac{|D_1 - D_2|}{\left(\frac{D_1 + D_2}{2}\right)}\right] \times 100 = \%RPD$$

where:

LFM = concentration determined for LFM,
$LFMD$ = concentration determined for LFMD,
D_1 = concentration determined for first duplicate, and
D_2 = concentration determined for second duplicate.

c. Initial calibration: See Section 1020B.12*a*.
d. Calibration verification: See Section 1020B.12*b*.
e. Laboratory-fortified blank recovery: See Section 1020B.12*c*.
f. Laboratory-fortified matrix: See Section 1020B.12*e*.
g. Standard additions: See Section 1020B.12*g*.

11. Control Charts

See Section 1020B.13.

12. Reference

1. U.S. Environmental Protection Agency. 2016. III.H. Changes to method detection limit (MDL) procedure. *In* Clean Water Act Methods Update Rule for the Analysis of Effluent. 40 CFR 136.

4110 DETERMINATION OF ANIONS BY ION CHROMATOGRAPHY*

4110 A. Introduction

Determination of the common anions such as bromide , chloride, fluoride, nitrate, nitrite, phosphate, and sulfate often is desirable to characterize a water and/or to assess the need for specific treatment. More recently, the need to measure the concentration of the disinfection by-products chlorite, chlorate, and bromate has arisen. Although conventional colorimetric, electrometric, or titrimetric methods are available for determining individual anions, ion chromatography provides a single instrumental technique that may be used for their rapid, sequential measurement. Ion chromatography eliminates the need to use hazardous reagents and it effectively distinguishes among the halides (Br^-, Cl^-, and F^-) and the oxyhalides (ClO_2^-, ClO_3^-, and BrO_3^-), and the oxy-ions (PO_4^{3-}, SO_4^{2-}, NO_2^-, and NO_3^-).

Methods 4110B and 4110C are applicable, after filtration to remove particles larger than 0.45 μm, to surface, ground, and wastewaters as well as drinking water. Some industrial process waters, such as boiler water and cooling water, also may be analyzed by this method. Method 4110D is applicable to untreated and finished drinking water as well as drinking water at various stages of treatment.

* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011. Joint Task Group: 21st Edition—Richard Mosher (chair), Bruce A. Hale, Daniel P. Hautman, Peter E. Jackson, S. V. Karmarkar, James Krol, James W. O'Dell, Roy-Keith Smith.

4110 B. Ion Chromatography with Chemical Suppression of Eluent Conductivity

1. General Discussion

a. Principle: A water sample is injected into a stream of eluent and passed through a series of ion exchangers. The anions of interest are separated on the basis of their relative affinities for a low-capacity, strongly basic anion exchanger (guard and analytical columns). The separated anions are directed through a suppressor device that provides continuous suppression of eluent conductivity and enhances analyte response. In the suppressor the separated anions are converted to their highly conductive acid forms while the conductivity of the eluent is greatly decreased. The separated anions in their acid forms are measured by conductivity. They are identified on the basis of retention time as compared to standards. Quantitation is by measurement of peak area or peak height.

b. Interferences: Any substance that has a retention time coinciding with that of any anion to be determined and produces a detector response will interfere. Low-molecular-weight organic acids, bromate, and chlorite may interfere with the determination of chloride and fluoride. A high concentration of any one ion also interferes with the resolution, and sometimes retention, of others. Sample dilution or gradient elution overcomes many interferences. To resolve uncertainties of identification or quantitation use the method of known additions. Spurious peaks may result from contaminants in reagent water, glassware, or sample processing apparatus. Modifications such as preconcentration of samples, gradient elution, or reinjection of portions of the eluted sample may alleviate some interferences but require individual validation for precision and bias and are beyond the scope of this method.

c. Method detection level: The detection level of an anion is a function of sample size. Table 4110:I presents detection levels obtained for reagent water with a 25-μL sample loop. Detection levels in natural waters may be substantially higher because of the presence of high levels of some of the anions.

Table 4110:I. Detection Level for Anions in Reagent Water.*

Anion	MDL $\mu g/L$
Fluoride	2.0
Chloride	4.0
Nitrite-N	3.7
Bromide	14
Nitrate-N	2.7
Orthophosphate-P	14
Sulfate	18

* See Figure 4110:1 for experimental conditions.

d. Limitations: Exercise caution if this method is used to determine F^- in unknown matrices. Two problems are commonly encountered: first, with some column/eluent combinations the fluoride peak elutes very close to the baseline depression caused by the elution of water, the so-called "water dip". This may cause difficulty in quantitating samples with low fluoride concentrations; second, the simple organic acids (formic, acetate) elute close to fluoride and may interfere. Determine precision and bias before analyzing samples. If fluoride is to be determined, preferably select a column/eluent combination that resolves water, fluoride, and simple organic acids.

Because of the utilization of nitrate, nitrite, and phosphate as nutrients by some species of bacteria, store samples at 4°C and analyze within 48 h. Disinfected samples to be analyzed for nitrate may be held for up to 14 d because all nitrite will already be converted to nitrate. Store samples to be analyzed for sulfate

at 4°C and analyze within 28 d. The other analytes do not require cold storage. Complete analysis within 28 d.

2. Apparatus

a. Ion chromatograph, including an injection valve, a sample loop, guard column, analytical column, suppressor device, a temperature-compensated small-volume conductivity cell and detector (6 μL or less), and an electronic peak integrator or chromatography data acquisition system. Use an ion chromatograph capable of delivering 2 to 5 mL eluent/min at a pressure of 5600 to 28 000 kPa (800 to 4000 psi).

b. Analytical column: Any commercially available anion-exchange column capable of resolving fluoride, bromide, chloride, nitrate, nitrite, phosphate, and sulfate is acceptable.*

c. Guard column, identical to separator column† to protect analytical column from fouling by particulates or organics.

d. Suppressor device:‡ Place this ion-exchange-based device between column and detector to reduce background conductivity of the eluent and enhance conductivity of the target analytes. Several such devices with different operational principles are available commercially; any that provides the required sensitivity and baseline stability may be used.

3. Reagents

a. Reagent water free from interferences at the method detection level of each constituent with 18 megohm resistivity and containing no particles larger than 0.2 μm. See Section 1080.

b. Eluent solution, appropriate to column used to resolve target anions. The eluent used to produce the chromatogram in Figure 4110:1 was sodium bicarbonate-sodium carbonate, 1.7 m*M* $NaHCO_3^-$, 1.8 m*M* Na_2CO_3. Dissolve 0.5712 g $NaHCO_3$ and 0.7632 g Na_2CO_3 in water and dilute to 4 L. Degas eluent before use either by vacuum filtration to simultaneously remove particles greater than 0.45 μm or by purging with helium for 10 min.

c. Regenerant solution: Required with some types of suppressors. See manufacturer's recommendations.

d. Standard anion solutions, 1000 mg/L: Purchase stock standard solutions as certified solutions or prepare from ACS reagent-grade salts. Prepare standard anion solutions by weighing the indicated amount of salt (Table 4110:II) , dried to a constant weight at 105°C, and diluting to 100 mL with distilled/deionized water. Store in plastic bottles at 4°C. These solutions are stable for at least 6 months except for the nitrite and phosphate standards, which should be discarded after 1 month. Prepare most dilute working standards monthly, nitrite and phosphate, daily.

4. Procedure

a. System equilibration: Turn on ion chromatograph and adjust eluent flow rate to manufacturer's recommendations for the column/eluent combination being used. A representative chromatogram is presented in Figure 4110:1. Adjust detector to desired setting (usually 10 to 30 μS) and let system come to equilibrium (15 to 20 min). A stable base line indicates equilibrium conditions. Adjust detector offset to zero out eluent conductivity. If regenerant is used with the suppressor, adjust flow rate to manufacturer's specifications.

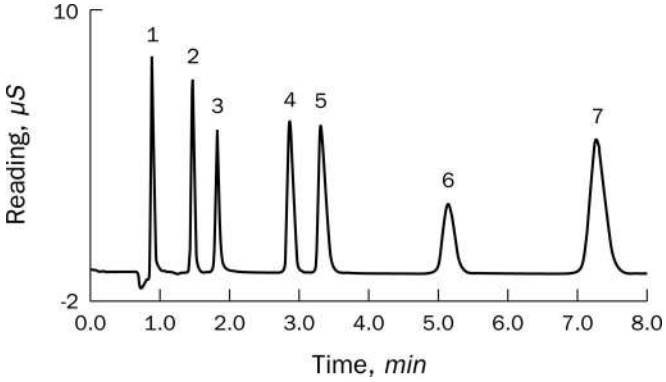


Figure 4110:1. Typical inorganic anion separation. Eluent: 1.7 m*M* $NaHCO_3$, 1.8 m*M* $NaCO_3$; sample loop: 25 μL; flow rate: 2.0 mL/min; column: Dionex AG4A-SC plus AS4A-SC.

Anion	Conc *mg/L*
1. Fluoride	2.0
2. Chloride	3.0
3. Nitrite	5.0
4. Bromide	10.0
5. Nitrate	10.0
6. Orthophosphate	15.0
7. Sulfate	15.0

TABLE 4110:II. STOCK STANDARD PREPARATIONS

Anion	Salt	Amount *g/100 mL*
Fluoride	NaF	0.2210
Chloride	NaCl	0.1649
Bromide	NaBr	0.1288
Nitrate-N*	$NaNO_3$	0.6068
Nitrite-N*	$NaNO_2$	0.4926
Phosphate-P	KH_2PO_4	0.4394
Sulfate	K_2SO_4	0.1814

* Do not oven-dry. Dry to constant weight in a dessicator.

b. Calibration: Inject standards containing a single anion or a mixture and determine approximate retention times. Observed times vary with conditions. If the analytical column and eluent mentioned in 4110B.2*b* and 3*b*, respectively, are used, retention always is in the order F^-, Cl^-, NO_2^-, Br^-, NO_3^-, HPO_4^{2-}, and SO_4^{2-}. Inject at least three different concentrations for each anion to be measured. Use concentrations that will bracket the expected analyte concentrations in samples. Construct a calibration curve by plotting peak height or area versus concentration using appropriate software. Verify calibration curves with a mid-range check standard from a source independent of that of the calibration standards. Check validity of existing calibration curves daily with a mid-range calibration standard. Results should be within 10% of original curve at mid-range.

* Dionex P/N 37041, Lachat P/N 28084, Waters P/N 26765, or equivalent.
† Dionex P/N 37042, Lachat P/N 28085, or equivalent.
‡ Dionex P/N 46081, Lachat P/N 28097, Alltech P/N 535101, or equivalent.

TABLE 4110:III. SINGLE-LABORATORY PRECISION (ONE STANDARD DEVIATION) AND BIAS DATA FOR 30 SETS OF SAMPLES OVER A 2-MONTH-PERIOD

Element	LFB Concentration *mg/L*	LFB Recovery and Precision *%*	Known Addition Concentration *mg/L*	Known Addition Recovery and Precision *%*
Chloride	25	104 ± 4.5	25	107 ± 10
Nitrite as N	1	97 ± 4	1	103 ± 7
Bromide	0.02	101 ± 8	0.1 to 0.5	106 ± 10
Bromide	0.3	102 ± 3	—	—
Nitrate as N	2.5	106 ± 2.6	2.5	113 ± 5
Orthophospate-P	10	101 ± 4	10	102 ± 4
Sulfate	50	105 ± 4	50	111 ± 6

Recalibrate whenever the detector setting, eluent, or regenerant is changed. To minimize the effect of the "water dip"§ on F^- analysis, analyze standards that bracket the expected result or eliminate the water dip by diluting the sample with eluent or by adding concentrated eluent to the sample to give the same concentration as in the eluent. If sample adjustments are made, adjust standards and blanks identically.

If linearity is established ($r \geq 0.99$) over the calibration range, the average response factor is acceptable. Record peak height or area for calculation of the response factor, RF. HPO_4^{2-} is nonlinear below 1.0 mg/L.

c. Sample analysis: If sample is collected with an autosampler that does not automatically filter samples, remove particulates by filtering through a prewashed 0.45-μm (or smaller) pore membrane. With either manual or automated injection, flush loop with several volumes of sample. Take care to prevent carryover of analytes from samples of high concentration. After last peak has appeared and detector signal has returned to base line, another sample can be injected.

d. Solid matrices: Soluble forms of the target anions may be determined in solid matrices (soils, sludges) after extraction and filtration of the extract. A slurry of the solid to be extracted is prepared with either reagent water or eluent and is either shaken or sonicated. (A representative standardized method for such extractions is available.[1]) Document the precision of the extraction process and the analyte recovery achieved by analyzing duplicate laboratory-fortified matrices for each distinct matrix.

5. Calculations

Determine the concentration of each anion, in milligrams per liter, by referring to the appropriate calibration curve. Alternatively, when the response is shown to be linear, use the following equation:

$$C = H \times RF \times D$$

where:

C = mg anion/L,
H = peak height or area,
RF = response factor = concentration of standard/height (or area) of standard, and
D = dilution factor.

§ Water dip occurs because water conductivity in sample is less than eluent conductivity (eluent is diluted by water).

6. Quality Control

The QC practices considered to be an integral part of each method are summarized in Table 4020:I. Preferably check recovery daily at reporting level using a reporting-level standard. Recovery should be between 75 and 125%. Alternate analysis of mid-range and high-range check standards after each 10 samples. Recovery should be between 90 and 110%. If the results are to be used for environmental compliance monitoring, document precision and accuracy of the method by the analysis of four replicates of a mid-range calibration standard and calculation of the average percent recovery, and the standard deviation of the recoveries, for each analyte. Additional QC may be required for regulatory purposes.

7. Precision and Bias

Multilaboratory data from a joint validation study with EPA and ASTM using older columns and hardware can be found in the 20th Edition of *Standard Methods*. Table 4110:III shows single-laboratory recoveries for laboratory-fortified blanks (LFB) and known additions to a variety of raw waters and finished drinking waters obtained with columns and equipment described herein.

8. Reference

1. AMERICAN SOCIETY FOR TESTING & MATERIALS. 1992. Standard Practice for Shake Extraction of Solid Waste with Water; D3987. *In* Annual Book of ASTM Standards, Vol. 11.01 Water. Philadelphia, Pa.

9. Bibliography

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PFAFF, J.D., D.F. HAUTMAN & D.J. MUNCH. 1997. Determination of Inorganic Anions in Drinking Water by Ion Chromatography; Method 300.1. U.S. Environmental Protection Agency, National Exposure Research Lab., Off. Research & Development, Cincinnati, Ohio.

4110 C. Single-Column Ion Chromatography with Direct Conductivity Detection

1. General Discussion

a. Principle: An aqueous sample is injected into an ion chromatograph consisting of an injector port, analytical column, and conductivity detector. The sample merges with the eluent stream and is pumped through the analytical column where the anions are separated on the basis of their affinity for the active sites of the column packing material. Direct conductivity detection without chemical suppression is used to determine concentrations.

b. Interferences: See 4110B.1*b*. Note that HCO_3^- elutes between F^- and Cl^- with this method. See Figure 4110:2.

Because method sensitivity is high, take care to avoid contamination by reagent water and equipment.

c. Method detection level: The detection level of an anion is a function of sample volume injected and the signal-to-noise ratio of the detector electronics. Generally, minimum detectable concentrations are about 0.02 to 0.12 mg/L (20 to 120 μg/L) for the anions with an injection volume of 100 μL (Table 4110:IV). Larger injection volumes can reduce detection levels. However, coelution is a possible problem with large injection volumes. Determine method detection level for each anion of interest under the conditions used to produce reportable data.

2. Apparatus

a. Ion chromatograph, complete with all required accessories including syringes, analytical columns, detector, and a data system. Required accessories are listed below.

b. Guard column, placed before analytical column to protect it from fouling by particulates or organic constituents.*

c. Analytical column, packed with low-capacity anion-exchange resin capable of resolving fluoride, chloride, nitrite, bromide, nitrate, orthophosphate, and sulfate.†

d. Conductivity detector, flow-through, with integral heat-exchange unit allowing direct temperature control and with separate working and reference electrodes.

e. Pump, constant flow between 0.5 and 5.0 mL/min at a pressure of 5600 to 28 000 kPa (800 to 4000 psi).

f. Data system, preferably computer with software capable of acquiring and processing chromatographic data. An integrator also may be used.

g. Sample injector: Either an automatic sample processor or a manual injector. The automatic device must be able to inject a minimum sample volume of 10 μL.

3. Reagents

a. Reagent water: See 4110B.3*a*.

b. Borate/gluconate concentrate: Place approximately 25 g of a strong cation-exchange resin‡ in the hydrogen form in a 60-mL plastic syringe fitted with a 0.45-μm filter. Wash resin with five 20-mL portions of deionized water. Dissolve 9.06 g sodium gluconate in 20 mL deionized water. Transfer this solution to the 60-mL syringe and slowly pass through the syringe into a 1-L volumetric flask. Wash resin with five 20-mL portions deionized water, adding washings to flask. Discard syringe and resin. Alternatively use commercially available 50% gluconic acid. This usually comes as a brown solution that when diluted gives a yellow tint to the eluent that may affect long-term performance. Remove color by passing the 50% gluconic acid through a C_{18} cartridge (5 mL/cartridge). Use 13.2 mL of the 50% gluconic acid for the eluent concentrate.

Adjust volume in the 1-L volumetric flask to approximately 500 mL with deionized water and add a stirring bar. Add 7.2 g lithium hydroxide monohydrate and 25.5 g boric acid. Stir until all reagents are dissolved. Add 94 mL 95% glycerol and mix well. Remove stirring bar and dilute to 1 L. This may be stored at room temperature for 6 months or at 4°C for 1 year (warm to ambient before use). Discard if evidence of microbial growth appears.

c. Eluent solution, 8.25 m*M* borate, 0.83 m*M* gluconate, 12% (v/v) acetonitrile: Combine 20 mL borate/gluconate concentrate and 120 mL HPLC-grade acetonitrile, and dilute to 1 L with reagent water. Vacuum filter through a 0.45-μm- (or smaller) pore size membrane before use.

d. Stock standard solutions: See 4110B.3*d*.

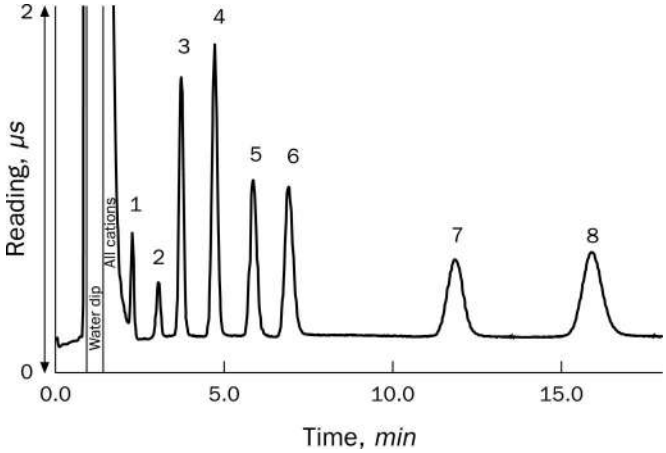


Figure 4110:2. Typical inorganic anion separation. Eluent: borate/gluconate containing 12% acetonitrile; flow rate: 1.0 mL/min; injection volume: 100 μL; column: Waters IC Pak A/HR; background conductivity: 240 μS.

Anion	Conc *mg/L*
1. Fluoride	1.0
2. Bicarbonate	—*
3. Chloride	2.0
4. Nitrite	4.0
5. Bromide	4.0
6. Nitrate	4.0
7. Orthophosphate	6.0
8. Sulfate	4.0

* Not quantified.

* Waters P/N WAT010551, or equivalent.
† Waters P/N WAT0026765, or equivalent.
‡ Bio-Rad AG-50W-X12, or equivalent.

Table 4110:IV. Detection Level for Anions in Reagent Water

Anion	MDL $\mu g/L$
Fluoride	40
Chloride	20
Nitrite-N	15
Bromide	75
Nitrate-N	17
Orthophosphate-P	40
Sulfate	75

4. Procedure

a. System equilibration: Set up ion chromatograph in accordance with the manufacturer's directions. Install guard and separator columns and begin pumping eluent until a stable base line is achieved. The background conductivity of the eluent solution is 240 ± 20 μS.

b. Calibration: Determine retention time for each anion by injecting a standard solution containing only the anion of interest and noting the time required for a peak to appear. Retention times may vary with operating conditions and with anion concentration. The order of elution is shown in Figure 4110:2.

Construct a calibration curve by injecting prepared standards including each anion of interest. Use at least three concentrations plus a blank. Bracket the range of concentrations expected for samples. Construct calibration curve by plotting either peak height or peak area versus concentration. If a data system is being used, make a hard copy of the calibration curve available.

After generating calibration curves, verify them with a mid-range check standard from a source independent of that of the calibration standards. Verify working calibration curves daily by injecting a mid-range standard. Agreement should be within 10%. Also verify curves with each batch of eluent. If linearity is established over the calibration range, the average response factor is acceptable. Record peak height or area for calculation of the response factor (RF).

c. Sample analysis: Inject sufficient sample (about two to three times the loop volume) to ensure that sample loop is properly flushed. Let all peaks elute before injecting another sample. Compare response in peak height or peak area and retention time to values obtained in calibration.

Table 4110:V. Single-Column Chromatography Single-Operator Precision and Bias*

Anion	Sample Type†	Amount Added *mg/L*	Mean Recovery %	SD *mg/L*
Cl^-	RW	16	105	1.6
	DW	16	98	1.9
NO_2^--N	RW	4	101	0.10
	DW	4	101	0.43
Br^-	RW	16	104	0.75
	DW	16	98	2.3
NO_3^--N	RW	8	103	0.75
	DW	8	87	1.9
PO_4^{3-}-P	RW	16	113	0.92
	DW	16	110	1.6
SO_4^{2-}	RW	32	101	0.42
	DW	32	94	4.8

* Data provided by EPA/EMSL (NERL), Cincinnati, OH 45268. Seven replicates were analyzed for each anion and sample type.
† RW=reagent water; DW=drinking water.

5. Calculation

See 4110B.5.

6. Quality Control

See 4110B.6.

7. Precision and Bias

Precision and bias data are given in Table 4110:V. These data were produced in 1988. Note: The columns and other hardware discussed herein were not available when the study was conducted. The advances in technology that have occurred since that time should be considered when using these data.

8. Bibliography

Glaser, J., D. Foerst, G. McKee, S. Quave & W. Budde. 1981. Trace analyses for wastewater. *Environ. Sci. Technol.* 15:1426.

4110 D. Ion Chromatographic Determination of Oxyhalides and Bromide

1. General Discussion

a. Principle: See 4110B.1*a*.

b. Interferences: The need to quantitate low levels of disinfection byproducts and their precursors in the presence of much higher levels of the common anions poses an analytical problem. Any ionic material that coelutes with a target analyte will interfere with the determination of that analyte. Bromate has been shown to be subject to positive interferences in some matrices. The interference is noticeable usually as a flattened peak. It often can be eliminated by passing the sample through an H^+ cartridge.* This problem may be addressed by selection of a different column/eluent combination or by dilution of the eluent, which will increase retention times and spread the chromato-

* Dionex PN 039596, or equivalent.

Table 4110:VI. Detection Level for Anions in Reagent Water*

Anion	MDL μg/L
Bromide	0.98
Bromate	1.32
Chlorate	2.55
Chlorite	1.44

* Determined under the following operating conditions: Eluent: 9 m*M* Na_2CO_3; flow rate: 1.25 mL/min; columns: Dionex AG9-HC and AS9-HC, 4 mm; sample loop: 200 μL; suppressor: Dionex ASRS-I, 300 mA in external water mode; detector: Dionex CD20.
Source: Pfaff, J.D., D.F. Hautman & D.J. Munch. 1997. Determination of Inorganic Ions in Drinking Water by Ion Chromatography. EPA Method 300.1, U.S. Environmental Protection Agency, National Exposure Research Lab., Off. Research & Development, Cincinnati, Ohio.

gram. Additionally, chloride or a non-target analyte present in unusually high concentration may overlap with a target analyte sufficiently to cause problems in quantitation or may cause retention-time shifts. Dilution of the sample may resolve this problem. Care must be exercised to avoid carryover from a sample of high concentration to the subsequent sample. Method interferences also may be caused by contamination of reagents, reagent water, glassware, syringes, and other equipment used to process samples.

c. Method detection level: The detection levels for bromide, bromate, chlorate, and chlorite in reagent water free of interfering anions are shown in Table 4110:VI.

d. Sample collection and storage: Collect samples to be analyzed for chlorite in opaque containers and store them at 4°C. All of the oxyhalides, including chlorite, require preservation with 50 mg/L ethylenediamine (EDA).

Residual chlorine dioxide present in the sample will result in the formation of additional chlorite between the times of sampling and analysis. If chlorine dioxide is suspected to be present, purge sample with an inert gas such as nitrogen, helium, or argon for approximately 4 min at time of collection, before adding ethylenediamine preservative.

2. Apparatus

a. Ion chromatograph: See 4110B.2*a*.

b. Analytical column: Any commercially available column† capable of resolving the oxyhalides chlorite, chlorate, and bromate, from the other anions that may be present in drinking water, including fluoride, chloride, nitrite, nitrate, phosphate, and sulfate, is acceptable.

c. Guard column: This is a short column‡ packed with the same stationary phase as the analytical column, used to protect the analytical column from particulates and organics.

d. Pretreatment cartridge for removal of interferences such as cations and carbonates.

e. Suppressor device: See 4110B.2*d*.

† Dionex P/N 51786, or equivalent.
‡ Dionex P/N 51791, or equivalent.

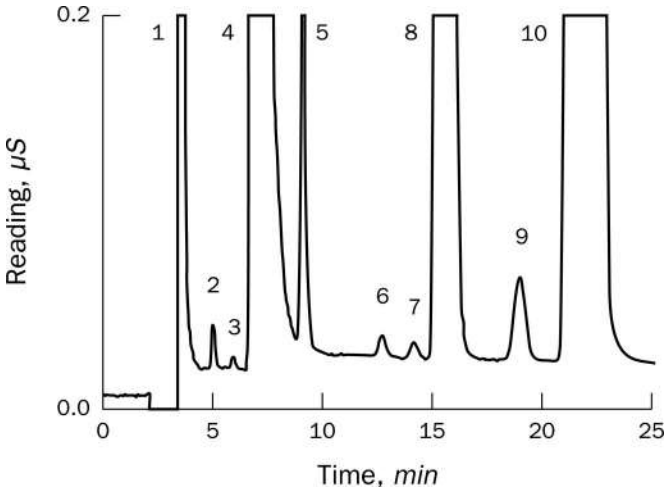


Figure 4110:3. Typical separation in a simulated drinking water sample. Eluent: 9 m*M* Na_2CO_3; flow rate: 1.0 mL/min; columns: Dionex AG9-HC and AS9-HC, 4 mm; sample loop: 200 μL; suppressor: Dionex ASRS, 100 mA in external water mode at 10 mL/min; detector: Dionex CD20 stabilized at 35°C.

Anion	Conc mg/L
1. Fluoride	1.0
2. Chlorite	0.01
3. Bromate	0.005
4. Chloride	50
5. Nitrite	0.1
6. Bromide	0.01
7. Chlorate	0.01
8. Nitrate	10
9. Phosphate	0.1
10. Sulfate	50

3. Reagents

a. Reagent water: See 4110B.3*a*.

b. Eluent solution, appropriate to the column used to resolve the target anions. Figure 4110:3 was obtained by using 9 m*M* sodium carbonate prepared as follows: Dissolve 1.91 g sodium carbonate in reagent water and dilute to 2 L. Vacuum filter eluent through a 0.45-μm or smaller filter to remove particles that could ultimately plug the column. This also degasses the eluent.

c. Regenerant solution (required with some suppressors): See manufacturers' recommendations.

d. Standard anion solutions, 1000 mg/L: Purchase stock standard solutions as certified solutions or prepare from ACS reagent-grade potassium or sodium salts. See Table 4110:VII.

e. Ethylenediamine (EDA) preservation solution, 100 mg/mL: Dilute 2.8 mL EDA (99%) to 25 mL with reagent water. This solution is stable for 1 month.

4. Procedure

a. System equilibration: See 4110B.4*a*.

TABLE 4110:VII. STOCK STANDARD PREPARATION

Anion	Salt	Amount *g/100 mL*
Bromide	NaBr	0.1288
Bromate	$NaBrO_3$	0.1180
Chlorate	$NaClO_3$	0.1275
Chlorite*	$NaClO_2$	0.1676

* High-purity sodium chlorite is not commercially available. The value assumes the use of a salt that is exactly 80% pure. Preferably confirm concentration of the chlorite stock standard by iodometric titration (Section 4500-ClO_2.C). Also demonstrate that this stock is free of the other target anions before preparing mixed working standards.

b. Calibration: Prepare a series of calibration standards by diluting the stock standards appropriately to bracket the expected analyte concentrations in the samples. If the analytical column and eluent described in 4110D.2*b* and 3*b* are used, retention is in the order chlorite, bromate, bromide, and chlorate (See Figure 4110:3). Use at least three concentrations for each anion and construct calibration curves by plotting peak height or area (recommended) against concentration using appropriate software. If linearity is established ($r \geq 0.99$) the average response factor may be used in place of a calibration curve. Verify calibration curves with a mid-range standard from a source independent of that of the calibration standards. Check validity of existing calibration curves daily with a mid-range calibration standard. Agreement should be within 10% at mid-range. Also verify existing calibration curves whenever eluent or regenerant is changed. A change in detector setting usually will require recalibration. Samples preserved with EDA may show a bias attributable to the presence of this compound. If such a bias is suspected, add EDA to each calibration standard.

c. Sample analysis: See 4110B.4*c*.

5. Calculations

See 4110B.5.

6. Quality Control

See 4110B.6.

NOTE: If any sample is pretreated, also pretreat QC samples.

7. Precision and Bias

The data in Table 4110:VIII provide single-laboratory precision and bias information for a variety of matrices. The HIW matrix is designed to simulate a high-ionic-strength field sample. It was prepared from reagent water that was fortified with chloride at 100 mg/L, carbonate at 100 mg/L, nitrate at 10 mg/L as nitrogen, phosphate at 10 mg/L as phosphorus, and sulfate at 100 mg/L.

TABLE 4110:VIII. SINGLE-OPERATOR PRECISION AND ACCURACY FOR BROMIDE, CHLORATE, CHLORITE, AND BROMATE*

Analyte	Matrix†	Unfortified Conc μg/L‡	Fortified Conc μg/L	Mean Conc μg/L	Mean Recovery§ %R	SD(n−1) μg/L	RSD %
Bromide	RW	<DL	20.0	20.9	104	0.50	3.82
			100	107	107	0.60	0.56
	HIW	3.24	20.0	21.8	92.5	0.79	3.63
			100	105	102	1.05	1
	SW	31.0	20.0	51.3	—§	0.97	1.9
			100	140	109	1.88	1.35
	GW	151	20.0	172	—	0.78	0.45
			100	265	—	2.18	0.82
	ClW	16.3	20.0	39.3	115	0.64	1.62
			100	125	109	2.00	1.6
	CDW	11.5	20.0	34.4	115	0.76	2.22
			100	125	113	1.24	0.99
	O_3W	39.8	20.0	65.4	—	3.67	5.61
			100	153	113	1.00	0.65
Chlorate	RW	<DL	100	98.3	98.3	0.80	0.82
			500	520	104	4.15	0.8
	HIW	<DL	100	86.1	86.1	1.47	1.7
			500	502	100	4.52	0.9
	SW	3.18	100	102	98.3	1.57	1.55
			500	513	102	7.11	1.39
	GW	<DL	100	93.5	93.5	2.00	2.14
			500	510	102	3.84	0.75
	ClW	34.4	100	136	102	1.01	0.74
			500	549	103	3.11	0.57
	CDW	121	100	223	—	3.20	1.44
			500	651	106	3.50	0.54
	O_3W	6.15	100	106	100	1.20	1.13
			500	523	103	2.45	0.47
Chlorite	RW	<DL	100	96.2	96.2	0.95	0.99
			500	523	105	3.13	0.60
	HIW	<DL	100	102	102	2.19	2.15
			500	520	104	3.64	0.70
	SW	<DL	100	91.4	91.4	1.22	1.33
			500	495	99.0	7.54	1.52
	GW	<DL	100	92.9	92.9	1.65	1.77
			500	490	98.1	3.40	0.69
	ClW	<DL	100	87.4	87.4	0.59	0.68
			500	485	97.1	6.36	1.31
	CDW	292	100	396	—	1.64	0.41
			500	811	104	4.00	0.49
	O3W	<DL	100	84.4	84.4	0.46	0.54
			500	481	96.1	3.24	0.67
Bromate	RW	<DL	5.00	5.04	101	0.45	8.86
			25.0	26.5	106	1.71	6.47
	HIW	<DL	5.00	4.88	97.5	0.95	19.5
			25.0	25.6	102	1.37	5.37
	SW	<DL	5.00	4.46	89.2	0.58	13.0
			25.0	26.3	105	1.10	4.18
	GW	<DL	5.00	5.10	102	0.50	9.75
			25.0	22.2	88.9	1.29	5.81
	ClW	<DL	5.00	4.63	92.6	0.77	16.7
			25.0	25.1	100	1.64	6.55
	CDW	<DL	5.00	4.14	82.7	0.62	15.1
			25.0	25.1	101	1.28	5.09
	O3W	1.45	5.00	5.49	80.9	0.61	11.1
			25.0	24.1	90.6	1.13	4.69

* Nine replicates were analyzed for each matrix/concentration combination. Analytical conditions: Ion chromatograph: Dionex DX500; columns: Dionex AG9-HC/AS9-HC, 2 mm; detector: Dionex CD20; suppressor: ASRS-I, external water electrolytic mode, 100 mA current; eluent: 9.0 m*M* Na_2CO_3; eluent flow: 0.40 mL/min; sample loop: 50 μL.

† RW = reagent water
HIW = high-ionic-strength water
SW = surface water
GW = ground water
ClW = chlorinated drinking water
CDW = chlorine-dioxide-treated drinking water
O_3W = ozonated drinking water

‡ <DL indicates less than minimum detection level.

§ Blank (—) indicates mean was not calculated since amount fortified was less than unfortified native matrix concentration.

SOURCE: PFAFF, J.D., D.F. HAUTMAN & D.J. MUNCH. 1997. Determination of Inorganic Anions in Drinking Water by Ion Chromatography. Method 300.1, U.S. Environmental Protection Agency, National Exposure Research Lab, Off. Research & Development, Cincinnati, Ohio.

4120 SEGMENTED CONTINUOUS FLOW ANALYSIS*

4120 A. Introduction

1. Background and Applications

Air-segmented flow analysis (SFA) is a method that automates a large number of wet chemical analyses. An SFA analyzer can be thought of as a "conveyor belt" system for wet chemical analysis, in which reagents are added in a "production-line" manner. Applications have been developed to duplicate manual procedures precisely. SFA was first applied to analysis of sodium and potassium in human serum, with a flame photometer as the detection device, by removing protein interferences with a selectively porous membrane (dialyzer).

The advantages of segmented flow, compared to the manual method, include reduced sample and reagent consumption, improved repeatability, and minimal operator contact with hazardous materials. A typical SFA system can analyze 30 to 120 samples/h. Reproducibility is enhanced by the precise timing and repeatability of the system. Because of this, the chemical reactions do not need to go to 100% completion. Decreasing the number of manual sample/solution manipulations reduces labor costs, improves workplace safety, and improves analytical precision. Complex chemistries using dangerous chemicals can be carried out in sealed systems. Unstable reagents can be made up *in situ*. An SFA analyzer uses smaller volumes of reagents and samples than manual methods, producing less chemical waste needing disposal.

SFA is not limited to single-phase colorimetric determinations. Segmented-flow techniques often include analytical procedures such as mixing, dilution, distillation, digestion, dialysis, solvent extractions, and/or catalytic conversion. In-line distillation methods are used for the determinations of ammonia, fluoride, cyanide, phenols, and other volatile compounds. In-line digestion can be used for the determination of total phosphorous, total cyanide, and total nitrogen (Kjeldahl + NO_2 + NO_3). Dialysis membranes are used to eliminate interferences such as proteins and color, and other types of membranes are available for various analytical needs. SFA also is well-suited for automated liquid/liquid extractions, such as in the determination of MBAS. Packed-bed ion exchange columns can be used to remove interferences and enhance sensitivity and selectivity of the detection.

Specific automated SFA methods are described in the sections for the analytes of interest.

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* Approved by Standard Methods Committee, 2004. Editorial revisions, 2011. Joint Task Group: 20th Edition—Theresa M. Wright (chair), Johan Menting, Lang Allen Reeves.

4120 B. Segmented Flow Analysis Method

1. General Discussion

a. Principle: A rudimentary system (Figure 4120:1) contains four basic components: a sampling device, a liquid transport device such as a peristaltic pump, the analytical cartridge where the chemistry takes place, and the detector to quantify the analyte.

In a generalized system, samples are loaded onto an automatic sampler. The sampler arm moves the sample pickup needle between the sample cup and a wash reservoir containing a solution closely matching the sample matrix and free of the analyte. The wash solution is pumped continuously through the reservoir to eliminate cross-contamination. The sample is pumped to the analytical cartridge as a discrete portion separated from the wash by an air-bubble created during the sampler arm's travel from wash reservoir to sample cup and back.

In the analytical cartridge, the system adds the sample to the reagent(s) and introduces proportionately identical air-bubbles to reagent or sample stream. Alternatively, another gas or immiscible fluid can be substituted for air. The analyzer then proportions the analyte sample into a number of analytical segments depending on sample time, wash time, and segmentation frequency. Relative flow and initial reagent concentration determine the amount and concentration of each reagent added. The micro-circulation pattern enhances mixing, as do mixing coils, which swirl the analytical system to utilize gravitational forces. Chemical reactions, solvent separation, catalytic reaction, dilution, distillation, heating, and/or special applications take place in their appropriate sections of the analytical cartridge as the segmented stream flows toward the detector.

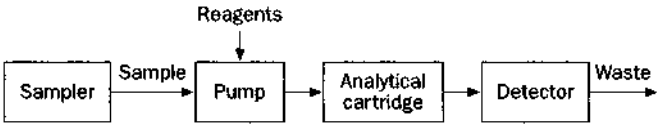


Figure 4120:1. Schematic of a segmented flow analyzer.

A typical SFA detector is a spectrophotometer that measures the color development at a specific wavelength. Other detectors, such as flame photometers and ion-selective electrodes, can be used. SFA detectors utilize flow-through cells, and typically send their output

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to a computerized data-collection system and/or a chart-recorder. The baseline is the reading when only the reagents and wash water are flowing through the system. Because gas bubbles are compressible, highly reflective, and electrically nonconductive, they severely distort the signal in the detector; therefore, many systems remove the bubbles before the optical light path. However, if the system removes the bubbles at any point within the system, the segregated liquids will be able to interact and pool. This interaction can cause cross-contamination or loss of wash, and decreases the rate at which samples can be processed. Real-time analog or digital data reconstruction techniques known as curve regeneration can remove the effect of pooling at the flow-cell debubbler and/or any other unsegmented zones of the system. "Bubble-gating" is a technique that does not remove the bubbles, but instead uses analog or digital processing to remove the distortion caused by the bubbles. Bubble-gating requires a sufficiently fast detector response time and requires that the volume of the measurement cell be smaller than the volume of the individual liquid segment.

b. Sample dispersion and interferences: Theoretically, the output of the detector is square-wave. Several carryover processes can deform the output exponentially. The first process, longitudinal dispersion, occurs as a result of laminar flow. Segmentation of the flow with air bubbles minimizes the dispersion and mixing between segments. The second process is axial or lag-phase dispersion. It arises from stagnant liquid film that wets the inner surfaces of the transmission tubing. Segmented streams depend on wet surfaces for hydraulic stability. The back-pressure within non-wet tubing increases in direct proportion to the number of bubbles it contains and causes surging and bubble breakup. Corrective measures include adding specific wetting agents (surfactants) to reagents and minimizing the length of transmission tubing.

Loose or leaking connections are another cause of carryover and can cause poor reproducibility. Wrap TFE tape around leaking screw fittings. When necessary, slightly flange the ends of types of tubing that require it for a tight connection. For other connections, sleeve one size of tubing over another size. Use a noninterfering lubricant for other tubing connections. Blockages in the tubing can cause back-pressure and leaks. Clean out or replace any blocked tubing or connection. A good indicator for problems is the bubble pattern; visually inspect the system for any abnormal bubble pattern that may indicate problems with flow.

For each analysis, check individual method for compounds that can interfere with color development and/or color reading. Other possible interferences include turbidity, color, and salinity. Turbid and/or colored samples may require filtration. In another interference-elimination technique, known as matrix correction, the solution is measured at two separate wavelengths, and the result at the interference wavelength is subtracted from that at the analytical wavelength.

2. Apparatus

a. Tubing and connections: Use mini- or micro-bore tubing on analytical cartridges. Replace flexible tubing that becomes discolored, develops a "sticky" texture, or loses ability to spring back into shape immediately after compression. Also see manufacturer's manual and specific methods.

b. Electrical equipment and connections: Make electrical connections with screw terminals or plug-and-socket connections. Use shielded electrical cables. Use conditioned power or a universal power supply if electrical current is subject to fluctuations. See manufacturer's manual for additional information.

c. Automated analytical equipment: Dedicate a chemistry manifold and tubing to each specific chemistry. See specific methods and manufacturer's manual for additional information.

d. Water baths: When necessary, use a thermostatically controlled heating/cooling bath to decrease analysis time and/or improve sensitivity. Several types of baths are available; the most common are coils heated or cooled by water or oil. Temperature-controlled laboratories reduce drift in temperature-sensitive chemistries if water baths are not used.

3. Reagents

Prepare reagents according to specific methods and manufacturer's instructions. If required, filter or degas a reagent. Use reagent water (see Section 1080) if available; if not, use a grade of water that is free of the analyte and interfering substances. Run blanks to demonstrate purity of the water used to prepare reagents and wash SFA system. Minimize exposure of reagents to air, and refrigerate if necessary. If reagents are made in large quantities, preferably decant a volume sufficient for one analytical run into a smaller container. If using a wetting agent, add it to the reagent just before the start of the run. Reagents and wetting agents have a limited shelf-life. Old reagents or wetting agents can produce poor reproducibility and distorted peaks. Do not change reagent solutions or add reagent to any reagent reservoirs during analysis. Always start with a sufficient quantity to last through the analytical run.

4. Procedure

For specific operating instructions, consult manufacturer's directions and methods for analytes of interest. At startup of a system, pump reagents and wash water through system until system has reached equilibrium (bubble pattern smooth and consistent) and base line is stable. Meanwhile, load samples and standards into sample cups or tubes and type corresponding tags into computer table. When ready, command computer to begin run. Most systems will run the highest standard to trigger the beginning of the run, followed by a blank to check return to base line, and then a set of standards covering the analytical range (sampling from lowest to highest concentration). Construct a curve plotting concentration against absorbance or detector reading and extrapolate results (many systems will do this automatically). Run a new curve daily immediately before use. Calculation and interpretation of results depend on individual chemistry and are analogous to the manual method. Insert blanks and standards periodically to check and correct for any drift of base line and/or sensitivity. Some systems will run a specific standard periodically as a "drift," and automatically will adjust sample results. At end of a run, let system flush according to manufacturer's recommendations.

5. Quality Control

The QC practices considered to be an integral part of each method are summarized in Table 4020:I. Also see individual methods for quality control methods and precision and bias data.

4130 INORGANIC NONMETALS BY FLOW INJECTION ANALYSIS*

4130 A. Introduction

1. Principle

Flow injection analysis (FIA) is an automated method of introducing a precisely measured portion of liquid sample into a continuously flowing carrier stream. The sample portion usually is injected into the carrier stream by either an injection valve with a fixed-volume sample loop or an injection valve in which a fixed time period determines injected sample volume. As the sample portion leaves the injection valve, it disperses into the carrier stream and forms an asymmetric Gaussian gradient in analyte concentration. This concentration gradient is detected continuously by either a color reaction or another analyte-specific detector through which the carrier and gradient flow.

When a color reaction is used as the detector, the color reaction reagents also flow continuously into the carrier stream. Each color reagent merges with the carrier stream and is added to the analyte gradient in the carrier in a proportion equal to the relative flow rates of the carrier stream and merging color reagent. The color reagent becomes part of the carrier after it is injected and has the effect of modifying or derivatizing the analyte in the gradient. Each subsequent color reagent has a similar effect, finally resulting in a color gradient proportional to the analyte gradient. When the color gradient passes through a flow cell placed in a flow-through absorbance detector, an absorbance peak is formed. The area of this peak is proportional to the analyte concentration in the injected sample. A series of calibration standards is injected to generate detector response data used to produce a calibration curve. It is important that the FIA flow rates, injected sample portion volume, temperature, and time the sample is flowing through the system ("residence time") be the same for calibration standards and unknowns. Careful selection of flow rate, injected sample volume, frequency of sample injection, reagent flow rates, and residence time determines the precise dilution of the sample's original analyte concentration into the useful concentration range of the color reaction. All of these parameters ultimately determine the sample throughput, dynamic range of the method, reaction time of the color reaction discrimination against slow interference reactions, signal-to-noise ratio, and method detection level (MDL).

2. Applications

FIA enjoys the advantages of all continuous-flow methods: There is a constantly measured reagent blank, the "base line" against which all samples are measured; high sample throughput encourages frequent use of quality control samples; large numbers of samples can be analyzed in batches; sample volume measurement, reagent addition, reaction time, and detection occur reproducibly without the need for discrete measurement and transfer vessels, such as cuvettes, pipets, and volumetric flasks; and all samples share a single reaction manifold or vessel consisting of inert flow tubing.

Specific FIA methods are presented as Sections 4500-Br^-.D, 4500-Cl^-.G, 4500-CN^-.N and O, 4500-F^-.G, 4500-NH_3.H, 4500-NO_3^-.I, 4500-N.B, 4500-N_{org}.D, 4500-P.G, H, and I, 4500-SiO_2.F, 4500-SO_4^{2-}.G, and 4500-S^{2-}.I.

*Approved by Standard Methods Committee, 2004.
Joint Task Group: 20th Edition—Scott Stieg (chair), Bradford R. Fisher, Owen B. Mathre, Theresa M. Wright.

4130 B. Quality Control

When FIA methods are used, follow a formal laboratory quality control program. The minimum requirements consist of an initial demonstration of laboratory capability and periodic analysis of laboratory reagent blanks, fortified blanks, and other laboratory solutions as a continuing check on performance. Maintain performance records that define the quality of the data generated.

See Sections 1020 and 4020 for the elements of such a quality control program.

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4140 INORGANIC ANIONS BY CAPILLARY ION ELECTROPHORESIS*

4140 A. Introduction

Determination of common inorganic anions such as fluoride, chloride, bromide, nitrite, nitrate, orthophosphate, and sulfate is a significant component of water quality analysis. Instrumental techniques that can determine multiple analytes in a single analysis [i.e., ion chromatography (Section 4110) and capillary ion electrophoresis] offer significant time and operating cost savings over traditional single-analyte wet chemical analysis.

Capillary ion electrophoresis is rapid (complete analysis in less than 5 min) and provides additional anion information (i.e., organic acids) not available with isocratic ion chromatography (IC). Operating costs are significantly less than those of ion chromatography. Capillary ion electrophoresis can detect all anions present in the sample matrix, providing an anionic "fingerprint."

Anion selectivity of capillary ion electrophoresis is different from that of IC and eliminates many of the difficulties present in the early portion of an IC chromatogram. For example, sample matrix neutral organics, water, and cations do not interfere with anion analysis, and fluoride is well resolved from monovalent organic acids. Sample preparation typically is dilution with reagent water and removal of suspended solids by filtration. If necessary, hydrophobic sample components such as oil and grease can be removed with the use of HPLC solid-phase extraction cartridges without biasing anion concentrations.

* Approved by Standard Methods Committee, 2004. Editorial revisions, 2011.
Joint Task Group: 20th Edition—Roy-Keith Smith (chair), James Krol, Yuefeng Xie.

4140 B. Capillary Ion Electrophoresis with Indirect UV Detection

1. General Discussion

a. Principle: A buffered aqueous electrolyte solution containing a UV-absorbing anion salt (sodium chromate) and an electroosmotic flow modifier (OFM) is used to fill a 75-μm-ID silica capillary. An electric field is generated by applying 15 kV of applied voltage using a negative power supply; this defines the detector end of the capillary as the anode. Sample is introduced at the cathodic end of the capillary and anions are separated on the basis of their differences in mobility in the electric field as they migrate through the capillary. Cations migrate in the opposite direction and are not detected. Water and neutral organics are not attracted towards the anode; they migrate after the anions and thus do not interfere with anion analysis. Anions are detected as they displace charge-for-charge the UV-absorbing electrolyte anion (chromate), causing a net decrease in UV absorbance in the analyte anion zone compared to the background electrolyte. Detector polarity is reversed to provide positive mv response to the data system (Figure 4140:1). As in chromatography, the analytes are identified by their migration time and quantitated by using time-corrected peak area relative to standards. After the analytes of interest are detected, the capillary is purged with fresh electrolyte, eliminating the remainder of the sample matrix before the next analysis.

b. Interferences: Any anion that has a migration time similar to the analytes of interest can be considered an interference. This method has been designed to minimize potential interference typically found in environmental waters, groundwater, drinking water, and wastewater.

Formate is a common potential interference with fluoride; it is a common impurity in reagent water, has a migration time similar to that of fluoride, and is an indicator of loss of water purification system performance and TOC greater than 0.1 mg/L. The addition of 5 mg formate/L in the mixed working anion standard, and to sample where identification of fluoride is in question, aids in the correct identification of fluoride.

Generally, a high concentration of any one ion may interfere with resolution of analyte anions in close proximity. Dilution in reagent water usually is helpful. Modifications in the electrolyte formulation can overcome resolution problems but require individual validation for precision and bias. This method is capable of interference-free resolution of a 1:100 differential of Br^- to Cl^-, and NO_2^- and NO_3^- to SO_4^{2-}, and 1:1000 differential of Cl^- and SO_4^{2-}.

Dissolved ferric iron in the mg/L range gives a low bias for PO_4. However, transition metals do not precipitate with chromate because of the alkaline electrolyte pH.

c. Minimum detectable concentrations: The minimum detectable concentration for an anion is a function of sample size. Generally, for a 30-s sampling time, the minimum detectable concentrations are 0.1 mg/L (Figure 4140:2). According to the method for calculating MDL given in Section 1030C, the calculated detection levels are below 0.1 mg/L. These detection levels can be compromised by analyte impurities in the electrolyte.

d. Limitations: Samples with high ionic strength may show a decrease in analyte migration time. This variable is addressed by using normalized migration time with respect to a reference peak, chloride, for identification, and using time-corrected area for quantitation. With electrophoresis, published data indicate that analyte peak area is a function of migration time. At high analyte anion concentrations, peak shape becomes asymmetrical; this phenomenon is typical and is different from that observed in ion chromatography.

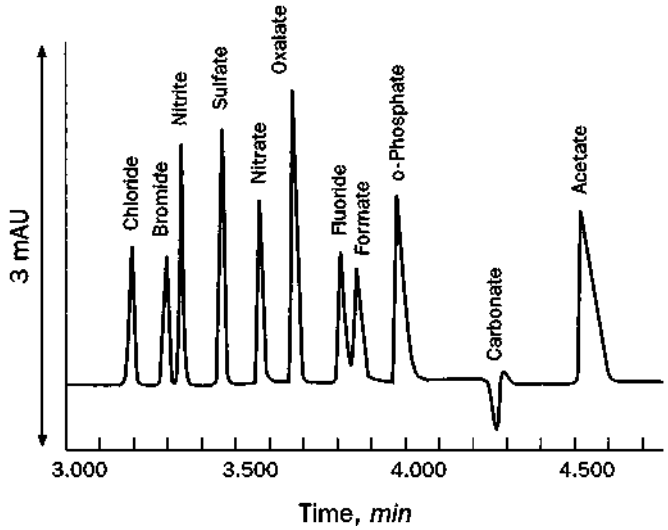


Figure 4140:1. Electropherogram of the inorganic anions and typically found organic acids using capillary ion electrophoresis and chromate electrolyte. Electrolyte: 4.7 m*N* Na_2CrO_4/4.0 m*M* TTAOH/10 m*M* CHES/0.1 m*M* calcium gluconate; capillary: 75-μm-ID × 375-μm-OD × 60-cm length, uncoated silica; voltage: 15 kV using a negative power supply; current: 14 ± 1 μA; sampling: hydrostatic at 10 cm for 30 s; detection: indirect UV with Hg lamp and 254-nm filter.

Anion	Conc *mg/L*	Migration Time *min*	Migration Time Ratio to Cl	Peak Area	Time-Corrected Peak Area
Chloride	2.0	3.200	1.000	1204	376.04
Bromide	4.0	3.296	1.030	1147	348.05
Nitrite	4.0	3.343	1.045	2012	601.72
Sulfate	4.0	3.465	1.083	1948	562.05
Nitrate	4.0	3.583	1.120	1805	503.69
Oxalate	5.0	3.684	1.151	3102	842.14
Fluoride	1.0	2.823	1.195	1708	446.65
Formate	5.0	3.873	1.210	1420	366.61
o-Phosphate	4.0	4.004	1.251	2924	730.25
Carbonate & bicarbonate		4.281	1.338		
Acetate	5.0	4.560	1.425	3958	868.01

2. Apparatus

*a. Capillary ion electrophoresis (CIE) system:** Various commercial instruments are available that integrate a negative high-voltage power supply, electrolyte reservoirs, covered sample carousel, hydrostatic sampling mechanism, capillary purge mechanism, self-aligning capillary holder, and UV detector capable of 254-nm detection in a single temperature-controlled compartment at 25°C. Optimal detection limits are attained with a fixed-wavelength UV detector with Hg lamp and 254-nm filter.

b. Capillary: 75-μm-ID × 375-μm-OD × 60-cm-long fused silica capillary with a portion of its outer coating removed to act as the UV detector window. Capillaries can be purchased premade* or on a spool and prepared as needed.

*c. Data system:** HPLC-based integrator or computer. Optimum performance is attained with a computer data system and electrophoresis-specific data processing including data acquisition at 20 points/s, migration times determined at midpoint of peak width, identification based on normalized migration times with respect to a reference peak, and time-corrected peak area.

* Waters Corp., or equivalent.

3. Reagents

a. Reagent water: See Section 1080. Ensure that water is analyte-free. The concentration of dissolved organic material will influence overall performance; preferably use reagent water with <50 μg TOC/L.

b. Chromate electrolyte solution: Prepare as directed from individual reagents, or purchase electrolyte preformulated.

1) *Sodium chromate concentrate,* 100 m*M*—In a 1-L volumetric flask dissolve 23.41 g sodium chromate tetrahydrate, $Na_2CrO_4 \cdot 4H_2O$, in 500 mL water and dilute to 1 L with water. Store in a capped glass or plastic container at ambient temperature; this reagent is stable for 1 year.

2) *Electroosmotic flow modifier concentrate,* 100 m*M*—In a 100-mL volumetric flask dissolve 3.365 g tetradecyltrimethyl ammonium bromide (TTAB), mol wt 336.4, in 50 mL water and

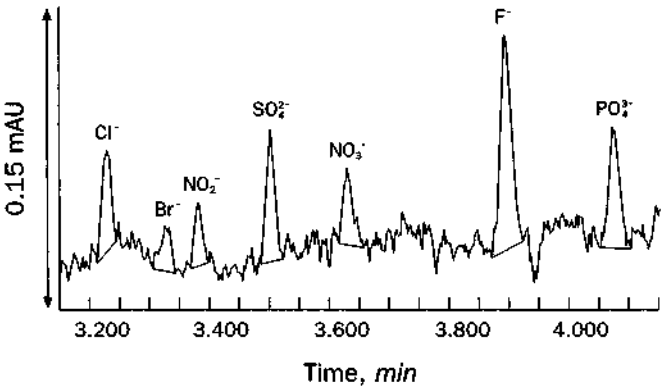


Figure 4140:2. Electropherogram of 0.1 mg/L inorganic anions at minimum detection level. Seven replicates of 0.1 mg/L inorganic anion standard used to calculate minimum detection levels, as mg/L, using analytical protocol described in Section 1030C.

Chloride	= 0.046	Nitrite	= 0.072
Nitrate	= 0.084	Phosphate	= 0.041
Bromide	= 0.090	Sulfate	= 0.032
Fluoride	= 0.020		

dilute to 100 mL. Store in a capped glass or plastic container at ambient temperature; this reagent is stable for 1 year.

3) *Buffer concentrate,* 100 m*M*—In a 1-L volumetric flask dissolve 20.73 g 2-[*N*-cyclohexylamino]-ethane sulfonate (CHES), mol wt 207.29, in 500 mL water and dilute to 1 L. Store in a capped glass or plastic container at ambient temperature; this reagent is stable for 1 year.

4) *Calcium gluconate concentrate,* 1 m*M*—In a 1-L volumetric flask dissolve 0.43 g calcium gluconate, mol wt 430.38, in 500 mL water and dilute to 1 L. Store in a capped glass or plastic container at ambient temperature; this reagent is stable for 1 year.

5) *Sodium hydroxide solution* (NaOH), 100 m*M*—In a 1-L plastic volumetric flask dissolve 4 g sodium hydroxide, NaOH, in 500 mL water and dilute to 1 L. Store in a capped plastic container at ambient temperature; this reagent is stable for 1 month.

6) *Chromate electrolyte solution*—Prerinse an anion exchange cartridge in the hydroxide form with 10 mL 100-m*M* NaOH followed by 10 mL water; discard the washings. Slowly pass 4 mL 100-m*M* TTAB concentrate through the cartridge into a 100-mL volumetric flask. Rinse cartridge with 10 mL water and add to flask. (NOTE: This step is needed to convert the TTAB from the bromide form into the hydroxide form TTAOH. The step can be eliminated if commercially available 100 m*M* TTAOH is used.)

To the 100-mL volumetric flask containing the TTAOH add 4.7 mL sodium chromate concentrate, 10 mL CHES buffer concentrate, and 10 mL calcium gluconate concentrate. Mix and dilute to 100 mL with water. The pH should be 9 ± 0.1; final solution is 4.7 m*M* sodium chromate, 4 m*M* TTAOH, 10 m*M* CHES, and 0.1 m*M* calcium gluconate. Filter and degas through a 0.45-μm aqueous membrane, using a vacuum apparatus. Store any remaining electrolyte in a capped plastic container at ambient temperature for up to 1 month.

c. Standard anion solution, 1000 mg/L: Prepare a series of individual standard anion solutions by adding the indicated amount of salt, dried to constant weight at 105°C, to 100 mL with water. Store in plastic bottles; these solutions are stable for 3 months. (Alternatively, purchase individual certified 1000-mg/L anion standards and store following manufacturer's directions.)

Anion	Salt	Amount *g/100mL*
Chloride	NaCl	0.1649
Bromide	NaBr	0.1288
Formate	$NaCO_2H$	0.1510
Fluoride	NaF	0.2210
Nitrite	$NaNO_2$	0.1499* (1000 mg NO_2^-/L = 304.3 mg NO_2^- −N/L)
Nitrate	$NaNO_3$	0.1371 (1000 mg NO_3^-/L = 225.8 mg NO_3^-−N/L)
Phosphate	Na_2HPO_4†	0.1500 (1000 mg PO_4^{3-}/L = 326.1 mg PO_4^{3-}−P/L)
Sulfate	Na_2SO_4†	0.1480 (1000 mg SO_4^{2-}/L = 676.3 mg SO_4^{2-}−S/L)

* Do not oven-dry, but dry to constant weight in a desiccator over phosphorous pentoxide.
† Potassium salts can be used, but with corresponding modification of salt amounts.

d. Mixed working anion standard solutions: Prepare at least three different working anion standard solutions that bracket the expected sample range, from 0.1 to 50 mg/L. Add 5 mg formate/L to all standards. Use 0.1 mL standard anion solution/100 mL working anion solution (equal to 1 mg anion/L). (Above 50 mg/L each anion, chloride, bromide, nitrite, sulfate, and nitrate are no longer baseline-resolved. Analytes that are not baseline-resolved may give a low bias. If the analytes are baseline-resolved, quantitation is linear to 100 mg/L.) Store in plastic containers in the refrigerator; prepare fresh standards weekly. Figure 4140:3 shows representative electropherograms of anion standards and Table 4140:I gives the composition of the standards.

e. Calibration verification sample: Use a certified performance evaluation standard, or equivalent, within the range of the mixed working anion standard solutions analyzed as an unknown. Refer to Section 4020B.2.

f. Analyte known-addition sample: To each sample matrix add a known amount of analyte, and use to evaluate analyte recovery.

4. Procedure

a. Capillary conditioning: Set up CIE system according to manufacturer's instructions. Rinse capillary with 100 m*M* NaOH for 5 min. Place fresh degassed electrolyte into both reservoirs and purge capillary with electrolyte for 3 min to remove all previous solutions and air bubbles. Apply voltage of 15 kV and note the current; if the expected 14 ± 1 μA is observed, the CIE system is ready for use. Zero UV detector to 0.000 absorbance.

b. Analysis conditions: Program CE system to apply constant current of 14 μA for the run time. Use 30 s hydrostatic sampling time for all standard and sample introduction. Analysis time is 5 min.

c. Analyte migration time calibration: Determine migration time of each analyte daily using the midrange mixed working anion standard. Perform duplicate analysis to ensure migration time stability. Use the midpoint of peak width, defined as midpoint between the start and stop integration marks, as the migration time for each analyte; this accounts for the observed

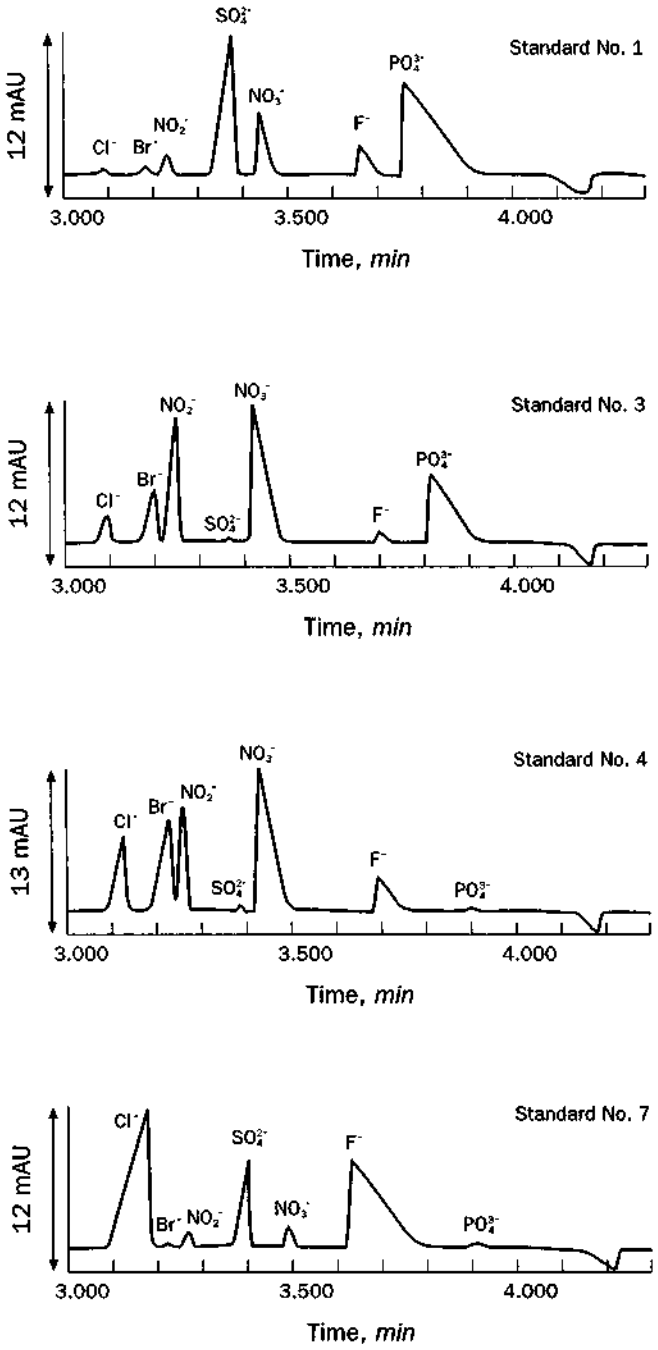


Figure 4140:3. Representative electropherograms of Youden anion standards. For composition of standards, see Table 4140:I.

Table 4140:I. Collaborative Design as Four Youden Pair Sets*

Anion	Anion Concentration in Individual Youden Pair Standards, *mg/L*							
	1	2	3	4	5	6	7	8
Cl^-	0.7	2.0	3.0	15.0	40.0	20.0	50.0	0.5
Br^-	2.0	3.0	15.0	40.0	20.0	50.0	0.7	0.5
NO_2^-	3.0	40.0	20.0	15.0	50.0	0.5	2.0	0.7
SO_4^{2-}	40.0	50.0	0.5	0.7	2.0	3.0	15.0	20.0
NO_3^-	15.0	20.0	40.0	50.0	0.5	0.7	2.0	3.0
F^-	2.0	0.7	0.5	3.0	10.0	7.0	20.0	25.0
PO_4^{3-}	50.0	40.0	20.0	0.5	3.0	2.0	0.7	15.0

* The collaborative design is intended to demonstrate performance between 0.1 and 50 mg anion/L, except for fluoride between 0.1 and 25 mg/L. The concentrations among anions are varied so as not to have any one standard at all low or all high anion concentrations.

Table 4140:II. Anion Migration Time Reproducibility from Youden Pair Standards

Youden Standard	Anion Midpoint Migration Time, Average of Triplicate Samplings, *min*						
	Cl^-	Br^-	NO_2^-	SO_4^{2-}	NO_3^-	F^-	PO_4^{3-}
1	3.132	3.226	3.275	3.405	3.502	3.761	3.906
2	3.147	3.239	3.298	3.431	3.517	3.779	3.931
3	3.138	3.231	3.283	3.411	3.497	3.771	3.925
4	3.158	3.244	3.307	3.434	3.510	3.781	3.963
5	3.184	3.271	3.331	3.435	3.551	3.787	3.981
6	3.171	3.260	3.312	3.418	3.537	3.776	3.964
7	3.191	3.272	3.315	3.437	3.544	3.773	3.978
8	3.152	3.248	3.294	3.418	3.526	3.739	3.954
SD*	0.021	0.015	0.018	0.012	0.20	0.015	0.027
%RSD*	0.67%	0.46%	0.55%	0.36%	0.56%	0.40%	0.68%

* Average SD = 0.018 min = 1.1 s; average %RSD = 0.53%.

non-symmetrical peak shapes. (Use of peak apex may result in analyte misidentification.) The migration order is always Cl^-, Br^-, NO_2^-, SO_4^{2-}, NO_3^-, F^-, and PO_4^{3-}. Dissolved HCO_3^- is the last peak in the standard (see Figure 4140:1). Set analyte migration time window as 2% of the migration time determined above, except for Cl^-, which is set at 10%. Chloride is always the first peak and is used as the reference peak for analyte qualitative identification; identify anions on the basis of normalized migration times with respect to the reference peak, or migration time ratio. (See Figure 4140:1 and Table 4140:II.)

d. Analyte response calibration: Analyze all three mixed working anion standards in duplicate. Plot time-corrected peak area for each analyte versus concentration using a linear regression through zero. (In capillary electrophoresis peak area is a function of analyte migration time, which may change during analyses. Time-corrected peak area is a well-documented CE normalization routine (i.e., peak area divided by migration time). (Note: Do not use analyte peak height.) Calibration is accepted as linear if regression coefficient of variation, R^2, is greater than 0.995. Linearity calibration curves for anions are shown in Figures 4140:4–6.

e. Sample analysis: After initial calibration run samples in the following order: calibration verification sample, reagent blank, 10 unknown samples, calibration verification sample, reagent blank, etc. Filter samples containing high concentrations of suspended solids. If peaks are not baseline-resolved, dilute sample 1:5 with water and repeat analysis for unresolved analyte quantitation. Resolved analytes in the undiluted sample are considered correct quantitation. Electropherograms of typical samples are shown in Figures 4140:7–9.

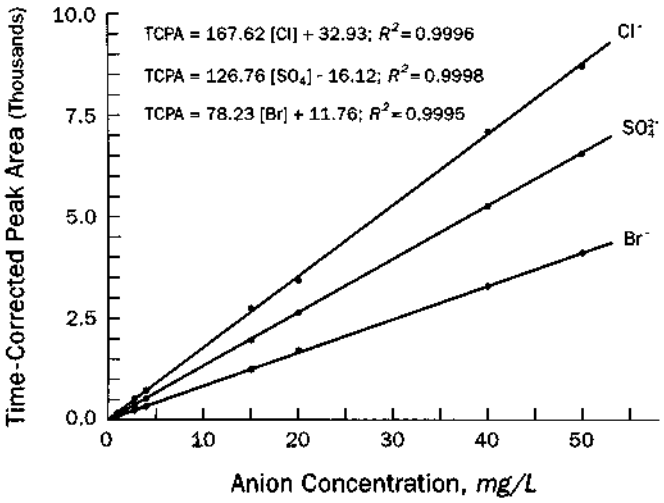


Figure 4140:4. Linearity calibration curve for chloride, bromide, and sulfate. Three data points were used per concentration; based on Youden pair design.

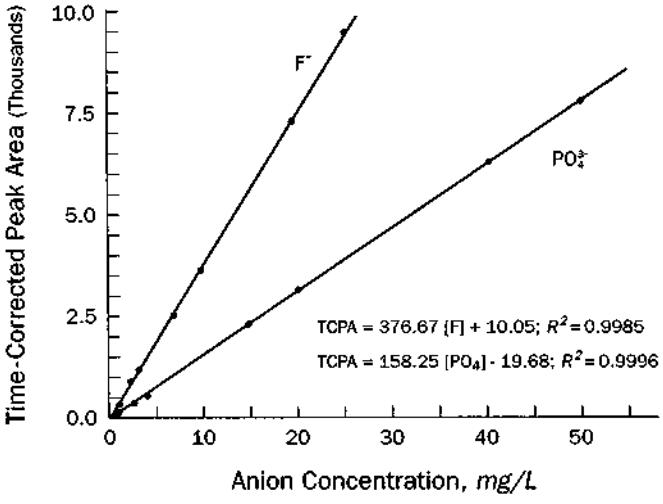


Figure 4140:5. Linearity calibration curve for fluoride and *o*-phosphate. Three data points were used per concentration; based on Youden pair design.

5. Calculation

Relate the time-corrected peak area for each sample analyte with the calibration curve to determine concentration of analyte. If the sample was diluted, multiply anion concentration by the dilution factor to obtain original sample concentration, as follows:

$$C = A \times F$$

where:

C = analyte concentration in original sample, mg/L,
A = analyte concentration from calibration curve, mg/L, and
F = scale factor or dilution factor (for a 1:5 sample dilution, $F = 5$).

6. Quality Control

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

a. Analytical performance check: Unless analyst has already demonstrated ability to generate data with acceptable precision and bias by this method, proceed as follows: Analyze seven replicates of a certified performance evaluation standard containing the analytes of interest. Calculate mean and standard deviation of these data. The mean must be within the performance evaluation standard's 95% confidence interval. Calculate percent relative standard deviation (RSD) for these data as (SD × 100) / mean; % RSD should conform to acceptance limit given in Section 1020B.

b. Calibration verification: Analyze an independent, certified performance evaluation standard at the beginning and end of the analyses, or if many samples are analyzed, after every 10 samples. The determined analyte concentration should be within ±10% of the true value, and the migration time of the Cl^- reference peak should be within 5% of the calibrated migration time. If the Cl^- reference peak differs by more than 5% of the calibrated migration time, repeat capillary conditioning and recalibrate before proceeding.

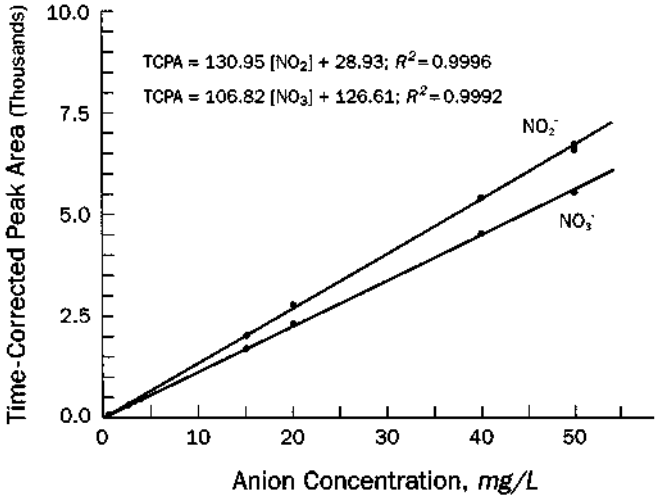


Figure 4140:6. Linearity calibration curve for nitrite and nitrate. Three data points were used per concentration; based on Youden pair design.

c. Water blank analysis: At the beginning of every set of analyses run a water blank to demonstrate that the water is free of analyte anions. Dissolved bicarbonate will always be observed as a positive or negative peak having a migration time greater than PO_4^{3-} and does not interfere with the analysis. Any negative peak indicates the presence of an anion impurity in the electrolyte; a positive peak indicates the presence of an impurity in the reagent water. If this is noted, discard electrolyte and prepare electrolyte and sample dilutions again with water from a different source.

d. Analyte recovery verification: For each sample matrix analyzed (e.g., drinking water, surface water, groundwater, or wastewater), analyze duplicate known-addition samples (4140B.3*f*). Analyte recoveries should conform to acceptance limits given in Section 1020B.

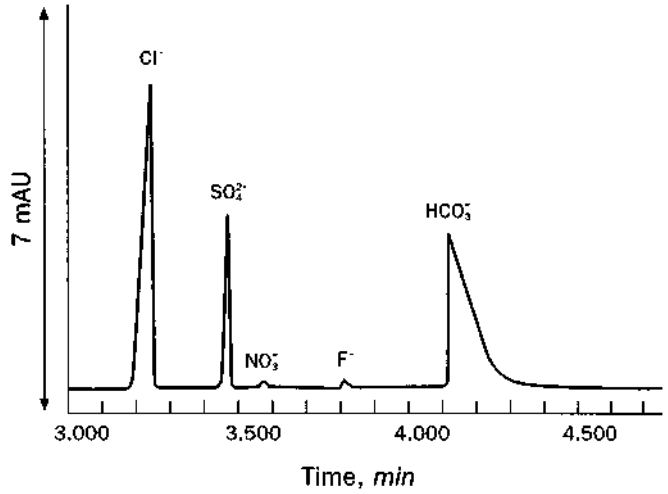


Figure 4140:7. Electropherogram of typical drinking water.

Chloride = 24.72 mg/L	Fluoride < 0.10 mg/L
Sulfate = 7.99 mg/L	Carbonate &
Nitrate = 0.36 mg/L	bicarbonate = natural

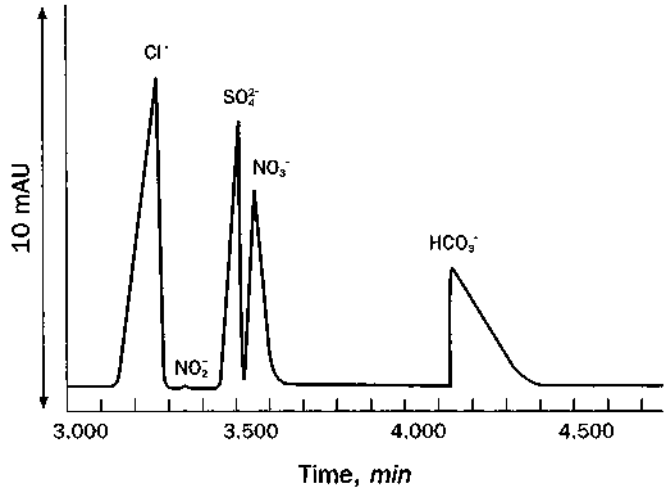


Figure 4140:8. Electropherogram of typical municipal wastewater discharge, undiluted.

Chloride = 93.3 mg/L	Nitrate < 40.8 mg/L
Nitrate = 0.46 mg/L	Carbonate &
Sulfate = 60.3 mg/L	bicarbonate = natural

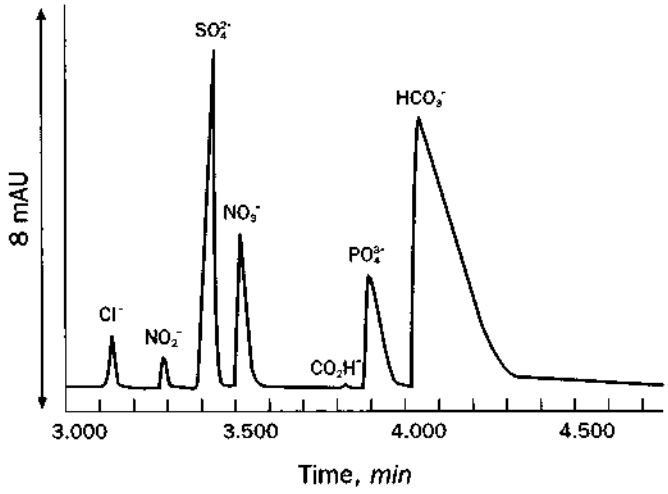


Figure 4140:9. Electropherogram of typical industrial wastewater discharge, undiluted.

Chloride = 2.0 mg/L	Formate < 0.05 mg/L
Nitrite = 1.6 mg/L	Phosphate = 12.3 mg/L
Sulfate = 34.7 mg/L	Carbonate &
Nitrate = 16.5 mg/L	bicarbonate = natural

e. Blind check sample: Analyze an unknown certified performance evaluation check sample at least once every 6 months to verify method accuracy.

f. Sample duplicates: Analyze one or more sample duplicates every 10 samples.

7. Precision and Bias

Table 4140:III compares results of capillary ion electrophoresis with those of other approved methods. Precision and bias data are given in Tables 4140:IV and 4140:V. Comparison of other methods and capillary ion electrophoresis for wastewater effluent, drinking water, and landfill leachates are given in Table 4140:VI.

8. Bibliography

JANDIK, P. & G. BONN. 1993. Capillary Electrophoresis of Small Molecules and Ions. VCH Publishers, New York, N.Y.

ROMANO, J. & J. KROL. 1993. Capillary electrophoresis, an environmental method for the determination of anions in water. *J. Chromatogr.* 640:403.

Table 4140:III. Comparison of Capillary Ion Electrophoresis and Other Methods

Source	Statistic	Value for Given Anion mg/L					
		Cl^-	NO_2^-	SO_4^{2-}	NO_3^-	F^-	PO_4^{3-}
Performance evaluation standard*	True value	43.00	1.77	37.20	15.37	2.69	6.29
Wet chemical and ion chromatography methods†	Measured mean	43.30	1.77	37.00	15.42	2.75	6.38
	Measured SD	3.09	0.07	2.24	1.15	0.26	0.21
CIE using chromate electrolyte‡	Average (n = 18)	43.34	1.64	37.11	14.41	2.64	6.34
	CIE/mean	1.003	0.927	1.003	0.935	0.959	0.993
	CIE/true value	1.008	0.927	0.996	0.938	0.981	1.008

* Purchased from APG Laboratories, June 1996; diluted 1:100 with deionized water.
† Measured result is the average from numerous laboratories using approved *Standard Methods* and EPA wet chemistry and ion chromatography methods.
‡ CIE results determined in July 1996 with proposed EPA and ASTM method, operationally identical to 4140; they are the average from four laboratories using the Youden pair standards for quantitation. These data can be considered known addition of the performance evaluation standard in reagent water; they conform to quality control acceptance limits given in Section 1020.

Table 4140:IV. Capillary Ion Electrophoresis Reproducibility and Precision*

Laboratory No.	Anion Concentration mg/L				
	Cl^-	NO_2^-	SO_4^{2-}	NO_3^-	F^-
1 (n = 5)	43.22 ± 0.22	1.58 ± 0.09	36.39 ± 0.33	14.57 ± 0.12	2.54 ± 0.10
2 (n = 5)	43.68 ± 0.61	1.58 ± 0.08	37.01 ± 0.37	13.94 ± 0.09	2.69 ± 0.02
3 (n = 5)	43.93 ± 0.39	1.60 ± 0.06	37.68 ± 0.24	15.05 ± 0.11	2.69 ± 0.03
4 (n = 3)	42.51 ± 0.22	1.78 ± 0.06	37.34 ± 0.19	14.06 ± 0.07	2.69 ± 0.02
Average (mean ± SD)	43.34 ± 0.36	1.64 ± 0.07	37.11 ± 0.28	14.41 ± 0.10	2.64 ± 0.04
%RSD	0.83%	4.5%	0.77%	0.67%	1.61%

* Results from four laboratories analyzing the performance evaluation standard using the Youden pair standards for quantitation. Only one laboratory reported results for PO_4^{3-} as 6.34 ± 0.02 mg/L on triplicate samplings yielding %RSD of 0.07%. Calculated replicate reproducibility and precision conform to the quality control acceptance limits given in Section 1020.

Table 4140:V. Capillary Ion Electrophoresis Known-Addition Recovery and Precision of Performance Evaluation Standard with Drinking Water

Variable	Value for Given Anion					
	Cl^-	NO_2^-	SO_4^{2-}	NO_3^-	F^-	PO_4^{3-}
Milford drinking water (MDW) n = 3, concentration as mg/L	24.72 ± 0.18	Not detected	7.99 ± 0.07	0.36 ± 0.05	Not detected	Not detected
%RSD	0.73	—	0.91	13.3	—	—
Performance evaluation standard (PES), concentration as mg/L	43.00	1.77	37.20	15.37	2.69	6.29
MDW + PES,* n = 3, concentration as mg/L	66.57 ± 0.34	1.74 ± 0.03	45.19 ± 0.17	15.42 ± 0.12	2.62 ± 0.07	5.55 ± 0.31
%RSD	0.51%	1.85%	0.38%	0.79%	2.69%	5.52%
% Recovery	97.9%	98.3%	100.2%	98.1%	97.4%	88.2%

* Performance evaluation standard diluted 1:100 with Milford drinking water. Calculated analyte recovery and precision conform to the quality control acceptance limits given in Section 1020.

TABLE 4140:VI. COMPARISON OF CAPILLARY ION ELECTROPHORESIS WITH CHROMATE ELECTROLYTE WITH OTHER METHODS FOR THE DETERMINATION OF ANIONS

Anion	Matrix	Sample No.	Concentration by Given Method* mg/L: Wet Chemical or Other Method†	IC‡	CIE
Chloride	Effluent	1	—	149	147
		2	—	162	161
		3	—	152	151
		4	—	139	139
		5	—	111	110
		6	—	109	107
		7	—	3.6	3.5
	Drinking water	1	5.5	5.1	5.0
		2	5.5	5.0	5.0
		3	5.3	5.2	5.2
		4	5.5	5.1	5.1
		5	5.3	5.0	5.1
		6	5.3	4.9	4.9
		7	5.5	4.9	4.9
	Landfill leachate	1	0.1	<0.1	ND
		2	230	245	240
Fluoride	Effluent	1	1.7	1.2	1.5
		2	0.9	0.6	0.6
		3	0.8	0.5	0.6
		4	0.8	0.4	0.7
		5	0.9	0.5	0.8
		6	0.9	0.5	0.7
		7	<0.1	ND	<0.1
	Drinking water	1	1.2	0.9	0.9
		2	1.3	0.9	0.9
		3	1.3	0.9	0.9
		4	1.3	0.9	0.9
		5	1.3	0.9	0.9
		6	0.9	0.6	0.6
		7	1.3	0.9	0.9
	Landfill leachate	1	<0.2	ND	ND
		2	16	10.6	10.9
Sulfate	Effluent	1	98	87.5	86.4
		2	110	95.3	95.9
		3	130	118	115
		4	130	139	136
		5	110	113	110
		6	100	107	106
		7	6	5.6	5.8
	Drinking water	1	6	5.8	6.0
		2	6	5.8	6.0
		3	6	5.9	6.1
		4	6	5.9	6.1
		5	5	5.8	6.2
		6	4	3.0	3.4
		7	5	5.8	6.1
	Landfill leachate	1	<1	ND	ND
		2	190	211	201

TABLE 4140:VI. CONT.

Anion	Matrix	Sample No.	Concentration by Given Method* mg/L: Wet Chemical or Other Method†	IC‡	CIE
Nitrite & nitrate§ (as N)	Effluent	1	0.3	ND	ND
		2	—	ND	ND
		3	—	ND	ND
		4	—	ND	0.5
		5	—	2.1	2.4
		6	2.4	1.9	2.2
		7	0.7	0.3	0.4
	Drinking water	1	0.6	0.3	0.4
		2	0.6	0.3	0.4
		3	0.4	0.3	0.4
		4	0.6	0.3	0.3
		5	0.6	0.3	0.4
		6	0.3	0.1	0.1
		7	0.5	0.3	0.4
	Landfill leachate	1	—	ND	ND
		2	—	ND	ND
Orthophosphate (as P)	Effluent	1	3.4	ND	2.8
		2	4.9	ND	4.4
		3	4.7	ND	4.5
		4	5.3	ND	4.2
		5	3.0	ND	3.0
		6	2.9	ND	2.3
		7	<0.1	ND	0.04
	Drinking water	1	<0.1	ND	ND
		2	<0.1	ND	ND
		3	—	ND	ND
		4	<0.1	ND	ND
		5	<0.1	ND	ND
		6	—	ND	ND
		7	—	ND	ND
	Landfill leachate	1	<0.1	ND	0.1
		2	2.2	1.6	1.4

* — = test not performed; ND = not detected.

† Methods used were: chloride—mercuric nitrate method (Section 4500-Cl^-.C); fluoride—ion-selective electrode method (Section 4500-F^-.C); sulfate—turbidimetric method (Section 4500-SO_4^{2-}.E); nitrite + nitrate (total)—cadmium reduction method (Section 4500-NO_3^-.E); orthophosphate—ascorbic acid method (Section 4500-P.E).

‡ Single-column ion chromatography with direct conductivity detection (Section 4110C).

§ Each technique gave separate nitrite and nitrate values; because of their interconvertability, results were added for comparison purposes.

4500-B BORON*

4500-B A. Introduction

1. Occurrence and Significance

Boron (B) is the first element in Group IIIA of the periodic table; it has an atomic number of 5, an atomic weight of 10.81, and a valence of 3. The average abundance of B in the earth's crust is 9 ppm; in soils it is 18 to 63 ppm; in streams it is 10 μg/L; and in groundwaters it is 0.01 to 10 mg/L. The most important mineral is borax, which is used in the preparation of heat-resistant glasses, detergents, porcelain enamels, fertilizers, and fiberglass.

The most common form of boron in natural waters is H_3BO_3. Although boron is an element essential for plant growth, in excess of 2.0 mg/L in irrigation water, it is deleterious to certain plants and some plants may be affected adversely by concentrations as low as 1.0 mg/L (or even less in commercial greenhouses). Drinking waters rarely contain more than 1 mg B/L and generally less than 0.1 mg/L, concentrations considered innocuous for human consumption. Seawater contains approximately 5 mg B/L and this element is found in saline estuaries in association with other seawater salts.

The ingestion of large amounts of boron can affect the central nervous system. Protracted ingestion may result in a clinical syndrome known as borism.

2. Selection of Method

Preferably, perform analyses by the inductively coupled plasma method (Section 3120). The inductively coupled plasma/mass spectrometric method (Section 3125) also may be applied successfully in most cases (with lower detection limits), even though boron is not specifically listed as an analyte in the method.

The curcumin method (4500-B.B) is applicable in the 0.10- to 1.0-mg/L range, while the carmine method (4500-B.C) is suitable for the determination of boron concentration in the 1- to 10-mg/L range. The range of these methods can be extended by dilution or concentration of the sample.

3. Sampling and Storage

Store samples in polyethylene bottles or alkali-resistant, boron-free glassware.

* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011.

4500-B B. Curcumin Method

1. General Discussion

a. Principle: When a sample of water containing boron is acidified and evaporated in the presence of curcumin, a red-colored product called rosocyanine is formed. The rosocyanine is taken up in a suitable solvent and the red color is compared with standards visually or photometrically.

b. Interference: NO_3^--N concentrations above 20 mg/L interfere. Significantly high results are possible when the total of calcium and magnesium hardness exceeds 100 mg/L as calcium carbonate ($CaCO_3$). Moderate hardness levels also can cause a considerable percentage error in the low boron range. This interference springs from the insolubility of the hardness salts in 95% ethanol and consequent turbidity in the final solution. Filter the final solution or pass the original sample through a column of strongly acidic cation-exchange resin in the hydrogen form to remove interfering cations. The latter procedure permits application of the method to samples of high hardness or solids content. Phosphate does not interfere.

c. Minimum detectable quantity: 0.2 μg B.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer,* for use at 540 nm, with a minimum light path of 1 cm.
2) *Filter photometer,* equipped with a green filter having a maximum transmittance near 540 nm, with a minimum light path of 1 cm.

b. Evaporating dishes, 100- to 150-mL capacity, of high-silica glass,* platinum, or other suitable material.

c. Water bath, set at 55 ± 2°C.

d. Glass-stoppered volumetric flasks, 25- and 50-mL capacity.

e. Ion-exchange column, 50 cm long by 1.3 cm in diameter.

3. Reagents

Store all reagents in polyethylene or boron-free containers.

a. Stock boron solution: Dissolve 571.6 mg anhydrous boric acid, H_3BO_3, in distilled water and dilute to 1000 mL; 1.00 mL = 100 μg B. Because H_3BO_3 loses weight on drying at 105°C, use a reagent meeting ACS specifications and keep the bottle tightly stoppered to prevent entrance of atmospheric moisture.

b. Standard boron solution: Dilute 10.00 mL stock boron solution to 1000 mL with distilled water; 1.00 mL = 1.00 μg B.

* Vycor, manufactured by Corning Glass Works, or equivalent.

c. Curcumin reagent: Dissolve 40 mg finely ground curcumin† and 5.0 g oxalic acid in 80 mL 95% ethyl alcohol. Add 4.2 mL conc HCl, make up to 100 mL with ethyl alcohol in a 100-mL volumetric flask, and filter if reagent is turbid (isopropyl alcohol, 95%, may be used in place of ethyl alcohol). This reagent is stable for several days if stored in a refrigerator.

d. Ethyl or isopropyl alcohol, 95%.

e. Reagents for removal of high hardness and cation interference:

1) *Strongly acidic cation-exchange resin*.

2) *Hydrochloric acid,* (HCl), 1 + 5.

4. Procedure

a. Precautions: Closely control such variables as volumes and concentrations of reagents, as well as time and temperature of drying. Use evaporating dishes identical in shape, size, and composition to ensure equal evaporation time because increasing the time increases intensity of the resulting color.

b. Preparation of calibration curve: Pipet 0 (blank), 0.25, 0.50, 0.75, and 1.00 μg boron into evaporating dishes of the same type, shape, and size. Add distilled water to each standard to bring total volume to 1.0 mL. Add 4.0 mL curcumin reagent to each and swirl gently to mix contents thoroughly. Float dishes on a water bath set at 55 ± 2°C and let them remain for 80 min, which is usually sufficient for complete drying and removal of HCl. Keep drying time constant for standards and samples. After dishes cool to room temperature, add 10 mL 95% ethyl alcohol to each dish and stir gently with a polyethylene rod to ensure complete dissolution of the red-colored product.

Wash contents of dish into a 25-mL volumetric flask, using 95% ethyl alcohol. Make up to mark with 95% ethyl alcohol and mix thoroughly by inverting. Read absorbance of standards and samples at a wavelength of 540 nm after setting reagent blank at zero absorbance. The calibration curve is linear from 0 to 1.00 μg boron. Make photometric readings within 1 h of drying samples.

c. Sample treatment: For waters containing 0.10 to 1.00 mg B/L, use 1.00 mL sample. For waters containing more than 1.00 mg B/L, make an appropriate dilution with boron-free distilled water, so that a 1.00-mL portion contains approximately 0.50 μg boron.

Pipet 1.00 mL sample or dilution into an evaporating dish. Unless the calibration curve is being determined at the same time, prepare a blank and a standard containing 0.50 μg boron and run with the sample. Proceed as in ¶ *b* above, beginning with "Add 4.0 mL curcumin reagent. . . ." If the final solution is turbid, filter through filter paper‡ before reading absorbance. Calculate boron content from calibration curve.

d. Visual comparison: The photometric method may be adapted to visual estimation of low boron concentrations, from 50 to 200 μg/L, as follows: Dilute the standard boron solution 1 + 3 with distilled water; 1.00 mL = 0.20 μg B. Pipet 0, 0.05, 0.10, 0.15, and 0.20 μg boron into evaporating dishes as indicated in ¶ *b* above. At the same time add an appropriate volume of sample (1.00 mL or portion diluted to 1.00 mL) to an identical evaporating dish. The total boron should be between 0.05 and 0.20 μg. Proceed as in ¶ *b* above, beginning with "Add 4.0 mL curcumin reagent. . . ." Compare color of samples with standards within 1 h of drying samples.

e. Removal of high hardness and cation interference: Prepare an ion-exchange column of approximately 20 cm × 1.3 cm diam. Charge column with a strongly acidic cation-exchange resin. Backwash column with distilled water to remove entrained air bubbles. Keep the resin covered with liquid at all times. Pass 50 mL 1 + 5 HCl through column at a rate of 0.2 mL acid/mL resin in column/min and wash column free of acid with distilled water.

Pipet 25 mL sample, or a smaller sample of known high boron content diluted to 25 mL, onto the resin column. Adjust rate of flow to about 2 drops/s and collect effluent in a 50-mL volumetric flask. Wash column with small portions of distilled water until flask is filled to mark. Mix and transfer 2.00 mL into evaporating dish. Add 4.0 mL curcumin reagent and complete the analysis as described in ¶ *b* above.

5. Calculation

Use the following equation to calculate boron concentration from absorbance readings:

$$\text{mg B/L} = \frac{A_2 \times C}{A_1 \times S}$$

where:

A_2 = absorbance of sample,
C = μg B in standard taken,
A_1 = absorbance of standard, and
S = mL sample.

6. Precision and Bias

A synthetic sample containing 240 μg B/L, 40 μg As/L, 250 μg Be/L, 20 μg Se/L, and 6 μg V/L in distilled water was analyzed in 30 laboratories by the curcumin method with a relative standard deviation of 22.8% and a relative error of 0%.

7. Bibliography

Silverman, L. & K. Trego. 1953. Colorimetric microdetermination of boron by the curcumin-acetone solution method. *Anal. Chem.* 25:1264.

Dirle, W.T., E. Truog & K.C. Berger. 1954. Boron determination in soils and plants—Simplified curcumin procedure. *Anal. Chem.* 26:418.

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Lishka, R.J. 1961. Comparison of analytical procedures for boron. *J. Amer. Water Works Assoc.* 53:1517.

Bunton, N.G. & B.H. Tait. 1969. Determination of boron in waters and effluents using curcumin. *J. Amer. Water Works Assoc.* 61:357.

† Eastman No. 1179, or equivalent.

‡ Whatman No. 30, or equivalent.

34500-B C. Carmine Method

1. General Discussion

a. Principle: In the presence of boron, a solution of carmine or carminic acid in concentrated sulfuric acid changes from a bright red to a bluish red or blue, depending on the concentration of boron present.

b. Interference: The ions commonly found in water and wastewater do not interfere.

c. Minimum detectable quantity: 2 μg B.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Colorimetric equipment: One of the following is required:

a. Spectrophotometer, for use at 585 nm, with a minimum light path of 1 cm.

b. Filter photometer, equipped with an orange filter having a maximum transmittance near 585 nm, with a minimum light path of 1 cm.

3. Reagents

Store all reagents in polyethylene or boron-free containers.

a. Standard boron solution: Prepare as directed in 4500-B.B.3*b*.

b. Hydrochloric acid (HCl), conc and 1 + 11.

c. Sulfuric acid (H_2SO_4), conc.

d. Carmine reagent: Dissolve 920 mg carmine N.F. 40, or carminic acid, in 1 L conc H_2SO_4. (If unable to zero spectrophotometer, dilute carmine 1 + 1 with conc H_2SO_4 to replace above reagent.)

4. Procedure

a. Low-level sample concentration: If sample contains less than 1 mg B/L, pipet a portion containing 2 to 20 μg B into a platinum dish, make alkaline with 1*N* NaOH plus a slight excess, and evaporate to dryness on a steam or hot water bath. If necessary, destroy any organic material by ignition at 500 to 550°C. Acidify cooled residue (ignited or not) with 2.5 mL 1 + 11 HCl and triturate with a rubber policeman to dissolve. Centrifuge if necessary to obtain a clear solution. Pipet 2.00 mL clear concentrate into a small flask or 30-mL test tube. Treat reagent blank identically.

b. Color development: Prepare a series of boron standard solutions (100, 250, 500, 750, and 1000 μg) in 100 mL with distilled water. Pipet 2.00 mL of each standard solution into a small flask or 30-mL test tube.

Treat blank and calibration standards exactly as the sample. Add 2 drops (0.1 mL) conc HCl, carefully introduce 10.0 mL conc H_2SO_4, mix, and let cool to room temperature. Add 10.0 mL carmine reagent, mix well, and after 45 to 60 min measure absorbance at 585 nm in a cell of 1-cm or longer light path, using the blank as reference.

To avoid error, make sure that no bubbles are present in the optical cell while photometric readings are being made. Bubbles may appear as a result of incomplete mixing of reagents. Because carmine reagent deteriorates, check calibration curve daily.

5. Calculation

$$\text{mg B/L} = \frac{\mu\text{g B}}{\text{mL sample}} \times \text{D}$$

where:

D = dilution correction (D = 1 for an undiluted sample, 1.25 if concentrated as in 4500-B.C4.*a*).

Other values determined by analyst.

6. Precision and Bias

A synthetic sample containing 180 μg B/L, 50 μg As/L, 400 μg Be/L, and 50 μg Se/L in distilled water was analyzed in nine laboratories by the carmine method with a relative standard deviation of 35.5% and a relative error of 0.6%.

7. Bibliography

HATCHER, J.T. & L.V. WILCOX. 1950. Colorimetric determination of boron using carmine. *Anal. Chem.* 22:567.

4500-Br^- BROMIDE*

4500-Br^- A. Introduction

1. Occurrence

Bromide occurs in varying amounts in ground and surface waters in coastal areas as a result of seawater intrusion and sea-spray-affected precipitation. The bromide content of ground waters and stream baseflows also can be affected by connate water. Industrial and oil-field brine discharges can contribute to the bromide in water sources. Under normal circumstances, the bromide content of most drinking waters is small, seldom exceeding 1 mg/L. Even levels of <100 μg/L can lead to formation of bromate or brominated by-products in disinfected waters.

2. Selection of Method

Described here are a colorimetric procedure suitable for the determination of bromide in most drinking waters and a flow injection analysis method. Bromide preferably is determined by the ion chromatography method (Section 4110) or by capillary ion electrophoresis (Section 4140).

* Approved by Standard Methods Committee, 2004. Editorial revisions, 2011. Joint Task Group: 20th Edition (4500-Br^-.D)—Scott Stieg (chair), Bradford R. Fisher, Owen B. Mathre, Theresa M. Wright.

4500-Br^- B. Phenol Red Colorimetric Method

1. General Discussion

a. Principle: When a sample containing bromide ions (Br^-) is treated with a dilute solution of chloramine-T in the presence of phenol red, the oxidation of bromide and subsequent bromination of the phenol red occur readily. If the reaction is buffered to pH 4.5 to 4.7, the color of the brominated compound will range from reddish to violet, depending on the bromide concentration. Thus, a sharp differentiation can be made among various concentrations of bromide. The concentration of chloramine-T and timing of the reaction before dechlorination are critical.

b. Interference: Most materials present in ordinary tap water do not interfere, but oxidizing and reducing agents and higher concentrations of chloride and bicarbonate can interfere. Free chlorine in samples should be destroyed as directed in Section 5210B.4*b*2); analyze bromide in a portion of dechlorinated sample. Addition of substantial chloride to the pH buffer solution (see 4500-Br^-.B3*a*) can eliminate chloride interference for waters with very low bromide/chloride ratios, such as those affected by dissolved road salt. Small amounts of dissolved iodide do not interfere, but small concentrations of ammonium ion interfere substantially. Sample dilution may reduce interferences to acceptable levels for some saline and waste waters. However, if two dilutions differing by a factor of at least five do not give comparable values, the method is inapplicable. Bromide concentration in diluted samples must be within the range of the method (0.1 to 1 mg/L).

c. Minimum detectable concentration: 0.1 mg Br^-/L.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer,* for use at 590 nm, providing a light path of at least 2 cm.

2) *Filter photometer,* providing a light path of at least 2 cm and equipped with an orange filter having a maximum transmittance near 590 nm.

3) *Nessler tubes,* matched, 100-mL, tall form.

b. Acid-washed glassware: Wash all glassware with 1 + 6 HNO_3 and rinse with distilled water to remove all trace of adsorbed bromide.

3. Reagents

a. Acetate buffer solution: Dissolve 90 g NaCl and 68 g sodium acetate trihydrate, $NaC_2H_3O_2 \cdot 3H_2O$, in distilled water. Add 30 mL conc (glacial) acetic acid and make up to 1 L. The pH should be 4.6 to 4.7.

b. Phenol red indicator solution: Dissolve 21 mg phenolsulfonephthalein sodium salt and dilute to 100 mL with distilled water.

c. Chloramine-T solution: Dissolve 500 mg chloramine-T, sodium *p*-toluenesulfonchloramide, and dilute to 100 mL with distilled water. Store in a dark bottle and refrigerate.

d. Sodium thiosulfate, 2M: Dissolve 49.6 g $Na_2S_2O_3 \cdot 5H_2O$ or 31.6 g $Na_2S_2O_3$ and dilute to 100 mL with distilled water.

e. Stock bromide solution: Dissolve 744.6 mg anhydrous KBr in distilled water and make up to 1000 mL; 1.00 mL = 500 μg Br^-.

f. Standard bromide solution: Dilute 10.00 mL stock bromide solution to 1000 mL with distilled water; 1.00 mL = 5.00 μg Br^-.

4. Procedure

a. Preparation of bromide standards: Prepare at least six standards, 0, 0.20, 0.40, 0.60, 0.80 and 1.00 mg Br^-/L, by diluting 0.0, 2.00, 4.00, 6.00, 8.00, and 10.00 mL standard bromide solution to 50.00 mL with distilled water. Treat standards the same as samples in ¶ *b* below.

b. Treatment of sample: Add 2 mL buffer solution, 2 mL phenol red solution, and 0.5 mL chloramine-T solution to

50.0 mL sample or two separate sample dilutions (see 4500-Br$^-$.B.1*b*) such that the final bromide concentration is in the range of 0.1 to 1.0 mg Br$^-$/L. Mix thoroughly immediately after each addition. Exactly 20 min after adding chloramine-T, dechlorinate by adding, with mixing, 0.5 mL $Na_2S_2O_3$ solution. Compare visually in nessler tubes against bromide standards prepared simultaneously, or preferably read in a photometer at 590 nm against a reagent blank. Determine the bromide values from a calibration curve of mg Br$^-$/L (in 55 mL final volume) against absorbance. A 2.54-cm light path yields an absorbance value of approximately 0.36 for 1 mg Br$^-$/L.

5. Calculation

mg Br$^-$/L = mg Br$^-$/L (from calibration curve) × dilution factor (if any). Results are based on 55 mL final volume for samples and standards.

6. Bibliography

STENGER, V.A. & I.M. KOLTHOFF. 1935. Detection and colorimetric estimation of microquantities of bromide. *J. Amer. Chem. Soc.* 57:831.

HOUGHTON, G.U. 1946. The bromide content of underground waters. *J. Soc. Chem. Ind.* (London) 65:227.

GOLDMAN, E. & D. BYLES. 1959. Suggested revision of phenol red method for bromide. *J. Amer. Water Works Assoc.* 51:1051.

SOLLO, F.W., T.E. LARSON & F.F. MCGURK. 1971. Colorimetric methods for bromine. *Environ. Sci. Technol.* 5:240.

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BASEL, C.L., J.D. DEFREESE & D.O. WHITTEMORE. 1982. Interferences in automated phenol red method for determination of bromide in water. *Anal. Chem.* 54:2090.

4500-Br$^-$ C. (Reserved)

4500-Br$^-$ D. Flow Injection Analysis

1. General Discussion

a. Principle: Bromide is oxidized to bromine by chloramine-T, followed by substitution of bromine on phenol red to produce bromphenol blue.[1] The absorbance measured at 590 nm is proportional to the concentration of bromide in the sample. Sodium thiosulfate is added to reduce interference from chloride.

This method is suitable for the determination of bromide in waters containing up to 20 000 mg Cl$^-$/L, including drinking, ground, and surface waters, and domestic and industrial wastes. The method determines total bromide, or, if the sample is filtered through a 0.45-μm-pore-size filter, the result is called "dissolved bromide." The difference between total bromide and dissolved bromide is called "insoluble bromide."

Also see 4500-Br$^-$.A and Section 4130, Flow Injection Analysis (FIA).

b. Interferences: Remove large or fibrous particulates by filtering sample through glass wool. Guard against contamination from reagents, water, glassware, and the sample preservation process.

Chloride interference is reduced by the addition of sodium thiosulfate. Chloramine-T dissociates in aqueous solution to form hypochlorous acid, which can then react with chloride, causing substitution of chloride at positions ortho to the hydroxy groups on phenol red, just as in bromination. Sodium thiosulfate reacts with chlorine to reduce this interferent to a selectivity (ratio of analyte to interferent concentration) of >28 000.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

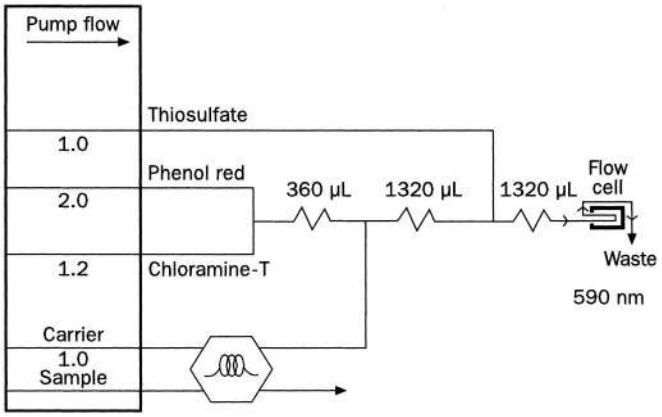


Figure 4500-Br$^-$:1. FIA bromide manifold.

2. Apparatus

Flow injection analysis equipment consisting of:

a. FIA injection valve with sample loop or equivalent.

b. Multichannel proportioning pump.

c. FIA manifold with flow cell (Figure 4500-Br$^-$:1). Relative flow rates only are shown. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material such as TFE.*

F1

* Teflon, or equivalent.

d. Absorbance detector, 590 nm, 10-nm bandpass.
e. Valve control and data acquisition system.

3. Reagents

Use reagent water (>10 megohm) to prepare carrier and all solutions. As an alternative to preparing reagents by weight/weight, use weight/volume.

a. Chloramine-T: To a tared 1-L container add 0.40 g chloramine-T hydrate (mol wt 227.65) and 999 g water. Cap and invert container to dissolve. Discard after 1 week.

b. Phenol red: To a tared 1-L container add 929 g water and 30.0 g glacial acetic acid. Swirl contents of container. Add 41.0 g sodium acetate and swirl container until it is dissolved. Add 0.040 g phenol red. Mix with a magnetic stirrer. Discard after 1 week.

c. Thiosulfate: To a tared 1-L container, add 724 g water and 500 g sodium thiosulfate pentahydrate, $Na_2S_2O_3 \cdot 5H_2O$. Dissolve by adding the solid slowly while stirring. The solid should be completely dissolved within 30 min. Gentle heating may be required. Discard after 1 week.

d. Stock bromide standard, 100.0 mg Br^-/L: To a 1-L volumetric flask add 0.129 g sodium bromide, NaBr. Dissolve in sufficient water, dilute to mark, and invert to mix.

e. Stock bromide standard, 10.0 mg Br^-/L: To a 500-mL volumetric flask add 50 mL stock standard (¶ *d* above). Dilute to mark and invert to mix. Prepare fresh monthly.

f. Standard bromide solutions: Prepare bromide standards for the calibration curve in the desired concentration range, using the stock standard (¶ *e* above), and diluting with water.

4. Procedure

Set up a manifold equivalent to that in Figure 4500-Br^-:1 and follow method supplied by manufacturer, or laboratory standard operating procedure for this method. Follow quality control guidelines outlined in Section 4020.

5. Calculations

Prepare standard curves by plotting absorbance of standards processed through the manifold vs. bromide concentration. The calibration curve gives a good fit to a second-order polynomial.

6. Precision and Bias

a. Precision: With a 300-μL sample loop, ten replicates of a 5.0-mg Br^-/L standard gave a mean of 5.10 mg Br^-/L and a relative standard deviation of 0.73%.

b. Bias: With a 300-μ/L sample loop, solutions of sodium chloride were fortified in triplicate with bromide and mean blanks and recoveries were measured. From a 10 000-mg Cl^-/L solution, a blank gave 0.13 mg Br^-/L. Corrected for this blank, a 1.0-mg Br^-/L known addition gave 98% recovery and a 5.0-mg Br^-/L known addition gave 102 % recovery. From a 20 000 mg Cl^-/L solution, a blank gave 0.27 mg Br^-/L. Corrected for this blank, a 1.0-mg Br^-/L known addition gave 100% recovery and a 5.0-mg Br^-/L known addition gave 101% recovery.

c. MDL: Using a published MDL method[2] and a 300-μL sample loop, analysts ran 21 replicates of a 0.5-mg Br^-/L standard. These gave a mean of 0.468 mg Br^-/L, a standard deviation of 0.030 mg Br^-/L, and an MDL of 0.07 mg Br^-/L. A lower MDL may be obtained by increasing the sample loop volume and increasing the ratio of carrier flow rate to reagents flow rate.

7. References

1. Anagnostopoulou, P. & M. Koupparis. 1986. Automated FIA phenol red method for determination of bromide. *Anal. Chem.* 58:322.
2. U.S. Environmental Protection Agency. 1989. Definition and procedure for the determination of method detection limits. Appendix B to CFR 136 rev. 1.11 amended June 30, 1986. 49 CFR 43430.

4500-CO_2 CARBON DIOXIDE*

4500-CO_2 A. Introduction

1. Occurrence and Significance

Surface waters normally contain less than 10 mg free carbon dioxide (CO_2) per liter, while some groundwaters may easily exceed that concentration. The CO_2 content of a water may contribute significantly to corrosion. Recarbonation of a supply during the last stages of water softening is a recognized treatment process. The subject of saturation with respect to calcium carbonate is discussed in Section 2330.

2. Selection of Method

A nomographic and a titrimetric method are described for the estimation of free CO_2 in drinking water. The titration may be performed potentiometrically or with phenolphthalein indicator. Properly conducted, the more rapid, simple indicator method is satisfactory for field tests and for control and routine applications if it is understood that the method gives, at best, only an approximation.

The nomographic method (4500-CO_2.B) usually gives a closer estimation of the total free CO_2 when the pH and alkalinity determinations are made immediately and correctly at the time of sampling. The pH measurement preferably should be made with an electrometric pH meter, properly calibrated with standard buffer solutions in the pH range of 7 to 8. The error resulting from inaccurate pH measurements grows with an increase in total alkalinity. For example, an inaccuracy of 0.1 in the pH determination causes a CO_2 error of 2 to 4 mg/L in the pH range of 7.0 to 7.3 and a total alkalinity of 100 mg $CaCO_3$/L. In the same pH range, the error approaches 10 to 15 mg/L when the total alkalinity is 400 mg as $CaCO_3$/L.

Under favorable conditions, agreement between the titrimetric and nomographic methods is reasonably good. When agreement is not precise and the CO_2 determination is of particular importance, state the method used.

The calculation of the total CO_2, free and combined, is given in 4500-CO_2.D.

* Approved by Standard Methods Committee, 2004. Editorial revisions, 2011.

4500-CO_2 B. Nomographic Determination of Free Carbon Dioxide and the Three Forms of Alkalinity*

1. General Discussion

Diagrams and nomographs enable the rapid calculation of the CO_2, bicarbonate, carbonate, and hydroxide content of natural and treated waters. These graphical presentations are based on equations relating the ionization equilibria of the carbonates and water. If pH, total alkalinity, temperature, and total mineral content are known, any or all of the alkalinity forms and CO_2 can be determined nomographically.

A set of charts, Figures 4500-CO_2:1–4,† is presented for use where their accuracy for the individual water supply is confirmed. The nomographs and the equations on which they are based are valid only when the salts of weak acids other than carbonic acid are absent or present in extremely small amounts.

Some treatment processes, such as superchlorination and coagulation, can affect significantly pH and total-alkalinity values of a poorly buffered water of low alkalinity and low total-dissolved-mineral content. In such instances, the nomographs may not be applicable.

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Precision and Bias

The precision possible with the nomographs depends on the size and range of the scales. With practice, the recommended nomographs can be read with a precision of 1%. However, the overall bias of the results depends on the bias of the analytical data applied to the nomographs and the validity of the theoretical equations and the numerical constants on which the nomographs are based. An approximate check of the bias of the calculations can be made by summing the three forms of alkalinity. Their sum should equal the total alkalinity.

3. Bibliography

MOORE, E.W. 1939. Graphic determination of carbon dioxide and the three forms of alkalinity. *J. Amer. Water Works Assoc.* 31:51.

* See also Alkalinity, Section 2320.

† Copies of the nomographs in Figures 4500-CO_2:1–4, enlarged to several times the size shown here, may be obtained as a set from the *Standard Methods* technical information manager at www.standardmethods.org/contact/.

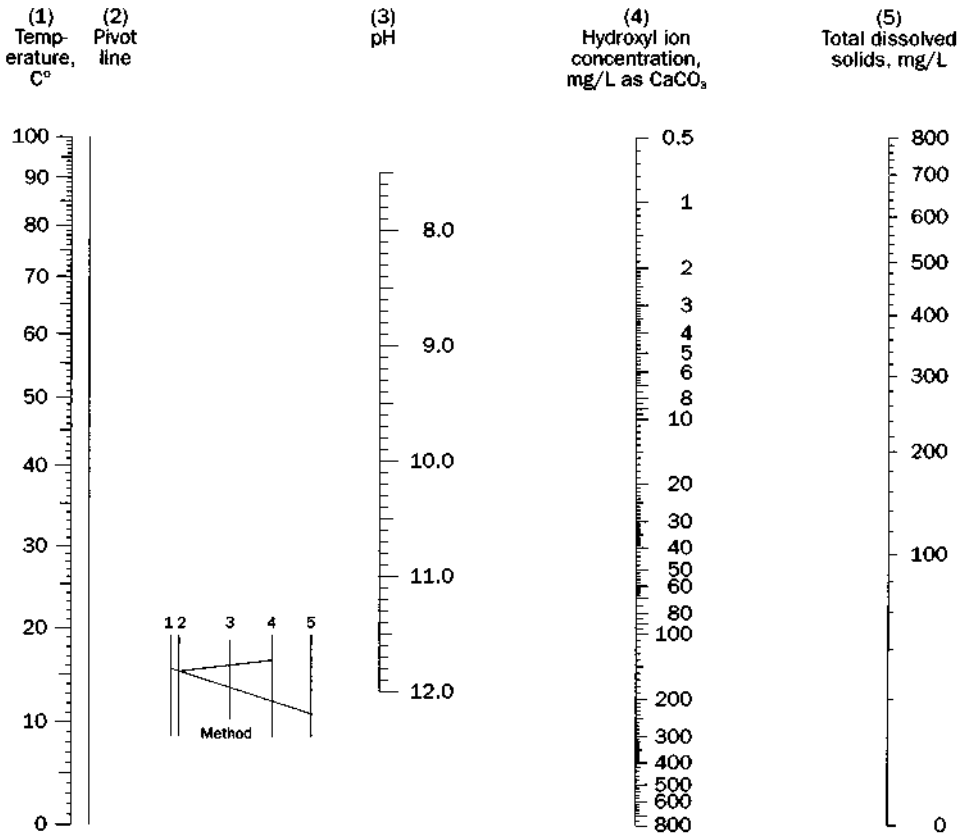


Figure 4500-CO_2:1. Nomograph for evaluation of hydroxide ion concentration. To use: Align temperature (Scale 1) and total dissolved solids (Scale 5); pivot on Line 2 to proper pH (Scale 3); read hydroxide ion concentration, as mg $CaCO_3$/L, on Scale 4. (Example: For 13°C temperature, 240 mg total dissolved solids/L, pH 9.8, the hydroxide ion concentration is found to be 1.4 mg as $CaCO_3$/L.)

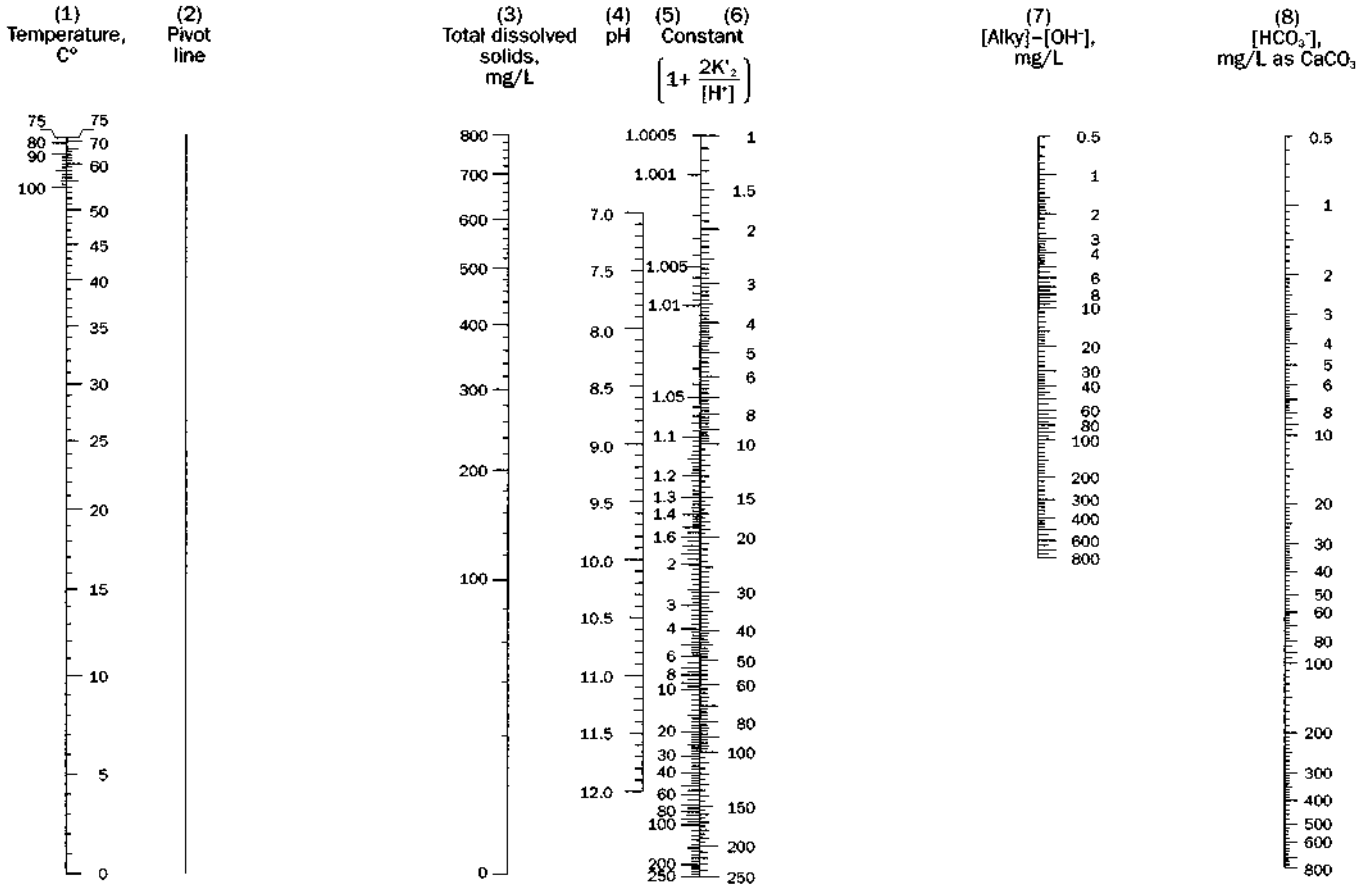


Figure 4500-CO_2:2. Nomograph for evaluation of bicarbonate alkalinity. To use: Align temperature (Scale 1) and total dissolved solids (Scale 3); pivot on Line 2 to proper pH (Scale 4) and read constant on Scale 5; locate constant on Scale 6 and align with nonhydroxide alkalinity (found with aid of Figure 4500-CO_2:1) on Scale 7; read bicarbonate alkalinity on Scale 8. (Example: For 13°C temperature, 240 mg total dissolved solids/L, pH 9.8, and 140 mg alkalinity/L, the bicarbonate content is found to be 90 mg as $CaCO_3$/L.)

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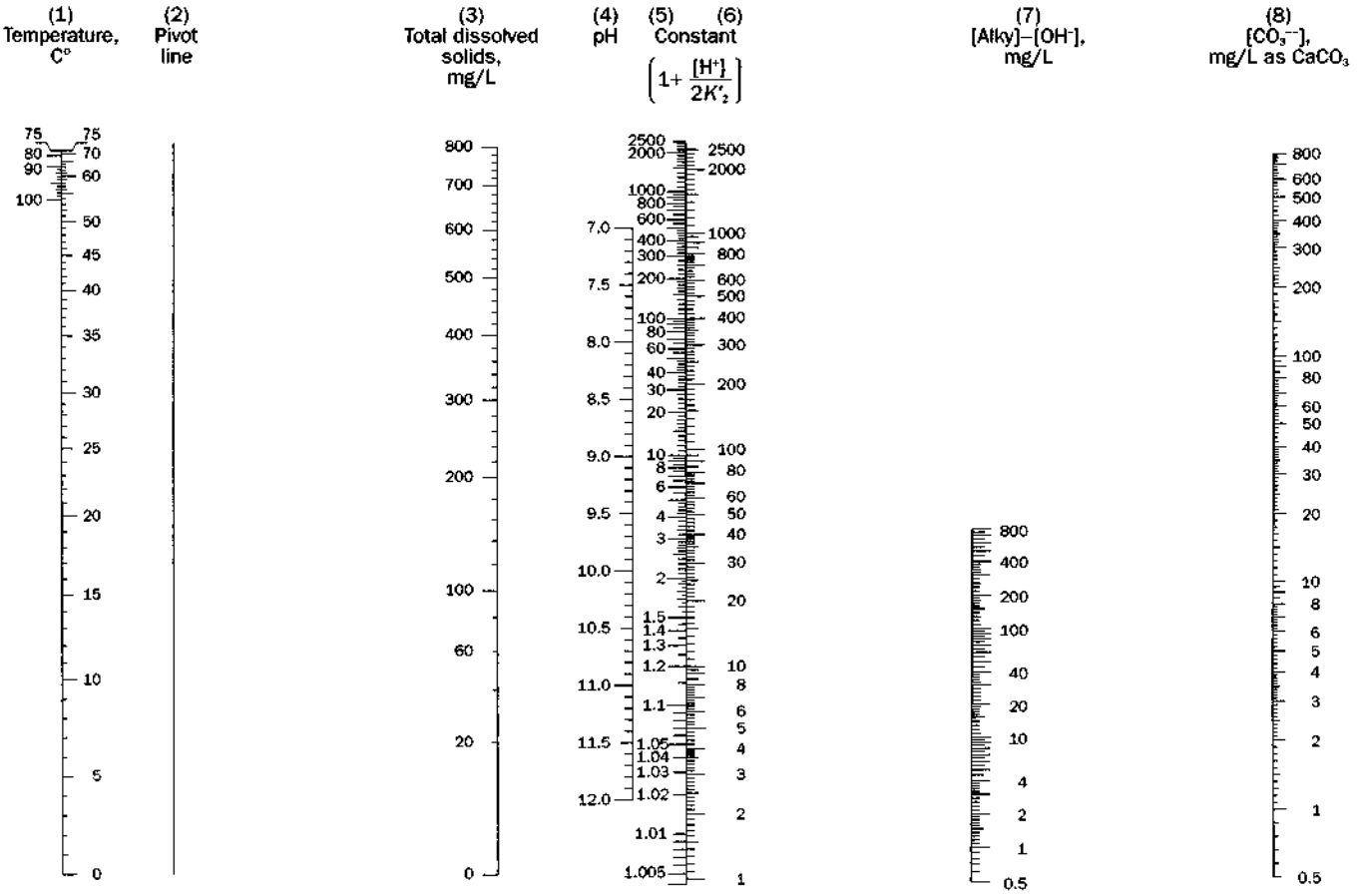


Figure 4500-CO_2:3. Nomograph for evaluation of carbonate alkalinity. To use: Align temperature (Scale 1) and total dissolved solids (Scale 3); pivot on Line 2 to proper pH (Scale 4) and read constant on Scale 5; locate constant on Scale 6 and align with nonhydroxide alkalinity (found with aid of Figure 4500-CO_2:1) on Scale 7; read carbonate alkalinity on Scale 8. (Example: For 13°C temperature, 240 mg total dissolved solids/L, pH 9.8, and 140 mg alkalinity/L, the carbonate content is found to be 50 mg as $CaCO_3$/L.)

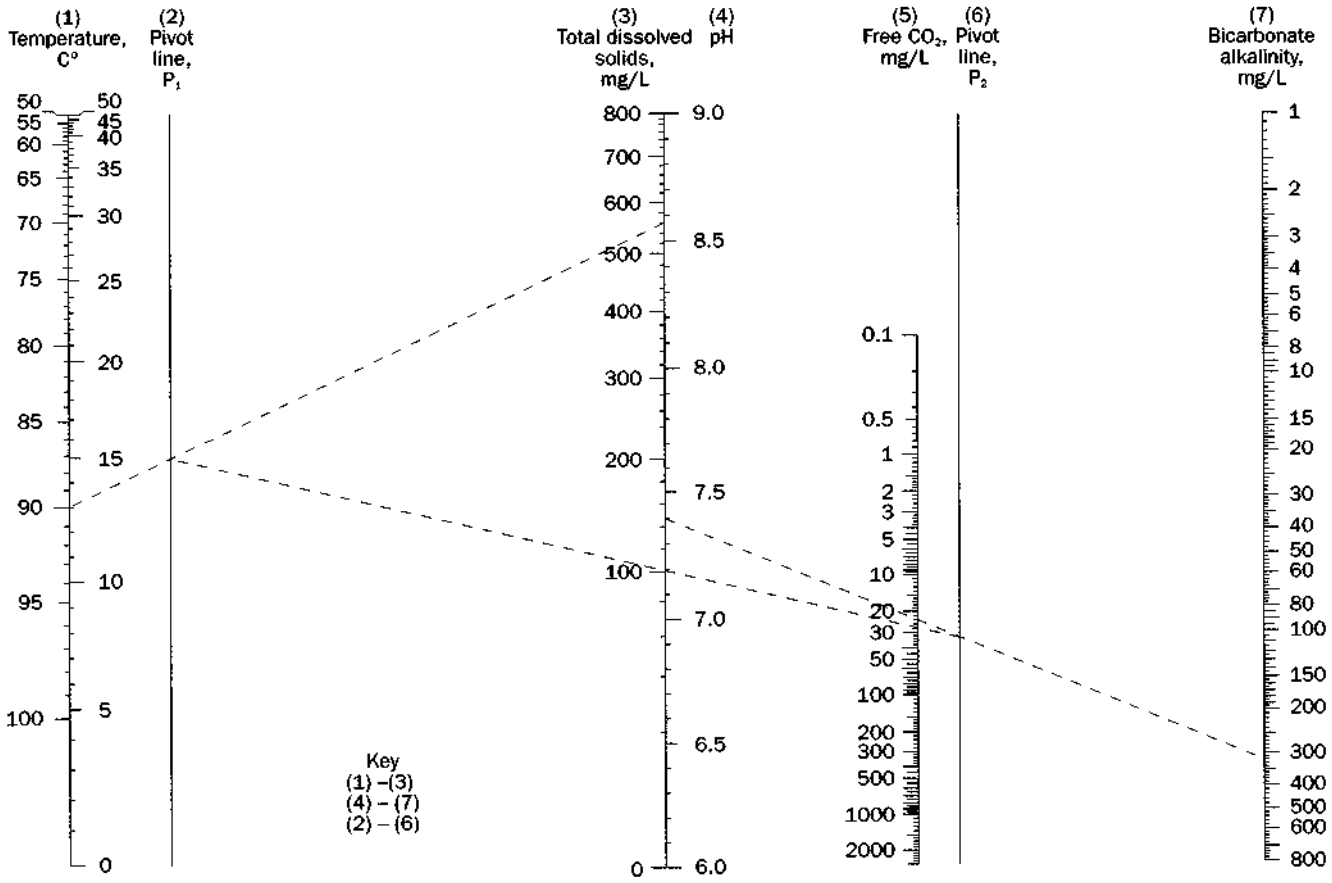


Figure 4500-CO_2:4. Nomograph for evaluation of free carbon dioxide content. To use: Align temperature (Scale 1) and total dissolved solids (Scale 3), which determines Point P_1 on Line 2; align pH (Scale 4) and bicarbonate alkalinity (Scale 7), which determines Point P_2 on Line 6; align P_1 and P_2 and read free carbon dioxide on Scale 5. (Example: For 13°C temperature, 560 mg total dissolved solids/L, pH 7.4, and 320 mg alkalinity/L, the free carbon dioxide content is found to be 28 mg/L.)

https://doi.org/10.2105/SMWW.2882.076

4500-CO_2 C. Titrimetric Method for Free Carbon Dioxide

1. General Discussion

a. Principle: Free CO_2 reacts with sodium carbonate or sodium hydroxide to form sodium bicarbonate. Completion of the reaction is indicated potentiometrically or by the development of the pink color characteristic of phenolphthalein indicator at the equivalence pH of 8.3. A 0.01*N* sodium bicarbonate ($NaHCO_3$) solution containing the recommended volume of phenolphthalein indicator is a suitable color standard until familiarity is obtained with the color at the endpoint.

b. Interference: Cations and anions that quantitatively disturb the normal CO_2–carbonate equilibrium interfere with the determination. Metal ions that precipitate in alkaline solution, such as aluminum, chromium, copper, and iron, contribute to high results. Ferrous ion should not exceed 1.0 mg/L. Positive errors also are caused by weak bases, such as ammonia or amines, and by salts of weak acids and strong bases, such as borate, nitrite, phosphate, silicate, and sulfide. Such substances should not exceed 5% of the CO_2 concentration. The titrimetric method for CO_2 is inapplicable to samples containing acid mine wastes and effluent from acid-regenerated cation exchangers. Negative errors may be introduced by high total dissolved solids, such as those encountered in seawater, or by addition of excess indicator.

c. Sampling and storage: Even with a careful collection technique, some loss in free CO_2 can be expected in storage and transit. This occurs more frequently when the gas is present in large amounts. Occasionally a sample may show an increase in free CO_2 content on standing. Consequently, determine free CO_2 immediately at the point of sampling. Where a field determination is impractical, fill completely a bottle for laboratory examination. Keep the sample, until tested, at a temperature lower than that at which the water was collected. Make the laboratory examination as soon as possible to minimize the effect of CO_2 changes.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

See Section 2310B.2.

3. Reagents

See Section 2310B.3.

4. Procedure

Follow the procedure given in Section 2310B.4*b*, phenolphthalein, or Section 2310B.4*d*, using endpoint pH 8.3.

5. Calculation

$$\text{mg } CO_2/\text{L} = \frac{A \times N \times 44\,000}{\text{mL sample}}$$

where:

A = mL titrant, and
N = normality of NaOH.

6. Precision and Bias

Precision and bias of the titrimetric method are on the order of ±10% of the known CO_2 concentration.

4500-CO_2 D. Carbon Dioxide and Forms of Alkalinity by Calculation

1. General Discussion

When the total alkalinity of a water (Section 2320) is due almost entirely to hydroxides, carbonates, or bicarbonates, and the total dissolved solids (Section 2540) is not greater than 500 mg/L, the alkalinity forms and free CO_2 can be calculated from the sample pH and total alkalinity. The calculation is subject to the same limitations as the nomographic procedure given above and the additional restriction of using a single temperature, 25°C. The calculations are based on the ionization constants:

$$K_1 = \frac{[H^+][HCO_3^-]}{[H_2CO_3^*]} \quad (K_1 = 10^{-6.36})$$

and

$$K_2 = \frac{[H^+][CO_3^{2-}]}{[HCO_3^-]} \quad (K_2 = 10^{-10.33})$$

where:

$$[H_2CO_3^*] = [H_2CO_3] + [CO_2(aq)]$$

Activity coefficients are assumed equal to unity.

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Calculation

Compute the forms of alkalinity and sample pH and total alkalinity using the following equations:

a. Bicarbonate alkalinity:

$$HCO_3^- \text{ as mg } CaCO_3/\text{L} = \frac{T - 5.0 \times 10^{(\text{pH}-10)}}{1 + 0.94 \times 10^{(\text{pH}-10)}}$$

where:

T = total alkalinity, mg $CaCO_3$/L.

b. Carbonate alkalinity:

$$CO_3^{2-} \text{ as mg } CaCO_3/L = 0.94 \times B \times 10^{(pH-10)}$$

where:

B = bicarbonate alkalinity, from *a*.

c. Hydroxide alkalinity:

$$OH^- \text{ as mg } CaCO_3/L = 5.0 \times 10^{(pH-10)}$$

d. Free carbon dioxide:

$$\text{mg } CO_2/L = 2.0 \times B \times 10^{(6-pH)}$$

where:

B = bicarbonate alkalinity, from *a*.

e. Total carbon dioxide:

$$\text{mg total } CO_2/L = A + 0.44\,(2B + C)$$

where:

A = mg free CO_2/L,
B = bicarbonate alkalinity from *a*, and
C = carbonate alkalinity from *b*.

3. Bibliography

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4500-Cl^- CHLORIDE*

4500-Cl^- A. Introduction

1. Occurrence

Chloride, in the form of chloride (Cl^-) ion, is one of the major inorganic anions in water and wastewater. The salty taste produced by chloride concentrations is variable and dependent on the chemical composition of water. Some waters containing 250 mg Cl^-/L may have a detectable salty taste if the cation is sodium. On the other hand, the typical salty taste may be absent in waters containing as much as 1000 mg/L when the predominant cations are calcium and magnesium.

The chloride concentration is higher in wastewater than in raw water because sodium chloride (NaCl) is a common article of diet and passes unchanged through the digestive system. Along the sea coast, chloride may be present in high concentrations because of leakage of salt water into the sewerage system. It also may be increased by industrial processes.

A high chloride content may harm metallic pipes and structures, as well as growing plants.

2. Selection of Method

Six methods are presented for the determination of chloride. Because the first two are similar in most respects, selection is largely a matter of personal preference. The argentometric method (4500-Cl^-.B) is suitable for use in relatively clear waters when 0.15 to 10 mg Cl^- are present in the portion titrated. The endpoint of the mercuric nitrate method (4500-Cl^-.C) is easier to detect. The potentiometric method (4500-Cl^-.D) is suitable for colored or turbid samples in which color-indicated endpoints might be difficult to observe. The potentiometric method can be used without a pretreatment step for samples containing ferric ions (if not present in an amount greater than the chloride concentration), chromic, phosphate, and ferrous and other heavy-metal ions. The ferricyanide method (4500-Cl^-.E) is an automated technique. Flow injection analysis (4500-Cl^-.G), an automated colorimetric technique, is useful for analyzing large numbers of samples. Preferably determine chloride by ion chromatography (Section 4110). Chloride also can be determined by the capillary ion electrophoresis method (Section 4140). Methods 4500-Cl^-.C and G in which mercury, a highly toxic reagent, is used require special disposal practices to avoid improper sewage discharges. Follow appropriate regulatory procedures (see Section 1090).

3. Sampling and Storage

Collect representative samples in clean, chemically resistant glass or plastic bottles. The maximum sample portion required is 100 mL. No special preservative is necessary if the sample is to be stored.

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* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011. Joint Task Group: 20th Edition (4500-Cl^-.G)—Scott Stieg (chair), Bradford R. Fisher, Owen B. Mathre, Theresa M. Wright.

4500-Cl^- B. Argentometric Method

1. General Discussion

a. Principle: In a neutral or slightly alkaline solution, potassium chromate can indicate the endpoint of the silver nitrate titration of chloride. Silver chloride is precipitated quantitatively before red silver chromate is formed.

b. Interference: Substances in amounts normally found in potable waters will not interfere. Bromide, iodide, and cyanide register as equivalent chloride concentrations. Sulfide, thiosulfate, and sulfite ions interfere but can be removed by treatment with hydrogen peroxide. Orthophosphate in excess of 25 mg/L interferes by precipitating as silver phosphate. Iron in excess of 10 mg/L interferes by masking the endpoint.

2. Apparatus

a. Erlenmeyer flask, 250-mL.
b. Buret, 50-mL.

3. Reagents

a. Potassium chromate indicator solution: Dissolve 50 g K_2CrO_4 in a little distilled water. Add $AgNO_3$ solution until a definite red precipitate is formed. Let stand 12 h, filter, and dilute to 1 L with distilled water.

b. Standard silver nitrate titrant, 0.0141*M* (0.0141*N*): Dissolve 2.395 g $AgNO_3$ in distilled water and dilute to 1000 mL. Standardize against NaCl by the procedure described in 4500-Cl^-.B.4*b*; 1.00 mL = 500 μg Cl^-. Store in a brown bottle.

c. Standard sodium chloride, 0.0141*M* (0.0141*N*): Dissolve 824.0 mg NaCl (dried at 140°C) in distilled water and dilute to 1000 mL; 1.00 mL = 500 μg Cl^-.

d. Special reagents for removal of interference:

1) *Aluminum hydroxide suspension*—Dissolve 125 g aluminum potassium sulfate or aluminum ammonium sulfate, $AlK(SO_4)_2 \cdot 12H_2O$ or $AlNH_4(SO_4)_2 \cdot 12H_2O$, in 1 L distilled water. Warm to 60°C and add 55 mL conc ammonium hydroxide (NH_4OH) slowly with stirring. Let stand about 1 h, transfer to a

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large bottle, and wash precipitate by successive additions, with thorough mixing and decanting with distilled water, until free from chloride. When freshly prepared, the suspension occupies a volume of approximately 1 L.

2) *Phenolphthalein indicator solution.*

3) *Sodium hydroxide* (NaOH), 1*N*.

4) *Sulfuric acid* (H_2SO_4), 1*N*.

5) *Hydrogen peroxide* (H_2O_2), 30%.

4. Procedure

a. Sample preparation: Use a 100-mL sample or a suitable portion diluted to 100 mL. If the sample is highly colored, add 3 mL $Al(OH)_3$ suspension, mix, let settle, and filter.

If sulfide, sulfite, or thiosulfate is present, add 1 mL H_2O_2 and stir for 1 min.

b. Titration: Directly titrate samples in the pH range 7 to 10. Adjust sample pH to 7 to 10 with H_2SO_4 or NaOH if it is not in this range. For adjustment, preferably use a pH meter with a non-chloride-type reference electrode. (If only a chloride-type electrode is available, determine amount of acid or alkali needed for adjustment and discard this sample portion. Treat a separate portion with required acid or alkali and continue analysis.) Add 1.0 mL K_2CrO_4 indicator solution. Titrate with standard $AgNO_3$ titrant to a pinkish yellow endpoint. Be consistent in endpoint recognition.

Standardize $AgNO_3$ titrant and establish reagent blank value by the titration method outlined above. A blank of 0.2 to 0.3 mL is usual.

5. Calculation

$$\text{mg Cl}^-\text{/L} = \frac{(A - B) \times N \times 35\,450}{\text{mL sample}}$$

where:

A = mL titration for sample,
B = mL titration for blank, and
N = normality of $AgNO_3$.

$$\text{mg NaCl/L} = (\text{mg Cl}^-\text{/L}) \times 1.65$$

6. Precision and Bias

A synthetic sample containing 241 mg Cl$^-$/L, 108 mg Ca/L, 82 mg Mg/L; 3.1 mg K/L, 19.9 mg Na/L, 1.1 mg NO_3^--N/L, 0.25 mg NO_2^-- N/L, 259 mg SO_4^{2-}/L, and 42.5 mg total alkalinity/L (contributed by $NaHCO_3$) in distilled water was analyzed in 41 laboratories by the argentometric method, with a relative standard deviation of 4.2% and a relative error of 1.7%.

7. Bibliography

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PAUSTIAN, P. 1987. A novel method to calculate the Mohr chloride titration. *In* Advances in Water Analysis and Treatment, Proc. 14th Annu. AWWA Water Quality Technology Conf., November 16–20, 1986, Portland, Ore., p. 673. American Water Works Assoc., Denver, Colo.

4500-Cl$^-$ C. Mercuric Nitrate Method

1. General Discussion

a. Principle: Chloride can be titrated with mercuric nitrate, $Hg(NO_3)_2$, because of the formation of soluble, slightly dissociated mercuric chloride. In the pH range 2.3 to 2.8, diphenylcarbazone indicates the titration endpoint by formation of a purple complex with the excess mercuric ions. Xylene cyanol FF serves as a pH indicator and endpoint enhancer. Increasing the strength of the titrant and modifying the indicator mixtures extend the range of measurable chloride concentrations.

b. Interference: Bromide and iodide are titrated with $Hg(NO_3)_2$ in the same manner as chloride. Chromate, ferric, and sulfite ions interfere when present in excess of 10 mg/L.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Erlenmeyer flask, 250-mL.

b. Microburet, 5-mL with 0.01-mL graduation intervals.

3. Reagents

a. Standard sodium chloride, 0.0141*M* (0.0141*N*): See 4500-Cl$^-$.B.3*c*.

b. Nitric acid (HNO_3), 0.1*N*.

c. Sodium hydroxide (NaOH), 0.1*N*.

d. Reagents for chloride concentrations below 100 mg/L:

1) *Indicator-acidifier reagent*—The HNO_3 concentration of this reagent is an important factor in the success of the determination and can be varied as indicated in ¶ a) or b) below to suit the alkalinity range of the sample. Reagent a) contains sufficient HNO_3 to neutralize a total alkalinity of

150 mg as $CaCO_3$/L to the proper pH in a 100-mL sample. Adjust amount of HNO_3 to accommodate samples of alkalinity different from 150 mg/L.

a) Dissolve, in the order named, 250 mg s-diphenylcarbazone, 4.0 mL conc HNO_3, and 30 mg xylene cyanol FF in 100 mL 95% ethyl alcohol or isopropyl alcohol. Store in a dark bottle in a refrigerator. This reagent is not stable indefinitely. Deterioration causes a slow endpoint and high results.

b) Because pH control is critical, adjust pH of highly alkaline or acid samples to 2.5 ± 0.1 with 0.1*N* HNO_3 or NaOH, not with sodium carbonate (Na_2CO_3). Use a pH meter with a nonchloride type of reference electrode for pH adjustment. If only the usual chloride-type reference electrode is available for pH adjustment, determine amount of acid or alkali required to obtain a pH of 2.5 ± 0.1 and discard this sample portion. Treat a separate sample portion with the determined amount of acid or alkali and continue analysis. Under these circumstances, omit HNO_3 from indicator reagent.

2) *Standard mercuric nitrate titrant,* 0.007 05*M* (0.0141*N*)—Dissolve 2.3 g $Hg(NO_3)_2$ or 2.5 g $Hg(NO_3)_2 \cdot H_2O$ in 100 mL distilled water containing 0.25 mL conc HNO_3. Dilute to just under 1 L. Make a preliminary standardization by following the procedure described in 4500-Cl⁻.C.4*a*. Use replicates containing 5.00 mL standard NaCl solution and 10 mg sodium bicarbonate ($NaHCO_3$) diluted to 100 mL with distilled water. Adjust titrant to 0.0141*N* and make a final standardization; 1.00 mL = 500 μg Cl⁻. Store away from light in a dark bottle.

e. Reagent for chloride concentrations greater than 100 mg/L—

1) *Mixed indicator reagent*—Dissolve 0.50 g diphenylcarbazone powder and 0.05 g bromphenol blue powder in 75 mL 95% ethyl or isopropyl alcohol and dilute to 100 mL with the same alcohol.

2) *Strong standard mercuric nitrate titrant,* 0.0705*M* (0.141*N*)—Dissolve 25 g $Hg(NO_3)_2 \cdot H_2O$ in 900 mL distilled water containing 5.0 mL conc HNO_3. Dilute to just under 1 L and standardize by following the procedure described in 4500-Cl⁻.C.4*b*. Use replicates containing 25.00 mL standard NaCl solution and 25 mL distilled water. Adjust titrant to 0.141*N* and make a final standardization; 1.00 mL = 5.00 mg Cl⁻.

4. Procedure

a. Titration of chloride concentrations less than 100 mg/L: Use a 100-mL sample or smaller portion so that the chloride content is less than 10 mg.

Add 1.0 mL indicator-acidifier reagent. (The color of the solution should be green-blue at this point. A light green indicates pH less than 2.0; a pure blue indicates pH more than 3.8.) For most potable waters, the pH after this addition will be 2.5 ± 0.1. For highly alkaline or acid waters, adjust pH to about 8 before adding indicator-acidifier reagent.

Titrate with 0.0141*N* $Hg(NO_3)_2$ titrant to a definite purple endpoint. The solution turns from green-blue to blue a few drops before the endpoint.

Determine blank by titrating 100 mL distilled water containing 10 mg $NaHCO_3$.

b. Titration of chloride concentrations greater than 100 mg/L: Use a sample portion (5 to 50 mL) requiring less than 5 mL titrant to reach the endpoint. Measure into a 150-mL beaker. Add approximately 0.5 mL mixed indicator reagent and mix well. The color should be purple. Add 0.1*N* HNO_3 dropwise until the color just turns yellow. Titrate with strong $Hg(NO_3)_2$ titrant to first permanent dark purple. Titrate a distilled water blank using the same procedure.

5. Calculation

$$\text{mg Cl}^-\text{/L} = \frac{(A - B) \times N \times 35\,450}{\text{mL sample}}$$

where:

A = mL titration for sample,
B = mL titration for blank, and
N = normality of $Hg(NO_3)_2$.

$$\text{mg NaCl/L} = (\text{mg Cl}^-\text{/L}) \times 1.65$$

6. Precision and Bias

A synthetic sample containing 241 mg Cl⁻/L, 108 mg Ca/L, 82 mg Mg/L, 3.1 mg K/L, 19.9 mg Na/L, 1.1 mg NO_3^--N/L, 0.25 mg NO_2^--N/L, 259 mg SO_4^{2-}/L, and 42.5 mg total alkalinity/L (contributed by $NaHCO_3$) in distilled water was analyzed in 10 laboratories by the mercurimetric method, with a relative standard deviation of 3.3% and a relative error of 2.9%.

7. Bibliography

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4500-Cl⁻ D. Potentiometric Method

1. General Discussion

a. Principle: Chloride is determined by potentiometric titration with silver nitrate solution with a glass and silver-silver chloride electrode system. During titration an electronic voltmeter is used to detect the change in potential between the two electrodes. The endpoint of the titration is that instrument reading at which the greatest change in voltage has occurred for a small and constant increment of silver nitrate added.

b. Interference: Iodide and bromide also are titrated as chloride. Ferricyanide causes high results and must be removed. Chromate and dichromate interfere and should be reduced to the chromic state

or removed. Ferric iron interferes if present in an amount substantially higher than the amount of chloride. Chromic ion, ferrous ion, and phosphate do not interfere.

Grossly contaminated samples usually require pretreatment. Where contamination is minor, some contaminants can be destroyed simply by adding nitric acid.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Glass and silver-silver chloride electrodes: Prepare in the laboratory or purchase a silver electrode coated with AgCl for use with specified instruments. Instructions on use and care of electrodes are supplied by the manufacturer.

b. Electronic voltmeter, to measure potential difference between electrodes: A pH meter may be converted to this use by substituting the appropriate electrode.

c. Mechanical stirrer, with plastic-coated or glass impeller.

3. Reagents

a. Standard sodium chloride solution, 0.0141*M* (0.0141*N*): See 4500-Cl⁻.B.3*c*.

b. Nitric acid (HNO_3), conc.

c. Standard silver nitrate titrant, 0.0141*M* (0.0141*N*): See 4500-Cl⁻.B.3*b*.

d. Pretreatment reagents:

1) *Sulfuric acid* (H_2SO_4), 1 + 1.

2) *Hydrogen peroxide* (H_2O_2), 30%.

3) *Sodium hydroxide* (NaOH), 1*N*.

4. Procedure

a. Standardization: The various instruments that can be used in this determination differ in operating details; follow the manufacturer's instructions. Make necessary mechanical adjustments. Then, after allowing sufficient time for warmup (10 min), balance internal electrical components to give an instrument setting of 0 mV or, if a pH meter is used, a pH reading of 7.0.

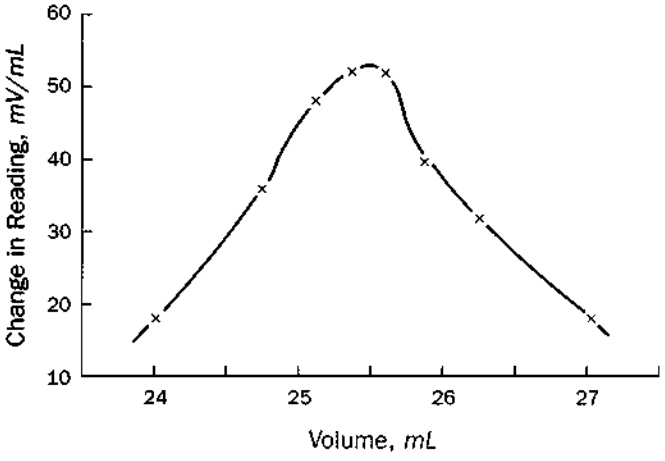
1) Place 10.0 mL standard NaCl solution in a 250-mL beaker, dilute to about 100 mL, and add 2.0 mL conc HNO_3. Immerse stirrer and electrodes.

2) Set instrument to desired range of millivolts or pH units. Start stirrer.

3) Add standard $AgNO_3$ titrant, recording scale reading after each addition. At the start, large increments of $AgNO_3$ may be added; then, as the endpoint is approached, add smaller and equal increments (0.1 or 0.2 mL) at longer intervals, so the exact endpoint can be determined. Determine volume of $AgNO_3$ used at the point at which there is the greatest change in instrument reading per unit addition of $AgNO_3$.

4) Plot a differential titration curve if the exact endpoint cannot be determined by inspecting the data. Plot change in instrument reading for equal increments of $AgNO_3$ against volume of $AgNO_3$ added, using average of buret readings before and after each addition. The procedure is illustrated in Figure 4500-Cl⁻:1.

b. Sample analysis:



Experimental Data Plotted Above

Volume, mL	23.50	24.50	25.00	25.25	25.50	25.75	26.00	26.50	27.50
Change, mV/mL		18	36	48	52	52	40	32	18

Figure 4500-Cl⁻:1. Example of differential titration curve (endpoint is 25.5 mL).

1) Pipet 100.0 mL sample, or a portion containing not more than 10 mg Cl⁻, into a 250-mL beaker. In the absence of interfering substances, proceed with ¶ *b*3) below.

2) In the presence of organic compounds, sulfite, or other interferences (such as large amounts of ferric iron, cyanide, or sulfide) acidify sample with H_2SO_4, using litmus paper. Boil for 5 min to remove volatile compounds. Add more H_2SO_4, if necessary, to keep solution acidic. Add 3 mL H_2O_2 and boil for 15 min, adding chloride-free distilled water to keep the volume above 50 mL. Dilute to 100 mL, add NaOH solution dropwise until alkaline to litmus, then 10 drops in excess. Boil for 5 min, filter into a 250-mL beaker, and wash precipitate and paper several times with hot distilled water.

3) Add conc HNO_3 dropwise until acidic to litmus paper, then 2.0 mL in excess. Cool and dilute to 100 mL if necessary. Immerse stirrer and electrodes and start stirrer. Make any necessary adjustments according to the manufacturer's instructions and set selector switch to appropriate setting for measuring the difference of potential between electrodes.

4) Complete determination by titrating according to ¶ *a*4) above. If an endpoint reading has been established from previous determinations for similar samples and conditions, use this predetermined endpoint. For the most accurate work, make a blank titration by carrying chloride-free distilled water through the procedure.

5. Calculation

$$\text{mg Cl}^-\text{/L} = \frac{(A - B) \times N \times 35\,450}{\text{mL sample}}$$

where:

A = mL $AgNO_3$,
B = mL blank, and
N = normality of titrant.

6. Precision and Bias

In the absence of interfering substances, the precision and bias are estimated to be about 0.12 mg for 5 mg Cl^-, or 2.5% of the amount present. When pretreatment is required to remove interfering substances, the precision and bias are reduced to about 0.25 mg for 5 mg Cl^-, or 5% of amount present.

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4500-Cl⁻ E. Automated Ferricyanide Method

1. General Discussion

a. Principle: Thiocyanate ion is liberated from mercuric thiocyanate by the formation of soluble mercuric chloride. In the presence of ferric ion, free thiocyanate ion forms a highly colored ferric thiocyanate, of which the intensity is proportional to the chloride concentration.

b. Interferences: Remove particulate matter by filtration or centrifugation before analysis. Guard against contamination from reagents, water, glassware, and sample preservation process. No chemical interferences are significant.

c. Application: The method is applicable to potable, surface, and saline waters, and domestic and industrial wastewaters. The concentration range is 1 to 200 mg Cl^-/L; it can be extended by dilution.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Automated analytical equipment: An example of the continuous-flow analytical instrument consists of the interchangeable components shown in Figure 4500-Cl⁻:2.

b. Filters, 480-nm.

3. Reagents

a. Stock mercuric thiocyanate solution: Dissolve 4.17 g $Hg(SCN)_2$ in about 500 mL methanol, dilute to 1000 mL with methanol, mix, and filter through filter paper.

b. Stock ferric nitrate solution: Dissolve 202 g $Fe(NO_3)_3 \cdot 9H_2O$ in about 500 mL distilled water, then carefully add 21 mL conc HNO_3. Dilute to 1000 mL with distilled water and mix. Filter through paper and store in an amber bottle.

c. Color reagent: Add 150 mL stock $Hg(SCN)_2$ solution to 150 mL stock $Fe(NO_3)_3$ solution. Mix and dilute to 1000 mL with distilled water. Add 0.5 mL polyoxyethylene 23 lauryl ether.*

d. Stock chloride solution: Dissolve 1.6482 g NaCl, dried at 140°C, in distilled water and dilute to 1000 mL; 1.00 mL = 1.00 mg Cl^-.

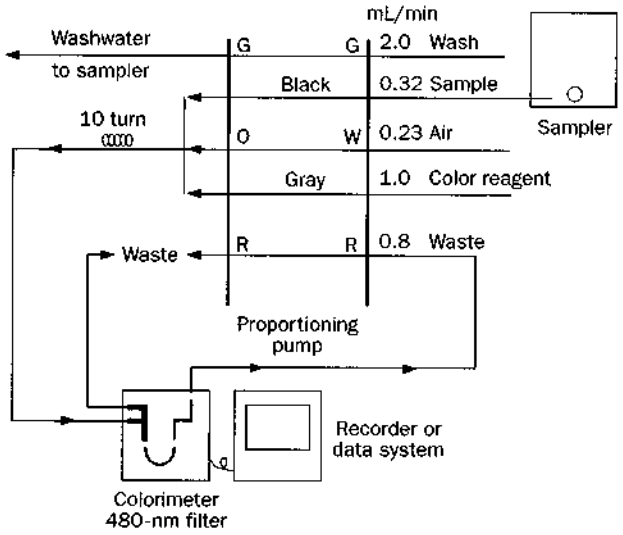


Figure 4500-Cl⁻:2. Flow scheme for automated chloride analysis.

e. Standard chloride solutions: Prepare chloride standards in the desired concentration range, such as 1 to 200 mg/L, using stock chloride solution.

4. Procedure

Set up manifold as shown in Figure 4500-Cl⁻:2 and follow general procedure described by the manufacturer.

5. Calculation

Prepare standard curves by plotting response of standards processed through the manifold against chloride concentrations in standards. Compute sample chloride concentration by comparing sample response with standard curve.

6. Precision and Bias

With an automated system in a single laboratory six samples were analyzed in septuplicate. At a concentration ranging

* Brij 35, available from ICI Americas, Wilmington, DE, or equivalent.

from about 1 to 50 mg Cl^-/L the average standard deviation was 0.39 mg/L. The coefficient of variation was 2.2%. In two samples with added chloride, recoveries were 104% and 97%.

7. Bibliography

ZALL, D.M., D. FISHER & M.D. GARNER. 1956. Photometric determination of chlorides in water. *Anal. Chem.* 28:1665.

O'BRIEN, J.E. 1962. Automatic analysis of chlorides in sewage. *Wastes Eng.* 33:670.

4500-Cl^- F. (Reserved)

4500-Cl^- G. Mercuric Thiocyanate Flow Injection Analysis

1. General Discussion

a. Principle: A water sample containing chloride is injected into a carrier stream to which mercuric thiocyanate and ferric nitrate are added. The chloride complexes with the Hg(II), displacing the thiocyanate anion, which forms the highly colored ferric thiocyanate complex anion. The resulting peak's absorbance is measured at 480 nm. The peak area is proportional to the concentration of chloride in the original sample.

Also see 4500-Cl^-.A and Section 4130, Flow Injection Analysis (FIA).

b. Interferences: Remove large or fibrous particulates by filtering sample through glass wool. Guard against contamination from reagents, water, glassware, and the sample preservation process.

Substances such as sulfite and thiosulfate, which reduce iron(III) to iron(II) and mercury(II) to mercury(I), can interfere. Halides, which also form strong complexes with mercuric ion (e.g., Br^-, I^-), give a positive interference.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Flow injection analysis equipment consisting of:

a. FIA injection valve with sample loop.

b. Multichannel proportioning pump.

c. FIA manifold with flow cell (Figure 4500-Cl^-:3). Relative flow rates only are shown. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material such as TFE.*

d. Absorbance detector, 480 nm, 10-nm bandpass.

e. Valve control and data acquisition system.

3. Reagents

Use reagent water (>10 megohm) to prepare carrier and all solutions.

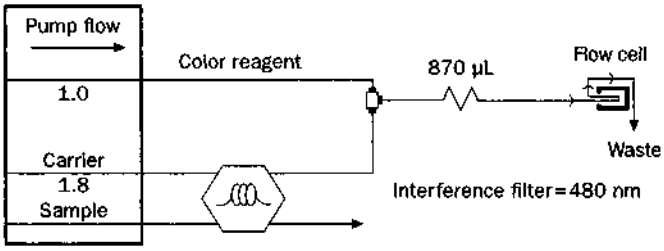


Figure 4500-Cl^-:3. FIA chloride manifold.

a. Stock mercuric thiocyanate solution: In a 1-L volumetric flask, dissolve 4.17 g mercuric thiocyanate, $Hg(SCN)_2$, in about 500 mL methanol. Dilute to mark with methanol and mix. **CAUTION: Mercuric thiocyanate is toxic. Wear gloves!**

b. Stock ferric nitrate reagent, 0.5*M:* In a 1-L volumetric flask, dissolve 202 g ferric nitrate, $Fe(NO_3)_3 \cdot 9H_2O$, in approximately 800 mL water. Add 25 mL conc HNO_3 and dilute to mark. Invert to mix.

c. Color reagent: In a 500-mL volumetric flask, mix 75 mL stock mercuric thiocyanate solution with 75 mL stock ferric nitrate reagent and dilute to mark with water. Invert to mix. Vacuum filter through a 0.45-μm membrane filter. The color reagent also is available as a commercially prepared solution that is stable for several months.

d. Stock chloride standard, 1000 mg Cl^-/L: In a 105°C oven, dry 3 g primary standard grade sodium chloride, NaCl, overnight. In a 1-L volumetric flask, dissolve 1.648 g primary standard grade sodium chloride in about 500 mL water. Dilute to mark and invert to mix.

e. Standard chloride solutions: Prepare chloride standards for the calibration curve in the desired concentration range, using the stock standard (¶ *d* above), and diluting with water.

4. Procedure

Set up a manifold equivalent to that in Figure 4500-Cl^-:3 and follow method supplied by manufacturer, or laboratory standard operating procedure for this method.

* Teflon, or equivalent.

5. Calculations

Prepare standard curves by plotting absorbance of standards processed through the manifold versus chloride concentration. The calibration curve gives a good fit to a second-order polynomial.

6. Precision and Bias

a. Recovery and relative standard deviation: The results of single-laboratory studies with various matrices are given in Table 4500-Cl⁻:I.

b. MDL: A 100-μL sample loop was used in the method described above. Using a published MDL method[1] analysts ran 21 replicates of a 1.0-mg Cl⁻/L standard. These gave a mean of 1.19 mg Cl⁻/L, a standard deviation of 0.027 mg Cl⁻/L, and an MDL of 0.07 mg Cl⁻/L. This is only an estimate because the ratio of standard to the MDL is above guidelines (see Section 1030C). A lower MDL may be obtained by increasing the sample loop volume and increasing the ratio of carrier flow rate to reagents flow rate. A higher MDL may be obtained by decreasing the sample loop volume and decreasing this ratio.

7. Reference

1. U.S. Environmental Protection Agency. 1989. Definition and Procedure for the Determination of Method Detection Limits. Appendix B to 40 CFR 136 rev. 1.11 amended June 30, 1986. 49 CFR 43430.

Table 4500-Cl⁻:I. Results of Single-Laboratory Studies with Selected Matrices

Matrix	Sample/Blank Designation	Known Addition *mg Cl⁻/L*	Recovery %	Relative Standard Deviation %
Wastewater treatment plant influent	Reference sample*	—	101	—
	Blank†	10	104	—
		20	102	—
	Site A‡	0	—	0.4
		10	92	—
		20	101	—
	Site B‡	0	—	0.2
		10	97	—
		20	106	—
	Site C‡	0	—	0.4
		10	102	—
		20	102	—
Wastewater treatment plant effluent	Reference sample*	—	101	—
	Blank†	10	104	—
		20	102	—
	Site A‡	0	—	0.3
		10	98	—
		20	101	—
	Site B‡	0	—	0.2
		10	99	—
		20	103	—
	Site C‡	0	—	0.4
		10	91	—
		20	97	—
Landfill leachate	Reference sample*	—	100	—
	Blank†	10	101	—
		20	100	—
	Site A§	0	—	0.3
		10	97	—
		20	103	—
	Site B§	0	—	0.2
		10	89	—
		20	103	—
	Site C§	0	—	0.5
		10	89	—
		20	103	—

* U.S. EPA nutrient QC sample, 51.7 mg Cl⁻/L.
† Determined in duplicate.
‡ Samples diluted fivefold. Samples without known additions determined four times; samples with known additions determined in duplicate. Typical relative difference between duplicates 0.2%.
§ Sample from Site A diluted 50-fold, those from B and C 100-fold. Samples without known additions determined four times; samples with known additions determined in duplicate; typical relative difference between duplicates 0.5%.

4500-Cl CHLORINE (RESIDUAL)*

4500-Cl A. Introduction

1. Effects of Chlorination

The chlorination of water supplies and polluted waters serves primarily to destroy or deactivate disease-producing microorganisms. A secondary benefit, particularly in treating drinking water, is the overall improvement in water quality resulting from the reaction of chlorine with ammonia, iron, manganese, sulfide, and some organic substances.

Chlorination may produce adverse effects. Taste and odor characteristics of phenols and other organic compounds present in a water supply may be intensified. Potentially carcinogenic chloroorganic compounds, such as chloroform, may be formed. Combined chlorine formed on chlorination of ammonia- or amine-bearing waters adversely affects some aquatic life. To fulfill the primary purpose of chlorination and to minimize any adverse effects, it is essential that proper testing procedures be used with a foreknowledge of the limitations of the analytical determination.

2. Chlorine Forms and Reactions

Chlorine applied to water in its molecular or hypochlorite form initially undergoes hydrolysis to form free chlorine consisting of aqueous molecular chlorine, hypochlorous acid, and hypochlorite ion. The relative proportion of these free chlorine forms is pH- and temperature-dependent. At the pH of most waters, hypochlorous acid and hypochlorite ion will predominate.

Free chlorine reacts readily with ammonia and certain nitrogenous compounds to form combined chlorine. With ammonia, chlorine reacts to form the chloramines: monochloramine, dichloramine, and nitrogen trichloride. The presence and concentrations of these combined forms depend chiefly on pH, temperature, initial chlorine-to-nitrogen ratio, absolute chlorine demand, and reaction time. Both free and combined chlorine may be present simultaneously. Combined chlorine in water supplies may be formed in the treatment of raw waters containing ammonia or by the addition of ammonia or ammonium salts. Chlorinated wastewater effluents, as well as certain chlorinated industrial effluents, normally contain only combined chlorine. Historically, the principal analytical problem has been to distinguish between free and combined forms of chlorine.

3. Selection of Method

In two separate but related studies, samples were prepared and distributed to participating laboratories to evaluate chlorine methods. Because of poor accuracy and precision and a high overall (average) total error in these studies, all orthotolidine procedures except one were dropped in the 14th Edition of this work. The useful stabilized neutral orthotolidine method was deleted from the 15th Edition because of the toxic nature of orthotolidine. The leuco crystal violet (LCV) procedure was dropped from the 17th Edition because of its relative difficulty and the lack of comparative advantages.

a. Natural and treated waters: The iodometric methods (4500-Cl.B and C) are suitable for measuring total chlorine concentrations greater than 1 mg/L, but the amperometric endpoint of 4500-Cl.C and D gives greater sensitivity. All acidic iodometric methods suffer from interferences, generally in proportion to the quantity of potassium iodide (KI) and H^+ added.

The amperometric titration method (4500-Cl.D) is a standard of comparison for the determination of free or combined chlorine. It is affected little by common oxidizing agents, temperature variations, turbidity, and color. The method is not as simple as the colorimetric methods and requires greater operator skill to obtain the best reliability. Loss of chlorine can occur because of rapid stirring in some commercial equipment. Clean and conditioned electrodes are necessary for sharp endpoints.

A low-level amperometric titration procedure (4500-Cl.E) has been added to determine total chlorine at levels below 0.2 mg/L. This method is recommended only when quantification of such low residuals is necessary. The interferences are similar to those found with the standard amperometric procedure (4500-Cl.D). The DPD methods (4500-Cl.F and G) are operationally simpler for determining free chlorine than the amperometric titration. Procedures are given for estimating the separate mono- and dichloramine and combined fractions. High concentrations of monochloramine interfere with the free chlorine determination unless the reaction is stopped with arsenite or thioacetamide. In addition, the DPD methods are subject to interference by oxidized forms of manganese unless compensated for by a blank.

The amperometric and DPD methods are unaffected by dichloramine concentrations in the range of 0 to 9 mg Cl as Cl_2/L in the determination of free chlorine. Nitrogen trichloride, if present, may react partially as free chlorine in the amperometric, DPD, and FACTS methods. The extent of this interference in the DPD methods does not appear to be significant.

The free chlorine test, syringaldazine (FACTS, 4500-Cl.H) was developed specifically for free chlorine. It is unaffected by significant concentrations of monochloramine, dichloramine, nitrate, nitrite, and oxidized forms of manganese.[1]

Sample color and turbidity may interfere in all colorimetric procedures.

Organic contaminants may produce a false free chlorine reading in most colorimetric methods (see ¶ *b* below). Many strong oxidizing agents interfere in the measurement of free chlorine in all methods. Such interferences include bromine, chlorine dioxide, iodine, permanganate, hydrogen peroxide, and ozone. However, the reduced forms of these compounds—bromide, chloride, iodide, manganous ion, and oxygen, in the absence of other oxidants, do not interfere. Reducing agents, such as ferrous compounds, hydrogen sulfide, and oxidizable organic matter, generally do not interfere.

* Approved by Standard Methods Committee, 2000. Editoral revisions, 2011.

https://doi.org/10.2105/SMWW.2882.078

b. Wastewaters: The determination of total chlorine in samples containing organic matter presents special problems. Because of the presence of ammonia, amines, and organic compounds, particularly organic nitrogen, residual chlorine exists in a combined state. A considerable residual may exist in this form, but at the same time there may be appreciable unsatisfied chlorine demand. Addition of reagents in the determination may change these relationships so residual chlorine is lost during the analysis. Only the DPD method for total chlorine is performed under neutral pH conditions. In wastewater, the differentiation between free chlorine and combined chlorine ordinarily is not made because wastewater chlorination seldom is carried far enough to produce free chlorine.

The determination of residual chlorine in industrial wastes is similar to that in domestic wastewater when the waste contains organic matter, but may be similar to the determination in water when the waste is low in organic matter.

None of these methods is applicable to estuarine or marine waters because the bromide is converted to bromine and bromamines, which are detected as free or total chlorine. A procedure for estimating this interference is available for the DPD method.

Although the methods given below are useful for the determination of residual chlorine in wastewaters and treated effluents, select the method in accordance with sample composition. Some industrial wastes, or mixtures of wastes with domestic wastewater, may require special precautions and modifications to obtain satisfactory results.

Determine free chlorine in wastewater by any of the methods provided that known interfering substances are absent or appropriate correction techniques are used. The amperometric method is the method of choice because it is not subject to interference from color, turbidity, iron, manganese, or nitrite nitrogen. The DPD method is subject to interference from high concentrations of monochloramine, which is avoided by adding thioacetamide immediately after reagent addition. Oxidized forms of manganese at all levels encountered in water will interfere in all methods except in the free chlorine measurement of amperometric titrations and FACTS, but a blank correction for manganese can be made in 4500-Cl.F and G.

The FACTS method is unaffected by concentrations of monochloramine, dichloramine, nitrite, iron, manganese, and other interfering compounds normally found in domestic wastewaters.

For total chlorine in samples containing significant amounts of organic matter, use either the DPD methods (4500-Cl.F and G), amperometric, or iodometric back titration method (4500-Cl.C) to prevent contact between the full concentration of liberated iodine and the sample. With 4500-Cl.C, do not use the starch-iodide endpoint if the concentration is less than 1 mg/L. In the absence of interference, the amperometric and starch-iodide endpoints give concordant results. The amperometric endpoint is inherently more sensitive and is free of interference from color and turbidity, which can cause difficulty with the starch-iodide endpoint. On the other hand, certain metals, surface-active agents, and complex anions in some industrial wastes interfere in the amperometric titration and indicate the need for another method for such wastewaters. Silver in the form of soluble silver cyanide complex, in concentrations of 1.0 mg Ag/L, poisons the cell at pH 4.0 but not at 7.0. The silver ion, in the absence of the cyanide complex, gives extensive response in the current at pH 4.0 and gradually poisons the cell at all pH levels. Cuprous copper in the soluble copper cyanide ion, in concentrations of 5 mg Cu/L or less, poisons the cell at pH 4.0 and 7.0. Although iron and nitrite may interfere with this method, minimize the interference by buffering to pH 4.0 before adding KI. Oxidized forms of manganese interfere in all methods for total chlorine including amperometric titration. An unusually high content of organic matter may cause uncertainty in the endpoint.

Regardless of endpoint detection, either phenylarsine oxide or thiosulfate may be used as the standard reducing reagent at pH 4. The former is more stable and is preferred.

The DPD titrimetric and colorimetric methods (4500-Cl.F and G, respectively) are applicable to determining total chlorine in polluted waters. In addition, both DPD procedures and the amperometric titration method allow for estimating monochloramine and dichloramine fractions. Because all methods for total chlorine depend on the stoichiometric production of iodine, waters containing iodine-reducing substances may not be analyzed accurately by these methods, especially where iodine remains in the solution for a significant time. This problem occurs in 4500-Cl.B and D. The back titration procedure (4500-Cl.C) and 4500-Cl.F and G cause immediate reaction of the iodine generated so it has little chance to react with other iodine-reducing substances.

In all colorimetric procedures, compensate for color and turbidity by using color and turbidity blanks.

A method (4500-Cl.I) for total residual chlorine using a potentiometric iodide electrode is proposed. This method is suitable for analysis of chlorine residuals in natural and treated waters and wastewater effluents. No differentiation of free and combined chlorine is possible. This procedure is an adaptation of other iodometric techniques and is subject to the same inferences.

4. Sampling and Storage

Chlorine in aqueous solution is not stable, and the chlorine content of samples or solutions, particularly weak solutions, will decrease rapidly. Exposure to sunlight or other strong light or agitation will accelerate the reduction of chlorine. Therefore, start chlorine determinations immediately after sampling, avoiding excessive light and agitation. Do not store samples to be analyzed for chlorine.

5. Reference

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6. Bibliography

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4500-Cl B. Iodometric Method I

1. General Discussion

a. Principle: Chlorine will liberate free iodine from potassium iodide (KI) solutions at pH 8 or less. The liberated iodine is titrated with a standard solution of sodium thiosulfate ($Na_2S_2O_3$) with starch as the indicator. Titrate at pH 3 to 4 because the reaction is not stoichiometric at neutral pH due to partial oxidation of thiosulfate to sulfate.

b. Interference: Oxidized forms of manganese and other oxidizing agents interfere. Reducing agents, such as organic sulfides, also interfere. Although the neutral titration minimizes the interfering effect of ferric and nitrite ions, the acid titration is preferred because some forms of combined chlorine do not react at pH 7. Use only acetic acid for the acid titration; sulfuric acid (H_2SO_4) will increase interferences; never use hydrochloric acid (HCl). See 4500-Cl.A.3 for discussion of other interferences.

c. Minimum detectable concentration: The minimum detectable concentration approximates 40 μg Cl as Cl_2/L if 0.01*N* $Na_2S_2O_3$ is used with a 1000-mL sample. Concentrations below 1 mg/L cannot be determined accurately by the starch-iodide endpoint used in this method. Lower concentrations can be measured with the amperometric endpoint in 4500-Cl.C and D.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Reagents

a. Acetic acid, conc (glacial).

b. Potassium iodide (KI), crystals.

c. Standard sodium thiosulfate, 0.1*N:* Dissolve 25 g $Na_2S_2O_3 \cdot 5H_2O$ in 1 L freshly boiled distilled water and standardize against potassium bi-iodate or potassium dichromate after at least 2 weeks storage. This initial storage is necessary to allow oxidation of any bisulfite ion present. Use boiled distilled water and add a few milliliters chloroform ($CHCl_3$) to minimize bacterial decomposition.

Standardize 0.1*N* $Na_2S_2O_3$ by one of the following:

1) Iodate method—Dissolve 3.249 g anhydrous potassium bi-iodate, $KH(IO_3)_2$, primary standard quality, or 3.567 g KIO_3 dried at 103 ± 2°C for 1 h, in distilled water and dilute to 1000 mL to yield a 0.1000*N* solution. Store in a glass-stoppered bottle.

   To 80 mL distilled water, add, with constant stirring, 1 mL conc H_2SO_4, 10.00 mL 0.1000*N* $KH(IO_3)_2$, and 1 g KI. Titrate immediately with 0.1*N* $Na_2S_2O_3$ titrant until the yellow color of the liberated iodine almost is discharged. Add 1 mL starch indicator solution and continue titrating until the blue color disappears.

2) Dichromate method—Dissolve 4.904 g anhydrous potassium dichromate, $K_2Cr_2O_7$, of primary standard quality, in distilled water and dilute to 1000 mL to yield a 0.1000*N* solution. Store in a glass-stoppered bottle.

Proceed as in the iodate method, with the following exceptions: Substitute 10.00 mL 0.1000*N* $K_2Cr_2O_7$ for iodate and let reaction mixture stand 6 min in the dark before titrating with 0.1*N* $Na_2S_2O_3$ titrant.

$$\text{Normality } Na_2S_2O_3 = \frac{1}{\text{mL } Na_2S_2O_3 \text{ consumed}}$$

d. Standard sodium thiosulfate titrant, 0.01*N* or 0.025*N:* Improve the stability of 0.01*N* or 0.025*N* $Na_2S_2O_3$ by diluting an aged 0.1*N* solution, made as directed above, with freshly boiled distilled water. Add 4 g sodium borate and 10 mg mercuric iodide/L solution. For accurate work, standardize this solution daily in accordance with the directions given above, using 0.01*N* or 0.025*N* iodate or $K_2Cr_2O_7$. Use sufficient volumes of these standard solutions so their final dilution is not greater than 1 + 4. To speed up operations where many samples must be titrated use an automatic buret of a type in which rubber does not come in contact with the solution. Standard titrants, 0.0100*N* and 0.0250*N*, are equivalent, respectively, to 354.5 μg and 886.3 μg Cl as Cl_2/1.00 mL.

e. Starch indicator solution: To 5 g starch (potato, arrowroot, or soluble), add a little cold water and grind in a mortar to a thin paste. Pour into 1 L of boiling distilled water, stir, and let settle overnight. Use clear supernate. Preserve with 1.25 g salicylic acid, 4 g zinc chloride, or a combination of 4 g sodium propionate and 2 g sodium azide/L starch solution. Some commercial starch substitutes are satisfactory.

f. Standard iodine, 0.1*N:* See 4500-Cl.C.3*g*.

g. Dilute standard iodine, 0.0282*N:* See 4500-Cl.C.3*h*.

3. Procedure

a. Volume of sample: Select a sample volume that will require no more than 20 mL 0.01*N* $Na_2S_2O_3$ and no less than 0.2 mL for the starch-iodide endpoint. For a chlorine range of 1 to 10 mg/L, use a 500-mL sample; above 10 mg/L, use proportionately less sample. Use smaller samples and volumes of titrant with the amperometric endpoint.

b. Preparation for titration: Place 5 mL acetic acid, or enough to reduce the pH to between 3.0 and 4.0, in a flask or white porcelain casserole. Add about 1 g KI estimated on a spatula. Pour sample in and mix with a stirring rod.

c. Titration: Titrate away from direct sunlight. Add 0.025*N* or 0.01*N* $Na_2S_2O_3$ from a buret until the yellow color of the liberated iodine almost is discharged. Add 1 mL starch solution and titrate until blue color is discharged.

If the titration is made with 0.025*N* $Na_2S_2O_3$ instead of 0.01*N*, then, with a 1-L sample, 1 drop is equivalent to about 50 μg/L. It is not possible to discern the endpoint with greater accuracy.

d. Blank titration: Correct result of sample titration by determining blank contributed by oxidizing or reducing reagent impurities. The blank also compensates for the concentration of iodine bound to starch at the endpoint.

Take a volume of distilled water corresponding to the sample used for titration in 4500-Cl.B.3*a–c*, add 5 mL acetic acid, 1 g KI, and 1 mL starch solution. Perform blank titration as in ¶s 1) or 2) below, whichever applies.

1) If a blue color develops, titrate with 0.01*N* or 0.025*N* $Na_2S_2O_3$ to disappearance of blue color and record result. *B* (see 4500-Cl.B.4) is negative.

2) If no blue color occurs, titrate with 0.0282*N* iodine solution until a blue color appears. Back-titrate with 0.01*N* or 0.025*N* $Na_2S_2O_3$ and record the difference. *B* is positive.

Before calculating the chlorine concentration, subtract the blank titration of ¶ 1) above from the sample titration; or, if necessary, add the net equivalent value of the blank titration of ¶ 2) above.

4. Calculation

For standardizing chlorine solution for temporary standards:

$$\text{mg Cl as } Cl_2/\text{mL} = \frac{(A \pm B) \times N \times 35.45}{\text{mL sample}}$$

For determining total available residual chlorine in a water sample:

$$\text{mg Cl as } Cl_2/\text{L} = \frac{(A \pm B) \times N \times 35\,450}{\text{mL sample}}$$

where:

A = mL titration for sample,
B = mL titration for blank (positive or negative), and
N = normality of $Na_2S_2O_3$.

5. Precision and Bias

Published studies[1,2] give the results of nine methods used to analyze synthetic water samples without interferences; variations of some of the methods appear in this edition. More current data are not now available.

6. References

1. Lishka, R.J., E.F. McFarren & J.H. Parker. 1969. Water Chlorine (Residual) No. 1: Report Number 35: Report of a Study Conducted by Analytical Reference Service. U.S. Public Health Service, Consumer Protection and Environmental Health Service, Environmental Control Administration, Cincinnati, Ohio.
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4500-Cl C. Iodometric Method II

1. General Discussion

a. Principle: In this method, used for wastewater analysis, the endpoint signal is reversed because the unreacted standard reducing agent (phenylarsine oxide or thiosulfate) remaining in the sample is titrated with standard iodine or standard iodate, rather than the iodine released being titrated directly. This indirect procedure is necessary regardless of the method of endpoint detection, to avoid contact between the full concentration of liberated iodine and the wastewater.

When iodate is used as a back titrant, use only phosphoric acid. Do not use acetate buffer.

b. Interference: Oxidized forms of manganese and other oxidizing agents give positive interferences. Reducing agents, such as organic sulfides, do not interfere as much as in 4500-Cl.B. Minimize iron and nitrite interference by buffering to pH 4.0 before adding potassium iodide (KI). An unusually high content of organic matter may cause some uncertainty in the endpoint. Whenever manganese, iron, and other interferences definitely are absent, reduce this uncertainty and improve precision by acidifying to pH 1.0. Control interference from more than 0.2 mg nitrite/L with phosphoric acid-sulfamic acid reagent. A larger fraction of organic chloramines will react at lower pH along with interfering substances. See 4500-Cl.A.3 for a discussion of other interferences.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

For a description of the amperometric endpoint detection apparatus and a discussion of its use, see 4500-Cl.D.2*a*.

3. Reagents

a. Standard phenylarsine oxide solution, 0.005 64*N:* Dissolve approximately 0.8 g phenylarsine oxide powder in 150 mL 0.3*N* NaOH solution. After settling, decant 110 mL into 800 mL distilled water and mix thoroughly. Bring to pH 6 to 7 with 6*N* HCl and dilute to 950 mL with distilled water. **Caution: Severe poison, cancer suspect agent.**

Standardization—Accurately measure 5 to 10 mL freshly standardized 0.0282*N* iodine solution into a flask and add 1 mL KI solution. Titrate with phenylarsine oxide solution, using the amperometric endpoint (4500-Cl.D) or starch solution (see 4500-Cl.B.2*e*) as an indicator. Adjust to 0.005 64*N* and recheck against the standard iodine solution; 1.00 mL = 200 μg available chlorine. (**Caution: Toxic—take care to avoid ingestion.**)

b. Standard sodium thiosulfate solution, 0.1*N:* See 4500-Cl.B.2*c*.

c. Standard sodium thiosulfate solution, 0.005 64*N:* Prepare by diluting 0.1*N* $Na_2S_2O_3$. For maximum stability of the dilute solution, prepare by diluting an aged 0.1*N* solution with freshly boiled distilled water (to minimize bacterial action) and add 4 g $Na_4B_4O_7$/L. To inhibit mold formation optionally add either 10 mg HgI_2 or 2 drops toluene per liter of solution. Standardize daily as directed in 4500-Cl.B.2*c* using 0.005 64*N* $K_2Cr_2O_7$ or iodate solution. Use sufficient volume of sample so the final dilution does not exceed 1 + 2. Use an automatic buret of a type in which rubber does not come in contact with the solution. 1.00 mL = 200 μg available chlorine.

d. Potassium iodide (KI), crystals.

e. Acetate buffer solution, pH 4.0: Dissolve 146 g anhydrous $NaC_2H_3O_2$, or 243 g $NaC_2H_3O_2 \cdot 3H_2O$, in 400 mL distilled water, add 480 g conc acetic acid, and dilute to 1 L with chlorine-demand-free water.

f. Standard arsenite solution, 0.1*N:* Accurately weigh a stoppered weighing bottle containing approximately 4.95 g arsenic trioxide, As_2O_3. Transfer without loss to a 1-L volumetric flask and again weigh bottle. Do not attempt to brush out adhering oxide. Moisten As_2O_3 with water and add 15 g NaOH and 100 mL distilled water. Swirl flask contents gently to dissolve. Dilute to 250 mL with distilled water and saturate with CO_2, thus converting all NaOH to $NaHCO_3$. Dilute to mark, stopper, and mix thoroughly. This solution will preserve its titer almost indefinitely. (**Caution: Severe poison. Cancer suspect agent.**)

$$\text{Normality} = \frac{\text{g } As_2O_3}{49.455}$$

g. Standard iodine solution, 0.1*N:* Dissolve 40 g KI in 25 mL chlorine-demand-free water, add 13 g resublimed iodine, and stir until dissolved. Transfer to a 1-L volumetric flask and dilute to mark.

Standardization—Accurately measure 40 to 50 mL 0.1*N* arsenite solution into a flask and titrate with 0.1*N* iodine solution, using starch solution as indicator. To obtain accurate results, ensure that the solution is saturated with CO_2 at end of titration by passing current of CO_2 through solution for a few minutes just before endpoint is reached, or add a few drops of HCl to liberate sufficient CO_2 to saturate solution. Alternatively, standardize against $Na_2S_2O_3$; see 4500-Cl.B.2*c*1).

Optionally, prepare 0.1000*N* iodine solution directly as a standard solution by weighing 12.69 g primary standard resublimed iodine. Because I_2 may be volatilized and lose from both solid and solution, transfer the solid immediately to KI as specified above. Never let solution stand in open containers for extended periods.

h. Standard iodine titrant, 0.0282*N:* Dissolve 25 g KI in a little distilled water in a 1-L volumetric flask, add correct amount of 0.1*N* iodine solution exactly standardized to yield a 0.0282*N* solution, and dilute to 1 L with chlorine-demand-free water. For accurate work, standardize daily according to directions in ¶ *g* above, using 5 to 10 mL of arsenite or $Na_2S_2O_3$ solution. Store in amber bottles or in the dark; protect solution from direct sunlight at all times and keep from all contact with rubber.

i. Starch indicator: See 4500-Cl.B.2*e*.

j. Standard iodate titrant, 0.005 64*N:* Dissolve 201.2 mg primary standard grade KIO_3, dried for 1 h at 103°C, or 183.3 mg primary standard anhydrous potassium bi-iodate in distilled water and dilute to 1 L.

k. Phosphoric acid solution (H_3PO_4), 1 + 9.

l. Phosphoric acid-sulfamic acid solution: Dissolve 20 g NH_2SO_3H in 1 L 1 + 9 phosphoric acid.

m. Chlorine-demand-free water: Prepare chlorine-demand-free water from good-quality distilled or deionized water by adding sufficient chlorine to give 5 mg/L free chlorine. After standing 2 d this solution should contain at least 2 mg/L free chlorine; if not, discard and obtain better-quality water. Remove remaining free chlorine by placing container in sunlight or irradiating with an ultraviolet lamp. After several hours take sample, add KI, and measure total chlorine with a colorimetric method using a nessler tube to increase sensitivity. Do not use before last trace of free and combined chlorine has been removed.

Distilled water commonly contains ammonia and also may contain reducing agents. Collect good-quality distilled or deionized water in a sealed container from which water can be drawn by gravity. To the air inlet of the container add an H_2SO_4 trap consisting of a large test tube half filled with 1 + 1 H_2SO_4 connected in series with a similar but empty test tube. Fit both test tubes with stoppers and inlet tubes terminating near the bottom of the tubes and outlet tubes terminating near the top of the tubes. Connect outlet tube of trap containing H_2SO_4 to the distilled water container, connect inlet tube to outlet of empty test tube. The empty test tube will prevent discharge to the atmosphere of H_2SO_4 due to temperature-induced pressure changes. Stored in such a container, chlorine-demand-free water is stable for several weeks unless bacterial growth occurs.

4. Procedure

a. Preparation for titration:

1) Volume of sample—For chlorine concentration of 10 mg/L or less, titrate 200 mL. For greater chlorine concentrations, use proportionately less sample and dilute to 200 mL with chlorine-demand-free water. Use a sample of such size that not more than 10 mL phenylarsine oxide solution is required.

2) Preparation for titration—Measure 5 mL 0.005 64*N* phenylarsine oxide or thiosulfate for chlorine concentrations from 2 to 5 mg/L, and 10 mL for concentrations of 5 to 10 mg/L, into a flask or casserole for titration with standard iodine or iodate. Start stirring. For titration by amperometry or standard iodine, also add excess KI (approximately 1 g) and 4 mL acetate buffer solution or enough to reduce the pH to between 3.5 and 4.2.

b. Titration: Use one of the following:

1) Amperometric titration—Add 0.0282*N* iodine titrant in small increments from a 1-mL buret or pipet. Observe meter needle response as iodine is added: the pointer remains practically stationary until the endpoint is approached, whereupon each iodine increment causes a temporary deflection of the microammeter, with the pointer dropping back to its original position. Stop titration at endpoint when a small increment of iodine titrant gives a definite pointer deflection upscale and the pointer does not return promptly to its original position. Record volume of iodine titrant used to reach endpoint.

2) Colorimetric (iodine) titration—Add 1 mL starch solution and titrate with 0.0282*N* iodine to the first appearance of blue color that persists after complete mixing.

3) Colorimetric (iodate) titration—To suitable flask or casserole add 200 mL chlorine-demand-free water and add, with agitation, the required volume of reductant, an excess of KI (approximately 0.5 g), 2 mL 10% H_3PO_4 solution, and 1 mL starch solution in the order given, and titrate immediately* with 0.005 64*N* iodate solution to the first appearance of a blue color that persists after complete mixing. Designate volume of iodate solution used as *A*. Repeat procedure, substituting 200 mL sample for the 200 mL chlorine-demand-free water. If sample is colored or turbid, titrate to the first change in color, using for comparison another portion of sample with H_3PO_4 added. Designate this volume of iodate solution as *B*.

5. Calculation

a. Titration with standard iodine:

$$\text{mg Cl as } Cl_2/\text{L} = \frac{(A - 5B) \times 200}{C}$$

where:

A = mL 0.005 64*N* reductant,
B = mL 0.0282 *N* I_2, and
C = mL sample.

b. Titration with standard iodate:

$$\text{mg Cl as } Cl_2/\text{L} = \frac{(A - B) \times 200}{C}$$

where:

A = mL $Na_2S_2O_3$,
B = mL iodate required to titrate $Na_2S_2O_3$, and
C = mL sample.

6. Bibliography

See 4500-Cl.B.7.

* Titration may be delayed up to 10 min without appreciable error if H_3PO_4 is not added until immediately before titration.

4500-Cl D. Amperometric Titration Method

1. General Discussion

Amperometric titration requires a higher degree of skill and care than the colorimetric methods. Chlorine residuals over 2 mg/L are measured best by means of smaller samples or by dilution with water that has neither residual chlorine nor a chlorine demand. The method can be used to determine total chlorine and can differentiate between free and combined chlorine. A further differentiation into monochloramine and dichloramine fractions is possible by control of KI concentration and pH.

a. Principle: The amperometric method is a special adaptation of the polarographic principle. Free chlorine is titrated at a pH between 6.5 and 7.5, a range in which the combined chlorine reacts slowly. The combined chlorine, in turn, is titrated in the presence of the proper amount of KI in the pH range 3.5 to 4.5. When free chlorine is determined, the pH must not be greater than 7.5 because the reaction becomes sluggish at higher pH values, nor less than 6.5 because at lower pH values some combined chlorine may react even in the absence of iodide. When combined chlorine is determined, the pH must not be less than 3.5 because of increased interferences at lower pH values, nor greater than 4.5 because the iodide reaction is not quantitative at higher pH values. The tendency of monochloramine to react more readily with iodide than does dichloramine provides a means for further differentiation. The addition of a small amount of KI in the neutral pH range enables estimation of monochloramine content. Lowering the pH into the acid range and increasing the KI concentration allows the separation determination of dichloramine.

Organic chloramines can be measured as free chlorine, monochloramine, or dichloramine, depending on the activity of chlorine in the organic compound.

Phenylarsine oxide is stable even in dilute solution and each mole reacts with two equivalents of halogen. A special amperometric cell is used to detect the endpoint of the residual chlorine-phenylarsine oxide titration. The cell consists of a nonpolarizable reference electrode that is immersed in a salt solution and a readily polarizable noble-metal electrode that is in contact with both the salt solution and the sample being titrated. In some applications, endpoint selectivity is improved by adding +200 mV to the platinum electrode versus silver, silver chloride. Another approach to endpoint detection uses dual platinum electrodes, a mercury cell with voltage divider to impress a potential across the electrodes, and a microammeter. If there is no chlorine residual in the sample, the microammeter reading will be comparatively low because of cell polarization. The greater the residual, the greater the microammeter reading. The meter acts merely as a null-point indicator—that is, the actual meter reading is not important, but rather the relative readings as the titration proceeds. The gradual addition of phenylarsine oxide causes the cell to become more and more polarized because of the decrease in chlorine. The endpoint is recognized when no further decrease in meter reading can be obtained by adding more phenylarsine oxide.

b. Interference: Accurate determinations of free chlorine cannot be made in the presence of nitrogen trichloride, NCl_3, or chlorine dioxide, which titrate partly as free chlorine. When present, NCl_3 can titrate partly as free chlorine and partly as dichloramine, contributing a positive error in both fractions at a rate of approximately 0.1%/min. Some organic chloramines also can be titrated in each step. Monochloramine can intrude into the

free chlorine fraction and dichloramine can interfere in the monochloramine fraction, especially at high temperatures and prolonged titration times. Free halogens other than chlorine also will titrate as free chlorine. Combined chlorine reacts with iodide ions to produce iodine. When titration for free chlorine follows a combined chlorine titration, which requires addition of KI, erroneous results may occur unless the measuring cell is rinsed thoroughly with distilled water between titrations.

Interference from copper has been noted in samples taken from copper pipe or after heavy copper sulfate treatment of reservoirs, with metallic copper plating out on the electrode. Silver ions also poison the electrode. Interference occurs in some highly colored waters and in waters containing surface-active agents. Very low temperatures slow response of measuring cell and longer time is required for the titration, but precision is not affected. A reduction in reaction rate is caused by pH values above 7.5; overcome this by buffering all samples to pH 7.0 or less. On the other hand, some substances, such as manganese, nitrite, and iron, do not interfere. The violent stirring of some commercial titrators can lower chlorine values by volatilization. When dilution is used for samples containing high chlorine content, take care that the dilution water is free of chlorine and ammonia and possesses no chlorine demand.

See 4500-Cl.A.3 for a discussion of other interferences.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Endpoint detection apparatus, consisting of a cell unit connected to a microammeter, with necessary electrical accessories. The cell unit includes a noble-metal electrode of sufficient surface area, a salt bridge to provide an electrical connection without diffusion of electrolyte, and a reference electrode of silver–silver chloride in a saturated sodium chloride solution connected into the circuit by means of the salt bridge. Numerous commercial systems are available.

Keep platinum electrode free of deposits and foreign matter. Vigorous chemical cleaning generally is unnecessary. Occasional mechanical cleaning with a suitable abrasive usually is sufficient. Keep salt bridge in good operating condition; do not allow it to become plugged nor permit appreciable flow of electrolyte through it. Keep solution surrounding reference electrode free of contamination and maintain it at constant composition by ensuring an adequate supply of undissolved salt at all times. A cell with two metal electrodes polarized by a small DC potential also may be used. (See 4500-Cl.D.7.)

b. Agitator, designed to give adequate agitation at the noble-metal electrode surface to ensure proper sensitivity. Thoroughly clean agitator and exposed electrode system to remove all chlorine- consuming contaminants by immersing them in water containing 1 to 2 mg/L free chlorine for a few minutes. Add KI to the same water and let agitator and electrodes remain immersed for 5 min. After thorough rinsing with chlorine-demand-free water or the sample to be tested, sensitized electrodes and agitator are ready for use. Remove iodide reagent completely from cell.

c. Buret: Commercial titrators usually are equipped with suitable burets (1 mL). Manual burets are available.*

d. Glassware, exposed to water containing at least 10 mg/L chlorine for 3 h or more before use and rinsed with chlorine-demand-free water.

3. Reagents

a. Standard phenylarsine oxide titrant: See 4500-Cl.C.3*a*.

b. Phosphate buffer solution, pH 7: Dissolve 25.4 g anhydrous KH_2PO_4 and 34.1 g anhydrous Na_2HPO_4 in 800 mL distilled water. Add 2 mL sodium hypochlorite solution containing 1% chlorine and mix thoroughly. Protect from sunlight for 2 d. Determine that free chlorine still remains in the solution. Then expose to sunlight until no chlorine remains. If necessary, carry out the final dechlorination with an ultraviolet lamp. Determine that no total chlorine remains by adding KI and measuring with one of the colorimetric tests. Dilute to 1 L with distilled water and filter if any precipitate is present.

c. Potassium iodide solution: Dissolve 50 g KI and dilute to 1 L with freshly boiled and cooled distilled water. Store in the dark in a brown glass-stoppered bottle, preferably in the refrigerator. Discard when solution becomes yellow.

d. Acetate buffer solution, pH 4: See 4500-Cl.C.3*e*.

4. Procedure

a. Sample volume: Select a sample volume requiring no more than 2 mL phenylarsine oxide titrant. Thus, for chlorine concentrations of 2 mg/L or less, take a 200-mL sample; for chlorine levels in excess of 2 mg/L, use 100 mL or proportionately less.

b. Free chlorine: Unless sample pH is known to be between 6.5 and 7.5, add 1 mL pH 7 phosphate buffer solution to produce a pH of 6.5 to 7.5. Titrate with standard phenylarsine oxide titrant, observing current changes on microammeter. Add titrant in progressively smaller increments until all needle movement ceases. Make successive buret readings when needle action becomes sluggish, signaling approach of endpoint. Subtract last very small increment that causes no needle response because of overtitration. Alternatively, use a system involving continuous current measurements and determine endpoint mathematically.

Continue titrating for combined chlorine as described in ¶ *c* below or for the separate monochloramine and dichloramine fractions as detailed in ¶s *e* and *f*.

c. Combined chlorine: To sample remaining from free-chlorine titration add 1.00 mL KI solution and 1 mL acetate buffer solution, in that order. Titrate with phenylarsine oxide titrant to the endpoint, as above. Do not refill buret but simply continue titration after recording figure for free chlorine. Again subtract last increment to give amount of titrant actually used in reaction with chlorine. (If titration was continued without refilling buret, this figure represents total chlorine. Subtracting free chlorine from total gives combined chlorine.) Wash apparatus and sample cell thoroughly to remove iodide ion to avoid inaccuracies when the titrator is used subsequently for a free chlorine determination.

* Kimax 17110-F, 5 mL, Kimble Products, Box 1035, Toledo, OH, or equivalent.

d. Separate samples: If desired, determine total chlorine and free chlorine on separate samples. If sample pH is between 3.5 and 9.5 and total chlorine alone is required, treat sample immediately with 1 mL KI solution followed by 1 mL acetate buffer solution, and titrate with phenylarsine oxide titrant as described in ¶ *c* above.

e. Monochloramine: After titrating for free chlorine, add 0.2 mL KI solution to same sample and, without refilling buret, continue titration with phenylarsine oxide titrant to endpoint. Subtract last increment to obtain net volume of titrant consumed by monochloramine.

f. Dichloramine: Add 1 mL acetate buffer solution and 1 mL KI solution to same sample and titrate final dichloramine fraction as described above.

5. Calculation

Convert individual titrations for free chlorine, combined chlorine, total chlorine, monochloramine, and dichloramine by the following equation:

$$\text{mg Cl as } Cl_2/L = \frac{A \times 200}{\text{mL sample}}$$

where:

A = mL phenylarsine oxide titration.

6. Precision and Bias

See 4500-Cl.B.5.

7. Bibliography

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4500-Cl E. Low-Level Amperometric Titration Method

1. General Discussion

Detection and quantification of chlorine residuals below 0.2 mg/L require special modifications to the amperometric titration procedure. With these modifications chlorine concentrations at the 10-μg/L level can be measured. It is not possible to differentiate between free and combined chlorine forms. Oxidizing agents that interfere with the amperometric titration method (4500-Cl.D) will interfere.

a. Principle: This method modifies 4500-Cl.D by using a more dilute titrant and a graphical procedure to determine the endpoint.

b. Interference: See 4500-Cl.D.1*b*.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

See 4500-Cl.D.2.

3. Reagents

a. Potassium bi-iodate, 0.002 256*N*: Dissolve 0.7332 g anhydrous potassium bi-iodate, $KH(IO_3)_2$, in 500 mL chlorine-free distilled water and dilute to 1000 mL. Dilute 10.00 mL to 100.0 mL with chlorine-free distilled water. Use only freshly prepared solution for the standardization of phenylarsine oxide.

b. Potassium iodide (KI), crystals.

c. Low-strength phenylarsine oxide titrant, 0.000 564*N:* Dilute 10.00 mL of 0.005 64*N* phenylarsine oxide (see 4500-Cl.C.3*a*) to 100.0 mL with chlorine-demand-free water (see 4500-Cl.C.3*m*).

Standardization—Dilute 5.00 mL 0.002 256*N* potassium bi-iodate to 200 mL with chlorine-free water. Add approximately 1.5 g KI and stir to dissolve. Add 1 mL acetate buffer and let stand in the dark for 6 min. Titrate using the amperometric titrator and determine the equivalence point as indicated below.

$$\text{Normality} = 0.002256 \times 5/A$$

where:

A = mL phenylarsine oxide titrant required to reach the equivalence point of standard bi-iodate.

d. Acetate buffer solution, pH 4: See 4500-Cl.C.3*e*.

4. Procedure

Select a sample volume requiring no more than 2 mL phenylarsine oxide titrant. A 200-mL sample will be adequate for samples containing less than 0.2 mg total chlorine/L.

Before beginning titration, rinse buret with titrant several times. Rinse sample container with distilled water and then with

sample. Add 200 mL sample to sample container and approximately 1.5 g KI. Dissolve, using a stirrer or mixer. Add 1 mL acetate buffer and place container in endpoint detection apparatus. When the current signal stabilizes, record the reading. Initially adjust meter to a near full-scale deflection. Titrate by adding small, known, volumes of titrant. After each addition, record cumulative volume added and current reading when the signal stabilizes. If meter reading falls to near or below 10% of full-scale deflection, record low reading, readjust meter to near full-scale deflection, and record difference between low amount and readjusted high deflection. Add this value to all deflection readings for subsequent titrant additions. Continue adding titrant until no further meter deflection occurs. If fewer than three titrant additions were made before meter deflection ceased, discard sample and repeat analysis using smaller titrant increments.

Determine equivalence point by plotting total meter deflection against titrant volume added. Draw straight line through the first several points in the plot and a second, horizontal straight line corresponding to the final total deflection in the meter. Read equivalence point as the volume of titrant added at the intersection of these two lines.

5. Calculation

$$\text{mg Cl as } Cl_2/L = \frac{A \times 200 \times N}{B \times 0.00564}$$

where:

A = mL titrant at equivalence point,
N = phenylarsine oxide normality, and
B = sample volume, mL.

6. Bibliography

BROOKS, A.S. & G.L. SEEGERT. 1979. Low-level chlorine analysis by amperometric titration. *J. Water Pollut. Control Fed.* 51:2636.

4500-Cl F. DPD Ferrous Titrimetric Method

1. General Discussion

a. Principle: N,N-diethyl-*p*-phenylenediamine (DPD) is used as an indicator in the titrimetric procedure with ferrous ammonium sulfate (FAS). Where complete differentiation of chlorine species is not required, the procedure may be simplified to give only free and combined chlorine or total chlorine.

In the absence of iodide ion, free chlorine reacts instantly with DPD indicator to produce a red color. Subsequent addition of a small amount of iodide ion acts catalytically to cause monochloramine to produce color. Addition of iodide ion to excess evokes a rapid response from dichloramine. In the presence of iodide ion, part of the nitrogen trichloride (NCl_3) is included with dichloramine and part with free chlorine. A supplementary procedure based on adding iodide ion before DPD permits estimating proportion of NCl_3 appearing with free chlorine.

Chlorine dioxide (ClO_2) appears, to the extent of one-fifth of its total chlorine content, with free chlorine. A full response from ClO_2, corresponding to its total chlorine content, may be obtained if the sample first is acidified in the presence of iodide ion and subsequently is brought back to an approximately neutral pH by adding bicarbonate ion. Bromine, bromamine, and iodine react with DPD indicator and appear with free chlorine.

Addition of glycine before determination of free chlorine converts free chlorine to unreactive forms, with only bromine and iodine residuals remaining. Subtractions of these residuals from the residual measured without glycine permits differentiation of free chlorine from bromine and iodine.

b. pH control: For accurate results careful pH control is essential. At the proper pH of 6.2 to 6.5, the red colors produced may be titrated to sharp colorless endpoints. **Titrate as soon as the red color is formed in each step.** Too low a pH in the first step tends to make the monochloramine show in the free-chlorine step and the dichloramine in the monochloramine step. Too high a pH causes dissolved oxygen to give a color.

c. Temperature control: In all methods for differentiating free chlorine from chloramines, higher temperatures increase the tendency for chloramines to react and lead to increased apparent free-chlorine results. Higher temperatures also increase color fading. Complete measurements rapidly, especially at higher temperature.

d. Interference: The most significant interfering substance likely to be encountered in water is oxidized manganese. To correct for this, place 5 mL buffer solution and 0.5 mL sodium arsenite solution in the titration flask. Add 100 mL sample and mix. Add 5 mL DPD indicator solution, mix, and titrate with standard FAS titrant until red color is discharged. Subtract reading from Reading *A* obtained by the normal procedure as described in 4500-Cl.F.3*a*1) of this method or from the total chlorine reading obtained in the simplified procedure given in 4500-Cl.F.3*a*4). If the combined reagent in powder form (see below) is used, first add KI and arsenite to the sample and mix, then add combined buffer-indicator reagent.

As an alternative to sodium arsenite use a 0.25% solution of thioacetamide, adding 0.5 mL to 100 mL sample.

Interference by copper up to approximately 10 mg Cu/L is overcome by the EDTA incorporated in the reagents. EDTA enhances stability of DPD indicator solution by retarding deterioration due to oxidation, and in the test itself, provides suppression of dissolved oxygen errors by preventing trace metal catalysis.

Chromate in excess of 2 mg/L interferes with endpoint determination. Add barium chloride to mask this interference by precipitation.

High concentrations of combined chlorine can break through into the free chlorine fraction. *If free chlorine will be measured in the presence of more than 0.5 mg/L combined chlorine, use the thioacetamide modification.* If this modification is not used, a color-development time in excess of 1 min leads to progressively greater interference from monochloramine. Adding thioacetamide (0.5 mL 0.25% solution to 100 mL) immediately after mixing DPD reagent with sample completely stops further reaction with combined chlorine in the free chlorine measurement. Continue immediately with FAS titration to obtain free chlorine.

Obtain total chlorine from the normal procedure (i.e., without thioacetamide).

Because high concentrations of iodide are used to measure combined chlorine and only traces of iodide greatly increase chloramine interference in free chlorine measurements, take care to avoid iodide contamination by rinsing between samples or using separate glassware.

See 4500-Cl.A.3 for a discussion of other interferences.

e. Minimum detectable concentration: Approximately 18 μg Cl as Cl_2/L. This detection limit is achievable under ideal conditions; normal working detection limits typically are higher.

f. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Reagents

a. Phosphate buffer solution: Dissolve 24 g anhydrous Na_2HPO_4 and 46 g anhydrous KH_2PO_4 in distilled water. Combine with 100 mL distilled water in which 800 mg disodium ethylenediamine tetraacetate dihydrate (EDTA) have been dissolved. Dilute to 1 L with distilled water and optionally add either 20 mg $HgCl_2$ or 2 drops toluene to prevent mold growth. Interference from trace amounts of iodide in the reagents can be negated by optional addition of 20 mg $HgCl_2$ to the solution. (**CAUTION: $HgCl_2$ is toxic—take care to avoid ingestion.**)

b. N,N-Diethyl-p-*phenylenediamine (DPD) indicator solution:* Dissolve 1 g DPD oxalate,* or 1.5 g DPD sulfate pentahydrate,† or 1.1 g anhydrous DPD sulfate in chlorine-free distilled water containing 8 mL 1 + 3 H_2SO_4 and 200 mg disodium EDTA. Make up to 1 L, store in a brown glass-stoppered bottle in the dark, and discard when discolored. Periodically check solution blank for absorbance and discard when absorbance at 515 nm exceeds 0.002/cm. (The buffer and indicator sulfate are available commercially as a combined reagent in stable powder form.) **CAUTION: The oxalate is toxic—take care to avoid ingestion.**

c. Standard ferrous ammonium sulfate (FAS) titrant: Dissolve 1.106 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in distilled water containing 1 mL 1 + 3 H_2SO_4 and make up to 1 L with freshly boiled and cooled distilled water. This standard may be used for 1 month, and the titer checked by potassium dichromate. For this purpose add 10 mL 1 + 5 H_2SO_4, 5 mL conc H_3PO_4, and 2 mL 0.1% barium diphenylamine sulfonate indicator to a 100-mL sample of FAS and titrate with potassium dichromate to a violet endpoint that persists for 30 s. FAS titrant equivalent to 100 μg Cl as Cl_2/1.00 mL requires 20.00 mL dichromate for titration.

d. Potassium iodide (KI), crystals.

e. Potassium iodide solution: Dissolve 500 mg KI and dilute to 100 mL, using freshly boiled and cooled distilled water. Store in a brown glass-stoppered bottle, preferably in a refrigerator. Discard when solution becomes yellow.

f. Potassium dichromate solution, 0.691 g to 1000 mL.

g. Barium diphenylaminesulfonate, 0.1%: Dissolve 0.1 g $(C_6H_5NHC_6H_4\text{-}4\text{-}SO_3)_2Ba$ in 100 mL distilled water.

h. Sodium arsenite solution: Dissolve 5.0 g $NaAsO_2$ in distilled water and dilute to 1 L. (**CAUTION: Toxic—take care to avoid ingestion.**)

i. Thioacetamide solution: Dissolve 250 mg CH_3CSNH_2 in 100 mL distilled water. (**CAUTION: Cancer suspect agent. Take care to avoid skin contact or ingestion.**)

j. Chlorine-demand-free water: See 4500-Cl.C.3*m*.

k. Glycine solution: Dissolve 20 g glycine (aminoacetic acid) in sufficient chlorine-demand-free water to bring to 100 mL total volume. Store under refrigerated conditions and discard if cloudiness develops.

l. Barium chloride crystals ($BaCl_2 \cdot 2H_2O$).

3. Procedure

The quantities given below are suitable for concentrations of total chlorine up to 5 mg/L. If total chlorine exceeds 5 mg/L, use a smaller sample and dilute to a total volume of 100 mL. Mix usual volumes of buffer reagent and DPD indicator solution, or usual amount of DPD powder, with distilled water *before* adding sufficient sample to bring total volume to 100 mL. (If sample is added before buffer, test does not work.)

If chromate is present (>2 mg/L) add and mix 0.2 g $BaCl_2 \cdot 2H_2O$/100 mL sample before adding other reagents. If, in addition, sulfate is >500 mg/L, use 0.4 g $BaCl_2 \cdot 2H_2O$/100 mL sample.

a. Free chlorine or chloramine: Place 5 mL each of buffer reagent and DPD indicator solution in titration flask and mix (or use about 500 mg DPD powder). Add 100 mL sample, or diluted sample, and mix.

1) Free chlorine—Titrate rapidly with standard FAS titrant until red color is discharged (Reading *A*).

2) Monochloramine—Add one very small crystal of KI (about 0.5 mg) or 0.1 mL (2 drops) KI solution and mix. Continue titrating until red color is discharged again (Reading *B*).

3) Dichloramine—Add several crystals KI (about 1 g) and mix to dissolve. Let stand for 2 min and continue titrating until red color is discharged (Reading *C*). For dichloramine concentrations greater than 1 mg/L, let stand 2 min more if color driftback indicates slightly incomplete reaction. When dichloramine concentrations are not expected to be high, use half the specified amount of KI.

4) Simplified procedure for free and combined chlorine or total chlorine—Omit ¶ *a*2) above to obtain monochloramine and dichloramine together as combined chlorine. To obtain total chlorine in one reading, add full amount of KI at the start, with the specified amounts of buffer reagent and DPD indicator, and titrate after 2 min standing.

b. Nitrogen trichloride: Place one very small crystal of KI (about 0.5 mg) or 0.1 mL KI solution in a titration flask. Add 100 mL sample and mix. Add contents to a second flask containing 5 mL each of buffer reagent and DPD indicator solution (or add about 500 mg DPD powder direct to the first flask). Titrate rapidly with standard FAS titrant until red color is discharged (Reading *N*).

c. Free chlorine in presence of bromine or iodine: Determine free chlorine as in ¶ *a*1) above. To a second 100-mL sample, add 1 mL glycine solution before adding DPD and buffer. Titrate according to ¶ *a*1) above. Subtract the second reading from the first to obtain Reading *A*.

* Eastman Chemical No. 7102, or equivalent.
† Available from Gallard-Schlesinger Chemical Mfg. Corp., 584 Mineloa Avenue, Carle Place, NY 11514, or equivalent.

4. Calculation

For a 100-mL sample, 1.00 mL standard FAS titrant = 1.00 mg Cl as Cl_2/L.

Reading	NCl_3 Absent	NCl_3 Present
A	Free Cl	Free Cl
$B - A$	NH_2Cl	NH_2Cl
$C - B$	$NHCl_2$	$NHCl_2 + ½NCl_3$
N	—	Free Cl + $½NCl_3$
$2(N - A)$	—	NCl_3
$C - N$	—	$NHCl_2$

In the event that monochloramine is present with NCl_3, it will be included in N, in which case obtain NCl_3 from $2(N-B)$.

Chlorine dioxide, if present, is included in A to the extent of one-fifth of its total chlorine content.

In the simplified procedure for free and combined chlorine, only A (free Cl) and C (total Cl) are required. Obtain combined chlorine from $C-A$.

The result obtained in the simplified total chlorine procedure corresponds to C.

5. Precision and Bias

See 4500-Cl.B.5.

6. Bibliography

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4500-Cl G. DPD Colorimetric Method

1. General Discussion

a. Principle: This is a colorimetric version of the DPD method and is based on the same principles. Instead of titration with standard ferrous ammonium sulfate (FAS) solution as in the titrimetric method, a colorimetric procedure is used.

b. Interference: See 4500-Cl.A.3 and F.1*d*. Compensate for color and turbidity by using sample to zero photometer. Minimize chromate interference by using the thioacetamide blank correction.

c. Minimum detectable concentration: Approximately 10 μg Cl as Cl_2/L. This detection limit is achievable under ideal conditions; normal working detection limits typically are higher.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Photometric equipment: One of the following is required:

1) *Spectrophotometer*, for use at a wavelength of 515 nm and providing a light path of 1 cm or longer.

2) *Filter photometer*, equipped with a filter having maximum transmission in the wavelength range of 490 to 530 nm and providing a light path of 1 cm or longer.

b. Glassware: Use separate glassware, including separate spectrophotometer cells, for free and combined (dichloramine) measurements, to avoid iodide contamination in free chlorine measurement.

3. Reagents

See 4500-Cl.F.2*a*, *b*, *c*, *d*, *e*, *h*, *i,* and *j*.

4. Procedure

a. Calibration of photometric equipment: Calibrate instrument with chlorine or potassium permanganate solutions.

1) Chlorine solutions—Prepare chlorine standards in the range of 0.05 to 4 mg/L from about 100 mg/L chlorine water standardized as follows: Place 2 mL acetic acid and 10 to 25 mL chlorine-demand-free water in a flask. Add about 1 g KI. Measure into the flask a suitable volume of chlorine solution. In choosing a convenient volume, note that 1 mL 0.025*N* $Na_2S_2O_3$ titrant (see 4500-Cl.B.2*d*) is equivalent to about 0.9 mg chlorine. Titrate with standardized 0.025*N* $Na_2S_2O_3$ titrant until the yellow iodine color almost disappears. Add 1 to 2 mL starch indicator solution and continue titrating to disappearance of blue color.

Determine the blank by adding identical quantities of acid, KI, and starch indicator to a volume of chlorine-demand-free water corresponding to the sample used for titration. Perform blank titration A or B, whichever applies, according to 4500-Cl.B.3*d*.

$$\text{mg Cl as } Cl_2/\text{mL} = \frac{(A + B) \times N \times 35.45}{\text{mL sample}}$$

where:

A = mL titrant for sample,
B = mL titrant for blank (to be added or subtracted according to required blank titration. See 4500-Cl.B.3*d*), and
N = normality of $Na_2S_2O_3$.

Use chlorine-demand-free water and glassware to prepare these standards. Develop color by first placing 5 mL phosphate buffer solution and 5 mL DPD indicator reagent in flask and then adding 100 mL chlorine standard with thorough mixing as described in ¶s *b* and *c* below. Fill photometer or colorimeter cell from flask and read color at 515 nm. Return cell contents to flask and titrate with standard FAS titrant as a check on chlorine concentration.

2) Potassium permanganate solutions—Prepare a stock solution containing 891 mg $KMnO_4$/1000 mL. Dilute 10.00 mL stock solution to 100 mL with distilled water in a volumetric flask. When 1 mL of this solution is diluted to 100 mL with distilled water, a chlorine equivalent of 1.00 mg/L will be produced in the DPD reaction. Prepare a series of $KMnO_4$ standards covering the chlorine equivalent range of 0.05 to 4 mg/L. Develop color by first placing 5 mL phosphate buffer and 5 mL DPD indicator reagent in flask and adding 100 mL standard with thorough mixing as described in ¶s *b* and *c* below. Fill photometer or colorimeter cell from flask and read color at 515 nm. Return cell contents to flask and titrate with FAS titrant as a check on any absorption of permanganate by distilled water.

Obtain all readings by comparison to color standards or the standard curve before use in calculation.

b. Volume of sample: Use a sample volume appropriate to the photometer or colorimeter. The following procedure is based on using 10-mL volumes; adjust reagent quantities proportionately for other sample volumes. Dilute sample with chlorine-demand-free water when total chlorine exceeds 4 mg/L.

c. Free chlorine: Place 0.5 mL each of buffer reagent and DPD indicator reagent in a test tube or photometer cell. Add 10 mL sample and mix. Read color immediately (Reading *A*).

d. Monochloramine: Continue by adding one very small crystal of KI (about 0.1 mg) and mix. If dichloramine concentration is expected to be high, instead of small crystal add 0.1 mL (2 drops) freshly prepared KI solution (0.1 g/100 mL). Read color immediately (Reading *B*).

e. Dichloramine: Continue by adding several crystals of KI (about 0.1 g) and mix to dissolve. Let stand about 2 min and read color (Reading *C*).

f. Nitrogen trichloride: Place a very small crystal of KI (about 0.1 mg) in a clean test tube or photometer cell. Add 10 mL sample and mix. To a second tube or cell add 0.5 mL each of buffer and indicator reagents; mix. Add contents to first tube or cell and mix. Read color immediately (Reading *N*).

g. Chromate correction using thioacetamide: Add 0.5 mL thioacetamide solution (F.2*i*) to 100 mL sample. After mixing, add buffer and DPD reagent. Read color immediately. Add several crystals of KI (about 0.1 g) and mix to dissolve. Let stand about 2 min and read color. Subtract the first reading from Reading *A* and the second reading from Reading *C* and use in calculations.

h. Simplified procedure for total chlorine: Omit Step *d* above to obtain monochloramine and dichloramine together as combined chlorine. To obtain total chlorine in one reading, add the full amount of KI at the start, with the specified amounts of buffer reagent and DPD indicator. Read color after 2 min.

5. Calculation

Reading	NCl_3 Absent	NCl_3 Present
A	Free Cl	Free Cl
$B - A$	NH_2Cl	NH_2Cl
$C - B$	$NHCl_2$	$NHCl_2 + ½NCl_3$
N	—	Free Cl + ½NCl_3
$2(N - A)$	—	NCl_3
$C - N$	—	$NHCl_2$

In the event that monochloramine is present with NCl_3, it will be included in Reading *N*, in which case obtain NCl_3 from $2(N-B)$.

6. Bibliography

See 4500-Cl.F.6.

4500-Cl H. Syringaldazine (FACTS) Method

1. General Discussion

a. Principle: The free (available) chlorine test, syringaldazine (FACTS) measures free chlorine over the range of 0.1 to 10 mg/L. A saturated solution of syringaldazine (3,5-dimethoxy-4-hydroxybenzaldazine) in 2-propanol is used. Syringaldazine is oxidized by free chlorine on a 1:1 molar basis to produce a colored product with an absorption maximum of 530 nm. The color product is only slightly soluble in water; therefore, at chlorine concentrations greater than 1 mg/L, the final reaction mixture must contain 2-propanol to prevent product precipitation and color fading.

The optimum color and solubility (minimum fading) are obtained in a solution having a pH between 6.5 and 6.8. At a pH <6, color development is slow and reproducibility is poor. At a pH >7, the color develops rapidly but fades quickly. A buffer is required to maintain the reaction mixture pH at approximately 6.7. Take care with waters of high acidity or alkalinity to assure that the added buffer maintains the proper pH.

Temperature has a minimal effect on the color reaction. The maximum error observed at temperature extremes of 5 and 35°C is ±10%.

b. Interferences: Interferences common to other methods for determining free chlorine do not affect the FACTS procedure. Monochloramine concentrations up to 18 mg/L, dichloramine concentrations up to 10 mg/L, and manganese concentrations (oxidized forms) up to 1 mg/L do not interfere. Trichloramine at levels above 0.6 mg/L produces an apparent free chlorine reaction. Very high concentrations of monochloramine (≥35 mg/L) and oxidized manganese (≥2.6 mg/L) produce a color with syringaldazine slowly. Ferric iron can react with syringaldazine; however, concentrations up to 10 mg/L do not interfere. Nitrite (≤250 mg/L), nitrate (≤100 mg/L), sulfate (≤1000 mg/L), and chloride (≤1000 mg/L) do not interfere. Waters with high hardness (≥500 mg/L) will produce a cloudy solution that can be compensated for by using a blank. Oxygen does not interfere.

Other strong oxidizing agents, such as iodine, bromine, and ozone, will produce a color.

c. Minimum detectable concentration: The FACTS procedure is sensitive to free chlorine concentrations of 0.1 mg/L or less.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Colorimetric equipment: One of the following is required:

a. Filter photometer, providing a light path of 1 cm for chlorine concentrations ≤1 mg/L or a light path from 1 to 10 mm for chlorine concentrations above 1 mg/L; also equipped with a filter having a band pass of 500 to 560 nm.

b. Spectrophotometer, for use at 530 nm, providing the light paths noted above.

3. Reagents

a. Chlorine-demand-free water: See 4500-Cl.C.3*m*. Use to prepare reagent solutions and sample dilutions.

b. Syringaldazine indicator: Dissolve 115 mg 3,5-dimethoxy-4-hydroxybenzaldazine* in 1 L 2-propanol.

* Aldrich No. 17, 753-9, Aldrich Chemical Company, Inc., 1001 West St. Paul Ave., Milwaukee, WI 53233, or equivalent.

c. 2-Propanol: To aid in dissolution use ultrasonic agitation or gentle heating and stirring. Redistill reagent-grade 2-propanol to remove chlorine demand. Use a 30.5-cm Vigreux column and take the middle 75% fraction. Alternatively, chlorinate good-quality 2-propanol to maintain a free residual overnight; then expose to UV light or sunlight to dechlorinate. **CAUTION: 2-Propanol is extremely flammable.**

d. Buffer: Dissolve 17.01 g KH_2PO_4 in 250 mL water; pH should be 4.4. Dissolve 17.75 g Na_2HPO_4 in 250 mL water; the pH should be 9.9. Mix equal volumes of these solutions to obtain FACTS buffer, pH 6.6. Verify pH with pH meter. For waters containing considerable hardness or high alkalinity other pH 6.6 buffers can be used, for example, 23.21 g maleic acid and 16.5 mL 50% NaOH per liter of water.

e. Hypochlorite solution: Dilute household hypochlorite solution, which contains about 30 000 to 50 000 mg Cl equivalent/L, to a strength between 100 and 1000 mg/L. Standardize as directed in 4500-Cl.G.4*a*1).

4. Procedure

a. Calibration of photometer: Prepare a calibration curve by making dilutions of a standardized hypochlorite solution (4500-Cl.H.3*e*). Develop and measure colors as described in ¶ *b* below. Check calibration regularly, especially as reagent ages.

b. Free chlorine analysis: Add 3 mL sample and 0.1 mL buffer to a 5-mL-capacity test tube. Add 1 mL syringaldazine indicator, cap tube, and invert twice to mix. Transfer to a photometer tube or spectrophotometer cell and measure absorbance. Compare absorbance value obtained with calibration curve and report corresponding value as milligrams free chlorine per liter.

5. Bibliography

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4500-Cl I. Iodometric Electrode Technique

1. General Discussion

a. Principle: This method involves the direct potentiometric measurement of iodine released on the addition of potassium iodide to an acidified sample. A platinum–iodide electrode pair is used in combination with an expanded-scale pH meter.

b. Interference: All oxidizing agents that interfere with other iodometric procedures interfere. These include oxidized manganese and iodate, bromine, and cupric ions. Silver and mercuric ions above 10 and 20 mg/L interfere.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Electrodes: Use either a combination electrode consisting of a platinum electrode and an iodide ion-selective electrode or two individual electrodes. Both systems are available commercially.

b. pH/millivolt meter: Use an expanded-scale pH/millivolt meter with 0.1 mV readability or a direct-reading selective ion meter.

3. Reagents

a. pH 4 buffer solution: See 4500-Cl.C.3*e*.

b. Chlorine-demand-free water: See 4500-Cl.C.3*m*.

c. Potassium iodide solution: Dissolve 42 g KI and 0.2 g Na_2CO_3 in 500 mL chlorine-demand-free, distilled water. Store in a dark bottle.

d. Standard potassium iodate 0.002 81*N:* Dissolve 0.1002 g KIO_3 in chlorine-demand-free, distilled water and dilute to 1000 mL. Each 1.0 mL, when diluted to 100 mL, produces a solution equivalent to 1 mg/L as Cl_2.

4. Procedure

a. Standardization: Pipet into three 100-mL stoppered volumetric flasks 0.20, 1.00, and 5.00 mL standard iodate solution. Add to each flask, and a fourth flask to be used as a reagent blank, 1 mL each of acetate buffer solution and KI solution. Stopper, swirl to mix, and let stand 2 min before dilution. Dilute each standard to 100 mL with chlorine-demand-free distilled water. Stopper, invert flask several times to mix, and pour into separate 150-mL beakers. Stir gently without turbulence, using a magnetic stirrer, and immerse electrode(s) in the 0.2-mg/L (0.2-mL) standard. Wait for the potential to stabilize and record potential in mV. Rinse electrodes with chlorine-demand-free water and repeat for each standard and for the reagent blank. Prepare a calibration curve by plotting, on semilogarithmic paper, potential (linear axis) against concentration. Determine apparent chlorine concentration in the reagent blank from this graph (Reading B).

b. Analysis: Select a volume of sample containing no more than 0.5 mg chlorine. Pipet 1 mL acetate buffer solution and 1 mL KI into a 100-mL glass-stoppered volumetric flask. Stopper, swirl and let stand for at least 2 min. Adjust sample pH to 4 to 5, if necessary (mid-range pH paper is adequate for pH measurement), by adding acetic acid. Add pH-adjusted sample to volumetric flask and dilute to mark. Stopper and mix by inversion several times. Let stand for 2 min. Pour into a 150-mL beaker, immerse the electrode(s), wait for the potential to stabilize, and record. If the mV reading is greater than that recorded for the 5-mg/L standard, repeat analysis with a smaller volume of sample.

5. Calculation

Determine chlorine concentration (mg/L) corresponding to the recorded mV reading from the standard curve. This is Reading *A*. Determine total residual chlorine from the following:

$$\text{Total residual chlorine} = A \times 100/V$$

where V = sample volume, mL. If total residual chlorine is below 0.2 mg/L, subtract apparent chlorine in reagent blank (Reading *B*) to obtain the true total residual chlorine value.

6. Bibliography

JENKINS, R.L. & R.B. BAIRD. 1979. Determination of total chlorine residual in treated wastewaters by electrode. *Anal. Letters* 12:125.

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SYNNOTT, J.C. & A.M. SMITH. 1985. Total Residual Chlorine by Ion-Selective Electrode—from Bench Top to Continuous Monitor. Paper presented at 5th International Conf. on Chemistry for Protection of the Environment, Leuven, Belgium.

4500-ClO_2 CHLORINE DIOXIDE*

4500-ClO_2 A. Introduction

Because the physical and chemical properties of chlorine dioxide resemble those of chlorine in many respects, read the entire discussion of Residual Chlorine (Section 4500-Cl) before attempting a chlorine dioxide determination.

1. Occurrence and Significance

Chlorine dioxide, ClO_2, has been used widely as a bleaching agent in the paper and pulp industry. It has been applied to water supplies to combat tastes and odors due to phenolic-type wastes, actinomycetes, and algae, as well as to oxidize soluble iron and manganese to a more easily removable form. It is a disinfectant, and some results suggest that it may be stronger than free chlorine or hypochlorite.

Chlorine dioxide is a deep yellow, volatile, unpleasant-smelling gas that is toxic and under certain conditions may react explosively. It should be handled with care in a vented area. The use of odor to warn of exposure to concentrations of health significance may not be adequate.

There are several methods of generating ClO_2; for laboratory purposes the acidification of a solution of sodium chlorite followed by suitable scrubbing and capture of the released gaseous ClO_2 is the most practical. **CAUTION: Sodium chlorite is a powerful oxidizer; keep out of direct contact with oxidizable material to avoid possibility of explosion.**

2. Selection of Method

The iodometric method (4500-ClO_2.B) gives a very precise measure of total available strength of a solution in terms of its ability to liberate iodine from iodide. However, ClO_2, chlorine, chlorite, and hypochlorite are not distinguished easily by this technique. It is designed primarily, and best used, for standardizing ClO_2 solutions needed for preparation of temporary standards. It often is inapplicable to industrial wastes.

The amperometric methods (4500-ClO_2.C and E) are useful when a knowledge of the various chlorine fractions in a water sample is desired. They distinguish various chlorine compounds of interest with good accuracy and precision, but require specialized equipment and considerable analytical skill.

3. Sampling and Storage

Determine ClO_2 promptly after collecting the sample. Do not expose sample to sunlight or strong artificial light and do not aerate to mix. Most of these methods can be performed onsite, with prior calibration in the laboratory. Minimum ClO_2 losses occur when the determination is completed immediately at the site of sample collection.

4. Bibliography

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MASSCHELEIN, W. 1966. Spectrophotometric determination of chlorine dioxide with acid chrome violet K. *Anal. Chem.* 38:1839.

MASSCHELEIN, W. 1969. Les Oxydes de Chlore et le Chlorite de Sodium, Chapter XI. Dunod, Paris.

* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011. Joint Task Group: 20th Edition—Robert P. Fisher (chair), James M. Gindelberger, Gilbert Gordon, Robert C. Hoehn, Wayne B. Huebner, Frances Y. Saunders.

4500-ClO_2 B. Iodometric Method

1. General Discussion

a. Principle: A pure solution of ClO_2 is prepared from gaseous ClO_2 by slowly adding dilute H_2SO_4 to a sodium chlorite ($NaClO_2$) solution. Contaminants such as chlorine are removed from the gas stream by a $NaClO_2$ scrubber; the gas is passed into distilled water in a steady stream of air. ***See*** **CAUTION, 4500-ClO_2.A.1.**

ClO_2 releases free iodine from a KI solution acidified with acetic acid or H_2SO_4. The liberated iodine is titrated with a standard solution of sodium thiosulfate ($Na_2S_2O_3$), with starch as the indicator.

b. Interference: There is little interference in this method, but temperature and strong light affect solution stability. Minimize ClO_2 losses by storing stock ClO_2 solution in a dark refrigerator and by preparing and titrating dilute ClO_2 solutions for standardization purposes at the lowest practicable temperature and in subdued light.

c. Minimum detectable concentration: One drop (0.05 mL) of 0.01*N* (0.01*M*) $Na_2S_2O_3$ is equivalent to 20 μg ClO_2/L (or 40 μg/L in terms of available chlorine) when a 500-mL sample is titrated.

2. Reagents

All reagents listed for the determination of residual chlorine in Section 4500-Cl.B.2*a–g* are required. Also needed are the following:

a. Stock chlorine dioxide solution: Prepare a gas generating and absorbing system as illustrated in Figure 4500-ClO_2:1. Con- F1

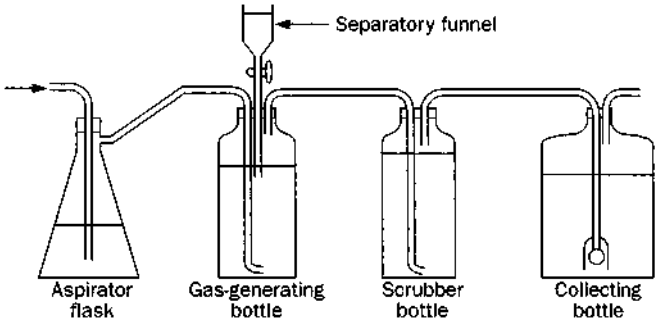


Figure 4500-ClO_2:1. Chlorine dioxide generation and absorption system.

nect aspirator flask, 500-mL capacity, with rubber tubing to a source of purified compressed air. Let air bubble through a layer of 300 mL distilled water in flask and then pass through a glass tube ending within 5 mm of the bottom of the 1-L gas-generating bottle. Conduct evolved gas via glass tubing through a scrubber bottle containing saturated $NaClO_2$ solution or a tower packed with flaked $NaClO_2$, and finally, via glass tubing, into a 2-L borosilicate glass collecting bottle where the gas is absorbed in 1500 mL distilled water. Provide an air outlet tube on collecting bottle for escape of air. Select for gas generation a bottle constructed of strong borosilicate glass and having a mouth wide enough to permit insertion of three separate glass tubes: the first leading almost to the bottom for admitting air, the second reaching below the liquid surface for gradual introduction of H_2SO_4, and the third near the top for exit of evolved gas and air. Fit to second tube a graduated cylindrical separatory funnel to contain H_2SO_4. Locate this system in a fume hood with an adequate shield.

Dissolve 10 g $NaClO_2$ in 750 mL distilled water and place in generating bottle. Carefully add 2 mL conc H_2SO_4 to 18 mL distilled water and mix. Transfer to funnel. Connect flask to generating bottle, generating bottle to scrubber, and the latter to collecting bottle. Pass a smooth current of air through the system, as evidenced by the bubbling rate in all bottles.

Introduce 5-mL increments of H_2SO_4 from funnel into generating bottle at 5-min intervals. Continue air flow for 30 min after last portion of acid has been added.

Store yellow stock solution in glass-stoppered dark-colored bottle in a dark refrigerator. The concentration of ClO_2 thus prepared varies between 250 and 600 mg/L, corresponding to approximately 500 to 1200 mg free chlorine/L.

b. Standard chlorine dioxide solution: Use this solution for preparing temporary ClO_2 standards. Dilute required volume of stock ClO_2 solution to desired strength with chlorine-demand-free water (see Section 4500-Cl.C.3*m*). Standardize solution by titrating with standard 0.01*N* (0.01*M*) or 0.025*N* (0.025*M*) $Na_2S_2O_3$ titrant in the presence of KI, acid, and starch indicator by following the procedure given in 4500-ClO_2.B.3. A full or nearly full bottle of chlorine or ClO_2 solution retains its titer longer than a partially full one. When repeated withdrawals reduce volume to a critical level, standardize diluted solution at the beginning, midway in the series of withdrawals, and at the end of the series. Shake contents thoroughly before drawing off needed solution from middle of the glass-stoppered dark-colored bottle. Prepare this solution frequently.

3. Procedure

Select volume of sample, prepare for titration, and titrate sample and blank as described in Section 4500-Cl.B.3. The only exception is the following: *Let ClO_2 react in the dark with acid and KI for 5 min before starting titration.*

4. Calculations

Express ClO_2 concentrations in terms of ClO_2 or as free chlorine content. Free chlorine is defined as the total oxidizing power of ClO_2 measured by titrating iodine released by ClO_2 from an acidic solution of KI. Calculate result in terms of chlorine itself.

For standardizing ClO_2 solution:

$$\text{mg } ClO_2/\text{mL} = \frac{(A \pm B) \times N \times 13.49}{\text{mL sample titrated}}$$

For determining ClO_2 temporary standards:

$$\text{mg } ClO_2 \text{ as } Cl_2/\text{mL} = \frac{(A \pm B) \times N \times 35.45}{\text{mL sample titrated}}$$

where:

A = mL titration for sample,
B = mL titration for blank (positive or negative, see Section 4500-Cl.B.3*d*), and
N = normality of $Na_2S_2O_3$ = molarity of $Na_2S_2O_3$.

5. Bibliography

POST, M.A. & W.A. MOORE. 1959. Determination of chlorine dioxide in treated surface waters. *Anal. Chem.* 31:1872.

4500-ClO_2 C. Amperometric Method I

1. General Discussion

a. Principle: The amperometric titration of ClO_2 is an extension of the amperometric method for chlorine. By performing four titrations with phenylarsine oxide, free chlorine (including hypochlorite and hypochlorous acid), chloramines, chlorite, and ClO_2 may be determined separately. The first titration step consists of conversion of ClO_2 to chlorite and chlorate through addition of sufficient NaOH to produce a pH of 12, followed by neutralization to a pH of 7 and titration of free chlorine. In the

second titration KI is added to a sample that has been treated similarly with alkali and had the pH readjusted to 7; titration yields free chlorine and monochloramine. The third titration involves addition of KI and pH adjustment to 7, followed by titration of free chlorine, monochloramine, and one-fifth of the available ClO_2. In the fourth titration, addition of sufficient H_2SO_4 to lower the pH to 2 enables all available ClO_2 and chlorite, as well as the total free chlorine, to liberate an equivalent amount of iodine from the added KI and thus be titrated.

b. Interference: The interferences described in Section 4500-Cl.D.1*b* apply also to determination of ClO_2.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

The apparatus required is given in Sections 4500-Cl.D.2*a–d*.

3. Reagents

All reagents listed for the determination of chlorine in Section 4500-Cl.D.3 are required. Also needed are the following:

a. Sodium hydroxide (NaOH), 6*N* (6*M*).

b. Sulfuric acid (H_2SO_4), 6*N* (3*M*), 1 + 5.

4. Procedure

Minimize effects of pH, time, and temperature of reaction by standardizing all conditions.

a. Titration of free available chlorine (hypochlorite and hypochlorous acid): Add sufficient 6*N* (6*M*) NaOH to raise sample pH to 12. After 10 min, add 6*N* (3*M*) H_2SO_4 to lower pH to 7. Titrate with standard phenylarsine oxide titrant to the amperometric endpoint as given in Section 4500-Cl.D. Record result as *A*.

b. Titration of free available chlorine and chloramine: Add 6*N* (6*M*) NaOH to raise sample pH to 12. After 10 min, add 6*N* (3*M*) H_2SO_4 to reduce pH to 7. Add 1 mL KI solution. Titrate with standard phenylarsine oxide titrant to the amperometric endpoint. Record result as *B*.

c. Titration of free available chlorine, chloramine, and one-fifth of available ClO_2: Adjust sample pH to 7 with pH 7 phosphate buffer solution. Add 1 mL KI solution. Titrate with standard phenylarsine oxide titrant to the amperometric endpoint. Record result as *C*.

d. Titration of free available chlorine, chloramines, ClO_2, and chlorite: Add 1 mL KI solution to sample. Add sufficient 6*N* (3*M*) H_2SO_4 to lower pH to 2. After 10 min, add sufficient 6*N* (6*M*) NaOH to raise pH to 7. Titrate with standard phenylarsine oxide titrant to the amperometric endpoint. Record result as *D*.

5. Calculation

Convert individual titrations (*A, B, C,* and *D*) into chlorine concentration by the following equation:

$$\text{mg Cl as } Cl_2/\text{L} = \frac{E \times 200}{\text{mL sample}}$$

where:

E = mL phenylarsine oxide titration for each individual sample *A, B, C,* or *D.*

Calculate ClO_2 and individual chlorine fractions as follows:

$$\text{mg } ClO_2 \text{ as } ClO_2/\text{L} = 1.9\,(C - B)$$
$$\text{mg } ClO_2 \text{ as } Cl_2/\text{L} = 5\,(C - B)$$
$$\text{mg free available chlorine/L} = A$$
$$\text{mg chloramine/L as chlorine} = B - A$$
$$\text{mg chlorite/L as chlorine} = 4B - 5C + D$$

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4500-ClO_2 D. (Reserved)

4500-ClO_2 E. Amperometric Method II

1. General Discussion

a. Principle: Like Amperometric Method I (Section 4500-ClO_2.C), this procedure entails successive titrations of combinations of chlorine species. Subsequent calculations determine the concentration of each species. The equilibrium for reduction of the chlorine species of interest by iodide is pH-dependent.

The analysis of a sample for chlorine, chlorine dioxide, chlorite, and chlorate requires the following steps: determination of all of the chlorine (free plus combined) and one-fifth of the chlorine dioxide at pH 7; lowering sample pH to 2 and determination of the remaining four-fifths of the ClO_2 and all of the chlorite (the chlorite measured in this step comes from the chlorite originally present in the sample and that formed in the first titration); preparation of a second sample by purging with nitrogen to remove ClO_2 and by reacting with iodide at pH 7 to remove any chlorine remaining; lowering latter sample pH to 2 and determination of all chlorite present (this chlorite only

comes from the chlorite originally present in the sample); and, in a third sample, determination of all of the relevant, oxidized chlorine species—chlorine, chlorine dioxide, chlorite, and chlorate—after reduction in hydrochloric acid.[1]

This procedure can be applied to concentrated solutions (10 to 100 mg/L) or dilute solutions (0.1 to 10 mg/L) by appropriate selection of titrant concentration and sample size.

b. Interferences: At pH values above 4, significant iodate formation is possible if iodine is formed in the absence of iodide;[2] this results in a negative bias in titrating the first and second samples. Acidification of these samples causes reduction of iodate to iodine and a positive bias. To prevent formation of iodate, add 1 g KI granules to stirred sample.

A positive bias results from oxidation of iodide to iodine by dissolved oxygen in strongly acidic solutions.[1] To minimize this bias, use bromide as the reducing agent in titrating the third sample (bromide is not oxidized by oxygen under these conditions). After reaction is completed, add iodide, which will be oxidized to iodine by the bromine formed from the reduction of the original chlorine species. Add iodide carefully so bromine gas is not lost. Rapid dilution of the sample with sodium phosphate decreases sample acidity and minimizes oxidation of iodide by oxygen. The pH of the solution to be titrated should be between 1.0 and 2.0. Carry a blank through the procedure as a check on iodide oxidation.

The potential for interferences from manganese, copper, and nitrate is minimized by buffering the sample to pH $\geq$4.[3,4] For the method presented here, the low pH required for the chlorite and chlorate analyses provides conditions favorable to manganese, copper, and nitrite interferences.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Titrators: See Section 4500-Cl.D.2*a–d*. Amperometric titrators with a platinum–platinum electrode system are more stable and require less maintenance. (NOTE: Chlorine dioxide may attack adhesives used to connect the platinum plate to the electrode, resulting in poor readings.)

If a potentiometric titrator is used, provide a platinum sensing electrode and a silver chloride reference electrode for endpoint detection.

b. Glassware: Store glassware used in this method separately from other laboratory glassware and do not use for other purposes because ClO_2 reacts with glass to form a hydrophobic surface coating. To satisfy any ClO_2 demand, before first use immerse all glassware in a strong ClO_2 solution (200 to 500 mg/L) for 24 h and rinse only with water between uses.

c. Sampling: ClO_2 is volatile and will vaporize easily from aqueous solution. When sampling a liquid stream, minimize contact with air by placing a flexible sample line to reach the bottom of the sample container, letting several container volumes overflow, slowly removing sample line, and capping container with minimum headspace. Protect from sunlight. Remove sample portions with a volumetric pipet with pipet tip placed at bottom of container. Drain pipet by placing its tip below the surface of reagent or dilution water.

3. Reagents

a. Standard sodium thiosulfate, 0.100*N* (0.100*M*): See Section 4500-Cl.B.2*c*.

b. Standard phenylarsine oxide, 0.005 64*N* (0.005 64*M*): See Section 4500-Cl.C.3*a*. (Weigh out 1.25 g phenylarsine oxide and standardize to 0.005 64*M*.)

c. Phosphate buffer solution, pH 7: See Section 4500-Cl.D.3*b*.

d. Potassium iodide (KI), granules.

e. Saturated sodium phosphate solution: Prepare a saturated solution of $Na_2HPO_4 \cdot 12H_2O$ with cold deionized-distilled water.

f. Potassium bromide solution, 5%: Dissolve 5 g KBr and dilute to 100 mL. Store in a brown glass-stoppered bottle. Make fresh weekly.

g. Hydrochloric acid (HCl), conc.

h. Hydrochloric acid (HCl), 2.5*N* (2.5*M*): Cautiously add 200 mL conc HCl, with mixing, to distilled water, diluting to 1000 mL.

i. Purge gas: Use nitrogen gas for purging ClO_2 from samples. Ensure that gas is free of contaminants and pass it through a 5% KI scrub solution. Discard solution at first sign of color.

4. Procedure

Use either sodium thiosulfate or phenylarsine oxide as titrant. Select concentration on basis of concentration range expected. The total mass of oxidant species should be no greater than about 15 mg. Make appropriate sample dilutions if necessary. A convenient volume for titration is 200 to 300 mL. Preferably analyze all samples and blanks in triplicate.

Minimize effects of pH, time, and temperature of reaction by standardizing all conditions.

a. Titration of residual chlorine and one-fifth of available ClO_2: Place 1 mL pH 7 phosphate buffer in beaker and add distilled-deionized dilution water if needed. Introduce sample with minimum aeration and add 1 g KI granules while stirring. Titrate to endpoint (see Section 4500-Cl.D). Record reading A = mL titrant/mL sample.

b. Titration of four-fifths of available ClO_2 and chlorite: Continuing with same sample, add 2 mL 2.5*N* (2.5*M*) HCl. Let stand in the dark for 5 min. Titrate to endpoint. Record reading B = mL titrant/mL sample.

c. Titration of nonvolatilized chlorine: Place 1 mL pH 7 phosphate buffer in purge vessel and add distilled-deionized dilution water if needed. Add sample and purge with nitrogen gas for 15 min. Use a gas-dispersion tube to give good gas–liquid contact. Add 1 g KI granules while stirring and titrate to endpoint. Record reading C = mL titrant/mL sample.

d. Titration of chlorite: Continuing with same sample, add 2 mL 2.5*N* (2.5*M*) HCl. Let stand in the dark for 5 min. Titrate to endpoint, and record reading D = mL titrant/mL sample.

e. Titration of chlorine, ClO_2, chlorate, and chlorite: Add 1 mL KBr and 10 mL conc HCl to 50-mL reaction flask and mix. Carefully add 15 mL sample, with minimum aeration. Mix and stopper immediately. Let stand in the dark for 20 min. Rapidly add 1 g KI granules and shake vigorously for 5 s. Rapidly transfer to titration flask containing 25 mL saturated Na_2HPO_4 solution. Rinse reaction flask thoroughly and add rinse water to

Table 4500-ClO_2:I. Equivalent Weights for Calculating Concentrations on the Basis of Mass

pH	Species	Molecular Weight *mg/mol*	Electrons Transferred	Equivalent Weight *mg/eq*
7	Chlorine dioxide	67 452	1	67 452
2, 0.1	Chlorine dioxide	67 452	5	13 490
7, 2, 0.1	Chlorine	70 906	2	35 453
2, 0.1	Chlorite	67 452	4	16 863
0.1	Chlorate	83 451	6	13 909

titration flask. Final titration volume should be about 200 to 300 mL. Titrate to endpoint.

Repeat procedure of preceding paragraph using distilled-deionized water in place of sample to determine blank value.

Record reading E = (mL titrant sample − mL titrant blank)/mL sample.

Note: The 15-mL sample volume can be adjusted to provide an appropriate dilution, but maintain the ratio of sample to HCl.

5. Calculations

Because the combining power of the titrants is pH-dependent, all calculations are based on the equivalents of reducing titrant required to react with equivalents of oxidant present. Use Table 4500-ClO_2:I to obtain the equivalent weights to be used in the calculations.

In the following equations, N is the normality of the titrant used in equivalents per liter and A through E are as defined previously.

$$\text{Chlorite, mg } ClO_2^-/L = D \times N \times 16\,863$$

$$\text{Chlorate, mg } ClO_3^-/L = [E - (A + B)] \times N \times 13\,909$$

$$\text{Chlorine dioxide, mg } ClO_2/L = (5/4) \times (B - D) \times N \times 13\,490$$

$$\text{Chlorine, mg } Cl_2/L = \{A - [(B - D)/4]\} \times N \times 35\,453$$

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4500-CN^- CYANIDE*

4500-CN^- A. Introduction

1. General Discussion

In this section, *cyanide* refers to inorganic cyanide (CN^-) in water. Cyanide can exist in aqueous media as undissociated hydrogen cyanide (HCN), free cyanide ion (CN^-), and anionic complexes of cyanide with various metal cations.

Hydrogen cyanide is a very weak acid with a dissociation pK_a of 9.2. In water samples with approximately neutral pH, HCN is the predominant form[1] rather than free CN^-.

Simple cyanide salts (NaCN and KCN) dissociate completely in water unless other metal cations are present; then, anionic cyanide complexes are formed. The stability of these complexes varies widely, with thermodynamic formation constants ranging from $10^{41.5}$ for $Hg(CN)_4^{2-}$ to $10^{16.7}$ for $Zn(CN)_4^{2-}$. Their stability depends on pH; most dissociate appreciably in acidic solutions. The most stable anionic cyanide complex is that formed with iron; when water contains excess cyanide, all available iron will be bound in either the ferrocyanide [$Fe(CN)_6^{4-}$] or ferricyanide [$Fe(CN)_6^{3-}$] complex.

Molecular HCN is highly toxic to aquatic life[2–5]; it is formed in solutions of cyanide by hydrolytic reaction of CN^- with water. CN^- is less toxic than HCN; it usually is unimportant because most of the free cyanide (CN group present as CN^- or as HCN) exists as HCN[2–5] because most natural waters have a substantially lower pH than the pK_a for molecular HCN. The toxicity to fish of most tested solutions of complex cyanides is attributable mainly to the HCN resulting from dissociation of the complexes.[2,4,5] Analytical distinction between HCN and other cyanide species in solutions of complex cyanides is possible.[2,5–10]

Most metallocyanide complexes achieve dissociation equilibrium slowly, and their toxicity is inversely related to their highly variable stability.[2,4,5] The degree of dissociation also is related to the dilution, and increases with decreasing pH. Cyanide complexes of zinc and cadmium are dissociated almost totally in very dilute solutions and can be acutely toxic to fish, even at pH characteristic of natural aquatic environments. In equally dilute solutions, there is much less dissociation of nickel and other more-stable cyanide complexes. Although the complexed cyanide exhibits much less toxicity than HCN, dilute solutions of copper and silver cyanide complexes may still demonstrate acute toxicity to fish.[2,5]

The iron cyanide complexes are very stable and generally regarded as not materially toxic. Acutely toxic levels of HCN, however, may be reached in aged solutions of moderate to high concentrations in the dark. Dilute solutions of iron cyanide complexes are subject to rapid and extensive photolysis when exposed to UV radiation.[2,11] The latter process is limited in deep, turbid, or shaded receiving waters.

Historically, the generally accepted physicochemical technique for industrial waste treatment of cyanide compounds is alkaline chlorination:

$$NaCN + Cl_2 \rightarrow CNCl + NaCl \quad (1)$$

The first reaction product on chlorination is cyanogen chloride (CNCl), a highly toxic gas of limited solubility. The toxicity of CNCl may exceed that of equal concentrations of cyanide.[2,3,12] At an alkaline pH, CNCl hydrolyzes to the cyanate ion (OCN^-), which has only limited toxicity.

There is no known natural reduction reaction that may convert OCN^- to CN^-.[13] On the other hand, breakdown of toxic CNCl is pH- and time-dependent. At pH 9, with no excess chlorine present, CNCl may persist for 24 h.[14,15]

$$CNCl + 2NaOH \rightarrow NaOCN + NaCl + H_2O \quad (2)$$

OCN^- can be oxidized further with chlorine at a nearly neutral pH to CO_2 and N_2:

$$2NaOCN + 4NaOH + 3Cl_2 \rightarrow 6NaCl + 2CO_2 + N_2 + 2H_2O \quad (3)$$

OCN^- also will be converted on acidification to NH_4^+:

$$2NaOCN + H_2SO_4 + 4H_2O \rightarrow (NH_4)_2SO_4 + 2NaHCO_3 \quad (4)$$

The alkaline chlorination of cyanide compounds is relatively fast, but depends equally on the dissociation constant, which also governs toxicity. Metal cyanide complexes, such as nickel, cobalt, silver, and gold, do not dissociate readily. The chlorination reaction, therefore, requires more time and a significant chlorine excess.[16] Iron cyanides, because they do not dissociate to any degree, are not oxidized by chlorination. There is correlation between the refractory properties of the noted complexes, in their resistance to chlorination and lack of toxicity.

Alkaline chlorination of wastewater with high nitrogen loads, followed by dechlorination with ascorbic acid, may form cyanide ions.[17] This generates negative values for the cyanide amenable to chlorination (4500-CN^-.G). Treating the sample with sulfamic acid before chlorination and judicious use of sodium thiosulfate for dechlorination may correct this issue.

It is advantageous to differentiate between *total cyanide* and *cyanides amenable to chlorination*. Total cyanide measures the almost nondissociable cyanides, dissociable complexes of cyanide, and complexes of intermediate stability. Cyanide compounds that are amenable to chlorination include free cyanide as well as those complex cyanides that are potentially dissociable, almost wholly or in large degree, and are therefore potentially toxic at low concentrations, even in the dark. The chlorination test procedure is carried out under

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rigorous conditions appropriate for measurement of the more dissociable forms of cyanide.

The free and potentially dissociable cyanides also may be estimated when using the *weak acid dissociable* procedure. While this method uses the same general rigorous distillation process as for total cyanide, it does so under mildly acidic conditions. Iron cyanide's interference in the measurement is minimized by the addition of precipitation chemicals to the distillation flask and by the avoidance of UV irradiation during processing.

The *cyanogen chloride* (CNCl) procedure is common with the colorimetric test for cyanides amenable to chlorination. This test is based on the addition of chloramine-T and subsequent color complex formation with a pyridine-barbituric acid solution. Without the addition of chloramine-T, only existing CNCl is measured. CNCl is a gas that hydrolyzes to OCN^-; sample preservation is not possible. Because of this, spot testing of CNCl levels may be best. This procedure can be adapted and used when the sample is collected.

There may be analytical requirements for the determination of OCN^-, even though the reported toxicity level is low. On acidification, OCN^- decomposes to ammonia (NH_3).[3] Molecular ammonia and metal–ammonia complexes are toxic to aquatic life.[18]

Thiocyanate (SCN^-) is not very toxic to aquatic life,[2,19] but when chlorinated, it forms toxic CNCl, as discussed above.[2,3,12] At least where subsequent chlorination is anticipated, the determination of SCN^- is desirable. Thiocyanate is biodegradable; ammonium ion is released in this reaction. Although the typical detoxifying agents used in cyanide poisoning induce thiocyanate formation, biochemical cyclic reactions with cyanide are possible, resulting in detectable levels of cyanide from exposure to thiocyanate.[19] Thiocyanate may be analyzed in samples properly preserved for determination of cyanide; however, thiocyanate also can be preserved in samples by acidification with H_2SO_4 to pH $\leq$2.

2. Cyanide in Solid Waste

a. Soluble cyanide: To determine the soluble cyanide concentration, the sample must first be leached with 0.01 *M* NaOH until solubility equilibrium is established. One hour of stirring should be satisfactory. Cyanide analysis is then performed on the leachate. Low cyanide concentration in the leachate may indicate presence of sparingly soluble metal cyanides. The leachate's cyanide content is indicative of residual solubility of insoluble metal cyanides in the waste.

High levels of cyanide in the leachate indicate soluble cyanide in the solid waste. For example, when 500 mL of 0.01 *M* NaOH are stirred into a 500-mg solid waste sample, the cyanide concentration (mg/L) of the leachate multiplied by 1000 will give the solubility level of the cyanide in the solid waste in milligrams per kilogram. The leachate may be analyzed for total cyanide and/or cyanide amenable to chlorination.

b. Insoluble cyanide: Insoluble cyanides in the solid should be leached out by stirring a weighed sample for 12 to 16 h in a 10% NaOH (2.5 *M*) solution. The leached solution of the solid waste will give the total cyanide, including the metal–metal cyanide content with the distillation procedure. Prechlorinating the extract will eliminate all cyanide amenable to chlorination. Do not expose sample to sunlight.

3. Method Selection

a. Total cyanide after distillation: After interferences are removed, cyanide is converted to HCN gas, which is distilled and absorbed in sodium hydroxide (NaOH) solution.[20] Due to cyanide's catalytic decomposition in the presence of cobalt at high temperature in a strong acid solution,[21,22] neither cobalticyanide nor cyanide complexes of noble metals (e.g., gold, platinum, and palladium) are recovered completely. Distillation also decomposes some organic cyanide compounds, such as cyanohydrins (the result of aldehydes reacting with cyanide). It also separates cyanide from other color-producing and possibly interfering organic or inorganic contaminants. Subsequent analysis is for the simple salt, sodium cyanide (NaCN).

The absorption liquid is analyzed by a titrimetric, colorimetric, or cyanide-ion-selective electrode procedure:

1) The titration method (4500-CN$^-$.D) is suitable for cyanide concentrations >1 mg/L.
2) The colorimetric methods (4500-CN$^-$.E, N, and O) are suitable for cyanide concentrations as low as 1 to 5 μg/L under ideal conditions. Method 4500-CN$^-$.N uses flow injection analysis of the distillate. Method 4500-CN$^-$.O uses flow injection analysis following transfer through a semipermeable membrane to separate gaseous cyanide, and colorimetric analysis. Method 4500-CN^2.E uses conventional colorimetric analysis of the distillate from 4500-CN$^-$.C.
3) The ion-selective electrode method (4500-CN$^-$.F) using the cyanide ion electrode is applicable for cyanide concentrations between 0.05 and 10 mg/L.

b. Cyanide amenable to chlorination:

1) Two samples (one unchlorinated and one chlorinated) must be distilled. Chlorination destroys all amenable cyanide present. Analyze absorption liquids from both tests for total cyanide. The observed difference is the percentage of cyanides amenable to chlorination.

2) Direct colorimetric methods, by converting CN^- and SCN^- to CNCl and developing the color complex with pyridine-barbituric acid solution, are used to determine the total of these cyanides (4500-CN$^-$.H). Repeating the test with formaldehyde addition to mask the cyanide provides a measure of SCN^- content. When subtracted from the earlier results, this provides an estimate of the amenable CN^- content. This method is useful for natural and ground waters, clean metal finishing, and heat treating effluents. Sanitary wastes may exhibit interference.

3) The weak and dissociable cyanides procedure (4500-CN$^-$.I) approximately measures the cyanide amenable to chlorination by freeing HCN from dissociable cyanide. After being collected in a NaOH absorption solution, CN^- may be determined by one of the finishing procedures given for the total cyanide determination. An automated procedure (4500-CN$^-$.O) also is presented.

Although cyanide amenable to chlorination and weak and dissociable cyanide in theory are identical, certain industrial effluents (e.g., pulp and paper, petroleum refining) contain some poorly understood substances that may produce interference. Using the procedure for cyanide amenable to chlorination often yields negative values. For natural waters and metal-finishing effluents, the direct colorimetric determination appears to be the simplest and most economical.

c. Cyanogen chloride: The colorimetric method may be used, but omit the chloramine-T addition. The spot test also may be used.

d. Spot test for sample screening: This procedure allows a quick sample screening to establish whether >50 µg/L cyanide amenable to chlorination is present. The test also may be used to estimate CNCl content at the time of sampling.

e. Cyanate: OCN^- is converted to ammonium carbonate [$(NH_4)_2CO_3$] by acid hydrolysis at elevated temperature. Ammonia (NH_3) is determined both before and after converting OCN^-; the OCN^- is estimated from the difference in NH_3 concentrations.[23–25] Measure NH_3 by either:

1) The selective electrode method, using the NH_3 gas electrode (Section 4500-NH_3.D) or
2) The colorimetric method, using the phenate method for NH_3 (Section 4500-NH_3.F or G).

f. Thiocyanate: Use the colorimetric determination with ferric nitrate as a color-producing compound.

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4500-CN⁻ B. Preliminary Treatment of Samples

Caution: Cyanide is toxic; avoid contact, inhalation, or ingestion. Use care in manipulating samples containing cyanide. Process in a hood or other well-ventilated area.

1. General Discussion

The preliminary treatment required depends on which interfering substances the samples contain. Distillation removes many interfering substances, but other pretreatment procedures will be needed for sulfides, fatty acids, oxidizing agents, nitrites, and nitrates.

Various chemical reactions can form or destroy cyanide, so take precautions to adequately maintain its integrity in samples. The preservation and pretreatment steps may need to be tailored to the site-specific sample matrix and the analytical method used. The U.S. Environmental Protection Agency (EPA) acknowledged this situation in its 2012 Methods Update Rule[1] by revising the footnote for cyanide preservation in wastewater samples as follows:

> There may be interferences that are not mitigated . . . any technique for removal or suppression of interference may be employed, provided the laboratory demonstrates that it more accurately measures cyanide through quality control measures described in the analytical test method.

Field spikes created at the time of sample collection are an effective way to demonstrate adequate preservation and treatment for interferences in both wastewater[2] and drinking water.[3]

2. Sample Preservation

Oxidizing agents, such as chlorine, decompose most cyanides. For chlorine residuals ≥2 mg/L, test by placing a drop of sample on a strip of fresh potassium iodide-starch paper moistened with acetate buffer (Section 4500-Cl.C.3*e*). A blue-purple color indicates that oxidants are present. Add 0.02 g/L sodium thiosulfate solution and re-test; repeat until the oxidizers are neutralized. Avoid using excess thiosulfate solution. Sodium arsenite solution (0.1 g/L) may be used in lieu of thiosulfate.

For chlorine residuals <2 mg/L, determine residual by titration with *N,N*-diethyl-*p*-phenylenediamine (DPD) and ferrous ammonium sulfate (FAS) (DPD-FAS titration) (Section 4500-Cl.F) or DPD colorimetric method (Section 4500-Cl.G) and add a stoichiometric amount of sodium thiosulfate solution (Section 4500-Cl.B.2*d*).

Ascorbic acid is no longer a recommended preservative for cyanide samples because it functions as a carbon donor in the presence of nitrite or nitrate, generating cyanide during distillation.[4] Sodium thiosulfate ($Na_2S_2O_3$) is an adequate dechlorinating agent as long as it is not used in excess. Sodium arsenite ($NaAsO_2$) also may be used, but it is a hazardous material. Sodium borohydride ($NaBH_4$) is another alternative. Generally, 0.1 g/L $NaBH_4$ can reduce 50 mg/L of chlorine.[5] If ascorbic acid must be used, first add sulfamic acid (2 g/500 mL sample), then add enough ascorbic acid to neutralize the oxidizing agent and test sample within 24 h.[6–8]

Oxidized products of sulfide convert cyanide to thiocyanate rapidly, especially at elevated pH.[9] Sulfides also interfere with several cyanide analysis methods.[10] If sulfides are suspected, test for S^{2-} by placing a drop of sample on lead acetate test paper previously moistened with acetic acid buffer solution at pH 4 (Section 4500-Cl.C.3*e*). If the paper darkens, then S^{2-} is present. Test suspect samples as soon as possible to avoid cyanide loss, and promptly dilute sulfide-positive samples with reagent water (to mitigate sulfide's effect). Avoid excessive dilution, which would raise the cyanide reporting limit above a regulatory limit.[6] Treating sulfide with lead acetate, lead carbonate, or cadmium carbonate is not recommended because the reactions may result in either cyanide loss or insoluble precipitate formation.[6,8]

Aldehydes convert cyanide to cyanohydrin. Longer contact times between cyanide and the aldehyde and higher ratios of aldehyde to cyanide both result in losses of cyanide. If analysts suspect that aldehydes are present, then stabilize with NaOH at time of collection and add 2 mL 3.5% ethylenediamine solution per 100 mL sample.

Most cyanides are very reactive and unstable, so analyze samples immediately. If that is impractical, add NaOH pellets or a strong NaOH solution to raise sample pH to 10 or 12–12.5 (5 mL of a 6 *N* NaOH solution per liter is usually sufficient for this), depending on the pertinent regulation. Keep in mind, however, that certain sample types tend to form cyanide when NaOH is added.[2,11,12] If such samples cannot be analyzed immediately, a field spike can be used to demonstrate that sample integrity has been maintained.[3] Field spikes of treated wastewater and raw surface water samples showed that <20% of free cyanide was lost during the first few days and more than half of free cyanide was retained up to 28 d. Follow the guidance in Section 1020B.7 for selecting spiking levels and evaluating spike recoveries.

A field dilution performed at the time of sample collection also can reduce interferences effectively. This is useful when the diluted sample's elevated reporting limit is still below the regulatory limit.[3]

If the sample contains residual disinfecting agent, remove it by adding a dechlorinating agent before adjusting the pH.. Then, store sample in a closed, dark bottle in a cool place and test as soon as possible.

To analyze for CNCl, collect a separate sample and omit the NaOH addition, because CNCl rapidly converts to OCN^- at high pH. Make colorimetric estimation immediately after sampling.

3. Interferences

a. Oxidizing agents may destroy most of the cyanide during storage and manipulation. Add $NaAsO_2$ or $Na_2S_2O_3$ as directed in 4500-CN$^-$.B.2. If $Na_2S_2O_3$ is used, a stoichiometric amount should be added to neutralize the oxidizing agent; avoid adding excess $Na_2S_2O_3$.

b. Sulfide will distill with cyanide and, therefore, adversely affect colorimetric, titrimetric, and electrode procedures. Test for and remove S^{2-} as directed in 4500-CN$^-$.B.2. Treat 25 mL more than required for the distillation to provide sufficient filtrate volume.

c. Fatty acids that distill and form soaps under alkaline titration conditions make the endpoint almost impossible to detect. Remove fatty acids by extraction.[13] Acidify sample with acetic acid (1 + 9) to pH 6.0 to 7.0. (**CAUTION: Perform this operation in a hood as quickly as possible.**) Immediately extract with iso-octane, hexane, or chloroform ($CHCl_3$) (preference in order named). Use a solvent volume equal to 20% of sample volume. Usually, one extraction will reduce the fatty acid concentration below the interference level. Avoid multiple extractions or a long contact time at low pH to minimize HCN loss. When extraction is completed, immediately raise pH >12 with NaOH solution.

d. Carbonate: Use caution if analyzing wastes containing high concentrations of carbonates. High carbonate concentrations may affect the distillation procedure by causing the violent release of CO_2 with excessive foaming when acid is added before distillation and by reducing the absorption solution's pH. To preserve such samples, stir and add calcium hydroxide slowly until pH is 10 or 12–12.5,[14] depending on the pertinent regulation. After precipitate settles, decant supernatant for use in determining cyanide.

Insoluble complex cyanide compounds will not be determined. If such compounds are present, filter a measured amount of well-mixed treated sample through a glass-fiber or membrane filter (47-mm dia or less). Rinse filter with dilute (1 + 9) acetic acid until effervescence ceases. Treat entire filter with insoluble material as insoluble cyanide (4500-CN$^-$.A.2*b*) or add to filtrate before distillation.

e. Aldehydes convert cyanide to cyanohydrin, which forms nitrile under the distillation conditions. Direct titration without distillation reveals only non-complex cyanides. Formaldehyde interference is noticeable when concentrations are >0.5 mg/L. Use the following spot test to establish the absence or presence of aldehydes (detection level = 0.05 mg/L):[15–17]

1) Reagents

a) *MBTH indicator solution*—Dissolve 0.05 g 3-methyl, 2-benzothiazolone hydrazone hydrochloride in 100 mL reagent water. Filter if turbid.

b) *Ferric chloride oxidizing solution*—Dissolve 1.6 g sulfamic acid and 1 g $FeCl_3 \cdot 6H_2O$ in 100 mL reagent water.

c) *Ethylenediamine solution,* 3.5%—Dilute 3.5 mL pharmaceutical-grade anhydrous $NH_2CH_2CH_2NH_2$ to 100 mL with reagent water.

2) Procedure—If the sample is alkaline, add 1 + 1 H_2SO_4 to 10 mL sample to adjust pH to <8. Place 1 drop sample and 1 drop reagent water (a blank) in separate cavities of a white spot plate. Add 1 drop MBTH solution and then 1 drop $FeCl_3$ oxidizing solution to each spot. Allow 10 min for color development. At higher concentrations of aldehyde, sample color will change from a faint green-yellow to a deeper green with blue-green to blue. The blank should remain yellow.

To minimize aldehyde interference, add 2 mL 3.5% ethylenediamine solution/100 mL sample. This quantity overcomes the interference caused by ≤50 mg/L formaldehyde.

When using a known addition in testing, 100% recovery of CN^- is not necessarily to be expected. Recovery depends on the aldehyde excess, time of contact, and sample temperature.

f. Other possible interferences: Distillation typically removes substances that might contribute color or turbidity.

The sulfuric acid and various reagents used in the strong-acid distillation procedure can cause various undesirable reactions, such as off-gassing or dehydrogenation, in certain wastes. As a QC measure, periodically conduct addition and recovery tests with industrial waste samples.

g. Glucose and other sugars, especially at the pH of preservation, lead to cyanohydrin formation via cyanide reaction with aldose.[18] Reduce cyanohydrin to cyanide with ethylenediamine (see ¶ *e* above). MBTH is not applicable.

h. Nitrite may form HCN during distillation in 4500-CN^-.C and G through reaction with organic compounds.[19,20] Nitrate also may reduce to nitrite, which can react further with thiocyanate. Add at least 2 g/500 mL sulfamic acid at time of sample collection and before dechlorination or basification to minimize nitrite interference.[7,8]

i. Some sulfur compounds may decompose during distillation, releasing S^{2-}, H_2S, or SO_2. Sulfur compounds may convert cyanide to thiocyanate and may interfere with analytical procedures for CN^-. To avoid this potential interference when it cannot be adequately mitigated by pretreatment, add 50 mg $PbCO_3$ to the absorption solution before distillation. Immediately filter the absorption solution before proceeding with the colorimetric or titrimetric determination.

Absorbed SO_2 forms Na_2SO_3, which consumes chloramine-T added in the colorimetric determination. The volume of chloramine-T added is sufficient to overcome 100 to 200 mg SO_3^{2-}/L. Test for presence of chloramine-T after adding it by placing a drop of sample on KI-starch test paper; add more chloramine-T if test paper remains blank, or use 4500-CN^-.F.

Some wastewaters, such as those from coal gasification or chemical extraction mining, contain high concentrations of sulfites. Pretreat sample to avoid overloading the absorption solution with SO_3^{2-}. Titrate a suitable sample iodometrically (Section 4500-O) with dropwise addition of 30% H_2O_2 solution to determine volume of H_2O_2 needed for the 500 mL distillation sample. Subsequently, add H_2O_2 dropwise while stirring until no more than 300 to 400 mg SO_3^{2-}/L remains. (Adding a lesser quantity than calculated is required to avoid oxidizing any CN^- that may be present.)

j. Alternate procedure: The strong-acid distillation procedure uses concentrated acid with magnesium chloride to dissociate metal–cyanide complexes. Some samples (e.g., certain industrial wastes) may be susceptible to interferences, such as those that occur when thiocyanate is converted to cyanide in the presence of an oxidant (e.g., nitrate). In those cases, use a ligand-displacement procedure with a mildly acidic medium that contains ethylenediaminetetraacetic acid (EDTA) to dissociate metal–cyanide complexes.[21] Under such conditions, thiocyanate is relatively stable and many oxidants, including nitrate, are weaker.

When a sample is from a source being tested for the first time, or if any cyanide procedure is revised or modified to meet specific requirements, obtain recovery data by performing a matrix spike (i.e., the addition of a known amount of cyanide) to check for the presence of interferences. Multiple interferences may be present in some samples, especially wastewater or process water from various industrial sources.

4. Quality Control

The QC practices considered to be an integral part of each method are summarized in Table 4020:I. These include daily use of reagent blanks, laboratory-fortified blanks, and known additions (e.g., matrix spikes). Analyses for regulatory purposes may require additional QC measures.

5. References

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4500-CN⁻ C. Total Cyanide after Distillation

1. General Discussion

Hydrogen cyanide (HCN) is liberated from an acidified sample by distillation and purging with air, with the HCN gas collected in a NaOH scrubbing solution. The cyanide concentration in the scrubbing solution is determined via titrimetric, colorimetric, or potentiometric procedures.

NOTE: The requirement to use magnesium chloride in the distillation first appeared in the 15th Edition of *Standard Methods*; subsequent data review demonstrates that it is not essential.[1] Use of magnesium chloride in the distillation is left to the analyst's discretion.

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

The apparatus described in ¶s *a–d* below is shown in Figure 4500-CN⁻:1.

NOTE: Other equivalent apparatus, including scaled-down apparatus (i.e., midi-distillation or micro-distillation systems), may be used if the scaled-down apparatus volumes preserve the same ratios of sample volume to digestion-reagents volume, as specified in the procedure (4500-CN⁻.C.4). Evaluate any apparatus being considered for use by experiments to show acceptable recovery of total cyanide.

a. Boiling flask, 1 L, with inlet tube and provision for water-cooled condenser.

b. Gas absorber, with gas-dispersion tube equipped with medium-porosity fritted outlet.

c. Heating element, adjustable.

d. Ground-glass ST joints, TFE-sleeved or with an appropriate lubricant for the boiling flask and condenser. Neoprene stopper and plastic threaded joints also may be used.

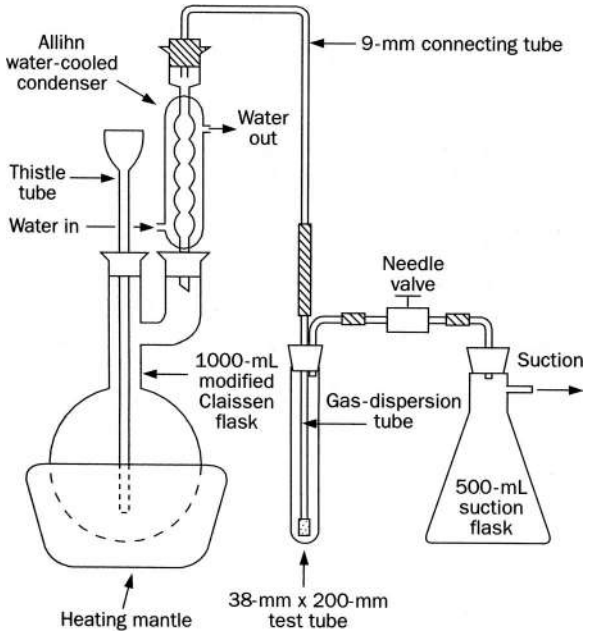


Figure 4500-CN⁻:1. Cyanide distillation apparatus.

3. Reagents (ACS grade or better)

a. Sodium hydroxide solution: Dissolve 40 g NaOH in reagent water and dilute to 1 L.

b. Magnesium chloride reagent (optional): Dissolve 510 g $MgCl_2 \cdot 6H_2O$ in water and dilute to 1 L.

c. Sulfuric acid (H_2SO_4), 1 + 1.

d. Lead carbonate ($PbCO_3$), powdered.

e. Sulfamic acid (NH_2SO_3H).

4. Procedure

The following procedure describes the process for macro-distillation; for midi- or micro-distillation procedures, follow the manufacturer's instructions and/or scale down the volume of sample and amounts of reagents added to maintain the same ratio(s) as for the macro-distillation method.

a. Add 500 mL sample, containing not more than 10 mg CN^-/L (diluted with reagent water, if necessary), to the boiling flask. If a higher CN^- content is anticipated, use the spot test (4500-CN^-.K) to approximate the required dilution. Add 10 mL NaOH solution to the gas scrubber and, if necessary, dilute with reagent water to obtain an adequate liquid depth in the absorber. Do not use more than 225 mL total volume of absorber solution. If the distilling flask is expected to generate S^{2-} that cannot be adequately mitigated by pretreatment, add 50 mg powdered $PbCO_3$ (or more) to the absorber solution to precipitate S^{2-}. Connect the train, consisting of boiling flask air inlet, flask, condenser, gas washer, suction flask trap, and aspirator. Adjust suction so at least 1 air bubble/s enters the boiling flask. This air rate will carry HCN gas from flask to absorber and usually will prevent a reverse flow of HCN through the air inlet. If this air rate does not prevent sample backup in the delivery tube, increase airflow rate to 2 air bubbles/s. Observe air purge rate in the absorber, where the liquid level should not be raised more than 6.5–10 mm. Maintain airflow throughout the reaction.

b. Add 2 g sulfamic acid through the air inlet tube and wash down with reagent water.

c. Add 50 mL 1 + 1 H_2SO_4 through the air inlet tube. Rinse tube with reagent water and let air mix flask contents for 3 min. Optionally, add 20 mL $MgCl_2$ reagent through air inlet and wash down with stream of reagent water. A precipitate may form that redissolves when heated.

d. Heat with rapid boiling, but do not flood condenser inlet or permit vapors to rise more than halfway into condenser. Reflux for at least 1 h at a rate of 40 to 50 drops/min from the condenser. Discontinue heating but continue airflow for 15 min. Cool and quantitatively transfer absorption solution to a 250-mL volumetric flask. Rinse absorber and its connecting tubing sparingly with reagent water and add to flask. Dilute to volume with reagent water and mix thoroughly.

e. Determine cyanide concentration in the absorption solution using 4500-CN^-.D, E, or F.

f. Distillation yields quantitative recovery of refractory cyanides, such as iron complexes. To obtain complete recovery of cobalticyanide, use UV radiation pretreatment.[2,3] If incomplete recovery is suspected, distill again by refilling the gas washer with a fresh charge of NaOH solution and refluxing 1 h more. The cyanide from the second reflux, if any, will indicate completeness of recovery.

g. As a QC measure, periodically (daily) test apparatus, reagents, and other potential variables in the concentration range of interest by distilling a laboratory QC sample containing a known amount of cyanide. Distillation of a complex cyanide, such as potassium ferricyanide, is preferred over the simple sodium or potassium cyanide.

5. References

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4500-CN^- D. Titrimetric Method

1. General Discussion

Principle: CN^- in the alkaline distillate from the preliminary treatment procedure is titrated with standard silver nitrate ($AgNO_3$) to form the soluble cyanide complex $Ag(CN)_2^-$. As soon as all CN^- has been complexed and a small excess of Ag^+ has been added, the silver-sensitive indicator, *p*-dimethylaminobenzalrhodanine, detects the excess Ag^+ and immediately changes color from yellow to salmon.[1] The distillation has provided a 2:1 concentration. The indicator is sensitive to about 0.1 mg Ag/L. If titration shows that CN^- is <1 mg/L, examine another portion colorimetrically or potentiometrically.

2. Apparatus

Koch microburet, 10-mL capacity.

3. Reagents

a. Indicator solution: Dissolve 20 mg *p*-dimethylaminobenzalrhodanine in 100 mL acetone.

b. Standard silver nitrate titrant: Dissolve 3.27 g $AgNO_3$ in 1 L reagent water. Standardize against standard NaCl solution, using the argentometric method with K_2CrO_4 indicator, as directed in Section 4500-Cl^-.B.

Dilute 500 mL $AgNO_3$ solution according to the titer found so 1.00 mL is equivalent to 1.00 mg CN^-.

c. Sodium hydroxide dilution solution: Dissolve 1.6 g NaOH in 1 L reagent water.

4. Procedure

a. Take a measured volume of sample from the absorption solution so the titration will require approximately 1 to 10 mL $AgNO_3$ titrant. Dilute to 100 mL (or some other convenient volume to be used for all titrations) using the NaOH dilution

solution. Do not dilute samples containing ≤5 mg/L cyanide. Add 0.5 mL indicator solution.

b. Titrate with standard $AgNO_3$ titrant to the first change in color from canary yellow to salmon. Titrate a blank containing the same amount of alkali and reagent water (e.g., 100 mL NaOH dilution solution or actual volume used for the sample). As the analyst becomes accustomed to the endpoint, blank titrations decrease from the high values usually experienced in the first few trials to 1 drop or less, with a corresponding improvement in precision.

5. Calculation

$$\text{mg } CN^-/L = \frac{(A - B) \times 1000}{\text{mL original sample}} \times 250/\text{mL portion used}$$

where:

A = mL standard $AgNO_3$ for sample and
B = mL standard $AgNO_3$ for blank.

6. Precision and Bias[2]

Based on the results of six operators in three laboratories, the overall and single-operator precision of this method within its designated range may be expressed as follows:

Reagent water: $S_T = 0.04x + 0.038$

$S_o = 0.01x + 0.018$

Selected water matrices: $S_T = 0.06x + 0.711$

$S_o = 0.04x + 0.027$

where:

S_T = overall precision, mg/L,
S_o = single-operator precision, mg/L, and
x = cyanide concentration, mg/L.

Recoveries of known amounts of cyanide from reagent water and selected water matrices are:

Medium	Added *mg/L*	Recovered *mg/L*	*n*	S_r	Bias	% Bias
Reagent water	2.00	2.10	18	0.1267	0.10	5
	5.00	4.65	18	0.2199	–0.35	–7
	5.00	5.18	18	0.2612	0.18	4
Selected water matrices	2.00	2.80	18	0.8695	0.80	40
	5.00	5.29	18	1.1160	0.29	6
	5.00	5.75	18	0.9970	0.75	15

7. References

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4500-CN⁻ E. Colorimetric Method

1. General Discussion

a. Principle: CN^- in the alkaline distillate from preliminary treatment is converted to CNCl by reaction with chloramine-T at pH <8 without hydrolyzing to CNO^-.[1] (**Caution: CNCl is a toxic gas; avoid inhalation.**) After the reaction is complete, adding a pyridine-barbituric acid reagent turns CNCl a red-blue color. Maximum color absorbance in aqueous solution is between 575 and 582 nm. To obtain colors of comparable intensity, have the same salt content in sample and standards.

b. Interference: All known interferences are eliminated or minimized via distillation.

c. Quality control: The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Colorimetric equipment: One of the following is required:

a. Spectrophotometer, for use at 578 nm, providing a light path of at least 10 mm.

b. Filter photometer, providing a light path of at least 10 mm and equipped with a red filter whose maximum transmittance is 570 to 580 nm.

3. Reagents

a. Chloramine-T solution: Dissolve 1.0 g white, water-soluble powder in 100 mL reagent water. Prepare fresh daily.

b. Stock cyanide solution: Dissolve approximately 1.6 g NaOH and 2.51 g KCN in 1 L reagent water. (**Caution: KCN is highly toxic; avoid contact or inhalation.**) Standardize against standard silver nitrate ($AgNO_3$) titrant as described in 4500-CN^- D.4, using 25 mL KCN solution. Check titer weekly because the solution gradually loses strength; 1 mL = 1 mg CN^-.

c. Standard cyanide solution: Based on the concentration determined for the KCN stock solution (¶ *b* above), calculate volume required (approximately 10 mL) to prepare 1 L of a 10 μg CN^-/ mL solution. Dilute with the NaOH dilution solution. Dilute 10 mL of the 10 μg CN^-/mL solution to 100 mL with the NaOH dilution solution; 1.0 mL = 1.0 μg CN^-. Prepare fresh daily and keep in a glass-stoppered bottle. (**Caution: Toxic; take care to avoid ingestion.**)

d. Pyridine-barbituric acid reagent: Place 15 g barbituric acid in a 250-mL volumetric flask and add just enough reagent water to wash sides of flask and wet barbituric acid. Add 75 mL pyridine and mix. Add 15 mL conc hydrochloric acid (HCl), mix, and cool to room temperature. Dilute to volume and mix until barbituric acid is dissolved. The solution is stable for

approximately 6 months if stored in an amber bottle under refrigeration; discard if precipitate develops.

e. Acetate buffer: Dissolve 410 g sodium acetate trihydrate ($NaC_2H_3O_2 \cdot 3H_2O$) in 500 mL reagent water. Add glacial acetic acid to adjust to pH 4.5, approximately 500 mL.

*f. Sodium hydroxide dilution solution (0.04*N*):* Dissolve 1.6 g NaOH in 1 L reagent water.

4. Procedure

a. Preparation of standard curve: Pipet a series of standards containing 1 to 10 μg CN^- into 50-mL volumetric flasks (0.02 to 0.2 μg CN^-/mL). Dilute to 40 mL with NaOH dilution solution. Use 40 mL of NaOH dilution solution as blank. Develop and measure absorbance in 10-mm cells as described in ¶ *b* below for both standards and blank. For concentrations <0.02 μg CN^-/mL, use 100-mm cells.

Recheck calibration curve periodically and each time a new reagent is prepared.

b. Color development: Pipet a portion of absorption solution into a 50-mL volumetric flask and dilute to 40 mL with NaOH dilution solution. Add 1 mL acetate buffer and 2 mL chloramine-T solution, stopper, and mix by inversion twice. Let stand exactly 2 min.

Add 5 mL pyridine-barbituric acid reagent, dilute to volume with reagent water, mix thoroughly, and let stand exactly 8 min. Measure absorbance against reagent water at 578 nm.

Measure absorbance of blank (0.0 mg CN^-/L) using 40 mL NaOH dilution solution and procedures for color development.

5. Calculation

Use the linear regression feature available on most scientific calculators, or compute slope and intercept of standard curve as follows:

$$m = \frac{n\sum ca - \sum c \sum a}{n\sum a^2 - \left(\sum a\right)^2}$$

$$b = \frac{\sum a^2 \sum c - \sum a \sum ac}{n\sum a^2 - \left(\sum a\right)^2}$$

where:

m = slope of standard curve,
n = number of standard solutions,
c = concentration of CN^- in standard, mg/L,
a = absorbance of standard solution, and
b = intercept on c axis.

Include the blank concentration, 0.0 mg CN^-/L, and blank absorbance in the calculations above.

$$CN^-, \text{mg/L} = (ma_1 + b) \times \frac{50}{X} \times \frac{250}{Y}$$

where:

a_1 = absorbance of sample solution,
X = absorption solution, mL, and
Y = original sample, mL.

6. Precision and Bias[2]

Based on the results of nine operators in nine laboratories, the overall and single-operator precision of this method within its designated ranges may be expressed as follows:

Reagent water: $S_T = 0.06x + 0.003$
$S_o = 0.11x + 0.010$

Selected water matrices: $S_T = 0.04x + 0.018$
$S_o = 0.04x + 0.008$

where:

S_T = overall precision, mg/L,
S_o = single-operator precision, mg/L, and
x = cyanide concentration, mg/L.

Recoveries of known amounts of cyanide from reagent water and selected water matrices (coke plant and refinery wastes, sewage, and surface water) are:

Medium	Added mg/L	Recovered mg/L	n	S_T	Bias	% Bias
Reagent water	0.060	0.060	26	0.0101	0.000	0
	0.500	0.480	23	0.0258	–0.020	–4
	0.900	0.996	27	0.0669	0.096	11
Selected water matrices	0.060	0.060	25	0.0145	0.000	0
	0.500	0.489	26	0.0501	–0.011	–3
	0.900	0.959	24	0.0509	0.059	7

7. References

1. Amus, E. & H. Garschagen. 1953. Über die Verwendung der Barbitsäure für die photometrische Bestimmund von Cyanid und Rhodanid. *Z. Anal. Chem.* 138:414.
2. American Society for Testing & Materials. 1987. Standard Test Methods for Cyanides in Water; Research Rep. D2036:19-1131. Philadelphia, Pa.

4500-CN⁻ F. Cyanide-Ion Selective Electrode Method

1. General Discussion

CN^- in the alkaline distillate from the preliminary treatment procedures can be determined potentiometrically by using a CN^--ion selective electrode (ISE), either a combination electrode or a half-cell CN^- ISE with a double-junction reference electrode, and a pH/ISE meter (concentration mode) or pH meter, expanded-scale or digital. This method can be used in place of either the colorimetric or titrimetric procedures to determine CN^- concentrations between 0.05 and 10 mg CN^-/L.[1–3] If the CN^- ISE is used, the previously described titration screening step can be omitted.

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. pH/ISE meter (concentration mode) or pH meter, expanded-scale or digital, capable of 0.1 mV resolution (when not in concentration mode).

b. Cyanide ISE, combination electrode or half-cell electrode with double-junction reference half-cell electrode:* Carefully follow manufacturer's instructions on care and storage.

c. Magnetic or motorized mixer with TFE-coated stirring bar or other inert material.

d. Koch microburet, 10-mL capacity.

3. Reagents

a. Stock standard cyanide solution: See 4500-CN^-.E.3*b*.

*b. Sodium hydroxide dilution solution (0.04*N*):* Dissolve 1.6 g NaOH in reagent water and dilute to 1 L.

c. Standard cyanide solution: Dilute a calculated volume (approximately 25 mL) of stock KCN solution, based on the determined concentration, to 1000 mL with NaOH diluent. Mix thoroughly; 1 mL = 25 μg CN^-.

d. Dilute standard cyanide solution: Dilute 100.0 mL standard CN^- solution to 1000 mL with NaOH diluent; 1.00 mL = 2.5 μg CN^-. Prepare daily and keep in a dark, glass-stoppered bottle.

e. Potassium nitrate solution: Dissolve 100 g KNO_3 in reagent water and dilute to 1 L. Adjust to pH 12 with KOH. This is the outer filling solution for the double-junction reference elecrode.

f. CN^- ISE filling solution for combination electrode: Use the filling solution recommended by the ISE manufacturer.

4. Procedure

Always progress from the lowest to the highest concentration of standard in order to reach equilibrium promptly. The electrode membrane dissolves in concentrated CN^- solutions; do not use when concentration is >25 mg CN^-/L. After taking measurements, remove electrode and soak in reagent water.

a. Calibration: Using Koch microburet and standard CN^- solution, prepare four (or more) additional solutions containing 2.5, 0.25, 0.125, and 0.025 mg CN^-/L in NaOH dilution solution. Transfer approximately 100 mL of each standard solution into a 250-mL beaker pre-rinsed with a small portion of standard being tested. Calibrate in decades using the 0.025, 0.25, and 2.5 mg/L standards. Use the 0.125 mg/L standard as the calibration verification, after the calibration is completed. Immerse CN^- combination ISE (or two half cells) about 1 in. into each solution. Mix well using the stirrer (magnetic or motorized) at 25°C, maintaining the same stirring rate as closely as possible for all solutions.

b. Calibration on an ISE or pH/ISE meter: Follow manufacturer's instructions to calibrate the meter in concentration mode. Set units to mg/L. Perform three-point calibration using the 0.025, 0.25, and 2.5 mg/L standards. The ideal slope should be between −54 and −60 mV/decade. Verify calibration by measuring the concentration of the 0.125 mg/L standard. Ideally, the reading will be within ±10% of the expected value.

c. Calibration on a pH meter: Perform a three-point calibration using the 0.025, 0.25, and 2.5 mg/L standards. Record the millivolt reading when stable (when reading's rate of change is <0.5 mV/min). Remove electrode(s), rinse, and blot dry. Repeat for all three standards. For millivolt readings, use electronic spreadsheet to plot the common logarithm (base 10) of CN^- concentration on the x-axis (abscissa) and potential (in mV) on the y-axis (ordinate). A straight line with a slope of −57 ± 3 mV/decade at 25°C is expected. If slope is outside the acceptable range, follow the electrode manufacturer's troubleshooting procedure. Verify calibration by measuring the concentration of the 0.125 mg/L standard. Ideally, the reading will be within ±10% of the expected value.

d. Sample measurement: Place 100 mL of absorption liquid obtained in 4500-CN^-.C.4*d* (or an accurately measured portion diluted to 100 mL with NaOH dilution solution) into a 250-mL beaker. When measuring low CN^- concentrations, first rinse beaker and electrodes with a small volume of sample. Immerse the CN^- combination ISE (or two half-cells) about 1 in. into each solution. Mix continuously using the stirrer (magnetic or motorized) at the same stirring rate used for calibration. After equilibrium is reached (≥5 but ≤10 min), record values indicated on ion meter or found from graph prepared as above. Calculate concentration as directed below.

5. Calculation

$$\text{mg } CN^-/L = \mu g\ CN^-/mL \text{ from graph or meter} \times \frac{100}{x} \times \frac{250}{y}$$

where:

x = volume of absorption solution, mL, and
y = volume of original sample, mL.

* Orion Model 94-06A, or equivalent.

6. Precision and Bias[4]

In collaborative testing, this method's precision has been found to be linear within its designated range.

Based on the results of six operators in five laboratories, the overall and single-operator precision of this method within its designated range may be expressed as follows:

$$\text{Reagent water: } S_T = 0.06x + 0.003$$
$$S_o = 0.03x + 0.008$$

$$\text{Selected water matrices: } S_T = 0.05x + 0.008$$
$$S_o = 0.03x + 0.012$$

where:

S_T = overall precision, mg/L,
S_o = single-operator precision, mg/L, and
x = cyanide concentration, mg/L.

Recoveries of known amounts of cyanide from reagent water and selected water matrices are:

Medium	Added *mg/L*	Recovered *mg/L*	*n*	S_T	Bias	% Bias
Reagent water	0.060	0.059	18	0.0086	–0.001	2
	0.500	0.459	18	0.0281	–0.041	–8
	0.900	0.911	18	0.0552	0.011	1
	5.00	5.07	18	0.297	0.07	1
Selected water matrices	0.060	0.058	14	0.0071	–0.002	–3
	0.500	0.468	21	0.0414	–0.032	–6
	0.900	0.922	19	0.0532	0.022	2
	5.00	5.13	20	0.2839	0.13	3

7. References

1. Orion Research, Inc. 1975. Cyanide Ion Electrode Instruction Manual. Cambridge, Mass.
2. Frant, M.S., J.W. Ross & J.H. Riseman. 1972. An electrode indicator technique for measuring low levels of cyanide. *Anal. Chem.* 44:2227.
3. Sekerka, J. & J.F. Lechner. 1976. Potentiometric determination of low levels of simple and total cyanides. *Water Res.* 10:479.
4. American Society for Testing & Materials. 1987. Standard Test Methods for Cyanides in Water; Research Rep. D2036:19-1131. Philadelphia, Pa.

4500-CN⁻ G. Cyanides Amenable to Chlorination after Distillation

1. General Discussion

This method applies to cyanides that are amenable to chlorination.

After part of the sample is chlorinated to decompose cyanides, both the chlorinated and the untreated sample are subjected to distillation, as described in 4500-CN⁻.C. The difference between the CN^- concentrations found in the two samples is expressed as cyanides amenable to chlorination.

Some unidentified organic chemicals may oxidize or form breakdown products during chlorination, making cyanide results higher after chlorination than before chlorination. This may lead to a negative value for cyanides amenable to chlorination after distillation for wastes from, for example, the steel industry, petroleum refining, and pulp and paper processing. Where such interferences are encountered, use 4500-CN⁻.I to determine dissociable cyanide.

Protect the sample from exposure to ultraviolet (UV) radiation by performing manipulations under incandescent light to prevent photodecomposition of some metal-cyanide complexes by UV light.

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Distillation apparatus: See 4500-CN⁻.C.2

b. Apparatus for determining cyanide by either the titrimetric method (4500-CN⁻.D.2), the colorimetric method (4500-CN⁻.E.2), or the electrode method (4500-CN⁻.F.2).

3. Reagents

a. All reagents listed in 4500-CN⁻.C.3.

b. All reagents listed in 4500-CN⁻.D.3, E.3, or F.3, depending on estimation method.

c. Calcium hypochlorite solution: Dissolve 5 g $Ca(OCl)_2$ in 100 mL reagent water. Store in an amber-colored glass bottle in the dark. Prepare monthly.

d. Potassium iodide (KI)-starch test paper.

4. Procedure

a. Divide sample into two equal portions of 500 mL (or equal portions diluted to 500 mL) and chlorinate one as in ¶ *b* below. Analyze both portions for CN^-. The difference in determined concentrations is the cyanide amenable to chlorination.

b. Place one portion in a 1-L beaker covered with aluminum foil or black paper. Keep beaker covered with a wrapped watch glass during chlorination. Add $Ca(OCl)_2$ solution dropwise to sample while agitating and maintain pH between 11 and 12 by adding NaOH solution. Test for chlorine by placing a drop of treated sample on a strip of KI-starch paper. A distinct blue color indicates sufficient chlorine (approximately 50 to 100 mg Cl_2/L). Maintain excess residual chlorine for 1 h while agitating; check every 15 min to ensure chlorine residual remains and, if necessary, add more $Ca(OCl)_2$.

c. After 1 h, remove any residual chlorine by dropwise addition of $NaAsO_2$ solution (2 g/100 mL) or by addition of 8 drops H_2O_2 (3%) followed by 4 drops $Na_2S_2O_3$ solution (500 g/L). Test with KI-starch paper until there is no color change.

d. Distill both chlorinated and unchlorinated samples as in 4500-CN⁻.C. Test according to 4500-CN⁻.D, E, or F.

5. Calculation

$$\text{mg CN}^- \text{ amenable to chlorination/L} = G - H$$

where:

G = mg CN⁻/L found in unchlorinated portion of sample, and
H = mg CN⁻/L found in chlorinated portion of sample.

Samples containing significant quantities of iron cyanides or other interferences may produce a second distillation (solution H) whose CN⁻ value is higher than the total cyanide value, leading to a negative result. If the difference is within the method's precision limit, report "no determinable quantities of cyanide amenable to chlorination." If the difference exceeds the precision limit, ascertain the cause (e.g., presence of interferences, manipulation of procedure, etc.) or use 4500-CN⁻.I.

6. Precision and Bias[1]

The precision and bias information given in this section may not apply to waters whose matrix type was not evaluated in this study.

a. Precision:

1) Colorimetric—Based on the results of eight operators in seven laboratories, the overall and single-operator precision of this method within its designated range may be expressed as follows:

$$\text{Reagent water: } S_T = 0.18x + 0.005$$
$$S_o = 0.06x + 0.003$$

$$\text{Selected water matrices: } S_T = 0.20x + 0.009$$
$$S_o = 0.05x + 0.005$$

where:

S_T = overall precision, mg/L,
S_o = single-operator precision, mg/L, and
x = cyanide concentration, mg CN⁻L.

2) Titrimetric—Based on the results of six operators in three laboratories, the overall and single-operator precision of this method within its designated range may be expressed as follows:

$$\text{Reagent water: } S_T = 0.01x + 0.439$$
$$S_o = 0.241 - 0.03x$$

$$\text{Selected water matrices: } S_T = 0.12x + 0.378$$
$$S_o = 0.209 - 0.01x$$

b. Bias: Recoveries of known amount of cyanide amenable to chlorination from reagent water and selected water matrices are shown below:

Medium	Technique	Added mg/L	Recovered mg/L	n	S_T	Bias	% Bias
Reagent water	Colorimetricc	0.008	0.009	21	0.0033	0.001	13
		0.019	0.023	20	0.0070	0.004	21
		0.080	0.103	20	0.0304	0.018	23
		0.191	0.228	21	0.0428	0.037	19
	Titrimetric	1.00	0.73	18	0.350	–0.27	–27
		1.00	0.81	18	0.551	–0.19	–19
		4.00	3.29	18	0.477	–0.71	–18
Selected water matrices	Colorimetric	0.008	0.013	17	0.0077	0.005	63
		0.019	0.025	18	0.0121	0.006	32
		0.080	0.100	18	0.0372	0.020	25
		0.191	0.229	18	0.0503	0.038	20
	Titrimetric	1.00	1.20	18	0.703	0.20	20
		1.00	1.10	18	0.328	0.10	10
		4.00	3.83	18	0.818	–0.17	–4

7. Reference

1. American Society for Testing & Materials. 1987. Standard Test Methods for Cyanides in Water; Research Rep. D2036:19-1131. Philadelphia, Pa.

4500-CN⁻ H. Cyanides Amenable to Chlorination without Distillation (Short-Cut Method)

1. General Discussion

This method can be used to determine HCN, CN complexes that are amenable to chlorination, and thiocyanates (SCN⁻). It does not measure cyanates (OCN⁻) or iron cyanide complexes, but does determine cyanogen chloride (CNCl). It does not require lengthy distillation or the chlorination of one sample before distillation. The recovery of CN⁻ from metal cyanide complexes will be comparable to that in 4500-CN⁻.G and I.

The cyanides are converted to CNCl by chloramine-T after the sample has been heated. In the absence of nickel, copper, silver, and gold cyanide complexes or SCN⁻, the CNCl may be developed at room temperature. The pyridine-barbituric acid reagent produces a red-blue color in the sample that can be estimated visually against standards or photometrically at 578 nm. The dissolved salt content in the standards used to develop the calibration curve should be near the sample's salt content, including the added NaOH and phosphate buffer.

The method's usefulness is limited by thiocyanate interference. So long as the ratio of SCN^- to CN^- is ≤3, the method can mask the CN^- content, establish a correction for the SCN^-content, and thereby allow the specific determination of CN^- amenable to chlorination (see 4500-CN⁻.H.2 and 5). When working with unknown samples, screen them for SCN^- via the spot test (4500-CN⁻.K).

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Interferences

a. Remove interfering agents as described in 4500-CN⁻.B.3, except NO_2- and NO_3-, which should be removed as described in 4500-CN⁻.B.3*h*.

b. The SCN^- ion reacts with chloramine-T to give a positive error equivalent to its concentration. Use the spot test (4500-CN⁻.K) for SCN^- when its presence is suspected. If the SCN^- content is more than three times the CN^- content, use 4500-CN⁻.G or I.

c. Reducing chemical compounds, such as SO_3^{2-}, may interfere by consuming chlorine in the chloramine-T addition. A significant excess of chlorine is provided, but the procedure prescribes a test (4500-CN⁻.G.4*b*) to avoid this interference.

d. Color and turbidity may interfere with the colorimetric determination. Overcome this interference via chloroform extraction (4500-CN⁻.B.3*c*) but omit pH reduction. Otherwise, use 4500-CN⁻.G or I.

Analysts also may compensate for color and turbidity by determining absorbance of a second sample solution to which all reagents except chloramine-T have been added.

e. Color intensity and absorption are affected by wide variations in the sample's total dissolved solids content. If samples contain 3000 to 10 000 mg/L of dissolved solids, add 6 g NaCl/L NaOH solution (1.6 g/L) used to prepare standards. If samples contain >10 000 mg/L of dissolved solids, add sufficient NaCl to the NaOH solution to approximate the dissolved solids content.

3. Apparatus

a. Apparatus listed in 4500-CN⁻.E.2.
b. Hot water bath.

4. Reagents

a. Reagents listed in 4500-CN⁻.B and E.3.
b. Sodium chloride (NaCl) crystals.
c. Sodium carbonate (Na_2CO_3) crystals.
d. Sulfuric acid solution (H_2SO_4), 1*N*.
e. EDTA solution, 0.05*M:* Dissolve 18.5 g disodium salt of ethylenediamine tetraacetic acid in reagent water and dilute to 1 L.
f. Formaldehyde solution, 10%: Dilute 27 mL formaldehyde (37% pharmaceutical grade) to 100 mL.
g. Phosphate buffer: Dissolve 138 g sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) in reagent water and dilute to 1 L. Refrigerate.

5. Procedure

a. Calibrate as directed in 4500-CN⁻.E.1*a* and 4*a*. For samples with >3000 mg/L total dissolved solids, prepare a calibration curve from standards and blank NaOH solutions containing 6 g NaCl/L. Samples containing >10 000 mg/L total dissolved solids require appropriate standards and a new calibration curve.

b. Adjust pH of 50 mL sample to between 11.4 and 11.8. To lower pH, add 0.2 to 0.4 g of sodium carbonate and stir to dissolve, then add HCl solution (1 + 9) dropwise while stirring. To raise pH, use a 1 *N* NaOH solution (40 g/L).

c. Pipet 20.0 mL of adjusted sample into a 50-mL volumetric flask. If the cyanide concentration is >0.3 mg/L, use a smaller portion and dilute to 20 mL with NaOH solution. Do not exceed the concentration limit of 0.3 mg/L.

d. To ensure uniform color development in both calibration and testing, maintain a uniform temperature. Immerse flasks in a water bath held at 27 ± 1°C for 10 min before adding reagents, and keep samples in water bath until all reagents have been added.

Add 4 mL phosphate buffer and swirl to mix. Add one drop of EDTA solution and mix.

e. Add 2 mL chloramine-T solution and swirl to mix. Place 1 drop of sample on potassium iodide-starch test paper already moistened with acetate buffer solution. Repeat the chloramine-T addition if required. After exactly 3 min, add 5 mL pyridine-barbituric acid reagent and swirl to mix.

f. Remove samples from water bath, dilute to volume, and mix. Allow 8 min from the addition of the pyridine-barbituric acid reagent for color development.

Determine absorbance at 578 nm in a 1.0-cm cell versus reagent water.

Calculate concentration of cyanide (mg/L in the original sample) following instructions given in 4500-CN⁻.E.5.

g. If the presence of thiocyanate is suspected, pipet a second 20-mL portion of pH-adjusted sample into a 50-mL volumetric flask. Add 3 drops 10% formaldehyde solution. Mix and let stand 10 min. Place in a water bath at 27 ± 1°C for another 10 min before adding reagent chemicals, and hold in the bath until all reagents have been added.

Continue with ¶ *b* above.

Calculate the cyanide concentration (mg/L in the original sample) following instructions given in 4500-CN⁻.E.5.

h. In the presence of thiocyanate, cyanide amenable to chlorination is equal to the difference between the cyanide concentrations obtained in ¶s *f* and *g* above.

6. Calculation

See 4500-CN⁻.E.5.

Deduct SCN^- value from the results found when CN^- was not masked by formaldehyde addition (total) for cyanide content.

7. Precision and Bias[1]

This precision and bias information may not apply to waters of untested matrices.

a. Precision: Based on the results of 14 operators in nine laboratories, the overall and single-operator precision of this test method within its designated range may be expressed as follows:

Reagent water: $S_T = 0.10x + 0.006$

$S_o = 0.07x + 0.005$

Selected water matrices: $S_T = 0.11x + 0.007$

$S_o = 0.02x + 0.005$

where:

S_T = overall precision, mg/L,
S_o = single-operator precision, mg/L, and
x = cyanide concentration, mg/L.

b. Bias: Recoveries of known amounts of cyanide from reagent water and selected water matrices, including creek waters, diluted sewage (1 to 20), and industrial wastewater, are shown below.

Medium	Added mg/L CN^-	Added mg/L SCN^-	Recovered mg/L	n	S_T	Bias	% Bias
Reagent water	0.005		0.007	42	0.0049	0.002	40
	0.027		0.036	41	0.0109	0.009	25
	0.09		0.1	42	0.0167	0.01	11
	0.09	0.08	0.08	39	0.0121	–0.010	11
	0.27		0.276	42	0.032	0.006	2
Selected water matrices	0.005		0.003	40	0.0042	–0.002	40
	0.027		0.026	42	0.0093	–0.001	4
	0.09		0.087	42	0.0202	–0.003	3
	0.09	0.08	0.068	37	0.0146	–0.022	24
	0.27		0.245	41	0.0319	–0.025	9

8. Reference

1. American Society for Testing & Materials. 1987. Standard Test Methods for Cyanides in Water; Research Rep. D2036:19-1074. Philadelphia, Pa.

4500-CN⁻ I. Weak Acid Dissociable Cyanide

1. General Discussion

Weak acid dissociable cyanides are sometimes called weak and dissociable, or available cyanide.[1,2]

In this method, hydrogen cyanide (HCN) is liberated from a slightly acidified (pH 4.5 to 6.0) sample under prescribed distillation conditions. The method does not recover CN^- from strong metal cyanide, such as iron cyanide complexes, that would not be amenable to oxidation by chlorine. The acetate buffer used contains zinc salts to precipitate iron cyanide as a further assurance of the method's selectivity; however, a 10-fold excess of iron cyanide concentration may still interfere. In other respects, the method is similar to 4500-CN⁻.C.

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Interferences

See 4500-CN⁻.B.3.

Protect sample and apparatus from UV light to prevent photodecomposition of some metal-cyanide complexes and an increase in the concentration of weak acid dissociable cyanide.

If procedure is used to determine low concentrations of cyanide in samples of ferri- and ferrocyanide, add more (e.g., fivefold excess) zinc acetate solution before adding acid and distilling. Check for interferences by spiking with ferri- and/or ferrocyanide.

3. Apparatus

See 4500-CN⁻.C.2 and Figure 4500-CN⁻:1, and also 4500-CN⁻.D.2, E.2, or F.2, depending on estimation method.

4. Reagents

a. Reagents listed in 4500-CN⁻.C.3.

b. Reagents listed in 4500-CN⁻.D.3, E.3, or F.3, depending on estimation method.

c. Acetic acid, 1 + 9: Mix 1 volume of glacial acetic acid with 9 volumes of reagent water.

d. Acetate buffer: Dissolve 410 g sodium acetate trihydrate ($NaC_2H_3O_2 \cdot 3H_2O$) in 500 mL reagent water. Add glacial acetic acid to yield a solution pH of 4.5 (approximately 500 mL).

e. Zinc acetate solution, 100 g/L: Dissolve 120 g $Zn(C_2H_3O_2)_2 \cdot 2H_2O$ in 500 mL reagent water. Dilute to 1 L.

f. Methyl red indicator.

5. Procedure

Follow procedure described in 4500-CN⁻.C.4, but with the following modifications:

a. Do not add sulfamic acid, because NO_2^- and NO_3^- do not interfere.

b. Instead of H_2SO_4 and $MgCl_2$ reagents, add 20 mL each of acetate buffer and zinc acetate solutions through air inlet tube. Also, add 2 to 3 drops methyl red indicator. Rinse air inlet tube with water and let air mix contents. If solution is not pink, add acetic acid (1 + 9) dropwise through air inlet tube until a pink color persists.

c. Follow instructions, beginning with 4500-CN⁻.C.4*d*.

d. To determine CN^- in the absorption solution, use the preferred finish method (4500-CN⁻.D, E, or F).

6. Precision and Bias[3]

The precision and bias information given in this section may not apply to waters of untested matrices.

a. Precision:

1) Colorimetric—Based on the results of nine operators in nine laboratories, the overall and single-operator precision of this test method within its designated range may be expressed as follows:

$$\text{Reagent water: } S_T = 0.09x + 0.010$$
$$S_o = 0.08x + 0.005$$
$$\text{Selected water matrices: } S_T = 0.08x + 0.012$$
$$S_o = 0.05x + 0.008$$

2) Electrode—Based on the results of six operators in five laboratories, the overall and single-operator precision of this test method within its designated range may be expressed as follows:

$$\text{Reagent water: } S_T = 0.09x + 0.004$$
$$S_o = 0.02x - 0.009$$
$$\text{Selected water matrices: } S_T = 0.08x + 0.005$$
$$S_o = 0.02x + 0.004$$

3) Titrimetric—Based on the results of six operators in three laboratories, the overall and single-operator precision of this test method within its designated range may be expressed as follows:

$$\text{Reagent water: } S_T = 0.532 - 0.10x$$
$$S_o = 0.151 - 0.01x$$
$$\text{Selected water matrices: } S_T = 0.604 - 0.06x$$
$$S_o = 0.092 + 0.02x$$

where:

S_T = overall precision,
S_o = single-operator precision, and
x = cyanide concentration, mg/L.

b. Bias: Recoveries of known amounts of cyanide from reagent water and selected water matrices are shown below.

Medium	Technique	Added mg/L	Recovered mg/L	n	S_T	Bias	% Bias
Reagent water	Colorimetric	0.030	0.030	25	0.0089	0.000	0
		0.100	0.117	27	0.0251	0.017	17
		0.400	0.361	27	0.0400	–0.039	–10
	Electrode	0.030	0.030	21	0.0059	0.000	0
		0.100	0.095	21	0.0163	–0.005	–5
		0.400	0.365	21	0.0316	–0.035	–9
		1.000	0.940	21	0.0903	–0.060	–6
	Titrimetric	1.00	1.35	18	0.4348	0.35	35
		1.00	1.38	18	0.3688	0.38	38
		4.00	3.67	18	0.1830	–0.33	–8
Selected water matrices	Colorimetric	0.030	0.029	15	0.0062	0.001	3
		0.100	0.118	24	0.0312	0.018	18
		0.400	0.381	23	0.0389	–0.019	–5
	Electrode	0.030	0.029	20	0.0048	–0.001	–3
		0.100	0.104	21	0.0125	0.004	4
		0.400	0.357	21	0.0372	–0.043	–11
		1.000	0.935	21	0.0739	–0.065	–7
	Titrimetric	1.00	1.55	18	0.5466	0.55	55
		1.00	1.53	18	0.4625	0.53	53
		4.00	3.90	18	0.3513	–0.10	–3

7. References

1. American Society for Testing & Materials. 2015. Standard Guide for Understanding Cyanide Species; ASTM D6696-15. W. Conshohocken, Pa.
2. U.S. Environmental Protection Agency. 2012. Table 1B—List of approved inorganic test procedures. *In* Guidelines Establishing Test Procedures for the Analysis of Pollutants; 40 CFR Part 136.3. Washington, D.C.
3. American Society for Testing & Materials. 1987. Standard Test Methods for Cyanides in Water; Research Rep. D2036:19-1131. Philadelphia, Pa.

4500-CN⁻ J. Cyanogen Chloride

1. General Discussion

Cyanogen chloride (CNCl) is the first reaction product when cyanide compounds are chlorinated. It is a volatile gas, only slightly soluble in water, but highly toxic even in low concentrations. (**Caution: Avoid inhalation or contact.**) A mixed pyridine-barbituric acid reagent produces a red-blue color with CNCl.

Because CNCl hydrolyzes to cyanate (OCN^-) at a pH of 12 or more, collect a separate sample for CNCl analysis (see 4500-CN⁻.B.2) in a closed container without sodium hydroxide (NaOH). A quick test with a spot plate or comparator as soon as sample is collected may be the only procedure for avoiding CNCl hydrolysis due to time lapse between sampling and analysis.

If starch-iodide (KI) test paper indicates presence of chlorine or other oxidizing agents, add sodium thiosulfate ($Na_2S_2O_3$) immediately as directed in 4500-CN⁻.B.2.

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

See 4500-CN⁻.E.2.

3. Reagents

a. Reagents listed in 4500-CN⁻.E.3 and H.4.

b. Phosphate buffer: Dissolve 138 g sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) in reagent water and dilute to 1 L. Refrigerate.

4. Procedure

a. Preparation of standard curve: Pipet a series of standards containing 1 to 10 μg CN^- into 50-mL volumetric flasks (0.02 to 0.2 μg CN^-/mL). Dilute to 20 mL with NaOH dilution solution. Use 20 mL NaOH dilution solution for the blank. Add 2 mL chloramine-T solution and 4 mL phosphate buffer; stopper and mix by inversion two or three times. Add 5 mL pyridine-barbituric acid reagent, dilute to volume with reagent water, mix thoroughly, and let stand exactly 8 min for color development. Measure absorbance at 578 nm in a 10-mm cell, using reagent water as a reference. Calculate slope and intercept of the curve.

b. If sample pH is >8, reduce it to between 8.0 and 8.5 by careful addition of phosphate buffer. Measure 20-mL sample portion into 50-mL volumetric flask. If >0.20 mg CNCl-CN^-/L is present, use a smaller portion diluted to 20 mL with reagent water. Add 1 mL phosphate buffer, stopper and mix by *one* inversion. Let stand 2 min. Add 5 mL pyridine-barbituric acid reagent, stopper and mix by *one* inversion. Let color develop 3 min, dilute to volume with reagent water, mix thoroughly, and let stand another 5 min. Measure absorbance at 578 nm in 10-mm cell, using reagent water as a reference.

5. Calculation

Compute slope (m) and intercept (b) of standard curve as directed in 4500-CN^-.E.5*e*.

$$\text{Cyanogen chloride as } CN^-, \text{ mg/L} = (ma_1 + b) \times \frac{50}{\text{mL sample}}$$

where:

a_1 = absorbance of sample solution.

6. Precision[1]

Cyanogen chloride is unstable, and round-robin testing is not possible. Single-operator precision is as follows:

Six operators made 70 duplicate analyses on samples of different concentrations within the method's applicable range. The overall single-operator precision within its designated range may be expressed as follows:

$$\log S_o = (0.5308 \log c) - 1.9842$$

$$\log R = (0.5292 \log c) - 1.8436$$

where:

S_o = single-operator precision in the method's range (precision depends on concentration),
c = mg CNCl-CN^-/L, and
R = range between duplicate determinations.

The collaborative test data were obtained on reagent-grade water. These data may not apply to other matrices.

7. Reference

1. American Society for Testing & Materials. 1989. Standard Test Methods for Cyanogen Chloride in Water; Research Rep. D4165:19-1100. Philadelphia, Pa.

4500-CN^- K. Spot Test for Sample Screening

1. General Discussion

This method permits quick screening to establish whether >50 μg/L of cyanide amenable to chlorination is present. It also establishes the presence or absence of cyanogen chloride (CNCl). (**Caution: CNCl is a toxic gas; avoid inhalation.**) With practice and dilution, the test reveals the approximate concentration range of these compounds by the color development compared with that of similarly treated standards.

When chloramine-T is added to cyanides amenable to chlorination, CNCl is formed. CNCl forms a red-blue color when mixed with pyridine-barbituric acid. When testing for CNCl, omit the chloramine-T addition.

The presence of >0.5 mg/L formaldehyde interferes with the test. A spot test for aldehydes and a method for removing this interference are given in 4500-CN^-.B.3.

Thiocyanate (SCN^-) reacts with chloramine-T, thereby creating a positive interference. The CN^- can be masked with formaldehyde, and the sample retested. This makes the spot test specific for SCN^-. This enables analysts to determine whether the spot discoloration is due to CN^-, SCN^-, or both.

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Porcelain spot plate with 6 to 12 cavities.
b. Dropping pipets.
c. Glass stirring rods.

3. Reagents

a. Chloramine-T solution: See 4500-CN^-.E.3*a*.
b. Stock cyanide solution: See 4500-CN^-.E.3*b*.

c. Pyridine-barbituric acid reagent: See 4500-CN⁻.E.3*d*.
d. Hydrochloric acid (HCl), 1 + 9.
e. Phenolphthalein indicator aqueous solution.
f. Sodium carbonate (Na_2CO_3), anhydrous.
g. Formaldehyde, 37%, pharmaceutical grade.

4. Procedure

If the solution to be tested has a pH >10, neutralize a 20- to 25-mL portion. Add about 250 mg Na_2CO_3 and swirl to dissolve. Add 1 drop phenolphthalein indicator. Add 1 + 9 HCl dropwise with constant swirling until the solution becomes colorless. Place 3 drops sample and 3 drops reagent water (for blanks) in separate cavities of the spot plate. To each cavity, add 1 drop chloramine-T solution and mix with a clean stirring rod. Add 1 drop pyridine-barbituric acid solution to each cavity and mix again. After 1 min, the sample spot will turn pink to red if ≥50 μg/L of CN^- is present. The blank spot will be faint yellow because of the reagents' color. Until familiar with the spot test, use a standard solution containing 50 μg CN^-/L instead of the reagent water blank for color comparison. This standard can be made by diluting the stock cyanide solution.

If SCN^- is suspected, test a second sample pretreated as follows: Heat a 20- to 25-mL sample in a water bath at 50°C; add 0.1 mL formaldehyde and hold for 10 min. This treatment will mask up to 5 mg CN^-/L, if present. Repeat spot-testing procedure. Color development indicates presence of SCN^-. Comparing color intensity in the two spot tests is useful in judging relative concentration of CN^- and SCN^-. If deep coloration is produced, serial dilution of sample and additional testing may allow closer approximation of the concentrations.

4500-CN⁻ L. Cyanates

1. General Discussion

Cyanate (OCN^-) may be of interest in analyzing industrial waste samples because the alkaline chlorination process used to oxidize cyanide yields cyanate in the second reaction.

Cyanate is unstable at neutral or low pH, so stabilize the sample as soon as collected by adding sodium hydroxide (NaOH) to pH 12. Remove residual chlorine by adding sodium thiosulfate ($Na_2S_2O_3$) (see 4500-CN⁻.B.2).

a. Principle: Cyanate hydrolyzes to ammonia when heated at low pH.

$$2NaOCN + H_2SO_4 + 4H_2O \rightarrow (NH_4)_2SO_4 + 2NaHCO_3$$

The ammonia concentration must be determined on one sample portion before acidification. The ammonia content before and after cyanate hydrolysis may be measured by phenate (Section 4500-NH_3.F) or ammonia-selective electrode (Section 4500-NH_3.D) method.[1] The test is applicable to cyanate compounds in natural waters and industrial waste.

b. Interferences:
1) Organic nitrogenous compounds may hydrolyze to ammonia (NH_3) upon acidification. To minimize this interference, control acidification and heating closely.
2) Reduce oxidants that oxidize cyanate to carbon dioxide and nitrogen with $Na_2S_2O_3$.
3) Industrial waste containing organic material may contain unknown interferences.

c. Detection limit: 1 to 2 mg CNO^-/L.

d. Quality control: The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Expanded-scale pH meter or selective-ion meter.
*b. Ammonia-selective electrode.**
c. Magnetic mixer, with TFE-coated stirring bar.
d. Heat barrier: Use a 3-mm-thick insulator under beaker to insulate against heat produced by stirrer motor.

3. Reagents

a. Stock ammonium chloride solution: Dissolve 3.819 g anhydrous NH_4Cl (dried at 100°C) in reagent water and dilute to 1 L; 1.00 mL = 1.00 mg N = 1.22 mg NH_3.

b. Standard ammonium chloride solution: From the stock NH_4Cl solution, prepare standard solutions containing 1.0, 10.0, and 100.0 mg NH_3-N/L by diluting with ammonia-free reagent water.

c. Sodium hydroxide, 10N: Dissolve 400 g NaOH in reagent water and dilute to 1 L.

d. Sulfuric acid solution (H_2SO_4), 1 + 1.

e. Ammonium chloride solution: Dissolve 5.4 g NH_4Cl in reagent water and dilute to 1 L. (Use only for soaking electrodes.)

4. Procedure

a. Calibration: Daily, calibrate the ammonia electrode as in Section 4500-NH_3.D.4*b* using standard NH_4Cl solutions.

b. Treatment of sample: Dilute sample, if necessary, so the OCN^- concentration is 1 to 200 mg/L or NH_3-N is 0.5 to 100 mg/L. Take or prepare at least 200 mL. From this 200 mL, take a 100-mL portion and, following the calibration procedure, establish the potential (millivolts) developed from the sample. Check electrode reading with prepared standards and adjust instrument calibration setting daily. Record NH_3-N content of untreated sample (*B* in equation below).

Acidify 100 mL of prepared sample by adding 0.5 mL 1 + 1 H_2SO_4 to a pH of 2.0 to 2.5. Heat sample to 90–95°C and maintain temperature for 30 min. Cool to room temperature and

* Orion Model 95-10, EIL Model 8002-2, Beckman Model 39565, or equivalent.

restore to original volume by adding ammonia-free reagent water. Pour into a 150-mL beaker, immerse electrode, start magnetic stirrer, then add 1 mL 10*N* NaOH solution. With pH paper check that pH is >11. If necessary, add more NaOH until pH 11 is reached.

After equilibrium has been reached (30 s), record the potential reading. Determine the NH_3-N concentration from the calibration curve.

5. Calculations

$$\text{mg } NH_3\text{-N derived from } OCN^-/L = A - B$$

where:

A = mg NH_3-N/L found in the acidified and heated sample portion, and
B = mg NH_3-N/L found in untreated portion.

$$\text{mg } OCN^-/L = 3.0 \times (A - B)$$

6. Precision

No data on this method's precision are available. See Section 4500-NH_3.D.6 for precision of ammonia-selective electrode method.

7. Reference

1. Thomas, R.F. & R.L. Booth. 1973. Selective electrode determination of ammonia in water and wastes. *Environ. Sci. Technol.* 7:523.

4500-CN^- M. Thiocyanate

1. General Discussion

When wastewater containing thiocyanate (SCN^-) is chlorinated, highly toxic cyanogen chloride (CNCl) is formed. At an acidic pH, ferric ion (Fe^{3+}) and SCN^- form an intense red color suitable for colorimetric determination.

a. Interferences:

- Hexavalent chromium (Cr^{6+}) interferes and is removed by adjusting to pH 1 to 2 with nitric acid (HNO_3) and then adding ferrous sulfate ($FeSO_4$). Raising the pH to 9 with 1*N* sodium hydroxide (NaOH) precipitates Fe^{3+} and Cr^{3+}, which are then filtered out.
- Agents that reduce Fe^{3+} to Fe^{2+}, thus preventing formation of ferric thiocyanate complex, are destroyed by adding a few drops of hydrogen peroxide (H_2O_2). Avoid excess H_2O_2 to prevent reaction with SCN^-.
- Industrial wastes may be highly colored or contain various interfering organic compounds. To eliminate these interferences,[1] pretreat sample as directed in 4500-CN^-.M.4*c*. It is the analyst's responsibility to validate the method's applicability without pretreatment. If in doubt, pretreat sample before proceeding with analysis.
- If sample contains cyanide amenable to chlorination and would be preserved for the cyanide determination at a high pH, sulfide could interfere by converting cyanide to SCN^-. To preserve SCN^- and CN^-, treat for sulfide according to 4500-CN^-.B.2 before adding alkali.
- Thiocyanate is biodegradable. Preserve samples at pH <2 by adding mineral acid (hydrochloric, nitric, or sulfuric acid) and refrigerating.
- If interferences from industrial wastes are not removed as directed in 4500-CN^-.M.4*c* below, consider adopting a solvent-extraction technique with colorimetric or atomic absorption analysis of the extract.[2,3]

b. Application: 0.1 to 2.0 mg SCN^-/L in natural or waste waters. For higher concentrations, use a portion of diluted sample.

c. Quality control: The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Spectrophotometer or filter photometer, for use at 460 nm, providing a light path of 5 cm.

b. Glass adsorption column: Use a 50-mL buret with a glass-wool plug, and pack with macroreticular resin (4500-CN^-.M.3*f*) approximately 40 cm high. For convenience, apply a powder funnel with the same diameter as the buret to the top with a short piece of plastic tubing.

3. Reagents

a. Ferric nitrate solution: Dissolve 404 g $Fe(NO_3)_3 \cdot 9H_2O$ in about 800 mL reagent water. Add 80 mL conc HNO_3 and dilute to 1 L.

b. Nitric acid solution, 0.1*N:* Mix 6.4 mL conc HNO_3 in about 800 mL reagent water and dilute to 1 L.

c. Stock thiocyanate solution: Dissolve 1.673 g potassium thiocyanate (KSCN) in reagent water and dilute to 1000 mL; 1.00 mL = 1.00 mg SCN^-.

d. Standard thiocyanate solution: Dilute 10 mL stock solution to 1 L with reagent water; 1.00 mL = 0.01 mg SCN^-.

e. Sodium hydroxide solution, 4 g/L: Dissolve 4 g NaOH in about 800 mL reagent water and dilute to 1 L.

f. Macroreticular resin, 18 to 50 mesh:* The available resin may not be purified. Some samples have shown contamination with waxes and oil, giving poor permeability and adsorption. Purify as follows:

Place sufficient resin to fill the column(s) in a beaker and add 5 times the resin volume of acetone. Stir gently for 1 h. Pour off fines and acetone from settled resin and add 5 times the resin volume of hexane. Stir for 1 h. Pour off fines and hexane and add 5 times the resin volume of methanol. Stir for 15 min. Pour off methanol and add 3 times the resin volume of 0.1*N* NaOH. Stir for 15 min. Pour off NaOH solution and add 3 times the resin volume of 0.1*N* HNO_3. Stir for 15 min. Pour off HNO_3 solution and add 3 times the resin volume of reagent water. Stir for

* Amberlite® XAD-7, or equivalent.

15 min. Drain excess water and use purified resin to fill the column. Store excess purified resin after covering it with reagent water. Keep in a closed jar.

g. Acetone.

h. Hexane.

i. Methyl alcohol.

4. Procedure

a. Preparation of calibration curve: Prepare a series of standards containing from 0.02 to 0.40 mg SCN^- by pipetting measured volumes of standard KSCN solution into 200-mL volumetric flasks and diluting with reagent water. Mix well. Develop color according to ¶ *b* below. Plot absorbance against SCN^- concentration expressed as mg/50 mL sample. The absorbance plot should be linear.

b. Color development: Use a filtered sample or portion from a diluted solution so the concentration of SCN^- is between 0.1 and 2 mg/L. Adjust pH to 2 with conc HNO_3 added dropwise. Pipet 50-mL portion to a beaker, add 2.5 mL ferric nitrate, and mix.

Fill a 5-cm absorption cell and measure absorbance against a reagent blank at 460 nm or close to the maximum absorbance found with the instrument being used. Measure the developed color's absorbance against a reagent blank within 5 min of adding the reagent. (The color develops within 30 s and fades when standing in light.)

c. Sample pretreatment:

1) Color and various organic compounds interfere with absorbance measurement. At pH 2, macroreticular resin removes these interfering materials via adsorption without affecting thiocyanate.

2) To prepare the adsorption column, fill it with resin, rinse with 100 mL methanol, and follow by rinses with 100 mL 0.1*N* NaOH, 100 mL 0.1*N* HNO_3, and finally with 100 mL reagent water. If previously purified resin is used, omit this step.

3) When washing, regenerating, or passing a sample through the column, as solution level approaches resin bed, add and drain five separate 5-mL volumes of solution or reagent water (depending on which is used in next step) to approximate bed height. After last 5-mL volume, fill column with remaining liquid. This procedure prevents undue mixing of solutions and helps void the column of the previous solution.

4) Acidify 150 mL sample (or a dilution) to pH 2 by adding conc HNO_3 dropwise while stirring. Pass it through the column at a flow rate ≤20 mL/min. If the resin becomes packed and the flow rate falls to 4 to 5 mL/min, use gentle pressure through a manually operated hand pump or squeeze bulb on the column. In this case, use a separator funnel for the liquid reservoir instead of the powder funnel. Alternatively, use a vacuum bottle as a receiver and apply gentle vacuum. Do not let liquid level drop below the adsorbent in the column.

5) When passing a sample through the column, measure 90 mL of sample in a graduated cylinder, and from this make the five 5-mL additions as directed in ¶ *c* 3) above, then pour the rest of the 90 mL into the column. Add remaining sample and collect 60 mL eluate to be tested after the first 60 mL has passed through the column.

6) Prepare a new calibration curve using standards prepared according to ¶ *a* above, but acidify standards according to ¶ *b* above, and pass them through the adsorption column. Develop color and measure absorbance according to ¶ *b* above against a reagent blank prepared by passing acidified reagent water through the adsorption column.

7) Pipet 50 mL from the collected eluate to a beaker, add 2.5 mL ferric nitrate solution, and mix. Measure absorbance according to ¶ *b* above against a reagent blank [see ¶ *c* 6) above].

8) From the measured absorbance value, determine the sample's or dilution's thiocyanate content using the absorbance plot.

9) Each day the column is in use, test a mid-range standard to check absorption curve.

10) Regenerate column between samples by rinsing with 100 mL 0.1*N* NaOH; 50 mL 0.1*N* HNO_3; and 100 mL reagent water. Ensure that the water has rinsed the buret's empty glass section. Occasionally rinse with 100 mL methanol for complete regeneration. Adsorbed weak organic acids and thiocyanate residuals from earlier tests are eluted by the NaOH rinse. Leave the column covered with the last rinse water for storage.

5. Calculation

Compute slope (m) and intercept (b) of standard curve as directed in 4500-CN^-.E.5.

Calculate thiocyanate concentration as follows:

$$\text{mg } SCN^-/\text{L} = (ma_1 + b) \times \text{dilution factor}$$

where:

a_1 = absorbance of sample solution.

6. Precision and Bias[4]

a. Precision: Based on the results of 12 operators in nine laboratories at four concentrations, the method's precision within its designated range is linear with concentration and may be expressed as follows:

$$\text{Reagent water: } S_T = 0.093x + 0.0426$$
$$S_o = 0.045x + 0.010$$

$$\text{Water matrix: } S_T = 0.055x + 0.0679$$
$$S_o = 0.024x + 0.182$$

where:

S_T = overall precision, mg/L,
S_o = pooled single-operator precision, mg/L, and
x = thiocyanate concentration, mg/L.

b. Bias: Recoveries of known amounts of thiocyanate from reagent water and selected water matrices, including natural waters, laboratory effluent, steel mill effluent, and dechlorinated and treated sanitary effluents, were as follows:

Medium	Added *mg/L*	Recovered *mg/L*	n	S_T	Bias	% Bias
Reagent water	1.42	1.411	30	0.181	–0.009	–0.6
	0.71	0.683	27	0.091	–0.027	–4
	0.35	0.329	30	0.084	–0.021	–6
	0.07	0.068	30	0.052	–0.002	–3
Selected water matrices	1.42	1.408	26	0.151	–0.012	–0.8
	0.71	0.668	29	0.096	–0.042	–6
	0.35	0.32	29	0.085	–0.030	–9
	0.07	0.05	29	0.079	–0.020	–29

For other matrices, these data may not apply.

7. References

1. Spencer, R.R., J. Leenheer & V.C. Marti. 1980. Automated colorimetric determination of thiocyanate, thiosulfate and tetrathionate in water. 94th Annual Meeting. Assoc. Official Agricultural Chemists, Washington, D.C. 1981.
2. Danchick, R.S. & D.F. Boltz. 1968. Indirect spectrophotometric and atomic absorption spectrometric methods in determination of thiocyanate. *Anal. Chem.* 43:2215.
3. Luthy, R.G. 1978. Manual of Methods: Preservation and Analysis of Coal Gasification Wastewaters; FE-2496-16. U.S. Dept. Energy, National Technical Information Serv., Springfield, Va.
4. American Society for Testing & Materials. 1989. Standard Test Methods for Thiocyanate in Water; Research Rep. D4193:19-1099. Philadelphia, Pa.

4500-CN⁻ N. Total Cyanide after Distillation, by Flow Injection Analysis

1. General Discussion

a. Principle: Total cyanides are digested and steam-distilled from the sample (4500-CN⁻.C), cyanides amenable to chlorination are digested and steam-distilled from the sample (4500-CN⁻.G), or weak acid dissociable cyanides are digested and steam-distilled from the sample (4500-CN⁻.I) via the apparatus described in 4500-CN⁻.C.2 or an equivalent distillation apparatus. In any case, the distillate should consist of cyanide in 0.25*M* NaOH. The cyanide in this distillate is converted to cyanogen chloride (CNCl) by reaction with chloramine-T at pH <8. The CNCl then forms a red-blue dye by reacting with pyridine-barbituric acid reagent. The absorbance of this red dye is measured at 570 nm and is proportional to the total or weak acid dissociable cyanide in the sample.

Also see 4500-CN⁻.A and E.

b. Interferences: Remove large or fibrous particulates by filtering sample through glass wool. Guard against contamination from reagents, water, glassware, and the sample-preservation process.

Nonvolatile interferences are eliminated or minimized by the distillation procedure. Some of the known interferences are aldehydes, nitrate-nitrite, and oxidizing agents (e.g., chlorine, thiocyanate, thiosulfate, and sulfide). Multiple interferences may require the analysis of a series of laboratory-fortified sample matrices (LFM) to verify the suitability of the chosen treatment. See 4500-CN⁻.B for a discussion of preliminary treatment of samples to be distilled.

2. Apparatus

Flow injection analysis equipment consisting of:

a. FIA injection valve with sample loop or equivalent.

b. Multichannel proportioning pump.

c. FIA manifold (Figure 4500-CN⁻:2) with tubing heater and flow cell. Figure 4500-CN⁻:2 shows relative flow rates and example tubing volumes; they may be scaled down proportionally. Follow the manufacturer's instructions. Use manifold tubing made of an inert material, such as TFE.*

d. Absorbance detector, 570 nm, 10-nm bandpass.

e. Injection valve control and data acquisition system.

* Teflon, or equivalent.

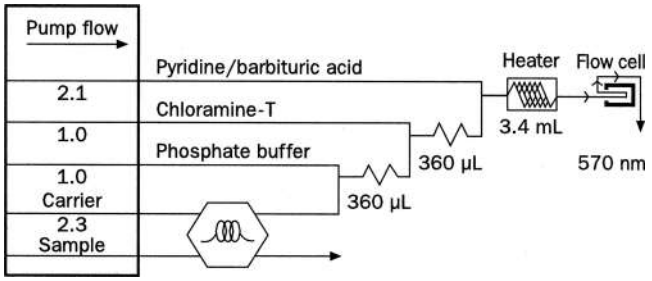


Figure 4500-CN⁻:2. FIA cyanide manifold.

3. Reagents

Use high-quality reagent water for all solutions. To prevent bubble formation, degas carrier and all reagents with helium. Pass He at 140 kPa (20 lb/in.^{2}) through a helium degassing tube. Bubble He through 1 L solution for 1 min. As an alternative to preparing reagents by weight/weight, use weight/volume. Degassing can also be accomplished by vacuum.

a. Carrier solution, 0.25*M:* In a 1-L plastic container, dissolve 10.0 g NaOH in 1.00 L water.

b. Phosphate buffer, 0.71*M:* To a 1-L tared container, add 97.0 g potassium phosphate, monobasic, anhydrous KH_2PO_4, and 975 g water. Stir or shake until dissolved. Prepare fresh monthly.

c. Chloramine-T: Dissolve 2.0 g chloramine-T hydrate (mol wt 227.65) in 500 mL water. Prepare fresh daily.

d. Pyridine/barbituric acid: In fume hood, place 15.0 g barbituric acid in a tared 1-L container and add 100 g water, rinsing down sides of beaker to wet the barbituric acid. Add 73 g pyridine (C_5H_5N) while stirring, and mix until barbituric acid dissolves. Add 18 g conc HCl, then another 825 g water, and mix. Prepare fresh weekly.

e. Stock cyanide standard, 100 mg CN⁻/L: In a 1-L container, dissolve 2.0 g potassium hydroxide (KOH) in approximately 800 mL water. Add 0.250 g potassium cyanide (KCN). **Caution: KCN is highly toxic. Avoid inhaling dust or contact with the solid or solutions.** Make to final weight of 1000 g with water and invert three times. Prepare fresh weekly or standardize weekly using procedure in 4500-CN⁻.D.4.

f. Standard cyanide solution: Prepare cyanide standards in the desired concentration range, using the stock cyanide standard (¶ *e* above) and diluting with the 0.25*M* NaOH carrier (¶ *a* above).

4. Procedure

Set up a manifold equivalent to that in Figure 4500-CN⁻:2 and follow method supplied by manufacturer or laboratory standard operating procedure for this method.

5. Calculation

Prepare standard curves by plotting absorbance of standards processed through manifold versus cyanide concentration. The calibration curve is linear. Some FIA systems allow first-order quadratic fits for the working curve range, as long as at least six standards and a blank are used for the curve fit over the method's working range.

6. Precision and Bias

a. Recovery and relative standard deviation: The results of single-laboratory studies with various matrices are given in Table 4500-CN⁻:I.

b. MDL without distillation: Using a published MDL method,[1] analysts ran 21 replicates of an undistilled 0.010-mg CN⁻/L standard with a 780-μL sample loop. These gave a mean of 0.010 mg CN⁻/L, a standard deviation of 0.00012 mg CN⁻/L, and an MDL of 0.0003 mg CN⁻/L. A lower MDL may be obtained by increasing the sample-loop volume and increasing the ratio of carrier flow rate to reagent flow rate.

c. MDL with distillation: Using a published MDL method,[1] analysts ran 21 replicates of a 0.0050-mg CN⁻/L standard distilled using the distillation device† equivalent to the apparatus specified in 4500-CN⁻.C.2. When the 0.25*M* NaOH distillates were determined with a 780-μL sample loop, they gave a mean of 0.0045 mg CN⁻/L, a standard deviation of 0.0002 mg CN⁻/L, and an MDL of 0.0006 mg CN⁻/L.

d. Precision study: Ten injections of an undistilled 0.050-mg CN⁻/L standard gave a relative standard deviation of 0.21%.

Table 4500-CN⁻:I. Results of Single-Laboratory Studies with Selected Matrices

Matrix	Sample/Blank Designation	Known Addition *mg CN⁻/L*	Recovery %	Relative Standard Deviation %
Wastewater treatment plant influent	Reference Sample*	—	94	—
	Blank†	0.050	96	—
		0.10	96	—
	Site A‡	0	—	<0.5
		0.010	104	—
		0.020	104	—
	Site B‡	0	—	<0.5
		0.010	101	—
		0.020	106	—
	Site C‡	0	—	<0.5
		0.010	103	—
		0.020	108	—
Wastewater treatment plant effluent	Reference sample*	—	95	—
	Blank†	0.050	88	—
		0.10	95	—
	Site A‡	0	—	<0.5
		0.010	112	—
		0.020	106	—
	Site B‡	0	—	<0.5
		0.010	110	—
		0.020	105	—
	Site C‡	0	—	<0.5
		0.010	101	—
		0.020	106	—
Landfill leachate	Reference sample*	—	98	—
	Blank†	0.050	96	—
		0.10	98	—
	Site A‡	0	—	<0.5
		0.050	114	—
		0.10	106	—
	Site B‡	0	—	<0.5
		0.050	104	—
		0.10	104	—
	Site C‡	0	—	<0.5
		0.050	103	—
		0.10	107	—

* EPA QC sample, 0.498 mg CN⁻/L, diluted fivefold.
† Determined in duplicate.
‡ Samples diluted fivefold. Samples without known additions determined four times; sample with known additions determined in duplicate; typical relative differences between duplicates <0.5%.

7. Reference

1. U.S. Environmental Protection Agency. 1989. Definition and procedure for the determination of method detection limits. Appendix B to 40 CFR 136 rev. 12.11 amended June 30, 1986. 49 CFR 43430.

† MICRO DIST, Lachat Instruments, Milwaukee, Wis.

4500-CN⁻ O. Total Cyanide and Weak Acid Dissociable Cyanide by Flow Injection Analysis

1. General Discussion

a. Principle: Total cyanide consists of various metal-cyanide complexes, which analysts break down (digest) by mixing a sample with heated phosphoric acid and then irradiating it with UV light to produce a "donor stream" containing aqueous HCN. Analysts then pass the donor stream over a silicone-rubber gas-permeation membrane, which extracts gaseous HCN. They then trap the gaseous HCN in a parallel "acceptor stream" that consists of dilute sodium hydroxide (equivalent to the distillate produced via digesting distillations in the sample-preparation steps of 4500-CN⁻.C, G, and I).

Analysts convert the cyanide in this acceptor stream (distillate) to cyanogen chloride (CNCl) via reaction with chloramine-T at pH <8 (as in 4500-CN⁻.N). They then add pyridine-barbituric acid reagent, which reacts with CNCl to form a red-blue dye. This dye's absorbance at 570 nm is proportional to the amount of total or weak acid dissociable (WAD) cyanide in the sample.

The WAD cyanide method is similar, except its donor stream is a solution of dihydrogen phosphate.

See also Sections 4130 and 4500-CN⁻.A, E, and N.

b. Interferences: Guard against contamination via reagents, water, glassware, and the sample-preservation process. Remove large or fibrous particulates by filtering sample through glass wool. The gas-permeable membrane minimizes or eliminates nonvolatile interferences.

If multiple interferences are present, analysts may need to analyze a series of sample matrices with known additions to verify the chosen treatment's suitability. See 4500-CN⁻.B for a discussion on preliminary treatment of samples to be distilled.

1) Total cyanide interferences—Sulfide concentrations ≤10 mg/L and thiocyanate concentrations ≤20 mg/L do not interfere in the determination of 100 μg CN⁻/L. When a sample known to contain nitrate (100 mg NO_3^--N/L), thiocyanate (20 mg/L), and cyanide (100 μg CN⁻/L) was pretreated with sulfamic acid, method results indicated that the sample contained 138.2 μg CN⁻/L. When a sample containing 50 mg formaldehyde/L was pretreated with ethylenediamine, the formaldehyde did not interfere with the cyanide determination.

2) WAD interferences—Sulfide concentrations ≤10 mg/L and thiocyanate concentrations ≤50 mg/L do not interfere in the determination of 0.1 mg/L cyanide.

c. Quality control: The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Flow injection analysis (FIA) equipment consisting of:

a. FIA injection valve with sample loop or equivalent.

b. Multichannel proportioning pump.

c. FIA manifold (Figure 4500-CN⁻:3) with tubing heater, in-line UV digestion fluidics, a gas-permeable silicone-rubber (or hydrophobic) membrane and its holder, and flow cell. Figure 4500-CN⁻:3 shows relative flow rates and example tubing volumes; they may be scaled down proportionally. Follow manufacturer's instructions. Use manifold tubing made of an inert material, such as TFE. The UV unit should consist of TFE tubing irradiated by a mercury-discharge UV lamp emitting radiation at 254 nm.

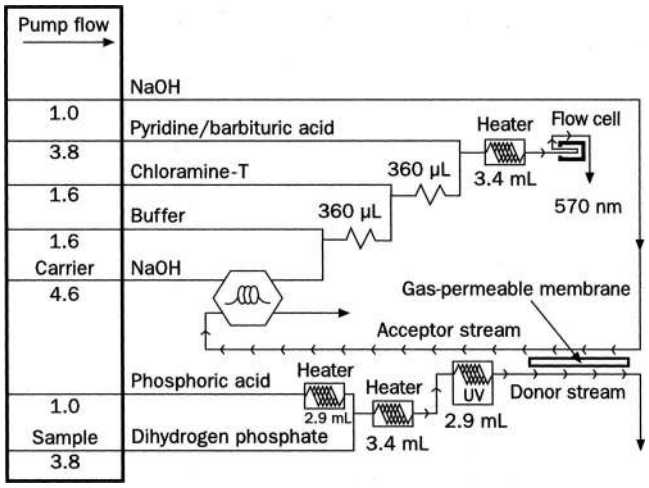


Figure 4500-CN⁻:3. FIA in-line total and WAD cyanide manifold.

d. Absorbance detector, 570 nm, 10-nm bandpass.

e. Injection valve control and data acquisition system.

3. Reagents

Use high-quality reagent water for all solutions. To prevent bubble formation, degas carrier and all reagents with helium as follows: Pass He at 140 kPa (20 lb/in.^{2}) through a helium-degassing tube and then bubble through 1 L solution for 1 min.

a. Phosphoric acid donor stream (total cyanide): To a 1-L volumetric flask, add approximately 700 mL water followed by 30 mL conc phosphoric acid (H_3PO_4). Mix and let solution cool. Dilute to mark. Prepare fresh monthly.

b. Dihydrogen phosphate donor stream (WAD cyanide): To a tared 1-L container, add 97 g anhydrous potassium dihydrogen phosphate (KH_2PO_4) and 975 g water. Stir for 2 h or until potassium phosphate completely dissolves. Degas with helium. Prepare fresh monthly.

c. NaOH acceptor stream, carrier, and diluent (total and WAD cyanide), 0.025*M* NaOH: To a 1-L container, add 1.0 g sodium hydroxide (NaOH) and 999 g water. Mix with a magnetic stirrer for about 5 min. Cover with laboratory film. Degas with helium. Prepare fresh daily.

d. Buffer (total and WAD cyanide), 0.71*M* phosphate: To a 1-L tared container, add 97.0 g potassium phosphate; monobasic, anhydrous KH_2PO_4; and 975 g water. Stir or shake until dissolved. Prepare fresh monthly.

e. Chloramine-T solution (total and WAD cyanide): Dissolve 3 g chloramine-T hydrate in 500 mL water. Degas with helium. Prepare fresh daily. NOTE: Chloramine-T is an air-sensitive solid. Preferably discard this chemical 6 months after opening.

f. Pyridine/barbituric acid solution (total and WAD cyanide): In the fume hood, place 15.0 g barbituric acid in a tared 1-L container and add 100 g water, rinsing down the sides of the beaker to wet the barbituric acid. Add 73 g pyridine (C_5H_5N) while stirring and mix until barbituric acid dissolves. Add 18 g conc HCl, then add another 825 mL water and mix. Prepare fresh weekly.

g. Stock cyanide standard, 100 mg CN^-/L: In a 1-L container, dissolve 2.0 g potassium hydroxide (KOH) in approximately 800 mL water. Add 0.250 g potassium cyanide (KCN). **CAUTION: KCN is highly toxic. Avoid inhalation of dust or contact with the solid or solutions.** Add water to make final weight of 1000 g, and invert three times to mix. Prepare fresh weekly or standardize weekly using the procedure in 4500-CN^-.D.4.

h. Standard cyanide solutions: Prepare cyanide standards in the desired concentration range, using the stock cyanide standard and diluting with NaOH diluent.

4. Procedure

Set up a manifold equivalent to that in Figure 4500-CN^-:3 and follow the method supplied by the manufacturer or laboratory standard operating procedure for this method.

5. Calculations

Prepare standard curves by plotting the absorbance(s) of standards processed through the manifold vs. cyanide concentration. The calibration curve is linear. Some FIA systems allow first-order quadratic fits for the working curve range, so long as at least six standards and a blank are used for the curve fit over the method's working range.

6. Precision and Bias

a. MDL (total cyanide): A 420-μL sample loop was used in the total cyanide method. Using a published MDL method,[1] analysts ran 21 replicates of a 10.0-μg CN^-/L standard. These gave a mean of 9.69 μg CN^-/L, a standard deviation of 0.86 μg CN^-/L, and an MDL of 2.7 μg CN^-/L.

b. MDL (WAD cyanide): A 420-μL sample loop was used in the WAD cyanide method. Using a published MDL method,[1] analysts ran 21 replicates of a 10.0-μg CN^-/L standard. These gave a mean of 11.5 μg CN^-/L, a standard deviation of 0.73 μg CN^-/L, and an MDL of 2.3 μg CN^-/L.

c. Precision study (total cyanide): Seven injections of a 100.0-μg CN^-/L standard gave a relative standard deviation (RSD) of 1.0%.

d. Precision study (WAD cyanide): Ten injections of a 200.0-μg CN^-/L standard gave an RSD of 1.3%.

e. Recovery of total cyanide: Two injections each of potassium ferricyanide and potassium ferrocyanide solutions, both at a concentration equivalent to 100 μg CN^-/L, gave an average recovery of 98%.

7. Reference

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1989. Definition and procedure for the determination of method detection limits. Appendix B to 40 CFR 136 rev. 12.11 amended June 30, 1986. 49 CFR 43430.

4500-F$^-$ FLUORIDE*

4500-F$^-$ A. Introduction

Fluoride may occur naturally in water or it may be added in controlled amounts. Some fluorosis may occur when the fluoride level exceeds the recommended limits. In rare instances, the naturally occurring fluoride concentration may approach 10 mg/L.

Accurate determination of fluoride has increased in importance with the growth of the practice of fluoridation of water supplies as a public health measure. Maintenance of an optimal fluoride concentration is essential in maintaining effectiveness and safety of the fluoridation procedure.

1. Preliminary Treatment

Among the methods suggested for determining fluoride ion (F^-) in water, the electrode and colorimetric methods are the most satisfactory. Because both methods are subject to errors due to interfering ions (Table 4500:F$^-$:I), it may be necessary to distill the sample as directed in 4500-F$^-$.B before making the determination. When interfering ions are not present in excess of the tolerance of the method, the fluoride determination may be made directly without distillation.

2. Selection of Method

The electrode methods (4500-F$^-$.C and G) are suitable for fluoride concentrations from 0.1 to more than 10 mg/L. Adding the prescribed buffer frees the electrode method from most interferences that adversely affect the SPADNS colorimetric method and necessitate preliminary distillation. Some substances in industrial wastes, such as fluoborates, may be sufficiently concentrated to present problems in electrode measurements and will not be measured without a preliminary distillation. Fluoride measurements can be made with an ion-selective electrode and either an expanded-scale pH meter or a specific ion meter, usually without distillation, in the time necessary for electrode equilibration.

The SPADNs method (4500-F$^-$.D) has a linear analytical range of 0 to 1.40 mg F$^-$/L. Use of a nonlinear calibration can extend the range to 3.5 mg F$^-$/L. Color development is virtually instantaneous. Color determinations are made photometrically, using either a filter photometer or a spectrophotometer. A curve developed from standards is used for determining the fluoride concentration of a sample.

Fluoride also may be determined by the automated complexone method, 4500-F$^-$.E.

Table 4500-F$^-$:I. Concentration of Some Substances Causing 0.1-mg/L Error at 1.0 mg F$^-$/L in Fluoride Methods

	4500-F$^-$.C (Electrode)		4500-F$^-$.D (SPADNS)	
Substance	Conc *mg/L*	Type of Error*	Conc *mg/L*	Type of Error*
Alkalinity ($CaCO_3$)	7 000	+	5 000	−
Aluminum (Al^{3+})	3.0	−	0.1†	−
Chloride (Cl^-)	20 000		7 000	+
Chlorine	5 000			Remove completely with arsenite
Color & turbidity				Remove or compensate for
Iron	200	−	10	−
Hexametaphosphate ($[NaPO_3]_6$)	50 000		1.0	+
Phosphate (PO_4^{3-})	50 000		16	+
Sulfate (SO_4^{2-})	50 000	−	200	−

* + = positive error
− = negative error
Blank = no measurable error.
† On immediate reading. Tolerance increases with time: after 2 h, 3.0; after 4 h, 30.

Ion chromatography (Section 4110) is an acceptable method if weaker eluents are used to separate fluoride from interfering peaks or fluoride can be determined by capillary ion electrophoresis (Section 4140).

The flow injection method (4500-F$^-$.G) is a convenient automated technique for analyzing large numbers of samples.

3. Sampling and Storage

Preferably use polyethylene bottles for collecting and storing samples for fluoride analysis. Glass bottles are satisfactory if previously they have not contained high-fluoride solutions. Always rinse bottle with a portion of sample.

For the SPADNs method, never use an excess of dechlorinating agent. Dechlorinate with sodium arsenite rather than sodium thiosulfate when using the SPADNS method because the latter may produce turbidity that causes erroneous readings.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011.
Joint Task Group: 20th Edition (4500-F$^-$.G)—Scott Stieg (chair), Bradford R. Fisher, Owen B. Mathre, Theresa M. Wright.

4500-F⁻ B. Preliminary Distillation Step

1. Discussion

Fluoride can be separated from other nonvolatile constituents in water by conversion to hydrofluoric or fluosilicic acid and subsequent distillation. The conversion is accomplished by using a strong, high-boiling acid. To protect against glassware etching, hydrofluoric acid is converted to fluosilicic acid by using soft glass beads. Quantitative fluoride recovery is approached by using a relatively large sample. Acid and sulfate carryover are minimized by distilling over a controlled temperature range.

Distillation will separate fluoride from most water samples. Some tightly bound fluoride, such as that in biological materials, may require digestion before distillation, but water samples seldom require such drastic treatment. Distillation produces a distillate volume equal to that of the original water sample, so usually it is not necessary to incorporate a dilution factor when expressing analytical results. The distillate will be essentially free of substances that might interfere with the fluoride determination if the apparatus used is adequate and distillation has been carried out properly. The only common volatile constituent likely to cause interference with colorimetric analysis of the distillate is chloride. When the concentration of chloride is high enough to interfere, add silver sulfate to the sulfuric acid distilling mixture to minimize the volatilization of hydrogen chloride.

CAUTION: Heating an acid–water mixture can be hazardous if precautions are not taken: Mix acid and water thoroughly before heating. Use of a quartz heating mantle and a magnetic stirrer in the distillation apparatus simplifies the mixing step.

2. Apparatus

a. Distillation apparatus consisting of a 1-L round-bottom long-neck borosilicate glass boiling flask, a connecting tube, an efficient condenser, a thermometer adapter, and a thermometer that can be read to 200°C. Use standard taper joints for all connections in the direct vapor path. Position the thermometer so the bulb always is immersed in boiling mixture. The apparatus should be disassembled easily to permit adding sample. Substituting a thermoregulator and necessary circuitry for the thermometer is acceptable and provides some automation.

Alternative types of distillation apparatus may be used. Carefully evaluate any apparatus for fluoride recovery and sulfate carryover. The critical points are obstructions in the vapor path and trapping of liquid in the adapter and condenser. (The condenser should have a vapor path with minimum obstruction. A double-jacketed condenser, with cooling water in the outer jacket and the inner spiral tube, is ideal, but other condensers are acceptable if they have minimum obstructions. Avoid using Graham-type condensers.) Avoid using an open flame as a heat source if possible, because heat applied to the boiling flask above the liquid level causes superheating of vapor and subsequent sulfate carryover.

CAUTION: Regardless of apparatus used, provide for thorough mixing of sample and acid; heating a non-homogenous acid–water mixture will result in bumping or possibly a violent explosion.

The preferred apparatus is illustrated in Figure 4500-F⁻:1.

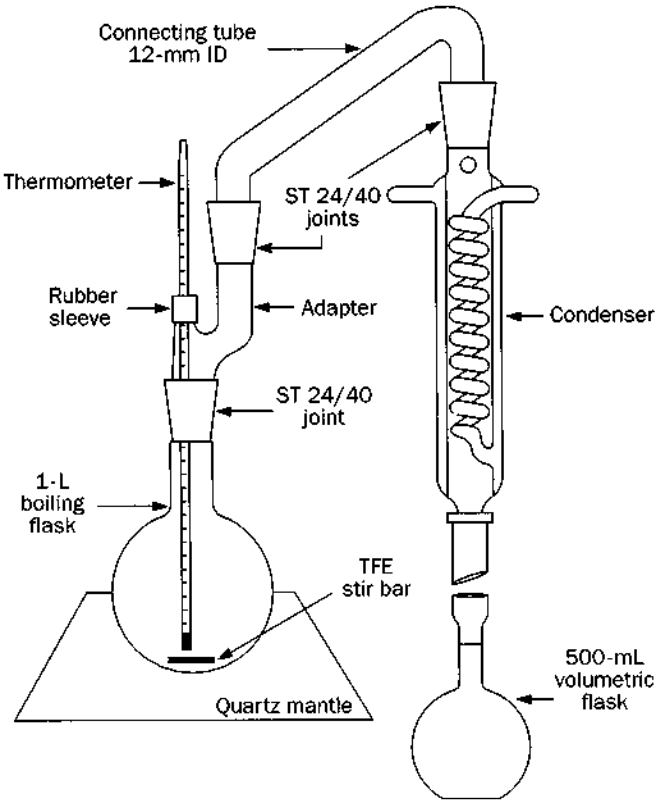


Figure 4500-F⁻:1. Direct distillation apparatus for fluoride.

b. Quartz hemispherical heating mantle, for full-voltage operation.

c. Magnetic stirrer, with TFE-coated stirring bar.

d. Soft glass beads.

3. Reagents

a. Sulfuric acid (H_2SO_4), conc, reagent grade.

b. Silver sulfate (Ag_2SO_4), crystals, reagent grade.

4. Procedure

a. Place 400 mL distilled water in the distilling flask and, with the magnetic stirrer operating, carefully add 200 mL conc H_2SO_4. Keep stirrer in operation throughout distillation. Add a few glass beads and connect the apparatus as shown in Figure 4500-F⁻:1, making sure all joints are tight. Begin heating and continue until flask contents reach 180°C (because of heat retention by the mantle, it is necessary to discontinue heating when the temperature reaches 178°C to prevent overheating). Discard distillate. This process removes fluoride contamination and adjusts the acid–water ratio for subsequent distillations.

b. After the acid mixture remaining in the steps outlined in ¶ *a* above, or previous distillations, has cooled to 80°C or below, add 300 mL sample, with stirrer operating, and distill until the temperature reaches 180°C. To prevent sulfate carryover, turn off heat before 178°C. Retain the distillate for analysis.

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c. Add Ag_2SO_4 to the distilling flask at the rate of 5 mg/mg Cl^- when the chloride concentration is high enough to interfere (see Table 4500-F⁻:I).

d. Use H_2SO_4 solution in the flask repeatedly until contaminants from samples accumulate to such an extent that recovery is affected or interferences appear in the distillate. Check acid suitability periodically by distilling standard fluoride samples and analyzing for both fluoride and sulfate. After distilling samples containing more than 3 mg F^-/L, flush still by adding 300 mL distilled water, redistill, and combine the two fluoride distillates. If necessary, repeat flushing until the fluoride content of the last distillate is at a minimum. Include additional fluoride recovered with that of the first distillation. After periods of inactivity, similarly flush still and discard distillate.

5. Interpretation of Results

The recovery of fluoride is quantitative within the accuracy of the methods used for its measurement.

6. Bibliography

BELLACK, E. 1958. Simplified fluoride distillation method. *J. Amer. Water Works Assoc.* 50:530.

BELLACK, E. 1961. Automatic fluoride distillation. *J. Amer. Water Works Assoc.* 53:98.

ZEHNPFENNIG, R.G. 1976. Letter to the editor. *Environ. Sci. Technol.* 10:1049.

4500-F⁻ C. Ion-Selective Electrode Method

1. General Discussion

a. Principle: The fluoride electrode is an ion-selective sensor. The key element in the fluoride electrode is the laser-type doped lanthanum fluoride crystal across which a potential is established by fluoride solutions of different concentrations. The crystal contacts the sample solution at one face and an internal reference solution at the other. The cell may be represented by:

$$\text{Ag}\,|\,\text{AgCl, Cl}^-(0.3M),\ \text{F}^-(0.001M)\,|\,\text{LaF}_3\,|\,\text{test solution}\,|\,\text{reference electrode}$$

The fluoride electrode can be used with a standard calomel reference electrode and almost any modern pH meter having an expanded millivolt scale. Calomel electrodes contain both metallic and dissolved mercury; therefore, dispose of them only in approved sites or recycle. For this reason, the Ag/AgCl reference electrode is preferred.

The fluoride electrode measures the ion activity of fluoride in solution rather than concentration. Fluoride ion activity depends on the solution total ionic strength and pH, and on fluoride complexing species. Adding an appropriate buffer provides a nearly uniform ionic strength background, adjusts pH, and breaks up complexes so, in effect, the electrode measures concentration.

b. Interference: Table 4500-F⁻:I lists common interferences. Fluoride forms complexes with several polyvalent cations, notably aluminum and iron. The extent to which complexation takes place depends on solution pH, relative levels of fluoride, and complexing species. However, CDTA (cyclohexylenediaminetetraacetic acid), a component of the buffer, preferentially will complex interfering cations and release free fluoride ions. Concentrations of aluminum, the most common interference, up to 3.0 mg/L can be complexed preferentially. In acid solution, F^- forms a poorly ionized HF · HF complex but the buffer maintains a pH above 5 to minimize hydrogen fluoride complex formation. In alkaline solution, hydroxide ion also can interfere with electrode response to fluoride ion whenever the hydroxide ion concentration is greater than one-tenth the concentration of fluoride ion. At the pH maintained by the buffer, no hydroxide interference occurs.

Fluoborates are widely used in industrial processes. Dilute solutions of fluoborate or fluoboric acid hydrolyze to liberate fluoride ion but in concentrated solutions, as in electroplating wastes, hydrolysis does not occur completely. Distill such samples or measure fluoborate with a fluoborate-selective electrode. Also distill the sample if the dissolved solids concentration exceeds 10 000 mg/L.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Expanded-scale or digital pH meter or ion-selective meter.

b. Sleeve-type reference electrode: Do not use fiber-tip reference electrodes because they exhibit erratic behavior in very dilute solutions.

c. Fluoride electrode.

d. Magnetic stirrer, with TFE-coated stirring bar.

e. Timer.

3. Reagents

a. Stock fluoride solution: Dissolve 221.0 mg anhydrous sodium fluoride, NaF, in distilled water and dilute to 1000 mL; 1.00 mL = 100 μg F^-.

b. Standard fluoride solution: Dilute 100 mL stock fluoride solution to 1000 mL with distilled water; 1.00 mL = 10.0 μg F^-.

c. Fluoride buffer: Place approximately 500 mL distilled water in a 1-L beaker and add 57 mL glacial acetic acid, 58 g NaCl, and 4.0 g 1,2 cyclohexylenediaminetetraacetic acid (CDTA).* Stir to dissolve. Place beaker in a cool water bath and add slowly 6*N* NaOH (about 125 mL) with stirring, until pH is between 5.0 and 5.5. Transfer to a 1-L volumetric flask and add distilled water to the mark. This buffer, as well as a more concentrated

* Also known as 1,2 cyclohexylenedinitrilotetraacetic acid.

version, is available commercially. In using the concentrated buffer, follow the manufacturer's directions.

4. Procedure

a. Instrument calibration: No major adjustment of any instrument normally is required to use electrodes in the range of 0.2 to 2.0 mg F^-/L. For those instruments with zero at center scale, adjust calibration control so the 1.0 mg F^-/L standard reads at the center zero (100 mV) when the meter is in the expanded-scale position. This cannot be done on some meters that do not have a millivolt calibration control. To use a selective-ion meter, follow the manufacturer's instructions.

b. Preparation of fluoride standards: Prepare a series of standards by diluting with distilled water 5.0, 10.0, and 20.0 mL of standard fluoride solution to 100 mL with distilled water. These standards are equivalent to 0.5, 1.0, and 2.0 mg F^-/L.

c. Treatment of standards and sample: In 100-mL beakers or other convenient containers, add by volumetric pipet from 10 to 25 mL standard or sample. Bring standards and sample to same temperature, preferably room temperature. Add an equal volume of buffer. The total volume should be sufficient to immerse the electrodes and permit operation of the stirring bar.

d. Measurement with electrode: Immerse electrodes in each of the fluoride standard solutions and measure developed potential while stirring on a magnetic stirrer. Check for air bubbles after immersing electrodes because entrapped air around the crystal can produce erroneous readings or needle fluctuations. Let electrodes remain in the solution 3 min (or until reading is constant) before taking a final millivolt reading. A layer of insulating material between stirrer and beaker minimizes solution heating. Withdraw electrodes, rinse with distilled water, and *blot dry* between readings. (**CAUTION: Blotting may poison electrode if not done gently.**) Repeat measurements with samples.

When using an expanded-scale pH meter or selective-ion meter, frequently recalibrate the electrode by checking potential reading of the 1.00-mg F^-/L standard and adjusting the calibration control, if necessary, until meter reads as before.

If a direct-reading instrument is not used, plot potential measurement of fluoride standards against concentration on two-cycle semilogarithmic graph paper. Plot milligrams F^- per liter on the logarithmic axis (ordinate), with the lowest concentration at the bottom of the graph. Plot millivolts on the abscissa. From the potential measurement for each sample, read the corresponding fluoride concentration from the standard curve.

The known-additions method may be substituted for the calibration method described. Follow the directions of the instrument manufacturer.

Selective-ion meters may necessitate using a slightly altered procedure, such as preparing 1.00 and 10.0 mg F^-/L standards or some other concentration. Follow the manufacturer's directions. Commercial standards, often already diluted with buffer, frequently are supplied with the meter. Verify the stated fluoride concentration of these standards by comparing them with standards prepared by the analyst.

5. Calculation

$$\text{mg } F^-/\text{L} = \frac{\mu\text{g } F^-}{\text{mL sample}}$$

6. Precision and Bias

A synthetic sample containing 0.850 mg F^-/L in distilled water was analyzed in 111 laboratories by the electrode method, with a relative standard deviation of 3.6% and a relative error of 0.7%.

A second synthetic sample containing 0.750 mg F^-/L, 2.5 mg $(NaPO_3)_6$/L, and 300 mg alkalinity/L added as $NaHCO_3$, was analyzed in 111 laboratories by the electrode method, with a relative standard deviation of 4.8% and a relative error of 0.2%.

A third synthetic sample containing 0.900 mg F^-/L, 0.500 mg Al/L, and 200 mg SO_4^{2-}/L was analyzed in 13 laboratories by the electrode method, with a relative standard deviation of 2.9% and a relative error of 4.9%.

7. Bibliography

FRANT, M.S. & J.W. ROSS, JR. 1968. Use of total ionic strength adjustment buffer for electrode determination of fluoride in water supplies. *Anal. Chem.* 40:1169.

HARWOOD, J.E. 1969. The use of an ion-selective electrode for routine analysis of water samples. *Water Res.* 3:273.

4500-F⁻ D. SPADNS Method

1. General Discussion

a. Principle: The SPADNS colorimetric method is based on the reaction between fluoride and a zirconium-dye lake. Fluoride reacts with the dye lake, dissociating a portion of it into a colorless complex anion (ZrF_6^{2-}); and the dye. As the amount of fluoride increases, the color produced becomes progressively lighter.

The reaction rate between fluoride and zirconium ions is influenced greatly by the acidity of the reaction mixture. If the proportion of acid in the reagent is increased, the reaction can be made almost instantaneous. Under such conditions, however, the effect of various ions differs from that in the conventional alizarin methods. The selection of dye for this rapid fluoride method is governed largely by the resulting tolerance to these ions.

b. Interference: Table 4500-F⁻:I lists common interferences. Because these are neither linear in effect nor algebraically additive, mathematical compensation is impossible. Whenever any

one substance is present in sufficient quantity to produce an error of 0.1 mg/L or whenever the total interfering effect is in doubt, distill the sample. Also distill colored or turbid samples. In some instances, sample dilution or adding appropriate amounts of interfering substances to the standards may be used to compensate for the interference effect. If alkalinity is the only significant interference, neutralize it with either hydrochloric or nitric acid. Chlorine interferes and provision for its removal is made.

Volumetric measurement of sample and reagent is extremely important to analytical accuracy. Use samples and standards at the same temperature or at least within 2°C. Maintain constant temperature throughout the color development period. Prepare different calibration curves for different temperature ranges.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Colorimetric equipment: One of the following is required:

a. Spectrophotometer, for use at 570 nm, providing a light path of at least 1 cm.

b. Filter photometer, providing a light path of at least 1 cm and equipped with a greenish yellow filter having maximum transmittance at 550 to 580 nm.

3. Reagents

a. Standard fluoride solution: Prepare as directed in the electrode method, 4500-F⁻.C.3*b*.

b. SPADNS solution: Dissolve 958 mg SPADNS, sodium 2-(parasulfophenylazo)-1,8-dihydroxy-3,6-naphthalene disulfonate, also called 4,5-dihydroxy-3-(parasulfophenylazo)-2,7-naphthalenedisulfonic acid trisodium salt, in distilled water and dilute to 500 mL. This solution is stable for at least 1 year if protected from direct sunlight.

c. Zirconyl-acid reagent: Dissolve 133 mg zirconyl chloride octahydrate, $ZrOCl_2 \cdot 8H_2O$, in about 25 mL distilled water. Add 350 mL conc HCl and dilute to 500 mL with distilled water.

d. Acid zirconyl-SPADNS reagent: Mix equal volumes of SPADNS solution and zirconyl-acid reagent. The combined reagent is stable for at least 2 years.

e. Reference solution: Add 10 mL SPADNS solution to 100 mL distilled water. Dilute 7 mL conc HCl to 10 mL and add to the diluted SPADNS solution. The resulting solution, used for setting the instrument reference point (zero), is stable for at least 1 year. Alternatively, use a prepared standard of 0 mg F⁻/L as a reference.

f. Sodium arsenite solution: Dissolve 5.0 g $NaAsO_2$ and dilute to 1 L with distilled water. (**Caution: Toxic—avoid ingestion.**)

4. Procedure

a. Preparation of standard curve: Prepare fluoride standards in the range of 0 to 1.40 mg F⁻/L by diluting appropriate quantities of standard fluoride solution to 50 mL with distilled water. Pipet 5.00 mL each of SPADNS solution and zirconyl-acid reagent, or 10.00 mL mixed acid-zirconyl-SPADNS reagent, to each standard and mix well. Avoid contamination. Set photometer to zero absorbance with the reference solution and obtain absorbance readings of standards. Plot a curve of the milligrams fluoride-absorbance relationship. Prepare a new standard curve whenever a fresh reagent is made or a different standard temperature is desired. As an alternative to using a reference, set photometer at some convenient point (0.300 or 0.500 absorbance) with the prepared 0 mg F⁻/L standard.

b. Sample pretreatment: If the sample contains residual chlorine, remove it by adding 1 drop (0.05 mL) $NaAsO_2$ solution/0.1 mg residual chlorine and mix. (Sodium arsenite concentrations of 1300 mg/L produce an error of 0.1 mg/L at 1.0 mg F⁻/L.)

c. Color development: Use a 50.0-mL sample or a portion diluted to 50 mL with distilled water. Adjust sample temperature to that used for the standard curve. Add 5.00 mL each of SPADNS solution and zirconyl-acid reagent, or 10.00 mL acid-zirconyl-SPADNS reagent; mix well and read absorbance, first setting the reference point of the photometer as above. If the absorbance falls beyond the range of the standard curve, repeat using a diluted sample.

5. Calculation

$$\text{mg F}^-\text{/L} = \frac{A}{\text{mL sample}} \times \frac{B}{C}$$

where:

A = μg F⁻ determined from plotted curve,
B = final volume of diluted sample, mL, and
C = volume of diluted sample used for color development, mL.

When the prepared 0 mg F⁻/L standard is used to set the photometer, alternatively calculate fluoride concentration as follows:

$$\text{mg F}^-\text{/L} = \frac{A_0 - A_x}{A_0 - A_1}$$

where:

A_0 = absorbance of the prepared 0 mg F⁻/L standard,
A_x = absorbance of the prepared sample, and
A_1 = absorbance of a prepared 1.0 mg F⁻/L standard.

6. Precision and Bias

A synthetic sample containing 0.830 mg F⁻/L and no interference in distilled water was analyzed in 53 laboratories by the SPADNS method, with a relative standard deviation of 8.0% and a relative error of 1.2%. After direct distillation of the sample, the relative standard deviation was 11.0% and the relative error 2.4%.

A synthetic sample containing 0.570 mg F⁻/L, 10 mg Al/L, 200 mg SO_4^{2-}/L, and 300 mg total alkalinity/L was analyzed in 53 laboratories by the SPADNS method without distillation, with a relative standard deviation of 16.2% and a relative error of 7.0%. After direct distillation of the sample, the relative standard deviation was 17.2% and the relative error 5.3%.

A synthetic sample containing 0.680 mg F⁻/L, 2 mg Al/L, 2.5 mg $(NaPO_3)_6$/L, 200 mg SO_4^{2-}/L, and 300 mg total alkalinity/L was analyzed in 53 laboratories by direct distillation and SPADNS methods with a relative standard deviation of 2.8% and a relative error of 5.9%.

7. Bibliography

Bellack, E. & P.J. Schouboe. 1968. Rapid photometric determination of fluoride with SPADNS-zirconium lake. *Anal. Chem.* 30:2032.

4500-F⁻ E. Complexone Method

1. General Discussion

a. Principle: The sample is distilled in the automated system, and the distillate is reacted with alizarin fluorine blue-lanthanum reagent to form a blue complex that is measured colorimetrically at 620 nm.

b. Interferences: Interferences normally associated with the determination of fluoride are removed by distillation.

c. Application: This method is applicable to potable, surface, and saline waters as well as domestic and industrial wastewaters. The range of the method, which can be modified by using the adjustable colorimeter, is 0.1 to 2.0 mg F^-/L.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

An example of the required continuous-flow analytical instrument consists of the interchangeable components in the number and manner indicated in Figure 4500-F^-:2.

3. Reagents

a. Standard fluoride solution: Prepare in appropriate concentrations from 0.10 to 2.0 mg F^-/L using the stock fluoride solution (see 4500-F^-.C.3*a*).

b. Distillation reagent: Add 50 mL conc H_2SO_4 to about 600 mL distilled water. Add 10.00 mL stock fluoride solution (see 4500-F^-.C.3*a*; 1.00 mL = 100 μg F^-) and dilute to 1000 mL.

c. Acetate buffer solution: Dissolve 60 g anhydrous sodium acetate, $NaC_2H_3O_2$, in about 600 mL distilled water. Add 100 mL conc (glacial) acetic acid and dilute to 1 L.

d. Alizarin fluorine blue stock solution: Add 960 mg alizarin fluorine,* $C_{14}H_7O_4 \cdot CH_2N(CH_2 \cdot COOH)_2$, to 100 mL distilled water. Add 2 mL conc NH_4OH and mix until dye is dissolved. Add 2 mL conc (glacial) acetic acid, dilute to 250 mL and store in an amber bottle in the refrigerator.

e. Lanthanum nitrate stock solution: Dissolve 1.08 g $La(NO_3)_3$ in about 100 mL distilled water, dilute to 250 mL, and store in refrigerator.

f. Working color reagent: Mix in the following order: 300 mL acetate buffer solution, 150 mL acetone, 50 mL tertiary butanol, 36 mL alizarin fluorine blue stock solution, 40 mL lanthanum nitrate stock solution, and 2 mL polyoxyethylene 23 lauryl ether.† Dilute to 1 L with distilled water. This reagent is stable for 2 to 4 d.

4. Procedure

No special handling or preparation of sample is required.

Set up manifold as shown in Figure 4500-F^-:2 and follow the manufacturer's instructions.

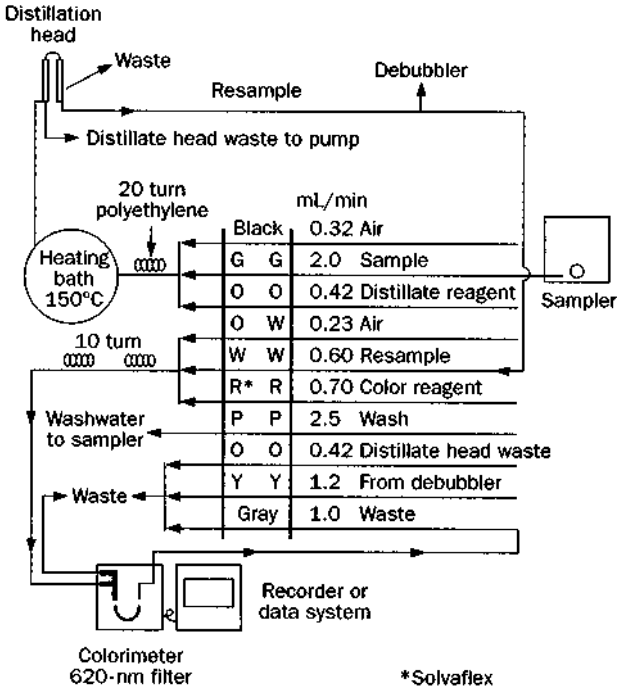


Figure 4500-F^-:2. Fluoride manifold.

5. Calculation

Prepare standard curves by plotting response of standards processed through the manifold against constituent concentrations in standards. Compute sample concentrations by comparing sample response with standard curve.

6. Precision and Bias

In a single laboratory, four samples of natural water containing from 0.40 to 0.82 mg F^-/L were analyzed in septuplicate. Average precision was ± 0.03 mg F^-/L. To two of the samples, additions of 0.20 and 0.80 mg F^-/L were made. Average recovery of the additions was 98%.

7. Bibliography

WEINSTEIN, L.H., R.H. MANDL, D.C. MCCUNE, J.S. JACOBSON & A.E. HITCHCOCK. 1963. A semi-automated method for the determination of fluorine in air and plant tissues. *Boyce Thompson Inst.* 22:207.

* J.T. Baker Catalog No. J-112, or equivalent.
† Brij-35, available from ICI Americas, Wilmington, DE, or equivalent.

4500-F⁻ F. (Reserved)

4500-F⁻ G. Ion-Selective Electrode Flow Injection Analysis

1. General Discussion

a. Principle: Fluoride is determined potentiometrically by using a combination fluoride-selective electrode in a flow cell. The fluoride electrode consists of a lanthanum fluoride crystal across which a potential is developed by fluoride ions. The reference cell is a Ag/AgCl/Cl^- cell. The reference junction is of the annular liquid-junction type and encloses the fluoride-sensitive crystal.

Also see 4500-F⁻.C and Section 4130, Flow Injection Analysis (FIA).

b. Interferences: Remove large or fibrous particulates by filtering sample through glass wool. Guard against contamination from reagents, water, glassware, and the sample preservation process.

The polyvalent cations Si^{4+}, Al^{3+}, and Fe^{3+} interfere by forming complexes with fluoride. As part of the buffer reagent, 1,2-cyclohexyldiaminetetraacetic acid (CDTA) is added to preferentially complex these cations and eliminate this interference when these concentrations do not exceed 3.0 mg Al^{3+}/L and 20 mg Fe^{3+}/L.

Some interferents are removed by distillation; see 4500-F⁻.B. Drinking water samples generally do not require sample distillation.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Flow injection analysis equipment consisting of:

a. FIA injection valve with sample loop or equivalent.

b. Multichannel proportioning pump.

c. FIA manifold (Figure 4500-F⁻:3) with tubing heater and ion-selective electrode flow cell. In Figure 4500-F⁻:3, relative flow rates only are shown. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material, such as TFE.

d. Combination ion-selective electrode.

e. Injection valve control and data acquisition system.

3. Reagents

Use reagent water (>10 megohm) for all solutions. To prevent bubble formation, degas carrier and buffer with helium. Pass He at 140 kPa (20 psi) through a helium degassing tube. Bubble He through 1 L solution for 1 min.

a. Carrier, 1.0 mg F⁻/L: Add 10 mL or 10 g stock fluoride standard (¶ *d* below) to 990 mL water and mix well.

b. Buffer: To a tared 1-L polyethylene container add 929.5 g water, 59.8 g glacial acetic acid, 30.0 g sodium hydroxide, NaOH, 58.0 g sodium chloride, NaCl, 0.5 g stock fluoride standard (¶ *d* below), and 4.0 g 1,2-cyclohexyldiaminetetraacetic acid (CDTA) (also called *trans*-1,2-diaminocyclohexane). Stir on a magnetic stir plate until all material has dissolved.

c. Electrode conditioning solution: To a tared 1-L container, add 534 g buffer (¶ *b* above) and 500 g carrier (¶ *a* above). Shake or stir to mix thoroughly. Store fluoride electrode in this solution when it is not in use.

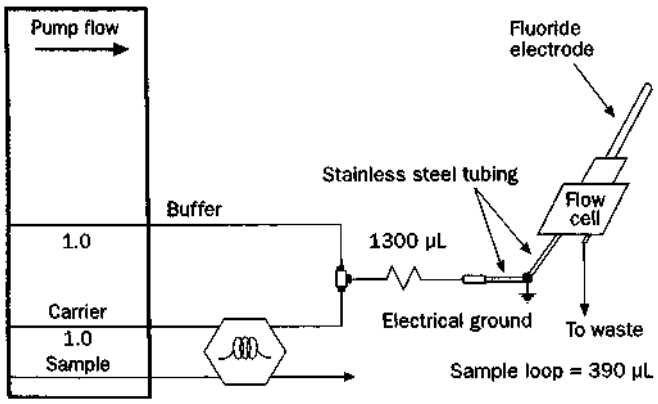


Figure 4500-F⁻:3. FIA fluoride manifold.

d. Stock fluoride standard, 100.0 mg F⁻/L: In a 1-L volumetric flask, dissolve 0.2210 g sodium fluoride, NaF, in approximately 950 mL water. Dilute to mark with water and mix well. Store in a polyethylene bottle.

e. Standard fluoride solutions: Prepare fluoride standards in the desired concentration range, using the stock standard (¶ *d* above), and diluting with water. A blank or zero concentration standard cannot be prepared for this method because it will give an undefined response from the fluoride electrode.

4. Procedure

Set up a manifold equivalent to that in Figure 4500-F⁻:3 and follow method supplied by manufacturer or laboratory standard operating procedure for this method.

5. Calculations

Prepare standard curves by plotting the electrode response to standards processed through the manifold vs. fluoride concentration. Standards greater than 1.0 mg F⁻/L will give positive peaks, standards less than 1.0 mg F⁻/L will give negative peaks, and the 1.0 mg F⁻/L standard having the same concentration as the carrier will give no peak. The calibration curve gives a good fit to a second-order polynomial.

It is not necessary to plot the response versus log[F⁻]; if this is done, the calibration curve will still be a second-order polynomial because there is a concentration-dependent kinetic effect in the flowing stream electrode system.

6. Precision and Bias

The samples used in the studies described below were not distilled.

https://doi.org/10.2105/SMWW.2882.081

a. Recovery and relative standard deviation: The results of single-laboratory studies with various matrices are given in Table 4500-F⁻:II.

b. MDL: A 390-μL sample loop was used in the method described above. Ten replicates of a 1.0-mg F⁻/L standard were run to obtain an MDL of 0.02 mg F⁻/L.

c. Precision: Ten replicate standards of 2.0 mg F⁻/L gave a % RSD of 0.5%.

TABLE 4500-F⁻:II. RESULTS OF SINGLE-LABORATORY STUDIES WITH SELECTED MATRICES

Matrix	Sample/Blank Designation	Known Addition *mg F⁻/L*	Recovery %	Relative Standard Deviation %
Wastewater treatment plant influent	Reference sample*	—	101	—
	Blank†	1.0	91	—
		2.0	97	—
	Site A‡	0	—	4.8
		1.0	93	—
		2.0	82	—
	Site B‡	0	—	6.4
		1.0	96	—
		2.0	86	—
	Site C‡§	0	—	15
		1.0	99	—
		2.0	86	—
Wastewater treatment plant effluent	Reference sample*	—	103	—
	Blank†	1.0	97	—
		2.0	97	—
	Site A‡	0	—	ND
		1.0	ND	—
		2.0	ND	—
	Site B‡	0	—	<0.1
		1.0	80	—
		2.0	78	—
	Site C‡	0	—	<0.1
		1.0	93	—
		2.0	91	—
Landfill leachate‖	Reference sample*	—	99	—
	Blank†	1.0	87	—
		2.0	88	—
	Site A‡	0	—	13
		1.0	74	—
		2.0	68	—
	Site B‡	0	—	10
		1.0	68	—
		2.0	73	—
	Site C‡	0	—	32
		1.0	66	—
		2.0	79	—

ND = not detectable.
* U.S. EPA QC sample, 1.81 mg F⁻/L.
† Determined in duplicate.
‡ Samples without known additions determined four times; samples with known additions determined in duplicate. Typical difference between duplicates for influent 5%, for effluent 6%.
§ Mean concentration 0.18 mg F⁻/L.
‖ All sites had mean concentration of <0.2 mg F⁻/L.

4500-H^+ pH VALUE*

4500-H^+ A. Introduction

1. Principles

Measurement of pH is one of the most important and frequently used tests in water chemistry. Practically every phase of water supply and wastewater treatment (e.g., acid–base neutralization, water softening, precipitation, coagulation, disinfection, and corrosion control) is pH-dependent. pH is used in alkalinity and carbon dioxide measurements and many other acid–base equilibria. At a given temperature the *intensity* of the acidic or basic character of a solution is indicated by pH or hydrogen ion activity. Alkalinity and acidity are the acid- and base-neutralizing capacities of a water and usually are expressed as milligrams $CaCO_3$ per liter. Buffer capacity is the amount of strong acid or base, usually expressed in moles per liter, needed to change the pH value of a 1-L sample by 1 unit. pH as defined by Sorenson[1] is $-\log [H^+]$; it is the "intensity" factor of acidity. Pure water is very slightly ionized and at equilibrium the ion product is

$$[H^+][OH^-] = K_w = 1.01 \times 10^{-14} \text{ at } 25°C \quad (1)$$

and

$$[H^+] = [OH^-] = 1.005 \times 10^{-7}$$

where:

$[H^+]$ = activity of hydrogen ions, moles/L,
$[OH^-]$ = activity of hydroxyl ions, moles/L, and
K_w = ion product of water.

Because of ionic interactions in all but very dilute solutions, it is necessary to use the "activity" of an ion and not its molar concentration. Use of the term pH assumes that the activity of the hydrogen ion, $a_{H}{}^{+}$, is being considered. The *approximate* equivalence to molarity, $[H^+]$ can be presumed only in very dilute solutions (ionic strength <0.1).

A logarithmic scale is convenient for expressing a wide range of ionic activities. Equation 1 in logarithmic form and corrected to reflect activity is:

$$(-\log_{10} a_{H^+}) + (-\log_{10} a_{OH^-}) = 14 \quad (2)$$

or

$$\text{pH} + \text{pOH} = \text{p}K_w$$

where:

pH† = $\log_{10} a_{H+}$ and
pOH = $\log_{10} a_{OH}{}^{-}$.

Equation 2 states that as pH increases pOH decreases correspondingly and vice versa because pK_w is constant for a given temperature. At 25°C, pH 7.0 is neutral, the activities of the hydrogen and hydroxyl ions are equal, and each corresponds to an approximate activity of 10^{-7} moles/L. The neutral point is temperature-dependent and is pH 7.5 at 0°C and pH 6.5 at 60°C.

The pH value of a highly dilute solution is approximately the same as the negative common logarithm of the hydrogen ion concentration. Natural waters usually have pH values in the range of 4 to 9, and most are slightly basic because of the presence of bicarbonates and carbonates of the alkali and alkaline earth metals.

2. Reference

1. Sorenson, S. 1909. Über die Messung und die Bedeutung der Wasserstoff ionen Konzentration bei Enzymatischen Prozessen. *Biochem. Z.* 21:131.

* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011, 2017.

† p designates $-\log_{10}$ of a number.

4500-H^+ B. Electrometric Method

1. General Discussion

a. Principle: The basic principle of electrometric pH measurement is determination of the activity of the hydrogen ions by potentiometric measurement using a standard hydrogen electrode and a reference electrode. The hydrogen electrode consists of a platinum electrode across which hydrogen gas is bubbled at a pressure of 101 kPa. Because of difficulty in its use and the potential for poisoning the hydrogen electrode, the glass electrode commonly is used. The electromotive force (emf) produced in the glass electrode system varies linearly with pH. This linear relationship is described by plotting the measured emf against the pH of different buffers. Sample pH is determined by extrapolation.

https://doi.org/10.2105/SMWW.2882.082

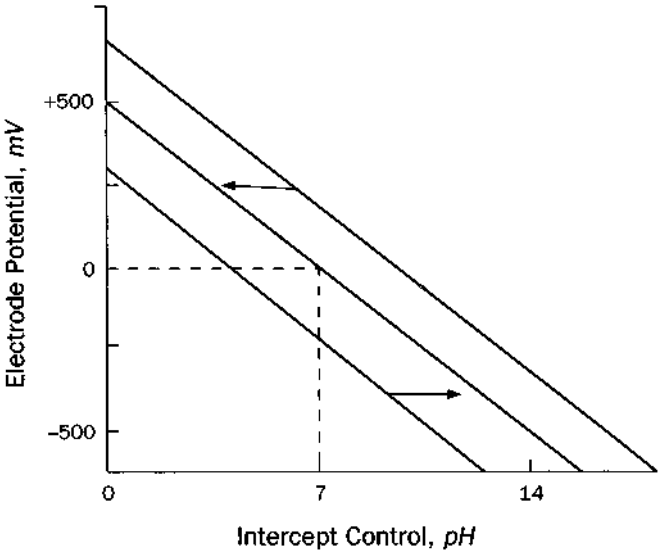


Figure 4500-H$^+$:1. Electrode potential vs. pH. Intercept control shifts response curve laterally.

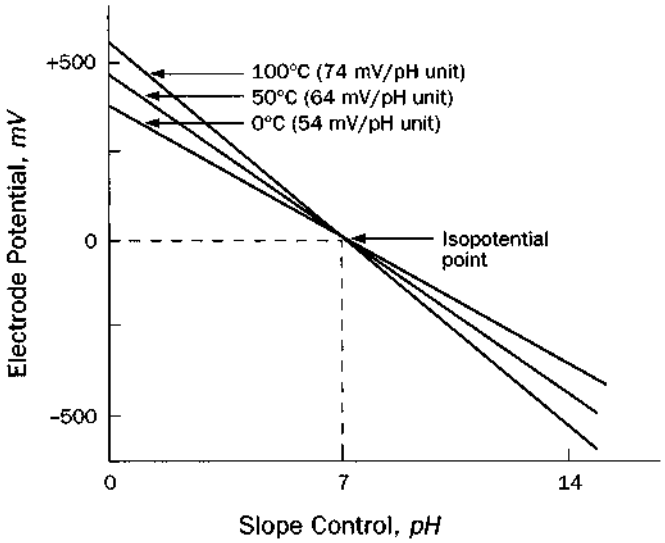


Figure 4500-H$^+$:2. Typical pH electrode response as a function of temperature.

Because single ion activities, such as a_{H^+}, cannot be measured, pH is defined operationally on a potentiometric scale. The pH measuring instrument is calibrated potentiometrically with an indicating (glass) electrode and a reference electrode using National Institute of Standards and Technology (NIST) buffers having assigned values so:

$$pH_B = -\log_{10} a_{H^+}$$

where:

pH_B = assigned pH of NIST buffer.

The operational pH scale is used to measure sample pH and is defined as:

$$pH_x = pH_B \pm \frac{F(E_x - E_s)}{2.303\,RT}$$

where:

pH_x = potentiometrically measured sample pH,
F = Faraday: 9.649×10^4 coulomb/mole,
E_x = sample emf, V,
E_s = buffer emf, V,
R = gas constant; 8.314 joule/(mole K), and
T = absolute temperature, K.

NOTE: Although the equation for pH_x appears in the literature with a plus sign, the sign of emf readings in millivolts for most pH meters manufactured in the United States is negative. The choice of negative sign is consistent with the IUPAC Stockholm convention concerning the sign of electrode potential.[1,2]

The activity scale gives values that are higher than those on Sorenson's scale by 0.04 units:

$$pH\ (activity) = pH\ (Sorenson) + 0.04$$

The equation for pH_x assumes that the emf of the cells containing the sample and buffer is due solely to hydrogen ion activity unaffected by sample composition. In practice, samples will have varying ionic species and ionic strengths, both affecting H^+ activity. This imposes an experimental limitation on pH measurement; thus, to obtain meaningful results, the differences between E_x and E_s should be minimal. Samples must be dilute aqueous solutions of simple solutes ($<0.2M$). (Choose buffers to bracket the sample.) Determination of pH cannot be made accurately in nonaqueous media, suspensions, colloids, or high-ionic-strength solutions.

b. Interferences: The glass electrode is relatively free from interference from color, turbidity, colloidal matter, oxidants, reductants, or high salinity, except for a sodium error at pH >10. Reduce this error by using special "low sodium error" electrodes.

pH measurements are affected by temperature in two ways: mechanical effects that are caused by changes in the properties of the electrodes and chemical effects caused by equilibrium changes. In the first instance, the Nernstian slope increases with increasing temperature and electrodes take time to achieve thermal equilibrium. This can cause long-term drift in pH. Because chemical equilibrium affects pH, standard pH buffers have a specified pH at indicated temperatures.

Always report temperature at which pH is measured.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. pH meter consisting of potentiometer, a glass electrode, a reference electrode, and a temperature-compensating device. A circuit is completed through the potentiometer when the electrodes are immersed in the test solution. Many pH meters are capable of reading pH or millivolts and some have scale expan-

TABLE 4500-H^+:I. PREPARATION OF pH STANDARD SOLUTIONS[3]

Standard Solution (molality)	pH at 25°C	Weight of Chemicals Needed/1000 mL Pure Water at 25°C
Primary standards:		
Potassium hydrogen tartrate (saturated at 25°C)	3.557	> 7 g $KHC_4H_4O_6$*
0.05 potassium dihydrogen citrate	3.776	11.41 g $KH_2C_6H_5O_7$
0.05 potassium hydrogen phthalate	4.004	10.12 g $KHC_8H_4O_4$
0.025 potassium dihydrogen phosphate + 0.025 disodium hydrogen phosphate	6.863	3.387 g KH_2PO_4 + 3.533 g Na_2HPO_4†
0.008 695 potassium dihydrogen phosphate + 0.030 43 disodium hydrogen phosphate	7.415	1.179 g KH_2PO_4 + 4.303 g Na_2HPO_4†
0.01 sodium borate decahydrate (borax)	9.183	3.80 g $Na_2B_4O_7 \cdot 10H_2O$†
0.025 sodium bicarbonate + 0.025 sodium carbonate	10.014	2.092 g $NaHCO_3$ + 2.640 g Na_2CO_3
Secondary standards:		
0.05 potassium tetroxalate dihydrate	1.679	12.61 g $KH_3C_4O_8 \cdot 2H_2O$
Calcium hydroxide (saturated at 25°C)	12.454	> 2 g $Ca(OH)_2$*

* Approximate solubility.
† Prepare with freshly boiled and cooled distilled water (carbon-dioxide-free).

sion that permits reading to 0.001 pH unit, but most instruments are not that precise.

For routine work, use a pH meter accurate and reproducible to 0.1 pH unit with a range of 0 to 14 and equipped with a temperature-compensation adjustment.

Although manufacturers provide operating instructions, the use of different descriptive terms may be confusing. For most instruments, there are two controls: intercept (set buffer, asymmetry, standardize) and slope (temperature, offset); their functions are shown diagramatically in Figures 4500-H^+:1 and 2. The intercept control shifts the response curve laterally to pass through the isopotential point with no change in slope. This permits bringing the instrument on scale (0 mV) with a pH 7 buffer that has no change in potential with temperature.

The slope control rotates the emf/pH slope about the isopotential point (0 mV/pH 7). To adjust slope for temperature without disturbing the intercept, select a buffer that brackets the sample with pH 7 buffer and adjust slope control to pH of this buffer. The instrument will indicate correct millivolt change per unit pH at the test temperature.

b. Reference electrode consisting of a half cell that provides a constant electrode potential. Commonly used are calomel and silver: silver-chloride electrodes. Either is available with several types of liquid junctions.

The liquid junction of the reference electrode is critical because at this point the electrode forms a salt bridge with the sample or buffer and a liquid junction potential is generated that in turn affects the potential produced by the reference electrode. Reference electrode junctions may be annular ceramic, quartz, or asbestos fiber, or the sleeve type. The quartz type is most widely used. The asbestos fiber type is not recommended for strongly basic solutions. Follow the manufacturer's recommendation on use and care of the reference electrode.

Refill nonsealed electrodes with the correct electrolyte to proper level and make sure junction is properly wetted.

c. Glass electrode: The sensor electrode is a bulb of special glass containing a fixed concentration of HCl or a buffered chloride solution in contact with an internal reference electrode. Upon immersion of a new electrode in a solution, the outer bulb surface becomes hydrated and exchanges sodium ions for hydrogen ions to build up a surface layer of hydrogen ions. This, together with the repulsion of anions by fixed, negatively charged silicate sites, produces at the glass–solution interface a potential that is a function of hydrogen ion activity in solution.

Several types of glass electrodes are available. Combination electrodes incorporate the glass and reference electrodes into a single probe. Use a "low sodium error" electrode that can operate at high temperatures for measuring pH > 10 because standard glass electrodes yield erroneously low values. For measuring pH <1 standard glass electrodes yield erroneously high values; use liquid membrane electrodes instead.

d. Beakers: Preferably use polyethylene or TFE* beakers.

e. Stirrer: Use either a magnetic, TFE-coated stirring bar or a mechanical stirrer with inert plastic-coated impeller.

f. Flow chamber: Use for continuous flow measurements or for poorly buffered solutions.

3. Reagents

a. General preparation: Calibrate the electrode system against standard buffer solutions of known pH. Because buffer solutions may deteriorate as a result of mold growth or contamination, prepare fresh as needed for accurate work by weighing the amounts of chemicals specified in Table 4500-H^+:I, dissolving in distilled water at 25°C, and diluting to 1000 mL. This is particularly important for borate and carbonate buffers.

Boil and cool distilled water having a conductivity of less than 2 μmhos/cm. To 50 mL, add 1 drop of saturated KCl solution suitable for reference electrode use. If the pH of this test solution is between 6.0 and 7.0, use it to prepare all standard solutions.

Dry KH_2PO_4 at 110 to 130°C for 2 h before weighing but do not heat unstable hydrated potassium tetroxalate above 60°C nor dry the other specified buffer salts.

* Teflon, or equivalent

TABLE 4500-H$^+$:II. STANDARD pH VALUES[3]

Temperature °C	Primary Standards							Secondary Standards	
	Tartrate (Saturated)	Citrate (0.05*M*)	Phthalate (0.05*M*)	Phosphate (1:1)	Phosphate (1:3.5)	Borax (0.01*M*)	Bicarbonate-Carbonate (0.025*M*)	Tetroxalate (0.05*M*)	Calcium Hydroxide (Saturated)
0			4.003	6.982	7.534	9.460	10.321	1.666	
5			3.998	6.949	7.501	9.392	10.248	1.668	
10			3.996	6.921	7.472	9.331	10.181	1.670	
15			3.996	6.898	7.449	9.276	10.120	1.672	
20			3.999	6.878	7.430	9.227	10.064	1.675	
25	3.557	3.776	4.004	6.863	7.415	9.183	10.014	1.679	12.454
30	3.552		4.011	6.851	7.403	9.143	9.968	1.683	
35	3.549		4.020	6.842	7.394	9.107	9.928	1.688	
37			4.024	6.839	7.392	9.093			
40	3.547		4.030	6.836	7.388	9.074	9.891	1.694	
45	3.547		4.042	6.832	7.385	9.044	9.859	1.700	
50	3.549		4.055	6.831	7.384	9.017	9.831	1.707	
55	3.554		4.070					1.715	
60	3.560		4.085					1.723	
70	3.580		4.12					1.743	
80	3.609		4.16					1.766	
90	3.650		4.19					1.792	
95	3.674		4.21					1.806	

Although ACS-grade chemicals generally are satisfactory for preparing buffer solutions, use certified materials available from the National Institute of Standards and Technology when the greatest accuracy is required. For routine analysis, use commercially available buffer tablets, powders, or solutions of tested quality. In preparing buffer solutions from solid salts, ensure complete solution.

As a rule, select and prepare buffer solutions classed as primary standards in Table 4500-H$^+$:I; reserve secondary standards for extreme situations encountered in wastewater measurements. Consult Table 4500-H$^+$:II for accepted pH of standard buffer solutions at temperatures other than 25°C. In routine use, store buffer solutions and samples in polyethylene bottles. Replace buffer solutions every 6 months.

b. Saturated potassium hydrogen tartrate solution: Shake vigorously an excess (5 to 10 g) of finely crystalline $KHC_4H_4O_6$ with 100 to 300 mL distilled water at 25°C in a glass-stoppered bottle. Separate clear solution from undissolved material by decantation or filtration. Preserve for 2 months or more by adding one thymol crystal (8 mm diam) per 200 mL solution.

c. Saturated calcium hydroxide solution: Calcine a well-washed, low-alkali grade $CaCO_3$ in a platinum dish by igniting for 1 h at 1000°C. Cool, hydrate by slowly adding distilled water with stirring, and heat to boiling. Cool, filter, and collect solid $Ca(OH)_2$ on a fritted glass filter of medium porosity. Dry at 110°C, cool, and pulverize to uniformly fine granules. Vigorously shake an excess of fine granules with distilled water in a stoppered polyethylene bottle. Let temperature come to 25°C after mixing. Filter supernatant under suction through a sintered glass filter of medium porosity and use filtrate as the buffer solution. Discard buffer solution when atmospheric CO_2 causes turbidity to appear.

d. Auxiliary solutions: 0.1*N* NaOH, 0.1*N* HCl, 5*N* HCl (dilute five volumes 6*N* HCl with one volume distilled water), and acid potassium fluoride solution (dissolve 2 g KF in 2 mL conc H_2SO_4 and dilute to 100 mL with distilled water).

4. Procedure

a. Instrument calibration: In each case, follow manufacturer's instructions for pH meter and for storage and preparation of electrodes for use. Recommended solutions for short-term storage of electrodes vary with type of electrode and manufacturer, but generally have a conductivity greater than 4000 μmhos/cm. A pH 4 buffer is best for the single glass electrode and saturated KCl is preferred for a calomel and Ag/AgCl reference electrode. Saturated KCl is the preferred solution for a combination electrode. Keep electrodes wet by returning them to storage solution whenever pH meter is not in use.

Before use, remove electrodes from storage solution, rinse, blot dry with a soft tissue, place in initial buffer solution, and set the isopotential point (4500-H$^+$.B.2*a*). Select a second buffer within 2 pH units of sample pH and bring sample and buffer to same temperature, which may be the room temperature; a fixed temperature, such as 25°C; or the temperature of a fresh sample. Remove electrodes from first buffer, rinse thoroughly with distilled water, blot dry, and immerse in second buffer. Record temperature of measurement and adjust temperature dial on meter so meter indicates pH value of buffer at test temperature (this is a slope adjustment).

Use the pH value listed in the tables for the buffer used at the test temperature. Remove electrodes from second buffer, rinse thoroughly with distilled water and dry electrodes as indicated above. Immerse in a third buffer below pH 10, approximately

3 pH units different from the second; the reading should be within 0.1 unit for the pH of the third buffer. If the meter response shows a difference greater than 0.1 pH unit from expected value, look for trouble with the electrodes or potentiometer (4500-H^+.B.5*a* and *b*).

The purpose of standardization is to adjust the response of the glass electrode to the instrument. When only occasional pH measurements are made, standardize instrument before each measurement. When frequent measurements are made and the instrument is stable, standardize less frequently. If sample pH values vary widely, standardize for each sample with a buffer having a pH within 1 to 2 pH units of the sample.

b. Sample analysis: Establish equilibrium between electrodes and sample by stirring sample to ensure homogeneity; stir gently to minimize carbon dioxide entrainment. For buffered samples or those of high ionic strength, condition electrodes after cleaning by dipping them into sample for 1 min. Blot dry, immerse in a fresh portion of the same sample, and read pH.

With dilute, poorly buffered solutions, equilibrate electrodes by immersing in three or four successive portions of sample. Take a fresh sample to measure pH.

5. Troubleshooting

a. Potentiometer: To locate trouble source, disconnect electrodes and, using a short-circuit strap, connect reference electrode terminal to glass electrode terminal. Observe change in pH when instrument calibration knob is adjusted. If potentiometer is operating properly, it will respond rapidly and evenly to changes in calibration over a wide scale range. A faulty potentiometer will fail to respond, will react erratically, or will show a drift upon adjustment. Switch to the millivolt scale on which the meter should read zero. If inexperienced, do not attempt potentiometer repair other than maintenance as described in instrument manual.

b. Electrodes: If potentiometer is functioning properly, look for the instrument fault in the electrode pair. Substitute one electrode at a time and cross-check with two buffers that are about 4 pH units apart. A deviation greater than 0.1 pH unit indicates a faulty electrode. Glass electrodes fail because of scratches, deterioration, or accumulation of debris on the glass surface. Rejuvenate electrode by alternately immersing it three times each in 0.1*N* HCl and 0.1*N* NaOH. If this fails, immerse tip in KF solution for 30 s. After rejuvenation, soak in pH 7.0 buffer overnight. Rinse and store in pH 7.0 buffer. Rinse again with distilled water before use. Protein coatings can be removed by soaking glass electrodes in a 10% pepsin solution adjusted to pH 1 to 2.

To check reference electrode, oppose the emf of a questionable reference electrode against another one of the same type that is known to be good. Using an adapter, plug good reference electrode into glass electrode jack of potentiometer; then plug questioned electrode into reference electrode jack. Set meter to read millivolts and take readings with both electrodes immersed in the same electrolyte (KCl) solution and then in the same buffer solution. The millivolt readings should be 0 ± 5 mV for both solutions. If different electrodes are used (i.e., silver:silver-chloride against calomel or vice versa) the reading will be 44 ± 5 mV for a good reference electrode.

Reference electrode troubles generally are traceable to a clogged junction. Interruption of the continuous trickle of electrolyte through the junction causes increase in response time and drift in reading. Clear a clogged junction by applying suction to the tip or by boiling tip in distilled water until the electrolyte flows freely when suction is applied to tip or pressure is applied to the fill hole. Replaceable junctions are available commercially.

6. Precision and Bias

By careful use of a laboratory pH meter with good electrodes, a precision of ±0.02 pH unit and an accuracy of ±0.05 pH unit can be achieved. However, ±0.1 pH unit represents the limit of accuracy under normal conditions, especially for measurement of water and poorly buffered solutions. For this reason, report pH values to the nearest 0.1 pH unit. A synthetic sample of a Clark and Lubs buffer solution of pH 7.3 was analyzed electrometrically by 30 laboratories with a standard deviation of ±0.13 pH unit.

7. References

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4500-I$^-$ IODIDE*

4500-I$^-$ A. Introduction

1. Occurrence

Iodide is found in natural waters at concentrations ranging from 40 μg I$^-$/L in coastal surface seawater to $<$1 μg I$^-$/L in deep ocean water and fresh water. Higher concentrations may be found in brines, certain industrial wastes, and waters treated with iodine. Iodide is thermodynamically unstable relative to iodate in oxygenated waters.

2. Selection of Method

The leuco crystal violet method (4500-I$^-$.B) is applicable to iodide concentrations of 50 to 6000 μg/L. The catalytic reduction method (4500-I$^-$.C) is applicable to iodide concentrations of 80 μg I$^-$/L or less. The voltammetric method (4500-I$^-$.D) is the most sensitive method. It can be used for samples with iodide concentrations of 0.13 to 10.2 μg I$^-$/L. It is also species-specific. It is insensitive to iodate, iodine, and most organic iodine compounds. It requires minimal sample manipulation, aside from an occasional dilution for samples with high concentrations of iodide. Thus, the concentrations of iodide in many types of water samples may be determined directly with the voltammetric method.

The choice of method depends on the sample and concentration to be determined. The high chloride concentrations of brines, seawater, and many estuarine waters will interfere with color development in the leuco crystal violet method. In the presence of iodine, the leuco crystal violet method gives the sum of iodine and iodide. Iodide may be determined by the difference after concentration of iodine has been estimated independently (see Section 4500-I). In the catalytic reduction method, As(III), under acidic conditions, is a strong reducing agent and will reduce the oxidized forms of iodine to iodide. Thus, this method measures not only iodide, but also the sum of all the inorganic iodine species, including iodide, iodate, hypoiodous acid, hypoiodite ion, and elemental iodine. Because iodate is the thermodynamically stable form of dissolved iodine in oxygenated natural waters and is frequently the dominant species of dissolved iodine, the catalytic reduction method is likely to overestimate the concentration of iodide. This method works well only under exactly reproducible conditions.

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4500-I$^-$ B. Leuco Crystal Violet Method

1. General Discussion

a. Principle: Iodide is selectively oxidized to iodine by the addition of potassium peroxymonosulfate, $KHSO_5$. The iodine produced reacts instantaneously with the colorless indicator reagent containing 4,4′,4″-methylidynetris (*N,N*-dimethylaniline), also known as leuco crystal violet, to produce the highly colored crystal violet dye. The developed color is sufficiently stable for the determination of an absorbance value and adheres to Beer's law over a wide range of iodine concentrations. Absorbance is highly pH-dependent, and must be measured within the pH range of 3.5 to 4.0 at a wavelength of 592 nm. Accurate control of pH is essential for maximum precision. (See Section 4500-I.B.1*a*.) Follow the general principles for quality control (Section 4020).

b. Interference: Chloride concentrations greater than 200 mg/ L may interfere with color development. Reduce these interferences by diluting sample to contain less than 200 mg Cl$^-$/L.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Colorimetric equipment: One of the following is required:

1) *Filter photometer,* providing a light path of 1 cm or longer, equipped with an orange filter having maximum transmittance near 592 nm.
2) *Spectrophotometer*, for use at 592 nm, providing a light path of 1 cm or longer.

b. Volumetric flasks: 100-mL with plastic caps or ground-glass stoppers.

c. Glassware: Completely remove any reducing substances from all glassware or plastic containers, including containers for storing reagent solutions (see Section 4500-Cl.D.2*d*).

3. Reagents

a. Iodine-demand-free water: Prepare a 1-m ion-exchange column of 2.5 to 5 cm diam, containing strongly acid cation and strong basic anion exchange resins. If a commercial analytical-grade mixed-bed resin is used, verify that compounds that react with iodine are removed. Pass distilled water at a slow rate through the resin bed and collect in clean container that will protect the treated water from undue exposure to the atmosphere.

Prepare all stock iodide and reagent solutions with iodine-demand-free water.

b. Stock iodide solution: Dissolve 1.3081 g KI in water and dilute to 1000 mL; 1 mL = 1 mg I$^-$.

c. Citric buffer solution, pH 3.8:

1) *Citric acid*—Dissolve 192.2 g $C_6H_8O_7$ or 210.2 g $C_6H_8O_7 \cdot H_2O$ and dilute to 1 L with water.

2) *Ammonium hydroxide,* 2*N*—Add 131 mL conc NH_4OH to about 700 mL water and dilute to 1 L. Store in a polyethylene bottle.

3) *Final buffer solution*—Slowly add, with mixing, 350 mL 2*N* NH_4OH solution to 670 mL citric acid. Add 80 g ammonium dihydrogen phosphate ($NH_4H_2PO_4$) and stir to dissolve.

d. Leuco crystal violet indicator: Measure 200 mL water and 3.2 mL conc sulfuric acid (H_2SO_4) into a brown glass container of at least 1-L capacity. Introduce a magnetic stirring bar and mix at moderate speed. Add 1.5 g 4,4′,4″-methylidynetris (*N,N*-dimethylaniline)* and with a small amount of water wash down any reagent adhering to neck or sides of container. Mix until dissolved.

To 800 mL water, add 2.5 g mercuric chloride ($HgCl_2$) and stir to dissolve. With mixing, add $HgCl_2$ solution to leuco crystal violet solution. For maximum stability, adjust pH of final solution to 1.5 or less, adding, if necessary, conc H_2SO_4 dropwise. Store in a brown glass bottle away from direct sunlight. Discard after 6 months. Do not use a rubber stopper.

e. Potassium peroxymonosulfate solution: Obtain $KHSO_5$ as a commercial product,† which is a stable powdered mixture containing 42.8% $KHSO_5$ by weight and a mixture of $KHSO_4$ and K_2SO_4. Dissolve 1.5 g powder in water and dilute to 1 L.

f. Sodium thiosulfate solution: Dissolve 5.0 g $Na_2S_2O_3 \cdot 5H_2O$ in water and dilute to 1 L.

4. Procedure

a. Preparation of temporary iodine standards: Add suitable portions of stock iodide solution, or of dilutions of stock iodide solution, to water to prepare a series of 0.1 to 6.0 mg I^-/L in increments of 0.1 mg/L or larger.

Measure 50.0 mL dilute KI standard solution into a 100-mL glass-stoppered volumetric flask. Add 1.0 mL citric buffer and 0.5 mL $KHSO_5$ solution. Swirl to mix and let stand approximately 1 min. Add 1.0 mL leuco crystal violet indicator, mix, and dilute to 100 mL. For best results, read absorbance as described below within 5 min after adding indicator solution.

b. Photometric calibration: Transfer colored temporary standards of known iodide concentrations to cells of 1-cm light path and read absorbance in a photometer or spectrophotometer at a wavelength of 592 nm against a water reference. Plot absorbance values against iodide concentrations to construct a curve that follows Beer's law.

c. Color development of sample: Measure a 50.0-mL sample into a 100-mL volumetric flask and treat as described for preparation of temporary iodine standards, ¶ *a* above. Read absorbance photometrically and refer to standard calibration curve for iodide equivalent.

d. Samples containing >6.0 mg I^-/L: Place approximately 25 mL water in a 100-mL volumetric flask. Add 1.0 mL citric buffer and a measured volume of 25 mL or less of sample. Add 0.5 mL $KHSO_5$ solution. Swirl to mix and let stand approximately 1 min. Add 1.0 mL leuco crystal violet indicator, mix, and dilute to 100 mL.

Read absorbance photometrically and compare with calibration curve from which the initial iodide concentration is obtained by applying the dilution factor. Select one of the following sample volumes to remain within the optimum iodide range:

Iodide *mg/L*	Sample Volume Required *mL*
6.0–12.0	25.0
12.0–30	10.0
30–60	5.0

e. Determination of iodide in the presence of iodine: On separate samples determine (1) total iodide and iodine, and (2) iodine. The iodide concentration is the difference between the iodine determined and the total iodine-iodide obtained. Determine iodine by not adding $KHSO_5$ solution in the iodide method and by comparing the absorbance value to the calibration curve developed for iodide.

f. Compensation for turbidity and color: Compensate for natural color or turbidity by adding 5 mL $Na_2S_2O_3$ solution to a 50-mL sample. Add reagents to sample as described previously and use as the blank to set zero absorbance on photometer. Measure all samples in relation to this blank and, from the calibration curve, determine concentrations of iodide or total iodine-iodide.

5. Bibliography

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* Eastman Chemical No. 3651, or equivalent.

† Oxone, E.I. duPont de Nemours and Co., Inc., Wilmington, DE, or equivalent.

4500-I^- C. Catalytic Reduction Method

1. General Discussion

a. Principle: Iodide can be determined by using its ability to catalyze the reduction of ceric ions by arsenious acid. The effect is nonlinearly proportional to the amount of iodide present. The reaction is stopped after a specific time interval by the addition of ferrous ammonium sulfate. The resulting ferric ions are directly proportional to the remaining ceric ions and develop a relatively stable color complex with potassium thiocyanate.

Pretreatment by digestion with chromic acid and distillation is necessary to estimate the nonsusceptible bound forms of iodine.

b. Interferences: The formation of noncatalytic forms of iodine and the inhibitory effects of silver and mercury are reduced by adding an excess of sodium chloride (NaCl) that sensitizes the reaction. Iodate, hypoiodous acid/hypoiodite ion, and ele-

mental iodine interfere. Under acidic conditions, As(III) may reduce these forms of inorganic iodine to iodide and include them as iodide in the subsequent detection of iodide.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Water bath, capable of temperature control to 30 ± 0.5°C.

b. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer,* for use at wavelengths of 510 or 525 nm and providing a light path of 1 cm.

2) *Filter photometer,* providing a light path of 1 cm and equipped with a green filter having maximum transmittance near 525 nm.

c. Test tubes, 2 × 15 cm.

d. Stopwatch.

3. Reagents

Store all stock solutions in tightly stoppered containers in the dark. Prepare all reagent solutions in distilled water.

a. Distilled water, containing less than 0.3 μg total I/L.

b. Sodium chloride solution: Dissolve 200.0 g NaCl in water and dilute to 1 L. Recrystallize the NaCl if an interfering amount of iodine is present, using a water–ethanol mixture.

c. Arsenious acid: Dissolve 4.946 g As_2O_3 in water, add 0.20 mL conc H_2SO_4, and dilute to 1000 mL.

d. Sulfuric acid (H_2SO_4), conc.

e. Ceric ammonium sulfate: Dissolve 13.38 g $Ce(NH_4)_4(SO_4)_4 \cdot 4H_2O$ in water, add 44 mL conc H_2SO_4, and make up to 1 L.

f. Ferrous ammonium sulfate reagent: Dissolve 1.50 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in 100 mL distilled water containing 0.6 mL conc H_2SO_4. Prepare daily.

g. Potassium thiocyanate solution: Dissolve 4.00 g KSCN in 100 mL water.

h. Stock iodide solution: Dissolve 261.6 mg anhydrous KI in water and dilute to 1000 mL; 1.00 mL = 200 μg I^-.

i. Intermediate iodide solution: Dilute 20.00 mL stock iodide solution to 1000 mL with water; 1.00 mL = 4.00 μg I^-.

j. Standard iodide solution: Dilute 25.00 mL intermediate iodide solution to 1000 mL with water; 1.00 mL = 0.100 μg I^-.

4. Procedure

a. Sample size: Add 10.00 mL sample, or a portion made up to 10.00 mL with water, to a 2- × 15-cm test tube. If possible, keep iodide content in the range 0.2 to 0.6 μg. Use thoroughly clean glassware and apparatus.

b. Color measurement: Add reagents in the following order: 1.00 mL NaCl solution, 0.50 mL As_2O_3 solution, and 0.50 mL conc H_2SO_4.

Place reaction mixture and ceric ammonium sulfate solution in 30°C water bath and let come to temperature equilibrium. Add 1.0 mL ceric ammonium sulfate solution, mix by inversion, and start stopwatch to time reaction. Use an inert clean test tube stopper when mixing. After 15 ± 0.1 min remove sample from water bath and add immediately 1.00 mL ferrous ammonium sulfate reagent with mixing, whereupon the yellow ceric ion color should disappear. Then add, with mixing, 1.00 mL KSCN solution. Replace sample in water bath. Within 1 h after adding thiocyanate, read absorbance in a photometric instrument. Maintain temperature of solution and cell compartment at 30 ± 0.5°C until absorbance is determined. If several samples are run, start reactions at 1-min intervals to allow time for additions of ferrous ammonium sulfate and thiocyanate. (If temperature control of cell compartment is not possible, let final solution come to room temperature and measure absorbance with cell compartment at room temperature.)

c. Calibration standards: Treat standards containing 0, 0.2, 0.4, 0.6, and 0.8 μg I^-/10.00 mL of solution as in ¶ *b* above. Run with each set of samples to establish a calibration curve.

5. Calculation

$$\text{mg } I^-/\text{L} = \frac{\mu\text{g I (in 15 mL final volume)}}{\text{mL sample}}$$

6. Precision and Bias

Results obtained by this method are reproducible on samples of Los Angeles source waters, and have been reported to be accurate to ±0.3 μg I^-/L on samples of Yugoslavian water containing from 0 to 14.0 μg I^-/L.

7. Bibliography

ROGINA, B. & M. DUBRAVCIC. 1953. Microdetermination of iodides by arresting the catalytic reduction of ceric ions. *Analyst* 78:594.

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4500-I^- D. Voltammetric Method

1. General Discussion

a. Principle: Iodide is deposited onto the surface of a static mercury drop electrode (SMDE) as mercurous iodide under an applied potential for a specified period of time. The deposited mercurous iodide is reduced by a cathodic potential scan. This reaction gives rise to a current peak at about −0.33 V relative to the saturated calomel electrode. The height of the current peak is directly proportional to the concentration of iodide in solution, which is quantified by the method of internal standard additions.

b. Interferences: Sulfide can interfere. Remove it as hydrogen sulfide by acidifying the sample and then purging it with air. Adjust pH of sample back to about pH 8 before analysis.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Voltammetric analyzer system, consisting of a potentiostat, static mercury drop electrode (SMDE), stirrer, and plotter, that can be operated in the cathodic stripping square wave voltammetry-SMDE mode with adjustable deposition potential, deposition time, equilibration time, scan rate, scan range, scan increment, pulse height, frequency, and drop size.

A saturated calomel electrode is used as the reference electrode through a salt bridge.

b. Glassware: Wash glassware and other surfaces contacting the sample or reagents with 10% (v/v) HCl (low in iodide); thoroughly rinse with reagent water (see Section 1080) before use.

3. Reagents

Use chemicals low in iodide whenever available.

a. Oxygen-free water: Remove oxygen in reagent water (see Section 1080) by bubbling it with argon gas while boiling it for 20 min in an Erlenmeyer flask. Let water cool while argon bubbling continues. Tightly stopper flask and store water under nitrogen. Prepare water immediately before use.

b. Alkaline pyrogallol solution: Dissolve 30 g pyrogallol in 200 mL oxygen-free water. Dissolve 120 g potassium hydroxide (KOH) in 400 mL oxygen-free water. Mix 300 mL KOH solution with 100 mL pyrogallol solution.

c. Sodium sulfite solution, 1*M:* Dissolve 1.26 g sodium sulfite, Na_2SO_3, in oxygen-free water and dilute to 10 mL.

d. Sodium sulfite solution, 0.1*M:* Dilute 5.0 mL 1*M* sodium sulfite solution to 50 mL. Prepare fresh daily.

e. Oxygen-free argon gas: Bubble argon gas (at least 99.99% pure) through a series of three traps containing, respectively, alkaline pyrogallol solution, 0.1*M* sodium sulfite solution, and oxygen-free water.

f. Standard iodide solution: Dry several grams potassium iodide, KI, in an oven at 80°C overnight. Dissolve 1.660 g dried KI in reagent water (see Section 1080) and dilute to 500 mL. Dilute 5 mL solution to 500 mL, and dilute 5 mL of the latter solution to 500 mL.

g. Polyethylene glycol p-*isooctylphenyl ether (PEG-IOPE) solution,* 0.2%: Dilute 0.2 mL commercially available reagent* to 100 mL in reagent water (see Section 1080).

4. Procedure

a. Sample measurement: Transfer 10 mL sample, 0.05 mL PEG-IOPE solution, and 0.2 mL 1*M* Na_2SO_3 solution (which also acts as the supporting electrolyte in fresh-water samples) to polarographic cell containing a magnetic stirrer. Purge solution with oxygen-free argon gas for 1 min. Set electrode at SMDE mode. Record a voltammogram in the cathodic stripping square wave voltammetry mode under the following conditions: deposition potential, −0.15 V; deposition time, 60 s; equilibration time, 5 s; scan rate, 200 mV/s; scan range, 0.15 to −0.6 V; scan increment, 2 mV; pulse height, 20 mV; frequency, 100 Hz; and the largest drop size. Measure magnitude of current peak above baseline at center of peak at an applied potential of about −0.33 V relative to saturated calomel electrode in the voltammogram.

b. Internal standard additions: Add 0.1 mL 2 μM standard KI solution to the cell. Purge solution with oxygen-free argon gas for 0.5 min. Record a voltammogram under conditions described in ¶ *a* above and again determine magnitude of current peak. Repeat procedure twice, for a total of three additions.

c. Blank determination: Determine method reagent blank by treating reagent water as a sample.

5. Calculation

For the *j*th addition of the standard KI ($j = 0, 1, 2, 3$), compute the following variables:

$$Y_j = I_j\,(V_x + jV_s + V_c)$$

$$X_j = jV_sC_s$$

where:

I_j = height of *j*th peak, nA,
V_x = sample volume, mL,
V_s = volume of standard KI added during each internal addition, mL,
V_c = total volume of PEG-IOPE solution and sodium sulfite added during analysis, mL, and
C_s = concentration of iodide in standard KI solution, n*M*.

Determine slope, *B*, and intercept, *A*, of line relating Y_j to X_j by linear least squares method. Calculate concentration of iodide in sample as:

$$C_x = \frac{A}{B \times V_x}$$

where:

C_x = concentration of iodide, n*M*, and other terms are as defined above.

If there is a reagent blank, subtract the reagent blank from C_x to get true concentration in sample.

Multiply C_x (or blank-corrected C_x) by 0.1269 to obtain concentration in μg/L.

6. Precision

In one laboratory, using seawater samples with a concentration of iodide of about 6 μg I/L, the precision was about ±5%.

7. Bibliography

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* Triton X-100, Catalog No. T9284, Sigma-Aldrich Corp., P.O. Box 14508, St. Louis, MO 63178.

4500-IO_3^- IODATE*

4500-IO_3^- A. Introduction

1. Occurrence

Iodate is found in natural waters at concentrations ranging from 60 μg I/L in deep ocean water to undetectable (3 μg I/L) in estuarine water and fresh water. Iodate is the thermodynamically stable form of dissolved inorganic iodine in waters containing dissolved oxygen; it is absent in anoxic waters.

2. Selection of Method

The differential pulse polarographic method is species-specific and highly sensitive. It is applicable to iodate concentrations of 3 to at least 130 μg I/L and can determine iodate in the presence of other iodine species, such as iodide and organic iodine. It can be used for the direct determination of iodate in many types of water samples.

3. Sampling and Storage

Collect representative samples in clean glass or plastic bottles. Clean sample bottles with 10% (v/v) hydrochloric acid (low in iodate) and thoroughly rinse them with reagent water (see Section 1080) before use. Most samples can be analyzed directly without further treatment. Highly turbid samples may be filtered through glass fiber filters before analysis. For storage of up to 2 d, refrigerate sample at 4°C. For longer storage, freeze sample and store at or below −5°C. Frozen samples can be stored for at least 1 month.

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4500-IO_3^- B. Polarographic Method

1. General Discussion

a. Principle: Under mildly basic conditions, iodate is reduced to iodide at a dropping mercury electrode by a cathodic potential scan. This reaction gives rise to a current peak centered around −1.1 V relative to the saturated calomel electrode. The height of the current peak is directly proportional to the concentration of iodate, which is quantified by the method of standard additions.

b. Interferences: Dissolved oxygen and zinc interfere. Remove dissolved oxygen by bubbling oxygen-free argon gas through sample and by reacting oxygen with added sodium sulfite. Remove interference from zinc by complexing with EDTA (ethylene diaminetetraacetate).

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Polarographic analyzer system: A polarographic analyzer system, consisting of a potentiostat, a static mercury drop electrode (SMDE), a stirrer, and a plotter, that can be operated in the difference pulse polarography-SMDE mode with adjustable drop time, scan increment, pulse height, scan range, and drop size.

Use a saturated calomel electrode as the reference electrode.

b. Glassware: Acid-wash glassware and other surfaces contacting sample or reagents with 10% (v/v) HCl (low in iodate); thoroughly rinse with reagent water (see Section 1080) before use.

3. Reagents

Use chemicals low in iodate whenever available.

a. Oxygen-free water: See Section 4500-I^-.D.3*a*.

b. Alkaline pyrogallol solution: See Section 4500-I^-.D.3*b*.

c. Oxygen-free argon gas: See Section 4500-I^-.D.3*e*.

d. Sodium sulfite solution: See Section 4500-I^-.D.3*c*.

e. Standard iodate solution, 25 μM*:* Dry several grams potassium iodate, KIO_3, in an oven at 80°C overnight. Dissolve 1.070 g dried KIO_3 in reagent water (see Section 1080) and dilute to 1000 mL. Dilute 5 mL of this solution to 1000 mL.

f. Na_2EDTA solution, 0.1*M:* Dissolve 3.722 g disodium ethylenediaminetetraacetate ($Na_2EDTA \cdot 2H_2O$) in reagent water (see Section 1080) and dilute to 100 mL.

g. Supporting electrolyte: Dissolve 54.8 g sodium chloride, 0.30 g potassium bromide, and 1.05 g sodium bicarbonate in reagent water (see Section 1080) to form a final volume of 250 mL.

4. Procedure

a. Sample measurement: Transfer 5 mL sample and 0.5 mL supporting electrolyte to polarographic cell containing a magnetic stirrer. Check pH of solution to make sure it is about 8. (For marine waters with salinities above 15, the supporting electrolyte is not needed.) Remove dissolved oxygen by bubbling sample rigorously with oxygen-free argon gas for 0.5 min with stirring. Add 0.1 mL 1*M* sodium sulfite solution to sample and purge, with stirring, with oxygen-free argon gas for one additional

minute. Add 0.01 mL 0.1*M* disodium EDTA and purge, with stirring, with oxygen-free argon for another 0.5 min. Set electrode in the SMDE mode. Record a polarogram in the differential pulse polarography mode under the following conditions: drop time, 1 s; scan increment, 6 mV; pulse height, 0.06 V; and scan range, −0.65 to −1.35 V. Use a medium drop size that allows mercury droplets to be formed and dislodged from the dropping mercury electrode at a steady and consistent rate. Measure height of current peak above baseline at an applied potential of about −1.1 V relative to the saturated calomel electrode.

b. Internal standard additions: Add 0.05 mL 25 μM standard iodate solution to cell. Purge solution, with stirring, with oxygen-free argon gas for 0.5 min.

Record a polarogram under conditions described in ¶ *a* above and determine height of current peak again. Repeat this procedure two additional times.

c. Blank determination: Determine method reagent blank by treating reagent water as a sample.

5. Calculation

Follow calculations given in Section 4500-I^-.D.5, with substitution of appropriate compounds in the definitions of terms.

6. Precision

In one laboratory, analyzing seawater samples with a concentration of iodate of 60 μg I/L, the precision was about ±3%.

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4500-N NITROGEN*

4500-N A. Introduction

In waters and wastewaters the forms of nitrogen of greatest interest are, in order of decreasing oxidation state: nitrate, nitrite, ammonia, and organic nitrogen. All these forms of nitrogen, as well as nitrogen gas (N_2), are biochemically interconvertible and are components of the nitrogen cycle. They are of interest for many reasons.

Organic nitrogen is defined functionally as organically bound nitrogen in the trinegative oxidation state. It does not include all organic nitrogen compounds. Analytically, organic nitrogen and ammonia can be determined together and have been referred to as "Kjeldahl nitrogen," a term that reflects the technique used in their determination. Organic nitrogen includes such natural materials as proteins and peptides, nucleic acids and urea, and numerous synthetic organic materials. Typical organic nitrogen concentrations vary from a few hundred micrograms per liter in some lakes to more than 20 mg/L in raw sewage.

Total oxidized nitrogen is the sum of nitrate and nitrite nitrogen. Nitrate generally occurs in trace quantities in surface water but may attain high levels in some groundwater. In excessive amounts, it contributes to the illness known as methemoglobinemia in infants. A limit of 10 mg nitrate as nitrogen/L has been imposed on drinking water to prevent this disorder. Nitrate is found only in small amounts in fresh domestic wastewater, but in the effluent of nitrifying biological treatment plants, nitrate may be found in concentrations of up to 30 mg nitrate as nitrogen/L. It is an essential nutrient for many photosynthetic autotrophs and in some cases has been identified as the growth-limiting nutrient.

Nitrite is an intermediate oxidation state of nitrogen, both in the oxidation of ammonia to nitrate and in the reduction of nitrate. Such oxidation and reduction may occur in wastewater treatment plants, water distribution systems, and natural waters. Nitrite can enter a water supply system through its use as a corrosion inhibitor in industrial process water. Nitrite is the actual etiologic agent of methemoglobinemia. Nitrous acid, which is formed from nitrite in acidic solution, can react with secondary amines (RR′NH) to form nitrosamines (RR′N-NO), many of which are known to be carcinogens. The toxicologic significance of nitrosation reactions *in vivo* and in the natural environment is the subject of much current concern and research.

Ammonia is present naturally in surface and wastewaters. Its concentration generally is low in groundwaters because it adsorbs to soil particles and clays and is not leached readily from soils. It is produced largely by deamination of organic nitrogen-containing compounds and by hydrolysis of urea. At some water treatment plants, ammonia is added to react with chlorine to form a combined chlorine residual. Ammonia concentrations encountered in water vary from less than 10 μg ammonia nitrogen/L in some natural surface and groundwaters to more than 30 mg/L in some wastewaters.

In this manual, organic nitrogen is referred to and reported as organic N, nitrate nitrogen as NO_3^--N, nitrite nitrogen as NO_2^--N, and ammonia nitrogen as NH_3-N.

Total nitrogen can be determined through oxidative digestion of all digestible nitrogen forms to nitrate, followed by quantitation of the nitrate. Two procedures, one using a persulfate/UV digestion (4500-N.B), and the other using persulfate digestion (4500-N.C) are presented. The procedures give good results for total nitrogen, composed of organic nitrogen (including some aromatic nitrogen-containing compounds), ammonia, nitrite, and nitrate. Molecular nitrogen is not determined and recovery of some industrial nitrogen-containing compounds is low.

Chloride ions do not interfere with persulfate oxidation, but the rate of reduction of nitrate to nitrite (during subsequent nitrate analysis by cadmium reduction) is significantly decreased by chlorides. Ammonium and nitrate ions adsorbed on suspended pure clay or silt particles should give a quantitative yield from persulfate digestion. If suspended matter remains after digestion, remove it before the reduction step.

If suspended organic matter is dissolved by the persulfate digestion reagent, yields comparable to those from true solutions are obtained; if it is not dissolved, the results are unreliable and probably reflect a negative interference. The persulfate method is not effective in wastes with high organic loadings. Dilute such samples and re-analyze until results from two dilutions agree.

A conductimetric method (4500-N.D) for the determination of inorganic nitrogen also is presented.

* Approved by Standard Methods Committee, A,B,C, 1997; D, 2001. Editorial revisions, 2011.
Joint Task Group: 21st Edition—(4500-N.D)—Thomas R. Holm (chair); 20th Edition—(4500-N.B)—Scott Stieg (chair), Bradford R. Fisher, Owen B. Mathre, Theresa M. Wright.

4500-N B. In-Line UV/Persulfate Digestion and Oxidation with Flow Injection Analysis

1. General Discussion

a. Principle: Nitrogen compounds are digested and oxidized in-line to nitrate by use of heated alkaline persulfate and ultraviolet radiation. The digested sample is injected onto the manifold, where its nitrate is reduced to nitrite by a cadmium granule column. The nitrite then is determined by diazotization with sulfanilamide under acidic conditions to form a diazonium ion. The diazonium ion is coupled with *N*-(1-naphthyl)ethylenediamine dihydrochloride. The result-

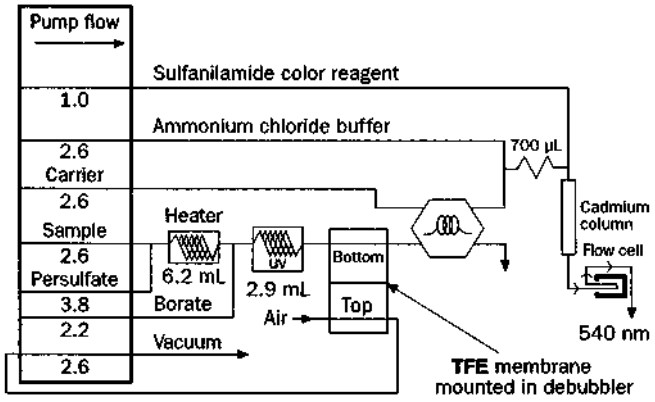


Figure 4500-N:1. FIA in-line total nitrogen manifold.

ing pink dye absorbs at 540 nm and is proportional to total nitrogen.

This method recovers nearly all forms of organic and inorganic nitrogen, reduced and oxidized, including ammonia, nitrate, and nitrite. It differs from the total Kjeldahl nitrogen method described in Section 4500-N_{org}.D, which does not recover the oxidized forms of nitrogen. This method recovers nitrogen components of biological origin, such as amino acids, proteins, and peptides as ammonia, but may not recover the nitrogenous compounds of some industrial wastes, such as amines, nitro-compounds, hydrazones, oximes, semicarbazones, and some refractory tertiary amines.

See 4500-N.A for a discussion of the various forms of nitrogen found in waters and wastewaters, Sections 4500-N_{org}.A and B for a discussion of total nitrogen methods, and Section 4130, Flow Injection Analysis (FIA). Also see 4500-N.C for a similar, batch total nitrogen method that uses only persulfate.

b. Interferences: Remove large or fibrous particulates by filtering sample though glass wool. Guard against contamination from reagents, water, glassware, and the sample preservation process.

2. Apparatus

Flow injection analysis equipment consisting of:

a. FIA injection valve with sample loop or equivalent.

b. Multichannel proportioning pump.

c. FIA manifold (Figure 4500-N:1) with tubing heater; in-line ultraviolet digestion fluidics, including a debubbler consisting of a gas-permeable TFE membrane and its holder; and flow cell. In Figure 4500-N:1, relative flow rates only are shown. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material, such as TFE. The block marked "UV" should consist of TFE tubing irradiated by a mercury discharge ultraviolet lamp emitting radiation at 254 nm.

d. Absorbance detector, 540 nm, 10-nm bandpass.

e. Injection valve control and data acquisition system.

3. Reagents

Use reagent water (>10 megohm) to prepare carrier and for all solutions. To prevent bubble formation, degas carrier and all reagents with helium. Pass He at 140 kPa (20 psi) through a helium degassing tube. Bubble He through 1 L solution for 1 min. As an alternative to preparing reagents by weight/weight, use weight/volume.

a. Borate solution ($Na_2B_4O_7 \cdot 10H_2O$): In a 1-L volumetric flask, dissolve 38.0 g $Na_2B_4O_7 \cdot 10H_2O$ and 3.0 g sodium hydroxide (NaOH) in approximately 900 mL water, using a magnetic stirring bar. Gentle heating will speed dissolution. Adjust to pH 9.0 with NaOH or conc hydrochloric acid (HCl). Dilute to mark and invert to mix.

b. Persulfate solution ($K_2S_2O_8$): Potassium persulfate solid reagent usually contains nitrogen contamination. Higher contamination levels result in larger blank peaks.

To a tared 1-L container, add 975 g water and 49 g $K_2S_2O_8$. Add a magnetic stirring bar, dissolve persulfate, and dilute to mark. Invert to mix.

c. Ammonium chloride buffer: **CAUTION: Fumes. Use a hood.** To a 1-L volumetric flask add 500 mL water, 105 mL conc HCl, and 95 mL conc ammonium hydroxide, NH_4OH. Dissolve, dilute to mark, and invert to mix. Adjust to pH 8.5 with 1*N* HCl or 1*N* NaOH solution.

d. Sulfanilamide color reagent: To a tared, dark, 1-L container add 876 g water, 170 g 85% phosphoric acid, H_3PO_4, 40.0 g sulfanilamide, and 1.0 g *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED). Shake to wet solids and stir for 30 min to dissolve. Store in a dark bottle and discard when solution turns dark pink.

e. Cadmium column: See Section 4500-NO_3^-.I.3*c*, *d*, and *e*.

f. Stock nitrate standard, 1000 mg N/L: In a 1-L volumetric flask, dissolve 7.221 g potassium nitrate, KNO_3 (dried at 60°C for 1 h), or 4.93 g sodium nitrite, $NaNO_2$, in about 800 mL water. Dilute to mark and invert to mix. When refrigerated, the standard may be stored for up to 3 months.

g. Standard solutions: Prepare nitrate standards in the desired concentration range, using stock nitrate standards (¶ *f* above), and diluting with water.

4. Procedure

Set up a manifold equivalent to that in Figure 4500-N:1 and follow method supplied by manufacturer, or laboratory standard operating procedure for this method.

Carry both standards and samples through this procedure. If samples have been preserved with sulfuric acid, preserve standards similarly. Samples may be homogenized. Turbid samples may be filtered, since digestion effectiveness on nitrogen-containing particles is unknown; however, organic nitrogen may be lost in the filtration.

5. Calculation

Prepare standard curves by plotting absorbance of standards processed through the manifold versus nitrogen concentration. The calibration curve is linear.

Verify digestion efficiency by determining urea, glutamic acid, or nicotinic acid standards (4500-N.C.3*d*) at regular intervals. In the concentration range of the method, the recovery of these compounds should be >95%.

6. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 4020:I. Also see Section 4130B.

7. Precision and Bias

a. MDL: Using a 70-μL sample loop and a published MDL method,[1] analysts ran 21 replicates of a 0.20-mg N/L standard. These gave a mean of 0.18 mg N/L, a standard deviation of 0.008 mg N/L, and MDL of 0.020 mg N/L.

b. Precision study: Ten injections each of a 4.00-mg N/L standard and of a 10.0-mg N/L standard both gave a relative standard deviation of 0.6%.

c. Recovery of total nitrogen: Table 4500-N:I shows recoveries for various nitrogen compounds determined at 10 and 4.0 mg N/L. All compounds were determined in triplicate.

d. Ammonia recoveries from wastewater treatment plant effluent with known additions: To a sample of wastewater treatment plant effluent, ammonium chloride was added at two concentrations, 2.50 and 5.00 mg N/L, and analyses were made in triplicate to give mean recoveries of 96 and 95%, respectively. A sample with no additions also was diluted twofold in triplicate to give a mean recovery of 99%.

Table 4500-N:I. Recoveries of Total Nitrogen

Compound	Mean Recovery %	
	10 mg N/L	4 mg N/L
Ammonium chloride	98.1	99.7
Sodium nitrite	100.5	101.8
Glycine	101.0	100.8
Glutamic acid	99.7	99.2
Ammonium *p*-toluenesulfonate	99.6	97.4
Glycine *p*-toluenesulfonate	101.4	102.3
Nicotinic acid	98.6	102.0
Urea	94.9	98.0
EDTA	89.4	89.4

8. Reference

1. U.S. Environmental Protection Agency. 1984. Definition and procedure for the determination of method detection limits. Appendix B to 40 CFR 136 Rev. 1.11 amended June 30, 1986. 49 CFR 43430, Oct. 26.

4500-N C. Persulfate Method

1. General Discussion

The persulfate method determines total nitrogen by oxidation of all nitrogenous compounds to nitrate. Should ammonia, nitrate, and nitrite be determined individually, "organic nitrogen" can be obtained by difference.

a. Principle: Alkaline oxidation at 100 to 110°C converts organic and inorganic nitrogen to nitrate. Total nitrogen is determined by analyzing the nitrate in the digestate.

b. Selection of nitrate measurement method: Automated or manual cadmium reduction may be used to determine total nitrogen levels below 2.9 mg N/L. Results summarized in Table 4500-N:II were obtained using automated cadmium reduction.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Autoclave, or hotplate and pressure cooker capable of developing 100 to 110°C for 30 min.

*b. Glass culture tubes:** 30-mL screw-capped (polypropylene linerless caps), 20 mm OD × 150 mm long. Clean before initial use by autoclaving with digestion reagent.

c. Apparatus for nitrate determination: See Section 4500-NO_3^-.E.2 or F.2.

d. Automated analytical equipment: An example of the continuous-flow analytical instrument consists of components shown in Figure 4500-NO_3^-:2.

Table 4500-N:II. Precision Data for Total Nitrogen, Persulfate Method, Based on Triplicate Analyses of Nicotinic Acid

Lab/ Analyst	Nicotinic Acid *mg N/L*	Recovery of N %	Standard Deviation *mg/L*	Relative Standard Deviation %
1/1	0.5	104	0.019	3.82
1/2	0.5	99.7	0.012	2.44
2/1	0.5	97.7	0.035	7.02
2/2	0.5	87.8	0.024	4.89
3/1	0.5	105	0.072	13.7
3/2	0.5	95.3	0.015	3.20
1/1	1.0	98.5	0.023	2.32
1/2	1.0	99.3	0.022	2.21
2/1	1.0	113	0.053	5.31
2/2	1.0	97.1	0.031	3.10
3/1	1.0	96.2	0.019	1.97
3/2	1.0	102	0.025	2.46
1/1	2.0	100	0.030	1.50
1/2	2.0	97.8	0.014	0.7
2/1	2.0	104	0.069	3.45
2/2	2.0	100	0.080	3.98
3/1	2.0	95.3	0.078	4.12
3/2	2.0	98.3	0.015	0.75

3. Reagents

a. Ammonia-free and nitrate-free water: Prepare by ion exchange or distillation methods as directed in Sections 4500-NH_3.B.3*a* and 4500-NO_3^-.B.3*a*.

* 18–415, Comar, Inc., Vineland, NJ, or equivalent.

b. Stock nitrate solution: Prepare as directed in Section 4500-NO_3^-.B.3*b*.

c. Intermediate nitrate solution: Prepare as directed in Section 4500-NO_3^-.B.3*c*.

d. Stock glutamic acid solution: Dry glutamic acid, $C_3H_5NH_2(COOH)_2$, in an oven at 105°C for 24 h. Dissolve 1.051 g in water and dilute to 1000 mL; 1.00 mL = 100 μg N. Preserve with 2 mL $CHCl_3$/L.

e. Intermediate glutamic acid solution: Dilute 100 mL stock glutamic acid solution to 1000 mL with water; 1.00 mL = 10.0 μg N. Preserve with 2 mL $CHCl_3$/L.

f. Digestion reagent: Dissolve 20.1 g low nitrogen (<0.001% N) potassium persulfate, $K_2S_2O_8$, and 3.0 g NaOH in water and dilute to 1000 mL just before use.

g. Borate buffer solution: Dissolve 61.8 g boric acid, H_3BO_3, and 8.0 g NaOH in water and dilute to 1000 mL.

h. Copper sulfate solution: Dissolve 2.0 g $CuSO_4 \cdot 5H_2O$ in 90 mL water and dilute to 100 mL.

i. Ammonium chloride solution: Dissolve 10.0 g NH_4Cl in 1 L water. Adjust to pH 8.5 by adding three or four NaOH pellets as necessary or NaOH solution before bringing to volume. This reagent is stable for 2 weeks when refrigerated.

j. Color reagent: Combine 1500 mL water, 200.0 mL conc phosphoric acid, H_3PO_4, 20.0 g sulfanilamide, and 1.0 g *N*-(1-naphthyl)-ethylenediamine dihydrochloride. Dilute to 2000 mL. Add 2.0 mL polyoxyethylene 23 lauryl ether.† Store at 4°C in the dark. Prepare fresh reagent every 6 weeks. Alternatively, prepare proportionally smaller volumes to minimize waste.

4. Procedure

a. Calibration curve: Prepare NO_3^- calibration standards in the range 0 to 2.9 mg NO_3^--N/L by diluting to 100 mL the following volumes of intermediate nitrate solution: 0, 1.00, 2.00, 4.00 . . . 29.0 mL. Treat standards in the same manner as samples.

b. Digestion check standard: Prepare glutamic acid digestion check standard of 2.9 mg N/L by diluting, to 100 mL, a 29.0-mL volume of intermediate glutamic acid solution. Treat digestion check standard in the same manner as samples.

† Brij-35, available from ICI Americas, Inc., Wilmington, DE, or equivalent.

c. Digestion: Samples preserved with acid cannot be analyzed by this method. To a culture tube, add 10.0 mL sample or standard or a portion diluted to 10.0 mL. Add 5.0 mL digestion reagent. Cap tightly. Mix by inverting twice. Heat for 30 min in an autoclave or pressure cooker at 100 to 110°C. Slowly cool to room temperature. Add 1.0 mL borate buffer solution. Mix by inverting at least twice.

d. Blank: Carry a reagent blank through all steps of the procedure and apply necessary corrections to the results.

e. Nitrate measurement: Determine nitrate by cadmium reduction. Set up manifold as shown in Figure 4500-NO_3^-:2, but use ammonium chloride and color reagents specified in 4500-N.C.3*i* and *j*.

5. Calculation

Prepare the standard curve by plotting the absorbances or peak heights of the nitrate calibration standards carried through the digestion procedure against their nitrogen concentrations. Compute organic N sample concentration by comparing sample absorbance or peak height with the standard curve.

6. Precision and Bias

See Table 4500-N:II.

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4500-N D. Conductimetric Determination of Inorganic Nitrogen

1. General Discussion

a. Principle: Aqueous ammonia nitrogen in samples is converted to dissolved ammonia gas by raising sample pH above 11 with alkaline solution. The sample/alkaline solution is placed in one cell of a two-cell diffusion chamber with a buffered absorbing solution in the second cell. A hydrophobic membrane, through which the dissolved ammonia gas diffuses, separates the cells. The pH of the absorbing solution is neutral, facilitating conversion of the dissolved ammonia gas to the ammonium ion form. Change in conductivity of the absorbing solution is determined in a conductivity cell. The method is best applied using a continuous-flow conductimetric system, such as shown in Figure 4500-N:2. In the continuous-flow system, samples are injected into a continuously fed stream of alkaline solution at selected time intervals, usually 1 to 2 min, and pass through the system as plugs. Response time usually is less than 1 min.

Total oxidized inorganic nitrogen (nitrate and/or nitrite) is measured by reducing to ammonia with zinc after mixing sample with the alkaline solution.[1] The reaction is

$$NO_3^- + 4Zn + 7OH^- \rightarrow NH_3 + 4ZnO_2^{2-} + 2H_2O$$

In the continuous-flow system, a zinc reduction cartridge is

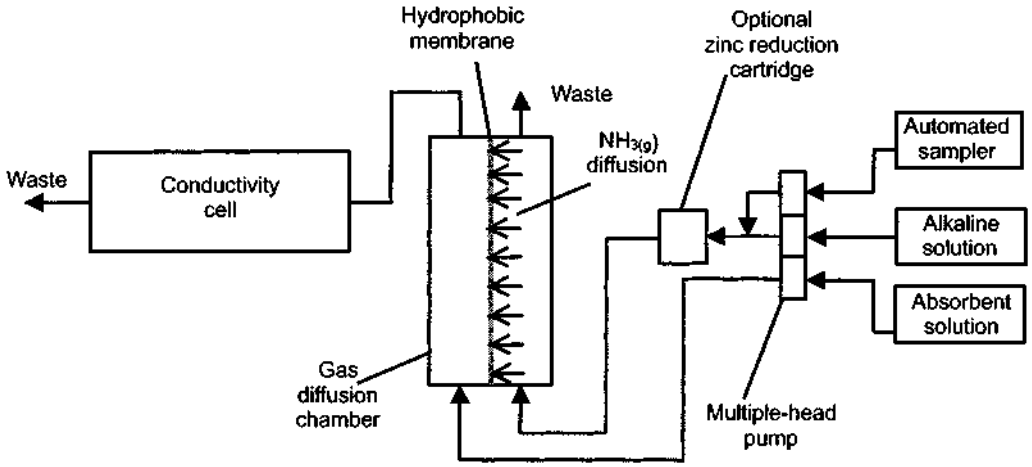


Figure 4500-N:2. Continuous-flow conductimetric analyzer system.

placed in the flow stream, as indicated in Figure 4500-N:2. The reduction reaction is catalyzed by copper; addition of copper sulfate to the alkaline solution is beneficial.

When both ammonia and oxidized forms of nitrogen are present in a sample, the total nitrogen and the ammonia nitrogen concentrations are determined separately and the oxidized nitrogen is calculated by difference. Because the reaction with zinc reduces both nitrite and nitrate to ammonia, differentiation between the two ions is not possible with this method.

b. Interferences: Remove particulate matter by filtration before analysis to prevent plugging of analyzer lines.

Calcium and magnesium precipitate formation can clog analyzer lines. Prevent precipitate formation by adding diethylenetriaminepentaacetic acid (DTPA) to the alkaline feed at a concentration up to 10 g/L.

Volatile amines, chloramines, or the amides of carboxylic acids can diffuse through the hydrophobic membrane to produce a conductivity response or react in the diffusion cell to form ammonia. Reduce or eliminate this response for volatile amines or the amides of carboxylic acids by adjusting pH of the alkaline solution below pK_a of the amine or amide but above pK_a of ammonia. Reduce or eliminate this response for chloramines by not reducing the chloramines over zinc to ammonia.

c. Minimum detectable concentration: The estimated minimum detectable concentration of NH_3-N or combined NO_3^--N and NO_2^--N is 0.01 mg/L.[2]

d. Storage of samples: Optional storage conditions for samples depend on the forms of nitrogen and interferences present. Refer to Sections 4500-NH_3.A.3, 4500-NO_2^-.B.1*c*, or 4500-NO_3^-.A.2 for specific conditions.

2. Apparatus

Automated analytical equipment: An example of the automated, continuous-flow analytical equipment required is shown in Figure 4500-N:2. Details of the requirements and current manufacturers of commercial apparatus* can be found in the literature.[3]

* Timberline Instruments, 1800 S. Flatiron Court, Boulder, CO 80301 (www.timberlineinstruments.com).

3. Reagents

Use ammonia-free, deionized water for making all reagents.

a. Ammonia standards: Prepare at least three standard ammonia nitrogen standards in the desired analysis range between 0.1 and 75 mg N/L by dissolving an appropriate quantity of ammonium sulfate, $(NH_4)_2SO_4$, in water and diluting to 1 L.

b. Nitrate standards: Prepare at least three standard nitrate-nitrogen standards in analysis range between 0.1 mg and 75 mg N/L by dissolving an appropriate quantity of sodium nitrate, $NaNO_3$, in water and diluting to 1 L.

c. Alkaline solution I (for determination of ammonia nitrogen): Dissolve 25 g potassium hydroxide, KOH, and 10 g diethylenetriaminepentaacetic acid, DTPA, precipitate inhibitor in 1 L water.

d. Copper catalyst solution: Dissolve 200 mg $CuSO_4 \cdot 5H_2O$ in 1 L water.

e. Alkaline solution II (for determination of nitrate and nitrite nitrogen): Dissolve 25 g potassium hydroxide, KOH, 10 g diethylenetriaminepentaacetic acid, DTPA, precipitate inhibitor, and 10 mL copper catalyst solution in 1 L water.

f. Absorbent solution: Dissolve 100 mg boric acid, H_3BO_3, in 1 L water. Adjust to pH 7 with ammonium hydroxide, NH_4OH.

g. Zinc reduction cartridges, containing 20/30 mesh zinc metal.

4. Procedure

Set up instrument equivalent to that in Figure 4500-N:2 and follow manufacturer's operating procedure. Calibrate instrument using appropriate standard solutions and insert a standard every tenth tube in the autosampler tray. Filter turbid samples before analysis.

5. Calculation

Prepare standard curves by plotting nitrogen concentration of standards versus conductivity of absorbent solution. The calibration is linear. Compute sample nitrogen concentration by comparing sample conductivity with standard curve.

6. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 4020:I. It is the analyst's responsibility to demonstrate method proficiency and to evaluate method bias. Certain regulatory programs may have more extensive QC requirements.

7. Precision and Bias

Ammonia standard concentrations of 0.1, 0.8, 5, 15, 30, 55, and 75 mg/L NH_3-N were analyzed. Recovery ranged from 99.31 and 101.64% at concentrations of 5 and 0.1 mg/L NH_3-N, respectively. Precision ranged from 0.33 to 3.57% for concentrations of 15 and 0.1 mg/L NH_3-N, respectively.

Nitrate standard concentrations of 0.1, 0.8, 5, 15, 30, 55, and 75 mg/L NO_3^--N were analyzed. Recovery ranged from 99.93 to 101.84% at concentrations of 75 and 0.8 mg/L NO_3^--N, respectively. Precision ranged from 0.64 to 4% at concentrations of 75 and 0.1 mg/L NO_3^--N, respectively.

Combined nitrogen standards were prepared using 50% portions of NH_3-N and NO_3^--N. Concentrations analyzed were 0.1, 0.8, 5, 15, 30, 50, and 75 mg N/L. Ranges of recovery and precision of the combined nitrogen analysis were 99.83 to 101.58% and 0.45 to 4.22%, respectively. Ranges of recovery and precision of the ammonia nitrogen analysis were 97.68 to 106.19% and 2.58 to 12.1%. Ranges of recovery and precision for the calculated nitrate nitrogen were 95.08 to 104.08% and 0.84 to 11%, respectively.[2]

8. References

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4500-NH_3 NITROGEN (AMMONIA)*

4500-NH_3 A. Introduction

1. Selection of Method

The two major factors that influence selection of the method to determine ammonia are concentration and presence of interferences. In general, direct manual determination of low concentrations of ammonia is confined to drinking waters, clean surface or groundwater, and good-quality nitrified wastewater effluent. In other instances, and where interferences are present or greater precision is necessary, a preliminary distillation step (B) is required.

A titrimetric method (C), an ammonia-selective electrode method (D), an ammonia-selective electrode method using known addition (E), a phenate method (F), and two automated versions of the phenate method (G and H) are presented. Methods D, E, F, G, and H may be used either with or without sample distillation. The data presented in Tables 4500-NH_3:I and III should be helpful in selecting the appropriate method of analysis.

Nesslerization has been dropped as a standard method, although it has been considered a classic water quality measurement for more than a century. The use of mercury in this test warrants its deletion because of the disposal problems.

The distillation and titration procedure is used especially for NH_3-N concentrations greater than 5 mg/L. Use boric acid as the absorbent following distillation if the distillate is to be titrated.

The ammonia-selective electrode method is applicable over the range from 0.03 to 1400 mg NH_3-N/L.

The manual phenate method is applicable to both fresh water and seawater and is linear to 0.6 mg NH_3-N/L. Distill into

* Approved by Standard Methods Committee, 1997.
Joint Task Group: 20th Edition (4500-NH_3.H)—Scott Stieg (chair), Bradford R. Fisher, Owen B. Mathre, Theresa M. Wright.

sulfuric acid (H_2SO_4) absorbent for the phenate method when interferences are present.

The automated phenate method is applicable over the range of 0.02 to 2.0 mg NH_3-N/L.

2. Interferences

Glycine, urea, glutamic acid, cyanates, and acetamide hydrolyze very slowly in solution on standing but, of these, only urea and cyanates will hydrolyze on distillation at pH of 9.5. Hydrolysis amounts to about 7% at this pH for urea and about 5% for cyanates. Volatile alkaline compounds such as hydrazine and amines will influence titrimetric results. Residual chlorine reacts with ammonia; remove by sample pretreatment. If a sample is likely to contain residual chlorine, immediately upon collection, treat with dechlorinating agent as in 4500-NH_3.B.3*d*.

3. Storage of Samples

Most reliable results are obtained on fresh samples. If samples are to be analyzed within 24 h of collection, refrigerate unacidified at 4°C. For preservation for up to 28 d, freeze at − 20°C unacidified, or preserve samples by acidifying to pH <2 and storing at 4°C. If acid preservation is used, neutralize samples with NaOH or KOH immediately before making the determination. CAUTION: Although acidification is suitable for certain types of samples, it produces interferences when exchangeable ammonium is present in unfiltered solids.

4. Bibliography

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SALLEY, B.A., J.G. BRADSHAW & B.J. NEILSON. 1986. Results of Comparative Studies of Preservation Techniques for Nutrient Analysis on Water Samples. Virginia Institute of Marine Science, Gloucester Point.

4500-NH_3 B. Preliminary Distillation Step

1. General Discussion

The sample is buffered at pH 9.5 with a borate buffer to decrease hydrolysis of cyanates and organic nitrogen compounds. It is distilled into a solution of boric acid when titration is to be used or into H_2SO_4 when the phenate method is used. The ammonia in the distillate can be determined either colorimetrically by the phenate method or titrimetrically with standard H_2SO_4 and a mixed indicator or a pH meter. The choice between the colorimetric and the acidimetric methods depends on the concentration of ammonia. Ammonia in the distillate also can be determined by the ammonia-selective electrode method, using 0.04*N* H_2SO_4 to trap the ammonia.

2. Apparatus

a. Distillation apparatus: Arrange a borosilicate glass flask of 800- to 2000-mL capacity attached to a vertical condenser so that the outlet tip may be submerged below the surface of the receiving acid solution. Use an all-borosilicate-glass apparatus or one with condensing units constructed of block tin or aluminum tubes.

b. pH meter.

3. Reagents

a. Ammonia-free water: Prepare by ion-exchange or distillation methods:

1) Ion exchange—Prepare ammonia-free water by passing distilled water through an ion-exchange column containing a strongly acidic cation-exchange resin mixed with a strongly basic anion-exchange resin. Select resins that will remove organic compounds that interfere with the ammonia determination. Some anion-exchange resins tend to release ammonia. If this occurs, prepare ammonia-free water with a strongly acidic cation-exchange resin. Regenerate the column according to the manufacturer's instructions. Check ammonia-free water for the possibility of a high blank value.

2) Distillation—Eliminate traces of ammonia in distilled water by adding 0.1 mL conc H_2SO_4 to 1 L distilled water and redistilling. Alternatively, treat distilled water with sufficient bromine or chlorine water to produce a free halogen residual of 2 to 5 mg/L and redistill after standing at least 1 h. Discard the first 100 mL distillate. Check redistilled water for the possibility of a high blank.

It is very difficult to store ammonia-free water in the laboratory without contamination from gaseous ammonia. However, if storage is necessary, store in a tightly stoppered glass container to which is added about 10 g ion-exchange resin (preferably a strongly acidic cation-exchange resin)/L ammonia-free water. For use, let resin settle and decant ammonia-free water. If a high blank value is produced, replace the resin or prepare fresh ammonia-free water.

Use ammonia-free distilled water for preparing all reagents, rinsing, and sample dilution.

b. Borate buffer solution: Add 88 mL 0.1*N* NaOH solution to 500 mL approximately 0.025*M* sodium tetraborate ($Na_2B_4O_7$) solution (9.5 g $Na_2B_4O_7 \cdot 10\ H_2O$/L) and dilute to 1 L.

c. Sodium hydroxide, 6*N*.

d. Dechlorinating reagent: Dissolve 3.5 g sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in water and dilute to 1 L. Prepare fresh weekly. Use 1 mL reagent to remove 1 mg/L residual chlorine in 500-mL sample.

e. Neutralization agent.

1) *Sodium hydroxide,* NaOH, 1*N*.

2) *Sulfuric acid,* H_2SO_4, 1*N*.

f. Absorbent solution, plain boric acid: Dissolve 20 g H_3BO_3 in water and dilute to 1 L.

g. Indicating boric acid solution: See Section 4500-NH_3.C.3*a* and *b*.

h. Sulfuric acid, 0.04*N:* Dilute 1.0 mL conc H_2SO_4 to 1 L.

4. Procedure

a. Preparation of equipment: Add 500 mL water and 20 mL borate buffer, adjust pH to 9.5 with 6*N* NaOH solution, and add to a distillation flask. Add a few glass beads or boiling chips and use this mixture to steam out the distillation apparatus until distillate shows no traces of ammonia.

b. Sample preparation: Use 500 mL dechlorinated sample or a known portion diluted to 500 mL with water. When NH_3-N concentration is less than 100 μg/L, use a sample volume of 1000 mL. Remove residual chlorine by adding, at the time of collection, dechlorinating agent equivalent to the chlorine residual. If necessary, neutralize to approximately pH 7 with dilute acid or base, using a pH meter.

Add 25 mL borate buffer solution and adjust to pH 9.5 with 6*N* NaOH using a pH meter.

c. Distillation: To minimize contamination, leave distillation apparatus assembled after steaming out and until just before starting sample distillation. Disconnect steaming-out flask and immediately transfer sample flask to distillation apparatus. Distill at a rate of 6 to 10 mL/min with the tip of the delivery tube below the surface of acid receiving solution. Collect distillate in a 500-mL erlenmeyer flask containing 50 mL indicating boric acid solution for titrimetric method. Distill ammonia into 50 mL 0.04*N* H_2SO_4 for the ammonia-selective electrode method and for the phenate method. Collect at least 200 mL distillate. Lower distillation receiver so that the end of the delivery tube is free of contact with the liquid and continue distillation during the last minute or two to cleanse condenser and delivery tube. Dilute to 500 mL with water.

When the phenate method is used for determining NH_3-N, neutralize distillate with 1*N* NaOH solution.

d. Ammonia determination: Determine ammonia by the titrimetric method (C), the ammonia-selective electrode methods (D and E), or the phenate methods (F and G).

5. Bibliography

NICHOLS, M.S. & M.E. FOOTE. 1931. Distillation of free ammonia from buffered solutions. *Ind. Eng. Chem.*, Anal. Ed. 3:311.

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TARAS, M.J. 1953. Effect of free residual chlorination of nitrogen compounds in water. *J. Amer. Water Works Assoc.* 45:47.

4500-NH_3 C. Titrimetric Method

1. General Discussion

The titrimetric method is used only on samples that have been carried through preliminary distillation (see Section 4500-NH_3.B). The following table is useful in selecting sample volume for the distillation and titration method.

Ammonia Nitrogen in Sample *mg/L*	Sample Volume *mL*
5–10	250
10–20	100
20–50	50.0
50–100	25.0

2. Apparatus

Distillation apparatus: See Section 4500-NH_3.B.2*a* and *b*.

3. Reagents

Use ammonia-free water in making all reagents and dilutions.

a. Mixed indicator solution: Dissolve 200 mg methyl red indicator in 100 mL 95% ethyl or isopropyl alcohol. Dissolve 100 mg methylene blue in 50 mL 95% ethyl or isopropyl alcohol. Combine solutions. Prepare monthly.

b. Indicating boric acid solution: Dissolve 20 g H_3BO_3 in water, add 10 mL mixed indicator solution, and dilute to 1 L. Prepare monthly.

c. Standard sulfuric acid titrant, 0.02*N:* Prepare and standardize as directed in Alkalinity, Section 2320B.3*c*. For greatest accuracy, standardize titrant against an amount of Na_2CO_3 that has been incorporated in the indicating boric acid solution to reproduce the actual conditions of sample titration; 1.00 mL = 14 × normality × 1000 μg N. (For 0.02*N*, 1.00 mL = 280 μg N.)

4. Procedure

a. Proceed as described in Section 4500-NH_3.B using indicating boric acid solution as absorbent for the distillate.

b. Sludge or sediment samples: Rapidly weigh to within ±1% an amount of wet sample, equivalent to approximately 1 g dry weight, in a weighing bottle or crucible. Wash sample into a 500-mL kjeldahl flask with water and dilute to 250 mL. Proceed as in ¶ 4*a* but add a piece of paraffin wax to distillation flask and collect only 100 mL distillate.

c. Titrate ammonia in distillate with standard 0.02*N* H_2SO_4 titrant until indicator turns a pale lavender.

d. Blank: Carry a blank through all steps of the procedure and apply the necessary correction to the results.

5. Calculation

a. Liquid samples:

$$\text{mg } NH_3\text{-N/L} = \frac{(A - B) \times 280}{\text{mL sample}}$$

b. Sludge or sediment samples:

$$\text{mg } NH_3\text{-N/kg} = \frac{(A - B) \times 280}{\text{g dry wt sample}}$$

where:

A = volume of H_2SO_4 titrated for sample, mL, and
B = volume of H_2SO_4 titrated for blank, mL.

6. Precision and Bias

Three synthetic samples containing ammonia and other constituents dissolved in distilled water were distilled and analyzed by titration.

Sample 1 contained 200 μg NH_3-N/L, 10 mg Cl^-/L, 1.0 mg NO_3^--N/L, 1.5 mg organic N/L, 10.0 mg PO_4^{3-}/L, and 5.0 mg silica/L. The relative standard deviation and relative error for the 21 participating laboratories were 69.8% and 20%, respectively.

Sample 2 contained 800 μg NH_3-N/L, 200 mg Cl^-/L, 1.0 mg NO_3^--N/L, 0.8 mg organic N/L, 5.0 mg PO_4^{3-}/L, and 15.0 mg silica/L. The relative standard deviation and relative error for the 20 participating laboratories were 28.6% and 5%, respectively.

Sample 3 contained 1500 μg NH_3-N/L, 400 mg Cl^-/L, 1.0 mg NO_3^--N/L, 0.2 mg organic N/L, 0.5 mg PO_4^{3-}/L, and 30.0 mg silica/L. The relative standard deviation and relative error for the 21 participating laboratories were 21.6%, and 2.6%, respectively.

7. Bibliography

MEEKER, E.W. & E.C. WAGNER. 1933. Titration of ammonia in the presence of boric acid. *Ind. Eng. Chem.,* Anal. Ed. 5:396.

WAGNER, E.C. 1940. Titration of ammonia in the presence of boric acid. *Ind. Eng. Chem.,* Anal. Ed. 12:711.

4500-NH_3 D. Ammonia-Selective Electrode Method

1. General Discussion

a. Principle: The ammonia-selective electrode uses a hydrophobic gas-permeable membrane to separate the sample solution from an electrode internal solution of ammonium chloride. Dissolved ammonia ($NH_{3(aq)}$ and NH_4^+) is converted to $NH_{3(aq)}$ by raising pH to above 11 with a strong base. $NH_{3(aq)}$ diffuses through the membrane and changes the internal solution pH that is sensed by a pH electrode. The fixed level of chloride in the internal solution is sensed by a chloride ion-selective electrode that serves as the reference electrode. Potentiometric measurements are made with a pH meter having an expanded millivolt scale or with a specific ion meter.

b. Scope and application: This method is applicable to the measurement of 0.03 to 1400 mg NH_3-N/L in potable and surface waters and domestic and industrial wastes. High concentrations of dissolved ions affect the measurement, but color and turbidity do not. Sample distillation is unnecessary. Use standard solutions and samples that have the same temperature and contain about the same total level of dissolved species. The ammonia-selective electrode responds slowly below 1 mg NH_3-N/L; hence, use longer times of electrode immersion (2 to 3 min) to obtain stable readings.

c. Interference: Amines are a positive interference. This may be enhanced by acidification. Mercury and silver interfere by complexing with ammonia, unless the NaOH/EDTA solution (3*c*) is used.

d. Sample preservation: Refrigerate at 4°C for samples to be analyzed within 24 h. Preserve samples high in organic and nitrogenous matter, and any other samples for longer storage, by lowering pH to 2 or less with conc H_2SO_4.

2. Apparatus

a. Electrometer: A pH meter with expanded millivolt scale capable of 0.1 mV resolution between −700 mV and +700 mV or a specific ion meter.

*b. Ammonia-selective electrode.**

c. Magnetic stirrer, thermally insulated, with TFE-coated stirring bar.

3. Reagents

a. Ammonia-free water: See Section 4500-NH_3.B.3*a*. Use for making all reagents.

b. Sodium hydroxide, 10*N*.

c. NaOH/EDTA solution, 10*N:* Dissolve 400 g NaOH in 800 mL water. Add 45.2 g ethylenediaminetetraacetic acid, tetrasodium salt, tetrahydrate ($Na_4EDTA \cdot 4\ H_2O$) and stir to dissolve. Cool and dilute to 1000 mL.

d. Stock ammonium chloride solution: Dissolve 3.819 g anhydrous NH_4Cl (dried at 100°C) in water, and dilute to 1000 mL; 1.00 mL = 1.00 mg N = 1.22 mg NH_3.

e. Standard ammonium chloride solutions: See ¶ 4*a* below.

4. Procedure

a. Preparation of standards: Prepare a series of standard solutions covering the concentrations of 1000, 100, 10, 1, and 0.1

* Orion Model 95-10, EIL Model 8002-2, Beckman Model 39565, or equivalent.

mg NH_3-N/L by making decimal dilutions of stock NH_4Cl solution with water.

b. Electrometer calibration: Place 100 mL of each standard solution in a 150-mL beaker. Immerse electrode in standard of lowest concentration and mix with a magnetic stirrer. Limit stirring speed to minimize possible loss of ammonia from the solution. Maintain the same stirring rate and a temperature of about 25°C throughout calibration and testing procedures. Add a sufficient volume of 10*N* NaOH solution (1 mL usually is sufficient) to raise pH above 11. If the presence of silver or mercury is possible, use NaOH/EDTA solution in place of NaOH solution. If it is necessary to add more than 1 mL of either NaOH or NaOH/EDTA solution, note volume used, because it is required for subsequent calculations. Keep electrode in solution until a stable millivolt reading is obtained. Do not add NaOH solution before immersing electrode, because ammonia may be lost from a basic solution. Repeat procedure with remaining standards, proceeding from lowest to highest concentration. Wait until the reading has stablized (at least 2 to 3 min) before recording millivolts for standards and samples containing $\leq$ 1 mg NH_3-N/L.

c. Preparation of standard curve: Using semilogarithmic graph paper, plot ammonia concentration in milligrams NH_3-N per liter on the log axis vs. potential in millivolts on the linear axis starting with the lowest concentration at the bottom of the scale. If the electrode is functioning properly a tenfold change of NH_3-N concentration produces a potential change of about 59 mV.

d. Calibration of specific ion meter: Refer to manufacturer's instructions and proceed as in ¶s 4*a* and *b*.

e. Measurement of samples: Dilute if necessary to bring NH_3-N concentration to within calibration curve range. Place 100 mL sample in 150-mL beaker and follow procedure in ¶ 4*b* above. Record volume of 10*N* NaOH added. Read NH_3-N concentration from standard curve.

5. Calculation

$$\text{mg } NH_3\text{-N/L} = A \times B \times \left[\frac{100 + D}{100 + C}\right]$$

where:

A = dilution factor,
B = concentration of NH_3-N/L, mg/L, from calibration curve,
C = volume of 10*N* NaOH added to calibration standards, mL, and
D = volume of 10*N* NaOH added to sample, mL.

4500-NH_3:I. PRECISION AND BIAS OF AMMONIA-SELECTIVE ELECTRODE

Level *mg/L*	Matrix	Mean Recovery %	Precision: Overall % *RSD*	Precision: Single Operator % *RSD*
0.04	Distilled water	200	125	25
	Effluent water	100	75	0
0.10	Distilled water	180	50	10
	Effluent water	470	610	10
0.80	Distilled water	105	14	5
	Effluent water	105	38	7.5
20	Distilled water	95	10	5
	Effluent water	95	15	10
100	Distilled water	98	5	2
	Effluent water	97	—	—
750	Distilled water	97	10.4	1.6
	Effluent water	99	14.1	1.3

Source: AMERICAN SOCIETY FOR TESTING AND MATERIALS. Method 1426-79. American Soc. Testing & Materials, Philadelphia, Pa.

6. Precision and Bias

For the ammonia-selective electrode in a single laboratory using surface water samples at concentrations of 1.00, 0.77, 0.19, and 0.13 mg NH_3-N/L, standard deviations were $\pm$0.038, $\pm$0.017, $\pm$0.007, and $\pm$0.003, respectively. In a single laboratory using surface water samples at concentrations of 0.10 and 0.13 mg NH_3-N/L, recoveries were 96% and 91%, respectively. The results of an interlaboratory study involving 12 laboratories using the ammonia-selective electrode on distilled water and effluents are summarized in Table 4500-NH_3:I.

7. Bibliography

BANWART, W.L., J.M. BREMNER & M.A. TABATABAI. 1972. Determination of ammonium in soil extracts and water samples by an ammonia electrode. *Comm. Soil Sci. Plant Anal.* 3:449.

MIDGLEY, C. & K. TERRANCE. 1972. The determination of ammonia in condensed steam and boiler feed-water with a potentiometric ammonia probe. *Analyst* 97:626.

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U.S. ENVIRONMENTAL PROTECTION AGENCY. 1979. Methods for Chemical Analysis of Water and Wastes. EPA-600/4-79-020, National Environmental Research Center, Cincinnati, Ohio.

AMERICAN SOCIETY FOR TESTING AND MATERIALS. 1979. Method 1426–79. American Soc. Testing & Materials, Philadelphia, Pa.

4500-NH_3 E. Ammonia-Selective Electrode Method Using Known Addition

1. General Discussion

a. Principle: When a linear relationship exists between concentration and response, known addition is convenient for measuring occasional samples because no calibration is needed. Because an accurate measurement requires that the concentration at least double as a result of the addition, sample concentration must be known within a factor of three. Total concentration of

ammonia can be measured in the absence of complexing agents down to 0.8 mg NH_3-N/L or in the presence of a large excess (50 to 100 times) of complexing agent. Known addition is a convenient check on the results of direct measurement.

b. See Section 4500-NH_3.D.1 for further discussion.

2. Apparatus

Use apparatus specified in Section 4500-NH_3.D.2.

3. Reagents

Use reagents specified in Section 4500-NH_3.D.3.

Add standard ammonium chloride solution approximately 10 times as concentrated as samples being measured.

4. Procedure

a. Dilute 1000 mg/L stock solution to make a standard solution about 10 times as concentrated as the sample concentrate.

b. Add 1 mL 10*N* NaOH to each 100 mL sample and immediately immerse electrode. When checking a direct measurement, leave electrode in 100 mL of sample solution. Use magnetic stirring throughout. Measure mV reading and record as E_1.

c. Pipet 10 mL of standard solution into sample. Thoroughly stir and immediately record new mV reading as E_2.

5. Calculation

a. $\Delta E = E_1 - E_2$.

b. From Table 4500-NH_3:II find the concentration ratio, Q, corresponding to change in potential, ΔE. To determine original total sample concentration, multiply Q by the concentration of the added standard:

$$C_o = Q\ C_s$$

where:

C_o = total sample concentration, mg/L,
Q = reading from known-addition table, and
C_s = concentration of added standard, mg/L.

c. To check a direct measurement, compare results of the two methods. If they agree within ±4%, the measurements probably are good. If the known-addition result is much larger than the direct measurement, the sample may contain complexing agents.

6. Precision and Bias

In 38 water samples analyzed by both the phenate and the known-addition ammonia-selective electrode method, the electrode method yielded a mean recovery of 102% of the values obtained by the phenate method when the NH_3-N concentrations varied between 0.30 and 0.78 mg/L. In 57 wastewater samples similarly compared, the electrode method yielded a mean recovery of 108% of the values obtained by the phenate method using distillation when the NH_3-N concentrations varied between 10.2 and 34.7 mg N/L. In 20 instances in which two to four replicates of these samples were analyzed, the mean standard deviation was 1.32 mg N/L. In three measurements at a sewer outfall, distillation did not change statistically the value obtained by the electrode method. In 12 studies using standards in the 2.5- to 30-mg N/L range, average recovery by the phenate method was 97% and by the electrode method 101%.

TABLE 4500-NH_3:II.* VALUES OF Q VS. ΔE (59 MV SLOPE) FOR 10% VOLUME CHANGE

ΔE	Q	ΔE	Q	ΔE	Q	ΔE	Q	ΔE	Q
5.0	0.297	9.0	0.178	16.0	0.0952	24.0	0.0556	32.0	0.0354
5.1	0.293	9.1	0.176	16.2	0.0938	24.2	0.0549	32.2	0.0351
5.2	0.288	9.2	0.174	16.4	0.0924	24.4	0.0543	32.4	0.0347
5.3	0.284	9.3	0.173	16.6	0.0910	24.6	0.0536	32.6	0.0343
5.4	0.280	9.4	0.171	16.8	0.0897	24.8	0.0530	32.8	0.0340
5.5	0.276	9.5	0.169	17.0	0.0884	25.0	0.0523	33.0	0.0335
5.6	0.272	9.6	0.167	17.2	0.0871	25.2	0.0517	33.2	0.0333
5.7	0.268	9.7	0.165	17.4	0.0858	25.4	0.0511	33.4	0.0329
5.8	0.264	9.8	0.164	17.6	0.0846	25.6	0.0505	33.6	0.0326
5.9	0.260	9.9	0.162	17.8	0.0834	25.8	0.0499	33.8	0.0323
6.0	0.257	10.0	0.160	18.0	0.0822	26.0	0.0494	34.0	0.0319
6.1	0.253	10.2	0.157	18.2	0.0811	26.2	0.0488	34.2	0.0316
6.2	0.250	10.4	0.154	18.4	0.0799	26.4	0.0482	34.4	0.0313
6.3	0.247	10.6	0.151	18.6	0.0788	26.6	0.0477	34.6	0.0310
6.4	0.243	10.8	0.148	18.8	0.0777	26.8	0.0471	34.8	0.0307
6.5	0.240	11.0	0.145	19.0	0.0767	27.0	0.0466	35.0	0.0304
6.6	0.237	11.2	0.143	19.2	0.0756	27.2	0.0461	36.0	0.0289
6.7	0.234	11.4	0.140	19.4	0.0746	27.4	0.0456	37.0	0.0275
6.8	0.231	11.6	0.137	19.6	0.0736	27.6	0.0450	38.0	0.0261
6.9	0.228	11.8	0.135	19.8	0.0726	27.8	0.0445	39.0	0.0249
7.0	0.225	12.0	0.133	20.0	0.0716	28.0	0.0440	40.0	0.0237
7.1	0.222	12.2	0.130	20.2	0.0707	28.2	0.0435	41.0	0.0226
7.2	0.219	12.4	0.128	20.4	0.0698	28.4	0.0431	42.0	0.0216
7.3	0.217	12.6	0.126	20.6	0.0689	28.6	0.0426	43.0	0.0206
7.4	0.214	12.8	0.123	20.8	0.0680	28.8	0.0421	44.0	0.0196
7.5	0.212	13.0	0.121	21.0	0.0671	29.0	0.0417	45.0	0.0187
7.6	0.209	13.2	0.119	21.2	0.0662	29.2	0.0412	46.0	0.0179
7.7	0.207	13.4	0.117	21.4	0.0654	29.4	0.0408	47.0	0.0171
7.8	0.204	13.6	0.115	21.6	0.0645	29.6	0.0403	48.0	0.0163
7.9	0.202	13.8	0.113	21.8	0.0637	29.8	0.0399	49.0	0.0156
8.0	0.199	14.0	0.112	22.0	0.0629	30.0	0.0394	50.0	0.0149
8.1	0.197	14.2	0.110	22.2	0.0621	30.2	0.0390	51.0	0.0143
8.2	0.195	14.4	0.108	22.4	0.0613	30.4	0.0386	52.0	0.0137
8.3	0.193	14.6	0.106	22.6	0.0606	30.6	0.0382	53.0	0.0131
8.4	0.190	14.8	0.105	22.8	0.0598	30.8	0.0378	54.0	0.0125
8.5	0.188	15.0	0.103	23.0	0.0591	31.0	0.0374	55.0	0.0120
8.6	0.186	15.2	0.1013	23.2	0.0584	31.2	0.0370	56.0	0.0115
8.7	0.184	15.4	0.0997	23.4	0.0576	31.4	0.0366	57.0	0.0110
8.8	0.182	15.6	0.0982	23.6	0.0569	31.6	0.0362	58.0	0.0105
8.9	0.180	15.8	0.0967	23.8	0.0563	31.8	0.0358	59.0	0.0101

* Orion Research Inc. Instruction Manual, Ammonia Electrode, Model 95-12, Boston, MA. 02129.

4500-NH_3 F. Phenate Method

1. General Discussion

a. Principle: An intensely blue compound, indophenol, is formed by the reaction of ammonia, hypochlorite, and phenol catalyzed by sodium nitroprusside.

b. Interferences: Complexing magnesium and calcium with citrate eliminates interference produced by precipitation of these ions at high pH. There is no interference from other trivalent forms of nitrogen. Remove interfering turbidity by distillation or filtration. If hydrogen sulfide is present, remove by acidifying samples to pH 3 with dilute HCl and aerating vigorously until sulfide odor no longer can be detected.

2. Apparatus

Spectrophotometer for use at 640 nm with a light path of 1 cm or greater.

3. Reagents

a. Phenol solution: Mix 11.1 mL liquified phenol (≥89%) with 95% v/v ethyl alcohol to a final volume of 100 mL. Prepare weekly. CAUTION: *Wear gloves and eye protection when handling phenol; use good ventilation to minimize all personnel exposure to this toxic volatile substance.*

b. Sodium nitroprusside, 0.5% w/v: Dissolve 0.5 g sodium nitroprusside in 100 mL deionized water. Store in amber bottle for up to 1 month.

c. Alkaline citrate: Dissolve 200 g trisodium citrate and 10 g sodium hydroxide in deionized water. Dilute to 1000 mL.

d. Sodium hypochlorite, commercial solution, about 5%. This solution slowly decomposes once the seal on the bottle cap is broken. Replace about every 2 months.

e. Oxidizing solution: Mix 100 mL alkaline citrate solution with 25 mL sodium hypochlorite. Prepare fresh daily.

f. Stock ammonium solution: See Section 4500-NH_3.D.3*d*.

g. Standard ammonium solution: Use stock ammonium solution and water to prepare a calibration curve in a range appropriate for the concentrations of the samples.

4. Procedure

To a 25-mL sample in a 50-mL erlenmeyer flask, add, with thorough mixing after each addition, 1 mL phenol solution, 1 mL sodium nitroprusside solution, and 2.5 mL oxidizing solution. Cover samples with plastic wrap or paraffin wrapper film. Let color develop at room temperature (22 to 27°C) in subdued light for at least 1 h. Color is stable for 24 h. Measure absorbance at 640 nm. Prepare a blank and at least two other standards by diluting stock ammonia solution into the sample concentration range. Treat standards the same as samples.

TABLE 4500-NH_3:III. PRECISION DATA FOR MANUAL PHENATE METHOD BASED ON TRIPLICATE ANALYSES OF AMMONIUM SULFATE

Lab/ Analyst	NH_3-N Concentration *mg/L*	Optical Density	Relative Standard Deviation *%*
1/1	0.1	0.129	1.55
1/2	0.1	0.114	9.66
2/1	0.1	0.100	10.2
2/2	0.1	0.122	2.36
3/1	0.1	0.112	3.61
3/2	0.1	0.107	1.94
1/1	0.3	0.393	0.39
1/2	0.3	0.364	0.32
2/1	0.3	0.372	2.64
2/2	0.3	0.339	0.90
3/1	0.3	0.370	0.31
3/2	0.3	0.373	0.46
1/1	0.5	0.637	0.77
1/2	0.5	0.630	0.56
2/1	0.5	0.624	1.65
2/2	0.5	0.618	0.86
3/1	0.5	0.561	0.27
3/2	0.5	0.569	0.91

5. Calculations

Prepare a standard curve by plotting absorbance readings of standards against ammonia concentrations of standards. Compute sample concentration by comparing sample absorbance with the standard curve.

6. Precision and Bias

For the manual phenate method, reagent water solutions of ammonium sulfate were prepared and analyzed by two analysts in each of three laboratories. Results are summarized in Table 4500-NH_3:III.

7. Bibliography

SOLORZANO, L. 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnol. Oceanogr.* 14:799.

PARSONS, T.R., Y. MAITA & C.M. LALLI. 1984. A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, Elmsford, N.Y.

4500-NH_3 G. Automated Phenate Method

1. General Discussion

a. Principle: Alkaline phenol and hypochlorite react with ammonia to form indophenol blue that is proportional to the ammonia concentration. The blue color formed is intensified with sodium nitroprusside.

b. Interferences: Seawater contains calcium and magnesium ions in sufficient concentrations to cause precipitation during analysis. Adding EDTA and sodium potassium tartrate reduces the problem. Eliminate any marked variation in acidity or alkalinity among samples because intensity of measured color is pH-dependent. Likewise, insure that pH of wash water and standard ammonia solutions approximates that of sample. For example, if sample has been preserved with 0.8 mL conc H_2SO_4/L, include 0.8 mL conc H_2SO_4/L in wash water and standards. Remove interfering turbidity by filtration. Color in the samples that absorbs in the photometric range used for analysis interferes.

c. Application: Ammonia nitrogen can be determined in potable, surface, and saline waters as well as domestic and industrial wastewaters over a range of 0.02 to 2.0 mg/L when photometric measurement is made at 630 to 660 nm in a 10- to 50-mm tubular flow cell at rates of up to 60 samples/h. Determine higher concentrations by diluting the sample.

2. Apparatus

Automated analytical equipment: An example of the required continuous-flow analytical instrument consists of the interchangeable components shown in Figure 4500-NH_3:1.

3. Reagents

a. Ammonia-free distilled water: See Section 4500-NH_3.B.3*a*. Use for preparing all reagents and dilutions.

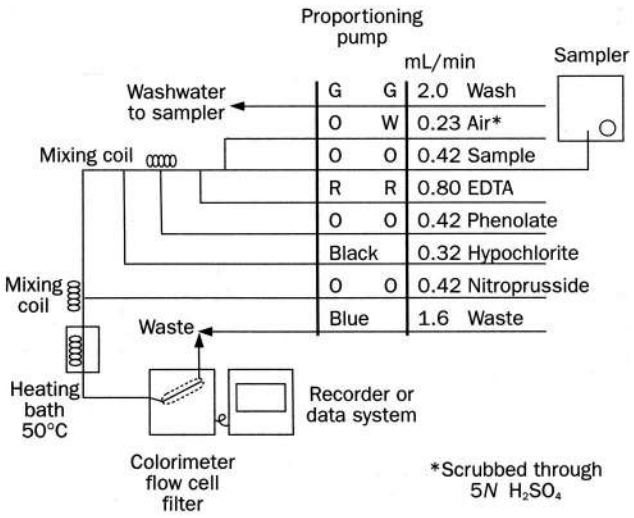


Figure 4500-NH_3:1. Ammonia manifold.

b. Sulfuric acid, H_2SO_4, 5*N*, air scrubber solution: Carefully add 139 mL conc H_2SO_4 to approximately 500 mL water, cool to room temperature, and dilute to 1 L.

c. Sodium phenate solution: In a 1-L erlenmeyer flask, dissolve 93 mL liquid (≥89%) phenol in 500 mL water. In small increments and with agitation, cautiously add 32 g NaOH. Cool flask under running water and dilute to 1 L. CAUTION: *Minimize exposure of personnel to this compound by wearing gloves and eye protection, and using proper ventilation.*

d. Sodium hypochlorite solution: Dilute 250 mL bleach solution containing 5.25% NaOCl to 500 mL with water.

e. EDTA reagent: Dissolve 50 g disodium ethylenediamine tetraacetate and approximately six pellets NaOH in 1 L water. For salt-water samples where EDTA reagent does not prevent precipitation of cations, use sodium potassium tartrate solution prepared as follows:

Sodium potassium tartrate solution: To 900 mL water add 100 g $NaKC_4H_4O_6 \cdot 4H_2O$, two pellets NaOH, and a few boiling chips, and boil gently for 45 min. Cover, cool, and dilute to 1 L. Adjust pH to 5.2 ± 0.05 with H_2SO_4. Let settle overnight in a cool place and filter to remove precipitate. Add 0.5 mL polyoxyethylene 23 lauryl ether* solution and store in stoppered bottle.

f. Sodium nitroprusside solution: Dissolve 0.5 g $Na_2(NO)Fe(CN)_5 \cdot 2H_2O$ in 1 L water.

g. Ammonia standard solutions: See Sections 4500-NH_3.D.3*d* and *e*. Use standard ammonia solution and water to prepare the calibration curve in the appropriate ammonia concentration range. To analyze saline waters use substitute ocean water of the following composition to prepare calibration standards:

Constituent	Concentration *g/L*
NaCl	24.53
$MgCl_2$	5.20
$CaCl_2$	1.16
KCl	0.70
$SrCl_2$	0.03
Na_2SO_4	4.09
$NaHCO_3$	0.20
KBr	0.10
H_3BO_3	0.03
NaF	0.003

Subtract blank background response of substitute seawater from standards before preparing standard curve.

4. Procedure

a. Eliminate marked variation in acidity or alkalinity among samples. Adjust pH of wash water and standard ammonia solutions to approximately that of sample.

b. Set up manifold and complete system as shown in Figure 4500-NH_3:1.

c. Obtain a stable base line with all reagents, feeding wash water through sample line.

* Brij-35, available from ICI Americas, Wilmington, DE.

d. Typically, use a 60/h, 6:1 cam with a common wash.

5. Calculation

Prepare standard curves by plotting response of standards processed through the manifold against NH_3-N concentrations in standards. Compute sample NH_3-N concentration by comparing sample response with standard curve.

6. Precision and Bias

For an automated phenate system in a single laboratory using surface water samples at concentrations of 1.41, 0.77, 0.59, and 0.43 mg NH_3-N/L, the standard deviation was $\pm$0.005 mg/L, and at concentrations of 0.16 and 1.44 mg NH_3-N/L, recoveries were 107 and 99%, respectively.

7. Bibliography

HILLER, A. & D. VAN SLYKE. 1933. Determination of ammonia in blood. *J. Biol. Chem.* 102:499.

FIORE, J. & J.E. O'BRIEN. 1962. Ammonia determination by automatic analysis. *Wastes Eng.* 33:352.

AMERICAN SOCIETY FOR TESTING AND MATERIALS. 1966. Manual on Industrial Water and Industrial Waste Water, 2nd ed. American Soc. Testing & Materials, Philadelphia, Pa.

O'CONNOR, B., R. DOBBS, B. VILLIERS & R. DEAN. 1967. Laboratory distillation of municipal waste effluents. *J. Water Pollut. Control Fed.* 39:25.

4500-NH_3 H. Flow Injection Analysis

1. General Discussion

a. Principle: A water sample containing ammonia or ammonium cation is injected into an FIA carrier stream to which a complexing buffer, alkaline phenol, and hypochlorite are added. This reaction, the Berthelot reaction, produces the blue indophenol dye. The blue color is intensified by the addition of nitroferricyanide. The resulting peak's absorbance is measured at 630 nm. The peak area is proportional to the concentration of ammonia in the original sample.

Also see Section 4500-NH_3.F and Section 4130, Flow Injection Analysis (FIA).

b. Interferences: Remove large or fibrous particulates by filtering sample through glass wool. Guard against contamination from reagents, water, glassware, and the sample preservation process.

Also see Section 4500-NH_3.A. Some interferents are removed by distillation; see Section 4500-NH_3.B.

2. Apparatus

Flow injection analysis equipment consisting of:

a. FIA injection valve with sample loop or equivalent.

b. Multichannel proportioning pump.

c. FIA manifold (Figure 4500-NH_3:2) with tubing heater and flow cell. In Figure 4500-NH_3:2, relative flow rates only are shown. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material such as TFE.*

d. Absorbance detector, 630 nm, 10-nm bandpass.

e. Injection valve control and data acquisition system.

* Teflon or equivalent.

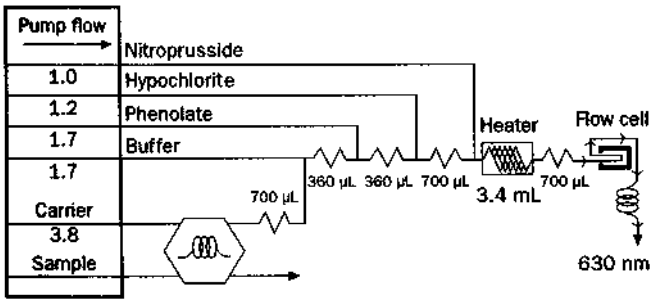


Figure 4500-NH_3:2. FIA ammonia manifold.

3. Reagents

Use reagent water (>10 megohm) to prepare carrier and all solutions. To prevent bubble formation, degas carrier and buffer with helium. Use He at 140 kPa (20 psi) through a helium degassing tube. Bubble He through 1 L solution for 1 min. As an alternative to preparing reagents by weight/weight, use weight/volume.

a. Buffer: To a 1-L tared container add 50.0 g disodium ethylenediamine tetraacetate and 5.5 g sodium hydroxide, NaOH. Add 968 mL water. Mix with a magnetic stirrer until dissolved.

b. Phenolate: CAUTION: *Wear gloves. Phenol causes severe burns and is rapidly absorbed into the body through the skin.* To a tared 1-L container, add 888 g water. Add 94.2 g 88% liquefied phenol or 83 g crystalline phenol, C_6H_5OH. While stirring, slowly add 32 g NaOH. Cool and invert to mix thoroughly. Do not degas.

c. Hypochlorite: To a tared 500-mL container add 250 g 5.25% sodium hypochlorite, NaOCl bleach solution† and 250 g water. Stir or shake to mix.

d. Nitroprusside: To a tared 1-L container add 3.50 g sodium nitroprusside (sodium nitroferricyanide), $Na_2Fe(CN)_5NO \cdot 2H_2O$, and 1000 g water. Invert to mix.

e. Stock ammonia standard, 1000 mg N/L: In a 1-L volumetric flask dissolve 3.819 g ammonium chloride, NH_4Cl, that has been dried for 2 h at 110°C, in about 800 mL water. Dilute to mark and invert to mix.

f. Standard ammonia solutions: Prepare ammonia standards in desired concentration range, using the stock standard (¶ 3*e*), and diluting with water.

4. Procedure

Set up a manifold equivalent to that in Figure 4500-NH_3:2 and follow method supplied by manufacturer or laboratory standard operating procedure for this method. Follow quality control procedures described in Section 4020.

5. Calculations

Prepare standard curves by plotting the absorbance of standards processed through the manifold versus ammonia concentration. The calibration curve is linear.

6. Precision and Bias

a. Recovery and relative standard deviation: The results of single-laboratory studies with various matrices are given in Table 4500-NH_3:IV.

b. MDL: A 650-μL sample loop was used in the method described above. Using a published MDL method,[1] analysts ran 21 replicates of a 0.020-mg N/L standard. These gave a mean of 0.0204 mg N/L, a standard deviation of 0.0007 mg N/L, and an MDL of 0.002 mg N/L.

7. Reference

1. U.S. Environmental Protection Agency. 1989. Definition and Procedure for the Determination of Method Detection Limits. Appendix B to 40 CFR 136 rev. 1.11 amended June 30, 1986. 49 CFR 43430.

Table 4500-NH_3:IV. Results of Single-Laboratory Studies with Selected Matrices

Matrix	Sample/Blank Designation	Known Addition *mg NH_3-N/L*	Recovery %	Relative Standard Deviation %
Wastewater treatment plant influent	Reference sample*	—	104	—
	Blank†	0.4	100	—
		0.8	102	—
	Site A‡§	0	—	0.5
		0.4	108	—
		0.8	105	—
	Site B‡§	0	—	0.5
		0.4	105	—
		0.8	105	—
	Site C‡§	0	—	1.1
		0.4	109	—
		0.8	107	—
Wastewater treatment plant effluent	Reference sample*	—	106	—
	Blank†	0.4	105	—
		0.8	105	—
	Site A‡‖	0	—	ND
		0.4	90	—
		0.8	88	—
	Site B‡‖	0	—	<0.1
		0.4	93	—
		0.8	94	—
	Site C‡‖	0	—	<0.1
		0.4	89	—
		0.8	91	—
Landfill leachate‖	Reference sample*	—	106	—
	Blank†	0.4	105	—
		0.8	106	—
	Site A‡#	0	—	1.9
		0.4	125	—
		0.8	114	—
	Site B‡#	0	—	0.4
		0.4	96	—
		0.8	106	—
	Site C‡#	0	—	0.2
		0.4	102	—
		0.8	107	—

ND = not detectable.
* U.S. EPA QC sample, 1.98 mg N/L.
† Determined in duplicate.
‡ Samples without known additions determined four times; samples with known additions determined in duplicate.
§ Site A and C samples diluted 20-fold, Site B sample diluted 100-fold. Typical relative difference between duplicates 0.2%.
‖ Samples not diluted. Typical relative difference between duplicates <1%.
Site A and C samples diluted 50-fold; Site B sample diluted 150-fold. Typical relative difference between duplicates 0.3%.

4500-NO_2^- NITROGEN (NITRITE)*

4500-NO_2^- A. Introduction

1. Occurrence and Significance

For a discussion of the chemical characteristics, sources, and effects of nitrite nitrogen, see Section 4500-N.

* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011.

2. Selection of Method

The colorimetric method (4500-NO_2^-.B) is suitable for concentrations of 5 to 1000 μg NO_2^--N/L (See 4500-NO_2^-.B.1*a*). Nitrite values can be obtained by the automated method given in Section 4500-NO_3^-.E with the Cu–Cd reduction step omitted. Additionally, nitrite nitrogen can be determined by ion chromatography (Section 4110), and by flow injection analysis (see Sections 4130 and 4500-NO_3^-.I).

4500-NO_2^- B. Colorimetric Method

1. General Discussion

a. Principle: Nitrite (NO_2^-) is determined through formation of a reddish purple azo dye produced at pH 2.0 to 2.5 by coupling diazotized sulfanilamide with *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NED dihydrochloride). The applicable range of the method for spectrophotometric measurements is 10 to 1000 μg NO_2^--N/L. Photometric measurements can be made in the range 5 to 50 μg N/L if a 5-cm light path and a green color filter are used. The color system obeys Beer's law up to 180 μg N/L with a 1-cm light path at 543 nm. Higher NO_2^- concentrations can be determined by diluting a sample.

b. Interferences: Chemical incompatibility makes it unlikely that NO_2^-, free chlorine, and nitrogen trichloride (NCl_3) will coexist. NCl_3 imparts a false red color when color reagent is added. The following ions interfere because of precipitation under test conditions and should be absent: Sb^{3+}, Au^{3+}, Bi^{3+}, Fe^{3+}, Pb^{2+}, Hg^{2+}, Ag^+, chloroplatinate ($PtCl_6^{2-}$), and metavanadate (VO_3^{2-}). Cupric ion may cause low results by catalyzing decomposition of the diazonium salt. Colored ions that alter the color system also should be absent. Remove suspended solids by filtration.

c. Storage of sample: Never use acid preservation for samples to be analyzed for NO_2^-. Make the determination promptly on fresh samples to prevent bacterial conversion of NO_2^- to NO_3^- or NH_3. For short-term preservation for 1 to 2 d, freeze at −20°C or store at 4°C.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Colorimetric equipment: One of the following is required:

a. Spectrophotometer, for use at 543 nm, providing a light path of 1 cm or longer.

b. Filter photometer, providing a light path of 1 cm or longer and equipped with a green filter having maximum transmittance near 540 nm.

3. Reagents

a. Nitrite-free water: If it is not known that the distilled or demineralized water is free from NO_2^-, use either of the following procedures to prepare nitrite-free water:

1) Add to 1 L distilled water one small crystal each of $KMnO_4$ and either $Ba(OH)_2$ or $Ca(OH)_2$. Redistill in an all-borosilicate-glass apparatus and discard the initial 50 mL of distillate. Collect the distillate fraction that is free of permanganate; a red color with DPD reagent (Section 4500-Cl.F.2*b*) indicates the presence of permanganate.

2) Add 1 mL conc H_2SO_4 and 0.2 mL $MnSO_4$ solution (36.4 g $MnSO_4 \cdot H_2O$/100 mL distilled water) to each 1 L distilled water, and make pink with 1 to 3 mL $KMnO_4$ solution (400 mg $KMnO_4$/L distilled water). Redistill as described in the preceding paragraph.

Use nitrite-free water in making all reagents and dilutions.

b. Color reagent: To 800 mL water, add 100 mL 85% phosphoric acid and 10 g sulfanilamide. After dissolving sulfanilamide completely, add 1 g *N*-(1-naphthyl)-ethylenediamine dihydrochloride. Mix to dissolve, then dilute to 1 L with water. Solution is stable for about a month when stored in a dark bottle in refrigerator.

c. Sodium oxalate, 0.025*M* (0.05*N*): Dissolve 3.350 g $Na_2C_2O_4$, primary standard grade, in water and dilute to 1000 mL.

d. Ferrous ammonium sulfate, 0.05*M* (0.05*N*): Dissolve 19.607 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ plus 20 mL conc H_2SO_4 in water and dilute to 1000 mL. Standardize as in Section 5220B.3*d.*

e. Stock nitrite solution: Commercial reagent-grade $NaNO_2$ assays at less than 99%. Because NO_2^- is oxidized readily in the

presence of moisture, use a fresh bottle of reagent for preparing the stock solution and keep bottles tightly stoppered against the free access of air when not in use. To determine $NaNO_2$ content, add a known excess of standard 0.05*N* $KMnO_4$ solution (see ¶ *h* below), discharge permanganate color with a known quantity of standard reductant, such as 0.025*M* $Na_2C_2O_4$ or 0.05*M* $Fe(NH_4)_2(SO_4)_2$, and back-titrate with standard permanganate solution.

1) Preparation of stock solution—Dissolve 1.232 g $NaNO_2$ in water and dilute to 1000 mL; 1.00 mL = 250 μg N. Preserve with 1 mL $CHCl_3$.

2) Standardization of stock nitrite solution—Pipet, in order, 50.00 mL standard 0.05*N* $KMnO_4$, 5 mL conc H_2SO_4, and 50.00 mL stock NO_2^- solution into a glass-stoppered flask or bottle. Submerge pipet tip well below surface of permanganate-acid solution while adding stock NO_2^- solution. Shake gently and warm to 70 to 80°C on a hot plate. Discharge permanganate color by adding sufficient 10-mL portions of standard 0.025*M* $Na_2C_2O_4$. Titrate excess $Na_2C_2O_4$ with 0.05*N* $KMnO_4$ to the faint pink endpoint. Carry a water blank through the entire procedure and make the necessary corrections in the final calculation as shown in the equation below.

If standard 0.05*M* ferrous ammonium sulfate solution is substituted for $Na_2C_2O_4$, omit heating and extend reaction period between $KMnO_4$ and Fe^{2+} to 5 min before making final $KMnO_4$ titration.

Calculate NO_2^--N content of stock solution by the following equation:

$$A = \frac{[(B \times C) - (D \times E)] \times 7}{F}$$

where:

A = mg NO_2^--N/mL in stock $NaNO_2$ solution,
B = total mL standard $KMnO_4$ used,
C = normality of standard $KMnO_4$,
D = total mL standard reductant added,
E = normality of standard reductant, and
F = mL stock $NaNO_2$ solution taken for titration.

Each 1.00 mL 0.05*N* $KMnO_4$ consumed by the $NaNO_2$ solution corresponds to 1725 μg $NaNO_2$ or 350 μg NO_2^--N.

f. Intermediate nitrite solution: Calculate the volume, *G*, of stock NO_2^- solution required for the intermediate NO_2^- solution from $G = 12.5/A$. Dilute the volume *G* (approximately 50 mL) to 250 mL with water; 1.00 mL = 50.0 μg N. Prepare daily.

g. Standard nitrite solution: Dilute 10.00 mL intermediate NO_2^- solution to 1000 mL with water; 1.00 mL = 0.500 μg N. Prepare daily.

h. Standard potassium permanganate titrant, 0.05*N:* Dissolve 1.6 g $KMnO_4$ in 1 L distilled water. Keep in a brown glass-stoppered bottle and age for at least 1 week. Carefully decant or pipet supernate without stirring up any sediment. Standardize this solution frequently by the following procedure:

Weigh to the nearest 0.1 mg several 100- to 200-mg samples of anhydrous $Na_2C_2O_4$ into 400-mL beakers. To each beaker, in turn, add 100 mL distilled water and stir to dissolve. Add 10 mL 1 + 1 H_2SO_4 and heat rapidly to 90 to 95°C. Titrate rapidly with permanganate solution to be standardized, while stirring, to a slight pink endpoint color that persists for at least 1 min. Do not let temperature fall below 85°C. If necessary, warm beaker contents during titration; 100 mg will consume about 6 mL solution. Run a blank on distilled water and H_2SO_4.

$$\text{Normality of } KMnO_4 = \frac{\text{g } Na_2C_2O_4}{(A - B) \times 0.067}$$

where:

A = mL titrant for sample and
B = mL titrant for blank.

Average the results of several titrations.

4. Procedure

a. Removal of suspended solids: If sample contains suspended solids, filter through a 0.45-μm-pore-diam membrane filter.

b. Color development: If sample pH is not between 5 and 9, adjust to that range with 1*N* HCl or NH_4OH as required. To 50.0 mL sample, or to a portion diluted to 50.0 mL, add 2 mL color reagent and mix.

c. Photometric measurement: Between 10 min and 2 h after adding color reagent to samples and standards, measure absorbance at 543 nm. As a guide use the following light paths for the indicated NO_2^--N concentrations:

Light Path Length *cm*	NO_2^--N *μg/L*
1	2–25
5	2–6
10	<2

5. Calculation

Prepare a standard curve by plotting absorbance of standards against NO_2^--N concentration. Compute sample concentration directly from curve.

6. Precision and Bias

In a single laboratory using wastewater samples at concentrations of 0.04, 0.24, 0.55, and 1.04 mg NO^-_3 + NO_2^--N/L, the standard deviations were ±0.005, ± 0.004, ± 0.005, and ± 0.01, respectively. In a single laboratory using wastewater samples at concentrations of 0.24, 0.55, and 1.05 mg NO_3^- + NO_2^--N/L, the recoveries were 100%, 102%, and 100%, respectively.[1]

7. Reference

1. U.S. Environmental Protection Agency. 1979. Methods for Chemical Analysis of Water and Wastes; Method 353.3. Washington, D.C.

8. Bibliography

Boltz, D.F., ed. 1958. Colorimetric Determination of Nonmetals. Interscience Publishers, New York, N.Y.

Nydahl, F. 1976. On the optimum conditions for the reduction of nitrate by cadmium. *Talanta* 23:349.

4500-NO_3^- NITROGEN (NITRATE)*

4500-NO_3^- A. Introduction

1. Selection of Method

Nitrate (NO_3^-) determination can be difficult because of the high probability that interfering constituents will be present in various matrices. This section includes numerous methods that can be used for NO_3^-. Select methods after considering the advantages and limitations of each. Consideration of a method should include sample matrix, concentration range, and data needs for the particular application.

The ultraviolet (UV) light technique (4500-NO_3^-.B), which measures NO_3^- absorbance at 220 nm, is suitable for screening uncontaminated water (low in organic matter).

Screen a sample if necessary, then select a method suitable for its concentration range and probable interferences. Nitrate can be determined by ion chromatography (Section 4110), capillary ion electrophoresis (Section 4140), or the methods shown here. Applicable ranges for the methods in this section are:

- 0.2–11 mg nitrate-nitrogen (NO_3-N)/L [Ultraviolet Spectrophotometric Method (4500-NO_3^-.B)]
- 0.5–2.5 mg NO_3-N/L [Second Derivative Ultraviolet Screening Method (4500-NO_3^-.C)]
- 1–50 mg NO_3-N/L [Nitrate Electrode Method (4500-NO_3^-.D)]
- 0.05–1.0 mg nitrate + nitrite-nitrogen (NO_3 + NO_2-N)/L [Cadmium Reduction Method (4500-NO_3^-.E)]
- 0.05–10 mg nitrate + nitrite-nitrogen (NO_3 + NO_2-N)/L [Automated Cadmium Reduction Method (4500-NO_3^-.F)]
- 0.05–10 mg nitrate + nitrite nitrogen (NO_3 + NO_2-N)/L [Automated Hydrazine Reduction Method (4500-NO_3^-.H)]
- 0.01–2.0 or 0.05–5.0 mg nitrate + nitrite (NO_3 + NO_2-N)/L [Cadmium Reduction Flow Injection Method (4500-NO_3^-.I)]

Other ranges may be possible for any of the above referenced methods. Refer to manufacturer's instructions.

For higher NO_3^--N concentrations, dilute into the range of the selected method. See Sections 4110B and C for the working range of ion chromatography methods.

Filter turbid samples. Test filters for NO_3^- contamination.

2. Collection and Storage of Samples

Collect samples in polyethylene, fluoropolymer, or glass containers. If possible, start NO_3^- determinations promptly after sampling. Samples can be stored un-acidified for up to 48 h at <6°C. Acidification converts any nitrite (NO_2^-) to NO_3^-. As a result, NO_3^- values are the sum of NO_3^- and NO_2^-. If samples must be stored for >48 h, acidify to pH <2 with sulfuric or hydrochloric acid (depending on the method) and store at <6°C (or at 2 to 6°C for SDWA compliance samples) for up to 28 d. Chlorinated samples are stable for at least 14 d without acid preservation.

3. Quality Control

Quality control (QC) practices and acceptance criteria are described in Sections 1020 and 4020. The following section applies to all NO_3^--N methods; however, some methods have additional QC measures. Complete the initial QC tasks, including initial demonstration of capability for each analyst, estimation of the method detection limit (MDL), and determination of the dynamic range, before analyzing any samples and at least annually thereafter. Apply the rest of these measures whenever samples are analyzed. If the acceptance criteria are not satisfied, stop and correct the problem. Regulators may specify different acceptance criteria than those given here.

Calibrate or verify the calibration of each instrument daily. Using a calculator, electronic spreadsheet, or instrument software, calculate the slope, intercept, and correlation coefficient (r) or coefficient of determination (r^2) of the calibration curve. The r value must be at least 0.995 ($r^2 \geq 0.99$). Back-calculate the apparent concentrations of the standards. For standards >10 times the MDL, the measured values must be 90 to 110% of the true values. The calibration requirements are different for 4500-NO_3^-.D; see instructions in 4500-NO_3^-.D.4*b* or 4500-NO_3^-.D.4*c*.

Prepare a calibration-verification standard (CVS) from a stock solution separate from that used to prepare the calibration standards. The CVS's NO_3^--N concentration should be 30 to 70% of the highest calibration standard; however, some QA/QC programs may require different concentrations. Run the CVS immediately after calibration; the result must be 90 to 110% of the expected value.

Run an initial calibration blank (ICB) immediately after the CVS to check for contamination. The ICB reading must be ≤½ the minimum reporting level (MRL).

Run a midpoint calibration standard as continuing calibration verification (CCV) and a continuing calibration blank (CCB) after every 10 samples and after the last sample. If the measured NO_3^--N concentration in the CCV is not 90 to 110% of the expected value, recalibrate and rerun all samples read since the last good CCV reading. The CCB must be ≤½MRL.

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4500-NO_3^- B. Ultraviolet Spectrophotometric Screening Method

1. General Discussion

a. Principle: Use this technique only to screen samples containing low organic matter (i.e., uncontaminated natural waters and potable water supplies). The NO_3^- calibration curve follows Beer's law up to 11 mg N/L.

Measuring UV absorption at 220 nm enables analysts to determine NO_3^- rapidly. Be aware that dissolved organic matter also may absorb at 220 nm but NO_3^- does not absorb at 275 nm, so a second measurement can be made at 275 nm and used to correct the NO_3^- value, if needed. The extent of this empirical correction is related to the nature and concentration of the organic matter and may vary from one water to another, so this method is not recommended if a significant correction is required. That said, it may be useful in monitoring NO_3^- levels in a waterbody with a constant type of organic matter.

b. Interferences: Potential interferences include dissolved organic matter, surfactants, NO_2^-, hexavalent chromium [Cr(VI)], and various inorganic ions not normally found in natural water, such as chlorite and chlorate. Correction factors for organic matter absorbance can be established by the method of additions combined with analysis of the original NO_3^- content via another method. Sample filtration eliminates interference by suspended particles. Acidification with 1*M* hydrochloric acid (HCl) to pH <2 prevents interference from hydroxide or carbonate concentrations up to 1000 mg calcium carbonate ($CaCO_3$)/L. Chloride does not affect the determination.

Inorganic substances can be compensated for by independently analyzing their concentrations and preparing individual correction curves. Filter turbid samples. Test filters for NO_3^- contamination.

2. Apparatus

Spectrophotometer, for use at 220 and 275 nm with matched silica cells of 1-cm or longer light path.

3. Reagents

a. Reagent water: Use reagent water as defined in Section 1080 to prepare all solutions and dilutions.

b. Stock nitrate solution: Dry potassium nitrate (KNO_3) in an oven at 103–105°C for 24 h. Dissolve 0.7218 g ± 0.0005g in water and dilute to 1000 mL; 1.00 mL = 100 μg NO_3^--N. Preserve with 2 mL chloroform ($CHCl_3$)/L. Solution is stable for at least 6 months. Alternatively, use a commercial NO_3^--N stock solution.

c. Intermediate nitrate solution: Dilute 100 mL stock NO_3^--N solution to 1000 mL with water; 1.00 mL = 10.0 μg NO_3^--N. Preserve with 2 mL $CHCl_3$/L. Solution is stable for 6 months.

d. Hydrochloric acid solution, (~1M): Dilute 83 mL concentrated HCl to 1L with reagent water. Store in a glass or high-density polyethylene (HDPE) bottle. Solution is stable for 1 year if kept closed.

4. Procedure

a. Treatment of sample: To 50 mL clear sample (filtered if necessary), add 1 mL 1*M* HCl solution and mix thoroughly.

b. Standards: Prepare NO_3^- calibration standards in the range 0 to 7 mg NO_3^--N/L by diluting to 50 mL the following volumes of intermediate NO_3^- solution: 0, 1.00, 2.00, 4.00, 7.00 . . . 35.0 mL. Other standard concentrations may also be used. Treat NO_3^--N standards in same manner as samples.

c. Spectrophotometric measurement: Read absorbance or transmittance against reagent water set at zero absorbance or 100% transmittance. Use a wavelength of 220 nm to obtain NO_3^--N reading and a wavelength of 275 nm to determine any interference due to dissolved organic matter.

5. Calculation

For samples and standards, subtract two times the absorbance reading at 275 nm from the reading at 220 nm to obtain absorbance due to NO_3^--N. If correction value is $>10\%$ of reading at 220 nm for a particular sample, then the NO_3^--N concentration is considered a rough estimate. Use an electronic spreadsheet, a calculator, or instrument software to find the slope and intercept of the calibration curve by least squares linear regression. Calculate the NO_3^--N concentration from the following equation:

$$C = \frac{A - I}{S}$$

where:

C = concentration,
A = absorbance,
I = intercept of the regression line, and
S = slope of the regression line.

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4500-NO_3^- C. Second-Derivative Ultraviolet Spectrophotometric Method

1. General Discussion

a. Principle: Even though NO_3^- strongly absorbs UV light, determining NO_3^- by measuring the absorbance at one wavelength may not be feasible because natural organic matter (NOM) and other solutes also absorb UV light. The spectra of NOM from different water sources usually are different, so if NOM is present the UV NO_3^- screening procedure (4500-NO_3^-.B) will not give reliable results. The UV spectrum of NO_3^- is quite different from that of NOM. In the NO_3^- spectrum, the absorbance increases rapidly from 230 to 210 nm, whereas in NOM spectra, the absorbance increases gradually in the same wavelength range. Computing the second derivative of a sample spectrum effectively eliminates the background NOM contribution.

b. Interferences: The NO_2^- and NO_3^- UV spectra are similar, but NO_2^- concentrations usually are much lower than NO_3^- concentrations. Bicarbonate absorbs weakly at wavelengths <210 nm but does not affect the second-derivative signal of NO_3^-. Bromide (Br^-) interferes at seawater concentrations (68 mg Br^-/L, salinity 35 ‰), so this method cannot be used to determine NO_3^- in seawater or samples containing high concentrations of Br^-. Neither iron (Fe) nor copper (Cu) interferes at 2 mg/L, but both metals seriously interfere at 20 mg/L.[1] For NO_3^--N concentrations up to 3 mg/L, the UV absorbance spectrum has a peak at 203 nm. For higher concentrations, the peak gradually shifts to 207 nm and the second-derivative maximum also shifts from 224 nm to higher wavelengths. The shape of the UV and second-derivative spectra may be affected by the spectrophotometer slit width. The method has been tested for potable water but not for wastewater.

2. Apparatus

a. Spectrophotometer capable of scanning from 250 to 200 nm. Some spectrophotometers can compute the second derivative of a spectrum; if the instrument does not, a computer interface is needed (see ¶ *d* below).

b. Cuvettes, matched 1-cm quartz.

c. Pipets: If adjustable pipets are used, calibrate according to manufacturer's directions.

d. Computer, cable, and software to transfer data from spectrophotometer and compute second-derivative spectra. Cable and software may be available from spectrophotometer manufacturer. These items are needed only if spectrophotometer cannot compute second-derivative spectra.

3. Reagents

a. Nitrate stock solution A, 100 mg NO_3^--N/L: Prepare as directed in 4500-NO_3^-.B.3*b* or obtain from a commercial source.

b. Nitrate stock solution B, 100 mg NO_3^--N/L: Purchase from a commercial source different from stock nitrate solution A or prepare as directed in 4500-NO_3^-.B.3*b* using KNO_3 from a commercial source different from that used to prepare stock nitrate solution A.

c. Reagent water (see 4500-NO_3^-.B.3*a*): Use to prepare standards and dilute samples if necessary.

4. Procedure

a. Sample treatment: Filter samples if turbid.

b. Standards: Dilute 0.5, 1.0, 1.5, 2.0, and 2.5 mL NO_3^--N stock solution A to 100 mL with reagent water to prepare standards containing 0.5, 1.0, 1.5, 2.0, and 2.5 mg NO_3^--N/L. Alternative concentrations are also acceptable. Prepare a mid-range standard from stock solution B to use as a CVS.

c. Measurement: Pour standard or sample into cuvette. Empty and refill cuvette. Scan from 250 to 200 nm. Dilute sample if needed to keep readings on-scale. Record any dilution. Compute second-derivative spectrum. Find maximum second derivative in the range of 230 to 220 nm and record value. If spectrophotometer cannot compute second-derivative spectra, then set it to read the absorbance every 1.0 nm and transfer spectra to the computer after each scan.

5. Calculation

a. Calibration: If spectrophotometer cannot compute second-derivative spectra, then calculate them and find maximum for each sample, blank, or standard. Use the simplified least-squares procedure[2,3] to simultaneously smooth and differentiate spectra.* Use an electronic spreadsheet, calculator, or instrument software to perform least-squares linear regression using the second derivatives of blank and standard spectra:

$$SD = I + C \times S$$

where:

SD = maximum second derivative,
I = intercept of regression line,
C = concentration, and
S = slope of regression line.

Thus,

$$C = \frac{SD - I}{S}$$

b. Samples: Calculate NO_3^- concentration (C) from the equation above.

6. Precision and Bias

Nitrate concentrations determined via UV second-derivative method were compared with those determined via ion chromatography for 79 potable-water samples with NO_3^--N concentrations of up to 40 mg NO_3^--N/L.[4] The slope of the regression line was 0.94, with a standard error of 0.005. For 65 of 79 samples, the difference between NO_3^--N concentrations determined by second-derivative UV spectrophotometry and ion chromatography was ≤0.5 mg N/L. The maximum difference for all samples was 1.6 mg NO_3^--N/L. The differences between NO_3^--N con-

* A Microsoft Excel® worksheet for this calculation can be obtained from Tom Holm, Illinois State Water Survey, 2204 Griffith Drive, Champaign, IL 61820.

centrations determined by the two methods appeared to be random, but they were not normally distributed.

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4500-NO_3^- D. Nitrate Electrode Method

1. General Discussion

a. Principle: The NO_3^- ion electrode is a selective sensor that develops a potential across a thin, inert membrane holding in place a water-immiscible liquid ion exchanger. The electrode responds to NO_3^- ion activity between about 10^{-5} and 10^{-1} *M* (0.14 to 1400 mg NO_3^--N/L). The electrode response is linearly related to the logarithm of NO_3^- activity. The lower limit of detection is determined by the small but finite solubility of the liquid ion exchanger.

b. Interferences: Standards and samples must all be at the same temperature ±1°C. Chloride (Cl^-) and bicarbonate (HCO_3^-) ions interfere when their weight ratios to NO_3^--N are >10 or >5, respectively. Ions that are potential interferences but do not normally occur at significant levels in potable waters are Br^-, chlorate (ClO_3^-), cyanide (CN^-), hydrosulfide (HS^-), iodide (I^-), NO_2^-, and perchlorate (ClO_4^-). Although the electrodes function satisfactorily in buffers ranging from pH 3 to 9, erratic responses have been noted when pH is not held constant. Because the electrode responds to NO_3^- activity rather than concentration, ionic strength must be constant in all samples and standards. Minimize these problems by using a buffer solution containing silver sulfate (Ag_2SO_4) to remove Br^-, Cl^-, CN^-, I^-, and HS^- interferences, sulfamic acid to remove NO_2^-, a buffer at pH 3 to eliminate HCO_3^- and maintain a constant pH and ionic strength, and aluminum sulfate [$Al_2(SO_4)_3$] to complex organic acids. For example, 10 mL of buffer will compensate for about 781 mg/L of chloride interference from the sample matrix.

2. Apparatus

a. pH/mV/ISE meter (concentration mode) or pH meter, expanded-scale or digital, capable of 0.1 mV resolution (no concentration mode).

b. Nitrate ion-selective electrode, combination or half-cell† with double-junction reference electrode‡* : Fill outer chamber with solution containing ammonium sulfate [$(NH_4)_2SO_4$]. Carefully follow manufacturer's instructions on care and storage.

c. Magnetic or motorized stirrer: Tetrafluoroethylene (TFE)-coated stirring bar.

d. Pipets: If adjustable pipets are used, calibrate according to manufacturer's directions.

3. Reagents

a. Reagent water: Prepare as described in 4500-NO_3^-.B.3*a*. Use for all solutions and dilutions.

b. Stock nitrate solution A, 100 mg/L: Purchase from a commercial source (shelf life as stated by manufacturer) or prepare by drying KNO_3 in a 103–105°C oven for 24 h. Dissolve 0.7218 g ± 0.0005g in water and dilute to 1000 mL; 1.00 mL = 100 µg NO_3^-N. Solution is stable for at least 6 months.

c. Stock nitrate solution B, 1 mL = 100 µg NO_3^--N: Purchase from a commercial source different from stock nitrate solution A or prepare as directed in ¶ *b* above using KNO_3 from a commercial source different from that used to prepare stock nitrate solution A.

d. Standard nitrate solutions: Dilute 1.0, 10.0, and 50.0 mL stock nitrate solution A to 100 mL with reagent water to obtain standard solutions of 1.0, 10.0, and 50.0 mg NO_3^--N/L, respectively. If calibration curve is nonlinear, consult the manual that came with the meter. Additional standards may be recommended. Some pH/ISE meters automatically perform nonlinear calibration.

e. Calibration verification standard: Dilute 10.0 mL stock nitrate solution B to 100 mL with reagent water. Nitrate concentration is 10 mg NO_3^--N/L.

f. Buffer solution: Dissolve 17.32 g $Al_2(SO_4)_3 \cdot 18H_2O$, 3.43 g Ag_2SO_4, 1.28 g H_3BO_3, and 2.52 g sulfamic acid (H_2NSO_3H) in about 800 mL reagent water. Adjust to pH 3.0 by slowly adding 0.10*N* NaOH. Dilute to 1000 mL and store in an amber glass bottle. Alternatively, obtain from a commercial source.§

g. Sodium hydroxide (NaOH), 0.1*M*.

h. Reference electrode filling solution: Dissolve 0.53 g $(NH_4)_2SO_4$ in reagent water and dilute to 100 mL, or obtain a

* Thermo Scientific Orion Model 97-07BN, or equivalent.
† Thermo Scientific Orion Model 93-07BNWP, or equivalent.
‡ Thermo Scientific Orion Model 90-0200, or equivalent.

§ Thermo Scientific Orion 930710, or equivalent.

filling solution containing $(NH_4)_2SO_4$ from a commercial source.‖

4. Procedure

a. Treatment of standards and samples: Pipet 10 mL of each NO_3^--N calibration standard or sample into 50-mL beakers. Pipet an equal volume of buffer into each standard and sample. Ensure that the total volume is sufficient to immerse the electrode(s) and permit stirring; if necessary, increase amount of sample and buffer in 1:1 ratio. If samples were refrigerated, allow time for standards and samples to reach the same temperature ±1°C.

b. Calibration on an ISE or pH/ISE meter: Follow manufacturer's directions to operate in concentration mode. Set units to mg/L and enable the nonlinear calibration feature (e.g., Autoblank). Perform three-point calibration using 1.0, 10.0, and 50.0 mg NO_3^--N/L nitrate standards. Immerse electrode tip(s) so junction(s) is/are submerged in the 1 mg NO_3^--N/L standard solution and start stirring. Remove electrode(s), rinse, and blot dry. Repeat for 10- and 50-mg NO_3^--N/L standards. The slope must be negative, with an absolute value ≥50 mV/decade.

c. Calibration on an ISE or pH/ISE meter in mV: Perform three-point calibration using 1, 10, and 50 mg NO_3^--N/L nitrate standards. Immerse electrode tip(s) so junction(s) is/are submerged in the 1 mg NO_3^--N/L standard solution and start stirring. Record the millivolt reading when stable (when reading's rate of change is <0.5 mV/min). Remove electrode(s), rinse, and blot dry. Repeat for 10- and 50-mg NO_3^--N/L standards. For millivolt readings, use an electronic spreadsheet to plot the common logarithm (base 10) of NO_3^--N concentration on the x-axis (abscissa) and potential (in millivolts) on the y-axis (ordinate). A straight line with a slope of –57 ± 3 mV/decade at 25°C is expected. If slope is outside the acceptable range, follow the electrode manufacturer's troubleshooting procedure. A nonlinear calibration is acceptable and may result in more accurate results. For a quadratic calibration curve, use standards of 1.0, 3.0, 10.0, 20.0, and 50.0 mg NO_3^--N/L. The r^2 value must be >0.99.

d. Measurement of sample on an ISE or pH/ISE meter: Immerse electrode tip(s) in a prepared sample so junction(s) is/are submerged and start stirring. Read sample concentration directly on meter's display. Dilute a sample if the reading is above the calibration range.

e. Measurement of sample on a pH meter: Immerse electrode tip(s) so junction(s) is/are submerged and start stirring. Record potential reading when it is stable ($\Delta E/\Delta t < 0.5$ mV/min, typically at least 1 min). Dilute a sample if the reading is above the calibration range.

‖ Thermo Scientific-Orion 900046, or equivalent.

5. Calculations

For ISE or pH/ISE meter operated in concentration mode, no calculations are required; read result directly from the meter. For mV measurements on a pH meter, find the slope, intercept, and correlation coefficient of the calibration line via least-squares linear regression using an electronic spreadsheet or calculator.

$$E = I + S^{*}Log_{10}(C)$$

where:

E = electrode potential,
I = intercept of calibration line,
S = slope of calibration line, and
C = NO_3^--N concentration in the standard.

Use I and S to calculate NO_3^--N in the samples:

$$C = 10^{\frac{E-I}{S}}$$

Use an electronic spreadsheet for quadratic calibration.

6. Quality Control

Analyze a fresh aliquot of 10-mg/L standard immediately after calibration. The calculated concentration must agree with the true value within ±10%. For mV readings on a pH meter, back-calculate NO_3^--N concentration from the reading for each standard. The back-calculated and true concentrations must agree within ±10%. If not, stop and correct the problem. For the matrix spike, select a sample for which the NO_3^--N concentration after spiking will be within the calibration range.

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4500-NO_3^- E. Cadmium Reduction Method

1. General Discussion

a. Principle: Nitrate (NO_3^-) is reduced almost quantitatively to nitrite (NO_2^-) in the presence of cadmium (Cd). This method uses commercially available Cd granules treated with copper sulfate ($CuSO_4$) and packed in a glass column.

The NO_2^- is then diazotized with sulfanilamide and coupled with *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NED) to form a highly colored azo dye that is measured colorimetrically. To correct for any NO_2^- present in the sample before NO_3^- reduction, samples must be analyzed without the reduction step. The applicable range of this method is 0.05 to 1.0 mg NO_3^--N/L. The method is recommended especially for NO_3^--N levels <0.1 mg N/L for which other methods lack adequate sensitivity.

b. Interferences: Suspended matter in the column may restrict sample flow, so filter turbid samples (see 4500-NO_3^-.A.l). Iron, copper, or other metals concentrations above several milligrams per liter lower reduction efficiency; add ethylenediaminetetraacetic acid (EDTA) to the buffer reagent to eliminate this interference. If necessary, pre-extract oil and grease with an organic solvent (Section 5520) to prevent it from coating the Cd surface. Hydrogen sulfide in water or wastewater samples from anoxic waterbodies may de-activate the column; treat acidified odorous samples by bubbling with air for 15 min. Residual chlorine can oxidize the Cd column, reducing its efficiency, so check samples for residual chlorine (see DPD methods in Section 4500-Cl) and, if needed, remove by adding sodium thiosulfate ($Na_2S_2O_3$) solution (Section 4500-NH_3.B.3*d*). Sample color that absorbs at about 540 nm interferes with results; dilute samples or measure absorbance of treated samples to which color reagent has not been added and subtract from the absorbance after addition of the color reagent.

2. Apparatus

a. Reduction column: Purchase or construct the column* (Figure 4500-NO_3^-:1) from a 100-mL volumetric pipet by removing the top portion. The column also can be constructed from two pieces of tubing joined end to end: join a 10-cm length of 3-cm-inner diameter (ID) tubing to a 25-cm length of 3.5-mm-ID tubing. Add a TFE stopcock with metering valve[1] to control flow rate.

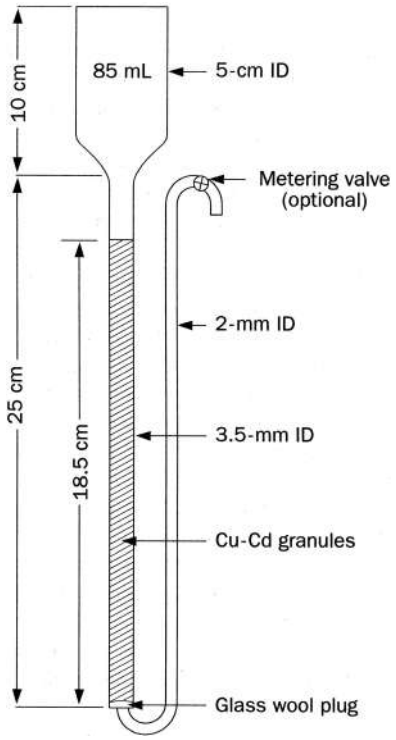


Figure 4500-NO_3^-:1. Reduction column.

b. Pipets: If adjustable pipets are used, verify the calibration according to manufacturer's directions.

c. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer*, for use at 543 nm, providing a light path of 1 cm or longer.

2) *Filter photometer*, with light path of 1 cm or longer and a filter whose maximum transmittance is near 540 nm.

3. Reagents

a. Reagent water: See 4500-NO_3^-.B.3*a*. Use for all solutions and dilutions.

b. Copper-cadmium granules: Wash 25 g new or used 20- to 100-mesh Cd granules† with 6*M* HCl and rinse with water. Swirl Cd with 100 mL 2% $CuSO_4$ solution for 5 min or until blue color partially fades. Decant and repeat with fresh $CuSO_4$ until a brown colloidal precipitate begins to develop. Gently flush with ammonium chloride-EDTA solution (¶ *d* below) to remove all precipitated Cu. Store activated Cd covered with dilute ammonium chloride–EDTA solution (¶ *e* below) .

c. Color reagent: To 800 mL reagent water, add 100 mL 85% phosphoric acid and 10 g sulfanilamide. After dissolving sulfanilamide completely, add 1 g NED. Mix to dissolve, then dilute to 1 L with water. Solution is stable for about a month when stored in an amber bottle in a refrigerator. Discard if the solution is highly colored or dark or if a precipitate forms.

d. Ammonium chloride-EDTA solution: Dissolve 13 g NH_4Cl and 1.7 g disodium ethylenediamine tetraacetate in 900 mL reagent water. Adjust to pH 8.5 with conc ammonium hydroxide (NH_4OH) and dilute to 1 L with reagent water. This solution is stable for 1 year.

e. Dilute ammonium chloride-EDTA solution: Dilute 300 mL NH_4Cl-EDTA solution to 500 mL with water. Use this reagent as a cadmium-reduction-column storage solution.

f. Hydrochloric acid, 6*M:* Carefully add 500 mL concentrated HCl to ~400 mL reagent water. Dilute to 1 L with reagent water. This solution is stable for 1 year.

* Tudor Scientific Glass Co., 555 Edgefield Road, Belvedere, SC 29841, Cat. No. TP-1730, or equivalent.

† EM Laboratories, Inc., 500 Exec. Blvd., Elmsford, NY, Cat. No. 2001, or equivalent.

g. Copper sulfate solution, 2%: Dissolve 20 g $CuSO_4 \cdot 5H_2O$ in 500 mL reagent water and dilute to 1 L with reagent water. This solution is stable for 1 year.

h. Stock nitrate solution A: Prepare as directed in 4500-NO_3^-.B.3*b* or obtain from a commercial source.

i. Stock nitrate solution B: Purchase from a commercial source different from stock nitrate solution A or prepare as directed in 4500-NO_3^-.B.3*b* using KNO_3 from a commercial source different from that used to prepare stock nitrate solution A.

j. Intermediate nitrate solution A: Prepare as directed in 4500-NO_3^-.B.3*c* using stock nitrate solution A.

k. Intermediate nitrate solution B: Prepare as directed in 4500-NO_3^-.B.3*c* using stock nitrate solution B.

l. Stock nitrite solution: See Section 4500-NO_2^-.B.3*e*.

m. Intermediate nitrite solution: See Section 4500-NO_2^-.B.3*f*.

n. Working nitrite solution: Dilute 50.0 mL intermediate NO_2^--N solution to 500 mL with reagent water; 1.00 mL = 5 μg NO_2^--N. Prepare fresh daily.

4. Procedure

a. Preparation of reduction column: Insert a glass wool plug into bottom of reduction column and fill with water. Add sufficient Cu-Cd granules to produce a column 18.5 cm long. Maintain water level above Cu-Cd granules to avoid entrapping air. Wash column with 200 mL dilute NH_4Cl-EDTA solution. Activate column by passing through it, at 7 to 10 mL/min, several 100-mL portions of a solution composed of 1 part 1.0 mg NO_3^--N/L standard and 3 parts NH_4Cl-EDTA solution.

CAUTION: Cadmium is toxic and carcinogenic. Collect and store all waste Cd. When handling Cd, wear gloves and follow the precautions described on Cd's safety data sheet.

b. Treatment of sample:

1) Turbidity removal—Filter turbid samples (see 4500-NO_3^-.A.1).

2) pH adjustment—Adjust pH to between 7 and 9, as necessary, with dilute HCl or NaOH. This ensures a pH of 8.5 after adding NH_4Cl-EDTA solution.

3) Sample reduction—To 25.0 mL sample or a portion diluted to 25.0 mL, add 75 mL NH_4Cl-EDTA solution and mix. Pour mixed sample into column and collect at a rate of 7 to 10 mL/min. Discard first 25 mL. Collect the rest in a clean sample flask. There is no need to wash columns between samples, but if columns will not be reused for several hours or longer, pour 50 mL dilute NH_4Cl-EDTA solution onto the top and let it pass through the system. Store Cu-Cd column in this solution and never let it dry.

4) Color development and measurement—As soon as possible (≤15 min after reduction), add 2.0 mL color reagent to 50 mL sample and mix. Between 10 min and 2 h afterward, measure absorbance at 543 nm against a distilled water-reagent blank. NOTE: If NO_3^--N concentration exceeds the standard curve range (about 1 mg N/L), use remainder of reduced sample to make an appropriate dilution and analyze again.

c. Calibration: Using intermediate nitrate solution A, prepare standards of 0.05, 0.10, 0.20, 0.50, and 1.0 mg NO_3^--N/L by diluting the following volumes to 100 mL in volumetric flasks: 0.5, 1.0, 2.0, 5.0, and 10.0 mL. (This calibration range is just a suggestion; other concentrations may be used as long as they meet reporting requirements). Prepare a CVS using intermediate nitrate solution B. To 25.0 mL of each standard, add 75 mL NH_4Cl-EDTA solution and mix. Pour mixed standard into column and collect at a rate of 7 to 10 mL/min. Discard first 50 mL. Collect the rest in a clean sample flask. There is no need to wash columns between samples, but if columns will not be reused for several hours or longer, pour 50 mL dilute NH_4Cl-EDTA solution onto the top and let it pass through the system. Store Cu-Cd column in this solution and never let it dry.

To verify reduction-column efficiency, compare at least one mid-range NO_2^- standard to a reduced NO_3^- standard at the same concentration. Reactivate Cu-Cd granules as described in 4500-NO_3^-.E.3*b* when reduction efficiency fails the quality control criterion (4500-NO_3^-.E.6).

5. Calculation

Use an electronic spreadsheet, calculator, or instrument software to find the slope, intercept, and correlation coefficient (r) or coefficient of determination (r^2) of the calibration curve by least squares linear regression. Calculate the NO_3^--N concentration from the following equation:

$$C = \frac{A - I}{S}$$

where:

C = concentration,
A = absorbance,
I = intercept of the regression line, and
S = slope of the regression line.

Report as milligrams oxidized N per liter (the sum of NO_3^--N plus NO_2^--N) unless the concentration of NO_2^--N is separately determined and subtracted.

6. Quality Control

Run a mid-level NO_2^-–N standard followed immediately by a NO_3^-–N standard of the same concentration. Calculate reduction efficiency as follows:

Efficiency = (NO_3^--N response/NO_2^--N response) × 100. The efficiency must be 90 to 110%. If not, stop and correct the problem by either following the manufacturer's instructions or passing 6 *M* HCl through the column followed by rinsing with dilute ammonium chloride-EDTA solution. Prepare or, if it cannot be reactivated, purchase a new column according to 4500-NO_3^-.E.3*b* and activate according to 4500-NO_3^-.E.4*a*.

7. Precision and Bias

In a single laboratory using wastewater samples at concentrations of 0.04, 0.24, 0.55, and 1.04 mg NO_3^- + NO_2^--N/L, the standard deviations were ±0.005, ±0.004, ±0.005, and ±0.01, respectively. In a single laboratory using wastewater with

additions of 0.24, 0.55, and 1.05 mg NO_3^- + NO_2^--N/L, the recoveries were 100, 102, and 100%, respectively.[2]

8. References

1. Wood, E.D., F.A.J. Armstrong & F.A. Richards. 1967. Determination of nitrate in sea water by cadmium-copper reduction to nitrite. *J. Mar. Biol. Assoc. U.K.* 47:23.
2. U.S. Environmental Protection Agency. 1979. Methods for Chemical Analysis of Water and Wastes; Method 353.3. Washington, D.C.

9. Bibliography

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Nydahl, F. 1976. On the optimum conditions for the reduction of nitrate by cadmium. *Talanta* 23:349.

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4500-NO_3^- F. Automated Cadmium Reduction Method

1. General Discussion

a. Principle: See 4500-NO_3^-.E.1*a*.

b. Interferences: Suspended matter may restrict sample flow in the column, so filter turbid samples (see 4500-NO_3^-.A.1). Iron, copper, or other metals concentrations above several milligrams per liter can lower reduction efficiency; ethylenediaminetetraacetic acid (EDTA) added to the buffer reagent eliminates this interference. If necessary, keep oil and grease from coating the Cd surface by pre-extracting it from samples with an organic solvent (see Section 5520). Hydrogen sulfide in water or wastewater samples from anoxic waterbodies may de-activate the column; if necessary, treat odorous acidified samples by bubbling with air for 15 min. Residual chlorine can oxidize the Cd column, reducing its efficiency, so check samples for residual chlorine (see DPD methods in Section 4500-Cl) and, if necessary, remove by adding $Na_2S_2O_3$ solution (Section 4500-NH_3.B.3*d*). Sample color that absorbs at about 540 nm interferes; measure absorbance of untreated samples and dilute, if necessary, to reduce the absorbance below that corresponding to the MDL.

c. Application: Nitrate and nitrite, singly or together in potable, surface, and saline waters and domestic and industrial wastewaters, can be determined over a range of 0.05 to 10 mg N/L.

2. Apparatus

F10 F2 *a. Automated analytical equipment:* See Figure 4500-NO_3^-:2 for an example of a continuous-flow analytical instrument. Tubing volumes and flow rates are given as examples only; follow the manufacturer's instructions.

b. Pipets: If adjustable pipets are used, verify the calibration according to manufacturer's directions.

3. Reagents

a. Reagent water: See 4500-NO_3^-.B.3*a*.

b. Copper sulfate solution: Dissolve 20 g $CuSO_4 \cdot 5H_2O$ in 500 mL reagent water and dilute to 1 L with reagent water. This solution is stable for 1 year.

c. Wash solution: Use reagent water.

d. Copper-cadmium granules: See 4500-NO_3^-.E.3*b*. Prepare the cadmium-reduction column according to the manufacturer's instructions or purchase a pre-made cadmium-reduction column or coil from the instrument manufacturer.

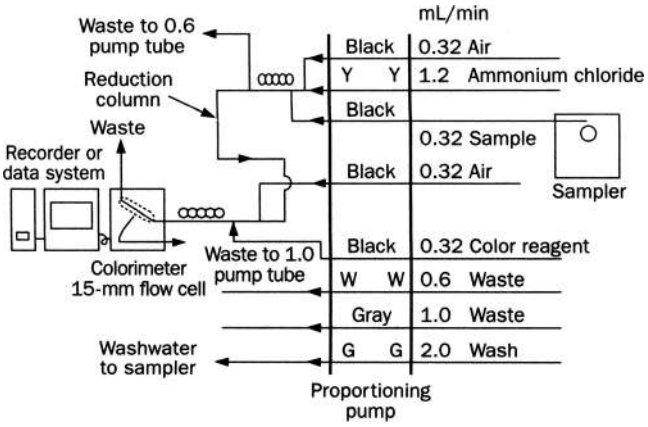


Figure 4500-NO_3^-:2. Nitrate-nitrite manifold.

Caution: Cadmium is toxic and carcinogenic. Collect and store all waste Cd. When handling Cd, wear gloves and follow the precautions described on Cd's safety data sheet.

e. Hydrochloric acid, conc.

f. Ammonium hydroxide, conc.

g. Color reagent: While stirring approximately 800 mL reagent water, add 100 mL conc phosphoric acid (H_3PO_4), 40 g sulfanilamide, and 2 g NED. Stir until dissolved and dilute to 1 L with reagent water. Store in an amber bottle and keep in the dark when not in use. Solution is stable for several months.

h. Ammonium chloride–EDTA solution: Dissolve 85 g NH_4Cl and 1 g EDTA disodium salt in reagent water and dilute to 1 L with reagent water. This solution is stable for 1 year. Add 0.5 mL polyoxyethylene 23 lauryl ether* per liter of reagent just before use.

i. Stock nitrate solution A: Prepare as directed in 4500-NO_3^-.B.3*b* or obtain from a commercial source.

j. Stock nitrate solution B: Purchase from a commercial source different from stock nitrate solution A or prepare as directed in 4500-NO_3^-.B.3*b* using KNO_3 from a commercial source different from that used to prepare stock nitrate solution A.

k. Intermediate nitrate solution A: Dilute 100 mL stock NO_3^- solution A to 1000 mL with water; 1.00 mL = 10.0 μg NO_3^--N. Preserve with 2 mL $CHCl_3$/L. Solution is stable for 6 months.

* Brij-35, available from ICI Americas, Inc., Wilmington, DE, or equivalent.

l. Intermediate nitrate solution B: Dilute 100 mL stock NO_3^- solution B to 1000 mL with water; 1.00 mL = 10.0 μg NO_3^--N. Preserve with 2 mL $CHCl_3$/L. Solution is stable for 6 months.

m. Standard nitrate solutions: Using intermediate NO_3^--N solution A and reagent water, prepare standards for calibration curve in appropriate NO_3^--N range. Compare a 1 mg/L NO_3^--N standard to a 1 mg/L NO_2^--N standard or follow instrument manufacturer's directions to verify column-reduction efficiency. To examine saline waters, prepare standard solutions with the substitute ocean water described in Section 4500-NH_3.G.3*g*.

n. Standard nitrite solution, 100 mg/L: Purchase from a commercial source (shelf-life as stated by manufacturer) or dissolve 0.6072 g ± 0.0005g KNO_2 in water and dilute to 1000 mL; 1.00 mL = 100 μg NO_2^--N. Store refrigerated and prepare fresh monthly.

o. Intermediate nitrite solution A: Dilute 1.0 mL stock NO_2^- solution 1 to 100 mL with water; 1.00 mL = 1.0 μg NO_2^--N. Prepare fresh daily.

4. Procedure

Set up manifold as shown in Figure 4500-NO_3^-:2 and follow general procedure described by the manufacturer.

If sample pH is <5 or >9, adjust to between 5 and 9 with either conc HCl or conc NH_4OH.

5. Calculation

Use an electronic spreadsheet, calculator, or instrument software to find the slope, intercept, and correlation coefficient (*r*) or coefficient of determination (r^2) of the calibration curve by least squares linear regression. Calculate the NO_3^--N concentration from the following equation:

$$C = \frac{A - I}{S}$$

where:

C = concentration,
A = absorbance,
I = intercept of the regression line, and
S = slope of the regression line.

6. Quality Control

Run a mid-level NO_2^--N standard or manufacturer-recommended standard followed immediately by a NO_3^--N standard of the same concentration. Calculate reduction efficiency as follows:

$$\text{Efficiency} = (NO_3^-\text{-N response}/NO_2^-\text{-N response}) \times 100$$

The efficiency must be 90 to 110%. If not, stop and correct the problem by either following the manufacturer's instruction or passing 6 *M* HCl through the column followed by rinsing with dilute ammonium chloride-EDTA solution. Prepare or, if it cannot be reactivated, purchase a new column according to 4500-NO_3^-E.3*b* and activate according to 4500-NO_3^-.E.4*a*.

7. Precision and Bias

Data obtained in three laboratories are given in the table below. The labs used automated systems based on identical chemical principles but with slightly different configurations. Analyses were conducted on four natural water samples containing exact increments of inorganic NO_3^--N:

Increment as NO_3^--N *µg/L*	Standard Deviation *µg N/L*	Bias *%*	Bias *µg N/L*
290	12	+5.75	+17
350	92	+18.10	+63
2310	318	+4.47	+103
2480	176	−2.69	−67

In a single laboratory using surface water samples at concentrations of 100, 200, 800, and 2100 μg N/L, the standard deviations were 0, ±40, ±50, and ±50 μg/L, respectively, and at concentrations of 200 and 2200 μg N/L, recoveries were 100 and 96%, respectively.

Precision and bias for the system described herein are believed to be comparable.

8. Bibliography

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4500-NO_3^- G. (Reserved)

4500-NO_3^- H. Automated Hydrazine Reduction Method

1. General Discussion

a. Principle: Reduce NO_3^--N to NO_2^--N with hydrazine sulfate. Then, diazotize all the NO_2^- (both the sample's initial NO_2^- content and its reduced NO_3^-) with sulfanilamide and couple it with NED to form a highly colored azo dye. Measure the dye colorimetrically to determine the sample's NO_2^- concentration. The reaction is catalyzed by a small concentration (about 0.5 to 1.0 mg/L) of Cu^{+2} ion.[1]

b. Interferences: Sample color that absorbs in the photometric range used will interfere. Sulfide ion concentrations <10 mg/L cause NO_3^- and NO_2^- concentrations to vary up to ±10%.

c. Application: NO_3^- + NO_2^- in potable and surface water and in domestic and industrial wastes can be determined over a range of 0.05 to 10 mg N/L.

2. Apparatus

F3 *a. Automated analytical equipment:* See Figure 4500-NO_3^-:3 for an example of the continuous-flow analytical instrument.

Tubing volumes, flow rates, and manifold configuration are given as reference only; follow the manufacturer's instructions.

b. Pipets: If using adjustable pipets, verify the calibration according to the manufacturer's directions.

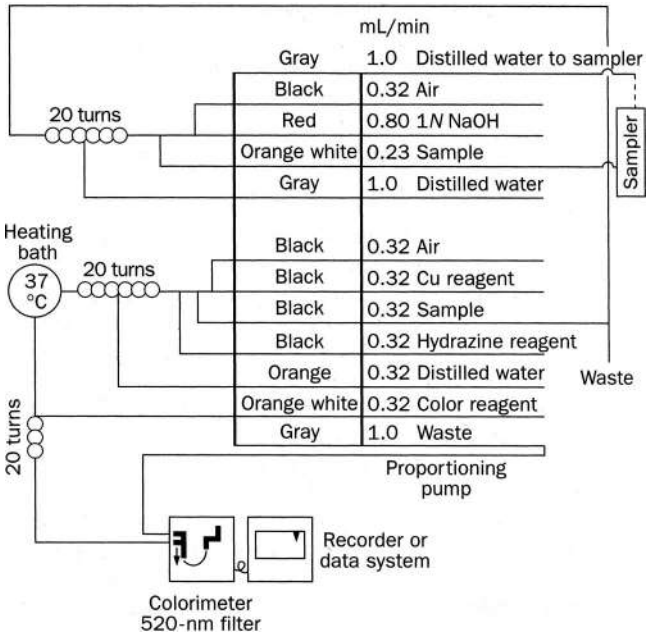


Figure 4500-NO_3^-:3. Nitrate-nitrite manifold.

3. Reagents

a. Color-developing reagent: To approximately 500 mL reagent water, add 200 mL conc phosphoric acid and 10 g sulfanilamide. After sulfanilamide is dissolved completely, add 0.8 g NED. Dilute to 1 L with reagent water, store in an amber bottle, and refrigerate. Solution is stable for approximately 1 month.

b. Copper sulfate stock solution: Dissolve 2.5 g $CuSO_4 \cdot 5H_2O$ in water and dilute to 1 L with reagent water.

c. Copper sulfate dilute solution: Dilute 20 mL stock solution to 2 L with reagent water.

d. Sodium hydroxide stock solution, 10 *M*: Dissolve 400 g NaOH in 750 mL reagent water, cool, and dilute to 1 L with reagent water.

e. Sodium hydroxide, 1.0M: Dilute 100 mL stock NaOH solution to 1 L with reagent water.

f. Hydrazine sulfate stock solution: Dissolve 27.5 g hydrazine sulfate ($N_2H_4 \cdot H_2SO_4$) in 900 mL water and dilute to 1 L with reagent water. Solution is stable for approximately 6 months. **CAUTION: Toxic if ingested. Mark container with appropriate warning.**

g. Hydrazine sulfate dilute solution: Dilute 22 mL stock solution to 1 L with reagent water.

h. Stock nitrate solution A: Dry KNO_3 in a 103–105°C oven for 24 h. Dissolve 0.7218 g ± 0.0005 g in reagent water and dilute to 1000 mL; 1.00 mL = 100 µg NO_3^--N. Preserve with 2 mL $CHCl_3$/L. Solution is stable for at least 6 months. Alternatively, use a commercial NO_3^--N stock solution.

i. Stock nitrate solution B: Using KNO_3 from a different source than that used for stock NO_3^- solution 1, dry it in a 103–105°C oven for 24 h. Dissolve 0.7218 g ± 0.0005g in water and dilute to 1000 mL; 1.00 mL = 100 µg NO_3^--N. Preserve with 2 mL $CHCl_3$/L. Solution is stable for at least 6 months. Alternatively, use a commercial NO_3^--N stock solution from a different source than that for stock nitrate solution A.

j. Standard nitrate solutions: Prepare NO_3^--N calibration standards in the range of 0 to 10 mg/L by diluting to 100 mL the following volumes of stock NO_3^--N solution A: 0, 0.5, 1.0, 2.0 . . . 10.0 mL. Prepare a mid-range CVS from stock NO_3^--N solution B. Compare at least one NO_2^--N standard to a NO_3^--N standard at the same concentration to verify the reduction's efficiency.

k. Standard nitrite solution: See Section 4500-NO_2^-.B.3*e*, *f*, and *g*.

4. Procedure

Set up manifold as shown in Figure 4500-NO_3^-:3 and follow general procedure described by manufacturer. Run a 2.0-mg NO_3^--N/L and a 2.0-mg NO_2^--N/L standard through the system to check for 100% reduction of NO_3^--N to NO_2^--N. The two peaks must agree within 10%; if not, adjust concentration of $N_2H_4 \cdot H_2SO_4$ solution. If NO_3^--N peak is lower than NO_2^--N peak, increase concentration of $N_2H_4 \cdot H_2SO_4$ until they are equal; if NO_3^--N peak is higher than NO_2^--N, reduce concentration of $N_2H_4 \cdot H_2SO_4$. Once correct concentration has been determined, no further adjustment is necessary.

5. Calculation

Use an electronic spreadsheet, calculator, or instrument software to find the slope, intercept, and correlation coefficient (r) or coefficient of determination (r^2) of the calibration curve by least squares linear regression. Calculate the NO_3^--N concentration from the following equation:

$$C = \frac{A - I}{S}$$

where:

C = concentration,
A = absorbance,
I = intercept of the regression line, and
S = slope of the regression line.

6. Precision and Bias

In a single laboratory using drinking water, surface water, and industrial waste at concentrations of 0.39, 1.15, 1.76, and 4.75 mg NO_3^--N/L, the standard deviations were ±0.02, ±0.01, ±0.02, and ±0.03, respectively. In a single laboratory using drinking water at concentrations of 0.75 and 2.97 mg NO_3^--N/L, the recoveries were 99 and 101%, respectively.[2]

7. References

1. Kamphake, L.J., S.A. Hannah & J.M. Cohen. 1966. Automated Analysis for Nitrate by Hydrazine Reduction. Water Res. Lab., Fed. Water Pollut. Ctrl. Admin., U.S. Environmental Protection Agency, Cincinnati, Ohio.
2. U.S. Environmental Protection Agency. 1979. Methods for Chemical Analysis of Water and Wastes. Washington, D.C.

8. Bibliography

Kamphake, L., S. Hannah & J. Cohen. 1967. Automated analysis for nitrate by hydrazine reduction. *Water Res.* 1:205.

4500-NO_3^- I. Cadmium Reduction Flow Injection Method

1. General Discussion

a. Principle: A sample is passed through a copperized Cd column to quantitatively reduce its NO_3^--N content to NO_2^--N. Then, the NO_2^--N is diazotized with sulfanilamide and coupled with NED to yield a water-soluble dye with a magenta color whose absorbance at 540 nm is proportional to the NO_3^- + NO_2^--N [total oxidized nitrogen (TON)] in the sample.

To determine the sample's nitrite content alone, remove the Cd column, recalibrate the method with nitrite standards, and repeat the sample analyses. Analysts also can run TON and NO_2^--N FIA methods in parallel for a set of samples. In this arrangement, subtract the concentrations determined via the FIA method from the corresponding concentrations determined via the TON method to calculate the samples' NO_3^--N concentrations.

See also Sections 4130 and 4500-NO_2^-.

b. Interferences: Suspended matter in the column may restrict sample flow; if necessary, filter turbid samples (see 4500-NO_3^-.A.1). Concentrations of Fe, Cu, or other metals above several milligrams per liter lower reduction efficiency. Add EDTA to the buffer to eliminate this interference. Oil and grease will coat the Cd surface, so if necessary remove it via pre-extraction with an organic solvent (see Section 5520). Hydrogen sulfide in water or wastewater samples from anoxic waterbodies may de-activate the column, so bubble odorous acidified samples with air for 15 min. Residual chlorine can oxidize the Cd column, so check samples for residual Cl (see DPD methods in Section 4500-Cl) and, if necessary, add $Na_2S_2O_3$ solution (Section 4500-NH_3.B.3*d*) to remove it. If the untreated sample color absorbs at about 540 nm, it will interfere with results. Measure absorbance of untreated samples and dilute, if necessary, to reduce the absorbance below that corresponding to the MDL.

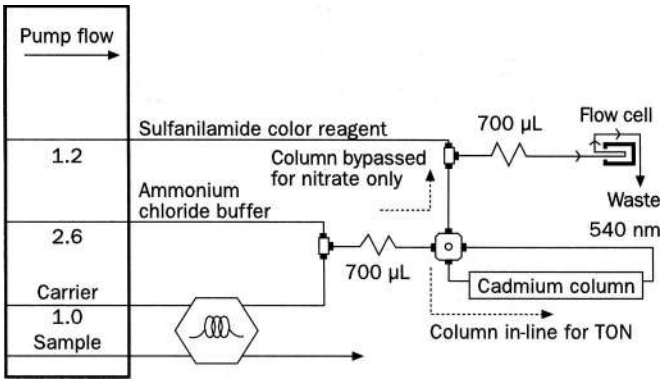


Figure 4500-NO_3^-:4. FIA nitrate + nitrite manifold.

2. Apparatus

Flow injection analysis equipment consisting of:

a. FIA injection valve with sample loop or equivalent.

b. Multichannel proportioning pump.

c. FIA manifold (Figure 4500-NO_3^-:4) with flow cell. Figure 4500-NO_3^-:4 only shows relative flow rates. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material, such as TFE. F4

d. Absorbance detector, 540 nm, 10-nm bandpass.

e. Injection valve control and data acquisition system.

f. Pipets: If adjustable pipets are used, calibrate according to the manufacturer's directions.

3. Reagents

Use reagent water to prepare carrier and all solutions. To prevent bubble formation in the manifold tubing, sparge carrier and buffer with helium (He). Pass He at 140 kPa (20 lb/in.^{2})

TABLE 4500-NO_3^-:I. RESULTS OF SINGLE-LABORATORY STUDIES WITH SELECTED MATRICES

Matrix	Sample/Blank Designation	Known Addition *mg NO_3^--N/L*	Recovery %	Relative Standard Deviation %
Wastewater treatment plant influent	Reference sample*	–	102	–
	Blank†	0.2	100	–
		0.4	100	–
	Site A‡	0	–	<1
		0.2	101	–
		0.4	96	–
	Site B‡	0	–	<1
		0.2	90	–
		0.4	88	–
	Site C‡	0	–	<1
		0.2	95	–
		0.4	95	–
Wastewater treatment plant effluent	Reference sample*	–	102	–
	Blank†	0.2	100	–
		0.4	95	–
	Site A‡	0	–	0.9
		0.2	95	–
		0.4	102	–
	Site B‡	0	–	0.7
		0.2	91	–
		0.4	101	–
	Site C‡	0	–	0.5
		0.2	91	–
		0.4	96	–
Landfill leachate	Reference sample*	–	98	–
	Blank†	0.2	100	–
		0.4	98	–
	Site A‡	0	–	<1
		0.2	104	–
		0.4	96	–
	Site B‡	0	–	<1
		0.2	95	–
		0.4	94	–
	Site C‡	0	–	<1
		0.2	91	<1
		0.4	93	–

* U.S. EPA QC sample, 1.98 mg N/L.
† Determined in duplicate.
‡ Samples without known additions determined four times; samples with known additions determined in duplicate. Typical difference between duplicates: influent, 2%; effluent, 1%; leachate, <1%.

through a He-degassing tube. Bubble He through 1 L solution for 1 min.

a. Ammonium chloride buffer: **CAUTION: Fumes. Use a hood.** To a 1-L container, add 800 mL reagent water, 107 mL conc HCl, 62 mL NH_4OH, and 1.0 g disodium EDTA. Mix until dissolved. The pH of this buffer must be 8.5. (Adjust the pH to 8.5 with NH_4OH or HCl.)

b. Sulfanilamide color reagent: To a tared 1-L amber container, add 850 mL water, 100 mL 85% H_3PO_4, 40.0 g sulfanilamide, and 1.0 g NED. Mix until wetted and stir with stir bar for 30 min until dissolved. Dilute to 1 L with reagent water. This solution is stable for 1 month. Discard the solution if it is highly colored or dark.

c. Hydrochloric acid, 1M: To a 1 L container, add 800 mL reagent water and then 83 mL conc HCl. Mix and dilute to 1 L with reagent water. This solution is stable for 1 year.

d. Copper sulfate solution, 2%: To a 1-L container, add 20 g $CuSO_4 \cdot 5H_2O$ to 900 mL reagent water. Stir to dissolve, dilute to 1 L with reagent water and mix. This solution is stable for 1 year.

e. Copperized cadmium granules: Place 10 to 20 g coarse Cd granules (0.3- to 1.5-mm-diam) in a 250-mL beaker. Wash with 50 mL acetone, then water, then two 50-mL portions 1*M* HCl (¶ *c* above). Rinse several times with water. **CAUTION: Cadmium is toxic and carcinogenic. Collect and store all waste Cd. When handling Cd, wear gloves and follow the precautions described on Cd's safety data sheet.**

Add 100 mL 2% copper sulfate solution (¶ *d* above) to cadmium prepared above. Swirl for about 5 min, then decant the liquid and repeat with a fresh 100-mL portion of the 2% $CuSO_4$ solution. Continue this process until the blue aqueous Cu color persists. Decant and wash with at least five portions of NH_4Cl buffer (see ¶ *a* above) to remove colloidal Cu. Properly activated Cd appears black or dark gray. The copperized Cd granules may be stored in a bottle under NH_4Cl buffer. Cd columns may also be purchased commercially.

f. Stock nitrate solution A, 200 mg NO_3^--N/L: In a 1-L volumetric flask, dissolve 1.444 g ± 0.0005 g KNO_3 in about 600 mL water. Add 2 mL chloroform. Dilute to mark and invert to mix. This solution is stable for 6 months.

g. Stock nitrate solution B, 200 mg NO_3^--N/L: In a 1-L volumetric flask, dissolve 1.444 g ± 0.0005 g KNO_3 in about 600 mL water. (Use KNO_3 from a source different from that for stock NO_3^- solution A.) Add 2 mL chloroform. Dilute to mark and invert to mix. This solution is stable for 6 months.

h. Stock nitrite standard, 200.0 mg NO_2^--N/L: In a 1-L volumetric flask, dissolve 0.986 g ±0.0005g sodium nitrite ($NaNO_2$) or 1.214 g ± 0.0005g potassium nitrite (KNO_2) in approximately 800 mL water. Add 2 mL chloroform. Dilute to mark and invert to mix. Refrigerate.

i. Standard solution: Prepare NO_3^- or NO_2^- standards in the desired concentration range, using stock nitrate solution A. Prepare a CVS with nitrate stock solution B.

4. Procedure

Set up a manifold equivalent to that in Figure 4500-NO_3^-:4 and pack column with copperized Cd granules. Follow methods supplied by column and instrument manufacturer or laboratory's standard operating procedure for this method. Follow QC procedures outlined in Section 4020.

5. Calculations

Use an electronic spreadsheet, calculator, or instrument software to find the slope, intercept, and correlation coefficient (*r*) or

coefficient of determination (r^2) of the calibration curve by least squares linear regression. Calculate the NO_3^--N concentration from the following equation:

$$C = \frac{A - I}{S}$$

where:

C = concentration,
A = response (peak height or area),
I = intercept of the regression line, and
S = slope of the regression line.

6. Quality Control

The NO_3^--N absorbance must be 90 to 110% of the NO_2^--N standard's absorbance at the same concentration. If not, stop and correct the problem by either regenerating (4500-NO_3^-.E.3*b*) and reactivating (4500-NO_3^-.E.4*a*) or replacing the column.

7. Precision and Bias

In the studies described below, NO_3^- was measured. There was no significant concentration of NO_2^--N in the samples.

Recovery and relative standard deviation: Table 4500-NO_3^-:I gives results of single-laboratory studies. T1

4500-N_{org} NITROGEN (ORGANIC)*

4500-N_{org} A. Introduction

1. Selection of Method

The Kjeldahl methods (4500-N_{org}.B and C) determine nitrogen in the trinegative state. They fail to account for nitrogen in the form of azide, azine, azo, hydrazone, nitrate, nitrite, nitrile, nitro, nitroso, oxime, and semi-carbazone. "Kjeldahl nitrogen" is the sum of organic nitrogen and ammonia nitrogen.

The major factor that influences the selection of a macro- or semi-micro-Kjeldahl method to determine organic nitrogen is its concentration. The macro-Kjeldahl method is applicable for samples containing either low or high concentrations of organic nitrogen but requires a relatively large sample volume for low concentrations. In the semi-micro-Kjeldahl method, which is applicable to samples containing high concentrations of organic nitrogen, the sample volume should be chosen to contain organic plus ammonia nitrogen in the range of 0.2 to 2 mg.

The block digestion method (4500-N_{org}.D) is a micro method with an automated analysis step capable of measuring organic nitrogen as low as 0.1 mg/L when blanks are carefully controlled.

2. Storage of Samples

The most reliable results are obtained on fresh samples. If an immediate analysis is not possible, preserve samples for Kjeldahl digestion by acidifying to pH 1.5 to 2.0 with concentrated H_2SO_4 and storing at 4°C. Do not use $HgCl_2$ because it will interfere with ammonia removal.

3. Interferences

a. Nitrate: During Kjeldahl digestion, nitrate in excess of 10 mg/L can oxidize a portion of the ammonia released from the digested organic nitrogen, producing N_2O and resulting in a negative interference. When sufficient organic matter in a low state of oxidation is present, nitrate can be reduced to ammonia, resulting in a positive interference. The conditions under which significant interferences occur are not well defined and there is no proven way to eliminate the interference with the Kjeldahl methods described herein.

b. Inorganic salts and solids: The acid and salt content of the Kjeldahl digestion reagent is intended to produce a digestion temperature of about 380°C. If the sample contains a very large quantity of salt or inorganic solids that dissolve during digestion, the temperature may rise above 400°C, at which point pyrolytic loss of nitrogen begins to occur. To prevent an excessive digestion temperature, add more H_2SO_4 to maintain the acid–salt balance. Not all salts cause precisely the same temperature rise, but adding 1 mL H_2SO_4/g salt in the sample gives reasonable results. Add the extra acid and the digestion reagent to both sample and reagent blank. Too much acid will lower the digestion temperature below 380°C and result in incomplete digestion and recovery. If necessary, add sodium hydroxide-sodium thiosulfate before the final distillation step to neutralize the excess acid.

Large amounts of salt or solids also may cause bumping during distillation. If this occurs, add more dilution water after digestion.

c. Organic matter: During Kjeldahl digestion, H_2SO_4 oxidizes organic matter to CO_2 and H_2O. If a large amount of organic matter is present, a large amount of acid will be consumed, the ratio of salt to acid will increase, and the digestion temperature will increase. If enough organic matter is present, the temperature will rise above 400°C, resulting in pyrolytic loss of nitrogen. To prevent this, add to the digestion flask 10 mL conc H_2SO_4/3 g COD. Alternately, add 50 mL more digestion reagent/g COD. Additional sodium hydroxide-sodium thiosulfate reagent may be necessary to keep the distillation pH high. Because reagents may contain traces of ammonia, treat the reagent blank identically with the samples.

4. Use of a Catalyst

Mercury has been the catalyst of choice for Kjeldahl digestion. Because of its toxicity and problems associated with legal disposal of mercury residues, a less toxic catalyst is recommended. Digestion of some samples may be complete or nearly complete without the use of a catalyst. Effective digestion results from the use of a reagent having a salt:acid ratio of 1 g/mL with copper as catalyst (4500-N_{org}.B.3*a*), and specified temperature (4500-N_{org}.B.2*a*) and time (4500-N_{org}.B.4*c*). If a change is made in the reagent formula, report the change and indicate percentage recovery relative to the results for similar samples analyzed using the previous formula.

Before results are considered acceptable, determine nitrogen recovery from samples with known additions of nicotinic acid, to test completeness of digestion; and with ammonium chloride to test for loss of nitrogen.

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* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011. Joint Task Group: 20th Edition—(4500-N_{org}.D)—Scott Stieg (chair), Bradford R. Fisher, Owen B. Mathre, Theresa M. Wright.

https://doi.org/10.2105/SMWW.2882.090

4500-N_{org} B. Macro-Kjeldahl Method

1. General Discussion

a. Principle: In the presence of H_2SO_4, potassium sulfate (K_2SO_4), and cupric sulfate ($CuSO_4$) catalyst, amino nitrogen of many organic materials is converted to ammonium. Free ammonia also is converted to ammonium. After addition of base, the ammonia is distilled from an alkaline medium and absorbed in boric or sulfuric acid. The ammonia may be determined colorimetrically, by ammonia-selective electrode, or by titration with a standard mineral acid.

b. Selection of ammonia measurement method: The sensitivity of colorimetric methods makes them particularly useful for determining organic nitrogen levels below 5 mg/L. The titrimetric and selective electrode methods of measuring ammonia in the distillate are suitable for determining a wide range of organic nitrogen concentrations. Selective electrode methods and automated colorimetric methods may be used for measurement of ammonia in digestate without distillation. Follow equipment manufacturer's instructions.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Digestion apparatus: Kjeldahl flasks with a total capacity of 800 mL yield the best results. Digest over a heating device adjusted so 250 mL water at an initial temperature of 25°C can be heated to a roiling boil in approximately 5 min. For testing, preheat heaters for 10 min if gas-operated or 30 min if electric. A heating device meeting this specification should provide the temperature range of 375 to 385°C for effective digestion.

b. Distillation apparatus: See Section 4500-NH_3.B.2*a*.

c. Apparatus for ammonia determination: See Section 4500-NH_3.C.2, D.2, F.2, or G.2.

3. Reagents

Prepare all reagents and dilutions in ammonia-free water.

All of the reagents listed for the determination of Nitrogen (Ammonia), Section 4500-NH_3.C.3, D.3, F.3, or G.3, are required, plus the following:

a. Digestion reagent: Dissolve 134 g K_2SO_4 and 7.3 g $CuSO_4$ in about 800 mL water. Carefully add 134 mL conc H_2SO_4. When it has cooled to room temperature, dilute the solution to 1 L with water. Mix well. Keep at a temperature close to 20°C to prevent crystallization.

b. Sodium hydroxide-sodium thiosulfate reagent: Dissolve 500 g NaOH and 25 g $Na_2S_2O_3 \cdot 5H_2O$ in water and dilute to 1 L.

c. Borate buffer solution: See Section 4500-NH_3.B.3*b*.

d. Sodium hydroxide (NaOH), 6*N*.

4. Procedure

a. Selection of sample volume and sample preparation: Place a measured volume of sample in an 800-mL Kjeldahl flask. Select sample size from the following tabulation:

Organic Nitrogen in Sample *mg/L*	Sample Size *mL*
0–1	500
1–10	250
10–20	100
20–50	50.0
50–100	25.0

If necessary, dilute sample to 300 mL, neutralize to pH 7, and dechlorinate as described in Section 4500-NH_3.B.4*b*.

b. Ammonia removal: Add 25 mL borate buffer and then 6*N* NaOH until pH 9.5 is reached. Add a few glass beads or boiling chips, such as Hengar Granules #12, and boil off 300 mL. If desired, distill this fraction and determine ammonia nitrogen. Alternately, if ammonia has been determined by the distillation method, use residue in distilling flask for organic nitrogen determination.

For sludge and sediment samples, weigh wet sample in a crucible or weighing bottle, transfer contents to a Kjeldahl flask, and determine Kjeldahl nitrogen. Follow a similar procedure for ammonia nitrogen and organic nitrogen determined by difference. Determinations of organic and Kjeldahl nitrogen on dried sludge and sediment samples are not accurate because drying results in loss of ammonium salts. Measure dry weight of sample on a separate portion.

c. Digestion: Cool and add carefully 50 mL digestion reagent (or substitute 6.7 mL conc H_2SO_4, 6.7 g K_2SO_4, and 0.365 g $CuSO_4$) to distillation flask. Add a few glass beads and, after mixing, heat under a hood or with suitable ejection equipment to remove acid fumes. Boil briskly until the volume is greatly reduced (to about 25 to 50 mL) and copious white fumes are observed (fumes may be dark for samples high in organic matter). Then continue to digest for an additional 30 min. As digestion continues, colored or turbid samples will become transparent and pale green. After digestion, let cool, dilute to 300 mL with water, and mix. Tilt flask away from personnel and carefully add 50 mL sodium hydroxide-thiosulfate reagent to form an alkaline layer at flask bottom. Connect flask to a steamed-out distillation apparatus and swirl flask to ensure complete mixing. The pH of the solution should exceed 11.0.

d. Distillation: Distill and collect 200 mL distillate. Use 50 mL indicating boric acid as absorbent solution when ammonia is to be determined by titration. Use 50 mL 0.04*N* H_2SO_4 solution as absorbent for manual phenate or electrode methods. Extend tip of condenser well below level of absorbent solution and do not let temperature in condenser rise above 29°C. Lower collected distillate free of contact with condenser tip and continue distillation during last 1 or 2 min to cleanse condenser.

e. Final ammonia measurement: Use the titration, ammonia-selective electrode, manual phenate, or automated phenate method, Sections 4500-NH_3.C, D, F, and G, respectively.

f. Standards: Carry a reagent blank and standards through all steps of the procedure.

5. Calculation

See Section 4500-NH_3.C.5, D.5, F.5, or G.5.

6. Precision and Bias

Two analysts in one laboratory prepared reagent water solutions of nicotinic acid and digested them by the macro-Kjeldahl method. Ammonia in the distillate was determined by titration. Results are summarized in Table 4500-N_{org}:I

T1

Table 4500-N_{org}:I. Precision Data for Kjeldahl Nitrogen Method Based on Mean of Triplicate Analyses of Nicotinic Acid

Lab/ Analyst	Nicotinic Acid *mg N/L*	Recovery of N %	Standard Deviation *mg/L*	Relative Standard Deviation %
1/1	5	93.3	0.16	3.46
1/2	5	101	0.16	3.17
1/1	10	87.7	0.16	1.84
1/2	10	91.5	0.28	3.06
1/1	20	95.7	0.16	0.84
1/2	20	95.7	0.58	3.03
2/1	0.5	97.4	0.005	1.04
2/2	0.5	95.3	0.027	5.46
3/1	0.5	87.3	0.130	29.9
4/1	0.5	113	0.235	41.7
2/1	1.0	103	0.012	1.15
2/2	1.0	101	0.046	4.63
3/1	1.0	84.3	0.081	9.66
4/1	1.0	99.3	0.396	39.9
2/1	2.0	104	0	0
2/2	2.0	99.2	0.029	1.44
3/1	2.0	89.2	0.071	3.98
4/1	2.0	112	0.139	6.18

7. Bibliography

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4500-N_{org} C. Semi-Micro-Kjeldahl Method

1. General Discussion

See 4500-N_{org}.B.1.

2. Apparatus

a. Digestion apparatus: Use Kjeldahl flasks with a capacity of 100 mL in a semi-micro-Kjeldahl digestion apparatus* equipped with heating elements to accommodate Kjeldahl flasks and a suction outlet to vent fumes. The heating elements should provide the temperature range of 375 to 385°C for effective digestion.

b. Distillation apparatus: Use an all-glass unit equipped with a steam-generating vessel containing an immersion heater† (Figure 4500-N_{org}:1).

F2

c. pH meter.

d. Apparatus for ammonia determination: See Section 4500-NH_3.C.2, D.2, F.2, or G.2.

3. Reagents

All of the reagents listed for the determination of Nitrogen (Ammonia) (Section 4500-NH_3.B.3) and Nitrogen (Organic) macro-Kjeldahl (4500-N_{org}.B.3) are required. Prepare all reagents and dilutions with ammonia-free water.

4. Procedure

a. Selection of sample volume: Determine the sample size from the following tabulation:

Organic Nitrogen in Sample *mg/L*	Sample Size *mL*
4–40	50
8–80	25
20–200	10
40–400	5

For sludge and sediment samples, weigh a portion of wet sample containing between 0.2 and 2 mg organic nitrogen in a crucible or weighing bottle. Transfer sample quantitatively to a 100-mL beaker by diluting it and rinsing the weighing dish several times with small quantities of water. Make the transfer using as small a quantity of water as possible and do not exceed a total volume of 50 mL. Measure dry weight of sample on a separate portion.

b. Ammonia removal: Pipet 50 mL sample or an appropriate volume diluted to 50 mL with water into a 100-mL beaker. Add 3 mL borate buffer and adjust to pH 9.5 with 6*N* NaOH, using a pH meter. Quantitatively transfer sample to a 100-mL Kjeldahl

* Rotary Kjeldahl digestion unit, Kontes, Model 551000-0000, or equivalent.
† ASTM E-147, or equivalent.

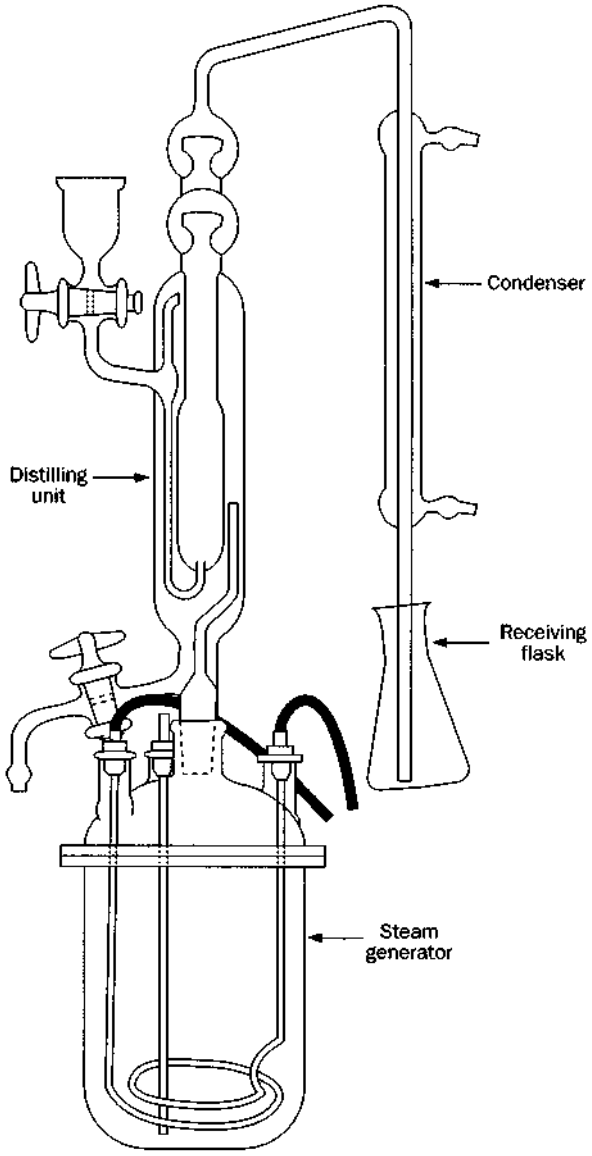


Figure 4500-N_{org}:1. Micro-Kjeldahl distillation apparatus.

flask and boil off 30 mL. Alternatively, if ammonia removal is not required, digest samples directly as described in ¶ *c* below. Distillation following this direct digestion yields Kjeldahl nitrogen concentration rather than organic nitrogen.

c. Digestion: Carefully add 10 mL digestion reagent to Kjeldahl flask containing sample. Add five or six glass beads (3- to 4-mm size) to prevent bumping during digestion. Set each heating unit on the micro-Kjeldahl digestion apparatus to its medium setting and heat flasks under a hood or with suitable ejection equipment to remove fumes of SO_3. Continue to boil briskly until solution becomes transparent and pale green and copious fumes are observed. Then turn each heating unit up to its maximum setting and digest for an additional 30 min. Cool. Quantitatively transfer digested sample by diluting and rinsing several times into micro-Kjeldahl distillation apparatus so total volume in distillation apparatus does not exceed 30 mL. Add 10 mL sodium hydroxide-thiosulfate reagent and turn on steam.

d. Distillation: Control rate of steam generation to boil contents in distillation unit so neither escape of steam from tip of condenser nor bubbling of contents in receiving flask occurs. Distill and collect 30 to 40 mL distillate below surface of 10 mL absorbent solution contained in a 125-mL Erlenmeyer flask. Use indicating boric acid for a titrimetric finish. Use 10 mL 0.04*N* H_2SO_4 solution for collecting distillate for the phenate or electrode methods. Extend tip of condenser well below level of absorbent solution and do not let temperature in condenser rise above 29°C. Lower collected distillate free of contact with condenser tip and continue distillation during last 1 or 2 min to cleanse condenser.

e. Standards: Carry a reagent blank and standards through all steps of procedure and apply necessary correction to results.

f. Final ammonia measurement: Use the titration, ammonia-selective electrode, manual phenate, or automated phenate method, Sections 4500-NH_3.C, D, F, and G, respectively.

5. Calculation

See Section 4500-NH_3.C.5, D.5, F.5, or G.5.

6. Precision and Bias

No data on the precision and bias of the semi-micro-Kjeldahl method are available.

7. Bibliography

See 4500-N_{org}.B.7.

4500-N_{org} D. Block Digestion and Flow Injection Analysis

1. General Discussion

a. Principle: Samples of drinking, ground, and surface waters and of domestic and industrial wastes are digested in a block digestor with sulfuric acid and copper sulfate as a catalyst. The digestion recovers nitrogen components of biological origin, such as amino acids, proteins, and peptides, as ammonia but may not recover the nitrogenous compounds of some industrial wastes, such as amines, nitro compounds, hydrazones, oximes, semicarbazones, and some refractory tertiary amines. Nitrate is not recovered. See Section 4500-N for a discussion of the various forms of nitrogen found in waters and wastewaters, 4500-N_{org}.B and C for a discussion of Kjeldahl nitrogen methods, and Section 4130, Flow Injection Analysis (FIA).

The digested sample is injected onto the FIA manifold, where its pH is controlled by raising it to a known, basic pH by

neutralization with a concentrated buffer. This in-line neutralization converts the ammonium cation to ammonia, and also prevents undue influence of the sulfuric acid matrix on the pH-sensitive color reaction that follows. The ammonia thus produced is heated with salicylate and hypochlorite to produce a blue color that is proportional to the ammonia concentration. The color is intensified by adding sodium nitroprusside. The presence of EDTA in the buffer prevents precipitation of calcium and magnesium. The resulting peak's absorbance is measured at 660 nm. The peak area is proportional to the concentration of total Kjeldahl nitrogen in the original sample.

b. Interferences: Remove large or fibrous particulates by filtering the sample through glass wool.

The main source of interference is ammonia. Ammonia is an airborne contaminant that is removed rapidly from ambient air by the digestion solution. Guard against ammonia contamination in reagents, water, glassware, and the sample preservation process. It is particularly important to prevent ammonia contamination in the sulfuric acid used for the digestion. Open sulfuric acid bottles away from laboratories in which ammonia or ammonium chloride have been used as reagents and store sulfuric acid away from such reagents. Ensure that the open ends of the block digestor's tubes can be covered to prevent ammonia from being scrubbed from the fume hood make-up air during the digestion.

If a sample consumes more than 10% of the sulfuric acid during digestion, the pH-dependent color reaction will show a matrix effect. The color reaction buffer will accommodate a range of 5.4 ± 0.4% H_2SO_4 (v/v) in the diluted digested sample. Sample matrices with a high concentration of carbohydrates or other organic material may consume more than 10% of the acid during digestion. If this effect is suspected, titrate digested sample with standardized sodium hydroxide to determine whether more than 10% of the sulfuric acid has been consumed during digestion. The block digestor also should have a means to prevent loss of sulfuric acid from the digestion tubes during the digestion period.

Also see 4500-N_{org}.A and B.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Digestion and flow injection analysis equipment consisting of:

a. Block digestor capable of maintaining a temperature of 380°C for 2 h.

b. Digestion tubes capable of being heated to 380°C for 2 h and having a cover to prevent ammonia contamination and loss of sulfuric acid.

c. FIA injection valve with sample loop or equivalent.

d. Multichannel proportioning pump.

F2 *e. FIA manifold* (Figure 4500-N_{org}:2) with tubing heater and flow cell. Relative flow rates only are shown in Figure 4500-N_{org}:2. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material, such as TFE.

f. Absorbance detector, 660 nm, 10-nm bandpass.

g. Injection valve control and data acquisition system.

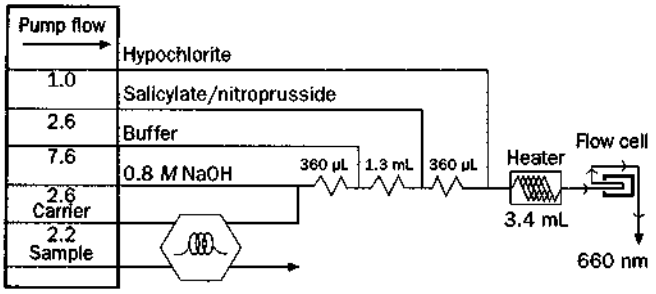


Figure 4500-N_{org}:2. FIA total Kjeldahl nitrogen manifold.

3. Reagents

Use reagent water (>10 megohm) for all solutions. To prevent bubble formation, degas carrier and buffer with helium. Pass He at 140 kPa (20 psi) through a helium degassing tube. Bubble He through 1 L solution for 1 min. As an alternative to preparing reagents by weight/weight, use weight/volume.

a. Digestion solution: In a 1-L volumetric flask, dissolve 134.0 g potassium sulfate, K_2SO_4, and 7.3 g copper sulfate, $CuSO_4$, in 800 mL water. Then add slowly while swirling 134 mL conc sulfuric acid, H_2SO_4. Let cool, dilute to mark, and invert to mix.

b. Carrier and diluent: To a tared 1-L container, add 496 g digestion solution (¶ *a* above) and 600 g water. Shake until dissolved.

c. Sodium hydroxide (NaOH), 0.8*M:* To a tared 1-L plastic container, add 32.0 g NaOH and 985.0 g water. Stir or shake until dissolved.

d. Buffer: To a tared 1-L container, add 941 g water. Add and completely dissolve 35.0 g sodium phosphate dibasic heptahydrate, $Na_2HPO_4 \cdot 7H_2O$. Add 20.0 g disodium EDTA (ethylenediaminetetracetic acid disodium salt). The EDTA will not dissolve but will form a turbid solution. Finally, add 50 g NaOH. Stir or shake until dissolved.

e. Salicylate/nitroprusside: To a tared 1-L dark container, add 150.0 g sodium salicylate (salicylic acid sodium salt), $C_6H_4(OH)(COO)Na$, 1.00 g sodium nitroprusside (sodium nitroferricyanide dihydrate), $Na_2Fe(CN)_5NO \cdot 2H_2O$, and 908 g water. Stir or shake until dissolved. Prepare fresh monthly.

f. Hypochlorite: To a tared 250-mL container, add 16 g commercial 5.25% sodium hypochlorite bleach solution* and 234 g deionized water. Shake to mix.

g. Stock standard, 250 mg N/L: In a 1-L volumetric flask, dissolve 0.9540 g ammonium chloride, NH_4Cl (dried for 2 h at 110°C), in about 800 mL water. Dilute to mark and invert to mix.

h. Standard ammonia solutions: Prepare ammonia standards in desired concentration range, using the stock standard (¶ *g* above) and diluting with water.

i. Simulated digested standards: To prepare calibration standards without having to digest the standards prepared in ¶ *h* above, proceed as follows:

j. Stock standard, 5.00 mg N/L: To a tared 250-mL container, add about 5.0 g stock standard (250 mg N/L). Divide actual

* Regular Clorox, The Clorox Company, Pleasanton, CA, or equivalent.

weight of solution added by 0.02 and make up to this resulting total weight with diluent (¶ *b* above). Shake to mix. Prepare working standards from this stock standard, diluting with the diluent (¶ *b* above), not water.

4. Procedure

a. Digestion procedure: Carry both standards and samples through this procedure.

To a 75-mL block digestor tube, add 25.0 mL sample or standard and then add 10 mL digestion solution (4500-N_{org}.D.3*a*) and mix. Add four alundum granules to each tube for smooth boiling. Place tubes in preheated block digestor for 1 h at 200°C. After 1 h, increase block temperature to 380°C and continue to digest for 1 h at 380°C. Remove tubes from block and let cool for about 10 min. Dilute each to 25.0 mL with water and mix with vortex mixer. Cover tubes to prevent ammonia contamination.

b. FIA analysis: Set up a manifold equivalent to that in Figure 4500-N_{org}:2 and analyze digested standards and samples by method supplied by manufacturer or laboratory standard operating procedure. Follow quality control protocols described in Section 4020.

5. Calculations

Prepare standard curves by plotting absorbance of standards processed through the manifold versus ammonia concentration. The calibration curve is linear.

6. Precision and Bias

a. Recovery and relative standard deviation: Table 4500-N_{org}:II gives results of single-laboratory studies.

b. MDL: A 130-μL sample loop was used in the method described above. Using a published MDL method,[1] analysts ran 21 replicates of a 0.1-mg N/L standard. These gave a mean of 0.103 mg N/L, a standard deviation of 0.014 mg N/L, and MDL of 0.034 mg N/L. A lower MDL may be obtained by increasing the sample loop volume and increasing the ratio of carrier flow rate to reagents flow rate.

7. Reference

1. U.S. Environmental Protection Agency. 1984. Definition and procedure for the determination of method detection limits. Appendix B to 40 CFR 136 rev. 1.11 amended June 30, 1986. 49 CFR 43430.

Table 4500-N_{org}:II. Results of Single-Laboratory Studies with Selected Matrices

Matrix	Sample/Blank Designation	Known Addition *mg N/L*	Recovery %	Relative Standard Deviation %
Wastewater treatment plant influent	Reference sample*	—	97	—
	Blank†	3.0	97	—
		6.0	99	—
	Site A‡§	0	—	3.3
		3.0	91	—
		6.0	95	—
	Site B‡§	0	—	3.6
		3.0	115	—
		6.0	93	—
	Site C‡§	0	—	5.1
		3.0	97	—
		6.0	107	—
Wastewater treatment plant effluent	Reference sample*	—	92	—
	Blank†	3.0	97	—
		6.0	100	—
	Site A‡‖	0	—	5.4
		3.0	94	—
		6.0	100	—
	Site B‡‖	0	—	4.1
		3.0	119	—
		6.0	81	—
	Site C‡‖	0	—	7.3
		3.0	93	—
		6.0	105	—
Landfill leachate	Reference sample*	—	96	—
	Blank†	3.0	101	—
		6.0	99	—
	Site A‡#	0	—	3.3
		3.0	95	—
		6.0	98	—
	Site B‡#	0	—	4.4
		3.0	134	—
		6.0	85	—
	Site C‡#	0	—	3.8
		3.0	98	—
		6.0	105	—

* U.S. EPA nutrient QC sample, 1.52 mg N/L.
† Determined in duplicate.
‡ Samples without known additions determined four times; samples with known additions determined in duplicate.
§ Sample dilutions: A - fivefold; B - 10-fold; C - fivefold. Typical relative difference between duplicates 3%.
‖ Sample dilutions: A - none; B - twofold; C - none. Typical relative difference between duplicates 1%.
Sample dilutions: A, B, and C - 25-fold. Typical relative difference between duplicates 4%.

4500-O OXYGEN (DISSOLVED)*

4500-O A. Introduction

1. Significance

The dissolved oxygen (DO) levels in natural and waste waters depend on the physical, chemical, and biochemical activities in a waterbody. DO analysis is a key test in water pollution and waste treatment process control.

2. Selection of Method

This section describes three methods for DO analysis: the iodometric (Winkler) method and its modifications, the membrane-electrode method, and the optical-probe method. The iodometric method[1] is a titrimetric procedure based on DO's oxidizing property. The membrane-electrode method is based on molecular oxygen's diffusion rate across a membrane.[2] The optical-probe method uses luminescence-based oxygen sensors to measure light emissions that correlate to the DO concentration.[3] The choice of procedure depends on the interferences present, the accuracy desired, and, in some cases, convenience or expedience.

3. References

1. Winkler, L.W. 1888. The determination of dissolved oxygen in water. *Berlin. Deut. Chem. Ges*. 21:2843.
2. Mancy, K.H. & T. Jaffe. 1966. Analysis of Dissolved Oxygen in Natural and Waste Waters; Pub. No. 999-WP-37. U.S. Public Health Serv., Washington, D.C.
3. Scott, E. 2007. Luminescence-based Measurement of Dissolved Oxygen in Natural Waters. Lecture, June 4–5. Hach Environmental, Loveland, Colo.

* Approved by Standard Methods Committee, 2016.
Joint Task Group: Michael A. Michaud (chair), Gregg L. Oelker, Marc Oliver D. Quijano.

4500-O B. Iodometric Methods

1. Principle

The iodometric test is the most precise and reliable titrimetric procedure for DO analysis. Analysts add divalent manganese solution and then strong alkali to a sample in a glass-stoppered bottle, and DO rapidly oxidizes an equivalent amount of the dispersed divalent manganous hydroxide precipitate into higher-valency hydroxides. Oxidized manganese reverts to the divalent state in the presence of iodide ions in an acidic solution, liberating an amount of iodine equivalent to the original DO content. The iodine is then titrated with a standard thiosulfate solution.

The titration endpoint can be detected visually with a starch indicator, or electrometrically with potentiometric or dead-stop techniques.[1] Experienced analysts can consistently detect endpoints with a precision of ±50 μg/L (visual) or ±5 μg/L (electrometric).[1,2]

The liberated iodine also can be determined directly by simple absorption spectrophotometers.[3] This method can be used routinely to provide accurate DO estimates in the microgram-per-liter range if interfering particulate matter, color, and chemical interferences are absent.

The quality control (QC) practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Selection of Method

Before selecting a method, consider the interferences that may be present in the sample. For example, certain oxidizing agents liberate iodine from iodides (positive interference) and some reducing agents reduce iodine to iodide (negative interference). Also, most organic matter is partially oxidized when the oxidized manganese precipitate is acidified, thus causing negative errors.

The following modifications of the iodometric method are designed to minimize the effect of interferences:[2]

- the azide modification (4500-O.C)[4] effectively removes nitrite interference, which is the most common interference in biologically treated effluents and incubated biochemical oxygen demand (BOD) samples.
- the permanganate modification (4500-O.D)[5] addresses ferrous iron [Fe(II)];
- the alum flocculation modification (4500-O.E)[6] minimizes suspended solids interference; and
- the copper sulfate-sulfamic acid flocculation modification (4500-O.F)[7,8] handles activated-sludge mixed liquor.

When the sample contains ≥5 mg ferric iron salts/L, add potassium fluoride (KF) as the first reagent in the azide modification or after the permanganate treatment for Fe(II). Another option is to eliminate ferric ion [Fe(III)] interference by using 85 to 87% phosphoric acid (H_3PO_4) instead of sulfuric acid (H_2SO_4) for acidification. This procedure has not been tested for Fe(III) concentrations >20 mg/L.

3. Collection of Samples

Collect samples very carefully. Sampling methods depend greatly on the source to be sampled and, to a certain extent, on the analytical method. Do not let sample remain in contact with air or be agitated; both will change its gaseous content. Boiler-

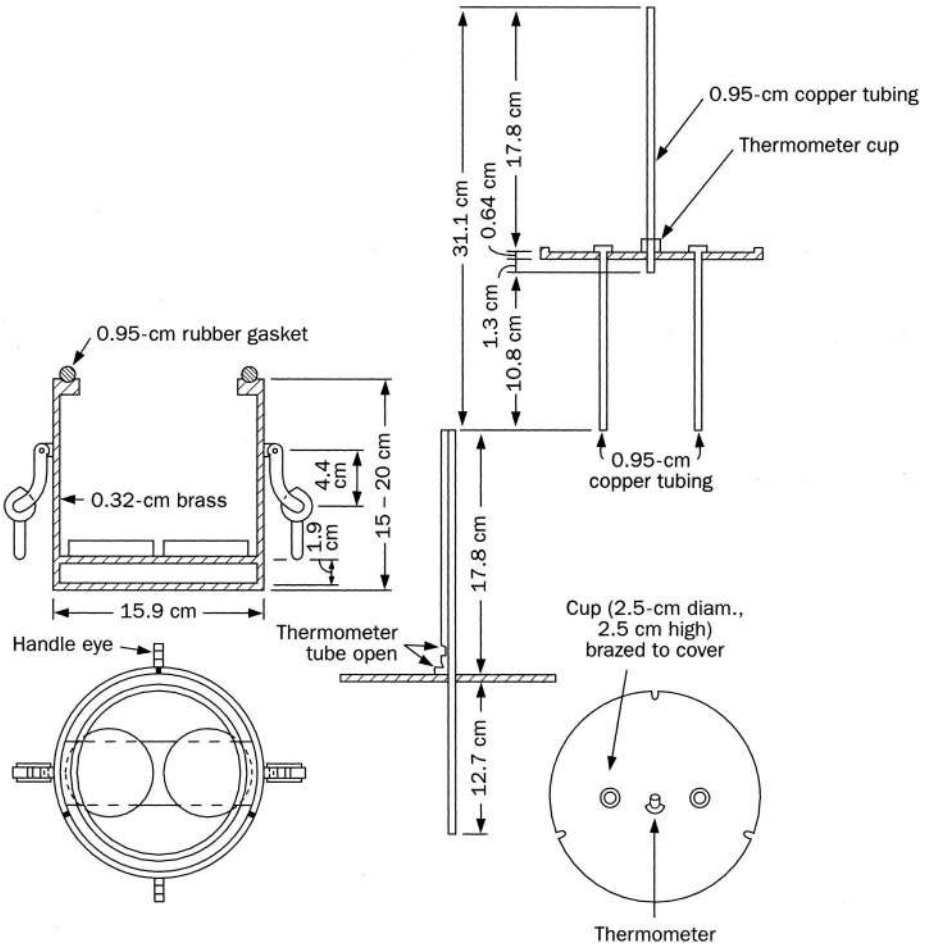


Figure 4500-O:1. DO and BOD sampler assembly.

water samples and samples from any depth in streams, lakes, or reservoirs require special equipment and procedures to eliminate changes in pressure and temperature that may affect sample results/DO concentration.[9,10]

Collect surface-water samples in BOD bottles with flared mouths and tapered-point ground-glass or acrylic stoppers. Avoid entraining or dissolving atmospheric oxygen. When sampling from a line under pressure, attach a glass or rubber tube to the tap and extend to bottom of bottle. Let bottle overflow two or three times its volume and replace stopper so no air bubbles are entrained.

American Public Health Association (APHA) suitable samplers for streams, ponds, or moderately deep tanks are shown in Figure 4500-O:1. Use a Kemmerer-type sampler for samples collected from depths >2 m. Bleed sample from bottom of sampler through a tube extending to bottom of a 250- to 300-mL BOD bottle. Fill bottle to overflowing (overflow for approximately 10 s), and prevent turbulence and bubble formation while filling. Record sample temperature to nearest degree Celsius or more precisely.

4. Preservation of Samples

Determine DO immediately in all samples with an appreciable oxygen or iodine demand. Samples with no iodine demand may be stored for a few hours without change after adding manganous sulfate ($MnSO_4$) solution, alkali-iodide solution, and H_2SO_4, and then shaking as described in the methods. Protect stored samples from strong sunlight, and titrate as soon as possible.

For samples with an iodine demand, preserve for 4 to 8 h by adding 0.7 mL conc H_2SO_4 and 1 mL sodium azide solution (2 g NaN_3/100 mL distilled water) to the BOD bottle. This will arrest biological activity and maintain DO if bottle is stored at collection temperature, or water-sealed and kept at 10 to 20°C. As soon as possible, complete the procedure using 2 mL $MnSO_4$ solution, 3 mL alkali-iodide solution, and 2 mL conc H_2SO_4.

5. References

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4500-O C. Azide Modification

1. General Discussion

Use the azide modification for most wastewater, effluent, and stream samples, especially if samples contain >50 μg nitrite nitrogen (NO_2^--N)/L and ≤1 mg Fe(II)/L. Other reducing or oxidizing materials should be absent. If analysts add 1 mL KF solution to the sample before acidifying and then promptly titrating it, then the method is applicable to samples containing 100 to 200 mg Fe(III)/L.

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Reagents

a. Manganous sulfate solution: Dissolve 480 g $MnSO_4 \cdot 4H_2O$, 400 g $MnSO_4 \cdot 2H_2O$, or 364 g $MnSO_4 \cdot H_2O$ in distilled water, filter, and dilute to 1 L. The $MnSO_4$ solution should not give a color with starch when added to an acidified potassium iodide (KI) solution.

b. Alkali-iodide-azide reagent:

1) For saturated or less-than-saturated samples—Dissolve 500 g sodium hydroxide (NaOH) [or 700 g potassium hydroxide (KOH)] and 135 g sodium iodide (NaI) (or 150 g KI) in distilled water and dilute to 1 L. Add 10 g NaN_3 dissolved in 40 mL distilled water. Potassium and sodium salts may be used interchangeably. This reagent should not give a color with starch solution when diluted and acidified .

2) For supersaturated samples—Dissolve 10 g NaN_3 in 500 mL distilled water. Add 480 g NaOH and 750 g NaI, and stir until dissolved. There will be a white turbidity due to sodium carbonate (Na_2CO_3), but this will do no harm. **CAUTION: Do not acidify this solution because toxic hydrazoic acid fumes may be produced.**

c. Sulfuric acid, conc: One milliliter is equivalent to about 3 mL alkali-iodide-azide reagent.

d. Starch: Use either an aqueous solution or soluble starch powder mixtures. To prepare an aqueous solution, dissolve 2 g laboratory-grade soluble starch and 0.2 g salicylic acid (as a preservative) in 100 mL hot distilled water.

e. Standard sodium thiosulfate ($Na_2S_2O_3$) titrant: Dissolve 6.205 g $Na_2S_2O_3 \cdot 5H_2O$ in reagent water. Add 1.5 mL 6*M* NaOH or 0.4 g solid NaOH and dilute to 1000 mL. Standardize with potassium bi-iodate [$KH(IO_3)_2$] solution.

f. Standard potassium bi-iodate solution, 0.0021*M:* Dissolve 812.4 mg $KH(IO_3)_2$ in reagent water and dilute to 1000 mL.

Standardization—Dissolve approximately 2 g KI, free from iodate, in an Erlenmeyer flask with 100 to 150 mL distilled water. Add 1 mL 3*M* H_2SO_4 or a few drops of conc H_2SO_4 and 20.00 mL standard bi-iodate solution. Dilute to 200 mL and titrate liberated iodine with thiosulfate titrant, adding starch toward end of titration, when a pale straw color is reached. When the solutions are of equal strength, 20.00 mL 0.025*M* $Na_2S_2O_3$ should be required. If not, adjust the $Na_2S_2O_3$ solution to 0.025*M*.

3. Procedure

a. To the sample collected in a 250- to 300-mL bottle, add 1 mL $MnSO_4$ solution followed by 1 mL alkali-iodide-azide reagent. If pipets are dipped into sample, rinse them before returning them to reagent bottles. Otherwise, hold pipet tips just above liquid surface when adding reagents. Stopper carefully to exclude air bubbles, and mix by inverting bottle a few times. When precipitate has settled enough (to approximately half the bottle volume) to leave clear supernate above the manganese hydroxide floc, add 1.0 mL conc H_2SO_4. Restopper and mix by inverting several times until the precipitate is completely dissolved. Titrate a volume corresponding to 200 mL original sample after correction for sample loss by displacement with reagents. Thus, for a total of 2 mL (1 mL each) of $MnSO_4$ and alkali-iodide-azide reagents in a 300-mL bottle, titrate $200 \times 300/(300 - 2) = 201$ mL.

b. Titrate with 0.025*M* $Na_2S_2O_3$ solution to a pale straw color. Add a few drops of starch solution and continue titration to first disappearance of blue color. If endpoint is overrun, back-titrate with 0.0021*M* bi-iodate solution added dropwise, or by adding a measured volume of treated sample. Correct for amount of bi-iodate solution or sample. Disregard subsequent recolorations due to the catalytic effect of nitrite or to traces of ferric salts that have not been complexed with fluoride.

4. Calculation

a. For titration of 200 mL sample, 1 mL 0.025*M* $Na_2S_2O_3$ = 1 mg DO/L.

b. To express results as percent saturation at 101.3 kPa, use the solubility data in Table 4500-O:I. Equations for correcting solubilities to barometric pressures other than mean sea level and for various chlorinities are given below the table.

TABLE 4500-O:I. SOLUBILITY OF OXYGEN IN WATER EXPOSED TO WATER-SATURATED AIR AT ATMOSPHERIC PRESSURE (101.3 kPa)[1]

Temperature	Oxygen Solubility *mg/L*						Temperature	Oxygen Solubility *mg/L*					
°C	Chlorinity: 0	5.0	10.0	15.0	20.0	25.0	°C	Chlorinity: 0	5.0	10.0	15.0	20.0	25.0
0.0	14.621	13.728	12.888	12.097	11.355	10.657	26.0	8.113	7.711	7.327	6.962	6.615	6.285
1.0	14.216	13.356	12.545	11.783	11.066	10.392	27.0	7.968	7.575	7.201	6.845	6.506	6.184
2.0	13.829	13.000	12.218	11.483	10.790	10.139	28.0	7.827	7.444	7.079	6.731	6.400	6.085
3.0	13.460	12.660	11.906	11.195	10.526	9.897	29.0	7.691	7.317	6.961	6.621	6.297	5.990
4.0	13.107	12.335	11.607	10.920	10.273	9.664	30.0	7.559	7.194	6.845	6.513	6.197	5.896
5.0	12.770	12.024	11.320	10.656	10.031	9.441	31.0	7.430	7.073	6.733	6.409	6.100	5.806
6.0	12.447	11.727	11.046	10.404	9.799	9.228	32.0	7.305	6.957	6.624	6.307	6.005	5.717
7.0	12.139	11.442	10.783	10.162	9.576	9.023	33.0	7.183	6.843	6.518	6.208	5.912	5.631
8.0	11.843	11.169	10.531	9.930	9.362	8.826	34.0	7.065	6.732	6.415	6.111	5.822	5.546
9.0	11.559	10.907	10.290	9.707	9.156	8.636	35.0	6.950	6.624	6.314	6.017	5.734	5.464
10.0	11.288	10.656	10.058	9.493	8.959	8.454	36.0	6.837	6.519	6.215	5.925	5.648	5.384
11.0	11.027	10.415	9.835	9.287	8.769	8.279	37.0	6.727	6.416	6.119	5.835	5.564	5.305
12.0	10.777	10.183	9.621	9.089	8.586	8.111	38.0	6.620	6.316	6.025	5.747	5.481	5.228
13.0	10.537	9.961	9.416	8.899	8.411	7.949	39.0	6.515	6.217	5.932	5.660	5.400	5.152
14.0	10.306	9.747	9.218	8.716	8.242	7.792	40.0	6.412	6.121	5.842	5.576	5.321	5.078
15.0	10.084	9.541	9.027	8.540	8.079	7.642	41.0	6.312	6.026	5.753	5.493	5.243	5.005
16.0	9.870	9.344	8.844	8.370	7.922	7.496	42.0	6.213	5.934	5.667	5.411	5.167	4.933
17.0	9.665	9.153	8.667	8.207	7.770	7.356	43.0	6.116	5.843	5.581	5.331	5.091	4.862
18.0	9.467	8.969	8.497	8.049	7.624	7.221	44.0	6.021	5.753	5.497	5.252	5.017	4.793
19.0	9.276	8.792	8.333	7.896	7.483	7.090	45.0	5.927	5.665	5.414	5.174	4.944	4.724
20.0	9.092	8.621	8.174	7.749	7.346	6.964	46.0	5.835	5.578	5.333	5.097	4.872	4.656
21.0	8.915	8.456	8.021	7.607	7.214	6.842	47.0	5.744	5.493	5.252	5.021	4.801	4.589
22.0	8.743	8.297	7.873	7.470	7.087	6.723	48.0	5.654	5.408	5.172	4.947	4.730	4.523
23.0	8.578	8.143	7.730	7.337	6.963	6.609	49.0	5.565	5.324	5.094	4.872	4.660	4.457
24.0	8.418	7.994	7.591	7.208	6.844	6.498	50.0	5.477	5.242	5.016	4.799	4.591	4.392
25.0	8.263	7.850	7.457	7.083	6.728	6.390							

NOTE:

1. The table provides three decimal places to aid interpolation. When computing saturation values to be used with measured values, such as in computing DO deficit in a receiving water, precision of measured values will control choice of decimal places to be used.
2. Equations are available to compute DO concentration in fresh water[1–3] and in seawater[1] at equilibrium with water-saturated air. Figures and tables also are available.[3]

   Calculate the equilibrium oxygen concentration, C^*, from equation:

$$\ln C^* = -139.344\ 11 + (1.575\ 701 \times 10^5/T) - (6.642\ 308 \times 10^7/T^2) + (1.243\ 800 \times 10^{10}/T^3) - (8.621\ 949 \times 10^{11}/T^4) - \text{Chl}\,[(3.1929) \times 10^{-2}) - (1.9428 \times 10^1/T) + (3.8673 \times 10^3/T^2)]$$

   where:

   C^* = equilibrium oxygen concentration at 101.325 kPa, mg/L,
   T = temperature (°K) = °C + 273.150 (°C is between 0.0 and 40.0 in the equation; the table is accurate up to 50.0), and
   Chl = Chlorinity (see definition in Note 4, below).

   Example 1: At 20°C and 0.000 Chl, ln C^* = 2.207 442 and C^* = 9.092 mg/L;
   Example 2: At 20°C and 15.000 ChL,

$$\ln C^* = (2.207\ 442) - 15.000\ (0.010\ 657) = 2.0476 \text{ and } C^* = 7.749 \text{ mg/L}.$$

   When salinity is used, replace the chlorinity term (−Chl[. . .]) by:
   $-S(1.7674 \times 10^{-2}) - (1.0754 \times 10^1/T) + (2.1407 \times 10^3/T^2)$

   where:

   S = salinity (see definition in Note 4, below).

3. For nonstandard conditions of pressure:

$$C_p = C^*P\left[\frac{(1 - P_{wv}/P)(1 - \theta P)}{(1 - P_{wv})(1 - \theta)}\right]$$

   where:

   C_p = equilibrium oxygen concentration at nonstandard pressure, mg/L,
   C^* = equilibrium oxygen concentration at standard pressure of 1 atm, mg/L.
   P = nonstandard pressure, atm,
   P_{wv} = partial pressure of water vapor, atm, computed from: $\ln P_{wv} = 11.8571 - (3840.70/T) - (216\ 961/T^2)$,
   T = temperature, K,
   $\theta = 0.000\ 975 - (1.426 \times 10^{-5}t) + (6.436 \times 10^{-8}t^2)$, and
   t = temperature, °C.

   N.B.: Although not explicit in the above, the quantity in brackets in the equation for C_p has dimensions of atm^{-1} per Reference 4, so P multiplied by this quantity is dimensionless.

   Also, the equation for ln P_{wv} is strictly valid for fresh water only, but for practical purposes no error is made by neglecting the effect of salinity. An equation for P_{wv} that includes the salinity factor may be found in Reference 1.

   Example 3: At 20°C, 0.000 Chl, and 0.700 atm,
   $C_p = C^*\ P\ (0.990\ 092) = 6.30$ mg/L.

4. Definitions:

   Salinity: Although salinity has been defined traditionally as the total solids in water after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized (see Section 2520), the new scale used to define salinity is based on the electrical conductivity of seawater relative to a specified solution of KCl in water.[5] The scale is dimensionless and the traditional dimension of parts per thousand (i.e., g/kg of solution) no longer applies.

   Chlorinity: Chlorinity is defined in relation to salinity as follows:

$$\text{Salinity} = 1.806\ 55 \times \text{chlorinity}$$

   Although chlorinity is not equivalent to chloride concentration, the factor for converting a chloride concentration in seawater to include bromide, for example, is only 1.0045 (based on the relative molecular weights and amounts of the two ions). Therefore, for practical purposes, chloride concentration (in g/kg of solution) is nearly equal to chlorinity in seawater. For wastewater, it is necessary to know the ions responsible for the solution's electrical conductivity to correct for their effect on oxygen solubility and use of the tabular value. If this is not done, the equation is inappropriate unless the relative composition of the wastewater is similar to that of seawater.

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5. Precision and Bias

DO can be determined with a precision (expressed as a standard deviation) of about 20 μg/L in reagent water and about 60 μg/L in wastewater and secondary effluents. Even with proper modifications, the standard deviation may be as high as 100 μg/L when appreciable interference is present. Still greater errors may occur when testing waters with organic suspended solids or heavy pollution. Avoid errors due to carelessness in collecting samples, prolonged test completion, or an unsuitable modification.

6. References

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4500-O D. Permanganate Modification

1. General Discussion

Use the permanganate modification only on samples containing Fe(II) (e.g., acid mine water). Interference from high concentrations of Fe(III) (up to several hundred milligrams per liter) may be overcome by adding 1 mL KF and alkali-iodide-azide so long as the final titration is made immediately after acidification.

This procedure is ineffective for oxidizing sulfite, thiosulfate, polythionate, or organic matter in wastewater. The error with samples containing 0.25% (by vol) digester waste from the manufacture of sulfite pulp may amount to 7 to 8 mg DO/L. With such samples, use the alkali-hypochlorite modification; however, this modification gives low results at best, the deviation amounting to 1 mg/L for samples containing 0.25% digester wastes.

2. Reagents

All the reagents required in 4500-O.C.2, as well as:

a. Potassium permanganate ($KMnO_4$) solution: Dissolve 6.3 g $KMnO_4$ in distilled water and dilute to 1 L.

b. Potassium oxalate ($K_2C_2O_4$) solution: Dissolve 2 g $K_2C_2O_4 \cdot H_2O$ in 100 mL distilled water; 1 mL will reduce about 1.1 mL permanganate solution.

c. Potassium fluoride (KF) solution: Dissolve 40 g $KF \cdot 2H_2O$ in distilled water and dilute to 100 mL.

3. Procedure

a. To a sample collected in a 250- to 300-mL bottle add, below the surface, 0.70 mL conc H_2SO_4, 1 mL $KMnO_4$ solution, and 1 mL KF solution. Stopper and mix by inverting. Never add >0.7 mL conc H_2SO_4 as the first step of pretreatment. Add acid with a 1-mL pipet graduated to 0.1 mL. Add enough $KMnO_4$ solution to obtain a violet tinge that persists for 5 min.

If the permanganate color disappears sooner, add more $KMnO_4$ solution but avoid large excesses.

b. Remove permanganate color completely by adding 0.5 to 1.0 mL $K_2C_2O_4$ solution. Mix well and let stand in the dark to facilitate the reaction. Excess oxalate causes low results; add only enough $K_2C_2O_4$ to decolorize $KMnO_4$ completely without an excess of >0.5 mL. Complete decolorization in 2 to 10 min. If sample cannot be decolorized without adding a large excess of oxalate, the DO result will be inaccurate.

c. From this point, the procedure closely parallels that in 4500-O.C.3. Add 1 mL $MnSO_4$ solution and 3 mL alkali-iodide-azide reagent. Stopper, mix, and let precipitate settle a short time; acidify with 2 mL conc H_2SO_4. When 0.7 mL acid, 1 mL KF solution, 1 mL $KMnO_4$ solution, 1 mL $K_2C_2O_4$ solution, 1 mL $MnSO_4$ solution, and 3 mL alkali-iodide-azide (or a total of 7.7 mL reagents) are used in a 300-mL bottle, take $200 \times 300/(300 - 7.7) = 205$ mL for titration.

This correction is slightly in error because the $KMnO_4$ solution is nearly saturated with DO and 1 mL would add about 0.008 mg oxygen to the DO bottle. However, because the method's precision (standard deviation, 0.06 mL thiosulfate titration, or 0.012 mg DO) is 50% greater than this error, a correction is unnecessary. When substantially more $KMnO_4$ solution is used routinely, use a solution several times more concentrated so 1 mL will satisfy the permanganate demand.

4. Bibliography

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4500-O E. Alum Flocculation Modification

1. General Discussion

Samples high in suspended solids may consume appreciable quantities of iodine in acid solution. The interference due to solids may be removed by alum flocculation.

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Reagents

All the reagents required for the azide modification (4500-O.C.2) as well as:

a. Alum solution: Dissolve 10 g aluminum potassium sulfate [$AlK(SO_4)_2 \cdot 12H_2O$] in distilled water and dilute to 100 mL.

b. Ammonium hydroxide: (NH_4OH) conc.

3. Procedure

Collect sample in a 500- to 1000-mL, glass-stoppered bottle, using the same precautions as for regular DO samples. Add 10 mL alum solution and 1 to 2 mL conc NH_4OH. Stopper and invert gently for about 1 min. Let sample settle for about 10 min and siphon clear supernate into a 250- to 300-mL DO bottle until it overflows. Avoid sample aeration and keep siphon submerged at all times. Continue procedure as in 4500-O.C.3 or an appropriate modification.

4500-O F. Copper Sulfate-Sulfamic Acid Flocculation Modification

1. General Discussion

This modification is used for biological flocs (e.g., activated sludge mixtures), which have high oxygen utilization rates.

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Reagents

All the reagents required for the azide modification (4500-O.C.2) as well as:

Copper sulfate-sulfamic acid inhibitor solution: Dissolve 32 g technical-grade sulfamic acid (NH_2SO_2OH) without heat in 475 mL distilled water. Dissolve 50 g copper sulfate ($CuSO_4 \cdot 5H_2O$) in 500 mL distilled water. Mix the two solutions and add 25 mL conc acetic acid.

3. Procedure

Add 10 mL $CuSO_4 \cdot NH_2SO_2OH$ inhibitor to a 1-L glass-stoppered bottle. Insert bottle in a special sampler designed so bottle fills from a tube near bottom and overflows only 25 to 50% of bottle capacity. Collect sample, stopper, and mix by inverting. Let suspended solids settle and siphon relatively clear supernatant liquor into a 250- to 300-mL DO bottle. Continue sample treatment as rapidly as possible via the azide procedure (4500-O.C.3) or other appropriate modification.

4500-O G. Membrane-Electrode Method

1. General Discussion

Although researchers have developed various modifications of the iodometric method to eliminate or minimize certain interferences, the method is still inapplicable to various industrial and domestic wastewaters.[1] Moreover, the iodometric method is not suited for field testing and cannot be easily adapted for continuous monitoring or determining DO *in situ*.

Polarographic methods using either dropping-mercury or rotating-platinum electrodes have been unreliable for DO analysis in domestic and industrial wastewaters because impurities in the test solution can cause electrode poisoning or other interferences.[2,3] These problems are minimized when using membrane-covered electrode systems because the sensing element is protected by an oxygen-permeable polymer membrane that serves as a diffusion barrier against impurities.[4–6] Under steady-state conditions, the current is directly proportional to the DO concentration.*

Polarographic[4] and galvanic[5] membrane electrodes have been used to measure DO in lakes and reservoirs[7] for stream survey and industrial-effluent control,[8,9] continuous DO monitoring in activated sludge units,[10] and estuarine and oceanographic studies.[11] Being completely submersible, membrane electrodes are suited for analysis *in situ*. Their portability and ease of operation and maintenance make them particularly convenient for field applications. In laboratory investigations, membrane electrodes

* Fundamentally, the current is directly proportional to molecular-oxygen activity.

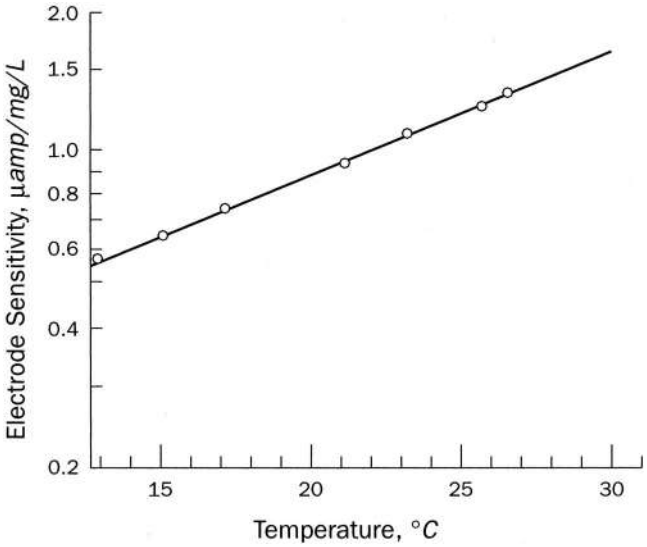


Figure 4500-O:2. Effect of temperature on electrode sensitivity.

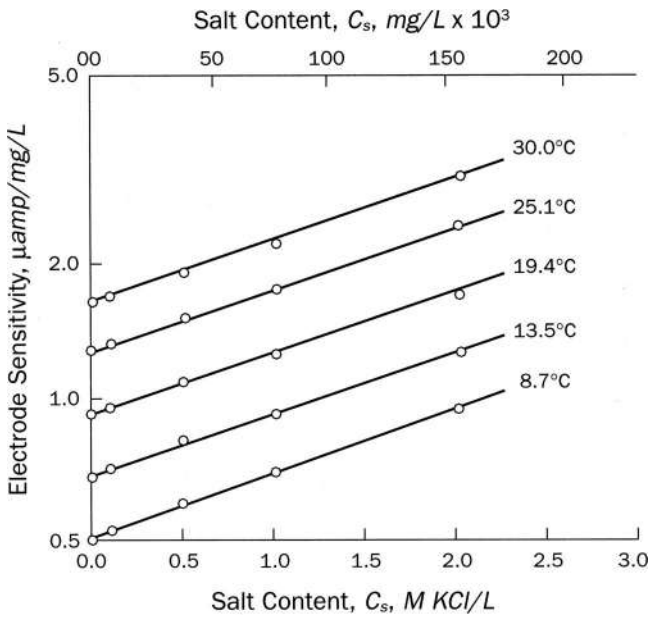


Figure 4500-O:3. The salting-out effect at different temperatures.

have been used for continuous DO analysis in bacterial cultures, including the BOD test.[5,12]

Membrane electrodes provide an excellent method for DO analysis in polluted waters, highly colored waters, and strong waste effluents. They are recommended for use especially when conditions are unfavorable for the iodometric method, or when that test and its modifications are subject to serious interference-related errors.

a. Principle: Oxygen-sensitive polarographic or galvanic membrane electrodes are composed of two solid metal electrodes in contact with supporting electrolyte separated from the test solution by a selective membrane. The basic difference between the galvanic and polarographic systems is that in the former the electrode reaction is spontaneous (similar to that in a fuel cell), while the latter requires an external source of applied voltage to polarize the indicator electrode. Polyethylene and fluorocarbon membranes are commonly used because they are permeable to molecular oxygen and are relatively rugged.

Membrane electrodes are commercially available in some variety. In all these instruments, the "diffusion current" is linearly proportional to the molecular-oxygen concentration. The measured current can be converted easily to concentration units (e.g., mg/L) by a number of calibration procedures.

Membrane electrodes have a relatively high temperature coefficient largely due to changes in membrane permeability.[6] The effect of temperature on electrode sensitivity, Ø (µA/mg/L), can be expressed by the following simplified relationship:

$$\log Ø = 0.43\,mt + b$$

where:

m = constant that depends on the membrane material,
t = temperature, °C, and
b = constant that largely depends on membrane thickness.

If Ø and m values are determined for one temperature ($Ø_0$ and t_0), the sensitivity at any desired temperature (Ø and t) can be calculated as follows:

$$\log Ø = \log Ø_0 + 0.43\,m\,(t - t_0)$$

Nomographic charts for temperature correction can be constructed easily[13] and are available from some manufacturers. An example is shown in Figure 4500-O:2, in which sensitivity is plotted versus temperature on semi-logarithmic coordinates. Check one or two points frequently to confirm original calibration. If calibration changes, the new calibration should be parallel to the original if the same membrane material is used.

Thermistors in an electrode circuit can automatically provide temperature compensation,[4] but they may not compensate fully over a wide temperature range. If accuracy is critical, use calibrated nomographic charts to correct for temperature effect.

To use the DO membrane-electrode method in estuarine waters or in wastewaters with varying ionic strength, correct for the salting-out effect on electrode sensitivity.[6,13] This effect is particularly significant for large changes in salt content. Electrode sensitivity varies with salt concentration according to the following relationship:

$$\log Ø_S = 0.43\,m_S C_S + \log Ø_0$$

where:

$Ø_S$, $Ø_0$ = sensitivities in salt solution and distilled water, respectively,
m_S = constant (salting-out coefficient), and
C_S = salt concentration (preferably ionic strength).

If $Ø_0$ and m_S are determined, then the sensitivity for any value of C_S can be calculated. Conductivity measurements can be used to approximate salt concentration (C_S). This is particularly applicable to estuarine waters. Figure 4500-O:3 shows calibration curves for the sensitivity of varying salt solutions at different temperatures.

b. Interference: Polymer films used with membrane-electrode systems are permeable to various gases besides oxygen, although none is depolarized easily at the indicator electrode .

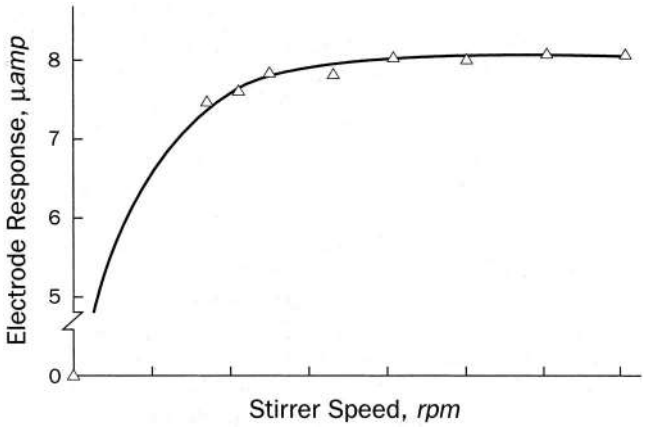


Figure 4500-O:4. Typical trend of effect of stirring on electrode response.

Prolonged use of membrane electrodes in waters containing such gases as hydrogen sulfide (H_2S) tends to lower cell sensitivity. Eliminate this interference by frequently changing the electrolyte and membrane and calibrating the membrane electrode.

c. Membrane fouling: Membrane fouling is an issue due to bacterial or algal growth, soaps, foam, polymers, grease, and non-aqueous-phase liquids.

d. Sampling: Because membrane electrodes provide analysis *in situ*, they eliminate errors due to sample handling and storage. If sampling is required, use the same precautions suggested for the iodometric method.

e. Quality control: The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Oxygen-sensitive membrane electrode, polarographic or galvanic, with appropriate meter.

3. Procedure

a. Calibration: Follow manufacturer's calibration procedure exactly to obtain guaranteed precision and accuracy. Generally, calibrate membrane electrodes by reading against air or a sample of known DO concentration (determined by iodometric method or see Table 4500-O:II), as well as in a sample with zero DO. [Add excess sodium sulfite (Na_2SO_3) and a trace of cobalt chloride ($CoCl_2$) to bring DO to zero.] Preferably calibrate with samples of water to be tested, but avoid iodometric calibration if interferences are suspected. The following illustrate the recommended procedures:

1) Fresh water—For unpolluted samples without interferences, calibrate in test solution or distilled water, whichever is more convenient.

2) Salt water—Calibrate directly with samples of seawater or waters whose salt concentration is constantly >1000 mg/L.

3) Fresh water containing pollutants or interferences—Calibrate with distilled water because erroneous results occur with sample.

4) Salt water containing pollutants or interferences—Calibrate with a sample of clean water with the same salt content as the sample. Add a concentrated potassium chloride (KCl) solution (see Section 2510 and Table 2510:I) to distilled water to produce the same specific conductance as that in the sample. For polluted ocean waters, calibrate with a sample of unpolluted seawater.

5) Estuary water containing varying quantities of salt—Calibrate with a sample of uncontaminated seawater or distilled or tap water. Determine sample chloride or salt concentration and revise calibration to account for change of oxygen solubility in the estuary water.[13]

b. Sample measurement: Follow all precautions recommended by manufacturer to ensure acceptable results. Change membrane carefully to avoid contaminating sensing element or trapping minute air bubbles under membrane, which can lead to lowered response and high residual current. Provide enough sample flow across membrane surface to overcome erratic response (see Figure 4500-O:4 for a typical example of stirring's effect).

c. Validation of temperature effect: Check one or two points frequently to verify temperature-correction data.

4. Precision and Bias

Most commercially available membrane-electrode systems can obtain an accuracy of 0.1 mg DO/L and a precision of 0.05 mg DO/L.

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TABLE 4500-O:II. DISSOLVED OXYGEN SATURATION IN WATER (MG/L)

Temp		Pressure in millimeters and inches Hg							
		mm							
		775	760	750	725	700	675	650	625
		in.							
°F	°C	30.51	29.92	29.53	28.45	27.56	26.57	25.59	24.61
32.0	0	14.9	14.6	14.4	13.9	13.5	12.9	12.5	12.0
33.8	1	14.5	14.2	14.1	13.6	13.1	12.6	12.2	11.7
35.6	2	14.1	13.8	13.7	13.2	12.9	12.3	11.8	11.4
37.4	3	13.8	13.5	13.3	12.9	12.4	12.0	11.5	11.1
39.2	4	13.4	13.1	13.0	12.5	12.1	11.7	11.2	10.8
41.0	5	13.2	12.8	12.6	12.2	11.8	11.4	10.9	10.5
42.8	6	12.7	12.4	12.3	11.9	11.5	11.1	10.7	10.3
44.6	7	12.4	12.1	12.0	11.6	11.2	10.8	10.4	10.0
46.4	8	12.1	11.8	11.7	11.3	10.9	10.5	10.1	9.8
48.2	9	11.8	11.6	11.5	11.1	10.7	10.3	9.9	9.5
50.0	10	11.6	11.3	11.2	10.8	10.4	10.1	9.7	9.3
51.8	11	11.3	11.0	10.9	10.6	10.2	9.8	9.5	9.1
53.6	12	11.1	10.8	10.7	10.3	10.0	9.6	9.2	8.9
55.4	13	10.8	10.5	10.5	10.1	9.8	9.4	9.1	8.7
57.2	14	10.6	10.3	10.2	9.9	9.5	9.2	8.9	8.5
59.0	15	10.4	10.1	10.0	9.7	9.3	9.0	8.7	8.3
60.8	16	10.1	9.9	9.8	9.5	9.1	8.8	8.5	8.1
62.6	17	9.9	9.7	9.6	9.3	9.0	8.6	8.3	8.0
64.4	18	9.7	9.5	9.4	9.1	8.8	8.4	8.1	7.8
66.2	19	9.5	9.3	9.2	8.9	8.6	8.3	8.0	7.6
68.0	20	9.3	9.1	9.1	8.7	8.4	8.1	7.7	7.5
69.8	21	9.2	8.9	8.9	8.6	8.3	8.0	7.5	7.4
71.6	22	9.0	8.7	8.7	8.4	8.1	7.8	7.4	7.2
73.4	23	8.8	8.6	8.5	8.2	8.0	7.7	7.2	7.1
75.2	24	8.7	8.4	8.4	8.1	7.8	7.5	7.1	7.0
77.0	25	8.5	8.3	8.3	8.0	7.7	7.4	7.0	6.8
78.8	26	8.4	8.1	8.1	7.8	7.6	7.3	6.9	6.7
80.6	27	8.2	8.0	8.0	7.7	7.4	7.1	6.8	6.6
82.4	28	8.1	7.8	7.8	7.6	7.3	7.0	6.7	6.5
84.2	29	7.9	7.7	7.7	7.4	7.2	6.9	6.6	6.4
86.0	30	7.8	7.6	7.6	7.3	7.0	6.8	6.5	6.2
87.8	31	7.7	7.4	7.4	7.2	6.9	6.7	6.4	6.1
89.6	32	7.6	7.3	7.3	7.0	6.8	6.6	6.3	6.0
91.4	33	7.4	7.2	7.2	6.9	6.7	6.4	6.2	5.9
93.2	34	7.3	7.1	7.1	6.8	6.6	6.3	6.	5.8
95.0	35	7.2	7.0	7.0	6.7	6.5	6.2	6.0	5.7
96.8	36	7.1	6.8	6.9	6.6	6.4	6.1	5.9	5.6
98.6	37	7.0	6.7	6.7	6.5	6.3	6.0	5.8	5.6
100.4	38	6.9	6.6	6.6	6.4	6.2	5.9	5.7	5.5
102.2	39	6.8	6.5	6.5	6.3	6.1	5.8	5.6	5.4
104.0	40	6.7	6.4	6.4	6.2	6.0	5.7	5.5	5.3
105.8	41	6.6	6.3	6.3	6.1	5.9	5.6	5.4	5.2
107.6	42	6.5	6.2	6.2	6.0	5.8	5.6	5.3	5.1
109.4	43	6.4	6.1	6.1	5.9	5.7	5.5	5.2	5.0
111.2	44	6.3	6.0	6.0	5.8	5.6	5.4	5.2	4.9
113.0	45	6.2	5.9	5.9	5.7	5.5	5.3	5.1	4.8
114.8	46	6.1	5.8	5.9	5.6	5.4	5.2	5.0	4.8
116.6	47	6.0	5.7	5.8	5.6	5.3	5.1	4.8	4.7
118.4	48	5.9	5.7	5.7	5.5	5.3	5.0	4.8	4.6
120.2	49	5.8	5.6	5.6	5.4	5.2	5.0	4.7	4.5
122.0	50	5.7	5.5	5.5	5.3	5.1	4.9	4.7	4.4

Courtesy of Hach Co., Loveland, Colo.

4500-O H. Optical-Probe Method

1. General Discussion

a. Principle: The optical probe uses luminescence-based oxygen sensors to measure the light-emission characteristics of a luminescent reaction; oxygen quantitatively quenches the luminescence. The change in the luminescence signal's lifetime correlates to the DO concentration.[1]

Lifetime-based measurements use modulated blue and red lights emitted from two light-emitting diode (LED) sources. Blue light hits the luminescent material on the probe sensor cap, which releases red light when excited. Oxygen quenches this red light, causing a phase shift in the returned signal detected by a photo diode. The higher the oxygen concentration, the faster the red light is quenched and the slower the red light is detected by the diode. The probe measures how long it takes the luminescent material to return to a relaxed state. Between flashes of blue light, a separate LED emits red light onto the probe sensor cap, thereby serving as an internal standard. The probe calculates the DO concentration by comparing the lifetime measurements of the blue and red light emissions.

b. Scope and application: This method is applicable to waters containing 0.05 to 20.00 mg/L DO.

c. Interferences: Chlorine dioxide interferes with this reaction at percent level concentrations. Biofouling due to bacteria or algal growth can prevent oxygen permeation through the window. Bacteria and algae may also generate or consume oxygen, resulting in erroneous readings; this can be minimized by rinsing the probe between readings to keep the sensor clean. Oils can clog the membrane and sensor cap, prohibiting oxygen from diffusing to the sensor; frequent rinsing between measurements can minimize this problem. Various alcohols and organic solvents could damage the probe's materials and permanently damage the probe.[2]

d. Sampling: This method provides analysis *in situ*, eliminating errors due to sample handling and storage. If sampling is required, use the same precautions suggested for the iodometric method (see 4500-O.B.3).

2. Apparatus

a. Oxygen-sensitive optical probe with appropriate meter.
b. Stirring device.

3. Reagents

a. Sodium sulfite (Na_2SO_3), 2M *solution* (to make a blank): See 4500-O.G.3*a*.

b. Cobalt chloride ($CoCl_2$), 0.03 g/L: Add to the 2 M sodium sulfite solution to make a zero DO blank solution. Blanks can also be prepared by aerating 1 to 2 L reagent water with an inert gas.

4. Procedure

Calibrate probe in accordance with manufacturer's instructions to obtain the guaranteed precision and accuracy. Some luminescence-based sensors have built-in multipoint calibration and do not require initial multipoint calibration. Verify the calibration with a blank and a control. The control can be either in air or in water. To prepare a water-saturated air sample, add 0.25 in. reagent water to clean 300-mL BOD bottle and seal with stopper. Shake vigorously for approximately 30 s. Allow about 30 min for the BOD bottle and its contents to equilibrate to room temperature (20 ± 2°C), then analyze. Alternatively, a manufacturer-supplied calibration sleeve may be used to provide a water-saturated air sample. To prepare air-saturated water, add approximately 1500 mL reagent water to a 2-L beaker. Allow water to equilibrate to room temperature (20 ± 2°C). Using a steady stream of clean compressed air* (approximately 10 to 40 mL/min flow rate), aerate the water for at least 30 min. Allow water to re-equilibrate to room temperature (20 ± 2°C) for approximately 45 min. Transfer aerated water to a clean BOD bottle until overflowing, then seal with stopper.[3] Note laboratory barometric pressure and sample temperature, and use Table 4500-O:I to determine the theoretical DO concentration. Analyze as soon as possible, but ≤4 h after transfer. The verification value should be ±10% of theoretical DO concentration. If verification values are outside acceptable ranges, recalibrate sensor and re-analyze controls.

Fill each BOD bottle to top of neck with sample. Be careful to pour sample slowly and make sure it flows down the side of bottle so oxygen is not introduced into the sample, resulting in erroneously high DO measurement. Place probe in sample, and turn on stirring mechanism if manufacturer recommends stirring. Record DO reading when the meter is finished analyzing sample. See 4500-O.G.3*a*.1)–5) for comments on analyzing different matrices.

5. Calculations

The meter and the probe perform the calculations automatically.

6. Quality Control

The QC practices considered to be an integral part of this method are summarized in Table 4020:I. Blanks should have a 0.0 ± 0.2 mg/L O_2 value. Controls should have a DO of 90 to 110% of the saturation level at the control temperature (see Table 4500-O:I). Duplicates should be within 15% RPD. Initial demonstration of capability (IDC) unknowns should be within 3 standard deviations of the certified value.

7. Precision and Bias

Most commercially available optical probe systems may have an accuracy of 0.1 mg DO/L and a precision of 0.01 mg DO/L.

Two studies were performed to verify this method:

- Four laboratories analyzed 46 proficiency samples, using laboratory reagent-water and wastewater matrices and opti-

* Allow compressed air to pass through activated carbon to remove oil from compressed air, if needed.

cal probes from three manufacturers, and sample results had an average deviation of 10.6% from assigned values; and
- A study of 85 simultaneous analysis events comparing an optical probe and a membrane-electrode system showed a 4.9% average difference in results.

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4500-O_3 OZONE (RESIDUAL)*

4500-O_3 A. Introduction

1. Sources

Ozone, a potent germicide, is used also as an oxidizing agent for the oxidation of organic compounds that produce taste and odor in drinking water, for the destruction of organic coloring matter, and for the oxidation of reduced iron or manganese salts to insoluble oxides.

2. Selection of Method

Ozone residual in water is determined by the indigo method. Residual ozone decays rapidly. Depending on water quality, the ozone residual half-life may be several seconds to a few minutes. Methods also are available for determining ozone in process gases.[1,2]

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011. Joint Task Group: 20th Edition—Kerwin Rakness (chair), Leo C. Fung, Gilbert Gordon, Jill T. Gramith, Willy J. Masschelein, Lawrence K. Wang.

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4500-O_3 B. Indigo Colorimetric Method

1. General Discussion

The indigo colorimetric method is quantitative, selective, and simple; it replaces methods based on the measurement of total oxidant. The method is applicable to lake water, river infiltrate, manganese-containing groundwaters, extremely hard groundwaters, and even biologically treated domestic wastewaters.

a. Principle: In acidic solution, ozone rapidly decolorizes indigo. The decrease in absorbance is linear with increasing concentration. The proportionality constant at 600 nm is 0.42 ± 0.01/cm/mg/L ($\Delta E = 20\,000/M \cdot$ cm) compared to the ultraviolet absorption of pure ozone of $E = 2950/M \cdot$ cm at 258 nm).[1]

b. Interferences: Hydrogen peroxide (H_2O_2) and organic peroxides decolorize the indigo reagent very slowly. H_2O_2 does not interfere if ozone is measured in less than 6 h after adding reagents. Organic peroxides may react more rapidly. Fe(III) does not interfere. Mn(II) does not interfere but it is oxidized by ozone to forms that decolorize the reagent. Correct for this decolorization by making the measurement relative to a blank in which the ozone has been destroyed selectively. Without the corrective procedure, 0.1 mg/L ozonated manganese gives a response of about 0.08 mg/L apparent ozone. Chlorine also interferes. Low concentrations of chlorine (<0.1 mg/L) can be masked by malonic acid. Bromine, which can be formed by oxidation of Br^-, interferes (1 mole HOBr corresponds to 0.4 mole ozone). In the presence of HOBr or chlorine in excess of 0.1 mg/L, an accurate measurement of ozone cannot be made with this method.

c. Minimum detectable concentration: For the spectrophotometric procedure using thermostated cells and a high-quality photometer, the low-range procedure will measure down to 2 μg O_3/L. The practical lower limit for residual measurement is 10 to 20 μg/L.

d. Sampling: React sample with indigo as quickly as possible, because the residual may decay rapidly. Avoid loss of ozone residual due to off-gassing during sample collection. Do not run sample down side of flask. Add sample so completely decolorized zones are eliminated quickly by swirling or stirring.

e. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Photometer: Spectrophotometer or filter colorimeter for use at 600 ± 10 nm.

3. Reagents

a. Indigo stock solution: Add about 500 mL distilled water and 1 mL conc phosphoric acid to a 1-L volumetric flask. With stirring, add 770 mg potassium indigo trisulfonate, $C_{16}H_7N_2O_{11}S_3K_3$ (use only high-grade reagent, commercially available at about 80 to 85% purity). Fill to mark with distilled water. A 1:100 dilution exhibits an absorbance of 0.20 ± 0.010 cm at 600 nm. The stock solution is stable for about 4 months when stored in the dark. Discard when absorbance of a 1:100 dilution falls below 0.16/cm. Do not change concentration of dye for higher ranges of ozone residual. Volume of dye used may be adjusted.

b. Indigo reagent I: To a 1-L volumetric flask add 20 mL indigo stock solution, 10 g sodium dihydrogen phosphate (NaH_2PO_4), and 7 mL conc phosphoric acid. Dilute to mark.

Prepare solution fresh when its absorbance decreases to less than 80% of its initial value (typically within a week).

c. Indigo reagent II: Proceed as with indigo reagent I, but add 100 mL indigo stock solution instead of 20 mL.

d. Malonic acid reagent: Dissolve 5 g malonic acid in water and dilute to 100 mL.

e. Glycine reagent: Dissolve 7 g glycine in water and dilute to 100 mL.

4. Procedure

a. Spectrophotometric, volumetric procedure:

1) Concentration range 0.01 to 0.1 mg O_3/L—Add 10.0 mL indigo reagent I to each of two 100-mL volumetric flasks. Fill one flask (blank) to mark with distilled water. Fill other flask to mark with sample. Measure absorbance of both solutions at 600 ± 10 nm as soon as possible but at least within 4 h. Preferably use 10-cm cells. Calculate the ozone concentration from the difference between the absorbances found in sample and blank (4500-O_3.B.5*a*). (NOTE: A maximum delay of 4 h before spectrophotometric reading can be tolerated only for drinking water samples. For other sample types that cannot be read immediately, determine the relationship between time and absorbance.)

2) Range 0.05 to 0.5 mg O_3/L—Proceed as above using 10.0 mL indigo reagent II instead of reagent I. Preferably measure absorbance in 4- or 5-cm cells.

3) Concentrations greater than 0.3 mg O_3/L—Proceed using indigo reagent II, but for these higher ozone concentrations use a correspondingly smaller sample volume. Dilute resulting mixture to 100 mL with distilled water.

4) Control of interferences—In presence of low chlorine concentration (<0.1 mg/L), place 1 mL malonic acid reagent in both flasks before adding sample and/or filling to mark. Measure absorbance as soon as possible, within 60 min (Br^-, Br_2, and HOBr are only partially masked by malonic acid).

In presence of manganese prepare a blank solution using sample, in which ozone is selectively destroyed by addition of glycine. Place 0.1 mL glycine reagent in 100-mL volumetric flask (blank) and 10.0 mL indigo reagent II in second flask (sample). Pipet exactly the same volume of sample into each flask. Adjust dose so decolorization in second flask is easily visible but complete bleaching does not result (maximum 80 mL).

Ensure that pH of glycine/sample mixture in blank flask (before adding indigo) is not below 6 because reaction between ozone and glycine becomes very slow at low pH. Stopper flasks and mix by carefully inverting. Add 10.0 mL indigo reagent II to blank flask only 30 to 60 s after sample addition. Fill both flasks to the mark with ozone-free water and mix thoroughly. Measure absorbance of both solutions at comparable contact times of approximately 30 to 60 min (after this time, residual manganese oxides further discolor indigo only slowly and the drift of absorbance in blank and sample become comparable). Reduced absorbance in blank flask results from manganese oxides, while that in sample flask is due to ozone plus manganese oxide.

5) Calibration—Because ozone is unstable, base measurements on known and constant loss of absorbance of the indigo reagent (f = 0.42 + 0.01/cm/mg O_3/L). For maximum accuracy, analyze the lot of potassium indigo trisulfonate (no commercial lot has been found to deviate from f = 0.42) using the iodometric procedure.

When using a filter photometer, re-adjust the conversion factor, f, by comparing photometer sensitivity with absorbance at 600 nm by an accurate spectrophotometer.

b. Spectrophotometric, gravimetric procedure:

1) Add 10.0 mL indigo reagent II to 100-mL volumetric flask and fill flask (blank) to mark with distilled water. Obtain tare weight of a second flask (volumetric or Erlenmeyer). Add 10.0 mL indigo reagent II to second flask. Fill directly with sample (do not run water down side), and swirl second flask until blue solution has turned to a light blue color. Weigh flask containing indigo and sample.

2) Preferably using 10-cm cells, measure absorbance of both solutions at 600 ± 10 nm as soon as possible, but at least within 4 h. NOTE: A maximum delay of 4 h before spectrophotometric reading is suitable only for drinking water samples. For other sample types, test the time drift.

5. Calculations

a. Spectrophotometric, volumetric method:

$$\text{mg } O_3/\text{L} = \frac{100 \times \Delta A}{f \times b \times V}$$

where:

ΔA = difference in absorbance between sample and blank,
f = 0.42,
b = path length of cell, cm, and
V = volume of sample, mL (normally 90 mL).

The factor f is based on a sensitivity factor of 20 000/cm for the change of absorbance (600 nm) per mole of added ozone per liter. It was calibrated by iodometric titration. The UV absorbance of ozone in pure water may serve as a secondary standard: the factor f = 0.42 corresponds to an absorption coefficient for aqueous ozone, $\mathrm{E} = 2950/M \cdot \text{cm}$ at 258 nm.

b. Spectrophotometric, gravimetric method:

$$\text{mg } O_3/\text{L} = \frac{(A_B \times 100) - (A_S \times V_T)}{f \times V_S \times b}$$

where:

A_B, A_S = absorbance of blank and sample, respectively,
V_T = total volume of sample plus indigo, mL = (final weight − tare weight) g × 1.0 mL/g,
f = 0.42 (see ¶ *a* above),
V_S = volume of sample, mL = [(final weight − tare weight) g × 1.0 mL/g] − 10 mL, and
b = path length of cell, cm.

6. Precision and Bias

For the spectrophotometric volumetric procedure in the absence of interferences, the relative error is less than 5% without special sampling setups. In laboratory testing, this may be reduced to 1%. No data are available for the spectrophotometric gravimetric procedure.

Because this method is based on the differences in absorbance between the sample and blank (ΔA) the method is not applicable

in the presence of chlorine. If the manganese content exceeds the ozone, precision is reduced. If the ratio of manganese to ozone is less than 10:1, ozone concentrations above 0.02 mg/L may be determined with a relative error of less than 20%.

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4500-P PHOSPHORUS*

4500-P A. Introduction

1. Occurrence

Phosphorus occurs in natural waters and in wastewaters almost solely as phosphates. These are classified as orthophosphates, condensed phosphates (pyro-, meta-, and other polyphosphates), and organically bound phosphates. They occur in solution, in particles or detritus, or in the bodies of aquatic organisms.

These forms of phosphate arise from a variety of sources. Small amounts of orthophosphate or certain condensed phosphates are added to some water supplies during treatment. Larger quantities of the same compounds may be added during laundering or other cleaning, because these materials are major constituents of many commercial cleaning preparations. Phosphates are used extensively in the treatment of boiler waters. Orthophosphates applied to agricultural or residential cultivated land as fertilizers are carried into surface waters with storm runoff and to a lesser extent with melting snow. Organic phosphates are formed primarily by biological processes. They are contributed to sewage by body wastes and food residues, and also may be formed from orthophosphates in biological treatment processes or by receiving-water biota.

Phosphorus is essential to the growth of organisms and can be the nutrient that limits the primary productivity of a body of water. In instances where phosphate is a growth-limiting nutrient, the discharge of raw or treated wastewater, agricultural drainage, or certain industrial wastes to that water may stimulate the growth of photosynthetic aquatic micro- and macroorganisms in nuisance quantities.

Phosphates also occur in bottom sediments and in biological sludges, both as precipitated inorganic forms and incorporated into organic compounds.

2. Terminology

Phosphorus analyses embody two general procedural steps:

- conversion of the phosphorus form of interest to dissolved orthophosphate, and
- colorimetric determination of dissolved orthophosphate.

The separation of phosphorus into its various forms is defined analytically but the analytical differentiations have been selected so they may be used for interpretive purposes.

Filtration through a 0.45-μm-pore-diam membrane filter separates dissolved from suspended forms of phosphorus. No claim is made that filtration through 0.45-μm filters is a true separation of suspended and dissolved forms of phosphorus; it is merely a convenient and replicable analytical technique designed to make a gross separation. Prefiltration through a glass fiber filter may be used to increase the filtration rate.

Phosphates that respond to colorimetric tests without preliminary hydrolysis or oxidative digestion of the sample are termed "reactive phosphorus." While reactive phosphorus is largely a measure of orthophosphate, a small fraction of any condensed phosphate present usually is hydrolyzed unavoidably in the procedure. Reactive phosphorus occurs in both dissolved and suspended forms.

Acid hydrolysis at boiling-water temperature converts dissolved and particulate condensed phosphates to dissolved orthophosphate. The hydrolysis unavoidably releases some phosphate from organic compounds, but this may be reduced to a minimum by judicious selection of acid strength and hydrolysis time and temperature. The term "acid-hydrolyzable phosphorus" is preferred over "condensed phosphate" for this fraction.

The phosphate fractions that are converted to orthophosphate only by oxidation destruction of the organic matter present are considered "organic" or "organically bound" phosphorus. The severity of the oxidation required for this conversion depends on the form—and to some extent on the amount—of the organic phosphorus present. Like reactive phosphorus and acid-hydrolyzable phosphorus, organic phosphorus occurs both in the dissolved and suspended fractions.

The total phosphorus, as well as the dissolved and suspended phosphorus fractions, each may be divided analytically into the three chemical types that have been described: reactive, acid-hydrolyzable, and organic phosphorus. Figure 4500-P:1 shows the steps for analysis of individual phosphorus fractions. As indicated, determinations usually are conducted only on the unfiltered and filtered samples. Suspended fractions generally are determined by difference; however, they may be determined directly by digestion of the material retained on a glass-fiber filter.

3. Selection of Method

a. Digestion methods: Because phosphorus may occur in combination with organic matter, a digestion method to determine total phosphorus must be able to oxidize organic matter effectively to release phosphorus as orthophosphate. Three digestion methods are given in 4500-P.B.3, 4, and 5. The perchloric acid method, the most drastic and time-consuming method, is recommended only for particularly difficult samples, such as sediments. The nitric acid-sulfuric acid method is recommended for most samples. By far the simplest method is the persulfate oxidation technique. Persulfate oxidation is coupled with ultraviolet light for a more efficient digestion in an automated in-line digestion/determination by flow injection analysis (4500-P.I).

The persulfate oxidation method in 4500-P.J renders a digestate that can be analyzed for both total nitrogen and total phos-

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* Approved by Standard Methods Committee, 2005. Editorial revisions, 2011, 2017.

Joint Task Group: 21st Edition—(4500-P.J)—William Nivens (chair), Prem H. Arora, Lori J. Emery, James G. Poff, Steven C. Schindler; 20th Edition—(4500-P.G, H, I)—Scott Stieg (chair), Bradford R. Fisher, Owen B. Mathre, Theresa M. Wright.

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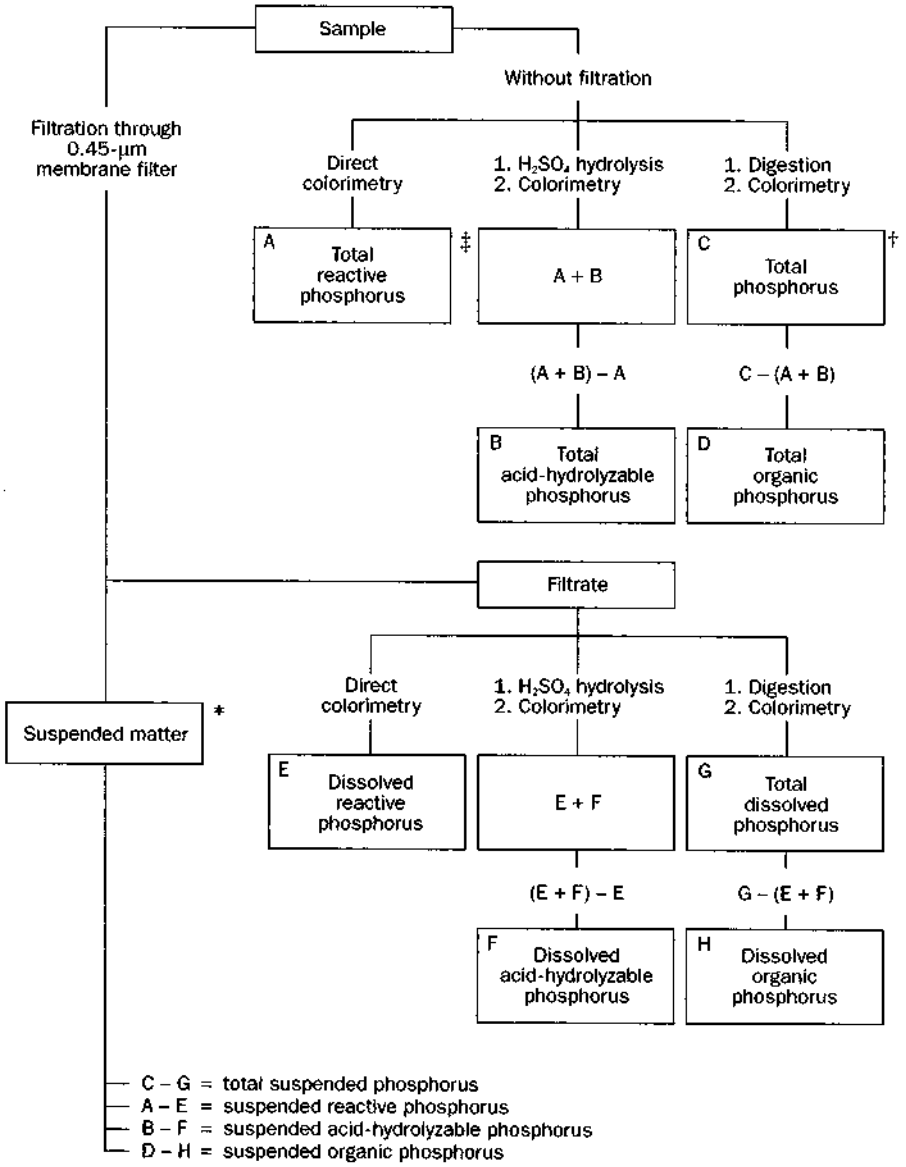


Figure 4500-P:1. Steps for analysis of phosphate fractions.

* Direct determination of phosphorus on the membrane filter containing suspended matter will be required where greater precision than that obtained by difference is desired. Digest filter with HNO_3 and follow by perchloric acid. Then perform colorimetry.

† Total phosphorus measurements on highly saline samples may be difficult because of precipitation of large quantities of salt as a result of digestion techniques that drastically reduce sample volume. For total phosphorus analyses on such samples, directly determine total dissolved phosphorus and total suspended phosphorus and add the results.

‡ In determination of total dissolved or total suspended reactive phosphorus, anomalous results may be obtained on samples containing large amounts of suspended sediments. Very often results depend largely on the degree of agitation and mixing to which samples are subjected during analysis because of a time-dependent desorption of orthophosphate from the suspended particles.

phorus. This procedure can be used for both parameters because it occurs over a broad pH range. During the initial stage of the digestion, sample pH is alkaline (pH>12); during the final stage, sample pH becomes acidic. As a result, nitrogenous compounds are oxidized to nitrate and phosphorus compounds to orthophosphate.

It is recommended that persulfate oxidation methods be checked against one or more of the more drastic digestion techniques and be adopted if identical recoveries are obtained.

b. Colorimetric method: Three methods of orthophosphate determination are described. Selection depends largely on the concentration range of orthophosphate. The vanadomolybdophosphoric acid method (4500-P.C) is most useful for routine analysis in the range of 1 to 20 mg P/L. The stannous chloride method (4500-P.D) or the ascorbic acid method (4500-P.E) is more suited for the range of 0.01 to 6 mg P/L. An extraction step is recommended for the lower levels of this range and when interferences must be overcome. Automated versions of the

Table 4500-P:I. Precision and Bias Data for Manual Phosphorus Methods

Method	Phosphorus Concentration			No. of Laboratories	Relative Standard Deviation %	Relative Error %
	Orthophosphate μg/L	Polyphosphate μg/L	Total μg/L			
Vanadomolybdophosphoric acid	100			45	75.2	21.6
	600			43	19.6	10.8
	7000			44	8.6	5.4
Stannous chloride	100			45	25.5	28.7
	600			44	14.2	8.0
	7000			45	7.6	4.3
Ascorbic acid	100			3	9.1	10.0
	600			3	4.0	4.4
	7000			3	5.2	4.9
Acid hydrolysis + vanadomolybdophosphoric acid		80		37	106.8	7.4
		300		38	66.5	14.0
		3000		37	36.1	23.5
Acid hydrolysis + stannous chloride		80		39	60.1	12.5
		300		36	47.6	21.7
		3000		38	37.4	22.8
Persulfate + vanadomolybdophosphoric acid			210	32	55.8	1.6
			990	32	23.9	2.3
			10 230	31	6.5	0.3
Sulfuric-nitric acids + vanadomolybdophosphoric acid			210	23	65.6	20.9
			990	22	47.3	0.6
			10 230	20	7.0	0.4
Perchloric acid + vanadomolybdophosphoric acid			210	4	33.5	45.2
			990	5	20.3	2.6
			10 230	6	11.7	2.2
Persulfate + stannous chloride			210	29	28.1	9.2
			990	30	14.9	12.3
			10 230	29	11.5	4.3
Sulfuric-nitric acids + stannous chloride			210	20	20.8	1.2
			990	17	8.8	3.2
			10 230	19	7.5	0.4

ascorbic acid method (4500-P.F, G, and H) also are presented. Careful attention to procedure may allow application of these methods to very low levels of phosphorus, such as those found in unimpaired fresh-water systems.

Ion chromatography (Section 4110) and capillary ion electrophoresis (Section 4140) are useful for determination of orthophosphate in undigested samples.

4. Precision and Bias

To aid in method selection, Table 4500-P:I presents the results of various combinations of digestions, hydrolysis, and colorimetric techniques for three synthetic samples of the following compositions:

- *Sample 1:* 100 μg orthosphosphate phosphorus (PO_4^{3-}-P/L), 80 μg acid-hydrolyzable phosphate phosphorus/L (sodium hexa-metaphosphate), 30 μg organic phosphorus/L (adenylic acid), 1.5 mg NH_3-N/L, 0.5 mg NO_3^--N/L, and 400 mg Cl^-/L.
- *Sample 2:* 600 μg PO_4^{3-}-P/L, 300 μg acid-hydrolyzable phosphate phosphorus/L (sodium hexametaphosphate), 90 μg organic phosphorus/L (adenylic acid), 0.8 mg NH_3-N/L, 5.0 mg NO_3^--N/L, and 400 mg Cl^-/L.
- *Sample 3:* 7.00 mg PO_4^{3-}-P/L, 3.00 μg acid-hydrolyzable phosphate phosphorus/L (sodium hexametaphosphate), 0.230 mg organic phosphorus/L (adenylic acid), 0.20 mg NH_3-N/L, 0.05 mg NO_3^--N/L, and 400 mg Cl^-/L.

5. Sampling and Storage

If dissolved phosphorus forms are to be differentiated, filter sample immediately after collection. Preserve by freezing at or below −10°C. In some cases 40 mg $HgCl_2$/L may be added

to the samples, especially when they are to be stored for long periods before analysis. **CAUTION: $HgCl_2$ is a hazardous substance; take appropriate precautions in disposal; use of $HgCl_2$ is not encouraged**. Do not add either acid or $CHCl_3$ as a preservative when phosphorus forms are to be determined. If total phosphorus alone is to be determined, add H_2SO_4 or HCl to pH<2 and cool to 4°C, or freeze without any additions.

Do not store samples containing low concentrations of phosphorus in plastic bottles unless kept in a frozen state because phosphates may be adsorbed onto the walls of plastic bottles.

Rinse all glass containers with hot dilute HCl, then rinse several times in reagent water. Never use commercial detergents containing phosphate for cleaning glassware used in phosphate analysis. More strenuous cleaning techniques may be used.

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4500-P B. Sample Preparation

For information on selection of digestion method (4500-P.B.3–5), see 4500-P.A.3*a*.

1. Preliminary Filtration

Filter samples for determination of dissolved reactive phosphorus, dissolved acid-hydrolyzable phosphorus, and total dissolved phosphorus through 0.45-μm membrane filters. A glass fiber filter may be used to prefilter hard-to-filter samples.

Wash membrane filters by soaking in distilled water before use because they may contribute significant amounts of phosphorus to samples containing low concentrations of phosphate. Use one of two washing techniques:

- soak 50 filters in 2 L distilled water for 24 h;
- soak 50 filters in 2 L distilled water for 1 h, change distilled water, and soak filters an additional 3 h.

Membrane filters also may be washed by running several 100-mL portions of distilled water through them. This procedure requires more frequent determination of blank values to ensure consistency in washing and to evaluate different lots of filters.

2. Preliminary Acid Hydrolysis

The acid-hydrolyzable phosphorus content of the sample is defined operationally as the difference between reactive phosphorus as measured in the untreated sample and phosphate found after mild acid hydrolysis. Generally, it includes such condensed phosphates as pyro-, tripoly-, and higher-molecular-weight species, such as hexametaphosphate. In addition, some natural waters contain organic phosphate compounds that are hydrolyzed to orthophosphate under the test conditions. Polyphosphates generally do not respond to reactive phosphorus tests but can be hydrolyzed to orthophosphate by boiling with acid.

After hydrolysis, determine reactive phosphorus by a colorimetric method (4500-P.C, D, or E). Interferences, precision, bias, and sensitivity will depend on the colorimetric method used.

a. Apparatus:

Autoclave or pressure cooker, capable of operating at 98 to 137 kPa.

b. Reagents:

1) *Phenolphthalein indicator aqueous solution.*

2) *Strong acid solution:* Slowly add 300 mL conc H_2SO_4 to about 600 mL distilled water. When cool, add 4.0 mL conc HNO_3 and dilute to 1 L.

3) *Sodium hydroxide* (NaOH), 6*N*.

c. Procedure: To 100-mL sample or a portion diluted to 100 mL, add 0.05 mL (1 drop) phenolphthalein indicator solution. If a red color develops, add strong acid solution dropwise, to just discharge the color. Then add 1 mL more.

Boil gently for at least 90 min, adding distilled water to keep the volume between 25 and 50 mL. Alternatively, heat for 30 min in an autoclave or pressure cooker at 98 to 137 kPa. Cool, neutralize to a faint pink color with NaOH solution, and restore to the original 100-mL volume with distilled water.

Prepare a calibration curve by carrying a series of standards containing orthophosphate (see 4500-P.C, D, or E) through the hydrolysis step. Do not use orthophosphate standards without hydrolysis, because the salts added in hydrolysis cause an increase in the color intensity in some methods.

Determine reactive phosphorus content of treated portions, using 4500-P.C, D, or E. This gives the sum of polyphosphate and orthophosphate in the sample. To calculate its content of acid-hydrolyzable phosphorus, determine reactive phosphorus in a sample portion that has not been hydrolyzed, using the same colorimetric method as for treated sample, and subtract.

3. Perchloric Acid Digestion

a. Apparatus:

1) *Hot plate*—A 30- × 50-cm heating surface is adequate.

2) *Safety shield.*

3) *Safety goggles.*

4) *Erlenmeyer flasks,* 125-mL, acid-washed and rinsed with distilled water.

b. Reagents:

1) *Nitric acid* (HNO_3), conc.

2) *Perchloric acid* ($HClO_4 \cdot 2H_2O$), purchased as 70 to 72% $HClO_4$, reagent-grade.

3) *Sodium hydroxide* (NaOH), 6*N*.

4) *Methyl orange indicator solution.*

5) *Phenolphthalein indicator aqueous solution.*

c. Procedure: **CAUTION: Heated mixtures of $HClO_4$ and organic matter may explode violently. Avoid this hazard by taking the following precautions:**

- **Do not add $HClO_4$ to a hot solution that may contain organic matter.**
- **Always initiate digestion of samples containing organic matter with HNO_3. Complete digestion using the mixture of HNO_3 and $HClO_4$.**
- **Do not fume with $HClO_4$ in ordinary hoods. Use hoods especially constructed for $HClO_4$ fuming or a glass fume eradicator* connected to a water pump.**
- **Never let samples being digested with $HClO_4$ evaporate to dryness.**

Measure sample containing the desired amount of phosphorus (this will be determined by whether 4500-P.C, D, or E is to be used) into a 125-mL Erlenmeyer flask. Acidify to methyl orange with conc HNO_3, add another 5 mL conc HNO_3, and evaporate on a steam bath or hot plate to 15 to 20 mL.

Add 10 mL each of conc HNO_3 and $HClO_4$ to the 125-mL conical flask, cooling the flask between additions. Add a few boiling chips, heat on a hot plate, and evaporate gently until dense white fumes of $HClO_4$ just appear. If solution is not clear, cover neck of flask with a watch glass and keep solution barely boiling until it clears. If necessary, add 10 mL more HNO_3 to aid oxidation.

Cool digested solution and add 1 drop aqueous phenolphthalein solution. Add 6*N* NaOH solution until the solution just turns pink. If necessary, filter neutralized solution and wash filter liberally with distilled water. Make up to 100 mL with distilled water.

Determine the PO_4^{3-}-P content of the treated sample by 4500-P.C, D, or E.

Prepare a calibration curve by carrying a series of standards containing orthophosphate (see 4500-P.C, D, or E) through digestion step. Do not use orthophosphate standards without treatment.

4. Sulfuric Acid-Nitric Acid Digestion

a. Apparatus:

1) *Digestion rack*—An electrically or gas-heated digestion rack with provision for withdrawal of fumes is recommended. Digestion racks typical of those used for micro-Kjeldahl digestions are suitable.

2) *Micro-Kjeldahl flasks.*

b. Reagents:

1) *Sulfuric acid* (H_2SO_4), conc.

2) *Nitric acid* (HNO_3), conc.

3) *Phenolphthalein indicator aqueous solution.*

4) *Sodium hydroxide* (NaOH), 1*N*.

c. Procedure: Into a micro-Kjeldahl flask, measure a sample containing the desired amount of phosphorus (this is determined by the colorimetric method used). Add 1 mL conc H_2SO_4 and 5 mL conc HNO_3.

Digest to a volume of 1 mL and then continue until solution becomes colorless to remove HNO_3.

Cool and add approximately 20 mL distilled water, 0.05 mL (1 drop) phenolphthalein indicator, and as much 1*N* NaOH solution as required to produce a faint pink tinge. Transfer neutralized solution, filtering if necessary to remove particulate material or turbidity, into a 100-mL volumetric flask. Add filter washings to flask and adjust sample volume to 100 mL with distilled water.

Determine phosphorus by 4500-P.C, D, or E, for which a separate calibration curve has been constructed by carrying standards through the acid digestion procedure.

5. Persulfate Digestion Method

a. Apparatus:

1) *Hot plate*—A 30- × 50-cm heating surface is adequate.

2) *Autoclave*—An autoclave or pressure cooker capable of developing 98 to 137 kPa may be used in place of a hot plate.

3) *Glass scoop,* to hold required amounts of persulfate crystals.

b. Reagents:

1) *Phenolphthalein indicator aqueous solution.*

2) *Sulfuric acid solution*—Carefully add 300 mL conc H_2SO_4 to approximately 600 mL distilled water and dilute to 1 L with distilled water.

3) *Ammonium persulfate* [$(NH_4)_2S_2O_8$], solid, or potassium persulfate, $K_2S_2O_8$, solid.

4) *Sodium hydroxide* (NaOH), 1*N*.

c. Procedure: Use 50 mL or a suitable portion of thoroughly mixed sample. Add 0.05 mL (1 drop) phenolphthalein indicator solution. If a red color develops, add H_2SO_4 solution dropwise to just discharge the color. Then add 1 mL H_2SO_4 solution and either 0.4 g solid $(NH_4)_2S_2O_8$ or 0.5 g solid $K_2S_2O_8$.

Boil gently on a preheated hot plate for 30 to 40 min or until a final volume of 10 mL is reached. Organophosphorus compounds, such as AMP, may require as much as 1.5 to 2 h for complete digestion. Cool, dilute to 30 mL with distilled water, add 0.05 mL (1 drop) phenolphthalein indicator solution, and neutralize to a faint pink color with NaOH. Alternatively, heat for 30 min in an autoclave or pressure cooker at 98 to 137 kPa. Cool, add 0.05 mL (1 drop) phenolphthalein indicator solution, and neutralize to a faint pink color with NaOH. Make up to 100 mL with distilled water. In some samples a precipitate may form at this stage, but do not filter. For any subsequent subdividing of the sample, shake well. The precipitate (which is possibly a calcium phosphate) redissolves under the acid conditions of the colorimetric reactive phosphorus test. Determine phosphorus by 4500-P.C, D, or E, for which a separate calibration curve has been constructed by carrying standards through the persulfate digestion procedure.

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* GFS Chemical Co., Columbus, OH, or equivalent.

4500-P C. Vanadomolybdophosphoric Acid Colorimetric Method

1. General Discussion

a. Principle: In a dilute orthophosphate solution, ammonium molybdate reacts under acid conditions to form a heteropoly acid, molybdophosphoric acid. In the presence of vanadium, yellow vanadomolybdophosphoric acid is formed. The intensity of the yellow color is proportional to phosphate concentration.

b. Interference: Positive interference is caused by silica and arsenate only if the sample is heated. Negative interferences are caused by arsenate, fluoride, thorium, bismuth, sulfide, thiosulfate, thiocyanate, or excess molybdate. Blue color is caused by ferrous iron but this does not affect results if ferrous iron concentration is less than 100 mg/L. Sulfide interference may be removed by oxidation with bromine water. Ions that do not interfere in concentrations up to 1000 mg/L are Al^{3+}, Fe^{3+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, Li^{+}, Na^{+}, K^{+}, NH_4^{+}, Cd^{2+}, Mn^{2+}, Pb^{2+}, Hg^{+}, Hg^{2+}, Sn^{2+}, Cu^{2+}, Ni^{2+}, Ag^{+}, U^{4+}, Zr^{4+}, AsO_3^{-}, Br^{-}, CO_3^{2-}, ClO_4^{-}, CN^{-}, IO_3^{-}, SiO_4^{4-}, NO_3^{-}, NO_2^{-}, SO_4^{2-}, SO_3^{2-}, pyrophosphate, molybdate, tetraborate, selenate, benzoate, citrate, oxalate, lactate, tartrate, formate, and salicylate. If HNO_3 is used in the test, Cl^{-} interferes at 75 mg/L.

c. Minimum detectable concentration: The minimum detectable concentration is 200 μg P/L in 1-cm spectrophotometer cells.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer,* for use at 400 to 490 nm.

2) *Filter photometer,* provided with a blue or violet filter exhibiting maximum transmittance between 400 and 470 nm.

The wavelength at which color intensity is measured depends on sensitivity desired, because sensitivity varies tenfold with wavelengths 400 to 490 nm. Ferric iron causes interference at low wavelengths, particularly at 400 nm. A wavelength of 470 nm usually is used. Concentration ranges for different wavelengths are:

P Range *mg/L*	Wavelength *nm*
1.0–5.0	400
2.0–10	420
4.0–18	470

b. Acid-washed glassware: Use acid-washed glassware for determining low concentrations of phosphorus. Phosphate contamination is common because of its absorption on glass surfaces. Avoid using commercial detergents containing phosphate. Clean all glassware with hot dilute HCl and rinse well with distilled water. Preferably, reserve the glassware only for phosphate determination, and after use, wash and keep filled with water until needed. If this is done, acid treatment is required only occasionally.

*c. Filtration apparatus and filter paper.**

3. Reagents

a. Phenolphthalein indicator aqueous solution.

b. Hydrochloric acid (HCl), 1 + 1. H_2SO_4, $HClO_4$, or HNO_3 may be substituted for HCl. The acid concentration in the determination is not critical, but a final sample concentration of 0.5*N* is recommended.

c. Activated carbon.† Remove fine particles by rinsing with distilled water.

d. Vanadate–molybdate reagent:

1) *Solution A:* Dissolve 25 g ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, in 300 mL distilled water.

2) *Solution B:* Dissolve 1.25 g ammonium metavanadate, NH_4VO_3, by heating to boiling in 300 mL distilled water. Cool and add 330 mL conc HCl. Cool Solution B to room temperature, pour Solution A into Solution B, mix, and dilute to 1 L.

e. Standard phosphate solution: Dissolve in distilled water 219.5 mg anhydrous KH_2PO_4 and dilute to 1000 mL; 1.00 mL = 50.0 μg PO_4^{3-}-P.

4. Procedure

a. Sample pH adjustment: If sample pH is greater than 10, add 0.05 mL (1 drop) phenolphthalein indicator to 50.0 mL sample and discharge the red color with 1 + 1 HCl before diluting to 100 mL.

b. Color removal from sample: Remove excessive color in sample by shaking about 50 mL with 200 mg activated carbon in an Erlenmeyer flask for 5 min and filter to remove carbon. Check each batch of carbon for phosphate because some batches produce high reagent blanks.

c. Color development in sample: Place 35 mL or less of sample, containing 0.05 to 1.0 mg P, in a 50-mL volumetric flask. Add 10 mL vanadate–molybdate reagent and dilute to the mark with distilled water. Prepare a blank in which 35 mL distilled water is substituted for the sample. After 10 min or more, measure absorbance of sample versus a blank at a wavelength of 400 to 490 nm, depending on sensitivity desired (see 4500-P.C.2*a*). The color is stable for days, and its intensity is unaffected by variation in room temperature.

d. Preparation of calibration curve: Prepare a calibration curve by using suitable volumes of standard phosphate solution and proceeding as in ¶ *c* above. When ferric ion is low enough not to interfere, plot a family of calibration curves of one series of standard solutions for various wavelengths. This permits a wide latitude of concentrations in one series of determinations. Analyze at least one standard with each set of samples.

5. Calculation

$$\text{mg P/L} = \frac{\text{mg P (in 50 mL final volume)} \times 1000}{\text{mL sample}}$$

* Whatman No. 42, or equivalent.

† Darco G60, or equivalent.

6. Precision and Bias

See Table 4500-P:I.

7. Bibliography

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4500-P D. Stannous Chloride Method

1. General Discussion

a. Principle: Molybdophosphoric acid is formed and reduced by stannous chloride to intensely colored molybdenum blue. This method is more sensitive than Method C and makes feasible measurements down to 7 μg P/L by use of increased light path length. Below 100 μg P/L an extraction step may increase reliability and lessen interference.

b. Interference: See 4500-P.C.1*b*.

c. Minimum detectable concentration: The minimum detectable concentration is about 3 μg P/L. The sensitivity at 0.3010 absorbance is about 10 μg P/L for an absorbance change of 0.009.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

The same apparatus is required as for 4500-P.C, except that a pipetting bulb is required for the extraction step. Set spectrophotometer at 625 nm in the measurement of benzene-isobutanol extracts and at 690 nm for aqueous solutions. If the instrument is not equipped to read at 690 nm, use a wavelength of 650 nm for aqueous solutions, with somewhat reduced sensitivity and precision.

3. Reagents

a. Phenolphthalein indicator aqueous solution.

b. Strong-acid solution: Prepare as directed in 4500-P.B.2*b*2).

c. Ammonium molybdate reagent I: Dissolve 25 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 175 mL distilled water. Cautiously add 280 mL conc H_2SO_4 to 400 mL distilled water. Cool, add molybdate solution, and dilute to 1 L.

d. Stannous chloride reagent I: Dissolve 2.5 g fresh $SnCl_2 \cdot 2H_2O$ in 100 mL glycerol. Heat in a water bath and stir with a glass rod to hasten dissolution. This reagent is stable and requires neither preservatives nor special storage.

e. Standard phosphate solution: Prepare as directed in 4500-P.C.3*e*.

f. Reagents for extraction:

1) *Benzene–isobutanol solvent*—Mix equal volumes of benzene and isobutyl alcohol. (**CAUTION: This solvent is highly flammable.**)

2) *Ammonium molybdate reagent II*—Dissolve 40.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in approximately 500 mL distilled water. Slowly add 396 mL ammonium molybdate reagent I. Cool and dilute to 1 L.

3) *Alcoholic sulfuric acid solution*—Cautiously add 20 mL conc H_2SO_4 to 980 mL methyl alcohol with continuous mixing.

4) *Dilute stannous chloride reagent II*—Mix 8 mL stannous chloride reagent I with 50 mL glycerol. This reagent is stable for at least 6 months.

4. Procedure

a. Preliminary sample treatment: To 100 mL sample containing not more than 200 μg P and free from color and turbidity, add 0.05 mL (1 drop) phenolphthalein indicator. If sample turns pink, add strong acid solution dropwise to discharge the color. If more than 0.25 mL (5 drops) is required, take a smaller sample and dilute to 100 mL with distilled water after first discharging the pink color with acid.

b. Color development: Add, with thorough mixing after each addition, 4.0 mL molybdate reagent I and 0.5 mL (10 drops) stannous chloride reagent I. Rate of color development and intensity of color depend on temperature of the final solution, each 1°C increase producing about 1% increase in color. Hence, hold samples, standards, and reagents within 2°C of one another and in the temperature range between 20 and 30°C.

c. Color measurement: After 10 min, but before 12 min, using the same specific interval for all determinations, measure color photometrically at 690 nm and compare with a calibration curve, using a distilled water blank. Light path lengths suitable for various concentration ranges are as follows:

Approximate P Range *mg/L*	Light Path *cm*
0.3–2	0.5
0.1–1	2

Approximate P Range *mg/L*	Light Path *cm*
0.007–0.2	10

Always run a blank on reagents and distilled water. Because the color at first develops progressively and later fades, maintain equal timing conditions for samples and standards. Prepare at least one standard with each set of samples or once each day that tests are made. The calibration curve may deviate from a straight line at the upper concentrations of the 0.3- to 2.0-mg/L range.

d. Extraction: When increased sensitivity is desired or interferences must be overcome, extract phosphate as follows: Pipet a 40-mL sample, or one diluted to that volume, into a 125-mL separatory funnel. Add 50.0 mL benzene-isobutanol solvent and 15.0 mL molybdate reagent II. Close funnel at once and shake vigorously for exactly 15 s. If condensed phosphate is present, any delay will increase its conversion to orthophosphate. Remove stopper and withdraw 25.0 mL of separated organic layer, using a pipet with safety bulb. Transfer to a 50-mL volumetric flask, add 15 to 16 mL alcoholic H_2SO_4 solution, swirl, add 0.50 mL (10 drops) dilute stannous chloride reagent II, swirl, and dilute to the mark with alcoholic H_2SO_4. Mix thoroughly. After 10 min, but before 30 min, read against the blank at 625 nm. Prepare blank by carrying 40 mL distilled water through the same procedure used for the sample. Read phosphate concentration from a calibration curve prepared by taking known phosphate standards through the same procedure used for samples.

5. Calculation

Calculate as follows:

a. Direct procedure:

$$\text{mg P/L} = \frac{\text{mg P (in approximately 104.5 mL final volume)} \times 1000}{\text{mL sample}}$$

b. Extraction procedure:

$$\text{mg P/L} = \frac{\text{mg P (in 50 mL final volume)} \times 1000}{\text{mL sample}}$$

6. Precision and Bias

See Table 4500-P:I.

4500-P E. Ascorbic Acid Method

1. General Discussion

a. Principle: Ammonium molybdate and antimony potassium tartrate react in acid medium with orthophosphate to form a heteropoly acid—phosphomolybdic acid—that is reduced to intensely colored molybdenum blue by ascorbic acid.

b. Interference: Arsenates react with the molybdate reagent to produce a blue color similar to that formed with phosphate. Concentrations as low as 0.1 mg As/L interfere with the phosphate determination. Hexavalent chromium and NO_2^- interfere to give results about 3% low at concentrations of 1 mg/L and 10 to 15% low at 10 mg/L. Sulfide (Na_2S) and silicate do not interfere at concentrations of 1.0 and 10 mg/L.

c. Minimum detectable concentration: Approximately 10 μg P/L. P ranges are as follows:

Approximate P Range *mg/L*	Light Path *cm*
0.30–2.0	0.5
0.15–1.30	1.0
0.01–0.25	5.0

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer,* with infrared phototube for use at 880 nm, providing a light path of 2.5 cm or longer.

2) *Filter photometer,* equipped with a red color filter and a light path of 0.5 cm or longer.

b. Acid-washed glassware: See 4500-P.C.2*b*.

3. Reagents

a. Sulfuric acid (H_2SO_4), 5*N:* Dilute 70 mL conc H_2SO_4 to 500 mL with distilled water.

b. Antimony potassium tartrate solution: Dissolve 1.3715 g $K(SbO)C_4H_4O_6 \cdot \frac{1}{2}H_2O$ in 400 mL distilled water in a 500-mL volumetric flask and dilute to volume. Store in a glass-stoppered bottle.

c. Ammonium molybdate solution: Dissolve 20 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 500 mL distilled water. Store in a glass-stoppered bottle.

d. Ascorbic acid, 0.1*M:* Dissolve 1.76 g ascorbic acid in 100 mL distilled water. The solution is stable for about 1 week at 4°C.

e. Combined reagent: Mix the above reagents in the following proportions for 100 mL of the combined reagent: 50 mL 5*N* H_2SO_4, 5 mL antimony potassium tartrate solution, 15 mL ammonium molybdate solution, and 30 mL ascorbic acid solution. *Mix after addition of each reagent.* Let all reagents reach room temperature before they are mixed and mix in the order given. If turbidity forms in the combined

TABLE 4500-P:II. COMPARISON OF PRECISION AND BIAS OF ASCORBIC ACID METHODS

Ascorbic Acid Method	Phosphorus Concentration, Dissolved Orthophosphate $\mu g/L$	No. of Laboratories	Relative Standard Deviation %		Relative Error %	
			Distilled Water	River Water	Distilled Water	River Water
13th Edition[1]	228	8	3.87	2.17	4.01	2.08
Current method[2]	228	8	3.03	1.75	2.38	1.39

reagent, shake and let stand for a few minutes until turbidity disappears before proceeding. The reagent is stable for 4 h.

f. Stock phosphate solution: See 4500-P.C.3*e*.

g. Standard phosphate solution: Dilute 50.0 mL stock phosphate solution to 1000 mL with distilled water; 1.00 mL = 2.50 μg P.

4. Procedure

a. Treatment of sample: Pipet 50.0 mL sample into a clean, dry test tube or 125-mL Erlenmeyer flask. Add 0.05 mL (1 drop) phenolphthalein indicator. If a red color develops add 5*N* H_2SO_4 solution dropwise to just discharge the color. Add 8.0 mL combined reagent and mix thoroughly. After at least 10 min but no more than 30 min, measure absorbance of each sample at 880 nm, using reagent blank as the reference solution.

b. Correction for turbidity or interfering color: Natural color of water generally does not interfere at the high wavelength used. For highly colored or turbid waters, prepare a sample blank by adding all reagents except ascorbic acid and antimony potassium tartrate to the sample. Subtract the sample blank absorbance from the absorbance of the sample.

c. Preparation of calibration curve: Prepare individual calibration curves from a series of four up to six standards, including a calibration blank, within the phosphate ranges indicated in 4500-P.E.1*c*. The calibration blank consists of reagent water with the combined reagent. Plot absorbance vs. phosphate concentration. Test at least one phosphate standard with each set of samples.

5. Calculation

$$\text{mg P/L} = \frac{\text{mg P (in approximately 58 mL final volume)} \times 1000}{\text{mL sample}}$$

6. Precision and Bias

The precision and bias values given in Table 4500-P:I are for a single-solution procedure given in the 13th Edition. The present procedure differs in reagent-to-sample ratios, no addition of solvent, and acidity conditions. It is superior in precision and bias to the previous technique in the analysis of both distilled water and river water at the 228-μg P/L level (Table 4500-P:II).

7. References

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2. MURPHY, J. & J. RILEY. 1962. A modified single solution method for the determination of phosphate in natural waters. *Anal. Chim. Acta* 27:31.

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4500-P F. Automated Ascorbic Acid Reduction Method

1. General Discussion

a. Principle: Ammonium molybdate and antimony potassium tartrate react with orthophosphate in an acid medium to form an antimony–phosphomolybdate complex, which, on reduction with ascorbic acid, yields an intense blue color suitable for photometric measurement.

b. Interferences: As much as 50 mg Fe^{3+}/L, 10 mg Cu/L, and 10 mg SiO_2/L can be tolerated. High silica concentrations cause positive interference.

In terms of phosphorus, the results are high by 0.005, 0.015, and 0.025 mg/L for silica concentrations of 20, 50, and 100 mg/L, respectively. Salt concentrations up to 20% (w/v) cause an error of less than 1%. Arsenate (AsO_4^{3-}) is a positive interference.

Eliminate interference from NO_2^- and S^{2-} by adding an excess of bromine water or a saturated potassium permanganate ($KMnO_4$) solution. Remove interfering turbidity by filtration before analysis. Filter samples for total or total hydrolyzable phosphorus only after digestion. Sample color that absorbs in the photometric range used for analysis also will interfere. See also 4500-P.E.1*b*.

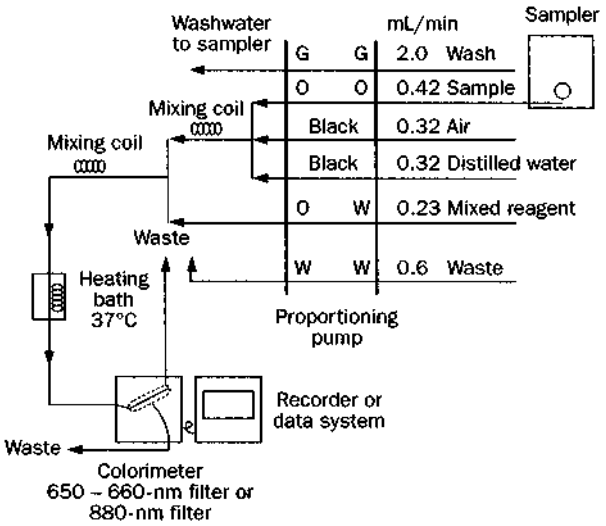


Figure 4500-P:2. Phosphate manifold for automated analytical system.

c. Application: Orthophosphate can be determined in potable, surface, and saline waters as well as domestic and industrial wastewaters over a range of 0.001 to 10.0 mg P/L when photometric measurements are made at 650 to 660 or 880 nm in a 15- or 50-mm tubular flow cell. Determine higher concentrations by diluting sample. Although the automated test is designed for orthophosphate only, other phosphorus compounds can be converted to this reactive form by various sample pretreatments described in 4500-P.B.1, 2, and 5.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Automated analytical equipment: An example of the continuous-flow analytical instrument consists of the interchangeable components shown in Figure 4500-P:2. A flow cell of 15 or 50 mm and a filter of 650 to 660 or 880 nm may be used.

b. Hot plate or autoclave.

c. Acid-washed glassware: See 4500-P.C.2*b*.

3. Reagents

a. Antimony potassium tartrate solution: Dissolve 0.3 g $K(SbO)C_4H_4O_6 \cdot \frac{1}{2}H_2O$ in approximately 50 mL distilled water and dilute to 100 mL. Store at 4°C in a dark, glass-stoppered bottle.

b. Ammonium molybdate solution: Dissolve 4 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 100 mL distilled water. Store in a plastic bottle at 4°C.

c. Ascorbic acid solution: See 4500-P.E.3*d*.

d. Combined reagent: See 4500-P.E.3*e*.

e. Dilute sulfuric acid solution: Slowly add 140 mL conc H_2SO_4 to 600 mL distilled water. When cool, dilute to 1 L.

f. Ammonium persulfate $[(NH_4)_2S_2O_8]$, crystalline.

g. Phenolphthalein indicator aqueous solution.

h. Stock phosphate solution: Dissolve 439.3 mg anhydrous KH_2PO_4, dried for 1 h at 105°C, in distilled water and dilute to 1000 mL; 1.00 mL = 100 μg P.

i. Intermediate phosphate solution: Dilute 100.0 mL stock phosphate solution to 1000 mL with distilled water; 1.00 mL = 10.0 μg P.

j. Standard phosphate solutions: Prepare a suitable series of standards by diluting appropriate volumes of intermediate phosphate solution.

4. Procedure

Set up manifold as shown in Figure 4500-P:2 and follow the general procedure described by the manufacturer.

Add 0.05 mL (1 drop) phenolphthalein indicator solution to approximately 50 mL sample. If a red color develops, add H_2SO_4 (4500-P.F.3*e*) dropwise to just discharge the color.

5. Calculation

Prepare standard curves by plotting response of standards processed through the manifold against P concentration in standards. Compute sample P concentration by comparing sample response with standard curve.

6. Precision and Bias

Six samples were analyzed in a single laboratory in septuplicate. At an average PO_4^{3-} concentration of 0.340 mg/L, the average deviation was 0.015 mg/L. The coefficient of variation was 6.2%. In two samples with added PO_4^{3-}, recoveries were 89 and 96%.

7. Bibliography

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4500-P G. Flow Injection Analysis for Orthophosphate

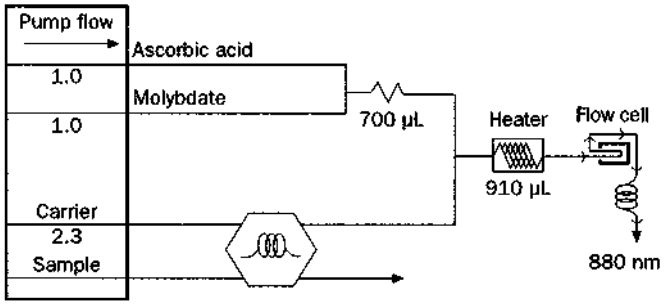


Figure 4500-P:3. FIA orthophosphate manifold.

1. General Discussion

a. Principle: The orthophosphate ion (PO_4^{3-}) reacts with ammonium molybdate and antimony potassium tartrate under acidic conditions to form a complex. This complex is reduced with ascorbic acid to form a blue complex that absorbs light at 880 nm. The absorbance is proportional to the concentration of orthophosphate in the sample.

Also see 4500-P.A, B, and F, and Section 4130, Flow Injection Analysis (FIA).

b. Interferences: Remove large or fibrous particulates by filtering sample through glass wool. Guard against contamination from reagents, water, glassware, and the sample preservation process.

Silica forms a pale blue complex that also absorbs at 880 nm. This interference is generally insignificant because a silica concentration of approximately 30 mg/L would be required to produce a 0.005 mg P/L positive error in orthophosphate.

Concentrations of ferric iron greater than 50 mg/L cause a negative error due to competition with the complex for the reducing agent ascorbic acid. Treat samples high in iron with sodium bisulfite to eliminate this interference, as well as the interference due to arsenates.

Glassware contamination is a problem in low-level phosphorus determinations. Wash glassware with hot dilute HCl and rinse with reagent water. Commercial detergents are rarely needed but, if they are used, use special phosphate-free preparations.

Also see 4500-P.F.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Flow injection analysis equipment consisting of:

a. FIA injection valve with sample loop or equivalent.

b. Multichannel proportioning pump.

c. FIA manifold (Figure 4500-P:3) with tubing heater and flow cell. Relative flow rates only are shown in Figure 4500-P:3. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material, such as TFE.

TABLE 4500-P:III. RESULTS OF SINGLE-LABORATORY STUDIES WITH SELECTED MATRICES

Matrix	Sample/Blank Designation	Known Addition *mg P/L*	Recovery %	Relative Standard Deviation %
Wastewater treatment plant influent	Reference sample*	—	101	—
	Blank†	0.05	96	—
		0.1	95	—
	Site A‡§	0	—	0.7
		0.05	98	—
		0.1	101	—
	Site B‡§	0	—	5
		0.05	75	—
		0.1	91	—
	Site C‡§	0	—	0.6
		0.05	88	—
		0.1	97	—
Wastewater treatment plant effluent	Reference sample*	—	100	—
	Blank†	0.05	96	—
		0.1	96	—
	Site A‡‖	0	—	0.7
		0.05	94	—
		0.1	96	—
	Site B‡‖	0	—	0.3
		0.05	94	—
		0.1	99	—
	Site C‡‖	0	—	0.5
		0.05	109	—
		0.1	107	—
Landfill leachate	Reference sample*	—	98	—
	Blank†	0.05	94	—
		0.1	95	—
	Site A‡#	0	—	0.9
		0.05	105	—
		0.1	106	—
	Site B‡#	0	—	6.7
		0.05	89	—
		0.1	94	—
	Site C‡#	0	—	0.9
		0.05	110	—
		0.1	109	—

* U.S. EPA QC sample, 0.109 mg P/L.
† Determined in duplicate.
‡ Samples without known additions determined four times; samples with known additions determined in duplicate.
§ Sample dilutions: A - fivefold; B - 100-fold; C - 10-fold. Typical relative difference between duplicates 0.5%.
‖ Sample dilutions: A - fivefold; B - 20-fold; C - 10-fold. Typical relative difference between duplicates 0.3%.
Sample dilutions: A - 20-fold; B - 10-fold; C - 20-fold. Typical relative difference between duplicates 1%.

d. Absorbance detector, 880 nm, 10-nm bandpass.

e. Injection valve control and data acquisition system.

3. Reagents

Use reagent water (>10 megohm) to prepare carrier and all solutions. To prevent bubble formation, degas carrier and buffer with helium. Pass He at 140 kPa (20 psi) through a helium degassing tube. Bubble He through 1 L solution for 1 min. As an alternative to preparing reagents by weight/weight, use weight/volume.

a. Stock ammonium molybdate solution: To a tared 1-L container add 40.0 g ammonium molybdate tetrahydrate [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$] and 983 g water. Mix with a magnetic stirrer for at least 4 h. Store in plastic and refrigerate.

b. Stock antimony potassium tartrate solution: To a 1-L dark, tared, container add 3.0 g antimony potassium tartrate (potassium antimonyl tartrate hemihydrate), $K(SbO)C_4H_4O_6 \cdot \frac{1}{2}H_2O$, and 995 g water. Mix with a magnetic stirrer until dissolved. Store in a dark bottle and refrigerate.

c. Working molybdate color reagent: To a tared 1-L container add 680 g water, then add 64.4 g conc sulfuric acid. **CAUTION: This solution becomes very hot!** Swirl to mix. When mixture can be handled comfortably, add 213 g stock ammonium molybdate solution (¶ *a* above) and 72.0 g stock antimony potassium tartrate solution (¶ *b* above). Shake and degas with helium.

d. Ascorbic acid solution: To a tared 1-L container, add 60.0 g granular ascorbic acid and 975 g water. Stir or shake until dissolved. Degas this reagent with helium, then add 1.0 g dodecyl sulfate, $CH_3(CH_2)_{11}OSO_3Na$, stirring gently to mix. Prepare fresh weekly.

e. Stock orthophosphate standard, 25.00 mg P/L: In a 1-L volumetric flask, dissolve 0.1099 g primary standard grade anhydrous potassium phosphate monobasic (KH_2PO_4) that has been dried for 1 h at 105°C in about 800 mL water. Dilute to mark with water and invert to mix.

f. Standard orthophosphate solutions: Prepare orthophosphate standards in desired concentration range, using stock standard (¶ *e* above) and diluting with water.

4. Procedure

Set up a manifold equivalent to that in Figure 4500-P:3 and follow method supplied by manufacturer or laboratory standard operating procedure.

5. Calculations

Prepare standard curves by plotting absorbance of standards processed through the manifold versus orthophosphate concentration. The calibration curve is linear.

6. Precision and Bias

a. Recovery and relative standard deviation: Table 4500-P:III gives results of single-laboratory studies.

b. MDL: A 700-μL sample loop was used in the method described above. Using a published MDL method,[1] analysts ran 21 replicates of a 5.0-μg P/L standard. These gave a mean of 5.26 μg P/L, a standard deviation of 0.264 μg P/L, and MDL of 0.67 μg P/L.

7. Reference

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1984. Definition and procedure for the determination of method detection limits. Appendix B to 40 CFR 136 Rev. 1.11 amended June 30, 1986. 49 CFR 43430.

4500-P H. Manual Digestion and Flow Injection Analysis for Total Phosphorus

1. General Discussion

a. Principle: Polyphosphates are converted to the orthophosphate form by a sulfuric acid digestion and organic phosphorus is converted to orthophosphate by a persulfate digestion. When the resulting solution is injected onto the manifold, the orthophosphate ion (PO_4^{3-}) reacts with ammonium molybdate and antimony potassium tartrate under acidic conditions to form a complex. This complex is reduced with ascorbic acid to form a blue complex that absorbs light at 880 nm. The absorbance is proportional to the concentration of total phosphorus in the sample.

See 4500-P.A for a discussion of the various forms of phosphorus found in waters and wastewaters, 4500-P.B for a discussion of sample preparation and digestion, and Section 4130, Flow Injection Analysis (FIA).

b. Interferences: See 4500-P.G.1*b*.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Digestion and flow injection analysis equipment consisting of:

a. Hotplate or autoclave.

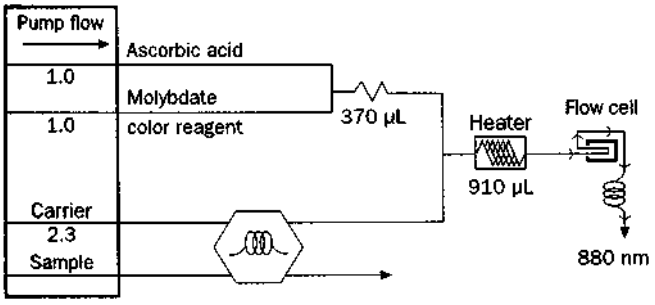


Figure 4500-P:4. FIA total phosphorus manifold.

b. FIA injection valve with sample loop or equivalent.

c. Multichannel proportioning pump.

d. FIA manifold (Figure 4500-P:4) with tubing heater and flow cell. Relative flow rates only are shown in Figure 4500-P:4. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material, such as TFE.

3. Reagents

Use reagent water (>10 megohm) for all solutions. To prevent bubble formation, degas carrier and buffer with helium. Pass He at 140 kPa (20 psi) through a helium degassing tube. Bubble He through 1 L solution for 1 min. As an alternative to preparing reagents by weight/weight, use weight/volume.

Prepare reagents listed in 4500-P.G.3*a*, *b*, *d*, *e*, and *f*, and in addition:

a. Sulfuric acid carrier (H_2SO_4), 0.13*M*: To a tared 1-L container add 993 g water, then add 13.3 g conc H_2SO_4. Shake carefully to mix. Degas daily. Prepare fresh weekly.

b. Molybdate color reagent: To a tared 1-L container add 694 g water, then add 38.4 g conc H_2SO_4. **CAUTION: The solution becomes very hot!** Swirl to mix. When mixture can be handled comfortably, add 72.0 g stock antimony potassium tartrate (4500-P.G.3*b*) and 213 g stock ammonium molybdate (4500-P.G.3*a*). Shake to mix, and degas.

4. Procedure

See 4500-P.B.4 or 5 for digestion procedures. Carry both standards and samples through the digestion. The resulting solutions should be about 0.13*M* in sulfuric acid to match the concentration of the carrier. If the solutions differ more than 10% from this concentration, adjust concentration of carrier's sulfuric acid to match that of digested samples.

Set up a manifold equivalent to that in Figure 4500-P:4 and analyze digested samples and standards by following method supplied by manufacturer or laboratory's standard operating procedure. Use quality control protocols outlined in Section 4020.

5. Calculations

Prepare standard curves by plotting absorbance of standards processed through the manifold versus phosphorus concentration. The calibration curve is linear.

6. Precision and Bias

a. MDL: A 780-μL sample loop was used in the method described above. Using a published MDL method,[1] analysts ran 21 replicates of a 3.5-μg P/L standard. These gave a mean of 3.53 μg P/L, a standard deviation of 0.82 μg P/L, and MDL of 2.0 μg P/L. The MDL is limited mainly by the precision of the digestion.

b. Precision study: Ten injections of a 100.0-μg P/L standard gave a percent relative standard deviation of 0.3%.

7. Reference

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1984. Definition and procedure for the determination of method detection limits. Appendix B to 40 CFR 136 Rev. 1.11 amended June 30, 1986. 49 CFR 43430.

4500-P I. In-line UV/Persulfate Digestion and Flow Injection Analysis for Total Phosphorus

1. General Discussion

a. Principle: Organic phosphorus is converted in-line to orthophosphate by heat, ultraviolet radiation, and persulfate digestion. At the same time, inorganic polyphosphates are converted to orthophosphate by in-line sulfuric acid digestion. The digestion processes occur before sample injection. A portion of the digested sample is then injected and its orthophosphate concentration determined by the flow injection method described in 4500-P.H.1.

See 4500-P.A for a discussion of the various forms of phosphorus found in waters and wastewaters, 4500-P.B for a discussion of sample preparation and digestion, and Section 4130, Flow Injection Analysis (FIA).

b. Interferences: See 4500-P.G.1*b*.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Flow injection analysis equipment consisting of:

a. FIA injection valve with sample loop or equivalent.

b. Multichannel proportioning pump.

c. FIA manifold (Figure 4500-P:5) with tubing heater; in-line ultraviolet digestion fluidics, including a debubbler consisting of a gas-permeable TFE membrane and its holder; and flow cell.

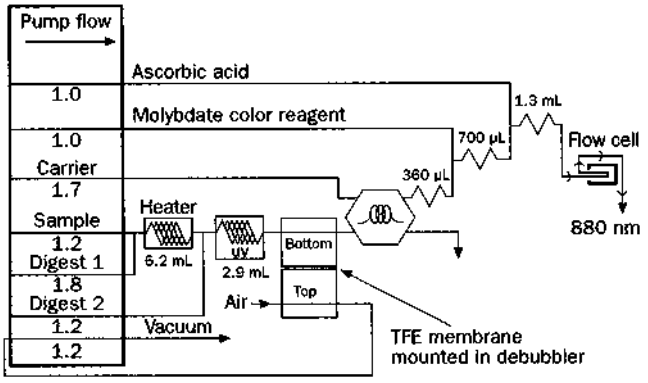


Figure 4500-P:5. FIA in-line total phosphorus manifold.

Relative flow rates only are shown in Figure 4500-P:5. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material, such as TFE. The block marked "UV" should consist of TFE tubing irradiated by a mercury discharge ultraviolet lamp emitting radiation at 254 nm.

d. Absorbance detector, 880 nm, 10-nm bandpass.

e. Injection valve control and data acquisition system.

3. Reagents

Use reagent water (>10 megohm) for all solutions. To prevent bubble formation, degas carrier and all reagents with helium. Pass He at 140 kPa (20 psi) through a helium degassing tube. Bubble He through 1 L solution for 1 min. As an alternative to preparing reagents by weight/weight, use weight/volume.

a. Digestion reagent 1: To a tared 1-L container, add 893.5 g water, then slowly add 196.0 g sulfuric acid, H_2SO_4. **CAUTION: This solution becomes very hot!** Prepare weekly. Degas before using.

b. Digestion reagent 2: To a tared 1-L container, add 1000 g water, then add 26 g potassium persulfate, $K_2S_4O_8$. Mix with a magnetic stirrer until dissolved. Prepare weekly. Degas before using.

c. Sulfuric acid carrier, 0.71*M*: To a tared 1-L container, slowly add 70 g H_2SO_4 to 962 g water. Add 5 g sodium chloride, NaCl. Let cool, then degas with helium. Add 1.0 g sodium dodecyl sulfate. Invert to mix. Prepare weekly.

d. Stock ammonium molybdate: To a tared 1-L container add 40.0 g ammonium molybdate tetrahydrate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 983 g water. Mix with a magnetic stirrer for at least 4 h. The solution can be stored in plastic for up to 2 months if refrigerated.

e. Stock antimony potassium tartrate: To a 1-L dark, plastic, tared container add 3.0 g antimony potassium tartrate (potassium antimonyl tartrate trihydrate), $C_8H_4K_2O_{12}Sb_2 \cdot 3H_2O$, and 995 g water. Mix with a magnetic stirrer until dissolved. The solution can be stored in a dark plastic container for up to 2 months if refrigerated.

f. Molybdate color reagent: To a tared 1-L container add 715 g water, then 213 g stock ammonium molybdate (¶ *d* above) and 72.0 g stock antimony potassium tartrate (¶ *e* above). Add and dissolve 22.8 g sodium hydroxide, NaOH. Shake and degas with helium. Prepare weekly.

g. Ascorbic acid: To a tared 1-L container add 70.0 g ascorbic acid and 975 g water. Mix with a magnetic stirrer until dissolved. Degas with helium. Add 1.0 g sodium dodecyl sulfate. Mix with a magnetic stirrer. Prepare fresh every 2 d.

h. Stock orthophosphate standard, 1000 mg P/L: In a 1-L volumetric flask dissolve 4.396 g primary standard grade anhydrous potassium phosphate monobasic, KH_2PO_4 (dried for 1 h at 105°C), in about 800 mL water. Dilute to mark with water and invert to mix. Prepare monthly.

i. Standard solutions: Prepare orthophosphate standards in desired concentration range, using stock orthophosphate standards (¶ *h* above), and diluting with water. If the samples are preserved with sulfuric acid, ensure that stock standard and diluted standards solutions are of the same concentration.

4. Procedure

Set up a manifold equivalent to that in Figure 4500-P:5 and follow method supplied by manufacturer or laboratory's standard operating procedure. Use quality control procedures described in Section 4020.

5. Calculations

Prepare standard curves by plotting absorbance of standards processed through manifold versus phosphorus concentration. The calibration curve is linear.

TABLE 4500-P:IV. RECOVERIES OF TOTAL PHOSPHORUS

Compound	Known Concentration *mg P/L*	Mean Concentration Recovered *mg P/L*	Recovery %	Relative Standard Deviation %
Sodium pyrophosphate	10	8.99	90.3	0.5
	2	1.81	90.2	0.6
	0.2	0.19	93.4	1.0
Phenylphosphate	10	10.10	101.5	0.2
	2	2.12	105.0	5.6
	0.2	0.20	101.3	1.0
Trimethylphosphate	10	8.99	90.3	0.2
	2	1.86	92.7	0.3
	0.2	0.18	95.3	1.1
Sodium tripolyphosphate	10	10.61	106.7	1.0
	2	2.14	106.6	0.2
	0.2	0.22	108.9	0.9

Verify digestion efficiency by determining tripolyphosphate and trimethylphosphate standards at regular intervals. In the concentration range of the method, the recovery of either of these compounds should be >95%.

6. Precision and Bias

a. MDL: A 390-µL sample loop was used in the method described above. Using a published MDL method,[1] analysts ran 21 replicates of a 0.10-mg P/L orthophosphate standard. These gave a mean of 0.10 mg P/L, a standard deviation of 0.003 mg P/L, and MDL of 0.007 mg P/L.

b. Precision of recovery study: Ten injections of a 10.0-mg P/L trimethylphosphate standard gave a mean percent recovery of 98% and a percent relative standard deviation of 0.8%.

c. Recovery of total phosphorus: Two organic and two inorganic complex phosphorus compounds were determined in triplicate at three concentrations. The results are shown in Table 4500-P:IV.

d. Comparison of in-line digestion with manual digestion method: Samples from a wastewater treatment plant influent and effluent and total phosphorus samples at 2.0 mg P/L were determined in duplicate with both manual persulfate digestion followed by the method in 4500-P.H and in-line digestion method. Table 4500-P:V gives the results of this comparison, and Figure 4500-P:6 shows the correlation between manual and in-line total phosphorus methods.

7. Reference

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1984. Definition and procedure for the determination of method detection limits. Appendix B to 40 CFR 136 Rev. 1.11 amended June 30, 1986. 49 CFR 43430.

TABLE 4500-P:V. COMPARISON OF MANUAL AND IN-LINE TOTAL PHOSPHORUS METHODS

Samples	Concentration by Manual Persulfate Digestion Method *mg P/L*	Concentration by In-Line Digestion Method *mg P/L*	Relative Difference %
Influent (I2)	5.93	5.52	−6.9
Influent (I3)	5.03	4.50	−10.5
Influent (I5)	2.14	2.11	−1.4
Influent (I6)	1.88	1.71	−9.0
Effluent (E1)	3.42	2.87	−16.1
Effluent (E2)	3.62	3.55	−1.9
Effluent (E3)	3.26	3.34	+2.4
Effluent (E4)	8.36	8.16	−2.4
Effluent (E5)	0.65	0.71	+9.2
Effluent (E6)	0.74	0.81	+9.5
Phenylphosphate	1.95	1.91	−2.1
Trimethylphosphate	1.87	1.80	−3.7
Sodium pyrophosphate	1.90	1.73	−8.9
Sodium tripolyphosphate	1.84	1.73	−6.0

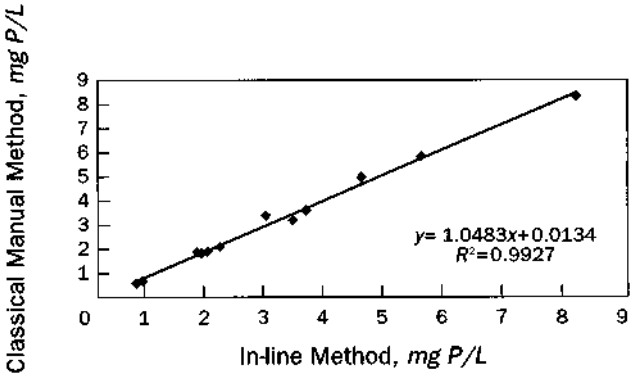


Figure 4500-P:6. Correlation between manual and in-line total phosphorus methods.

4500-P J. Persulfate Method for Simultaneous Determination of Total Nitrogen and Total Phosphorus

1. General Discussion

a. Principle: The oxidation of nitrogenous compounds for determining total nitrogen must occur in an alkaline medium. Conversely, the oxidation of phosphorus compounds for determining total phosphorus must occur under acidic conditions. Methods determining total nitrogen have used a persulfate-sodium hydroxide system to oxidize nitrogenous compounds to nitrate. Accordingly, methods determining total phosphorus have used persulfate in an acidic medium.

During the initial stage of the digestion, sample pH is alkaline (pH>12). In the final stage of the digestion, the sodium hydroxide is consumed, causing sample pH to become acidic (pH<2). By means of this broad pH range, the method allows for the oxidation of both nitrogen and phosphorus compounds. The digested sample is analyzed for nitrate and orthophosphate, yielding total nitrogen and total phosphorus results.

b. Selection of nitrate/orthophosphate measurement methods: Using a dual-channel autoanalyzer that performs nitrate-nitrite by the cadmium reduction method and orthophosphate by the ascorbic acid reduction method, total nitrogen and total phosphorus can be measured simultaneously. Alternatively, other methods for orthophosphate and nitrate can be used.

2. Apparatus

Clean all glassware with HCl before use.

a. Autoclave, capable of achieving a temperature of 120°C for a minimum of 120 min.

b. Glass culture tubes, 13-mm-OD ×100-mm-long with autoclavable caps.

c. Autopipettor, capable of pipetting a 6.0-mL portion.

d. Repeating pipettor, capable of pipetting 1.25-mL portion.

e. Erlenmeyer flask, 3000-mL.

f. Aluminum foil.

g. Automated continuous-flow instrument system for nitrate and phosphate determination: The suggested analytical instruments are described in Sections 4500-NO_3^-.F.2 and 4500-P.F.2*a*.

3. Reagents

a. Deionized water, high-quality, free of phosphorus and nitrogen compounds. Prepare by ion-exchange or distillation methods as directed in Sections 4500-NH_3.B.3*a* and 4500-NO_3^-.B.3*a*.

b. Sodium hydroxide, 3N: Dissolve 120 g low-nitrogen NaOH in 800 mL deionized water in a 1000-mL volumetric flask. Cool and dilute to volume.

c. Oxidizing reagent: Dissolve 64 g low-nitrogen (<0.001% N) potassium persulfate, $K_2S_2O_8$, in 500 mL deionized water. Use low heat if necessary. Add 80 mL 3*N* NaOH, prepared from low-nitrogen sodium hydroxide, and dilute to 1000 mL. Store in a brown bottle at room temperature.

d. All of the reagents listed for determining nitrate + nitrite as indicated in Section 4500-NO_3^-.F.3.

e. All of the reagents listed for determining phosphate as indicated in 4500-P.F.3.

f. Nicotinic acid p-*toluenesulfonate stock and working standards:* Dry nicotinic acid *p*-toluenesulfonate in an oven at 105°C for 24 h. Dissolve 2.1084 g in deionized water and dilute to 100 mL; 1 mL = 1 mg N. To prepare a working standard, dilute 2.0 mL stock solution to 1000 mL; 1 mL = 2 μg N.

g. Adenosine triphosphate stock and working standards: Dissolve 0.6514 g adenosine triphosphate in deionized water and dilute to 1000 mL; 1 mL = 0.1 mg P. To prepare a working standard, dilute 20.0 mL stock solution to 1000 mL; 1 mL = 2 μg P. To prepare a low-range working standard, dilute 1.0 mL stock solution to 1000 mL; 1 mL = 0.1 μg P.

4. Procedure

a. Calibration curve: Prepare a minimum of five standards over the desired calibration ranges using a stock calibration standard containing both nitrate and orthophosphate. Treat standards in the same manner as samples. Include blanks in calibration curves.

b. Sample preparation: If necessary, dilute sample with deionized water so expected nitrogen and phosphorus concentrations fall within the range of the calibration standards. Samples preserved with acid cannot be analyzed by this digestion method.

c. Digestion check standards: Analyze quality-control standards containing organic nitrogen and phosphorus on each analytical run (see 4500-P.J.3*f* and *g* for suggested standards and preparation procedures). These standards provide reference checks on the calibration and test the efficiency of the digestion.

d. Digestion: Pipet 6.0 mL of sample or standard into the culture tubes. Add 1.25 mL oxidizing reagent to each tube using a repeating pipettor. Cover the tubes with loose-fitting plastic caps. Prepare an autoanalyzer wash water in an Erlenmeyer flask by adding oxidation reagent to deionized water in the same proportion as was added to the samples. Cover flask with foil. Autoclave samples and wash water for 55 min at 120°C. Cool to room temperature. Add 0.05 mL of 3*N* NaOH to each tube before proceeding to nitrate + nitrite and phosphate analyses. Shake to mix. Add same proportion of 3*N* NaOH to digested wash water.

e. Final nitrate + nitrite measurement: Use the automated cadmium reduction method for the determination of nitrate-nitrite after digestion. See Section 4500-NO_3^-.F. Other nitrate analysis methods may be applicable; however, precision and bias data do not exist for these methods on this matrix at this time.

f. Final phosphate measurement: Use the automated ascorbic acid reduction method for the determination of phosphate after digestion. See 4500-P.F. Other phosphate analysis methods may be applicable; however, precision and bias data do not exist for these methods at this time.

5. Calculation

Prepare nitrogen and phosphorus standard curves by plotting the instrument response of standards against standard concentrations. Compute the nitrogen and phosphorus concentrations by comparing the sample response with the standard curve. Where necessary, multiply sample concentration by the appropriate dilution factor to determine final concentration.

6. Quality Control

The QC practices considered to be an integral part of each method are summarized in Table 4020:I. Use protocols specified in Section 4020 to verify performance. These include daily use of reagent blanks, laboratory-fortified blanks, and known additions. Regulatory analysis may require additional quality control procedures.

7. Precision and Bias

a. Total nitrogen:

1) Nitrogen digestion check standards—Four different organic nitrogen standards (2 mg N/L) were analyzed by a single laboratory on three separate analytical runs yielding the following results:

Nitrogen Compound	Mean *mg N/L*	Recovery of N %	Standard Deviation *mg N/L*	Relative Standard Deviation %
Urea	2.03	102	0.021	1.0
Ammonium *p*-toluenesulfonate	1.93	97	0.037	1.9
Glycine *p*-toluenesulfonate	1.94	97	0.034	1.8
Nicotinic acid *p*-toluenesulfonate	1.86	93	0.044	2.4

2) Mixed ammonia-nitrate solution—A mixed ammonia-nitrate sample (0.55 mg N/L) was analyzed nine times, yielding the following results:

Nitrogen Compounds	Mean *mg/L*	Recovery of N %	Standard Deviation *mg/L*	Relative Standard Deviation %
Ammonium sulfate & potassium nitrate	0.557	101	0.012	2.1

3) Samples—A single laboratory analyzed five samples in quadruplicate on three separate occasions. Samples analyzed for total nitrogen included surface water and diluted wastewater

samples. At an average concentration of 15.0 mg N/L, the average standard deviation was 0.143 mg/L.

b. Phosphorus:

1) Adenosine triphosphate solutions—Two concentrations (2 mg P/L and 100 μg P/L) adenosine triphosphate were analyzed by a single laboratory on two separate analytical runs yielding the following results:

Phosphorus Compound	Mean *mg P/L*	Recovery of P %
Adenosine triphosphate, 2 mg P/L	2.05	103
Adenosine triphosphate, 0.100 μg/L	0.103	103

2) Samples—A single laboratory analyzed five samples in quadruplicate on three separate occasions. Samples were analyzed for total phosphorus over two different calibration ranges. Surface water and diluted wastewater samples were analyzed using a low calibration range [0 to 250 μg P/L, method detection level (MDL) = 2 μg P/L]. At an average concentration of 1670 μg P/L, the average standard deviation was 29.4 μg/L. Surface water and undiluted wastewater samples were analyzed using a high calibration range (0 to 6 mg P/L, MDL = 0.05 mg P/L). At an average concentration of 1.97 mg P/L, the average standard deviation was 0.028 mg/L.

8. Bibliography

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4500-$KMnO_4$ POTASSIUM PERMANGANATE*

4500-$KMnO_4$ A. Introduction

1. Occurrence and Significance

Potassium permanganate, $KMnO_4$, has been widely used in both potable and nonpotable water sources. It has been applied to water supplies to remove taste, odor, color, iron, manganese, and sulfides and to control trihalomethanes (THMs) and zebra mussels. Municipal and industrial waste treatment facilities use potassium permanganate for odor control, toxic pollutant destruction, bio-augmentation, and grease removal.

Potassium permanganate is produced as a dark black-purple crystalline material. It has a solubility in water of 60 g/L at 20°C. The color of potassium permanganate solutions ranges from faint pink (dilute) to deep purple (concentrated). Under normal conditions, the solid material is stable. However, as with all oxidizing agents, avoid contact with acids, peroxides, and all combustible organic or readily oxidizable materials.

2. Sampling and Storage

If kept dry, solid potassium permanganate may be stored indefinitely. Potassium permanganate solutions, made in oxidant-demand-free water, are stable for long periods of time if kept in an amber bottle out of direct sunlight.[1] For samples obtained from other water sources (those having an oxidant demand), analyze potassium permanganate onsite, as soon as possible after sample collection.

3. Reference

1. DAY, R.A. & A.L. UNDERWOOD. 1986. Quantitative Analysis, 5th ed. Prentice-Hall, Englewood Cliffs, N.J.

* Approved by Standard Methods Committee, 2007. Editorial revisions, 2011. Joint Task Group: 20th Edition—Philip A. Vella (chair), Bernard Bubnis, Richard E. DeBlois, Gilbert Gordon.

4500-$KMnO_4$ B. Spectrophotometric Method

1. General Discussion

a. Principle: This method is a direct determination of aqueous potassium permanganate solutions. The concentration (pink to violet color) is directly proportional to the absorbance as measured at 525 nm. The concentrations are best determined from the light-absorbing characteristics of a filtered sample by means of a spectrophotometer.

b. Application: This method is applicable to ground and surface waters.

c. Interference: Turbidity and manganese dioxide interfere. Remove by the filtration methods described below. Other color-producing compounds also interfere. Compensate for color by using an untreated sample to zero the spectrophotometer.

d. Minimum detectable concentration: As a guide, use the following light paths for the indicated $KMnO_4$ concentrations measured at 525 nm. Sample dilution may be required, depending on the initial concentration.

Cell Path Length *cm*	Range *mg $KMnO_4$*/L	Expected Absorbance for 1-mg/L $KMnO_4$ Solution
1	0.5–100	0.016
2.5	0.2–25	0.039
5	0.1–20	0.078

e. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Photometric equipment: Use one of the following:

1) *Spectrophotometer,* for use at a wavelength of 525 nm and providing a light path of 1 cm or longer.

2) *Filter photometer,* equipped with a filter having a maximum transmittance at or near 525 nm and providing a light path of 1 cm or longer.

*b. Filtration apparatus.**

c. Filters: Use 0.22-μm filters that do not react with $KMnO_4$ (or smallest glass fiber filters available).

3. Reagents

Use potassium-permanganate-demand-free water (¶ *e* below) for all reagent preparation and dilutions.

a. Calcium chloride solution ($CaCl_2$), 1*M:* Dissolve 111 g $CaCl_2$ in water and dilute to 1 L.

b. Sulfuric acid (H_2SO_4), 20%: Add 20 g conc H_2SO_4 slowly, with stirring, to 80 mL water. After cooling, adjust final volume to 100 mL.

c. Sodium oxalate ($Na_2C_2O_4$), primary standard.

d. Sodium thiosulfate solution ($Na_2S_2O_3 \cdot 5H_2O$), 0.019*M:* Dissolve 0.471 g $Na_2S_2O_3 \cdot 5H_2O$ in water and dilute to 100 mL.

e. Potassium-permanganate-demand-free water: Add one small crystal $KMnO_4$ to 1 L distilled or deionized water; let

* Millipore or equivalent.

stand. After 1 to 2 d, a residual pink color should be present; if not, discard and obtain better-quality water or increase permanganate added. Redistill in an all-borosilicate-glass apparatus and discard initial 50 mL distillate. Collect distillate fraction that is free of permanganate: a red color with DPD reagent (see Section 4500-Cl.F.2*b*) indicates presence of permanganate.

f. Potassium permanganate standard ($KMnO_4$), 0.006*M:* Dissolve 1.000 g $KMnO_4$ in water and dilute to 1000 mL. Standardize as follows: Weigh, to the nearest mg, about 0.1 g primary standard grade sodium oxalate and dissolve it in 150 mL water in a 250-mL Erlenmeyer flask. Add 20 mL 20% H_2SO_4 and heat to 70 to 80°C. Titrate the warm oxalate solution with the potassium permanganate standard until a pink coloration persists (60 s). Calculate the potassium permanganate concentration:

$$\text{mg } KMnO_4/\text{L} = \frac{W \times 1000}{2.1197 \times V}$$

where:

W = weight of sodium oxalate, mg, and
V = mL $KMnO_4$ titrant.

4. Procedure

a. Calibration of photometric equipment: Prepare calibration curve by diluting standardized potassium permanganate solution. Make dilutions appropriate for the cell path length and range desired. Plot absorbance (y axis) versus $KMnO_4$ concentration (x axis). Calculate a best-fit line through the points. Preferably perform a calibration check with a known $KMnO_4$ standard before any analysis to ensure that equipment is in proper working order.

b. Potassium permanganate analysis: Check zero on spectrophotometer at 525 nm with deionized water. If the water is soft (i.e.,<40 mg/L hardness as $CaCO_3$), add 1 mL $CaCl_2$ solution/L sample (111 mg/L as $CaCl_2$) to aid in removal of any colloidal manganese dioxide and suspended solids. Pass 50 mL sample through a 0.22-μm filter. Rinse spectrophotometer cell with two or three portions of filtrate. Fill cell and check that no air bubbles are present in the solution or on the sides of the cell. Measure absorbance at 525 nm (Reading A). For best results, minimize time between filtration and reading absorbance. To 100 mL sample, add 0.1 mL $CaCl_2$ solution. Add 0.1 mL sodium thiosulfate solution per 1 mg/L $KMnO_4$ (based on Reading A). Pass through a 0.22-μm filter and measure absorbance (Reading B).

5. Calculation

$$\text{Correct absorbance} = A - B$$

where:

A = absorbance of sample, and
B = absorbance of blank.

Compare corrected absorbance value obtained with the calibration curve and report the corresponding value as milligrams potassium permanganate per liter.

6. Precision and Bias

Based on the results obtained by eight analysts in a single laboratory, the overall precision (pooled standard deviation) was determined to be 0.035 mg/L for a 1-cm cell. The mean recovery for 12 measurements at 4 initial concentrations was 98%. The method detection level (MDL), as determined using Section 1030C, was 0.083 mg $KMnO_4$/L.

4500-SiO_2 SILICA*

4500-SiO_2 A. Introduction

1. Occurrence and Significance

Silicon does not occur free in nature, but rather as free silica (SiO_2) in coarsely crystalline (quartz, rock crystal, amethyst, etc.) and microcrystalline (flint, chert, jasper, etc.) varieties of quartz, the major component of sand and sandstone. Silicon is found in combination with other elements in silicates, represented by feldspar, hornblende, mica, asbestos, and other clay minerals. Silicates also occur in rocks, such as granite, basalt, and shale. Silicon therefore is usually reported as silica (SiO_2) when rocks, sediments, soils, and water are analyzed. The average abundance of silica in different rock types is 7 to 80%, in typical soils 50 to 80%, and in surface and groundwater 14 mg/L.

The common aqueous forms of silica are H_4SiO_4 and $H_3SiO_4^-$. In the presence of magnesium, it can form scale deposits in boilers and in steam turbines. It is considered a nonessential trace element for most plants, but essential for most animals. Chronic exposure to silica dust can be toxic. There is no U.S. EPA drinking water standard MCL for silica.

A more complete discussion of the occurrence and chemistry of silica in natural waters is available.[1]

2. Selection of Method

Perform analyses by the electrothermal atomic absorption method (Section 3113B) or one of the colorimetric methods (4500-SiO_2.C, D, E, or F), depending on the fraction to be measured. The inductively coupled plasma mass spectrometric method (Section 3125) or the inductively coupled plasma method (Section 3120) also may be applied successfully in most cases (with lower detection limits), even though silica is not specifically listed as an analyte in the method.

Methods 3120 and 3125 determine total silica. 4500-SiO_2.C, D, E, and F determine molybdate-reactive silica. As noted in 4500-SiO_2.C.4, it is possible to convert other forms of silica to the molybdate-reactive form for determination by these methods. Method 3111D determines more than one form of silica. It will determine all dissolved silica and some colloidally dispersed silica. The determination of silica present in micrometer and submicrometer particles will depend on the size distribution, composition, and structure of the particles; thus 3111D cannot be said to determine total silica.

Method 4500-SiO_2.C is recommended for relatively pure waters containing from 0.4 to 25 mg SiO_2/L. As with most colorimetric Methods, the range can be extended, if necessary, by diluting, by concentrating, or by varying the light path. Interferences due to tannin, color, and turbidity are more severe with this method than with 4500-SiO_2.D. Moreover, the yellow color produced by 4500-SiO_2.C has a limited stability and attention to timing is necessary. When applicable, however, it offers greater speed and simplicity than 4500-SiO_2.D because one reagent fewer is used; one timing step is eliminated; and many natural waters can be analyzed without dilution, which is not often the case with 4500-SiO_2.D. Method 4500-SiO_2.D is recommended for the low range, from 0.04 to 2 mg SiO_2/L. This range also can be extended if necessary. Such extension may be desirable if interference is expected from tannin, color, or turbidity. A combination of factors renders 4500-SiO_2.D, E, and F less susceptible than 4500-SiO_2.C to those interferences; also, the blue color in 4500-SiO_2.D, E, and F is more stable than the yellow color in 4500-SiO_2.C. However, many samples will require dilution because of the high sensitivity of the method. Permanent artificial color standards are not available for the blue color developed in 4500-SiO_2.D.

The yellow color produced by 4500-SiO_2.C and the blue color produced by 4500-SiO_2.D, E, and F are affected by high concentrations of salts. With seawater the yellow color intensity is decreased by 20 to 35% and the blue color intensity is increased by 10 to 15%. When waters of high ionic strength are analyzed by these methods, use silica standards of approximately the same ionic strengths.[2]

Method 4500-SiO_2.E or F may be used where large numbers of samples are analyzed regularly. Method 3111D is recommended for broad-range use. Although Method 3111D is usable from 1 to 300 mg SiO_2/L, optimal results are obtained from about 20 to 300 mg/L. The range can be extended upward by dilution if necessary. This method is rapid and does not require any timing step.

The inductively coupled plasma method (Section 3120) also may be used in analyses for silica.

3. Sampling and Storage

Collect samples in bottles of polyethylene, other plastic, or hard rubber, especially if there will be a delay between collection and analysis. Borosilicate glass is less desirable, particularly with waters of pH above 8 or with seawater, in which cases a significant amount of silica in the glass can dissolve. Freezing to preserve samples for analysis of other constituents can lower soluble silica values by as much as 20 to 40% in waters that have a pH below 6. Do not acidify samples for preservation because silica precipitates in acidic solutions.

4. References

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* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011, 2017.
Joint Task Group: 20th Edition—(4500-SiO_2.F)—Scott Stieg (chair), Bradford R. Fisher, Owen B. Mathre, Theresa M. Wright.

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4500-SiO_2 B. (Reserved)

4500-SiO_2 C. Molybdosilicate Method

1. General Discussion

a. Principle: Ammonium molybdate at pH approximately 1.2 reacts with silica and any phosphate present to produce heteropoly acids. Oxalic acid is added to destroy the molybdophosphoric acid but not the molybdosilicic acid. Even if phosphate is known to be absent, the addition of oxalic acid is highly desirable and is a mandatory step in both this method and 4500-SiO_2.D The intensity of the yellow color is proportional to the concentration of "molybdate-reactive" silica. In at least one of its forms, silica does not react with molybdate even though it is capable of passing through filter paper and is not noticeably turbid. It is not known to what extent such "unreactive" silica occurs in waters. Terms such as "colloidal," "crystalloidal," and "ionic" have been used to distinguish among various forms of silica but such terminology cannot be substantiated. "Molybdate-unreactive" silica can be converted to the "molybdate-reactive" form by heating or fusing with alkali. Molybdate-reactive or unreactive does not imply reactivity, or lack of it, toward *other* reagents or processes.

b. Interference: Because both apparatus and reagents may contribute silica, avoid using glassware as much as possible and use reagents low in silica. Also, make a blank determination to correct for silica so introduced. In both this method and 4500-SiO_2.D, tannin, large amounts of iron, color, turbidity, sulfide, and phosphate interfere. Treatment with oxalic acid eliminates interference from phosphate and decreases interference from tannin. If necessary, use photometric compensation to cancel interference from color or turbidity.

c. Minimum detectable concentration: Approximately 1 mg SiO_2/L can be detected in 50-mL nessler tubes.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Platinum dishes, 100-mL.

b. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer,* for use at 410 nm, providing a light path of 1 cm or longer. See Table 4500-SiO_2:I for light path selection.

2) *Filter photometer,* providing a light path of 1 cm or longer and equipped with a violet filter having maximum transmittance near 410 nm.

3) *Nessler tubes,* matched, 50-mL, tall form.

TABLE 4500-SiO_2.I. SELECTION OF LIGHT PATH LENGTH FOR VARIOUS SILICA CONCENTRATIONS

	4500-SiO_2.C	4500-SiO_2.D	
		Silica in 55 mL Final Volume μg	
Light Path *cm*	Silica in 55 mL Final Volume μg	650 nm Wavelength	815 nm Wavelength
1	200–1300	40–300	20–100
2	100–700	20–150	10–50
5	40–250	7–50	4–20
10	20–130	4–30	2–10

3. Reagents

For best results, set aside and use batches of chemicals low in silica. Use distilled reagent water in making reagents and dilutions. Store all reagents in plastic containers to guard against high blanks.

a. Sodium bicarbonate ($NaHCO_3$), powder.

b. Sulfuric acid (H_2SO_4), 1*N*.

c. Hydrochloric acid (HCl), 1 + 1.

d. Ammonium molybdate reagent: Dissolve 10 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in water, with stirring and gentle warming, and dilute to 100 mL. Filter if necessary. Adjust to pH 7 to 8 with silica-free NH_4OH or NaOH and store in a polyethylene bottle to stabilize. (If the pH is not adjusted, a precipitate gradually forms. If the solution is stored in glass, silica may leach out and cause high blanks.) If necessary, prepare silica-free NH_4OH by passing gaseous NH_3 into distilled water contained in a plastic bottle.

e. Oxalic acid solution: Dissolve 7.5 g $H_2C_2O_4 \cdot 2H_2O$ in water and dilute to 100 mL.

f. Stock silica solution: Dissolve 4.73 g sodium metasilicate nonahydrate, $Na_2SiO_3 \cdot 9H_2O$, in water and dilute to 1000 mL. For work of highest accuracy, analyze 100.0-mL portions by the gravimetric method.[1] Store in a tightly stoppered plastic bottle.

g. Standard silica solution: Dilute 10.00 mL stock solution to 1000 mL with water; 1.00 mL = 10.0 μg SiO_2. Calculate exact concentration from concentration of stock silica solution. Store in a tightly stoppered plastic bottle.

h. Permanent color solutions:

1) *Potassium chromate solution*—Dissolve 630 mg K_2CrO_4 in water and dilute to 1 L.

2) *Borax solution*—Dissolve 10 g sodium borate decahydrate, $Na_2B_4O_7 \cdot 10H_2O$, in water and dilute to 1 L.

4. Procedure

a. Color development: To 50.0 mL sample add in rapid succession 1.0 mL 1 + 1 HCl and 2.0 mL ammonium molybdate reagent. Mix by inverting at least six times and let stand for 5 to 10 min. Add 2.0 mL oxalic acid solution and mix thoroughly. Read color after 2 min but before 15 min, measuring time from addition of oxalic acid. Because the yellow color obeys Beer's law, measure photometrically or visually.

b. To detect the presence of molybdate-unreactive silica, digest sample with $NaHCO_3$ before color development. This digestion is not necessarily sufficient to convert all molybdate-unreactive silica to the molybdate-reactive form. Complex silicates and higher silica polymers may require extended fusion with alkali at high temperatures or digestion under pressure for complete conversion. Omit digestion if all the silica is known to react with molybdate.

Prepare a clear sample by filtration if necessary. Place 50.0 mL, or a smaller portion diluted to 50 mL, in a 100-mL platinum dish. Add 200 mg silica-free $NaHCO_3$ and digest on a steam bath for 1 h. Cool and add slowly, with stirring, 2.4 mL 1*N* H_2SO_4. Do not interrupt analysis but proceed *at once* with remaining steps. Transfer quantitatively to a 50-mL nessler tube and make up to mark with water. (Tall-form 50-mL nessler tubes are convenient for mixing even if the solution subsequently is transferred to an absorption cell for photometric measurement.)

c. Preparation of standards: If $NaHCO_3$ pretreatment is used, add to the standards (approximately 45 mL total volume) 200 mg $NaHCO_3$ and 2.4 mL 1*N* H_2SO_4, to compensate both for the slight amount of silica introduced by the reagents and for the effect of the salt on color intensity. Dilute to 50.0 mL.

d. Correction for color or turbidity: Prepare a special blank for every sample that needs such correction. Carry two identical portions of each such sample through the procedure, including $NaHCO_3$ treatment if this is used. To one portion, add all reagents as directed in ¶ *a* above. To the other portion, add HCl and oxalic acid but no molybdate. Adjust photometer to zero absorbance with the blank containing no molybdate before reading absorbance of molybdate-treated sample.

e. Photometric measurement: Prepare a calibration curve from a series of approximately six standards to cover the optimum ranges cited in Table 4500-SiO_2:I. Follow direction of ¶ *a* above on suitable portions of standard silica solution diluted to 50.0 mL in nessler tubes. Set photometer at zero absorbance with water and read all standards, including a reagent blank, against water. Plot micrograms silica in the final (55 mL) developed solution against photometer readings. Run a reagent blank and at least one standard with each group of samples to confirm that the calibration curve previously established has not shifted.

f. Visual comparison: Make a set of permanent artificial color standards, using K_2CrO_4 and borax solutions. Mix liquid volumes specified in Table 4500-SiO_2:II and place them in well-stoppered, appropriately labeled 50-mL nessler tubes. Verify correctness of these permanent artificial standards by comparing them visually against standards prepared by analyzing portions of the standard silica solution. Use permanent artificial color standards only for visual comparison.

Table 4500-SiO_2:II. Preparation of Permanent Color Standards for Visual Determination of Silica

Values in Silica *μg*	Potassium Chromate Solution *mL*	Borax Solution *mL*	Water *mL*
0	0.0	25	30
100	1.0	25	29
200	2.0	25	28
400	4.0	25	26
500	5.0	25	25
750	7.5	25	22
1000	10.0	25	20

5. Calculation

$$\text{mg } SiO_2/\text{L} = \frac{\mu\text{g } SiO_2 \text{ (in 55 mL final volume)}}{\text{mL sample}}$$

Report whether $NaHCO_3$ digestion was used.

6. Precision and Bias

A synthetic sample containing 5.0 mg SiO_2/L, 10 mg Cl^-/L, 0.20 mg NH_3-N/L, 1.0 mg NO_3^--N/L, 1.5 mg organic N/L, and 10.0 mg PO_4^{3-}/L in distilled water was analyzed in 19 laboratories by the molybdosilicate method with a relative standard deviation of 14.3% and a relative error of 7.8%.

Another synthetic sample containing 15.0 mg SiO_2/L, 200 mg Cl^-/L, 0.800 mg NH_3-N/L, 1.0 mg NO_3^--N/L, 0.800 mg organic N/L, and 5.0 mg PO_4^{3-}/L in distilled water was analyzed in 19 laboratories by the molybdosilicate method, with a relative standard deviation of 8.4% and a relative error of 4.2%.

A third synthetic sample containing 30.0 mg SiO_2/L, 400 mg Cl^-/L, 1.50 mg NH_3-N/L, 1.0 mg NO_3^--N/L, 0.200 mg organic N/L, and 0.500 mg PO_4^{3-}/L, in distilled water was analyzed in 20 laboratories by the molybdosilicate method, with a relative standard deviation of 7.7% and a relative error of 9.8%.

All results were obtained after sample digestion with $NaHCO_3$.

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4500-SiO_2 D. Heteropoly Blue Method

1. General Discussion

a. Principle: The principles outlined under 4500-SiO_2.C.1*a*, also apply to this method. The yellow molybdosilicic acid is reduced by means of aminonaphtholsulfonic acid to heteropoly blue. The blue color is more intense than the yellow color of 4500-SiO_2.C provides increased sensitivity.

b. Interference: See 4500-SiO_2.C.1*b*.

c. Minimum detectable concentration: Approximately 20 μg SiO_2/L can be detected in 50-mL nessler tubes and 50 μg SiO_2/L spectrophotometrically with a 1-cm light path at 815 nm.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Platinum dishes, 100-mL.

b. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer*—for use at approximately 815 nm. The color system also obeys Beer's law at 650 nm, with appreciably reduced sensitivity. Use light path of 1 cm or longer. See Table 4500-SiO_2:I for light path selection.
2) *Filter photometer*—provided with a red filter exhibiting maximum transmittance in the wavelength range of 600 to 815 nm. Sensitivity improves with increasing wavelength. Use light path of 1 cm or longer.
3) *Nessler tubes*—matched, 50-mL, tall form.

3. Reagents

For best results, set aside and use batches of chemicals low in silica. Store all reagents in plastic containers to guard against high blanks. Use distilled water that does not contain detectable silica after storage in glass.

All of the reagents listed in 4500-SiO_2.C.3 are required, and in addition:

Reducing agent: Dissolve 500 mg 1-amino-2-naphthol-4-sulfonic acid and 1 g Na_2SO_3 in 50 mL distilled water, with gentle warming if necessary; add this to a solution of 30 g $NaHSO_3$ in 150 mL distilled water. Filter into a plastic bottle. Discard when solution becomes dark. Prolong reagent life by storing in a refrigerator and away from light. Do not use aminonaphtholsulfonic acid that is incompletely soluble or that produces reagents that are dark even when freshly prepared.*

4. Procedure

a. Color development: Proceed as in 4500-SiO_2.C.4*a* up to and including the words, "Add 2.0 mL oxalic acid solution and mix thoroughly." Measuring time from the moment of adding oxalic acid, wait at least 2 min but not more than 15 min, add 2.0 mL reducing agent, and mix thoroughly. After 5 min, measure blue color photometrically or visually. If $NaHCO_3$ pretreatment is used, follow 4500-SiO_2.C.4*b*.

b. Photometric measurement: Prepare a calibration curve from a series of approximately six standards to cover the optimum range indicated in Table 4500-SiO_2:I. Carry out the steps described above on suitable portions of standard silica solution diluted to 50.0 mL in nessler tubes; pretreat standards if $NaHCO_3$ digestion is used (see 4500-SiO_2.C.4*b*). Adjust photometer to zero absorbance with distilled water and read all standards, including a reagent blank, against distilled water. If necessary to correct for color or turbidity in a sample, see 4500-SiO_2.C.4*d*. To the special blank add HCl and oxalic acid, but no molybdate or reducing agent. Plot micrograms silica in the final 55 mL developed solution against absorbance. Run a reagent blank and at least one standard with each group of samples to check the calibration curve.

c. Visual comparison: Prepare a series of not less than 12 standards, covering the range 0 to 120 μg SiO_2, by placing the calculated volumes of standard silica solution in 50-mL nessler tubes, diluting to mark with distilled water, and developing color as described in ¶ *a* above.

5. Calculation

$$\text{mg } SiO_2\text{/L} = \frac{\mu\text{g } SiO_2 \text{ (in 55 mL final volume)}}{\text{mL sample}}$$

Report whether $NaHCO_3$ digestion was used.

6. Precision and Bias

A synthetic sample containing 5.0 mg SiO_2/L, 10 mg Cl^-/L, 0.200 mg NH_3-N/L, 1.0 mg NO_3^--N/L, 1.5 mg organic N/L, and 10.0 mg PO_4^{3-}/L in distilled water was analyzed in 11 labora-

* Eastman No. 360 has been found satisfactory.

tories by the heteropoly blue method, with a relative standard deviation of 27.2% and a relative error of 3.0%.

A second synthetic sample containing 15 mg SiO_2/L, 200 mg Cl^-/L, 0.800 mg NH_3-N/L, 1.0 mg NO_3^--N/L, 0.800 mg organic N/L, and 5.0 mg PO_4^{3-}/L in distilled water was analyzed in 11 laboratories by the heteropoly blue method, with a relative standard deviation of 18.0% and a relative error of 2.9%.

A third synthetic sample containing 30.0 mg SiO_2/L, 400 mg Cl^-/L, 1.50 mg NH_3-N/L, 1.0 mg NO_3^--N/L, 0.200 mg organic N/L, and 0.500 mg PO_4^{3-}/L in distilled water was analyzed in 10 laboratories by the heteropoly blue method with a relative standard deviation of 4.9% and a relative error of 5.1%.

All results were obtained after sample digestion with $NaHCO_3$.

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4500-SiO_2 E. Automated Method for Molybdate-Reactive Silica

1. General Discussion

a. Principle: This method is an adaptation of the heteropoly blue method (4500-SiO_2.D) using a continuous-flow analytical instrument.

b. Interferences: See 4500-SiO_2.C.1*b*. If particulate matter is present, filter sample or use a continuous filter as an integral part of the system.

c. Application: This method is applicable to potable, surface, domestic, and other waters containing 0 to 20 mg SiO_2/L. The range of concentration can be broadened to 0 to 80 mg/L by substituting a 15-mm flow cell for the 50-mm flow cell shown in Figure 4500-SiO_2:1.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Automated analytical equipment: An example of the continuous-flow analytical instrument consists of the interchangeable components shown in Figure 4500-SiO_2:1.

3. Reagents

a. Sulfuric acid (H_2SO_4), 0.05*M* (0.1*N*).

b. Ammonium molybdate reagent: Dissolve 10 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 1 L 0.05*M* H_2SO_4. Filter and store in an amber plastic bottle.

c. Oxalic acid solution: Dissolve 50 g $H_2C_2O_4 \cdot 2H_2O$ in 900 mL distilled water and dilute to 1 L.

d. Reducing agent: Dissolve 120 g $NaHSO_3$ and 4 g Na_2SO_3 in 800 mL warm distilled water. Add 2 g 1-amino-2-naphthol-4-sulfonic acid, mix well, and dilute to 1 L. Filter into amber plastic bottle for storage.

To prepare working reagent, dilute 100 mL to 1 L with distilled water. Make working reagent daily.

e. Standard silica solution: See 4500-SiO_2.C.3*g*.

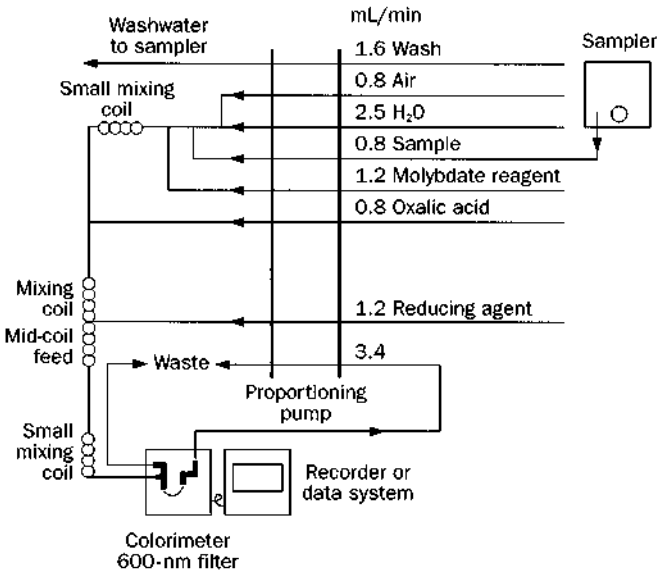


Figure 4500-SiO_2:1. Silica manifold.

4. Procedure

Set up manifold as shown in Figure 4500-SiO_2:1 and follow the general procedure described by the manufacturer. Determine absorbance at 660 nm. Use quality control procedures given in Section 4020.

5. Calculation

Prepare standard curves by plotting response of standards processed through the manifold against SiO_2 concentration in standards. Compute sample SiO_2 concentration by comparing sample response with standard curve.

6. Precision and Bias

For 0 to 20 mg SiO_2/L, when a 50-mm flow cell was used at 40 samples/h, the detection limit was 0.1 mg/L, sensitivity (concentration giving 0.398 absorbance) was 7.1 mg/L, and the coefficient of variation (95% confidence level at 7.1 mg/L) was 1.6%. For 0 to 80 mg SiO_2/L, when a 15-mm flow cell was used at 50 samples/h, detection limit was 0.5 mg/L, sensitivity was 31 mg/ L, and coefficient of variation at 31 mg/L was 1.5%.

4500-SiO_2 F. Flow Injection Analysis for Molybdate-Reactive Silicate

1. General Discussion

Silicate reacts with molybdate under acidic conditions to form yellow beta-molybdosilicic acid. This acid is subsequently reduced with stannous chloride to form a heteropoly blue complex that has an absorbance maximum at 820 nm. Oxalic acid is added to reduce the interference from phosphate.

Collect samples in polyethylene or other plastic bottles and refrigerate at 4°C. Chemical preservation for silica is not recommended. Adding acid may cause polymerization of reactive silicate species. Freezing decreases silicate concentrations, especially at concentrations greater than 100 μg SiO_2/L. If filtration is required, preferably use a 0.45-μm TFE filter. Samples may be held for 28 d.

Also see 4500-SiO_2.A, D, and E, and Section 4130, Flow Injection Analysis (FIA).

Interferences: Remove large or fibrous particulates by filtering sample through inert filter.

The interference due to phosphates is reduced by the addition of oxalic acid as a reagent on the flow injection manifold. By the following method, a solution of 1000 μg P/L was determined as 20 μg SiO_2/L. Verify extent of phosphate interference by determining a solution of phosphate at the highest concentration that is expected to occur.

Tannin and large amounts of iron or sulfides are interferences. Remove sulfides by boiling an acidified sample. Add disodium EDTA to eliminate interference due to iron. Treat with oxalic acid to decrease interference from tannin.

Sample color and turbidity can interfere. Determine presence of these interferences by analyzing samples without the presence of the molybdate.

Avoid silica contamination by storing samples, standards, and reagents in plastic. Do not use glass-distilled water for reagents or standards.

2. Apparatus

Flow injection analysis equipment consisting of:

a. FIA injection valve with sample loop or equivalent.

b. Multichannel proportioning pump.

c. FIA manifold (Figure 4500-SiO_2:2) with tubing heater and flow cell. Relative flow rates only are shown in Figure 4500-SiO_2:2. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material, such as TFE.*

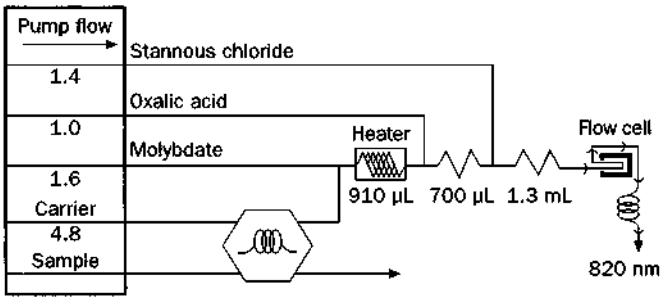


Figure 4500-SiO_2:2. FIA manifold.

d. Absorbance detector, 820 nm, 10-nm bandpass.

e. Injection valve control and data acquisition system.

3. Reagents

Use reagent water (>10 megohm) to prepare carrier and all solutions. To prevent bubble formation, degas carrier and buffer with helium. Pass He at 140 kPa (20 psi) through a helium degassing tube. Bubble He through 1 L solution for 1 min. All reagents can also be prepared on a weight/volume basis if desired.

a. Molybdate: To a tared 500-mL container, add 20.0 g ammonium molybdate tetrahydrate [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$]. Add 486 g warm water and 14.8 g conc sulfuric acid (H_2SO_4). Stir or shake until dissolved. Store in plastic and refrigerate. Prepare fresh monthly and discard if precipitate or blue color is observed.

b. Oxalic acid: To a tared 500-mL container, add 50.0 g $H_2C_2O_4 \cdot 2H_2O$ and 490 g water. Stir or shake until dissolved. Store in plastic.

c. Stannous chloride: To a tared 1-L container, add 978 g water. Add 40.0 g conc H_2SO_4. Dissolve 2.0 g hydroxylamine hydrochloride in this solution. Then dissolve 0.30 g stannous chloride. Prepare fresh weekly.

d. Stock silicate standard, 100 mg SiO_2/L: In a 1-L volumetric flask dissolve 0.473 g sodium metasilicate nonahydrate ($Na_2SiO_3 \cdot 9H_2O$) in approximately 800 mL water. Dilute to mark and invert three times. Alternatively, use a commercially prepared standard solution, especially if nonstoichiometry of the solid metasilicate is suspected; the original degree of polymerization of the sodium metasilicate, which depends on storage time, can affect free silica concentration of the resulting solution.

e. Standard silicate solutions: Prepare silicate standards in the desired concentration range, using the stock standard (¶ *d* above), and diluting with water.

* Teflon, or equivalent.

4. Procedure

Set up a manifold equivalent to that in Figure 4500-SiO_2:2 and follow method supplied by manufacturer or laboratory standard operating procedure.

5. Calculations

Prepare standard curves by plotting absorbance of standards processed through the manifold versus silicate concentration. The calibration curve is linear.

6. Precision and Bias

Twenty-one replicates of a 5.0-μg SiO_2/L standard were analyzed with a 780-μL sample loop by a published MDL method.[1] These gave a mean of 4.86 μg SiO_2/L, a standard deviation of 0.31 μg SiO_2/L, and an MDL of 0.78 μg SiO_2/L.

7. Quality Control

The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

8. Reference

1. U.S. Environmental Protection Agency. 1989. Definition and Procedure for the Determination of Method Detection Limits. Appendix B to 40 CFR 136 rev. 1.11 amended June 30, 1986. 49 CFR 43430.

4500-SO_4^{2-} SULFATE*

4500-SO_4^{2-} A. Introduction

1. Occurrence

Sulfate (SO_4^{2-}) is widely distributed in nature and may be present in natural waters in concentrations ranging from a few to several thousand milligrams per liter. Mine drainage wastes may contribute large amounts of SO_4^{2-} through pyrite oxidation. Sodium and magnesium sulfate exert a cathartic action.

2. Selection of Method

The ion chromatographic method (Section 4110) and capillary ion electrophoresis (CIE—see Section 4140) are suitable for sulfate concentrations above 0.1 mg/L. The gravimetric methods (4500-SO_4^{2-}.C and D) are suitable for SO_4^{2-} concentrations above 10 mg/L. The turbidimetric method (4500-SO_4^{2-}.E) is applicable in the range of 1 to 40 mg SO_4^{2-} /L. The automated methylthymol blue methods (4500-SO_4^{2-}.F and G) are the procedures for analyzing large numbers of samples for sulfate alone when the equipment is available; over 30 samples can be analyzed per hour. Methods 4500-SO_4^{2-}.C, D, F, G, Section 4110, or CIE (Section 4140) are preferred for accurate results.

3. Sampling and Storage

In the presence of organic matter certain bacteria may reduce SO_4^{2-} to S^{2-}. To avoid this, store samples at 4°C.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011. Joint Task Group: 20th Edition—(4500-SO_4^{2-}.G)—Scott Stieg (chair), Bradford R. Fisher, Owen B. Mathre, Theresa M. Wright.

4500-SO_4^{2-} B. (Reserved)

4500-SO_4^{2-} C. Gravimetric Method with Ignition of Residue

1. General Discussion

a. Principle: Sulfate is precipitated in a hydrochloric acid (HCl) solution as barium sulfate ($BaSO_4$) by the addition of barium chloride ($BaCl_2$).

The precipitation is carried out near the boiling temperature, and after a period of digestion the precipitate is filtered, washed with water until free of Cl^-, ignited or dried, and weighed as $BaSO_4$.

b. Interference: The gravimetric determination of SO_4^{2-} is subject to many errors, both positive and negative. In potable waters where the mineral concentration is low, these may be of minor importance.

1) Interferences leading to high results—Suspended matter, silica, $BaCl_2$ precipitant, NO_3^-, SO_3^{2-} and occluded mother liquor in the precipitate are the principal factors in positive errors. Suspended matter may be present in both the sample and the precipitating solution; soluble silicate may be rendered insoluble and SO_3^{2-} may be oxidized to SO_4^{2-} during analysis. Barium nitrate [$Ba(NO_3)_2$], $BaCl_2$, and water are occluded to some extent with the $BaSO_4$ although water is driven off if the temperature of ignition is sufficiently high.

2) Interferences leading to low results—Alkali metal sulfates frequently yield low results. This is true especially of alkali hydrogen sulfates. Occlusion of alkali sulfate with $BaSO_4$ causes substitution of an element of lower atomic weight than barium in the precipitate. Hydrogen sulfates of alkali metals act similarly and, in addition, decompose on being heated. Heavy metals, such as chromium and iron, cause low results by interfering with the complete precipitation of SO_4^{2-} and by formation of heavy metal sulfates. $BaSO_4$ has small but significant solubility, which is increased in the presence of acid. Although an acid medium is necessary to prevent precipitation of barium carbonate and phosphate, it is important to limit its concentration to minimize the solution effect.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Steam bath.

b. Drying oven, equipped with thermostatic control.

c. Muffle furnace, with temperature indicator.

d. Desiccator.

e. Analytical balance, capable of weighing to 0.1 mg.

f. Filter: Use one of the following:

1) *Filter paper,* acid-washed, ashless hard-finish, sufficiently retentive for fine precipitates.

2) *Membrane filter,* with a pore size of about 0.45 μm.

g. Filtering apparatus, appropriate to the type of filter selected. (Coat membrane filter holder with silicone fluid to prevent precipitate from adhering.)

3. Reagents

a. Methyl red indicator solution: Dissolve 100 mg methyl red sodium salt in distilled water and dilute to 100 mL.

b. Hydrochloric acid (HCl), 1 + 1.

c. Barium chloride solution: Dissolve 100 g $BaCl_2 \cdot 2H_2O$ in 1 L distilled water. Filter through a membrane filter or hard-finish filter paper before use; 1 mL is capable of precipitating approximately 40 mg SO_4^{2-}.

d. Silver nitrate-nitric acid reagent: Dissolve 8.5 g $AgNO_3$ and 0.5 mL conc HNO_3 in 500 mL distilled water.

*e. Silicone fluid.**

4. Procedure

a. Removal of silica: If the silica concentration exceeds 25 mg/L, evaporate sample nearly to dryness in a platinum dish on a steam bath. Add 1 mL HCl, tilt, and rotate dish until the acid comes in complete contact with the residue. Continue evaporation to dryness. Complete drying in an oven at 180°C and if organic matter is present, char over flame of a burner. Moisten residue with 2 mL distilled water and 1 mL HCl, and evaporate to dryness on a steam bath. Add 2 mL HCl, take up soluble residue in hot water, and filter. Wash insoluble silica with several small portions of hot distilled water. Combine filtrate and washings. Discard residue.

b. Precipitation of barium sulfate: Adjust volume of clarified sample to contain approximately 50 mg SO_4^{2-} in a 250-mL volume. Lower concentrations of SO_4^{2-} may be tolerated if it is impracticable to concentrate sample to the optimum level, but in such cases limit total volume to 150 mL. Adjust pH with HCl to pH 4.5 to 5.0, using a pH meter or the orange color of methyl red indicator. Add 1 to 2 mL HCl. Heat to boiling and, while stirring gently, slowly add warm $BaCl_2$ solution until precipitation appears to be complete; then add about 2 mL in excess. If amount of precipitate is small, add a total of 5 mL $BaCl_2$ solution. Digest precipitate at 80 to 90°C, preferably overnight but for not less than 2 h.

c. Filtration and weighing: Mix a small amount of ashless filter paper pulp with the $BaSO_4$, quantitatively transfer to a filter, and filter at room temperature. The pulp aids filtration and reduces the tendency of the precipitate to creep. Wash precipitate with small portions of warm distilled water until washings are free of Cl^- as indicated by testing with $AgNO_3$-HNO_3 reagent. Place filter and precipitate in a weighed platinum crucible and ignite at 800°C for 1 h. Do not let filter paper flame. Cool in desiccator and weigh.

5. Calculation

$$\text{mg } SO_4^{2-}/\text{L} = \frac{\text{mg } BaSO_4 \times 411.6}{\text{mL sample}}$$

6. Precision and Bias

A synthetic sample containing 259 mg SO_4^{2-}/L, 108 mg Ca^{2+}/L, 82 mg Mg^{2+}/L, 3.1 mg K^+/L, 19.9 mg Na^+/L, 241 mg Cl^-/L, 0.250 mg NO_2^--N/L, 1.1 mg NO_3^--N/L, and 42.5 mg total alkalinity/L (contributed by $NaHCO_3$) was analyzed in 32 laboratories by the gravimetric method, with a relative standard deviation of 4.7% and a relative error of 1.9%.

7. Bibliography

HILLEBRAND, W.F. *et al.* 1953. Applied Inorganic Analysis, 2nd ed. John Wiley & Sons, New York, N.Y.

KOLTHOFF, I.M., E.J. MEEHAN, E.B. SANDELL & S. BRUCKENSTEIN. 1969. Quantitative Chemical Analysis, 4th ed. Macmillan Co., New York, N.Y.

* "Desicote" (Beckman), or equivalent.

4500-SO_4^{2-} D. Gravimetric Method with Drying of Residue

1. General Discussion

See 4500-SO_4^{2-}.C.1.

2. Apparatus

With the exception of the filter paper, all of the apparatus cited in 4500-SO_4^{2-}.C.2 is required, plus the following:

a. Filters: Use one of the following:

1) *Fritted-glass filter,* fine ("F") porosity, with a maximum pore size of 5 μm.

2) *Membrane filter,* with a pore size of about 0.45 μm.

b. Vacuum oven.

3. Reagents

All the reagents listed in 4500-SO_4^{2-}.C.3 are required.

4. Procedure

a. Removal of interference: See 4500-SO_4^{2-}.C.4*a.*

b. Precipitation of barium sulfate: See 4500-SO_4^{2-}.C.4*b.*

c. Preparation of filters:

1) Fritted glass filter—Dry to constant weight in an oven maintained at 105°C or higher, cool in desiccator, and weigh.

2) Membrane filter—Place filter on a piece of filter paper or a watch glass and dry to constant weight* in a vacuum oven at 80°C, while maintaining a vacuum of at least 85 kPa or in a conventional oven at a temperature of 103 to 105°C. Cool in desiccator and weigh membrane only.

d. Filtration and weighing: Filter $BaSO_4$ at room temperature. Wash precipitate with several small portions of warm

* *Constant weight* is a change of not more than 0.5 mg in two successive operations consisting of heating, cooling in desiccator, and weighing.

distilled water until washings are free of Cl^-, as indicated by testing with $AgNO_3$-HNO_3 reagent. If a membrane filter is used add a few drops of silicone fluid to the suspension before filtering, to prevent adherence of precipitate to holder. Dry filter and precipitate by the same procedure used in preparing filter. Cool in a desiccator and weigh.

5. Calculation

$$\text{mg } SO_4^{2-}/\text{L} = \frac{\text{mg } BaSO_4 \times 411.6}{\text{mL sample}}$$

6. Bibliography

See 4500-SO_4^{2-}.C.7.

4500-SO_4^{2-} E. Turbidimetric Method

1. General Discussion

a. Principle: Sulfate ion (SO_4^{2-}) is precipitated in an acetic acid medium with barium chloride ($BaCl_2$) so as to form barium sulfate ($BaSO_4$) crystals of uniform size. Light absorbance of the $BaSO_4$ suspension is measured by a photometer and the SO_4^{2-} concentration is determined by comparison of the reading with a standard curve.

b. Interference: Color or suspended matter in large amounts will interfere. Some suspended matter may be removed by filtration. If both are small in comparison with the SO_4^{2-} concentration, correct for interference as indicated in 4500-SO_4^{2-}.E.4*d* below. Silica in excess of 500 mg/L will interfere, and in waters containing large quantities of organic material it may not be possible to precipitate $BaSO_4$ satisfactorily.

In potable waters there are no ions other than SO_4^{2-} that will form insoluble compounds with barium under strongly acid conditions. Make determination at room temperature; variation over a range of 10°C will not cause appreciable error.

c. Minimum detectable concentration: Approximately 1 mg SO_4^{2-}/L.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Magnetic stirrer: Use a constant stirring speed. It is convenient to incorporate a fixed resistance in series with the motor operating the magnetic stirrer to regulate stirring speed. Use magnets of identical shape and size. The exact speed of stirring is not critical, but keep it constant for each run of samples and standards and adjust it to prevent splashing.

b. Photometer: One of the following is required, with preference in the order given:

1) *Nephelometer.*
2) *Spectrophotometer,* for use at 420 nm, providing a light path of 2.5 to 10 cm.
3) *Filter photometer,* equipped with a violet filter having maximum transmittance near 420 nm and providing a light path of 2.5 to 10 cm.

c. Stopwatch or electric timer.

d. Measuring spoon, capacity 0.2 to 0.3 mL.

3. Reagents

a. Buffer solution A: Dissolve 30 g magnesium chloride, $MgCl_2 \cdot 6H_2O$, 5 g sodium acetate, $CH_3COONa \cdot 3H_2O$, 1.0 g potassium nitrate, KNO_3, and 20 mL acetic acid, CH_3COOH (99%), in 500 mL distilled water and make up to 1000 mL.

b. Buffer solution B (required when the sample SO_4^{2-} concentration is less than 10 mg/L): Dissolve 30 g $MgCl_2 \cdot 6H_2O$, 5 g $CH_3COONa \cdot 3H_2O$, 1.0 g KNO_3, 0.111 g sodium sulfate, Na_2SO_4, and 20 mL acetic acid (99%) in 500 mL distilled water and make up to 1000 mL.

c. Barium chloride ($BaCl_2$), crystals, 20 to 30 mesh: In standardization, uniform turbidity is produced with this mesh range and the appropriate buffer.

d. Standard sulfate solution: Prepare a standard sulfate solution as described in 1) or 2) below; 1.00 mL = 100 μg SO_4^{2-}.

1) Dilute 10.4 mL standard 0.0200*N* H_2SO_4 titrant specified in Alkalinity, Section 2320B.3*c*, to 100 mL with distilled water.

2) Dissolve 0.1479 g anhydrous Na_2SO_4 in distilled water and dilute to 1000 mL.

4. Procedure

a. Formation of barium sulfate turbidity: Measure 100 mL sample, or a suitable portion made up to 100 mL, into a 250-mL Erlenmeyer flask. Add 20 mL buffer solution and mix in stirring apparatus. While stirring, add a spoonful of $BaCl_2$ crystals and begin timing immediately. Stir for 60 ± 2 s at constant speed.

b. Measurement of barium sulfate turbidity: After stirring period has ended, pour solution into absorption cell of photometer and measure turbidity at 5 ± 0.5 min.

c. Preparation of calibration curve: Estimate SO_4^{2-} concentration in sample by comparing turbidity reading with a calibration curve prepared by carrying SO_4^{2-} standards through the entire procedure. Space standards at 5-mg/L increments in the 0- to 40-mg/L SO_4^{2-} range. Above 40 mg/L accuracy decreases and $BaSO_4$ suspensions lose stability. Check reliability of calibration curve by running a standard with every three or four samples.

d. Correction for sample color and turbidity: Correct for sample color and turbidity by running blanks to which $BaCl_2$ is not added.

5. Calculation

$$\text{mg } SO_4^{2-}/\text{L} = \frac{\text{mg } SO_4^{2-} \times 1000}{\text{mL sample}}$$

If buffer solution A was used, determine SO_4^{2-} concentration directly from the calibration curve after subtracting sample absorbance before adding $BaCl_2$. If buffer solution B was used subtract SO_4^{2-} concentration of blank from apparent SO_4^{2-} concentration as determined above; because the calibration curve is not a straight line, this is not equivalent to subtracting blank absorbance from sample absorbance.

6. Precision and Bias

With a turbidimeter,* in a single laboratory with a sample having a mean of 7.45 mg SO_4^{2-}/L, a standard deviation of 0.13 mg/L and a coefficient of variation of 1.7% were obtained. Two samples dosed with sulfate gave recoveries of 85 and 91%.

* Hach 2100 A.

7. Bibliography

SHEEN, R.T., H.L. KAHLER & E.M. ROSS. 1935. Turbidimetric determination of sulfate in water. *Ind. Eng. Chem.,* Anal. Ed. 7:262.

THOMAS, J.F. & J.E. COTTON. 1954. A turbidimetric sulfate determination. *Water Sewage Works* 101:462.

ROSSUM, J.R. & P. VILLARRUZ. 1961. Suggested methods for turbidimetric determination of sulfate in water. *J. Amer. Water Works Assoc.* 53:873.

4500-SO_4^{2-} F. Automated Methylthymol Blue Method

1. General Discussion

a. Principle: Barium sulfate is formed by the reaction of the SO_4^{2-} with barium chloride ($BaCl_2$) at a low pH. At high pH excess barium reacts with methylthymol blue to produce a blue chelate. The uncomplexed methylthymol blue is gray. The amount of gray uncomplexed methylthymol blue indicates the concentration of SO_4^{2-}.

b. Interferences: Because many cations interfere, use an ion-exchange column to remove interferences.

Molybdenum, often used to treat cooling waters, has been shown to cause a strong positive bias with this method, even with as little as 1 mg Mo/L.

c. Application: This method is applicable to potable, ground, surface, and saline waters as well as domestic and industrial wastewaters over a range from about 10 to 300 mg SO_4^{2-}/L.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Automated analytical equipment: An example of the required continuous-flow analytical instrument consists of the interchangeable components shown in Figure 4500-SO_4^{2-}:1.

b. Ion-exchange column: Fill a piece of 2-mm-ID glass tubing about 20 cm long with the ion-exchange resin.* To simplify filling column put resin in distilled water and aspirate it into the tubing, which contains a glass-wool plug. After filling, plug other end of tube with glass wool. Avoid trapped air in the column.

3. Reagents

a. Barium chloride solution: Dissolve 1.526 g $BaCl_2 \cdot 2H_2O$ in 500 mL distilled water and dilute to 1 L. Store in a polyethylene bottle.

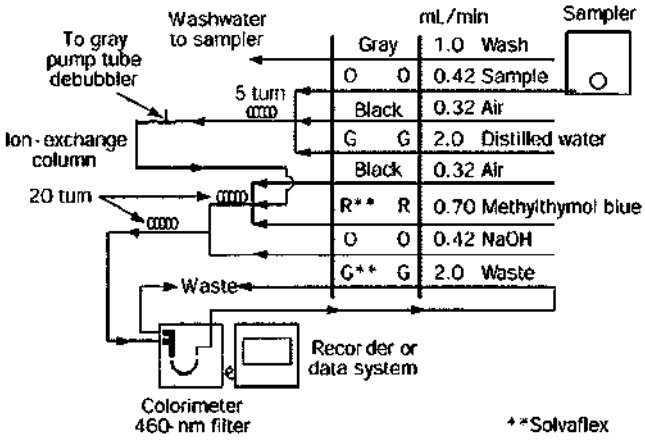


Figure 4500-SO_4^{2-}:1. Sulfate manifold.

b. Methylthymol blue reagent: Dissolve 118.2 mg methylthymol blue† in 25 mL $BaCl_2$ solution. Add 4 mL 1*N* HCl and 71 mL distilled water and dilute to 500 mL with 95% ethanol. Store in a brown glass bottle. Prepare fresh daily.

c. Buffer solution, pH 10.1: Dissolve 6.75 g NH_4Cl in 500 mL distilled water. Add 57 mL conc NH_4OH and dilute to 1 L with distilled water. Adjust pH to 10.1 and store in a polyethylene bottle. Prepare fresh monthly.

d. EDTA reagent: Dissolve 40 g tetrasodium ethylenediaminetetraacetate in 500 mL pH 10.1 buffer solution. Dilute to 1 L with pH 10.1 buffer solution and store in a polyethylene bottle.

e. Sodium hydroxide solution, 0.36*N:* Dissolve 7.2 g NaOH in 250 mL distilled water. Cool and make up to 500 mL with distilled water.

* Ion-exchange resin Bio-Rex 70, 20-50 mesh, sodium form, available from Bio-Rad Laboratories, Richmond, CA 94804, or equivalent.

† Eastman Organic Chemicals, Rochester, NY 14615. No. 8068 3′,3″ bis [*N,N*-bis(carboxymethyl)-aminolmethyl] thymolsulfonphthalein pentasodium salt.

f. Stock sulfate solution: Dissolve 1.479 g anhydrous Na_2SO_4 in 500 mL distilled water and dilute to 1000 mL; 1.00 mL = 1.00 mg SO_4^{2-}.

g. Standard sulfate solutions: Prepare in appropriate concentrations from 10 to 300 mg SO_4^{2-}/L, using the stock sulfate solution.

4. Procedure

Set up the manifold as shown in Figure 4500-SO_4^{2-}:1 and follow the general procedure described by the manufacturer.

After use, rinse methylthymol blue and NaOH reagent lines in water for a few minutes, rinse them in the EDTA solution for 10 min, and then rinse in distilled water.

5. Calculation

Prepare standard curves by plotting peak heights of standards processed through the manifold against SO_4^{2-} concentrations in standards. Compute sample SO_4^{2-} concentration by comparing sample peak height with standard curve.

6. Precision and Bias

In a single laboratory a sample with an average concentration of about 28 mg SO_4^{2-}/L had a standard deviation of 0.68 mg/L and a coefficient of variation of 2.4%. In two samples with added SO_4^{2-}, recoveries were 91% and 100%.

7. Bibliography

LAZRUS, A.L., K.C. HILL & J.P. LODGE. 1965. A new colorimetric microdetermination of sulfate ion. *In* Automation in Analytical Chemistry. Technicon Symposium.

COLOROS, E., M.R. PANESAR & F.P. PERRY. 1976. Linearizing the calibration curve in determination of sulfate by the methylthymol blue method. *Anal. Chem.* 48:1693.

4500-SO_4^{2-} G. Methylthymol Blue Flow Injection Analysis

1. General Discussion

a. Principle: At pH 13.0 barium forms a blue complex with methylthymol blue (MTB). This gives a dark blue base line. The sample is injected into a low, but known, concentration of sulfate. The sulfate from the sample then reacts with the ethanolic barium-MTB solution and displaces the MTB from the barium to give barium sulfate and uncomplexed MTB. Uncomplexed MTB has a grayish color. The pH is raised with NaOH and the color of the gray uncomplexed MTB is measured at 460 nm. The intensity of gray color is proportional to the sulfate concentration.

Also see 4500-SO_4^{2-}.A and F, and Section 4130, Flow Injection Analysis (FIA).

b. Interferences: Remove large or fibrous particulates by filtering sample through glass wool. Guard against nitrate and nitrite contamination from reagents, water, glassware, and the sample preservation process.

A cation-exchange column removes multivalent cations such as Ca^{2+} and Mg^{2+}. A midrange sulfate standard containing a typical level of hardness as $CaCO_3$ can be run periodically to check the performance of the column. Any decrease in peak height from that of a sulfate standard without added $CaCO_3$ indicates the need to regenerate or replace the resin.

Neutralize samples that have pH less than 2. High acid concentrations can displace multivalent cations from the column.

Orthophosphate forms a precipitate with barium at high pH. If samples are known to be high in orthophosphate, make a recovery study using added amounts of sulfate, or run a sample blank containing only the orthophosphate matrix.

Also see 4500-SO_4^{2-}.F.1*b*.

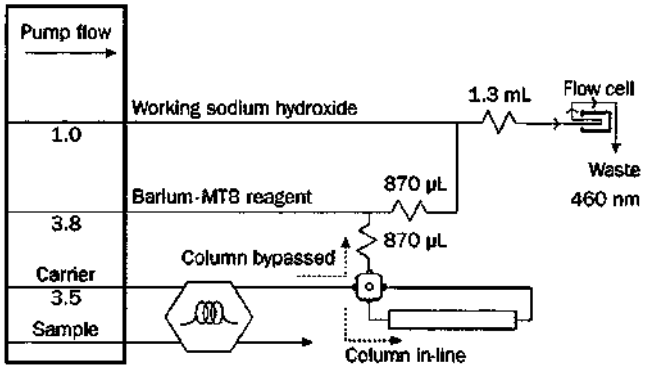


Figure 4500-SO_4^{2-}:2. FIA manifold.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

Flow injection analysis equipment consisting of:

a. FIA injection valve with sample loop or equivalent.

b. Multichannel proportioning pump.

c. FIA manifold (Figure 4500-SO_4^{2-}:2) with cation-exchange column and flow cell. Relative flow rates only are shown in Figure 4500-SO_4^{2-}:2. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material such as TFE.

d. Absorbance detector, 460 nm, 10-nm bandpass.

e. Injection valve control and data acquisition system.

3. Reagents

Use reagent water (>10 megohm) for all solutions. To prevent bubble formation, degas carrier and buffer with helium. Pass He at 140 kPa (20 psi) through a helium degassing tube. Bubble He through 1 L solution for 1 min. As an alternative to preparing reagents by weight/weight, use weight/volume.

a. Carrier solution, 0.30 mg SO_4^{2-}/L: To a tared 1-L container, add 0.30 g 1000 mg/L stock sulfate standard (¶ *h* below) and 999.7 g water. Shake or stir to mix. Degas with helium.

b. Barium chloride solution, 6.24m*M:* To a tared 1-L container, add 1.526 g barium chloride dihydrate, $BaCl_2 \cdot 2H_2O$, and 995 g water. Shake or stir until dissolved. Degas with helium.

c. Hydrochloric acid (HCl), 1.0*M:* To a tared 1-L container, add 913 g water and 99.6 g conc HCl (specific gravity 1.20, 37%). **Caution: Fumes.** Shake or stir to mix well. Degas with helium.

d. Barium—MTB color reagent: Note: The purity of the methylthymol blue and the denaturants in the alcohol are critical. Use the sources specified below, or test the material from alternative sources for suitability before using.

To a tared 500-mL dry brown plastic bottle, place 0.236 g methylthymol blue, 3,3′-bis[*N,N*-di(carboxymethyl)aminomethyl]-thymol-sulfonephthalein, pentasodium salt. Add 50 g barium chloride solution (¶ *b* above), which may be used to aid in transfer of the dye. Swirl to dissolve. Add 4.0 g of 1.0*M* HCl (¶ *c* above) and mix. The solution should turn orange. Add 71 g water and 321 g ethanol (ethyl alcohol, specially denatured anhydrous alcohol*). Stir or shake to mix well. The pH should be 2.5. Prepare solution the day before use and store, refrigerated, in a brown plastic bottle. Let warm to room temperature before using, then degas with helium.

e. Stock sodium hydroxide solution (NaOH), 50% (w/v) solution: To a glass 1-L container add 500 g water and 500.0 g NaOH. Dilute to 1 L. **Caution: The solution becomes very hot.** Shake or stir until dissolved. Cool to ambient. Store in a plastic bottle.

f. Working sodium hydroxide solution (NaOH), 0.18*M:* To a tared plastic 1-L container add 982 g water and 19.8 g stock NaOH solution (¶ *e* above). Shake or stir to mix. Degas with helium.

g. Cation exchange column preparation: Prepare approximately 0.5 g ion exchange resin,† 50 to 100 mesh, by mixing with sufficient water to make a slurry. Remove one end fitting from the threaded glass column. Fill column with water and aspirate slurry into column or let it settle into column by gravity. Take care to avoid trapping air bubbles in column or fittings at this point and during all subsequent operations. When resin has settled, replace end fitting. To ensure a good seal, remove any resin particles from the threads of glass, column end, and end fitting. To store column, join ends of the TFE tubing.

To test column effectiveness, make up two midrange standards, one of only sodium sulfate and the other with an identical concentration of sodium sulfate but with hardness typical of the samples. If the column is depleted, the standard with hardness will give a lower response because the divalent Mg^{2+} and Ca^{2+} cations are complexing with the free MTB. If depletion has occurred, repack column with fresh resin.

h. Stock sulfate standard, 1000 mg SO_4^{2-}/L: Dry approximately 2 g sodium sulfate, Na_2SO_4, at 105° overnight. Cool in a desiccator. In a 1-L volumetric flask, add 1.479 g of dried sodium sulfate to about 800 mL water. Dissolve by swirling, dilute to mark, and mix by inversion.

i. Standard solutions: Prepare sulfate standards in desired concentration range, using the stock standard (¶ *h* above), and diluting with water.

4. Procedure

Set up a manifold equivalent to that in Figure 4500-SO_4^{2-}:2 and follow method supplied by manufacturer or laboratory standard operating procedure. Follow quality control protocols outlined in Section 4020.

5. Calculations

Prepare standard curves by plotting adsorbance of standards processed through the manifold versus sulfate concentration.

6. Precision and Bias

a. Recovery and relative standard deviation: Table 4500-SO_4^{2-}:I gives results of single-laboratory studies.

b. MDL: A 180-μL sample loop was used in the method described above. Using a published MDL method,[1] analysts ran 21 replicates of a 5.00-mg SO_4^{2-}/L standard. These gave a mean of 4.80 mg SO_4^{2-}/L, a standard deviation of 0.69 mg SO_4^{2-}/L, and MDL of 1.8 mg SO_4^{2-}/L.

7. Reference

1. U.S. Environmental Protection Agency. 1984. Definition and procedure for the determination of method detection limits. Appendix B to 40 CFR 136 rev. 1.11 amended June 30, 1986. 49 CFR 43430.

* Aldrich 24, 511-9, or equivalent.
† BioRex 70, or equivalent.

TABLE 4500-SO_4^{2-}:I. RESULTS OF SINGLE-LABORATORY STUDIES WITH SELECTED MATRICES

Matrix	Sample/Blank Designation	Known Addition *mg* SO_4^{2-}/*L*	Recovery %	Relative Standard Deviation %
Wastewater treatment plant influent	Reference sample*	—	99	—
	Blank†	10.0	99	—
		20.0	99	—
	Site A‡	0	—	0.7
		10.0	109	—
		20.0	110	—
	Site B‡	0	—	0.7
		10.0	106	—
		20.0	112	—
	Site C‡	0	—	1.9
		10.0	104	—
		20.0	107	—
Wastewater treatment plant effluent	Reference sample*	—	99	—
	Blank†	10.0	95	—
		20.0	99	—
	Site A‡	0	—	0.9
		10.0	108	—
		20.0	108	—
	Site B‡	0	—	2.4
		10.0	107	—
		20.0	107	—
	Site C‡	0	—	0.6
		10.0	97	—
		20.0	104	—
Landfill leachate	Reference sample*	—	100	—
	Blank†	10.0	100	—
		20.0	99	—
	Site A‡	0	—	0.7
		10.0	106	—
		20.0	110	—
	Site B‡	0	—	0.5
		10.0	106	—
		20.0	107	—
	Site C‡	0	—	0.9
		10.0	101	—
		20.0	103	—

* U.S. EPA QC sample, 20.0 mg SO_4^{2-}/L.
† Determined in duplicate.
‡ Samples without known additions determined four times; samples with known additions determined in duplicate. Typical relative difference between duplicates 1%. Sample dilutions: Influent and effluent, all sites - fivefold; leachate A - 100-fold; B - 50-fold; C - 10-fold.

4500-S^{2-} SULFIDE*

4500-S^{2-} A. Introduction

1. Occurrence and Significance

Sulfide is often present in groundwater and sediment. It is produced by decomposition of organic matter and bacterial reduction of sulfate. It is sometimes found in industrial or municipal wastewater. Hydrogen sulfide escaping into the air from sulfide-containing wastewater causes odor nuisances. The threshhold odor concentration of H_2S in clean water is between 0.025 and 0.25 μg/L. Gaseous H_2S is very toxic and has claimed the lives of numerous workers. At levels toxic to humans it interferes with the olfactory system, giving a false sense of the safe absence of H_2S. It attacks metals directly, and indirectly has caused serious corrosion of concrete sewers because it is oxidized biologically in the presence of oxygen to H_2SO_4 on the pipe wall. Dissolved H_2S is toxic to fish and other aquatic organisms.

Hydrogen sulfide combines with iron and other metals in natural sediments and sludges to form slightly-soluble precipitates. Acid-volatile sulfide (AVS) is an important class of metal sulfides in these anoxic environments. The determination of AVS concentrations has become more prevalent because AVS is considered to be a key binding phase for controlling bioavailability of toxic metals in anoxic sediments.

AVS typically is determined by a purge-and-trap method in which hydrochloric acid is used to volatilize AVS at room temperature. The metals associated with the sulfides can be determined from the supernatant of the purged sample solution by using methods such as those in Part 3000. The hydrogen sulfide produced is trapped in zinc-acetate absorbing solution. AVS concentrations are measured by iodometric titration of the ZnS precipitated in the trap (4500-S^{2-}.F). Certain minerals, including iron pyrite, are partially digested by the AVS reagents at elevated temperatures, which may result in a significant overestimation of AVS. Iron pyrite can be partially digested to the extent of less than 10% of the total pyrite present. The addition of stannous chloride ($SnCl_2$) prevents the oxidation of sulfides by any liberated ferric iron.

2. Categories of Sulfides

Four categories of sulfide in water, wastewater, and sediment can be operationally defined:

a. Total sulfide includes dissolved H_2S and HS^- and acid-volatile metallic sulfides present in particulate matter. The pK_{a2} of H_2S is so high that the concentration of S^{2-} is negligible at all pH values. Copper and silver sulfides are so insoluble that they do not respond in ordinary sulfide determinations; they can be ignored for practical purposes.

b. Dissolved sulfide is that remaining after suspended solids have been removed by flocculation and settling. Flocculation and settling are used to separate dissolved and particulate sulfide because sulfide may be oxidized during filtration. Centrifugation also may be used.

c. Acid-volatile sulfide includes amorphous iron monosulfides, including mackinawite (FeS), greigite (Fe_3S_4), and pyrrhotite (FeS), and amorphous monosulfides of other metals. Pyrite, another iron sulfide mineral, is not included in the acid-volatile sulfides.

d. Un-ionized hydrogen sulfide can be calculated from the concentration of dissolved sulfide, the sample pH, and the conditional ionization constant of H_2S.

Figure 4500-S^{2-}:1 shows analytical flow paths for sulfide determinations under various conditions and options. None of the operationally defined sulfide categories includes pyrite or marcasite (FeS_2).

3. Sampling and Storage

Collect water samples with minimum aeration. Either analyze samples immediately after collection or preserve with zinc acetate solution for later analysis. To preserve a sample for a total sulfide determination, put zinc acetate and sodium hydroxide solutions into sample bottle before filling it with sample. Use 0.2 mL 2*M* zinc acetate solution per 100 mL sample. Increase volume of zinc acetate solution if the sulfide concentration is expected to be greater than 64 mg/L. The final pH should be at least 9. Add more NaOH if necessary. Fill bottle completely and stopper.

Sample sediments and sludges under nitrogen atmosphere if possible. Store samples at 4°C or frozen, and analyze within 2 weeks (1 month for frozen samples) of collection. Do not freeze-dry because acid-volatile sulfide may decompose as a result of oxidation artifacts.

4. Qualitative Tests

A qualitative test for sulfide often is useful. It is advisable in the examination of industrial wastes containing interfering substances that may give a false negative result in the methylene blue method (4500-S^{2-}.D).

a. Antimony test: To about 200 mL sample, add 0.5 mL saturated solution of antimony potassium tartrate and 0.5 mL 6*N* HCl in excess of phenolphthalein alkalinity.

Yellow antimony sulfide (Sb_2S_3) is discernible at a sulfide concentration of 0.5 mg/L. Comparisons with samples of known sulfide concentration make the technique roughly quantitative. The only known interferences are metallic ions, such as lead, which hold the sulfide so firmly that it does not produce Sb_2S_3, and dithionite, which decomposes in acid solution to produce sulfide.

b. Silver-silver sulfide electrode test: Dilute sample 1:1 with alkaline antioxidant reagent (see 4500-S^{2-}.G.3*a*). Measure electrode potential relative to a double-junction reference electrode and estimate the sulfide concentration from an old calibration

* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011.
Joint Task Group: 21st Edition—(4500-S^{2-}.A, H, I, J)—Thomas R. Holm (chair), Robert P. Fisher, Martin S. Frant, Christian Gagnon, Lorne R. Goodwin; 20th Edition—(4500-S^{2-}.I)—Scott Stieg (chair), Bradford R. Fisher, Owen B. Mathre, Theresa M. Wright.

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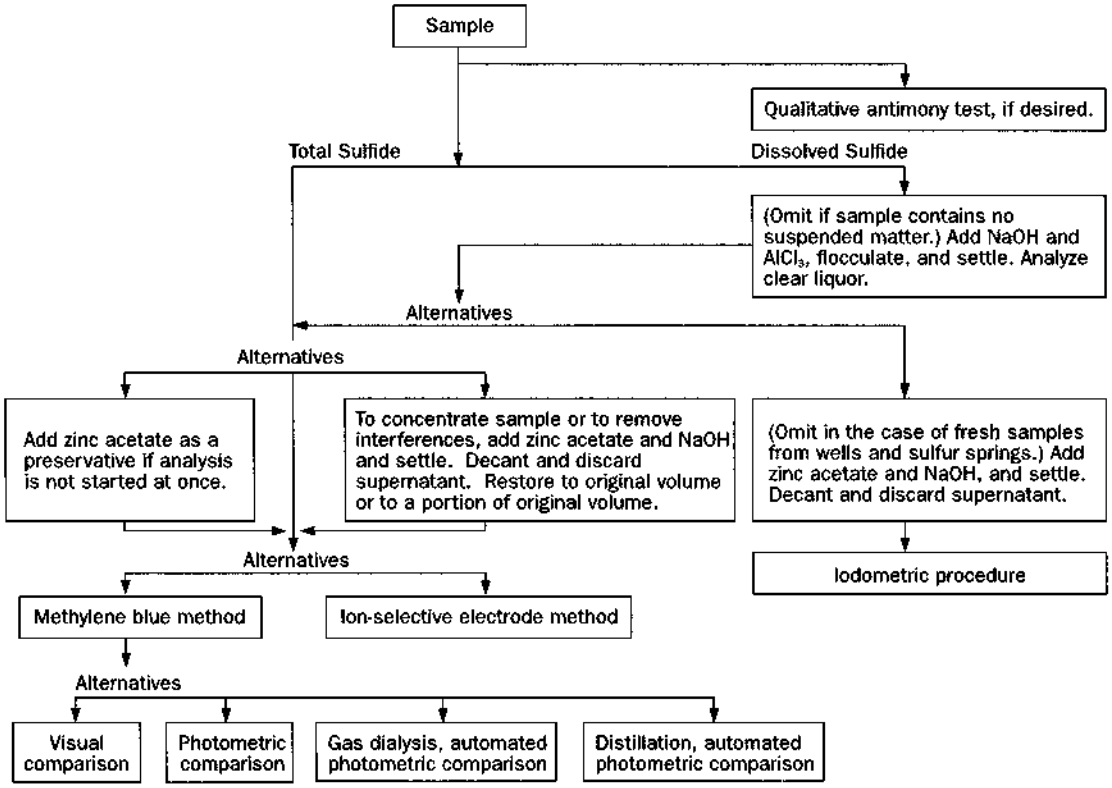


Figure 4500-S^{2-}:1. Analytical flow paths for sulfide determination.

curve or the example calibration curve in the electrode manual. This gives a reasonable estimate of sulfide concentration if the electrode is in good condition.

c. Lead acetate paper and silver foil tests: Confirm odors attributed to H_2S with lead acetate paper. On exposure to the vapor of a slightly acidified sample, the paper becomes blackened by formation of PbS. A strip of silver foil is more sensitive than lead acetate paper. Clean the silver by dipping in NaCN solution and rinse. **CAUTION: NaCN is toxic; handle with care.** Silver is suitable particularly for long-time exposure in the vicinity of possible H_2S sources because black Ag_2S is permanent, whereas PbS slowly oxidizes.

5. Selection of Quantitative Methods

Iodine oxidizes sulfide in acid solution. A titration based on this reaction is an accurate method for determining sulfide at concentrations above 1 mg/L if interferences are absent† and if loss of H_2S is avoided. The iodometric method (F) is useful for standardizing the methylene blue colorimetric methods (4500-S^{2-}.D, E, and I) and is suitable for analyzing samples freshly taken from wells or springs. The method can be used for wastewater and partly oxidized water from sulfur springs if interfering substances are removed first. The automated methylene blue method with distillation (4500-S^{2-}.I) is useful for a variety of samples containing more than 1 mg S^{2-}/L.

The methylene blue method (4500-S^{2-}.D) is based on the reaction of sulfide, ferric chloride, and dimethyl-*p*-phenylenediamine to produce methylene blue. Ammonium phosphate is added after color development to remove ferric chloride color. The procedure is applicable at sulfide concentrations between 0.1 and 20.0 mg/L. The automated methylene blue method (4500-S^{2-}.E) is similar to 4500-S^{2-}.D. A gas dialysis technique separates the sulfide from the sample matrix. Gas dialysis eliminates most interferences, including turbidity and color. The addition of the antioxidant ascorbic acid improves sulfide recoveries. The method is applicable at sulfide concentrations between 0.002 and 0.100 mg/L.

Potentiometric methods using a silver/sulfide ion-selective electrode (4500-S^{2-}.G) may be suitable. The sulfide concentration can be estimated from the potential of the electrode relative to a reference electrode, but careful attention to details of procedures and frequent standardizations are needed to secure good results. The electrode is useful particularly as an endpoint indicator for titration of dissolved sulfide with silver nitrate. The ion-selective electrode method is unaffected by sample color or turbidity and is applicable for concentrations greater than 0.03 mg/L.

6. Preparation of Sulfide Standards

Take care in preparing reliable stock solutions of sulfide for calibration and quality control. Prepare sulfide standards from

† Many substances can reduce iodine; all of these are potential interferences in procedures using this chemistry.

sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$) crystals. These crystals usually have excess water present on the surface, in addition to a layer of contamination from oxidation products (polysulfides, polythionates, and sulfate) of sulfide reacting with atmospheric oxygen. Further, solutions of sulfide are prone to ready oxidation by dissolved and atmospheric oxygen. Use reagent water to prepare sulfide standards and sample dilutions. Degas the water with either argon or nitrogen. Purchase the smallest amount of solid standards possible and keep no longer than 1 year. Preferably handle and store solid sulfide standards and stock solutions in an inert atmosphere glove bag or glove box to reduce contamination due to oxidation.

Preferably remove single crystals of $Na_2S \cdot 9H_2O$ from reagent bottle with nonmetallic tweezers; quickly rinse in degassed reagent water to remove surface contamination. Blot crystal dry with a tissue, then rapidly transfer to a tared, stoppered weighing bottle containing 5 to 10 mL degassed reagent water. Repeat procedure until desired amount of sodium sulfide is in weighing bottle. Avoid excess agitation and mixing of the solution with atmospheric oxygen. Quantitatively transfer and dilute entire contents of weighing bottle to an appropriate size volumetric flask with degassed reagent water to prepare a known concentration sulfide stock solution (3.75 g $Na_2S \cdot 9H_2O$ diluted to a final volume of 500 mL will give a stock solution of which 1.00 mL = 1.00 mg S^{2-}). Standardize stock solution using the iodometric method, 4500-S^{2-}.F. Alternatively, purchase precertified stock solutions of sulfide. Verify concentration of stock solution daily using the iodometric method (4500-S^{2-}.F). Store stock solution with minimum headspace for no more than 1 week.

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4500-S^{2-} B. Separation of Soluble and Insoluble Sulfides

Unless the sample is entirely free from suspended solids (dissolved sulfide equals total sulfide), to measure dissolved sulfide first remove insoluble matter. This can be done by producing an aluminum hydroxide floc that is settled, leaving a clear supernatant for analysis.

1. Apparatus

Glass bottles with stoppers: Use 100 mL if sulfide will be determined by the methylene blue method and 500 to 1000 mL if by the iodometric method.

2. Reagents

a. Sodium hydroxide solution (NaOH), 6*N*.

b. Aluminum chloride solution: Because of the hygroscopic and caking tendencies of this chemical, purchase 100-g bottles of $AlCl_3 \cdot 6H_2O$. Dissolve contents of a previously unopened 100-g bottle in 144 mL distilled water.

3. Procedure

a. To a 100-mL glass bottle, add 0.2 mL (nominally 4 drops) 6*N* NaOH. Fill bottle with sample and immediately add 0.2 mL (4 drops) $AlCl_3$ solution. Stopper bottle with no air under stopper. Rotate back and forth about a transverse axis vigorously for 1 min or longer to flocculate contents. Vary volumes of these added chemicals to get good clarification without using excessively large amounts and to produce a pH of 6 to 9. If a 500- or 1000-mL bottle is used, add proportionally larger amounts of reagents.

b. Let settle until reasonably clear supernatant can be drawn off. With proper flocculation, this may take 5 to 15 min. Do not wait longer than necessary.

c. Either analyze the supernatant immediately or preserve with 2*N* zinc acetate (see 4500-S^{2-}.C).

4500-S^{2-} C. Sample Pretreatment to Remove Interfering Substances or to Concentrate the Sulfide

The iodometric method suffers interference from reducing substances that react with iodine, including thiosulfate, sulfite, and various organic compounds, both solid and dissolved.

Strong reducing agents also interfere in the methylene blue method (4500-S^{2-}.D) by preventing formation of the blue color. Thiosulfate at concentrations about 10 mg/L may retard color formation or completely prevent it. Ferrocyanide produces a blue

color. Sulfide itself prevents the reaction if its concentration is very high, in the range of several hundred milligrams per liter. To avoid the possibility of false negative results, use the antimony method to obtain a qualitative result in industrial wastes likely to contain sulfide but showing no color by the methylene blue method. Iodide, which is likely to be present in oil-field wastewaters, may diminish color formation if its concentration exceeds 2 mg/L. Many metals (e.g., Hg, Cd, Cu) form insoluble sulfides and give low recoveries.

Eliminate interferences due to sulfite, thiosulfate, iodide, and many other soluble substances, but not ferrocyanide, by first precipitating ZnS, removing the supernatant, and replacing it with distilled water. Use the same procedure, even when not needed for removal of interferences, to concentrate sulfide. The automated methylene blue method (4500-S^{2-}.E) is relatively free from interferences because gas dialysis separates the sulfide from the sample matrix.

1. Apparatus

Glass bottles with stoppers: See 4500-S^{2-}.B.1.

2. Reagents

a. Zinc acetate solution: Dissolve 220 g $Zn(C_2H_3O_2)_2 \cdot 2H_2O$ in 870 mL water; this makes 1 L solution.

b. Sodium hydroxide solution (NaOH), 6*N*.

3. Procedure

a. Put 0.20 mL (4 drops) zinc acetate solution and 0.10 mL (2 drops) 6*N* NaOH into a 100-mL glass bottle, fill with sample, and add 0.10 mL (2 drops) 6*N* NaOH solution. Stopper with no air bubbles under stopper and mix by rotating back and forth vigorously about a transverse axis. For the iodometric procedure, use a 500-mL bottle or other convenient size, with proportionally larger volumes of reagents. Vary volume of reagents added according to sample so the resulting precipitate is not excessively bulky and settles readily. Add enough NaOH to raise the pH above 9. Let precipitate settle for 30 min. The treated sample is relatively stable and can be held for several hours. However, if much iron is present, oxidation may be fairly rapid.

b. If the iodometric method is to be used, collect precipitate on a glass fiber filter and continue at once with titration according to the procedure of 4500-S^{2-}.F. If the methylene blue method (4500-S^{2-}.D) is used, let precipitate settle for 30 min and decant as much supernatant as possible without loss of precipitate. Refill bottle with distilled water, shake to resuspend precipitate, and quickly withdraw a sample. If interfering substances are present in high concentration, settle, decant, and refill a second time. If sulfide concentration is known to be low, add only enough water to bring volume to one-half or one-fifth of original volume. Use this technique for analyzing samples of very low sulfide concentrations. After determining the sulfide concentration colorimetrically, multiply the result by the ratio of final to initial volume. No concentration or pretreatment steps to remove interferences are necessary for 4500-S^{2-}.E.

4500-S^{2-} D. Methylene Blue Method

1. Apparatus

a. Matched test tubes, approximately 125 mm long and 15 mm OD.

b. Droppers, delivering 20 drops/mL methylene blue solution. To obtain uniform drops hold dropper in a vertical position and let drops form slowly.

c. If photometric rather than visual color determination will be used, either:

1) *Spectrophotometer,* for use at a wavelength of 664 nm with cells providing light paths of 1 cm and 1 mm, or other path lengths, or

2) *Filter photometer,* with a filter providing maximum transmittance near 660 nm.

2. Reagents

a. Amine-sulfuric acid stock solution: Dissolve 27 g *N*,*N*-dimethyl-*p*-phenylenediamine oxalate* in an iced mixture of 50 mL conc H_2SO_4 and 20 mL distilled water. Cool and dilute to 100 mL with distilled water. Use fresh oxalate because an old supply may be oxidized and discolored to a degree that results in interfering colors in the test. Store in a dark glass bottle. When this stock solution is diluted and used in the procedure with a sulfide-free sample, it first will be pink but then should become colorless within 3 min.

b. Amine-sulfuric acid reagent: Dilute 25 mL amine-sulfuric acid stock solution with 975 mL 1 + 1 H_2SO_4. Store in a dark glass bottle.

c. Ferric chloride solution: Dissolve 100 g $FeCl_3 \cdot 6H_2O$ in 40 mL water.

d. Sulfuric acid solution (H_2SO_4), 1 + 1.

e. Diammonium hydrogen phosphate solution: Dissolve 400 g $(NH_4)_2HPO_4$ in 800 mL distilled water.

f. Methylene blue solution I: Use USP grade dye or one certified by the Biological Stain Commission. The dye content should be reported on the label and should be 84% or more. Dissolve 1.0 g in distilled water and make up to 1 L. This solution will be approximately the correct strength, but because of variation between different lots of dye, standardize against sulfide solutions of known strength and adjust its concentration so 0.05 mL (1 drop) = 1.0 mg sulfide/L.

Standardization—Prepare five known-concentration sulfide standards ranging from 1 to 8 mg/L as described in 4500-S^{2-}.A.6, or proceed as follows: Put several grams of clean,

* Eastman Cat. No. 5672 has been found satisfactory for this purpose.

washed crystals of $Na_2S \cdot 9H_2O$ into a small beaker. Add somewhat less than enough water to cover crystals. Stir occasionally for a few minutes, then pour solution into another vessel. This solution reacts slowly with oxygen but the change is insignificant if analysis is performed within a few hours. Prepare solution daily. To 1 L distilled water, add 1 drop of Na_2S solution and mix. Immediately determine sulfide concentration by the meth-ylene blue procedure and by the iodometric procedure. Repeat, using more than 1 drop Na_2S solution or smaller volumes of water, until at least five tests have been made, with a range of sulfide concentrations between 1 and 8 mg/L. Calculate average percent error of the methylene blue result as compared to the iodometric result. If the average error is negative (i.e., methylene blue results are lower than iodometric results), dilute methylene blue solution by the same percentage, so a greater volume will be used in matching colors. If methylene blue results are high, increase solution strength by adding more dye.

g. Methylene blue solution II: Dilute 10.00 mL of adjusted methylene blue solution I to 100 mL with reagent water.

3. Procedure

a. Color development: Transfer 7.5 mL sample to each of two matched test tubes, using a special wide-tip pipet or filling to marks on test tubes. If sample has been preserved with zinc acetate, shake vigorously before taking subsample. Add to Tube A 0.5 mL amine-sulfuric acid reagent and 0.15 mL (3 drops) $FeCl_3$ solution. Mix immediately by inverting slowly, only once. (Excessive mixing causes low results by loss of H_2S as a gas before it has had time to react). To Tube B add 0.5 mL 1 + 1 H_2SO_4 and 0.15 mL (3 drops) $FeCl_3$ solution and mix. The presence of S^{2-} will be indicated by the appearance of blue color in Tube A. Color development usually is complete in about 1 min, but a longer time often is required for fading out of the initial pink color. Wait 3 to 5 min and add 1.6 mL $(NH_4)_2HPO_4$ solution to each tube. Wait 3 to 15 min and make color comparisons. If zinc acetate was used, wait at least 10 min before making a visual color comparison.

b. Color determination:

1) Visual color estimation—Add methylene blue solution I or II, depending on sulfide concentration and desired accuracy, dropwise, to the second tube, until color matches that developed in first tube. If the concentration exceeds 20 mg/L, repeat test with a portion of sample diluted tenfold.

With methylene blue solution I, adjusted so 0.05 mL (1 drop) = 1.0 mg S^{2-}/L when 7.5 mL of sample are used:

$$\text{mg } S^{2-}/\text{L} = \text{no. drops solution I} + 0.1\ (\text{no. drops solution II})$$

2) Photometric color measurement—A cell with a light path of 1 cm is suitable for measuring sulfide concentrations from 0.1 to 2.0 mg/L. Use shorter or longer light paths for higher or lower concentrations. This method is suitable for sample concentrations up to 20 mg/L. Zero instrument with a portion of treated sample from Tube B. Prepare calibration curves on basis of colorimetric tests made on Na_2S solutions simultaneously analyzed by the iodometric method, plotting concentration vs. absorbance. A linear relationship between concentration and absorbance can be assumed from 0 to 1.0 mg/L.

Read sulfide concentration from calibration curve.

4. Precision and Bias

In a study by two chemists working in the same laboratory, the standard deviation estimated from 34 sets of duplicate sulfide measurements was 0.04 mg/L for concentrations between 0.2 and 1.5 mg/L. The average recoveries of known additions were 92% for 40 samples containing 0.5 to 1.5 mg/L and 89% for samples containing less than 0.1 mg/L.

5. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 4020:I.

6. Bibliography

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4500-S^{2-} E. Gas Dialysis, Automated Methylene Blue Method

1. Apparatus

Automated analytical equipment: An example of the continuous-flow analytical instrument consists of the interchangeable components shown in Figure 4500-S^{2-}:2.

The sampler is equipped with a mixer to stir samples before analysis and the gas dialysis membrane, which is maintained at room temperature, separates H_2S from the sample matrix.

2. Reagents

a. N,N-*dimethyl*-p-*phenylenediamine stock solution:* Dissolve 1 g *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride in 500 mL 6*N* HCl. Prepare fresh monthly. Store in an amber bottle.

b. N,N-*dimethyl*-p-*phenylenediamine working solution:* Dilute 190 mL *N,N*-dimethyl-*p*-phenylenediamine stock solution to 1 L. Store in an amber bottle. Prepare weekly.

c. Ferric chloride stock solution: Dissolve 13.5 g $FeCl_3 \cdot 6H_2O$ in 500 mL 5*N* HCl. Store in an amber bottle. Prepare fresh monthly.

d. Working ferric chloride solution: Dilute 190 mL ferric chloride stock solution to 1 L. Store in an amber bottle. Prepare fresh weekly.

e. Hydrochloric acid (HCl), 6*N*.

f. Sodium hydroxide stock solution (NaOH), 1*N*.

g. Sodium hydroxide (NaOH), 0.01*N*: Dilute 10 mL NaOH stock solution to 1 L.

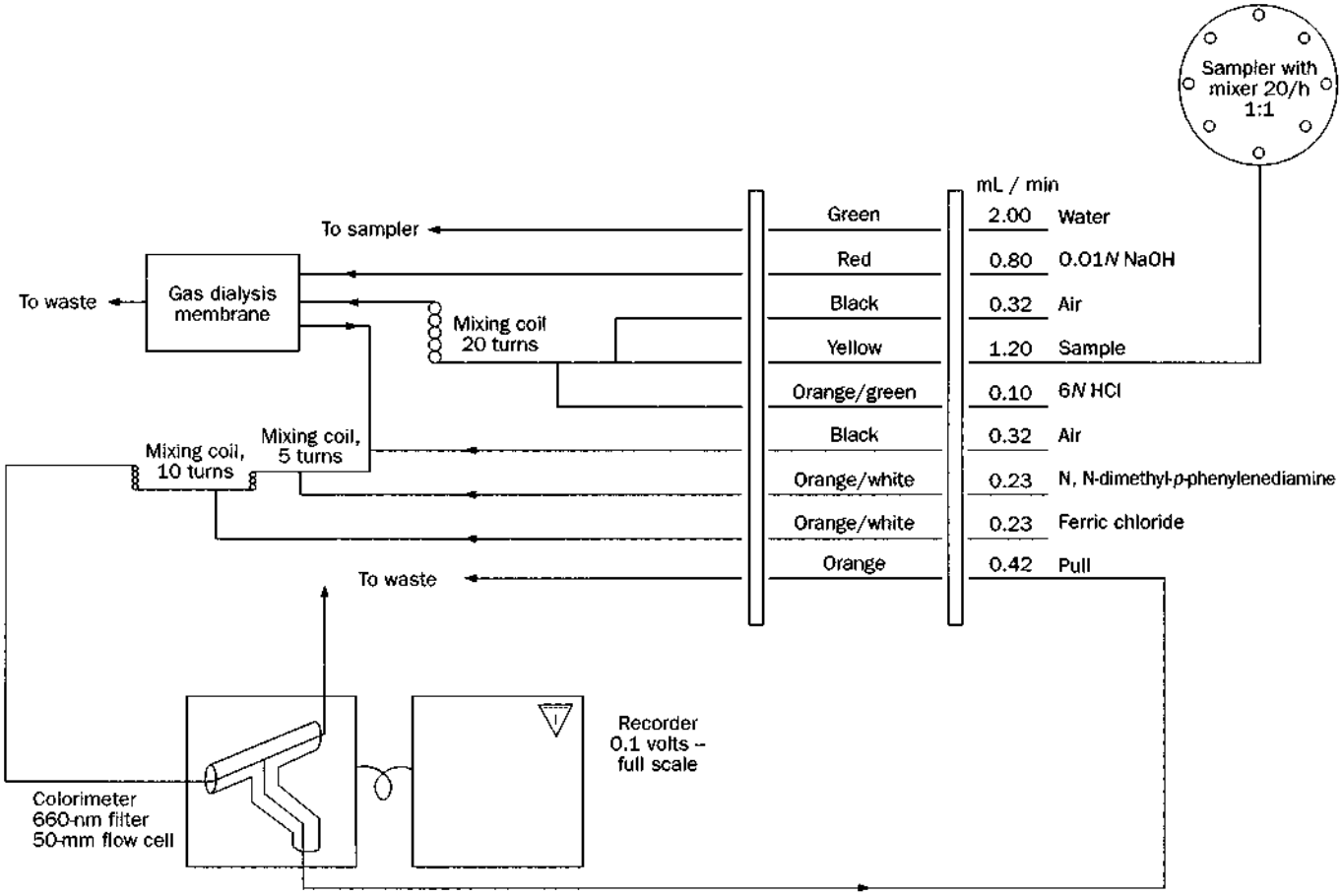


Figure 4500-S^{2-}:2. Sulfide manifold.

h. Sulfide stock solution, 1.00 mg S^{2-}/1.00 mL: See 4500-S^{2-}.A.6.

i. Sulfide intermediate standard solution: Dilute 10 mL sulfide stock solution to 1 L with water. Prepare fresh daily. Standardize by iodometric titration method, 4500-S^{2-}.F. 1 mL ≈ 0.01 mg S^{2-}.

j. Sulfide tertiary standard solution: Dilute 50 mL sulfide intermediate solution to 500 mL with 0.01*N* NaOH. Prepare fresh daily. Use standardization value from ¶ *i* above to determine exact concentration. 1.00 mL ≈ 0.001 mg S^{2-}.

k. Working sulfide standard solutions: Prepare a suitable series of standards by diluting appropriate volumes of sulfide tertiary standing solutions with 0.01*N* NaOH. Prepare fresh daily.

l. Zinc acetate preservative solution: Dissolve 220 g $Zn(C_2H_3O_2)_2 \cdot 2H_2O$ in 870 mL water (this makes 1 L solution).

3. Procedure

For unpreserved, freshly collected samples and sulfide working standards, add, in order, 4 drops 2*N* zinc acetate, 0.5 mL 6*N* NaOH, and 400 mg ascorbic acid/100 mL. For preserved samples, add 0.5 mL 6*N* NaOH and 400 mg ascorbic acid/100 mL. Shake well.

Let precipitate settle for at least 30 min. Pour a portion of well-mixed sample or working standard into a sample cup. Set up manifold as shown in Figure 4500-S^{2-}:2 and follow the general procedure described by the manufacturer. Determine absorbance at 660 nm.

4. Calculation

Prepare standard curves by plotting peak heights of standards processed through the manifold against S^{2-} concentration in the standards. Compute S^{2-} sample concentration by comparing sample response with standard curve.

5. Precision and Bias

In a single laboratory, samples with S^{2-} concentrations of 0.012, 0.015, 0.034, and 0.085 mg/L had standard deviations of 0.001, 0.001, 0.001, and 0.001 mg/L, respectively, with coefficients of variation of 8.3%, 6.3%, 2.9%, and 1.2%, respectively. In two environmental samples with added S^{2-}, recoveries were 104.2 and 97.6%.

6. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 4020:I.

7. Bibliography

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4500-S^{2-} F. Iodometric Method

1. Reagents

a. Hydrochloric acid (HCl), 6*N*.

b. Standard iodine solution, 0.0250*N:* Dissolve 20 to 25 g KI in a little water and add 3.2 g iodine. After iodine has dissolved, dilute to 1000 mL and standardize against 0.0250*N* $Na_2S_2O_3$, using starch solution as indicator.

c. Standard sodium thiosulfate solution, 0.0250*N:* See Section 4500-O.C.2*e*.

d. Starch solution: See Section 4500-O.C.2*d*.

2. Procedure

a. Measure from a buret into a 500-mL flask an amount of iodine solution estimated to be an excess over the amount of sulfide present. Add distilled water, if necessary, to bring volume to about 20 mL. Add 2 mL 6*N* HCl. Pipet 200 mL sample into flask, discharging sample under solution surface. If iodine color disappears, add more iodine until color remains. Back-titrate with $Na_2S_2O_3$ solution, adding a few drops of starch solution as endpoint is approached, and continuing until blue color disappears.

b. If sulfide was precipitated with zinc and ZnS filtered out, return filter with precipitate to original bottle and add about 100 mL water. Add iodine solution and HCl and titrate as in ¶ *a* above.

3. Calculation

One milliliter 0.0250*N* iodine solution reacts with 0.4 mg S^{2-}:

$$\text{mg } S^{2-}/\text{L} = \frac{[(A \times B) - (C \times D)] \times 16\,000}{\text{mL sample}}$$

where:

A = mL iodine solution,
B = normality of iodine solution,
C = mL $Na_2S_2O_3$ solution, and
D = normality of $Na_2S_2O_3$ solution.

4. Precision

The precision of the endpoint varies with the sample. In clean waters it should be determinable within 1 drop, which is equivalent to 0.1 mg/L in a 200-mL sample.

5. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 4020:I.

4500-S^{2-} G. Ion-Selective Electrode Method

1. General Discussion

a. Principle: The potential of a silver/sulfide ion-selective electrode (ISE) is related to the sulfide ion activity. An alkaline antioxidant reagent (AAR) is added to samples and standards to inhibit oxidation of sulfide by oxygen and to provide a constant ionic strength and pH. Use of the AAR allows calibration in terms of total dissolved sulfide concentration. All samples and standards must be at the same temperature. Sulfide concentrations between 0.032 mg/L ($1 \times 10^{-6}M$) and 100 mg/L can be measured without preconcentration. For lower concentrations, preconcentration is necessary.

b. Interferences: Humic substances may interfere with Ag/S-ISE measurements. For highly colored water (high concentration of humic substances), use the method of standard additions to check results. Sulfide is oxidized by dissolved oxygen. Sulfide oxidation may cause potential readings to drift in the direction of decreasing concentration (i.e., to more positive values). Flush surface of samples and standards with nitrogen to minimize contact with atmospheric oxygen for low-level measurements. Temperature changes may cause potentials to drift either upward or downward. Therefore, let standards and samples come to the same temperature. If samples cannot be analyzed immediately, preserve dissolved sulfide by precipitating with zinc acetate (4500-S^{2-}.C).

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

*a. Silver/sulfide electrode.**

b. Double-junction reference electrode.

* Orion 941600, or equivalent.

c. Electrode polishing strips.†

d. pH meter with millivolt scale, capable of 0.1-mV resolution. Meters that can be calibrated in concentration and that perform standard-additions calculations are available.

e. Electrochemical cell: Make suitable cell from a 150-mL beaker and a sheet of rigid plastic (PVC or acrylic) with holes drilled to allow insertion of the electrodes and a tube for flushing the headspace with nitrogen. Alternatively, purchase a polarographic cell with gas transfer tube.‡

f. Gas dispersion tube: Use to deaerate water for preparing reagents and standards.

g. Magnetic stirrer and stirring bar: Use a piece of styrofoam or cardboard to insulate the cell from the magnetic stirrer.

3. Reagents

a. Alkaline antioxidant reagent (AAR): To approximately 600 mL deaerated reagent water (DRW) in a 1-L volumetric flask, add 80 g NaOH, 35 g ascorbic acid, and 67 g Na_2H_2EDTA. Swirl to dissolve and dilute to 1 L. The color of freshly prepared AAR will range from colorless to yellow. Store in a tightly capped brown glass bottle. Discard when solution becomes brown.

b. Lead perchlorate, 0.1*M:* Dissolve 4.60 g $Pb(ClO_4)_2 \cdot 3H_2O$ in 100 mL reagent water. Standardize by titrating with Na_2H_2EDTA. Alternatively, use commercially available 0.1*M* $Pb(ClO_4)_2$ solutions.

c. Sulfide stock solution, 130 mg/L: See 4500-S^{2-}.A.6, and dilute 13.0 mL of 1.00 mg S^{2-}/mL stock to 100.0 mL with AAR. Alternatively, add 500 mL AAR and 1 g $Na_2S \cdot 9H_2O$ to a 1-L volumetric flask; dissolve. Dilute to 1 L with DRW. Use deaerated artificial seawater (DASW), Table 8010:III, or 0.7*M* NaCl if sulfide concentrations are to be determined in seawater. Standardize stock solution by titrating with 0.1*M* $Pb(ClO_4)_2$. Pipet 50 mL sulfide stock solution into the electrochemical cell. (Use 10 mL with a small-volume polarographic cell.) Insert Ag/S electrode and reference electrode and read initial potential. Titrate with 0.1*M* $Pb(ClO_4)_2$. Let electrode potential stabilize and record potential after each addition. Locate equivalence point as in Section 4500-Cl^-.D.4*a*. Alternatively, linearize the titration curve.[1] Calculate the function F_1 for points before the equivalence point.

$$F_1 = (V_o + V)10^{\frac{E}{m}}$$

where:

V_o = volume of stock solution, mL,
V = titrant volume, mL,
E = potential, mV, and
m = slope of calibration curve, mV/log unit.

Plot F_1 as a function of titrant volume. Extrapolate to find the intersection with the x-axis; that is, the equivalence point. Calculate sulfide concentration in the stock solution from:

$$C = \frac{V_{eq}[Pb]}{V_o}$$

where:

C = sulfide concentration, mg/L,
V_{eq} = equivalence volume, mL,
$[Pb]$ = concentration of Pb in titrant, mg/L, and
V_o = volume of stock solution, mL.

Store stock solution in a tightly capped bottle for 1 week or less. The stock solution also can be standardized iodometrically (see 4500-S^{2-}.F). **Caution: Store in a fume hood.**

d. Sulfide standards: Prepare sulfide standards daily by serial dilution of stock. Add AAR and $Zn(C_2H_3O_2)_2$ solutions to 100-mL volumetric flasks. Add sulfide solutions and dilute to volume with DRW (or DASW). Refer to Table 4500-S^{2-}:I for volumes. Prepare at least one standard with a concentration less than the lowest sample concentration.

4. Procedure

Check electrode performance and calibrate daily. Check electrode potential in a sulfide standard every 2 h. The procedure depends on the sulfide concentration and the time between sample collection and sulfide determination. If the total sulfide concentration is greater than 0.03 mg/L ($1 \times 10^{-6}M$) and the time delay is only a few minutes, sulfide can be determined directly. Otherwise, precipitate ZnS and filter as described in 4500-S^{2-}.C.

a. Check electrode performance: Pipet 50 mL AAR, 50 mL DWR, and 1 mL sulfide stock solution into the measurement cell. Place Ag/S and reference electrodes in the solution and read potential. Add 10 mL stock solution and read potential. The change in potential should be -28 ± 2 mV. If it is not, follow the troubleshooting procedure in the electrode manual.

b. Calibration: Place electrodes in the most dilute standard but use calibration standards that bracket the sulfide concentrations in the samples. Record potential when the rate of change is less than 0.3 mV/min. [This may take up to 30 min for very low sulfide concentrations (i.e., $<$ 0.03 mg/L.)] Rinse electrodes, blot dry with a tissue, and read potential of the next highest standard. For a meter that can be calibrated directly in concentration, follow manufacturer's directions. For other meters, plot potential as a function of the logarithm (base 10) of the sulfide concentration. For potentials in the linear range, calculate the slope and intercept of the linear portion of the calibration plot.

c. Sulfide determination by comparison with calibration curve, no ZnS precipitation: Add 40 mL AAR, 0.15 mL (3 drops) zinc

Table 4500-S^{2-}:I. Dilution of Sulfide Stock Solution for Preparation of Standards (100 mL Total Volume)

Dilution	Alkaline Antioxidant Reagent *mL*	Sulfide Solution	Sulfide Solution *mL*	1*M* Zinc Acetate *mL*
1:10	45	Stock	10	0.15
1:100	50	Stock	1	0.15
1:1 000	45	1:100	10	0.14
1:10 000	50	1:100	1	0.15

† Orion 948201, or equivalent.
‡ EG&G Princeton Applied Research K0066, K0060, G0028, or equivalent.

acetate, and 50 mL sample to a 100-mL volumetric flask. Dilute to 100 mL with AAR. Pour into the electrochemical cell and insert the electrodes. Record potential when the rate of change is less than 0.3 mV/min. Read sulfide concentration from the calibration curve. Alternatively, for potentials in the linear range, calculate the sulfide concentration from:

$$S_{Tot} = 10^{\frac{E-b}{m}}$$

where:

E = electrode potential, and
b and m are the intercept and slope of the calibration curve.

For a meter that can be calibrated directly in concentration, follow the manufacturer's directions.

d. Sulfide determination by comparison with calibration curve, with ZnS precipitation: Place filter with ZnS precipitate in a 150-mL beaker containing a stir bar. Wash sample bottle with 50 mL AAR and 20 mL DRW and pour the washings into the beaker. Stir to dissolve precipitate. Remove filter with forceps while rinsing it into the beaker with a minimum amount of DRW. Quantitatively transfer to a 100-mL volumetric flask and dilute to mark with DRW. Pour into the electrochemical cell and place the electrodes in the solution. Measure potential as in ¶ *c* above. Calculate sulfide concentration (¶ *c* above).

e. Sulfide determination by standard addition with or without ZnS precipitation: Measure the Ag/S-ISE electrode potential as in ¶s *c* or *d* above. Add sulfide stock solution and measure potential again. Calculate sulfide concentration as follows:

$$C_o = \frac{fC_s}{(1+f)10^{\frac{E_s-E_o}{m}} - 1}$$

where:

C_o, C_s = sulfide concentrations in sample and known addition,
f = ratio of known-addition volume to sample volume,
E_o, E_s = potentials measured for sample and known addition, and
m = slope of calibration curve (approximately 28 mV/log S^{2-}.

f. Sulfide determination by titration: Use the same procedure as for standardizing the sulfide stock solution (4500-S^{2-}.G.3*c*). The minumum sulfide concentration for determination by titration is 0.3 mg/L ($10^{-5}M$).

5. Precision

For sulfide determination by comparison with the calibration curve, the relative standard deviation varies with the sulfide concentration. RSD values of 23% for 0.0091 mg/L and 5% for 0.182 mg/L have been reported.[2] [0.0091 μg/L was below the range for which the potential varied linearly with the logarithm of the sulfide concentration (i.e., the Nernstian range.)] For sulfide determination by standard addition, the precision is greatest if the amount of sulfide added is as large as possible while staying within the linear range.[3]

6. References

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2. Baumann, E. 1974. Determination of parts per billion sulfide in water with the sulfide-selective electrode. *Anal. Chem.* 46:1345.
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4500-S^{2-} H. Calculation of Un-ionized Hydrogen Sulfide

Hydrogen sulfide (H_2S) and bisulfide ion (HS^-), which together constitute dissolved sulfide, are in equilibrium with hydrogen ions:

$$H_2S \rightleftharpoons H^+ + HS^-$$

The fraction of sulfide present as un-ionized H_2S can be estimated with an error of less than 40% from Figure 4500-S^{2-}:3. If more accuracy is needed, use the methods given below. For both fresh water and seawater, it is convenient to define "conditional" dissociation constants, which are valid for the temperature and ionic strength of the water of interest. In the following mass-action equation for fresh water, K'_{FW} is a mixed equilibrium constant that relates the hydrogen ion activity (calculated from the pH) and the concentrations of H_2S and HS^-:

$$K'_{FW} = \frac{[H^+]\,[HS^-]}{[H_2S]}$$

The square brackets indicate concentrations and the braces indicate activity. The value of pK'_{FW} for H_2S is approximately 7.0 ± 0.3 for the ionic strengths and temperatures likely to be encountered in water-quality monitoring. For seawater, it is convenient to use a stoichiometric equilibrium constant (K'_{SW}), which relates the concentrations of H^+, HS^-, and H_2S:

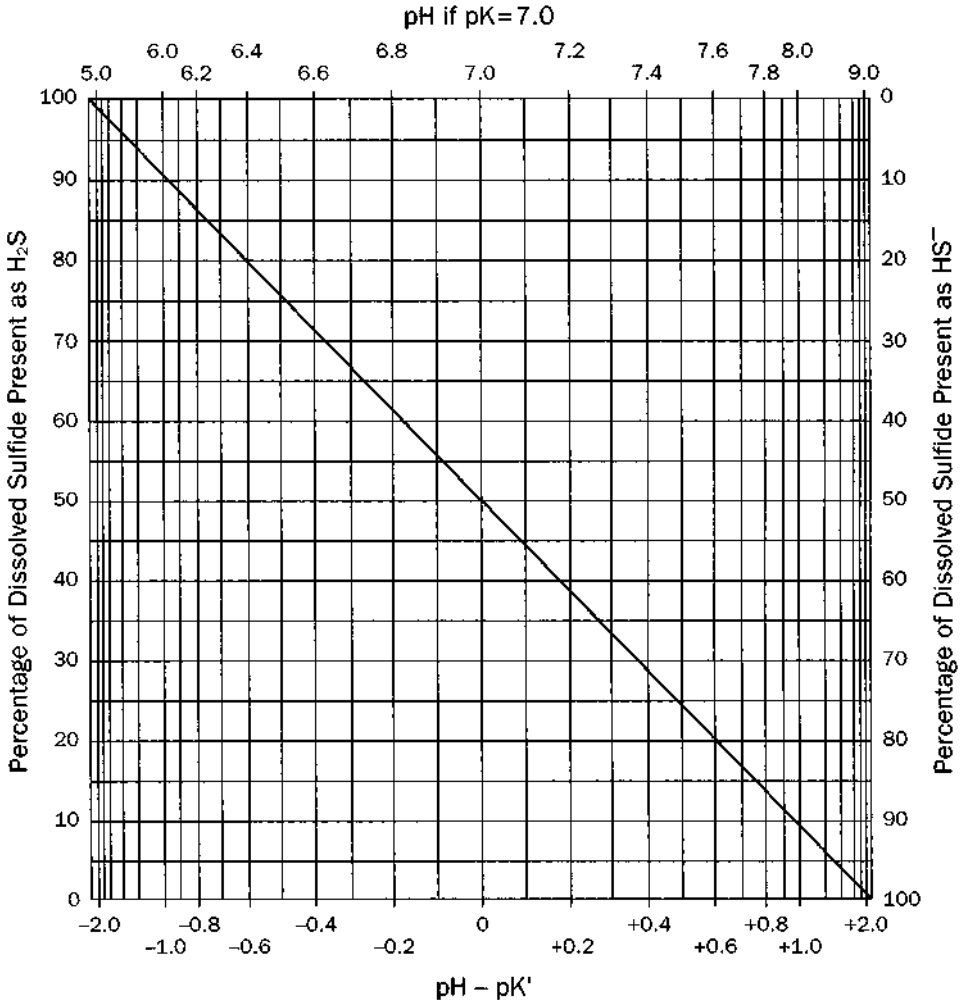


Figure 4500-S^{2-}:3. Proportion of H_2S and HS^- in dissolved sulfide.

$$K'_{SW} = \frac{[H^+][HS^-]}{[H_2S]}$$

The mass-action equations can be rearranged to give:

$$pH - pK' = \log \frac{[HS^-]}{[H_2S]}$$

In this equation, pK' can be either pK'_{FW} or pK'_{SW}. The fraction of un-ionized H_2S can either be read from Figure 4500-S^{2-}:3 or calculated with the following equation:

$$\alpha_{H_2S} = \frac{[H_2S]}{S_T} = \frac{1}{10^{pH - pK'} + 1}$$

where:

S_T = total dissolved sulfide concentration.

1. Calculation for Fresh Water ($I \leq 0.01M$)

Calculate ionic strength I as in Table 2330:I. Read value of pK'_{FW} from Table 4500-S^{2-}:II.

Sample calculation: Total sulfide concentration, 1.5 mg S^{2-}/L; pH, 6.87; temperature, 10°C; ionic strength, 0.04. From Table 4500-S^{2-}:II, $pK'_{FW} = 7.11$.

$$pH - pK'_{FW} = -0.24$$

$$10^{pH - pK'_{FW}} = 10^{-0.24} = 0.575$$

$$\alpha_{H_2S} = \frac{1}{1 + 0.575} = 0.63$$

$$0.63 \times 1.5 = 0.95$$

The concentration of un-ionized H_2S is 0.95 mg S^{2-}/L.

TABLE 4500-S^{2-}:II. CONDITIONAL FIRST DISSOCIATION CONSTANT OF HYDROGEN SULFIDE, FRESH WATER*

Temperature °C	pK'_{FW} at Given Ionic Strength						
	0.00 mol/L	0.005 mol/L	0.01 mol/L	0.02 mol/L	0.03 mol/L	0.05 mol/L	0.10 mol/L
0	7.36	7.33	7.32	7.30	7.29	7.27	7.24
5	7.28	7.25	7.23	7.22	7.21	7.19	7.16
10	7.20	7.16	7.15	7.13	7.12	7.10	7.07
15	7.12	7.09	7.08	7.06	7.05	7.03	7.00
20	7.05	7.02	7.00	6.99	6.97	6.96	6.92
25	6.98	6.95	6.94	6.92	6.91	6.89	6.86
30	6.92	6.89	6.87	6.86	6.84	6.83	6.79

* Values calculated according to Millero.[1]

2. Calculation for Seawater, Estuarine Water, and Brackish Water

This procedure is the same as that for fresh water. A potential source of error is the determination of the hydrogen ion concentration. If the pH electrode is calibrated using NIST buffers as in Section 4500-H^+, then a correction factor[2] must be determined. Add acid (HNO_3, HCl, or $HClO_4$, not H_2SO_4) to artificial seawater diluted to the salinity of interest and at the temperature of interest to give an acid concentration of 0.001*N*. (Prepare artificial seawater as in Table 8010:III, substituting NaCl for Na_2SO_4 on an equimolar basis and omitting NaF, $SrCl_2 \cdot 6H_2O$, H_3BO_3, KBr, $Na_2SiO_3 \cdot 9H_2O$, Na_4EDTA, and $NaHCO_3$.) Measure the pH. The difference between the negative logarithm of the known acid concentration and the measured pH is the correction factor. For example, if the acid concentration is 0.001*N* and the measured pH is 3.15, the correction factor is 0.15. Subtract 0.15 from measured pH values to get p^cH, the negative logarithm of the hydrogen ion *concentration.* (The pH in fresh water corresponds to the negative logarithm of the hydrogen ion *activity.*) Alternatively, calibrate the pH electrode with Tris* buffer in artificial seawater diluted to the salinity of interest and at the temperature of interest.[3] Read pK'_{SW} from Table 4500-S^{2-}:III. Calculate the fraction of un-ionized H_2S as for fresh water.

Sample calculation: Total sulfide concentration, 1.5 mg S^{2-}/L; temperature 10°C; pH, 7.15; salinity 25‰. From Table 4500-S^{2-}:III, $pK'_{SW} = 6.87$.

$$pH - pK'_{SW} = 0.28$$

$$10^{pH-pK'_{SW}} = 10^{0.28} = 1.91$$

$$\alpha_{H_2S} = \frac{1}{1 + 1.91} = 0.34$$

$$0.34 \times 1.5 = 0.51$$

The concentration of un-ionized H_2S is 0.51 mg S^{2-}/L.

* Trishydroxymethylaminomethane.

3. References

1. MILLERO, F.J. 1986. The thermodynamics and kinetics of the hydrogen sulfide system in natural waters. *Mar. Chem.* 18:121.
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3. MILLERO, F.J. 1986. The pH of estuarine waters. *Limnol. Oceanogr.* 31:839.

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TABLE 4500-S^{2-}:III. CONDITIONAL FIRST DISSOCIATION CONSTANT OF HYDROGEN SULFIDE, SEAWATER*

Temperature °C	pK'_{SW} at Given Salinity						
	5‰	10‰	15‰	20‰	25‰	30‰	35‰
0	7.17	7.12	7.09	7.07	7.06	7.06	7.06
5	7.08	7.02	6.99	6.97	6.96	6.96	6.96
10	6.99	6.93	6.90	6.88	6.87	6.86	6.86
15	6.91	6.85	6.82	6.80	6.78	6.78	6.77
20	6.83	6.77	6.74	6.72	6.70	6.69	6.69
25	6.76	6.70	6.66	6.64	6.63	6.62	6.61
30	6.70	6.63	6.60	6.57	6.56	6.55	6.54

* Values calculated according to Millero.[1]

4500-S^{2-} I. Distillation, Methylene Blue Flow Injection Analysis Method

1. General Discussion

a. Principle: Water and wastewater samples are distilled into a sodium hydroxide trapping solution and the distillate is analyzed. Hydrogen sulfide (H_2S) reacts in acid media and in the presence of ferric chloride with two molecules of *N*, *N*-dimethyl-*p*-phenylenediamine to form methylene blue. The resulting color is read at 660 nm.

b. Sample preservation: Because H_2S oxidizes rapidly, analyze samples and standards without delay. To preserve samples, add 4 drops 2*M* zinc acetate to 100 mL sample and adjust pH to >9 with 6*M* NaOH, then cool to 4°C. Samples are distilled into a trapping solution resulting in 0.25*M* NaOH matrix.

Also see 4500-S^{2-}.A, B, and E, and Section 4130, Flow Injection Analysis (FIA).

c. Interferences: This method measures total sulfide, which is defined as the acid-soluble sulfide fraction of a sample. Total sulfide includes both acid-soluble sulfides, such as H_2S, and acid-soluble metal sulfides present in suspended matter. This method does not measure acid-insoluble sulfides, such as CuS.

Most nonvolatile interferences are eliminated by distillation. Strong reducing agents inhibit color formation at concentrations of several hundred milligrams per liter. Iodide interferes at concentrations greater than 2 mg I/L.

Also see 4500-S^{2-}.A and B.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Distillation apparatus consisting of a glass or polypropylene micro-distillation device* capable of distilling 6 mL or more of sample into a 0.25*M* NaOH final concentration trapping solution.

b. Flow injection analysis equipment consisting of:

1) *FIA injection valve* with sample loop or equivalent.
2) *Multichannel proportioning pump.*
3) *FIA manifold* (Figure 4500-S^{2-}:4) with cation exchange column and flow cell. Relative flow rates only are shown in Figure 4500-S^{2-}:4. Tubing volumes are given as an example only; they may be scaled down proportionally. Use manifold tubing of an inert material, such as TFE.
4) *Absorbance detector,* 660 nm, 10-nm bandpass.
5) *Injection valve control and data acquisition system.*

3. Reagents

Use reagent water (>10 megohm) to prepare carrier for all solutions. To prevent bubble formation, degas carrier and buffer with helium. Pass He at 140 kPa (20 psi) through a helium degassing tube. Bubble He through 1 L solution for 1 min.

a. Sodium hydroxide diluent (NaOH), 0.25*M:* In a 2-L volumetric flask, dissolve 20 g NaOH in approximately 1800 mL water. Dilute to mark and mix with a magnetic stirrer until dissolved. Store in a plastic container.

* Lachat Instruments MICRO DIST, or equivalent.

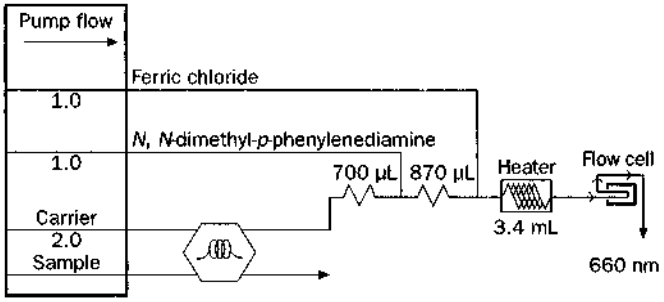


Figure 4500-S^{2-}:4. FIA sulfide manifold.

b. Hydrochloric acid (HCl), 3*M:* To a tared 1-L container, add 752 g water and then slowly add 295 g conc HCl. Invert to mix.

c. Hydrochloric acid (HCl), 0.20*M:* To a tared 1-L container, add 983.5 g water. Then add 19.7 g conc HCl. Invert to mix.

d. N,N-*dimethyl*-p-*phenylenediamine:* In a 1-L volumetric flask dissolve 1.0 g *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride, $(CH_3)_2NC_6H_4NH_2 \cdot 2HCl$, in about 800 mL 3*M* HCl (¶ *b* above). Dilute to mark and invert to mix. If solution appears dark, it is likely that the *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride is decomposed; discard, and use fresh reagent.

e. Ferric chloride: In a 500-mL volumetric flask dissolve 6.65 g ferric chloride hexahydrate, $FeCl_3 \cdot 6H_2O$, in about 450 mL 0.20*M* HCl (¶ *c* above). Dilute to mark with water and invert to mix.

f. Stock sulfide standard, 100 mg S^{2-}/L: In a 1-L volumetric flask dissolve 0.750 g sodium sulfide nonahydrate, $Na_2S \cdot 9H_2O$, in approximately 900 mL NaOH diluent (¶ *a* above). Dilute to mark and invert to mix. Standardize as in 4500-S^{2-}.F; also see 4500-S^{2-}.A.6.

g. Standard solutions: Prepare sulfide standards in desired concentration range, using stock standard (¶ *f* above), and diluting with NaOH diluent (¶ *a* above).

h. Sulfuric acid distillation releasing solution (H_2SO_4), 9*M:* To a tared 500-mL container, add 150.0 g water, then add slowly while swirling, in increments of 40 g, 276 g conc H_2SO_4. **CAUTION: Solution will become very hot.** Allow to cool before using.

4. Procedure

a. Distillation: This procedure is designed for the determination of sulfides in aqueous solutions, solid waste materials, or effluents. To preserve and remove sulfide from interfering substances, distill samples immediately after collection.

Follow manufacturer's instructions for use of distillation apparatus. Add sufficient 9*M* H_2SO_4 (4500-S^{2-}.I.3*h*) to sample to dissolve ZnS (s), digest total sulfides, and release the sulfide as hydrogen sulfide gas. Immediately place sample on-line with the receiving vessel or collector tube and distill hydrogen sulfide and water in the sample into a 0.25*M* trapping solution.

b. Flow injection analysis: Set up a manifold equivalent to that in Figure 4500-S^{2-}:4 and follow method supplied by the

manufacturer or laboratory standard operating procedure. Follow quality control protocols outlined in Section 4020.

5. Calculations

Prepare standard curves by plotting absorbance of standards processed through the manifold versus sulfide concentration.

6. Precision and Bias (MDL)

a. Method detection level (MDL): A 200-μL sample loop was used in the method described above. Using a published method,[1] analysts ran 21 replicates of 0.02-mg S^{2-}/L standard. These gave a mean of 0.024 mg S^{2-}/L, a standard deviation of 0.0021 mg S^{2-}/L, and MDL of 0.006 mg S^{2-}/L.

b. Precision: Ten injections of a distilled 0.8 mg S^{2-}/L standard gave a mean of 0.82 mg S^{2-}/L, a standard deviation of 0.0054 mg S^{2-}/L, and percent relative standard deviation of 0.66.

7. Reference

1. U.S. Environmental Protection Agency. 1984. Definition and procedure for the determination of method detection limits. Appendix B to 40 CFR 136 Rev. 1.11 Amended June 30, 1986. 49 CFR 43430.

4500-S^{2-} J. Acid-Volatile Sulfide

1. Apparatus

See Figure 4500-S^{2-}:5.

a. Reaction vessel, 250-mL, 3-neck flask, standard taper.

b. Gas traps, 125-mL gas scrubbers.

c. Dropping funnel, standard taper to fit reaction vessel.

d. Purge-gas tube, standard taper to fit reaction vessel.

e. Tubing, TFE or polypropylene, 0.635 cm (0.25-in.) OD, to connect reaction vessel to gas traps.

f. Syringe, plastic, 5 mL, lower end cut off to inject sediment into reaction vessel.

g. Compressed gas, cylinder of high-purity nitrogen, regulator, needle valve, rotameter or flow meter (optional).

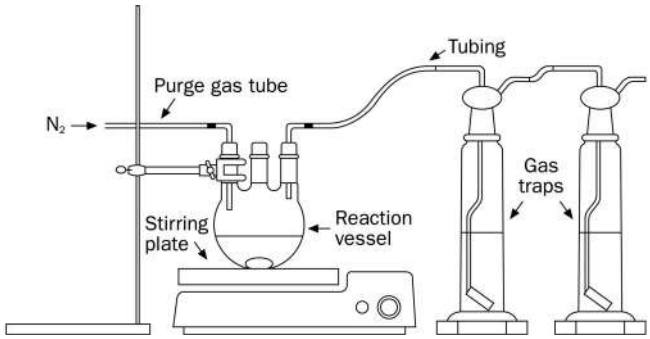


Figure 4500-S^{2-}:5. Apparatus for acid-volatile sulfide analysis.

2. Reagents

a. Hydrochloric acid solution, 9N: Add 186 mL conc HCl to about 50 mL distilled or deionized water. Dilute to 250 mL.

b. Stannous chloride solution, 0.53M: Dissolve 50 g $SnCl_2$ in 250 mL 9*N* HCl solution.

c. Sodium hydroxide solution (NaOH), 2*N*.

d. Zinc acetate solution, 1M, 22%: Dissolve 220 g $Zn(CH_3COO)_2 \cdot 2H_2O$ in 1 L distilled or deionized water.

e. Alkaline zinc solution: Add 150 mL 22% zinc acetate solution to 850 mL 2*N* NaOH solution.

f. Reagents for the iodometric titration method: See 4500-S^{2-}.F.

3. Procedure

Add 15 mL stannous chloride/hydrochloric acid solution to digestion vessel and 100 mL alkaline zinc solution to each trap. Adjust nitrogen flow rate to approximately 40 to 60 mL/min and flush system for 10 min. Add approximately 6 g fresh wet sediment to digestion vessel using a 5-mL syringe with its distal ends removed. Close outlets with clamps and gently stir the suspension. Allow H_2S generation to take place at room temperature (25 ± 3°C) for 3 h while stirring and with the nitrogen flowing. Combine the solutions from the two traps and titrate as described in 4500-S^{2-}.F.

4. Precision and Bias

For marine sediment samples (n=6), an average of 97% of the added ferrous monosulfide was recovered by the diffusion method. Reproducibility of this measurement performed on four samples with concentrations of around 40 to 150 μmol/g dry weight (1.3 to 4.8 mg S/g) was better than 5%.

5. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 4020:I.

6. Bibliography

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4500-SO_3^{2-} SULFITE*

4500-SO_3^{2-} A. Introduction

1. Occurrence

Sulfite ions (SO_3^{2-}) may occur in boilers and boiler feedwaters treated with sulfite for dissolved oxygen control, in natural waters or wastewaters as a result of industrial pollution, and in treatment plant effluents dechlorinated with sulfur dioxide (SO_2). Excess sulfite ion in boiler waters is deleterious because it lowers the pH and promotes corrosion. Control of sulfite ion in wastewater treatment and discharge may be important environmentally, principally because of its toxicity to fish and other aquatic life and its rapid oxygen demand.

2. Selection of Method

The iodometric titration method is suitable for relatively clean waters with concentrations above 2 mg SO_3^{2-}/L. The phenanthroline colorimetric determination, following evolution of sulfite from the sample matrix as SO_2, is preferred for low levels of sulfite.

* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011.

4500-SO_3^{2-} B. Iodometric Method

1. General Discussion

a. Principle: An acidified sample containing sulfite (SO_3^{2-}) is titrated with a standardized potassium iodide–iodate titrant. Free iodine, liberated by the iodide–iodate reagent, reacts with SO_3^{2-}. The titration endpoint is signalled by the blue color resulting from the first excess of iodine reacting with a starch indicator.

b. Interferences: The presence of other oxidizable materials, such as sulfide, thiosulfate, and Fe^{2+} ions, can cause apparently high results for sulfite. Some metal ions, such as Cu^{2+}, may catalyze the oxidation of SO_3^{2-} to SO_4^{2-} when the sample is exposed to air, thus leading to low results. NO_2^- will react with SO_3^{2-} in the acidic reaction medium and lead to low sulfite results unless sulfamic acid is added to destroy nitrite. Addition of EDTA as a complexing agent at the time of sample collection inhibits Cu^{2+} catalysis and promotes oxidation of ferrous to ferric iron before analysis. Sulfide and thiosulfate ions normally would be expected only in samples containing certain industrial discharges, but must be accounted for if present. Sulfide may be removed by adding about 0.5 g zinc acetate and analyzing the supernatant of the settled sample. However, thiosulfate may have to be determined by an independent method (e.g., the formaldehyde/iodometric method[1]), and then the sulfite determined by difference.

c. Minimum detectable concentration: 2 mg SO_3^{2-}/L.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Reagents

a. Sulfuric acid: (H_2SO_4), 1 + 1.

b. Standard potassium iodide–iodate titrant, 0.002083*M:* Dissolve 0.4458 g primary-grade anhydrous KIO_3 (dried for 4 h at 120°C), 4.35 g KI, and 310 mg sodium bicarbonate ($NaHCO_3$) in distilled water and dilute to 1000 mL; 1.00 mL = 500 μg SO_3^{2-}.

c. Sulfamic acid (NH_2SO_3H), crystalline.

d. EDTA reagent: Dissolve 2.5 g disodium EDTA in 100 mL distilled water.

e. Starch indicator: To 5 g starch (potato, arrowroot, or soluble) in a mortar, add a little cold distilled water and grind to a paste. Add mixture to 1 L boiling distilled water, stir, and let settle overnight. Use clear supernatant. Preserve by adding either 1.3 g salicylic acid, 4 g $ZnCl_2$, or a combination of 4 g sodium propionate and 2 g sodium azide to 1 L starch solution.

3. Procedure

a. Sample collection: Collect a fresh sample, taking care to minimize contact with air. Fix cooled samples (<50°C) immediately by adding 1 mL EDTA solution/100 mL sample. Cool hot samples to 50°C or below. Do not filter.

b. Titration: Add 1 mL H_2SO_4 and 0.1 g NH_2SO_3H crystals to a 250-mL Erlenmeyer flask or other suitable titration vessel. Accurately measure 50 to 100 mL EDTA-stabilized sample into flask, keeping pipet tip below liquid surface. Add 1 mL starch indicator solution. Titrate immediately with standard KI–KIO_3 titrant, while swirling flask, until a faint permanent blue color develops. Analyze a reagent blank using distilled water instead of sample.

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4. Calculation

$$\text{mg } SO_3^{2-}/\text{L} = \frac{(A - B) \times M \times 6 \times 40\,000}{\text{mL sample}}$$

where:

A = mL titrant for sample,
B = mL titrant for blank, and
M = molarity of KI-KIO_3 titrant.

5. Precision and Bias

Three laboratories analyzed five replicate portions of a standard sulfite solution and of secondary treated wastewater effluent to which sulfite was added. The data are summarized below. Individual analyst's precision ranged from 0.7 to 3.6% standard deviation (N = 45).

Sample	$\bar{X}$ mg/L	Standard Deviation, σ mg/L	Relative Standard Deviation %
Standard, 6.3 mg SO_3^{2-}/L	4.5	0. 25	5.5
Secondary effluent with 2.0 mg SO_3^{2-}/L	2.1	0.28	13.4
Secondary effluent with 4.0 mg SO_3^{2-}/L	3.6	0.17	4.8

6. Reference

1. KURTENACKER, A. 1924. The aldehyde-bisulfite reaction in mass analysis. *Z. Anal. Chem.* 64:56.

4500-SO_3^{2-} C. Phenanthroline Method

1. General Discussion

a. Principle: An acidified sample is purged with nitrogen gas, and the liberated SO_2 is trapped in an absorbing solution containing ferric ion and 1,10-phenanthroline. Ferric iron is reduced to the ferrous state by SO_2, producing the orange tris(1,10-phenanthroline) iron(II) complex. After excess ferric iron is removed with ammonium bifluoride, the phenanthroline complex is measured colorimetrically at 510 nm.[1]

b. Interferences: See Section 4500-SO_3^{2-}.B.1*b*.

c. Minimum detectable concentration: 0.01 mg SO_3^{2-}/L.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 4020:I.

2. Apparatus

a. Apparatus for evolution of SO_2: Figure 4500-SO_3^{2-}:1 shows the following components:

1) *Gas flow meter,* with a capacity to measure 2 L/min of pure nitrogen gas.

2) *Gas washing bottle,* 250-mL, with coarse-porosity, 12-mm-diam fritted cylinder gas dispersion tube.

3) *Tubing connectors,* quick-disconnect, polypropylene.

4) *Tubing,* flexible PVC, for use in all connections.

5) *Nessler tube,* 100-mL.

b. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer,* for use at 510 nm, providing a light path of 1 cm or longer.

2) *Filter photometer,* providing a light path of 1 cm or longer and equipped with a green filter having maximum transmittance near 510 nm.

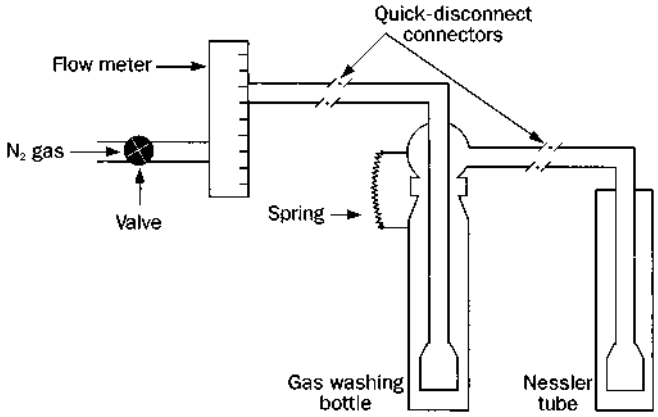


Figure 4500-SO_3^{2-}:1. Apparatus for evolution of SO_2 from samples for colorimetric analysis.

3. Reagents

a. 1,10-phenanthroline solution, 0.03*M:* Dissolve 5.95 g 1,10-phenanthroline in 100 mL 95% ethanol. Dilute to 1 L with distilled water. Discard if solution becomes colored.

b. Ferric ammonium sulfate solution, 0.01*M:* Dissolve 4.82 g $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 1 L distilled water to which has been added 1 mL conc H_2SO_4 to suppress ferric hydrolysis. Filter through a glass fiber filter if insoluble matter is visible. If necessary, adjust volume of acid so a mixture of 10 parts of

phenanthroline solution and one part of ferric ammonium sulfate solution will have a pH between 5 and 6.

c. Ammonium bifluoride, 5%: Dissolve 25 g NH_4HF_2 in 500 mL distilled water. Store in a polyethylene bottle and dispense with a plastic pipet.

d. Potassium tetrachloromercurate (TCM) (K_2HgCl_4), 0.04*M:* Dissolve 10.86 g $HgCl_2$, 5.96 g KCl, and 0.066 g disodium EDTA in distilled water and dilute to 1 L. Adjust pH to 5.2. This reagent normally is stable for 6 months, but discard if a precipitate forms.[2]

e. Dilute TCM-stabilized sulfite standard: Dissolve 0.5 g Na_2SO_3 in 500 mL distilled water. Standardize on the day of preparation, but wait at least 30 min to allow the rate of oxidation to slow. Determine molarity by titrating with standard 0.0125*M* potassium iodide–iodate titrant using starch indicator (see 4500-SO_3^{2-}.B.3*b*). Calculate molarity of working standard as follows:

$$\text{Molarity of } SO_3^{2-}\text{standard} = \frac{(A - B) \times M}{\text{mL sample}}$$

where:

A = titrant for sample, mL,
B = titrant for blank, mL,
M = molarity of potassium iodide–iodate titrant.

Because stock Na_2SO_3 solution is unstable, immediately after standardization, pipet 10 mL into a 500-mL volumetric flask partially filled with TCM and dilute to mark with TCM. Calculate the concentration of this dilute sulfite solution by multiplying the stock solution concentration by 0.02. This TCM-stabilized standard is stable for 30 d if stored at 5°C. Discard as soon as any precipitate is noticed at the bottom.

f. Standard potassium iodide-iodate titrant, 0.002083*M:* See 4500-SO_3^{2-}.B.2*b*.

g. Hydrochloric acid, 1 + 1.

h. Octyl alcohol, reagent-grade.

i. Sulfamic acid, 10%: Dissolve 10 g NH_2SO_3H in 100 mL distilled water. This reagent can be kept for a few days if protected from air.

j. EDTA reagent: See 4500-SO_3^{2-}.B.2*d*.

4. Procedure

a. Sample collection: Collect a fresh sample, taking care to minimize contact with air. Fix cooled samples (<50°C) immediately by adding 1 mL EDTA solution for each 100 mL sample.

b. SO_2 evolution: Prepare the absorbing solution by adding 5 mL 1,10-phenanthroline solution, 0.5 mL ferric ammonium sulfate solution, 25 mL distilled water, and 5 drops octyl alcohol (to act as defoamer) to a 100-mL nessler tube; insert a gas dispersion tube. Add 1 mL sulfamic acid solution to the gas washing bottle and 100 mL of sample or a portion containing less than 100 μg SO_3^{2-} diluted to 100 mL. Add 10 mL 1 + 1 HCl and immediately connect the gas washing bottle to the gas train as shown in Figure 4500-SO_3^{2-}:1. Place a spring or rubber band on the gas washing bottle to keep the top securely closed during gas flow. Adjust nitrogen flow to 2.0 L/min and purge for 60 min.

c. Colorimetric measurement: After exactly 60 min, turn off nitrogen flow, disconnect nessler tube, and immediately add 1 mL ammonium bifluoride solution. Remove gas dispersion tube after rinsing it with distilled water into the tube and forcing the rinse water into the nessler tube with a rubber bulb. Dilute to 50 mL in the nessler tube and mix by rapidly moving the tube in a circular motion. Do not let rubber stoppers or PVC tubing come in contact with the absorbing solution. After at least 5 min from the time of adding ammonium bifluoride, read the absorbance versus distilled water at 510 nm using either a 5-cm cell for a range of 0 to 30 μg SO_3^{2-} per portion or a 1-cm cell for a range of 0 to 100 μg SO_3^{2-}. Avoid transferring octyl alcohol into the cell by letting it rise to the surface of the absorbing solution and transferring the clear lower solution to the cell with a pipet. Make a calibration curve by analyzing a procedure blank and at least three standards. Run at least one standard with each set of samples. For maximum accuracy hold samples and standards at the same temperature and keep the time interval from start of purging to the addition of ammonium bifluoride constant. This is easier to do if several gas trains are used simultaneously in parallel. If ambient temperatures are subject to frequent fluctuation, a water bath may be used to control color development at a fixed temperature.

5. Calculation

$$\text{mg } SO_3^{2-}\text{/L} = \frac{\mu\text{g } SO_3^{2-} \text{ from calibration curve}}{\text{mL sample}}$$

6. Precision and Bias

Three laboratories analyzed five replicate portions of a standard sulfite solution and of secondary treated wastewater effluent to which sulfite was added. The data are summarized below. Individual analyst's precision ranged from 4.1 to 10.5% standard deviation (N = 45).

Sample	$\bar{X}$ *mg/L*	Standard Deviation, σ *mg/L*	Relative Standard Deviation *%*
Standard, 4.7 mg SO_3^{2-}/L	3.7	0.78	21
Secondary effluent with 0.12 mg SO_3^{2-}/L	0.12	0.03	25
Secondary effluent with 4.0 mg SO_3^{2-}/L	3.7	0.30	8.0

7. References

1. Stephens, B.G. & F. Lindstrom. 1964. Spectrophotometric determination of sulfur dioxide suitable for atmospheric analysis. *Anal. Chem.* 36:1308.
2. West, P.W. & G.C. Gaeke. 1956. Fixation of sulfur dioxide as sulfitomercurate and subsequent colorimetric determination. *Anal. Chem.* 28:1816.
3. Haskins, J.E., H. Kendall & R.B. Baird. 1984. A low-level spectrophotometric method for the determination of sulfite in water. *Water Res.* 18(6):751.

PART 5000

AGGREGATE ORGANIC CONSTITUENTS

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Joint Task Group Chairs for the 23rd Edition

5020 Quality Assurance/Quality Control.....Robin S. Parnell
5210 Biochemical Oxygen Demand (BOD).....James C. Young
5310 Total Organic Carbon (TOC).....Edward W.D. Huffman, Jr.
5910 UV- Absorbing Organic Constituents.....Suzanne M. Teague

Summary of Major Changes since 2012

Quality Assurance/Quality Control (5020) was revised to be consistent with Sections 1020, 2020, 3020, 4020, and 6020. Biochemical Oxygen Demand (5210) clarifies specific procedural steps and QA/QC practices. More sampling, filtering, and QA/QC requirements were added to Total Organic Carbon (5310). The Wet-Oxidation Method (5310D) was dropped from this edition. UV-Absorbing Organic Constituents (5910) includes new information on sampling and storage, filter blanks, QC, and buffer preparation.

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5010 INTRODUCTION

5010 A. General Discussion

Analyses for organic matter in water and wastewater can be classified into two general types of measurements: those that quantify an aggregate amount of organic matter comprising organic constituents with a common characteristic and those that quantify individual organic compounds. The latter can be found in Part 6000. The former, described here in Part 5000, have been grouped into four categories: oxygen-demanding substances, organically bound elements, classes of compounds, and formation potentials.

Methods for total organic carbon and chemical oxygen demand are used to assess the total amount of organics present. Gross fractions of the organic matter can be identified analytically, as in the measurement of BOD, which is an index of the biodegradable organics present, oil and grease, which represents material extractable from a sample by a nonpolar solvent, or dissolved organic halide (DOX), which measures organically bound halogens. Trihalomethane formation potential is an aggregate measure of the total concentration of trihalomethanes formed upon chlorination of a water sample.

Analyses of organics are made to assess the concentration and general composition of organic matter in raw water supplies, wastewaters, treated effluents, and receiving waters; and to determine the efficiency of treatment processes.

5010 B. Sample Collection and Preservation

The sampling, field treatment, preservation, and storage of samples taken for organic matter analysis are covered in detail in the individual introductions to the methods. If possible, analyze samples immediately because preservatives often interfere with the tests. Otherwise, store at a low temperature (4°C) immediately after collection to preserve most samples. Use chemical preservatives only when they are shown not to interfere with the examinations to be made (see Section 1060C.2). Never use preservatives for samples to be analyzed for BOD. When preservatives are used, add them to the sample bottle initially so that all portions are preserved as soon as collected. No single method of preservation is entirely satisfactory; choose the preservative with due regard to the determinations that are to be made. All methods of preservation may be inadequate when applied to samples containing significant amounts of suspended matter.

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5020 QUALITY ASSURANCE/QUALITY CONTROL*

5020 A. Introduction

Quality assurance (QA) is a laboratory operations program that specifies the measures required to produce defensible data with known precision and accuracy. This program is defined in a QA manual, written procedures, work instructions, and records. The manual should include a policy that defines the statistical level of confidence used to express data precision and bias, as well as method detection levels (MDLs) and reporting limits. The overall system includes all QA policies and quality control (QC) processes needed to demonstrate the laboratory's competence and to ensure and document the quality of its analytical data. Quality systems are essential for laboratories seeking accreditation under state or federal laboratory certification programs. Refer to Section 1020 for details on establishing a Quality Assurance Plan.

As described in Part 1000, essential QC measures may include method calibration, reagent preparation and/or standardization, assessment of each analyst's capabilities, analysis of blind check samples, determination of the method's sensitivity [method detection level (MDL), limit of detection (LOD), level of quantification (LOQ), or minimum reporting level (MRL)], and daily evaluation of bias, precision, and laboratory contamination or other analytical interference.

Some methods in Part 5000 include specific QC procedures, frequencies, and acceptance criteria. These are considered the minimum QCs needed to perform the method successfully. When the words *should* or *preferably* are used, the QC is recommended; when *must* is used, the QC is mandatory. Additional QC procedures should be used when necessary to ensure that results are valid. Some regulatory programs may require additional QC or have alternative acceptance limits. In those cases, the laboratory should follow the more stringent requirements.

The QC program consists of at least the following elements, as applicable to specific methods:

- calibration,
- continuing calibration verification (CCV),
- operational range and MDL determination,
- initial demonstration of capability (IDC),
- ongoing demonstration of capability,
- method blank/reagent blank,
- laboratory-fortified blank (LFB),
- laboratory-fortified matrix (LFM),
- duplicate sample/laboratory-fortified matrix duplicate (LFMD),
- verification of MDL and MRL,
- QC calculations,
- control charts,
- corrective action,
- frequency of QC,
- QC acceptance criteria, and
- definitions of prep and analytical batches.

Sections 1010 and 1030 describe calculations for evaluating data quality.

* Reviewed by Standard Methods Committee, 2010. Additional revisions, 2017. Robin S. Parnell (chair), Rodger B. Baird, Andrew D, Eaton, William C. Lipps.

5020 B. Quality Control Practices

At a minimum, analysts must use the QC practices specified here unless a method specifies alternative, more stringent practices. Laboratories may save time and money by purchasing premade standards, titrants, and reagents, but they still must perform the QC checks on these materials required by the analytical methods.

1. Calibration

a. Instrument calibration (not applicable to non-instrumental methods): Perform both instrument calibration and maintenance according to the manufacturer's instructions and recommendations. Conduct instrument-performance checks according to method or standard operating procedure (SOP) instructions.

b. Initial calibration: Perform initial calibration using

- at least three concentrations of standards and one blank (for linear calibrations),
- at least five concentrations of standards and one blank (for nonlinear calibrations), or
- as many concentrations as the method specifies.

The lowest concentration must be at or below the MRL, and the highest concentration should be at the upper end of the calibration range. Make sure the calibration range encompasses the concentrations expected in method samples or required dilutions. For the most accurate results, choose calibration standard concentrations no more than one order of magnitude apart. Some methods and instruments respond better to more orders of magnitude between concentrations.

Apply response-factor, linear, or quadratic-curve-fitting statistics (depending on what the method allows) to analyze the concentration–instrument response relationship. If the relative standard deviation (%RSD) of the response factors is $\leq$15%, then the average response factor may be used. Otherwise, use a regression equation. The appropriate linear or nonlinear correlation coefficient for standard concentration-to-instrument response should be $\geq$0.995 for linear calibrations and $\geq$0.990 for quadratic calibrations. Weighting factors (e.g., 1/x or $1/x^2$) may be used to give more weight to the lower concentration points of the calibration. Depending on the method, calibration curves may be

- linear through the origin,
- linear not through the origin,
- nonlinear through the origin, or
- nonlinear not through the origin.

Some nonlinear functions can be linearized via mathematical transformations (e.g., log). The following acceptance criteria are recommended for various calibration functions.

Compare each calibration point to the curve and recalculate its concentration. If any recalculated values are not within the method's acceptance criteria—≤3 times the MRL ±50%; between 3 and 5 times the MRL ±20%; or >5 times the MRL ±10%, unless otherwise specified in individual methods—identify the source of any outlier(s) and correct before sample quantitation.

NOTE: Do not use the correlation coefficient to verify a calibration's accuracy. However, many methods require verification of the correlation.

Verify the initial calibration by analyzing a standard prepared from a different stock standard than that used to create the calibration curve; its concentration should be near the midpoint of the calibration range. The analytical results for this second-source mid-range standard must be within 10% of its true value. If not, determine the cause of the error, and take corrective action.

Use the initial calibration to quantitate analytes of interest in samples. Use CCV (¶ *c* below) only for calibration checks, not for sample quantitation. Perform initial calibration when the instrument is set up and whenever CCV criteria are not met.

c. Continuing calibration verification: In CCV, analysts periodically use a calibration standard to confirm that instrument performance has not changed significantly since initial calibration. Base the CCV interval on the number of samples analyzed (e.g., after every 10 samples and at least once per batch). Verify calibration by analyzing one standard whose concentration is at or near the midpoint of the calibration range. The results must be within allowable deviations from either initial-calibration values or specific points on the calibration curve. If the CCV is out of control, then take corrective action—including re-analysis of any samples analyzed since the last acceptable CCV.

Refer to the method for CCV frequency and acceptance criteria; if not specified, use the criteria given here. Other concentrations (e.g., one near the MRL) may be used, but be aware that the acceptance criteria may vary depending on the standard's concentration.

2. Operational Range and MDL Determination

Before using a new method or instrument, you should determine its operational (calibration) range (upper and lower limits). Calibrate according to 5020B.1, or verify the calibration by analyzing prepared standard solutions ranging from low to high concentrations. Determine the maximum concentration that can be measured within 10% of its true value based on the calibration curve: this is the *limit of linearity*. All samples whose concentrations are above the limit of linearity or the highest calibration point, whichever is lower, must be diluted.

If reporting results <MRL, initially estimate the MDL as a concentration about 3 to 5 times lower than the minimum calibration standard. The method for determining the MDL is based on the procedure outlined by the U.S. Environmental Protection Agency (EPA).[1]

To determine an MDL, prepare and analyze at least seven portions of a solution spiked at or near the minimum calibration concentration and an equal number of blanks. Analysts should prepare and analyze the spikes and blanks over ≥3 d rather than doing them all in one batch. If one MDL will be used for multiple instruments, then the MDL analysis must be performed across all of them (however, it is unnecessary to analyze all samples on all instruments). Analysts must prepare and analyze at least two spikes and two blanks on different calendar dates for each instrument. If evaluating more than three instruments, then one set of spikes and blanks can be analyzed on multiple instruments, so long as at least seven sets of spikes and blanks total are used. Alternatively, determine instrument-specific MDLs.

Calculate the estimated sample standard deviation, s_s, of the 7 replicates, and multiply by 3.14 to compute the MDL_s. Calculate MDL_b (MDL based on method blanks) using the following procedure.

If none of the method blanks give a numerical result (positive or negative), then MDL_b is not applicable, and MDL = MDL_s. If some give numerical results, then MDL_b equals the highest method blank result. If all of the method blanks give numerical results, calculate MDL_b as

$$MDL_b = X + 3.14S_b$$

where:

X = mean of blank results (set negative values to 0), and
S_b = standard deviation of blank results.

The MDL then equals whichever is greater: MDL_s or MDL_b.

If using more than 7 replicates, adjust the t value from 3.14 using student t tables with n-1 degrees of freedom.

For methods in this section, spike recovery for MDL determinations must be within 50 to 150%, with a %RSD of <20%. If it does not meet these criteria, the MRL spiking level and calculated MDL are too low and must be repeated at a higher concentration.

3. Initial Demonstration of Capability

Each analyst in the laboratory should conduct an IDC at least once before analyzing any sample to demonstrate proficiency in performing the method and obtaining acceptable results for each analyte. The IDC also is used to demonstrate that a laboratory's modifications to a method will produce results as precise and accurate as those produced by the reference method. As a minimum, include a reagent blank and at least four LFBs at a concentration between one and 10 times the MDL and the midpoint of the calibration curve (or other level specified in the method). Run the IDC after analyzing all required calibration standards. Ensure that the reagent blank does not contain any analyte of interest at a concentration greater than half the lowest calibration point (or other level specified in the method). Ensure that precision and accuracy (percent recovery) calculated for LFBs are within the acceptance criteria listed in the method of choice or generated by the laboratory (if there are no established mandatory criteria).

To establish laboratory-generated accuracy and precision limits, calculate the upper and lower control limits from the mean and standard deviation of percent recovery for ≥20 data points:

$$\text{Upper control limit} = \text{Mean} + 3(\text{Standard deviation})$$

$$\text{Lower control limit} = \text{Mean} - 3(\text{Standard deviation})$$

In the absence of established mandatory criteria, use laboratory-generated acceptance criteria for the IDC or else obtain acceptance criteria from a proficiency testing (PT) provider on PT studies and translate the data to percent recovery limits per analyte and method of choice. Ensure that lab-generated criteria are at least as tight as PT-study criteria, which are typically based on either multiple lab results.

4. Ongoing Demonstration of Capability

The ongoing demonstration of capability, sometimes called a *laboratory control sample* (LCS), *laboratory control standard, QC check sample,* or *laboratory-fortified blank,* is used to ensure that the laboratory analysis remains in control while samples are analyzed and separates laboratory performance from method performance on the sample matrix. This standard should be preserved in accordance with method requirements and carried through the entire procedure, including any digestions, extraction, or filtration. Purchase an external QC standard (if available) from a reputable supplier and use the certified acceptance limits as the laboratory acceptance criteria.

Acceptance criteria will vary depending on the method, matrix, and concentration. The concentration range should be either near the middle of the calibration range or near the maximum contaminant level (MCL), whichever is lower. Alternatively, prepare your own QC standard and calculate acceptance limits as ± 2 standard deviations based on analysis of ≥ 20 replicates, unless the method specifies acceptance limits.

5. Reagent Blank

A *reagent blank* (method blank) consists of reagent water (see Section 1080) and all reagents (including preservatives) that normally are in contact with a sample during the entire analytical procedure. The reagent blank is used to determine whether and how much reagents and the preparative analytical steps contribute to measurement uncertainty. As a minimum, include one reagent blank with each sample set (batch) or on a 5% basis, whichever is more frequent. Analyze a blank after the initial CCV standard and before analyzing samples. Evaluate reagent-blank results for contamination; if contamination levels are unacceptable, identify and eliminate the source.

Positive sample results are suspect if analyte(s) in the reagent blank are >1/2MRL, unless the method specifies otherwise. Samples analyzed with a contaminated blank must be re-prepared and re-analyzed unless concentrations are ≥10 times those of the blank, concentrations are nondetect, or data user will accept qualified data. See method for specific reagent-blank acceptance criteria. General guidelines for qualifying sample results with regard to reagent-blank quality are as follows:

- If reagent blank is <MDL and sample results are >MRL, then no qualification is required.
- If reagent blank is >1/2MRL but <MRL and sample results are >MRL, then qualify results to indicate that analyte was detected in the reagent blank.
- If reagent blank is >MRL, then further corrective action and qualification is required.

6. Laboratory-Fortified Blank

A *laboratory-fortified blank* (LFB) is a reagent-water sample (with associated preservatives) to which a known concentration of the analyte(s) of interest has been added. The LFB may be used as the LCS (5020B.4) if the method requires a preliminary sample extraction or digestion.

An LFB is used to evaluate laboratory performance and analyte recovery in a blank matrix. Its concentration should be high enough to be measured precisely, but not high enough to be irrelevant to measured environmental concentrations. The analyst should rotate LFB concentrations to cover different parts of the calibration range. As a minimum, include one LFB with each sample set (batch) or on a 5% basis, whichever is more frequent. (The definition of a batch is typically project-specific.)

Process the LFB through all sample-preparation and -analysis steps. Use an added concentration of at least 10 × MDL, at or below the midpoint of the calibration curve, a method-specified level, or a level specified in a project plan's data quality objectives. Ideally, the LFB concentration should be less than the MCL (if the contaminant has one). Depending on method requirements, prepare the addition solution from either the same reference source used for calibration or an independent source. Evaluate the LFB for percent recovery of the added analytes by comparing results to method-specified limits, control charts, or other approved criteria. If LFB results are out of control, take corrective action, including re-preparation and re-analysis of associated samples if required. Use LFB results to evaluate batch performance, calculate recovery limits, and plot control charts.

7. Laboratory-Fortified Matrix

A *laboratory-fortified matrix* (LFM) is an additional portion of a sample to which a known amount of the analyte(s) of interest is added before sample preparation. Some analytes are not appropriate for LFM analysis; see Table 5020:I and specific methods for guidance on when an LFM is relevant. T1

The LFM is used to evaluate analyte recovery in a sample matrix. If an LFM is feasible and the method does not specify LFM frequency requirements, then include at least one LFM with each sample set (batch) or on a 5% basis, whichever is more frequent. Add a concentration that is at least 10 × MRL, less than or equal to the midpoint of the calibration curve, or method-specified level to the selected sample(s). The analyst should use the same concentration as for LFB (5020B.6) to allow analysts to separate the matrix's effect from laboratory performance. Prepare LFM from the same reference source used for LFB. Make the addition such that sample background levels do not adversely affect recovery (preferably adjust LFM concentrations if the known sample is more than five times the background level). For example, if the sample contains the analyte of interest, then add approximately as much analyte to the LFM sample as the concentration found in the known sample.

Evaluate LFM results for percent recovery; if they are not within control limits, then take corrective action to rectify the matrix effect, use another method, use the method of standard addition, or flag the data if reported. See method for specific LFM-acceptance criteria until the laboratory develops statistically valid, laboratory-specific performance criteria. If the method does not provide limits, use the calculated preliminary limits from the IDC (5020B.3). LFM control limits may be wider than for LFB or LCS, and batch acceptance generally is not contingent upon LFM results.

8. Duplicate Sample/Laboratory-Fortified Matrix Duplicate

Duplicate samples are analyzed to estimate precision. If an analyte is rarely detected in a matrix type, use an LFM duplicate. An *LFM duplicate* is a second portion of the sample described in 5020B.7 to which a known amount of the analyte(s) of interest is added before sample preparation. If sufficient sample volume is collected, this second portion of sample is added and processed in the same way as the LFM. As a minimum, include one duplicate sample or one LFM duplicate with each sample set (batch) or on a 5% basis, whichever is more frequent, and process it independently through the entire sample preparation and analysis.

Evaluate LFM duplicate results for precision and accuracy (precision alone for duplicate samples). If LFM duplicate results are out of control, then take corrective action to rectify the matrix effect, use another method, use the method of standard addition, or flag the data if reported. If duplicate results are out of control, then re-prepare and re-analyze the sample and take additional corrective action, as needed. When the value of one or both duplicate samples is $\leq 5 \times$ MRL, the laboratory may use the MRL as the control limit for percent recovery, and the duplicate results are not used. See method for specific acceptance criteria for LFM duplicates or duplicate samples until the laboratory develops statistically valid, laboratory-specific performance criteria. If the method does not provide limits, use the calculated preliminary limits from the IDC. In general, batch acceptance is not contingent upon LFM duplicate results.

9. Verification of MDL and MRL

With each analytical batch, analyze a reagent-water sample spiked at MRL and ensure that it meets MRL acceptance criteria (generally $\pm 50\%$). If not, re-analyze the entire batch or flag results for all samples in the batch.

If reporting to the MDL, then verify the MDL at least quarterly by analyzing a sample spiked at the same level used to determine the MDL and ensure that the result is positive. If two consecutive MDL-verification samples do not produce positive results, then recalculate the MDL using the most recent set of at least 7 blanks and MRL level spikes, following the protocols outlined in 5020B.2.

10. QC Calculations

The following is a compilation of equations frequently used in QC calculations.

a. Initial calibration: See Section 1020B.12*a*.
b. Calibration verification: See Section 1020B.12*b*.
c. Laboratory-fortified blank recovery: See Section 1020B.12*c*.
d. Laboratory-fortified matrix: See Section 1020B.12*e*.
e. Relative percent deviation: See Section 1020B.12*f*.
f. Standard additions: See Section 1020B.12*g*.

11. Control Charts

See Section 1020B.13.

12. Reference

1. U.S. Environmental Protection Agency. 2016. III.H. Changes to method detection limit (MDL) procedure. *In* Clean Water Act Methods Update Rule for the Analysis of Effluent. 40 CFR 136.

Table 5020:I. Minimum Quality Control for Methods in Part 5000

Section	Analyte	Method Blank	LFB*	LFM† & LFMD‡	Other
5210B	BOD	–	–	–	1,2,3
5210C		–	–	–	1,2,3
5210D		–	–	–	1,2,3
5220B	COD	×	×	×	1,2,3
5220C		×	×	×	1,2,3
5220D		×	×	×	1,2,3
5310B	TOC	×	×	×	1,2,3
5310C		×	×	×	1,2,3
5310D		×	×	×	1,2,3
5320B	Dissolved Organic Halogen	×	×	×	1,2,3
5510B	Aquatic Humic Substances	×	×	×	1,2,3
5510C		×	×	×	1,2,3
5520B	Oil and Grease	×	×	×	1,2,3
5520C		×	×	×	1,2,3
5520D		×	×	×	1,2,3
5520E		×	×	×	1,2,3
5520F		×	×	×	1,2,3
5520G		×	×	×	1,2,3
5530B§	Phenols	×	×	×	1,2,3
5530C		×	×	×	1,2,3
5530D		×	×	×	1,2,3
5540B§	Surfactants	×	×	×	1,2,3
5540C		×	×	×	1,2,3
5540D		×	×	×	1,2,3
5550B	Tannin and Lignin	×	×	×	1,2,3
5560B§	Organic/Volatile Acids	×	×	×	1,2,3
5560C§		×	×	×	1,2,3
5560 D		×	×	×	1,2,3
5710B	THMs and DBPs	–	–	–	1,2,3
5710C		–	–	–	1,2,3
5710D		–	–	–	1,2,3
5910B	UV-Absorbing Organic Constituents	×	–	–	1,2,3

* Laboratory-fortified blank.
† Laboratory-fortified matrix.
‡ Laboratory-fortified matrix duplicate.
§ A sample-preparation technique that is normally combined with a subsequent determinative technique
× indicates that a QC type is mandatory for the method.
– indicates that a QC type is not mandatory for the method.
1. Additional QC guidelines in method.
2. Duplicates or LFMD of the sample will be run.
3. Refer to 5020B for further QC requirements.
This table is not comprehensive; refer to the specific method and 5020B for further details.

5210 BIOCHEMICAL OXYGEN DEMAND (BOD)*

5210 A. Introduction

1. General Discussion

Biochemical oxygen demand (BOD) testing is used to determine the relative oxygen requirements of wastewaters, effluents, and polluted waters; its widest application is in measuring waste loadings to treatment plants and in evaluating the plants' BOD-removal efficiency. BOD testing measures the molecular oxygen used during a specified incubation period to

- biochemically degrade organic material (carbonaceous demand),
- oxidize inorganic material (e.g., sulfides and ferrous iron), and/or
- measure the amount of oxygen used to oxidize reduced forms of nitrogen (nitrogenous demand) unless an inhibitor is added to prevent such reduction.

The seeding and dilution procedures provide an estimate of BOD at pH 6 to 8.

The methods below measure oxygen consumed in a 5-d period (5210B), oxygen consumed after 60 to 90 d of incubation (5210C), and continuous oxygen uptake (5210D). Other BOD methods published elsewhere may use shorter or longer incubation periods; tests to determine oxygen-uptake rates; and/or alternative seeding, dilution, and incubation conditions to mimic receiving-water conditions, thereby estimating the environmental effects of wastewaters and effluents.

The ultimate BOD (UBOD) test measures the oxygen required to totally degrade organic material (ultimate carbonaceous demand) and/or to oxidize reduced nitrogen compounds (ultimate nitrogenous demand). UBOD values and appropriate kinetic descriptions are needed in water-quality modeling studies [e.g., UBOD:BOD_5 ratios for relating stream assimilative capacity to regulatory requirements; definition of river, estuary, or lake deoxygenation kinetics; and instream ultimate carbonaceous BOD (UCBOD) values for model calibration].

A number of factors (e.g., soluble versus particulate organics, settleable and floatable solids, oxidation of reduced iron and sulfur compounds, or lack of mixing) may affect the accuracy and precision of BOD measurements. Presently, there are no effective adjustments or corrections to compensate for these factors.

2. Carbonaceous Versus Nitrogenous BOD

Microorganisms can oxidize reduced forms of nitrogen, such as ammonia and organic nitrogen, thus exerting nitrogenous demand. Nitrogenous demand historically has been considered an interference in BOD testing; adding ammonia to dilution water contributes an external source of nitrogenous demand. The interference from nitrogenous demand can now be prevented by an inhibitory chemical,[1] but if it isn't used, the measured oxygen demand is the sum of carbonaceous and nitrogenous demands.

Measurements that include nitrogenous demand generally are not useful for assessing the oxygen demand associated with organic material. Nitrogenous demand can be estimated directly from ammonia nitrogen, and carbonaceous demand can be estimated by subtracting the theoretical equivalent of the nitrite and nitrate produced in uninhibited test results. However, this method is cumbersome and subject to considerable error. Chemical inhibition of nitrogenous demand provides a more direct, reliable measure of carbonaceous demand.

How much nitrogenous compounds oxidize during the 5-d incubation period depends on the concentration and type of microorganisms that can carry out this oxidation. Such organisms quite often are present in raw or settled primary sewage in adequate numbers to oxidize enough reduced nitrogen forms to contribute oxygen demand in the 5-d BOD test. Most biological treatment plant effluents contain enough nitrifying organisms to cause nitrification in BOD tests. Because nitrogenous compounds can oxidize in such samples, nitrification inhibition (as directed in 5210B.5*e*) is recommended for secondary-effluent samples, samples seeded with secondary effluent, and polluted-water samples.

3. Reference

1. YOUNG, J.C. 1973. Chemical methods for nitrification control. *J. Water Pollut. Control Fed.* 45:637.

* Approved by Standard Methods Committee, 2016.
Joint Task Group: James C. Young (chair), Victor D. Hahn, Robert V. Menegotto, Devon A. Morgan, Robin S. Parnell, Lisa M. Ramirez, Debra A. Waller.

5210 B. 5-Day BOD Test

1. General Discussion

The BOD test is an indirect measurement of organic matter; it measures the change in DO concentration caused by microorganisms as they degrade organic matter in a sample held in a stoppered bottle incubated for 5 d in the dark at 20°C. Analysts measure DO before and after incubation, and compute BOD using the difference between DO measurements. Because initial DO is determined shortly after dilution, all oxygen uptake occurring after this measurement is included in the BOD measurement.

For sampling and storage procedures, see 5210B.4*a*.

2. Apparatus

a. Incubation bottles: Use 60-mL glass bottles or larger (300-mL bottles with a flared mouth and ground-glass stopper are preferred). Clean bottles with a detergent, rinse thoroughly,

and drain before use. Alternatively, use disposable plastic BOD bottles that are capable of meeting all method quality-control (QC) checks.

b. Air incubator or water bath, thermostatically controlled at 20 ± 1°C. Exclude all light to prevent the possibility of photosynthetic production of DO.

c. Oxygen-sensitive membrane electrode, polarographic or galvanic, or *oxygen-sensitive optical probe* with appropriate meter.

3. Reagents

Discard reagents if there is any sign of precipitation or biological growth in the stock bottles. Commercial equivalents of these reagents are acceptable, and different stock concentrations may be used if doses are adjusted proportionally. Use reagent grade or better for all chemicals and use distilled or equivalent reagent-grade water (see Section 1080) to make all solutions.

a. Phosphate buffer solution: Dissolve 8.5 g monopotassium phosphate (KH_2PO_4), 21.75 g dipotassium phosphate (K_2HPO_4), 33.4 g disodium phosphate ($Na_2HPO_4 \cdot 7H_2O$, and 1.7 g ammonium chloride (NH_4Cl) in about 500 mL reagent-grade water and dilute to 1 L. The pH should be 7.2 without further adjustment. Alternatively, dissolve 42.5 g KH_2PO_4 and 1.7 g NH_4Cl in about 700 mL reagent-grade water. Adjust pH to 7.2 with 30% sodium hydroxide (NaOH) and dilute to 1 L.

b. Magnesium sulfate ($MgSO_4$) solution: Dissolve 22.5 g $MgSO_4 \cdot 7H_2O$ in reagent-grade water and dilute to 1 L.

c. Calcium chloride ($CaCl_2$) solution: Dissolve 27.5 g $CaCl_2$ in reagent-grade water and dilute to 1 L.

d. Ferric chloride ($FeCl_3$) solution: Dissolve 0.25 g $FeCl_3 \cdot 6H_2O$ in reagent-grade water and dilute to 1 L.

e. Acid and alkali solutions, 1N, to neutralize caustic or acidic waste samples.

1) Acid—Slowly and while stirring, add 28 mL conc sulfuric acid (H_2SO_4) to reagent-grade water. Dilute to 1 L.

2) Alkali—Dissolve 40 g NaOH in distilled water. Dilute to 1 L.

f. Sodium sulfite (Na_2SO_3) solution: Dissolve 1.575 g Na_2SO_3 in 1000 mL reagent-grade water. This solution is unstable; prepare daily.

g. Nitrification inhibitor:

1) 2-chloro-6-(trichloromethyl) pyridine (TCMP)—Use pure TCMP or commercial preparations.*

2) Allylthiourea (ATU) solution—Dissolve 2.0 g allylthiourea ($C_4H_8N_2S$) in about 500 mL reagent-grade water and dilute to 1 L. Store at 4°C. The solution is stable for 2 weeks when stored at ≤6°C without freezing.

h. Glucose–glutamic acid (GGA) solution: Dry reagent-grade glucose and reagent-grade glutamic acid at 103°C for 1 h. Add 150 mg glucose and 150 mg glutamic acid to reagent-grade water and dilute to 1 L. Prepare fresh immediately before use unless solution is maintained in a sterile container. Store all GGA mixtures at ≤6°C without freezing unless manufacturer recommendations state otherwise. Commercial preparations may be used but concentrations may vary. Discard solutions if evidence of contamination is indicated (e.g., growth occurs in the stock bottle or GGA test results are consistently low).

i. Ammonium chloride solution: Dissolve 1.15 g NH_4Cl in about 500 mL reagent-grade water, adjust pH to 7.2 with NaOH solution, and dilute to 1 L. Solution contains 0.3 mg N/mL.

j. Source water for preparing BOD dilution water: Use demineralized, distilled, or equivalent reagent-grade water, tap, or natural water to make sample dilutions (see 5210B.4*c*).

4. Preparatory Procedures

a. Sampling and storage: Samples for BOD analysis may degrade significantly during storage between collection and analysis, resulting in low BOD values.

1) Grab samples—If analysis begins within 2 h of collection, cold storage is unnecessary. Otherwise, keep sample at ≤6°C between collection and analysis. Ideally, begin analysis within 6 h of sample collection; if impossible due to distance between sampling site and laboratory, then begin analysis within 24 h of collection. The recommended holding time is 24 h; however, the U.S. Environmental Protection Agency (EPA) allows for a 48-h holding time.

2) Composite samples—Limit compositing period to 24 h, and keep samples at ≤6°C during process. Store for the same time and temperature as grab samples, although in this case, holding time begins when the compositing period ends.

b. Sample preparation and pretreatment:

1) All samples—Check pH; if it is not between 6.0 and 8.0, adjust sample temperature to 20 ± 3°C, then adjust pH to between 6.5 and 7.5 using an H_2SO_4 or NaOH solution strong enough not to dilute sample by >0.5%. Exceptions may be justified with natural waters when BOD will be measured at *in situ* pH values. Dilution-water pH should not be affected by the lowest sample dilution. Always seed samples that have been pH-adjusted.

2) Samples containing residual chlorine compounds—If possible, avoid samples containing residual chlorine by sampling ahead of chlorination processes. If residual chlorine is present, dechlorinate sample. Sometimes chlorine will dissipate from sample within 1 to 2 h of standing in the light; this often occurs during transport and handling. If the chlorine residual does not dissipate in a reasonably short time, destroy it by adding Na_2SO_3 solution. Determine required volume of Na_2SO_3 solution on a 100- to 1000-mL portion of neutralized sample by adding 10 mL 1 + 1 acetic acid or 1 + 50 H_2SO_4 and 10 mL potassium iodide (KI) solution (10 g/100 mL) per 1000 mL sample, and then titrating with Na_2SO_3 solution to the starch-iodine endpoint for residual. Add to neutralized sample the proportional volume of Na_2SO_3 solution determined by the above test, mix, and check sample for residual chlorine after 10 to 20 min. (NOTE: Excess Na_2SO_3 exerts an oxygen demand and reacts slowly with certain organic chloramine compounds that may be present in chlorinated samples.) Do not test chlorinated/dechlorinated samples without seeding.

3) Samples containing other toxic substances—Certain industrial wastes (e.g., plating wastes) contain toxic metals. Such samples often require special study and treatment.

4) Samples supersaturated with DO (Table 4500-O:I)—Samples with DO concentrations above saturation at 20°C may be collected in cold waters or in water where photosynthesis occurs. To prevent oxygen loss when incubating such samples, reduce

* Nitrification Inhibitor Formula 2533 (2% TCMP on sodium sulfate), Hach Co., Loveland, CO, or equivalent.

DO to saturation by bringing sample to about 20 ± 3°C in partially filled bottle while agitating by vigorous shaking or aerating with clean, filtered compressed air.

5) Samples containing hydrogen peroxide—Hydrogen peroxide remaining in samples from some industrial bleaching processes (e.g., those used at paper mills and textile plants) can cause supersaturated oxygen levels in samples collected for BOD testing. Mix such samples vigorously in open containers long enough to allow hydrogen peroxide to dissipate before setting up BOD tests. Check adequacy of peroxide removal by observing DO concentrations over time during mixing or by using peroxide-specific test strips. Mixing times can vary from 1 to 2 h, depending on the amount of hydrogen peroxide present. The peroxide reaction can be considered complete when DO no longer increases during a 30-min period without mixing.

c. Selection and storage of source water for BOD sample dilution: Obtain water from suitable source (i.e., distilled, tap, or reagent-grade water). Make sure water is free of heavy metals, specifically copper (<0.05 mg/L) and toxic substances [e.g., chlorine (<0.10 mg/L)] that can interfere with BOD measurements. Protect source-water quality by using clean glassware, tubing, and bottles. Deionized (DI) water often contains enough organics and microorganisms to cause the dilution-water QC check to fail (5210B.6*c*). DI water is not recommended unless dilution-water blanks consistently meet QC limits. Source water may be stored before use as long as the prepared dilution water (5210B.5*a*) meets QC criteria in the dilution-water blank (5210B.6*c*). Such storage may improve the quality of some source waters but may allow biological growth to deteriorate others. Storing prepared dilution water (5210B.5*h*) for >24 h after adding nutrients, minerals, and buffer is not recommended unless dilution-water blanks consistently meet QC limits. Discard stored source water if dilution-water blank shows >0.2 mg/L DO depletion in 5 d (5210B.6*c*).

d. Preparation of seed suspension: Each BOD bottle must contain a microorganism population that can oxidize biodegradable organic matter in the sample. Domestic wastewater, unchlorinated or other undisinfected effluents from biological wastewater treatment plants, and surface waters receiving wastewater discharges usually contain satisfactory microbial populations. Some samples (e.g., some untreated industrial wastes, disinfected wastes, high-temperature wastes, wastes with pH values <6 or >8, or wastes stored >6 h after collection) do not contain a sufficient microbial population. Seed such samples by adding a population of suitable microorganisms; the preferred seed comes from a sample-related biological wastewater treatment system or receiving water. In this case, use supernatant from settled domestic wastewater, effluent from primary clarifiers, diluted mixed liquor from an aeration basin, undisinfected effluent, or receiving water from below the discharge point. If using effluent or mixed liquor from a biological treatment process as a seed source, nitrification inhibition is recommended. Do not use seed from effluents that have been disinfected by chlorine or other means. Commercial seed sources may be used according to manufacturer's preparation instructions but are more likely to be unadapted to the wastewater constituents. Do not filter seed sources; filtering removes the seed microorganisms.

If adapted seed sources are unavailable, develop an acclimated seed in the laboratory by continuously aerating a sample of settled domestic wastewater and adding small daily increments of sample from the waste in question. Use a soil suspension, activated sludge, or a commercial seed preparation to obtain the initial microbial population. Determine the existence of a satisfactory population by testing the seed's performance in BOD tests on the sample. BOD values that increase during adaptation to a steady high value indicate successful seed acclimation.

5. Testing Procedure

a. Preparation of dilution water: Transfer desired working volume of source water (5210B.4*c*) to a suitably sized bottle (glass is preferred). Check to ensure that the DO concentration is at least 7.5 mg/L before using water for BOD tests. If not, add DO by shaking bottle or aerating it with organic-free filtered air. Alternatively, store the water in cotton-plugged bottles long enough for the DO concentration to approach saturation. Add 1 mL each of phosphate buffer, $MgSO_4$, $CaCl_2$, and $FeCl_3$ solution/L to prepared source water (5210B.4*c*). Mix thoroughly and bring temperature to 20 ± 3°C. Prepare dilution water immediately before use, unless dilution-water blanks (5210B.6*c*) show that the water is acceptable after longer storage times. If dilution-water blanks show a DO depletion >0.2 mg/L, then improve purification or use water from another source. Do not add oxidizing agents or expose dilution water to ultraviolet light to try to bring the dilution blank into range.

b. Sample temperature adjustment: Bring sample temperature to 20 ± 3°C before making dilutions.

c. Preparation of dilutions: Using dilution water prepared as in ¶ *a* above, make at least three dilutions of prepared sample estimated to produce, at the end of the test, at least one dilution that would result in a residual DO of ≥1.0 mg/L and a DO uptake of ≥2.0 mg/L after a 5-d incubation. Two dilutions are allowed if experience with a particular sample source produces at least one bottle with acceptable minimum DO depletions and residual limits (5210B.6*a*). Individual laboratories should evaluate the need for more than three dilutions when historical sample data are unavailable. A more rapid analysis, such as COD (Section 5220), may be correlated approximately with BOD and serve as a guide in selecting dilutions. In the absence of prior knowledge, use the following percentages of wastewater when preparing dilutions: 0.01 to 1.0% for strong industrial wastes, 1 to 5% for raw and settled wastewater, 5 to 25% for biologically treated effluent, and 25 to 100% for polluted river waters. The number of bottles to be prepared for each dilution depends on DO technique and number of replicates desired. Prepare dilutions in volumetric containers (Class A glass or equivalent) and then transfer to BOD bottles, or else prepare directly in BOD bottles. Either dilution method can be used to transfer sample to respective BOD bottles.

1) Dilutions prepared in volumetric containers—Using a wide-tipped pipet or graduated cylinder, add desired amount of prepared sample to individual volumetric cylinders or flasks. Mix sample well immediately before pipetting to avoid solids loss via settling. For dilutions greater than 1:300, make a primary dilution before making final dilution in volumetric cylinders or flasks. Fill cylinders or flasks at least two-thirds full with dilution water and sample without entraining air. Add appropriate amounts of seed suspension (¶ *d* below) and nitrification inhibitor (¶ *e* below). Dilute to final level with dilution water (¶ *a* above). Mix well but avoid entraining air. Siphon mixed dilution

into a suitable number of BOD bottles, taking care not to let solids settle in cylinder or flask during transfer. When a cylinder or flask contains >67% of sample after dilution, nutrients may be limited in the diluted sample and subsequently reduce biological activity. In such samples, add the nutrient, mineral, and buffer solutions (5210B.3*a–d*) directly to diluted sample at a rate of 1 mL/L (0.30 mL/300-mL bottle), or use commercially prepared solutions designed to dose the appropriate container size.

2) Dilutions prepared directly in BOD bottles—Using a wide-tip volumetric pipet or graduated cylinder, add desired sample volume to individual BOD bottles. Mix sample well immediately before pipetting to avoid solids loss via settling. For dilutions greater than 1:300, make a primary dilution before making final dilution in the bottle. Fill each BOD bottle approximately two-thirds full with dilution water and/or sample without entraining air. Add appropriate amounts of seed suspension (¶ *d* below) and nitrification inhibitor (¶ *e* below) to individual BOD bottles. Fill remainder of BOD bottle with dilution water. When a bottle contains >67% of sample after dilution, nutrients may be limited in the diluted sample and subsequently reduce biological activity. In such samples, add the nutrient, mineral, and buffer solutions (5210B.3*a–d*) directly to diluted sample at a rate of 1 mL/L (0.30 mL/300-mL bottle), or use commercially prepared solutions designed to dose the appropriate bottle size.

d. Addition of seed suspension: If seeding is used, add seed suspensions to dilution vessels or individual BOD bottles before final dilution, as described in ¶ *c* above. Do not add seed directly to wastewater samples before dilution if they contain toxic materials. Generally, 1 to 3 mL of settled raw wastewater or primary effluent or 1 to 2 mL of a 1:10 dilution of mixed liquor/300-mL bottle will provide enough microorganisms. Do not filter seed suspension before use. Agitate seed suspension during transfer to ensure that the same quantity of microorganisms is added to each BOD bottle. Always record the exact volume of seed suspension added to each bottle. The DO uptake attributable to added seed generally should be between 0.6 and 1.0 mg/L, but adjust seed amount as needed to provide GGA check results of 198 ± 30.5 mg/L. For example, if 1 mL seed suspension is required to achieve 198 ± 30.5 mg/L BOD in the GGA check, then use 1 mL in each BOD bottle receiving the test wastewater.

e. Addition of nitrification inhibitor: Samples that may require nitrification inhibition[1] include, but are not limited to, biologically treated effluents, samples seeded with biologically treated effluents, and river waters. Note the use of nitrification inhibition and the related chemical used when reporting results. (NOTE: TCMP is the preferred nitrification inhibitor but requires handling and transfer in a solid form. ATU is not always effective in inhibiting nitrification within the 5-d incubation period, and concentrations >2 mg/L may increase carbonaceous BOD (CBOD) measurements and/or adversely affect the azide modification of the iodometric method.) Seed all samples to which nitrification inhibitor has been added.

1) Nitrification inhibition using TCMP—Add 10 mg TCMP/L to diluted sample, 3 mg TCMP to each 300-mL bottle, or proportional amounts to other sized bottles after initial sample dilution but before final filling of bottles with dilution water. Do not add TCMP to BOD bottles before they are at least two-thirds filled with diluted sample. (NOTE: TCMP dissolves slowly and can float on top of sample if not mixed well.) Some commercial TCMP formulations are not 100% TCMP; adjust dosage appropriately.

2) Nitrification inhibition using ATU—Add 1 mL ATU solution [5210B.3*g*2)]/L diluted sample or 0.3 mL/300-mL test bottle. Do not add ATU to BOD bottles until they are at least two-thirds filled with diluted sample.

f. Sealing bottles: Completely fill each bottle by adding enough dilution water so insertion of stopper leaves no bubbles in the bottle. Mix sample by turning bottle manually several times unless immediately using a DO probe with a stirrer to measure initial DO concentration. As a precaution against drawing air into the dilution bottle during incubation, use a water seal. Obtain satisfactory water seals by inverting bottles in a water bath or by adding water to the flared mouth of special BOD bottles. Place a paper or plastic cup or foil cap over flared mouth of bottle to reduce evaporation of water seal during incubation.

g. Determination of initial DO: Use the azide modification of the iodometric method (Section 4500-O.C), membrane-electrode method (Section 4500-O.G), or optical-probe method (Section 4500-O.H) to determine initial DO on all sample dilutions, dilution-water blanks, and, where appropriate, seed controls. Replace any displaced contents with enough diluted sample or dilution water to fill the bottle, stopper all bottles tightly, and water seal before beginning incubation. After preparing dilution, measure initial DO within 30 min. If using the membrane-electrode method or optical probe method, calibrate DO probe daily by following the manufacturer's calibration procedure. Make frequent calibration checks daily to ensure accurate DO readings and, ideally, perform a Winkler titration as needed to verify calibration. If using the azide modification of the titrimetric iodometric method, prepare an extra bottle for initial DO determination for each sample dilution.

h. Sample incubation: Incubate at 20 ± 1°C the stoppered and sealed BOD bottles containing desired dilutions (¶ *c* above), seed controls (5210B.6*d*), dilution-water blanks (5210B.6*c*), and GGA checks (5210B.6*b*). Exclude light to avoid algae growth in bottles during incubation.

i. Determination of final DO: After 5 d ± 6 h of incubation, determine DO in all sample dilutions, blanks, and checks as in 5210B.6*g*, using the azide modification of the titrimetric method (Section 4500-O.C), membrane-electrode method (Section 4500-O.G), or optical-probe method (Section 4500-O.H).

6. Quality Control Checks

The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

a. Minimum residual DO and minimum DO depletion: Only bottles (including seed controls) whose DO depletion is ≥2.0 mg/L and residual DO is ≥1.0 mg/L after 5 d of incubation are considered to produce valid data, because ≥2.0 mg oxygen uptake/L is required to give a meaningful measure of oxygen uptake and ≥1.0 mg/L must remain to ensure that waste constituents' oxidation rates were not limited by insufficient DO. However, there are exceptions—for reporting purposes only—when testing undiluted samples and all bottles' DO depletion is <2.0 mg/L and residual DO is <1.0 mg/L (see 5210B.7).

b. Glucose–glutamic acid check: The GGA check is the primary basis for establishing the BOD test's accuracy and precision, as well as the principal measure of seed quality and set-up procedure. Together with each batch of BOD or CBOD samples, check seed effectiveness and analytical technique by using pro-

cedures in 5210B.5 to make BOD measurements on an equal weight mixture of glucose and glutamic acid as follows: Add sufficient amounts of standard glucose and glutamic acid solutions (5210B.3*h*) to give 3.0 mg glucose/L and 3.0 mg glutamic acid/L in each of three test bottles (20 mL GGA solution/L seeded dilution water, or 6.0 mL/300-mL bottle). Commercial solutions may contain other GGA concentrations; adjust doses accordingly. Add nitrification inhibitor if seed is obtained from a source that is nitrifying, and also to all CBOD GGA checks. Evaluate data as described in 5210B.8. The resulting average BOD/CBOD for the three bottles, after correcting for dilution and seeding, must fall into the control-limit range established in 5210B.8*a*. If the average value falls outside this range, evaluate the cause and make appropriate corrections. Consistently high values can indicate too much seed suspension, contaminated dilution water, or nitrification; consistently low values can indicate poor seed quality or quantity or else the presence of a toxic material. If low values persist, prepare a new GGA mixture and check the dilution-water and seed sources.

c. Dilution-water-quality check: With each batch of dilution water, incubate two or more bottles of dilution water containing nutrient, mineral, and buffer solutions but no seed or nitrification inhibitor. Dilution water checks must be analyzed with each batch of samples; the dilution-water blank serves as a check on the quality of unseeded dilution water and cleanliness of incubation bottles. Determine initial and final DO for each bottle (5210B.5*e* and *i*), and average results. The average DO uptake in 5 d must not be >0.2 mg/L and preferably ≤0.1 mg/L before making seed corrections. If average dilution-water blank is >0.2 mg/L, record the data and clearly identify such samples in data records.

d. Seed control: Determine the seed suspension's BOD as for any other sample. This is the *seed control.* Ideally, make three dilutions of seed so the smallest quantity depletes ≥2.0 mg/L DO and the largest quantity leaves ≥1.0 mg/L DO residual after 5 d of incubation. Determine DO uptake per milliliter of seed by dividing the DO depletion by the volume of seed in milliliters for each seed control bottle with a 2.0 mg/L depletion and >1.0 mg/L minimum residual DO, and averaging the results. Seed dilutions showing widely varying depletions per milliliter of seed (±30%) suggest the presence of toxic substances or large particulates in the seed suspension; check or change the seed source.

7. Data Analysis and Reporting

a. Calculations:

1) For each test bottle with at least 2.0 mg/L DO depletion and at least 1.0 mg/L residual DO—before seed correction, calculate BOD as follows:

$$\text{BOD}_5, \text{mg/L} = \frac{(D_1 - D_2) - (S)V_S}{P}$$

where:

D_1 = DO of diluted sample immediately after preparation, mg/L,
D_2 = DO of diluted sample after 5 d incubation at 20°C, mg/L,
S = oxygen uptake of seed [Δ DO/mL seed suspension added per bottle (5210B.6*d*) ($S = 0$ if samples are unseeded)],
V_s = volume of seed in respective test bottle, mL, and
P = decimal volumetric fraction of sample used; $1/P$ = dilution factor.

2) If DO depletion is <2.0 mg/L and sample concentration is 100% (no dilution except for seed, nutrient, mineral, and buffer solutions), actual seed-corrected DO depletion may be reported as the BOD even if it is <2.0 mg/L.

3) When all dilutions result in a residual DO <1.0, select the bottle with the highest DO concentration (usually the greatest dilution) and report:

$$\text{BOD}_5, \text{mg/L} > \frac{(D_1 - D_2) - (S)V_S}{P}$$

4) If all dilutions result in DO depletion <2.0 mg/L and the sample was diluted, select the bottle with the largest volume of sample (the least dilution) and calculate the report as if the dilution had depleted 2.0 mg/L:

$$\text{BOD}_5, \text{mg/L} < \frac{(D_1 - D_2) - (S)V_S}{P}$$

In the above calculations, do not make corrections for DO uptake by the dilution-water blank during incubation.

b. Reporting: Average test results for all qualified bottles in each dilution series. Report the result as BOD_5 if nitrification is not inhibited; report it as CBOD_5 if nitrification is inhibited. Samples with large differences between the computed BOD for different dilutions (e.g., the highest value is >30% larger than the lowest value) may indicate a toxic substance or analytical problems. When the effect becomes repetitive, investigate to identify the cause. Toxicity should be claimed only after thorough investigation using respirometric (5210D) or equivalent methods. Identify results in the test reports when any of the following QC conditions occur:

- dilution-water blank average is >0.2 mg/L (5210B.6*c*),
- GGA check falls outside acceptable limits (5210B.6*b*),
- test replicates show >30% difference between highest and lowest values,
- none of the seed control samples meet the above criteria (5210B.6*d*), or
- all dilutions result in a residual DO <1.0 mg/L [5210B.7*a*3)].

8. Precision and Bias

There is no measurement for establishing the BOD test's bias. The GGA check prescribed in 5210B.6*b* is intended to be a reference point for evaluating dilution-water quality, seed effectiveness, and analytical technique. Single-laboratory tests using a 300-mg/L mixed GGA solution provided the following results:

Number of months:	14
Number of triplicates:	421
Average monthly recovery:	204 mg/L
Average monthly standard deviation:	10.4 mg/L

In a series of interlaboratory studies,[2] each involving 2 to 112 laboratories (and as many analysts and seed sources), 5-d BOD measurements were made on synthetic-water samples containing a 1:1 mixture of GGA ranging from 3.3 to 231 mg/L total concentration. The regression equations for mean value, X, and standard deviation, S, from these studies were:

$$X = 0.658 \text{ (added concentration, mg/L)} + 0.280 \text{ mg/L}$$

$$S = 0.100 \text{ (added concentration, mg/L)} + 0.547 \text{ mg/L}$$

a. Control limits: Applying the above equations to the 300-mg/L GGA primary standard yields an average 5-d BOD of 198 mg/L with a standard deviation of 30.5 mg/L. Because many factors affect BOD tests in multi-laboratory studies, resulting in extremely variable test results, one standard deviation (as determined by interlaboratory tests) is recommended as a control limit for individual laboratories. Alternatively, each laboratory may establish its own control limits by performing at least 25 GGA checks (5210B.6*b*) over several weeks or months and calculating the mean and standard deviation. Use the mean ±3 standard deviations as the control limit for future GGA checks. Compare calculated control limits to the single-laboratory tests presented above and to interlaboratory results. If any GGA test results are outside the acceptable control-limit range, identify them clearly in all data records, investigate source of the problem, and make appropriate corrections.

When nitrification inhibitors are used, GGA test results outside the control-limit range often indicate that incorrect amounts of seed were used. Adjust the amount of seed added to the GGA test so results fall within range (5210B.6*b*).

b. Working range and reporting limit: The *working range* is equal to the difference between the maximum initial DO (7 to 9 mg/L) and minimum DO residual of 1 mg/L corrected for seed and multiplied by the dilution factor, including any intermediate dilutions performed (5210B.5*c*).

Reporting limits are established by the minimum DO depletion and minimum DO residuals as follows:

- The lower reporting limit for unseeded samples that require no dilution—except for nutrient, mineral, and buffer solutions ($S = 0$; $P = 1.0$)—is equal to the DO measurement method's detection limit (~0.1 mg/L).
- The lower reporting limit for seeded samples that require no dilution—except for seed, nutrient, mineral, and buffer solutions ($S > 0$; $P = 1.0$)—is the difference between sample DO depletion and seed correction.

9. References

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5210 C. Ultimate BOD Test

1. General Discussion

The ultimate BOD test is an extension of the 5-d dilution BOD test (5210B) but with a number of specific test requirements and differences in application. Be familiar with the 5210B procedure before conducting tests for UBOD.

a. Principle: The method consists of placing a single sample dilution in full, airtight bottles and incubating under specified conditions for an extended period, depending on wastewater, effluent, river, or estuary quality.[1] DO is measured (with probes) initially and intermittently during the test. From the DO versus time series, UBOD is calculated by an appropriate statistical technique. For more accuracy, run tests in triplicate.

Bottle size and incubation time are flexible to accommodate individual sample characteristics and laboratory limitations. Incubation temperature, however, is 20°C. Most effluents and some naturally occurring surface waters contain materials whose oxygen demands exceed the DO available in air-saturated water; in such cases, either dilute sample or monitor DO frequently to ensure that low DO or anaerobic conditions do not occur. Reaerate sample when DO concentrations approach 2 mg/L.

Because bacterial growth requires nutrients (e.g., nitrogen, phosphorus, and trace metals), the necessary amounts may be added to dilution water, along with a buffer to keep pH in the bacterial-growth range and enough seed for an adequate bacterial population. (No specific nutrient or buffer formulations are included here because of the wide range of water and wastewater characteristics and varied applications of UBOD data.) That said, if the result will be used to estimate the oxidation rate of naturally occurring surface waters, adding nutrients and seed probably will accelerate the decay rate and produce misleading results. If only UBOD is desired, adding supplemental nutrients that accelerate decay and shorten test duration may be advantageous. When using nutrients, also add them to the dilution-water blank.

How much nitrogenous compounds will oxidize during the prescribed incubation period depends on how many relevant oxidizing microorganisms are present. These organisms may be too scarce in wastewaters to oxidize significant quantities of reduced nitrogen, but abundant in naturally occurring surface waters. Results may be erratic when a nitrification inhibitor is used,[2] so do NOT use one unless prior experimental evidence on

the particular sample suggests that it is acceptable.* Monitor nitrite nitrogen (NO_2^--N) and nitrate nitrogen (NO_3^--N) to compute the oxygen equivalency of the nitrification reaction. When these values are subtracted from the DO versus time series, the CBOD time series can be constructed.[3]

b. Sampling and storage: See 5210B.4*a*.

c. Quality control: The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Incubation bottles: 2-L or larger glass bottles with ground-glass stoppers;† 4- to 10-L glass serum bottles are available. Alternatively, use nonground-glass bottles with nonbiodegradable plastic caps as a plug insert. Do not reuse plugs because they become discolored with continued use. Replace plugs every 7 to 14 d. Do not use rubber stoppers that may exert an oxygen demand.

Clean bottles with a detergent and wash with dilute hydrochloric acid (HCl) (3*N*) to remove surface films and precipitated inorganic salts; rinse thoroughly with DI water before use. Cover top of bottles with paper after rinsing to prevent dust from collecting.

Use a water seal to avoid drawing air into sample bottle during incubation. If bottle does not have a flared mouth, construct a water seal by making a watertight dam around the stopper (or plug) and fill with water from the reservoir as necessary. Cover dam with clean aluminum foil to retard evaporation. If using a 2-L BOD bottle, fill reservoir with sample and cover with a polyethylene cap before incubation.

Place a clean magnetic stirring bar in each bottle to mix contents before making DO measurement or taking a subsample. Do not remove magnets until test is complete.

Alternatively, use a series of 300-mL BOD bottles (5210B.2*a*) if larger bottles are unavailable or incubation space is limited.

b. Reservoir bottle: 4-L or larger glass bottle. Close with plastic screw cap or non-rubber plug.

c. Incubator or water bath, thermostatically controlled at 20 ± 1°C. Exclude all light to prevent the possibility of photosynthetic production of DO.

d. Oxygen-sensitive membrane electrode: See Section 4500-O.G.2.

3. Procedure

a. River water samples: Preferably fill large BOD bottle (>2 L, or else 6 or more 300-mL BOD bottles) with sample at 20°C. Add no nutrients, seed, or nitrification inhibitor if in-bottle decay rates will be used to estimate in-stream rates. Do not dilute sample unless pretesting or experience shows that ultimate BOD will be high (>20 mg/L).

Measure DO in each bottle, stopper it, and make an airtight seal. Incubate at 20°C in the dark.

Measure DO in each bottle at intervals of at least 2 to 5 d over 30 to 60 d (minimum of 6 to 8 readings), or longer under special circumstances. To avoid oxygen depletion in samples containing ammonia nitrogen (NH_3-N), measure DO more frequently until nitrification has taken place. If DO falls to about 2 mg/L, re-aerate as directed below. Replace sample lost by the cap and DO-probe displacement by adding 1 to 2 mL sample from the reservoir bottle.

When DO approaches 2 mg/L, re-aerate. Pour a small amount of sample into a clean vessel and re-aerate the remainder directly in the bottle by vigorous shaking or bubbling with purified air (medical grade). Refill bottle from the storage reservoir and measure DO. This concentration becomes the initial DO for the next measurement. If using 300-mL BOD bottles, empty all of the bottles into a clean vessel, re-aerate, and refill the small bottles.

Analyze for NO_2^--N + NO_3^--N (see Sections 4500-NO_2^- and 4500-NO_3^-) on Days 0, 5, 10, 15, 20, and 30. Alternatively, determine NO_2^--N and NO_3^--N each time DO is determined, thereby producing corresponding BOD and nitrogen determinations. If the ultimate demand occurs after 30 d, make additional analyses at 30-d intervals. Remove 10 to 20 mL from the bottle for these analyses, and refill bottle as necessary from the reservoir bottle. Preserve NO_2^--N + NO_3^--N subsample with H_2SO_4 to pH <2 and refrigerate. If the UBOD test's goal is to assess UBOD and not to provide data for rate calculations, measure NO_3^--N concentration only at Day 0 and on the last day of the test (kinetic rate estimates are not useful when the nitrification reaction is not followed).

Calculate oxygen consumption during each time interval and make appropriate corrections for nitrogenous oxygen demand. Correct by using 3.43 × the NH_3^--N to NO_2^--N conversion plus 1.14 × the NO_2^--N to NO_3^--N conversion to reflect the stoichiometry of NH_4^+ oxidation to NO_2^- or NO_3^-.

When using a dilution-water blank, subtract the blank's DO uptake from the total DO consumed. High-quality reagent water without nutrients typically will consume a maximum of 1 mg DO/L in a 30- to 90-d period. If the dilution water's DO uptake is >0.5 mg/L for a 20-d period or 1 mg/L for a 90-d period, report the magnitude of the correction and try to obtain higher-quality dilution water for subsequent UBOD tests.

When weekly DO consumption drops below 1 to 2% of the total accumulative consumption, calculate UBOD using a nonlinear regression method.

b. Wastewater treatment plant samples: Use high-quality reagent water (see Section 1080) for dilution water. Add no nitrification inhibitors if decay rates are desired. If seed and nutrients are necessary, add the same amounts of each to the dilution-water blank. Use minimal sample dilution. As a general rule, the diluted sample's UBOD should be in the range of 20 to 30 mg/L. Dilution to this level probably will require two or three sample re-aerations during incubation to prevent DO concentrations from falling below 2 mg/L.

Use 2-L or larger BOD bottles (alternatively, multiple 300-mL BOD bottles) for each dilution. Add desired volume of sample to each bottle and fill with dilution water.

Fill a BOD bottle with dilution water to serve as a dilution-water blank. Treat blank the same as all samples. Follow procedure given in ¶ *a* above and incubate for at least as long as UBOD test.

* Some analysts have reported satisfactory results with 2-chloro-6-(trichloromethyl) pyridine (Nitrification Inhhibitor, Formula 2533, Hach Co., Loveland, CO, or equivalent).

† Wheaton 2-L BOD bottle No. 227580, 1000 North Tenth St., Millville, NJ, or equivalent.

TABLE 5210:I. UBOD RESULTS FOR WASTEWATER SAMPLE

Day	(1) Average DO* *mg/L*	(2) Average Blank DO† *mg/L*	(3) Accumulated DO Consumed by Sample‡ *mg/L*	(4) Average NO_3-N *mg/L*	(5) NBOD *mg/L*§	(6) CBOD *mg/L*‖
0	8.1	–	0	0.0	0	0
3	5.6	–	2.5	–	0	2.5
5	3.5/8.0	–	4.6	0.0	0	4.6
7	6.2	–	6.4	–	0.23	6.2
10	3.2/8.2	–	9.4	0.10	0.46	8.9
15	4.3	–	13.3	–	0.58	12.7
18	2.7/8.1	–	14.9	0.15	0.69	14.2
20	6.6	–	16.4	–	0.80	15.6
25	5.4	–	17.6	0.20	0.92	16.7
30	2.6/8.2	–	20.4	–	0.92	19.5
40	5.3	–	23.3	0.20	0.92	22.4
50	3.1/8.0	–	25.5	–	0.92	24.6
60	4.5	–	29.0	–	0.92	28.1
70	3.3/8.1	–	30.2	–	0.92	29.3
80	5.4	–	32.9	0.20	0.92	32.0

* Two readings indicate concentrations before and after re-aeration.
† None was used.
‡ Column (1) – blank correction (none needed in the example).
§ Column (4) × 4.57 (linear interpolation between values).
‖ [Column (3) – Column (5)] × dilution factor.
UCBOD = 34.5 mg/L; CBOD decay rate = 0.03/d (calculated with first-order equation from 5210C.4).

4. Calculations

An example of results obtained for an undiluted wastewater sample, without seed and nutrients, is given in Table 5210:I .

UBOD can be estimated by using a first-order model described as follows:

$$BOD_t = UBOD(1 - e^{-kt})$$

where:

BOD_t = oxygen uptake measured at time t, mg/L, and
k = first-order oxygen uptake rate.

The data in Table 5210:I were analyzed via a nonlinear regression technique applied to the above first-order model.[4] However, a first-order kinetic model may not always be the best choice. Significantly better statistical fits usually are obtained with alternative kinetic models, including sum of two first-order and logistic function models.[1,3–8]

5. Precision and Bias

UBOD-test precision was assessed via a series of replicate tests in a single laboratory. Interlaboratory studies have not been conducted.

Reference	Replicate No.	UBOD *mg/L*	Precision Summary*
2	1	154	μ = 151 mg/L
	2	154	
	3	145	CV = 3.5%
5	1	10.3	
	2	11.1	
	3	9.6	μ = 10.0 mg/L
	4	9.9	
	5	9.8	CV = 5.8%
	6	9.6	
6	1	12.8	μ = 12.4 mg/L
	2	12.6	
	3	12.6	CV = 4.4%
	4	11.6	

* μ = mean.
CV = coefficient of variation.

Bias was assessed by determining the BOD of a known concentration of glucose (150 mg/L) and glutamic acid (150 mg/L). This solution has a UBOD of 321 to 308 mg/L, depending on the extent of nitrification. The results of the study, conducted in triplicate, were:

Estimated* UBOD *mg/L*	Theoretical BOD *mg/L*	Percent Difference
276	308/321	–10/–14
310	308/321	+1/–3
303	308/321	–2/–6

* By statistical model.

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5210 D. Respirometric Method

1. General Discussion

a. Principle: Respirometric methods directly measure the oxygen consumed by microorganisms in an air- or oxygen-enriched environment in a closed vessel under constant temperature and agitation.

b. Uses: Respirometry measures oxygen uptake more or less continuously over time. Respirometric methods are useful for assessing the biodegradation of specific chemicals; the treatability of organic industrial wastes; the effect of known amounts of toxic compounds on a test wastewater's or organic chemical's oxygen-uptake reaction; the concentration at which a pollutant or a wastewater measurably inhibits biological degradation; the effect of various treatments (e.g., disinfection, nutrient addition, and pH adjustment) on oxidation rates; the oxygen requirement for essentially complete oxidation of biologically oxidizable matter; the need for using adapted seed in other biochemical oxygen-uptake measurements (e.g., the dilution BOD test); or a sludge's stability.

Respirometric data typically will be used comparatively (i.e., direct comparisons of oxygen-uptake rates in two test samples or in a test sample and a control). Because of inherent differences among uses, seed cultures, instruments, and applications of results, no single procedure for respirometric tests is applicable to all cases. Therefore, only basic recommendations and guidelines for overall test setup and procedure are given. Follow manufacturer's instructions for operating specific commercial instruments.

c. Types of respirometers: Four principal types of commercial respirometers are available: manometric, volumetric, electrolytic, and direct-input. Manometric respirometers relate oxygen uptake to the pressure change due to oxygen consumption when volume is constant. Volumetric respirometers measure oxygen uptake in incremental gas-volume changes when pressure is constant (at the time of reading). Electrolytic respirometers monitor how much oxygen is produced when water electrolyzes to maintain constant oxygen pressure in the reaction vessel. Direct-input respirometers deliver oxygen from a pure-oxygen supply to a sample via on-demand metering in response to minute pressure differences.

Most respirometers have been instrumented to permit data collection and processing via computer. Reaction-vessel contents are mixed by a magnetic or mechanical stirring device or by bubbling the reaction vessel's gaseous phase through its liquid phase. All respirometers remove carbon dioxide (CO_2) produced during biological growth by suspending a concentrated adsorbent (granular or solution) in the closed reaction chamber or by recirculating the gas phase through an external scrubber.

d. Interferences: Evolution of gases other than CO_2 may introduce errors in pressure or volume measurements; this is uncommon in the presence of DO. Incomplete CO_2 absorption will introduce errors if appropriate amounts and concentrations of alkaline absorbent are not used. Temperature fluctuations or inadequate mixing will introduce error. Fluctuations in barometric pressure can cause errors with some respirometers. Become familiar with the limits of the instrument used.

e. Minimum detectable concentration: Most commercial respirometers can detect oxygen demand in increments as small as 0.1 mg, but test precision depends on the total amount of oxygen consumed at the time of reading, the precision of pressure or volume measurement, and the effect of temperature and barometric-pressure changes. The upper limits of oxygen-uptake rate are determined by the ability to transfer oxygen into solution from the gas phase, which typically is related to mixing intensity. Transfer limits typically range from $<$10 mg O_2/L/h for low-intensity mixing to $>$100 mg O_2/L/h for high-intensity mixing.

f. Relationship to dilution BOD: Variations in waste composition, substrate concentration, mixing, and oxygen concentrations from one wastewater source to another generally preclude use of a general relationship between oxygen uptake by respirometers and the 5-d BOD at 20°C (see 5210B). Reasonably accurate correlations may be possible for a specific wastewater. The incubation period for respirometric measurements need not be 5 d because equally valid correlations can be made between the 5-d BOD and respirometric oxygen uptake at any time after 2 d.[1,2] Correlations between respirometric measurements and 5-d BOD for municipal wastewaters seem to occur at about 2 to 3 d incubation; however, correlations between respirometric measurements and 5-d BOD for industrial wastes and specific chemicals are less certain. Respirometric measurements also can

provide an indication of UBOD (see 5210C). In many cases, it is reasonable to consider that the 28- to 30-d oxygen uptake is essentially equal to UBOD.[3]

More commonly, respirometers are used as a diagnostic tool. The continuous oxygen-consumption readout in respirometric measurements indicates lag, toxicity, or any abnormalities in the biodegradation reaction. A change in the normal shape of an oxygen-uptake curve in the first few hours may help identify a toxic or unusual waste entering a treatment plant in time to adjust operations appropriately.

g. Relationship to other test methods and protocols: This method supports most of the protocols and guidelines established by the European Organization for Economic Co-operation and Development[3] (OECD) that require oxygen-uptake measurements.

h. Sampling and storage:

1) Grab samples—If analysis is begun within 2 h of sample collection, cold storage is unnecessary. Otherwise, keep sample ≤6°C from the time of collection. Begin analysis within 6 h of collection; when this is not possible, store ≤6°C and report storage temperature and duration. Never start analysis >24 h after grab-sample collection.

2) Composite samples—Keep samples ≤6°C during compositing. Limit compositing period to 24 h. Store using the same criteria as for grab samples; holding time begins when the compositing period ends.

2. Apparatus

a. Respirometer system: Use commercial apparatus and check manufacturer's instructions for specific system requirements, reaction vessel type and volume, and instrument operating characteristics.

b. Incubator or water bath: Use a constant-temperature room, incubator chamber, or water bath to control temperature to ±1°C. Exclude all light to prevent any algae in sample from forming oxygen. Use red, actinic-coated bottles for analysis outside of a darkened incubator.

3. Reagents

The following reagent formulations produce 1-L solutions, but smaller or larger volumes may be prepared according to need. Discard any reagent showing signs of biological growth or chemical precipitation. Stock solutions can be sterilized by autoclaving to provide longer shelf life.

a. Distilled water: Use only high-quality water distilled from a block tin or all-glass still (see Section 1080) or equivalent reagent-grade water. DI water may be used but often contains high bacterial counts. The water must contain <0.01 mg heavy metals/L and be free of chlorine, chloramines, caustic alkalinity, organic material, or acids. Make all reagents with this water. When other waters are required for special-purpose testing, state clearly their source and quality characteristics.

b. Phosphate buffer solution, 1.5*N:* Dissolve 207 g sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) in water. Neutralize to pH 7.2 with 6*N* potassium hydroxide (KOH) (¶ *g* below) and dilute to 1 L.

c. Ammonium chloride solution, 0.71*N:* Dissolve 38.2 g NH_4Cl in water. Neutralize to pH 7.0 with KOH. Dilute to 1.0 L; 1 mL = 10 mg N.

d. Calcium chloride solution, 0.25*M:* Dissolve 27.7 g $CaCl_2$ in water and dilute to 1 L; 1 mL = 10 mg Ca.

e. Magnesium sulfate ($MgSO_4$) solution, 0.41M: Dissolve 101 g $MgSO_4 \cdot 7H_2O$ in water and dilute to 1 L; 1 mL = 10 mg Mg.

f. Ferric chloride solution, 0.018*M:* Dissolve 4.84 g $FeCl_3 \cdot 6H_2O$ in water and dilute to 1 L; 1 mL = 1.0 mg Fe.

g. Potassium hydroxide solution, 6*N:* Dissolve 336 g KOH in about 700 mL water and dilute to 1 L. **CAUTION: Add KOH to water slowly and use constant mixing to prevent excessive heat buildup. Alternatively, use commercial solutions containing 30 to 50% KOH by weight.**

h. Acid solutions, 1*N:* Add 28 mL conc H_2SO_4 or 83 mL conc HCl to about 700 mL water. Dilute to 1 L.

i. Alkali solution, 1*N:* Add 40 g NaOH to 700 mL water. Dilute to 1 L.

j. Nitrification inhibitor: Reagent-grade TCMP or equivalent.[3]*

k. Glucose–glutamic acid solution: Dry reagent-grade glucose and reagent-grade glutamic acid at 103°C for 1 h. Add 15.0 g glucose and 15.0 g glutamic acid to distilled water and dilute to 1 L. Neutralize to pH 7.0 using 6*N* KOH (¶ *g* above). This solution may be stored for up to 1 week at 4°C.

l. Electrolyte solution (for electrolytic respirometers): Use manufacturer's recommended solution.

m. Sodium sulfite solution, 0.025*N:* Dissolve 1.575 g Na_2SO_3 in about 800 mL water. Dilute to 1 L. This solution is not stable; prepare daily or as needed.

n. Trace element solution: Dissolve 40 mg $MnSO_4 \cdot 4H_2O$, 57 mg H_3BO_3, 43 mg $ZnSO_4 \cdot 7H_2O$, 35 mg $(NH_4)_6 Mo_7O_{24}$, and 100 mg Fe-chelate ($FeCl_3$-EDTA) in about 800 mL water. Dilute to 1 L. Sterilize at 120°C and 200 kPa (2 atm) pressure for 20 min.

o. Yeast extract solution:[3] Add 15 mg laboratory- or pharmaceutical-grade brewer's yeast extract to 100 mL water. Make this solution fresh immediately before each test in which it is used.

p. Nutrient solution:[3] Add 2.5 mL phosphate buffer solution (¶ *b* above), 0.65 mL ammonium chloride solution (¶ *c* above), 1.0 mL calcium chloride solution (¶ *d* above), 0.22 mL magnesium sulfate solution (¶ *e* above), 0.1 mL ferric chloride solution (¶ *f* above), 1 mL trace element solution (¶ *n* above), and 1 mL yeast extract solution (¶ *o* above) to about 900 mL water. Dilute to 1 L. This solution and those of ¶s *n* and *o* above are specifically formulated for use with the OECD method.[3] (NOTE: A 10:1 concentrated nutrient solution can be made and diluted accordingly.)

4. Procedure

a. Instrument operation: Follow respirometer manufacturer's instructions for assembling, testing, calibrating, and operating the instrument. NOTE: The manufacturer's stated maximum and minimum measurement limits are not always the same as the

* Formula 2533, Hach Chemical Co., Loveland, Colo., or equivalent. NOTE: Some commercial formulations are not pure TCMP. Check with supplier to verify compound purity and adjust dosages accordingly.

instrument's output limits. Make sure test conditions are within measurement limits.

b. Sample volume: The sample volume or concentration of organic chemicals to be added to test vessels is a function of expected oxygen-uptake characteristics and the instrument's oxygen-transfer capability. Small volumes or low concentrations may be required for high-strength wastes. Large volumes may be required for low-strength wastes to improve accuracy.

c. Data recording interval: Set instrument to give data readings at suitable intervals. Intervals typically range from 15 min to 6 h.

d. Sample preparation:

1) Homogenization—If sample contains large settleable or floatable solids, homogenize it with a blender and transfer representative test portions while all solids are in suspension. If concerned about changing sample characteristics, skip this step.

2) pH adjustment—Neutralize samples to pH 7.0 using H_2SO_4 (5210D.3*h*) or NaOH (5210D.3*i*) without diluting the sample >0.5%.

3) Dechlorination—Avoid analyzing samples containing residual chlorine by collecting them ahead of chlorination processes. If residual chlorine is present, aerate as described in ¶ *d*5) below or let stand in light for 1 to 2 h. If a chlorine residual persists, add Na_2SO_3 solution. Determine required volume of Na_2SO_3 solution by adding 10 mL 1 + 1 acetic acid or 1 + 50 H_2SO_4 and 10 mL potassium iodide solution (10 g/100 mL) to a portion of sample. Titrate with 0.025*N* Na_2SO_3 solution to the starch-iodine endpoint (see Section 4500-Cl.B). Add to the neutralized sample a proportional volume of Na_2SO_3 solution determined above, mix, and after 10 to 20 min check for residual chlorine. Reseed the sample (see ¶ *h* below).

4) Samples containing toxic substances—Certain industrial wastes contain toxic metals or organic compounds. These often require special study and treatment.[3]

5) Initial oxygen concentration—If samples contain DO concentrations smaller or larger than the desired concentration, agitate or aerate with clean and filtered compressed air for about 1 h immediately before testing. Minimum and maximum actual DO concentrations will vary with test objectives. In some cases, pure oxygen may be added to respirometer vessels to increase oxygen levels above ambient.

6) Temperature adjustment—Bring samples and dilution water to desired test temperature (±1°C) before making dilutions or transferring to test vessels.

e. Sample dilution: Use distilled water or water from other appropriate sources free of organic matter. In some cases, receiving-stream water may be used for dilution. Add desired sample volume to test vessels using a wide-tip volumetric pipet or other suitable volumetric glassware. Add dilution water to bring sample to about 80% of desired final volume. Add appropriate amounts of nutrients, minerals, buffer, nitrification inhibitor (if desired), and seed culture as described in ¶s *f–h* below. Dilute sample to desired final volume. The number of test vessels needed to prepare for each dilution depends on test objectives and number of replicates desired.

f. Nutrients, minerals, and buffer: Add enough ammonia nitrogen to provide a COD:N:P ratio of 100:5:1 or a TOC:N:P ratio of 30:5:1. Add 2 mL each of calcium, magnesium, ferric chloride, and trace mineral solutions to each liter of diluted sample, unless sufficient amounts of these minerals are present in the original sample. Phosphorus requirements will be met by the phosphate buffer, if used (1 mL/50 mg/L COD or UBOD of diluted sample usually is sufficient to maintain pH between 6.8 and 7.2). Be cautious in adding phosphate buffer to samples containing metal salts because metal phosphates may precipitate and show less toxic or beneficial effect than when phosphate is not present. For OECD-compatible tests, substitute the nutrient, mineral, and buffer amounts listed in 5210D.3*p* for the above nutrient/mineral/buffer quantities.

g. Nitrification inhibition: If nitrification inhibition is desired, add 10 mg TCMP/L sample in the test vessel. Samples that may nitrify readily include biologically treated effluents, samples seeded with biologically treated effluents, and river waters.[4]

h. Seeding: See 5210B.4*d* for seed preparation. Use enough seed culture to prevent major lags in the oxygen-uptake reaction but not so much that the seed's oxygen uptake exceeds about 10% of the seeded sample's oxygen uptake.

Determine the seeding material's oxygen uptake in the same way as for any other sample. This is the seed control. Typically, seed volume in the seed control should be 10 times the volume used in seeded samples.

i. Incubation: Incubate samples at 20°C or other suitable temperature ±1.0°C. Take care that the stirring device does not raise sample temperature.

5. Calculations

To convert instrument readings to oxygen uptake, refer to manufacturer's procedures.

Correct oxygen uptake for seed and dilution as follows:

$$C = [A - B(S_A/S_B)](1000/N_A)$$

where:

C = corrected oxygen uptake of sample, mg/L,
A = measured oxygen uptake in seeded sample, mg,
B = measured oxygen uptake in seed control, mg,
S_A = volume of seed in Sample A, mL,
S_B = volume of seed in Sample B, mL, and
N_A = volume of undiluted sample in Sample A, mL.

6. Quality Control

The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

Periodically use the following procedure to check distilled water quality, instrument quality, instrument function, and analytical technique by making oxygen-uptake measurements using a GGA mixture as a standard check solution.

Adjust water for sample formulation to test temperature and saturate with DO by aerating with clean, organic-free filtered air. Protect water quality by using clean glassware, tubing, and bottles.

Prepare a *test solution* by adding 10 mL GGA solution (5210D.3*k*); 6 mL phosphate buffer (5210D.3*b*); 2 mL each of ammonium chloride (5210D.3*c*), magnesium sulfate (5210D.3*e*), calcium chloride (5210D.3*d*), ferric chloride (5210D.3*f*), and trace element solution (5210D.3*n*) to approximately 800 mL water. Add 10 mg TCMP/L to inhibit nitrification. Add sufficient

seed from a suitable source (5210D.4*h*) to give a lag time <6 h (usually 25 mL supernatant from settled primary effluent/L test solution is sufficient). Dilute to 1 L. Adjust temperature to 20 ± 1°C.

Prepare a *seed blank* by diluting 500 mL or more of seed solution to 800 mL with distilled water. Add the same amount of buffer, nutrients, and TCMP as in the test solution, and dilute to 1 L. Adjust temperature to 20 ± 1°C.

Place test solution and seed-blank solution in separate reaction vessels of respirometer and incubate for 5 d at 20°C. Run at least three replicates of each. The seed-corrected oxygen uptake after 5 d incubation should be 260 ± 30 mg/L; if the value is outside this range, repeat the test using a fresh seed culture and seek the cause of the problem.

7. Precision and Bias

a. Precision: No standard is available to check the accuracy of respirometric oxygen uptake measurements. To obtain laboratory precision data, use a GGA mixture (5210D.6) with a known theoretical maximum oxygen-uptake value. Tests with this and similar organic compound mixtures have shown that the standard deviation (expressed as the coefficient of variation, C_v) is approximately 5% for samples with total oxygen uptakes of 50 to 100 mg/L and 3% for more concentrated samples.[1,2] Individual instruments have different readability limits that can affect precision. The minimum response or sensitivity of most commercial respirometers ranges from 0.05 to 1 mg oxygen. Check manufacturer's specifications for a given instrument's sensitivity.

b. Control limits: To establish laboratory control limits, perform a minimum of 25 GGA checks over several weeks or months and calculate the mean and standard deviation. If measured oxygen uptake in 5 d at 20°C is outside the 260 ± 30 mg/L range, re-evaluate procedure to identify the source of error. Forother samples, use the mean ±3 standard deviations as the control limit.

c. Working range and detection limits: The working range and detection limits are established by each commercial instrument's limits. Refer to manufacturer's specifications.

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5220 CHEMICAL OXYGEN DEMAND (COD)*

5220 A. Introduction

Chemical oxygen demand (COD) is defined as the amount of a specified oxidant that reacts with the sample under controlled conditions. The quantity of oxidant consumed is expressed in terms of its oxygen equivalence. Because of its unique chemical properties, the dichromate ion ($Cr_2O_7^{2-}$) is the specified oxidant in Methods 5220B, C, and D; it is reduced to the chromic ion (Cr^{3+}) in these tests. Both organic and inorganic components of a sample are subject to oxidation, but in most cases the organic component predominates and is of the greater interest. If it is desired to measure either organic or inorganic COD alone, additional steps not described here must be taken to distinguish one from the other. COD is a defined test; the extent of sample oxidation can be affected by digestion time, reagent strength, and sample COD concentration.

COD often is used as a measurement of pollutants in wastewater and natural waters. Other related analytical values are biochemical oxygen demand (BOD), total organic carbon (TOC), and total oxygen demand (TOD). In many cases it is possible to correlate two or more of these values for a given sample. BOD is a measure of oxygen consumed by microorganisms under specific conditions; TOC is a measure of organic carbon in a sample; TOD is a measure of the amount of oxygen consumed by all elements in a sample when complete (total) oxidation is achieved.

In a COD analysis, hazardous wastes of mercury, hexavalent chromium, sulfuric acid, silver, and acids are generated. Methods 5220C and D reduce these waste problems but may be less accurate and less representative (5220A.2).

1. Selection of Method

The open reflux method (5220B) is suitable for a wide range of wastes where a large sample size is preferred. The closed reflux methods (5220C and D) are more economical in the use of metallic salt reagents and generate smaller quantities of hazardous waste, but require homogenization of samples containing suspended solids to obtain reproducible results. Ampules and culture tubes with premeasured reagents are available commercially. Measurements of sample volumes as well as reagent volumes and concentrations are critical. Consequently, obtain specifications as to limits of error for premixed reagents from manufacturer before use.

Determine COD values of >50 mg O_2/L by using procedures 5220B.4*a*, C.4, or D.4. Use procedure 5220B.4*b* to determine, with lesser accuracy, COD values from 5 to 50 mg O_2/L.

2. Interferences and Limitations

Oxidation of most organic compounds is 95 to 100% of the theoretical value. Pyridine and related compounds resist oxidation and volatile organic compounds will react in proportion to their contact with the oxidant. Straight-chain aliphatic compounds are oxidized more effectively in the presence of a silver sulfate catalyst.

The most common interferent is the chloride ion. Chloride reacts with silver ion to precipitate silver chloride, and thus inhibits the catalytic activity of silver. Bromide, iodide, and any other reagent that inactivates the silver ion can interfere similarly. Such interferences are negative in that they tend to restrict the oxidizing action of the dichromate ion itself. However, under the rigorous digestion procedures for COD analyses, chloride, bromide, or iodide can react with dichromate to produce the elemental form of the halogen and the chromic ion. Results then are in error on the high side. The difficulties caused by the presence of the chloride can be overcome largely, though not completely, by complexing with mercuric sulfate ($HgSO_4$) before the refluxing procedure. Although 1 g $HgSO_4$ is specified for 50 mL sample, a lesser amount may be used where sample chloride concentration is known to be less than 2000 mg/L, as long as a 10:1 weight ratio of $HgSO_4$:Cl^- is maintained. Do not use the test for samples containing more than 2000 mg Cl^-/L. Techniques designed to measure COD in saline waters are available.[1,2]

Halide interferences may be removed by precipitation with silver ion and filtration before digestion. This approach may introduce substantial errors due to the occlusion and carrydown of COD matter from heterogenous samples.

Ammonia and its derivatives, in the waste or generated from nitrogen-containing organic matter, are not oxidized. However, elemental chlorine reacts with these compounds. Hence, corrections for chloride interferences are difficult.

Nitrite (NO_2^-) exerts a COD of 1.1 mg O_2/mg NO_2^--N. Because concentrations of NO_2^- in waters rarely exceed 1 or 2 mg NO_2^--N/L, the interference is considered insignificant and usually is ignored. To eliminate a significant interference due to NO_2^-, add 10 mg sulfamic acid for each mg NO_2^--N present in the sample volume used; add the same amount of sulfamic acid to the reflux vessel containing the distilled water blank.

Reduced inorganic species such as ferrous iron, sulfide, manganous manganese, etc., are oxidized quantitatively under the test conditions. For samples containing significant levels of these species, stoichiometric oxidation can be assumed from known initial concentration of the interfering species and corrections can be made to the COD value obtained.

The silver, hexavalent chromium, and mercury salts used in the COD determinations create hazardous wastes. The greatest problem is in the use of mercury. If the chloride contribution to COD is negligible, $HgSO_4$ can be omitted. Smaller sample sizes (see Table 5220:I) reduce the waste. Recovery of the waste material may be feasible if allowed by regulatory authority.[3]

3. Sampling and Storage

Preferably collect samples in glass bottles. Test unstable samples without delay. If delay before analysis is unavoidable, preserve sample by acidification to pH ≤2 using conc H_2SO_4. Blend (homogenize) all samples containing suspended solids before analysis. If COD is to be related to BOD, TOC, etc., ensure that all tests receive identical pretreatment. Make prelim-

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inary dilutions for wastes containing a high COD to reduce the error inherent in measuring small sample volumes.

4. References

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5220 B. Open Reflux Method

1. General Discussion

a. Principle: Most types of organic matter are oxidized by a boiling mixture of chromic and sulfuric acids. A sample is refluxed in strongly acid solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). After digestion, the remaining unreduced $K_2Cr_2O_7$ is titrated with ferrous ammonium sulfate to determine the amount of $K_2Cr_2O_7$ consumed and the oxidizable matter is calculated in terms of oxygen equivalent. Keep ratios of reagent weights, volumes, and strengths constant when sample volumes other than 50 mL are used. The standard 2-h reflux time may be reduced if it has been shown that a shorter period yields the same results. Some samples with very low COD or with highly heterogeneous solids content may need to be analyzed in replicate to yield the most reliable data. Results are further enhanced by reacting a maximum quantity of dichromate, provided that some residual dichromate remains.

b. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Reflux apparatus, consisting of 500- or 250-mL Erlenmeyer flasks with ground-glass 24/40 neck and 300-mm jacket Liebig, West, or equivalent condenser with 24/40 ground-glass joint, and a hot plate having sufficient power to produce at least 1.4 W/cm^2 of heating surface, or equivalent.

b. Blender.

c. Pipets, Class A and wide-bore.

3. Reagents

a. Standard potassium dichromate solution, 0.04167*M:* Dissolve 12.259 g $K_2Cr_2O_7$, primary standard grade, previously dried at 150°C for 2 h, in distilled water and dilute to 1000 mL. This reagent undergoes a six-electron reduction reaction; the equivalent concentration is $6 \times 0.04167M$ or 0.2500*N*.

b. Sulfuric acid reagent: Add Ag_2SO_4, reagent or technical grade, crystals or powder, to conc H_2SO_4 at the rate of 5.5 g Ag_2SO_4/kg H_2SO_4. Let stand 1 to 2 d to dissolve. Mix.

c. Ferroin indicator solution: Dissolve 1.485 g 1,10-phenanthroline monohydrate and 695 mg $FeSO_4 \cdot 7H_2O$ in distilled water and dilute to 100 mL. This indicator solution may be purchased already prepared.*

* GFS Chemicals, Inc., Columbus, OH, or equivalent.

d. Standard ferrous ammonium sulfate (FAS) titrant, approximately 0.25*M:* Dissolve 98 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in distilled water. Add 20 mL conc H_2SO_4, cool, and dilute to 1000 mL. Standardize this solution daily against standard $K_2Cr_2O_7$ solution as follows:

Dilute 25.00 mL standard $K_2Cr_2O_7$ to about 100 mL. Add 30 mL conc H_2SO_4 and cool. Titrate with FAS titrant using 0.10 to 0.15 mL (2 to 3 drops) ferroin indicator.

$$\text{Molarity of FAS solution} = \frac{\text{Volume } 0.04167M \text{ } K_2Cr_2O_7 \text{ solution titrated, mL}}{\text{Volume FAS used in titration, mL}} \times 0.2500$$

e. Mercuric sulfate ($HgSO_4$), crystals or powder.

f. Sulfamic acid: Required only if the interference of nitrites is to be eliminated (see 5220A.2).

g. Potassium hydrogen phthalate (KHP) standard, $HOOCC_6H_4COOK$: Lightly crush and then dry KHP to constant weight at 110°C. Dissolve 425 mg in distilled water and dilute to 1000 mL. KHP has a theoretical COD[1] of 1.176 mg O_2/mg and this solution has a theoretical COD of 500 μg O_2/ mL. This solution is stable when refrigerated, but not indefinitely. Be alert to development of visible biological growth. If practical, prepare and transfer solution under sterile conditions. Weekly preparation usually is satisfactory.

4. Procedure

a. Treatment of samples with COD of >50 mg O_2/L: Blend sample if necessary and pipet 50.00 mL into a 500-mL refluxing flask. For samples with a COD of >900 mg O_2/L, use a smaller portion diluted to 50.00 mL. Add 1 g $HgSO_4$, several glass beads, and very slowly add 5.0 mL sulfuric acid reagent, with mixing to dissolve $HgSO_4$. Cool while mixing to avoid possible loss of volatile materials. Add 25.00 mL 0.04167*M* $K_2Cr_2O_7$ solution and mix. Attach flask to condenser and turn on cooling water. Add remaining sulfuric acid reagent (70 mL) through open end of condenser. Continue swirling and mixing while adding sulfuric acid reagent. **CAUTION: Mix reflux mixture thoroughly before applying heat to prevent local heating of flask bottom and a possible blowout of flask contents.**

Cover open end of condenser with a small beaker to prevent foreign material from entering refluxing mixture and reflux for 2 h. Cool and wash down condenser with distilled water. Disconnect reflux condenser and dilute mixture to about twice its volume with distilled water. Cool to room temperature and titrate excess

$K_2Cr_2O_7$ with FAS, using 0.10 to 0.15 mL (2 to 3 drops) ferroin indicator. Although the quantity of ferroin indicator is not critical, use the same volume for all titrations. Take as the endpoint of the titration the first sharp color change from blue-green to reddish brown that persists for 1 min or longer. Duplicate determinations should agree within 5% of their average. Samples with suspended solids or components that are slow to oxidize may require additional determinations. The blue-green may reappear. In the same manner, reflux and titrate a blank containing the reagents and a volume of distilled water equal to that of sample.

b. Alternate procedure for low-COD samples: Follow procedure of ¶ *a* above, with two exceptions: (*i*) use standard 0.004167*M* $K_2Cr_2O_7$, and (*ii*) titrate with standardized 0.025*M* FAS. Exercise extreme care with this procedure because even a trace of organic matter on the glassware or from the atmosphere may cause gross errors. If a further increase in sensitivity is required, concentrate a larger volume of sample before digesting under reflux as follows: Add all reagents to a sample larger than 50 mL and reduce total volume to 150 mL by boiling in the refluxing flask open to the atmosphere without the condenser attached. Compute amount of $HgSO_4$ to be added (before concentration) on the basis of a weight ratio of 10:1, $HgSO_4$:Cl^-, using the amount of Cl^- present in the original volume of sample. Carry a blank reagent through the same procedure. This technique has the advantage of concentrating the sample without significant losses of easily digested volatile materials. Hard-to-digest volatile materials such as volatile acids are lost, but an improvement is gained over ordinary evaporative concentration methods. Duplicate determinations are not expected to be as precise as in ¶ *a* above.

c. Determination of standard solution: Evaluate the technique and quality of reagents by conducting the test on a standard potassium hydrogen phthalate solution.

5. Calculation

$$\text{COD as mg } O_2/\text{L} = \frac{(B - A) \times M \times 8000}{\text{mL sample}}$$

where:

B = mL FAS used for sample,
A = mL FAS used for blank,
M = molarity of FAS, and
8000 = milliequivalent weight of oxygen × 1000 mL/L.

6. Precision and Bias

A set of synthetic samples containing potassium hydrogen phthalate and NaCl was tested by 74 laboratories. At a COD of 200 mg O_2/L in the absence of chloride, the standard deviation was ±13 mg/L (coefficient of variation, 6.5%). At COD of 160 mg O_2/L and 100 mg Cl^-/L, the standard deviation was ±14 mg/L (coefficient of variation, 10.8%).

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5220 C. Closed Reflux, Titrimetric Method

1. General Discussion

a. Principle: See 5220B.1*a*.

b. Interferences and limitations: See 5220A.2. Volatile organic compounds are more completely oxidized in the closed system because of longer contact with the oxidant. Before each use inspect culture-tube caps for breaks in the TFE liner. Select culture-tube size according to block heater capacity and degree of sensitivity desired. Use the 25- × 150-mm tube for samples with low COD content because a larger volume sample can be treated.

This procedure is applicable to COD values between 40 and 400 mg/L. Obtain higher values by dilution. Alternatively, use higher concentrations of dichromate digestion solution to determine greater COD values. COD values of 100 mg/L or less can be obtained by using a more dilute dichromate digestion solution or a more dilute FAS titrant. Overall accuracy can be improved by using an FAS titrant which is less than the 0.10*M* solution specified below. Higher dichromate concentrations or reduced FAS concentrations probably require titrations to be done in a separate vessel, rather than in the digestion vessel, because of the volumes of titrant required.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Digestion vessels: Preferably use borosilicate culture tubes, 16- × 100-mm, 20- × 150-mm, or 25- × 150-mm, with TFE-lined screw caps. Alternatively, use borosilicate ampules, 10-mL capacity, 19- to 20-mm diam.

Digestion vessels with premixed reagents and other accessories are available from commercial suppliers. Contact supplier for specifications.*

b. Block heater or similar device to operate at 150 ± 2°C, with holes to accommodate digestion vessels. Use of culture

* Hach Co., Bioscience, Inc., or equivalent.

Table 5220:I. Sample and Reagent Quantities for Various Digestion Vessels

Digestion Vessel	Sample *mL*	Digestion Solution *mL*	Sulfuric Acid Reagent *mL*	Total Final Volume *mL*
Culture tubes:				
16 × 100 mm	2.50	1.50	3.5	7.5
20 × 150 mm	5.00	3.00	7.0	15.0
25 × 150 mm	10.00	6.00	14.0	30.0
Standard 10-mL ampules	2.50	1.50	3.5	7.5

tubes probably requires the caps to be outside the vessel to protect caps from heat. **Caution: Do not use an oven because of the possibility of leaking samples generating a corrosive and possibly explosive atmosphere. Also, culture tube caps may not withstand the 150°C temperature in an oven.**

c. Microburet.

d. Ampule sealer: Use only a mechanical sealer to ensure strong, consistent seals.

3. Reagents

a. Standard potassium dichromate digestion solution, 0.01667*M:* Add to about 500 mL distilled water 4.903 g $K_2Cr_2O_7$, primary standard grade, previously dried at 150°C for 2 h, 167 mL conc H_2SO_4, and 33.3 g $HgSO_4$. Dissolve, cool to room temperature, and dilute to 1000 mL.

b. Sulfuric acid reagent: See 5220B.3*b*.

c. Ferroin indicator solution: See 5220B.3*c*. Dilute this reagent by a factor of 5 (1 + 4).

d. Standard ferrous ammonium sulfate titrant (FAS), approximately 0.10*M:* Dissolve 39.2 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in distilled water. Add 20 mL conc H_2SO_4, cool, and dilute to 1000 mL. Standardize solution daily against standard $K_2Cr_2O_7$ digestion solution as follows:

Pipet 5.00 mL digestion solution into a small beaker. Add 10 mL reagent water to substitute for sample. Cool to room temperature. Add 1 to 2 drops diluted ferroin indicator and titrate with FAS titrant.

$$\text{Molarity of FAS solution} = \frac{\text{Volume 0.01667}M\ K_2Cr_2O_7\text{ solution titrated, mL}}{\text{Volume FAS used in titration, mL}} \times 0.1000$$

e. Sulfamic acid: See 5220B.3*f*.

f. Potassium hydrogen phthalate standard: See 5220B.3*g*.

4. Procedure

Wash culture tubes and caps with 20% H_2SO_4 before first use to prevent contamination. Refer to Table 5220:I for proper sample and reagent volumes. Make volumetric measurements as accurate as practical; use Class A volumetric ware. The most critical volumes are of the sample and digestion solution. Use a microburet for titrations. Measure H_2SO_4 to ±0.1 mL. The use of hand-held pipettors with non-wetting (polyethylene) pipet tips is practical and adequate. Place sample in culture tube or ampule and add digestion solution. Carefully run sulfuric acid reagent down inside of vessel so an acid layer is formed under the sample-digestion solution layer. Tightly cap tubes or seal ampules, and invert each several times to mix completely. **Caution: Wear face shield and protect hands from heat produced when contents of vessels are mixed. Mix thoroughly before applying heat to prevent local heating of vessel bottom and possible explosive reaction**.

Place tubes or ampules in block digester preheated to 150°C and reflux for 2 h behind a protective shield. **Caution: These sealed vessels may be under pressure from gases generated during digestion. Wear face and hand protection when handling. If sulfuric acid is omitted or reduced in concentration, very high and dangerous pressures will be generated at 150°C**. Cool to room temperature and place vessels in test tube rack. Some mercuric sulfate may precipitate out but this will not affect the analysis. Remove culture tube caps and add small TFE-covered magnetic stirring bar. If ampules are used, transfer contents to a larger container for titrating. Add 0.05 to 0.10 mL (1 to 2 drops) ferroin indicator and stir rapidly on magnetic stirrer while titrating with standardized 0.10*M* FAS. The endpoint is a sharp color change from blue-green to reddish brown, although the blue-green may reappear within minutes. In the same manner reflux and titrate a blank containing the reagents and a volume of distilled water equal to that of the sample.

5. Calculation

$$\text{COD as mg } O_2\text{/L} = \frac{(B-A) \times M \times 8000}{\text{mL sample}}$$

where:

B = mL FAS used for sample,
A = mL FAS used for blank,
M = molarity of FAS, and
8000 = milliequivalent weight of oxygen × 1000 mL/L.

Preferably analyze samples in duplicate because of small sample size. Samples that are inhomogeneous may require multiple determinations for accurate analysis. Results should agree within ±5% of their average unless the condition of the sample dictates otherwise.

6. Precision and Bias

Sixty synthetic samples containing potassium hydrogen phthalate and NaCl were tested by six laboratories. At an average COD of 195 mg O_2/L in the absence of chloride, the standard deviation was ±11 mg O_2/L (coefficient of variation, 5.6%). At an average COD of 208 mg O_2/L and 100 mg Cl^-/L, the standard deviation was ±10 mg O_2/L (coefficient of variation, 4.8%).

5220 D. Closed Reflux, Colorimetric Method

1. General Discussion

a. Principle: See 5220B.1*a*. When a sample is digested, the dichromate ion oxidizes COD material in the sample. This results in the change of chromium from the hexavalent (VI) state to the trivalent (III) state. Both of these chromium species are colored and absorb in the visible region of the spectrum. The dichromate ion ($Cr_2O_7^{2-}$) absorbs strongly in the 400-nm region, where the chromic ion (Cr^{3+}) absorption is much less. The chromic ion absorbs strongly in the 600-nm region, where the dichromate has nearly zero absorption. In 9*M* sulfuric acid solution, the approximate molar extinction coefficients for these chromium species are as follows: Cr^{3+} − 50 L/mole cm at 604 nm; $Cr_2O_7^{2-}$ − 380 L/mole cm at 444 nm; Cr^{3+} − 25 L/mole cm at 426 nm. The Cr^{3+} ion has a minimum in the region of 400 nm. Thus a working absorption maximum is at 420 nm.

For COD values between 100 and 900 mg/L, increase in Cr^{3+} in the 600-nm region is determined. Higher values can be obtained by sample dilution. COD values of 90 mg/L or less can be determined by following the decrease in $Cr_2O_7^{2-}$ at 420 nm. The corresponding generation of Cr^{3+} gives a small absorption increase at 420 nm, but this is compensated for in the calibration procedure.

b. Interferences and limitations: See 5220C.1*b*.

For this procedure to be applicable, all visible light-absorbing interferents must be absent or be compensated for. This includes insoluble suspended matter as well as colored components. If either type of interference occurs, the test is not necessarily lost because COD can be determined titrimetrically as in 5220C.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. See 5220C.2. Ensure that reaction vessels are of optical quality. Other types of absorption cells with varying path lengths may be used. Use the extinction coefficients of the ions of interest for this approach.

b. Spectrophotometer, for use at 600 nm and/or 420 nm with access opening adapter for ampule or 16-, 20-, or 25-mm tubes. Verify that the instrument operates in the region of 420 nm and 600 nm. Values slightly different from these may be found, depending on the spectral bandpass of the instrument.

3. Reagents

a. Digestion solution, high range: Add to about 500 mL distilled water 10.216 g $K_2Cr_2O_7$, primary standard grade, previously dried at 150°C for 2 h, 167 mL conc H_2SO_4, and 33.3 g $HgSO_4$. Dissolve, cool to room temperature, and dilute to 1000 mL.

b. Digestion solution, low range: Prepare as in ¶ *a* above, but use only 1.022 g potassium dichromate.

c. Sulfuric acid reagent: See 5220B.3*b*.

d. Sulfamic acid: See 5220B.3*f*.

e. Potassium hydrogen phthalate standard: See 5220B.3*g*.

4. Procedure

a. Treatment of samples: Measure suitable volume of sample and reagents into tube or ampule as indicated in Table 5220:I. Prepare, digest, and cool samples, blank, and one or more standards as directed in 5220C.4. **Note the safety precautions.** It is critical that the volume of each component be known and that the total volume be the same for each reaction vessel. If volumetric control is difficult, transfer digested sample, dilute to a known volume, and read. Premixed reagents in digestion tubes are available commercially.

b. Measurement of dichromate reduction: Cool sample to room temperature slowly to avoid precipitate formation. Once samples are cooled, vent, if necessary, to relieve any pressure generated during digestion. Mix contents of reaction vessels to combine condensed water and dislodge insoluble matter. Let suspended matter settle and ensure that optical path is clear. Measure absorption of each sample blank and standard at selected wavelength (420 nm or 600 nm). At 600 nm, use an undigested blank as reference solution. Analyze a digested blank to confirm good analytical reagents and to determine the blank COD; subtract blank COD from sample COD. Alternately, use digested blank as the reference solution once it is established that the blank has a low COD.

At 420 nm, use reagent water as a reference solution. Measure all samples, blanks, and standards against this solution. The absorption measurement of an undigested blank containing dichromate, with reagent water replacing sample, will give initial dichromate absorption. Any digested sample, blank, or standard that has a COD value will give lower absorbance because of the decrease in dichromate ion. Analyze a digested blank with reagent water replacing sample to ensure reagent quality and to determine the reagents' contribution to the decrease in absorbance during a given digestion. The difference between absorbances of a given digested sample and the digested blank is a measure of the sample COD. When standards are run, plot differences of digested blank absorbance and digested standard absorbance versus COD values for each standard.

c. Preparation of calibration curve: Prepare at least five standards from potassium hydrogen phthalate solution with COD equivalents to cover each concentration range. Make up to volume with reagent water; use same reagent volumes, tube, or ampule size, and digestion procedure as for samples. Prepare calibration curve for each new lot of tubes or ampules or when standards prepared in ¶ *a* above differ by ≥5% from calibration curve. Curves should be linear. However, some nonlinearity may occur, depending on instrument used and overall accuracy needed.

5. Calculation

If samples, standards, and blanks are run under same conditions of volume and optical path length, calculate COD as follows:

$$\text{COD as mg } O_2\text{/L} = \frac{\text{mg } O_2 \text{ in final volume} \times 1000}{\text{mL sample}}$$

Preferably analyze samples in duplicate because of small sample size. Samples that are inhomogeneous may require multiple determinations for accurate analysis. These should

not differ from their average by more than ±5% for the high-level COD test unless the condition of the sample dictates otherwise. In the low-level procedure, results below 25 mg/L may tend to be qualitative rather than quantitative.

6. Precision and Bias

Forty-eight synthetic samples containing potassium hydrogen phthalate and NaCl were tested by five laboratories. At an average COD of 193 mg O_2/L in the absence of chloride, the standard deviation was ±17 mg O_2/L (coefficient of variation 8.7%). At an average COD of 212 mg O_2/L and 100 mg Cl^-/L, the standard deviation was ±20 mg O_2/L (coefficient of variation, 9.6%). Additional QA/QC data for both high- and low-level procedures may be found elsewhere.[1]

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5310 TOTAL ORGANIC CARBON (TOC)*

5310 A. Introduction

1. General Discussion

The organic carbon in water and wastewater consists of multiple organic compounds in various oxidation states. Some of these compounds can be oxidized further via biological or chemical processes. Biochemical oxygen demand (BOD), assimilable organic carbon (AOC), and chemical oxygen demand (COD) methods may be used to characterize these fractions. Total organic carbon (TOC) is a more convenient and direct expression of total organic content than BOD, AOC, or COD but does not provide the same kind of information. TOC is independent of the organic matter's oxidation state and does not measure other organically bound elements (e.g., nitrogen and hydrogen) or inorganics that can contribute to the oxygen demand measured by BOD and COD. The amount of TOC in water sources varies widely from <0.1 to >25 mg/L in drinking water to >100 mg/L in wastewater. Although TOC is not a direct substitute for BOD, AOC, or COD testing, several studies have demonstrated that correlations can be developed and TOC can be used as a surrogate for some of these other parameters in certain cases. This may be acceptable under some regulatory frameworks. These relationships must be established independently for each set of matrix conditions (e.g., various points in a treatment process and water types). Correlations should be developed with caution and validated seasonally.

Organic contaminants in water may degrade ion-exchange capacity, serve as a carbon or energy source for undesired biological growth, react with disinfectants to form byproducts that could harm human health or aquatic organisms, or be otherwise detrimental to the water's purpose. Before organically bound carbon can be analyzed, the molecules must be converted into carbon dioxide (CO_2), a molecular form that can be measured quantitatively. TOC methods use either high temperature (typically 680 to 950°C) with catalysts and oxygen or air, or lower temperatures (<100°C) with ultraviolet irradiation and/or one or more chemical oxidants and catalysts to convert organic carbon into carbon dioxide. The CO_2 may be purged from the sample, dried, and transferred via carrier gas to a nondispersive infrared analyzer or coulometric titrator. Alternatively, CO_2 may be separated from the sample via a CO_2-selective membrane into high-purity water, where the increase in conductivity corresponds to the amount of CO_2 passing through the membrane.

2. Fractions of Total Carbon

The methods and instruments used to measure TOC analyze fractions of total carbon (TC) and measure TOC via two or more determinations. These fractions of total carbon are defined as:

- inorganic carbon (IC)—carbonate, bicarbonate, and dissolved CO_2;
- total organic carbon (TOC)—all carbon atoms covalently bonded in organic molecules;
- dissolved organic carbon (DOC)—the fraction of TOC that passes through a 0.45-μm-pore-diameter filter;
- suspended organic carbon (also called *particulate organic carbon*)—the fraction of TOC retained by a 0.45-μm filter;
- purgeable organic carbon (also called *volatile organic carbon*)—the fraction of TOC removed from an aqueous solution via gas stripping under specified conditions; and
- nonpurgeable organic carbon (NPOC)—the fraction of TOC not removed by gas stripping. In most cases, inorganic carbon is purged and not "determined," in which case only NPOC is determined and purgeable organic carbon is assumed to be negligible.

In many water samples, the IC fraction is many times larger than the TOC fraction. IC can be eliminated by adjusting samples to pH 4 or less to convert IC species to CO_2. Samples are normally adjusted to pH 2 or less; however, this may maximize hydrophobic molecule loss to the sample vessel walls. Adjusting to pH 4 minimizes such losses, but IC-removal efficiency must be checked and carbonate minerals in unfiltered samples may not completely dissolve. The CO_2 is then removed by purging the sample with a purified gas (free of both CO_2 and organic contaminants) or by vacuum degassing. Sample purging also removes purgeable organic carbon, so the organic carbon measurement made after eliminating IC interferences is actually an NPOC determination. In many surface and ground waters, the purgeable organic carbon contribution to TOC is negligible, so in practice the NPOC determination is commonly substituted for TOC.

Alternatively, TOC may be determined by separately measuring TC and IC, and calculating the difference. If the sample contains far more IC than TOC, subtracting IC from TC is not the preferred approach to determining TOC because of the relatively large error involved. The purgeable fraction of TOC is a function of the specific conditions and equipment used. Sample temperature and salinity, gas-flow rate, type of gas diffuser, purging-vessel dimensions, volume purged, and purging time affect the division of TOC into purgeable and nonpurgeable fractions. When separately measuring purgeable organic carbon and NPOC in the same sample, use identical purging conditions for both fractions. Also, consider purging conditions when comparing purgeable organic carbon or NPOC data from different laboratories or instruments.

3. Method Selection

The high-temperature combustion method (5310B) is suitable for samples with higher TOC levels, which otherwise would have to be diluted for some of the persulfate methods (5310C). Generally, 5310B determines organic carbon levels in com-

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pounds that are chemically refractory. These compounds cannot be determined by 5310C. High-temperature combustion may be desirable for samples containing high levels of suspended organic carbon or more than 500 mg/L of chloride or other halides, where carbon may not be oxidized efficiently by persulfate and/or ultraviolet (UV) irradiation methods. When analyzing saline waters, care must be taken when using the combustion method. Manufacturers may offer different catalyst packing schemes that will increase the life of the column and minimize the formation of salt cakes. Interlaboratory studies have shown biases on the order of 1 mg C/L using older high-temperature instruments. With newer instruments, bias has been reduced and detection limits as low as 0.010 mg C/L have been reported. Some high-temperature combustion instruments are not designed for levels <1 mg C/L. Method 5310B accumulates nonvolatile residues in the analyzer, while in 5310C residuals are drained from the analyzer after each analysis. Method 5310C generally provides better sensitivity for lower-level samples (<1 mg C/L); it is useful for TOC as low as 0.010 mg C/L.

Because the sensitivity range of the methods overlaps, other factors may dictate method choice. A method may be chosen based on desired precision, ease of use, cost, or other factors.

Samples high in particulate organic carbon are problematic for any TOC technique. Even if the particulates can be homogenously suspended, they may selectively adhere to sample introduction portions of the analyzer. The best approach is to filter the samples using carbon-free filters and analyze the filtrate for DOC, then analyze the filters for particulate organic carbon using an appropriate carbon analyzer.

To qualify a particular instrument for use, demonstrate that the single-user precision and bias given in each method can be reproduced. Preferably, demonstrate overall precision by conducting in-house studies with more than one operator.

Evaluate the selected method to ensure that data quality objectives are attained. Evaluate the method detection level in a matrix as similar as possible to the unknowns. Be aware that TOC analyzers handle instrument blanks in a variety of ways and that the true magnitude of the blank may not be readily apparent to the analyst. Some instruments "zero out" much of the blank by adjusting the detector's baseline reading. Some enter blank values in units (e.g., mv responses) rather than absolute concentrations, and others accumulate the total blank in the system during a blank run. Carefully observe the variability of low-level measurements, and both the method detection level and the blank whenever reagents or instrument operations are changed.

Methods 5310B and C note that when a water blank is analyzed, the carbon level in the blank water contributes to the observed blank value. However, an instrument blank should only represent the contribution of reagents or system upset (injection blank). Organic carbon-free water exposed to the atmosphere can rapidly gain TOC. Modern instrumentation provides schemes to recycle previously combusted water or allows reagent blanks to be run in the absence of injected water. Some instruments have on-line capability or sample-introduction procedures so low-TOC water can be injected without exposure to the atmosphere. Blank values should be based on internally recycled water or reagent blanks, and external blank water injections should not be subtracted from determined sample values. Determine the background TOC level in the reagent water used to prepare standards. Handle this water similarly to the standards and subtract the background TOC level from the TOC level of the standards. Do not subtract the background TOC level from the samples unless they are diluted; in which case, subtract the dilution water's contribution to the TOC concentration. Evaluate each instrument and/or external sparging system for IC removal. IC removal efficiency can be significantly altered by minor changes in the sparging system or by changes in sample temperature. Whenever the sparging system is altered, re-evaluate IC-removal efficiency. The methods show expected single-operator and multiple-laboratory precision. These equations are based on referenced interlaboratory studies that, in some cases, were performed on older equipment. Observe the testing range because the error and bias generally will be some significant fraction of the low standard. Consult references to determine the type of equipment and conditions of the interlaboratory study. Determine instrument performance by analyzing waters with matrices similar to those of the unknowns.

4. Filtering Samples for DOC Determinations

Ideally, a 0.45-μm pore-size filter is required to determine DOC; however in practice, glass fiber filters with larger nominal pore sizes are often used.

a. Filter apparatus: Glass, tetrafluoroethylene (TFE), stainless steel, acetyl resin, or other material that neither adsorbs nor leaches organic substances and is capable of holding the selected filters may be used. Polypropylene syringes with syringe-type filter holders or peristaltic pumps with in-line filter holders may be used as an alternative to vacuum filtration.

b. Filters: Use a glass fiber filter without organic binder or else filters made of TFE, polycarbonate, silver, polyethersulfone, or hydrophilic polypropylene. Other filters that neither adsorb nor leach organic carbon into water may be used, especially if colloidal matter must be removed. The specific filter used must be documented along with the data. Filters commonly used are 2.2 to 4.7 cm in diameter, although this may vary. Syringe filters are also common. Alternatively, centrifugation may be used to remove colloidal material.

Pre-rinse the filter with organic-free water to remove soluble impurities. Glass fiber filters may be combusted at high temperatures (450 to 500°C for at least 1 h) before rinsing. If alternate separation techniques, filters, or filter preparations are used, demonstrate that equivalent results are produced. Filter pore size may influence test results, especially in raw waters. When filtering samples, check applicable regulations to see if the filter must have an absolute pore size of 0.45 μm because glass fiber filters do not meet this requirement. For highly turbid samples, pre-filters (including glass fiber) may be used to remove interferences and minimize clogging of 0.45-μm filters. Studies have shown that hydrophilic polyethersulfone or polypropylene filters are the best options.[1]

c. Filter blanks: Filters can both release and adsorb DOC. Before use, a filter must be rinsed to remove any potential DOC. Also, for each filter type and manufacturer, determine and document the volume required so the resulting filtrate analysis yields a TOC result <1/2 MRL. Begin with three successive rinses of 25 mL each, and analyze the third rinse for TOC. If analysis of the third aliquot yields a result <1/2 MRL, then the volume used is acceptable. If the value exceeds 1/2 MRL, continue with

another 25 mL rinse and analyze. Continue the process until a sufficient rinsing volume has been determined.

d. Filter to waste: If possible, pass a portion of the sample through the filter to waste before beginning to collect filtrate. For the recommended filters, 25 mL was generally found to be an adequate amount to discard before collecting sample. The filter-to-waste volume can be determined on a low suspended solids sample by collecting aliquots of filtrate and determining DOC on the aliquots until the difference in DOC values between sample and filtrate is insignificant.

e. Filter plugging: Filter plugging is a common problem. A glass prefilter that has been heated to 500°C for 1 hour in a muffle furnace or an organic-free glass fiber filter that has been demonstrated to be free of interferences and pre-rinsed immediately before use has been shown to be effective in reducing plugging.

5. Quality Control Applicable to All Methods

a. Initial demonstration of performance: This should be done whenever a new instrument is set up, a method or instrument is modified, or a new operator is trained.

1) Initial demonstration of calibration and precision—Analyze at least 4 replicates of a mid-range calibration check sample prepared from a different source than that used for the calibration. The average obtained value should be within 15% of the theoretical, and the relative standard deviation (RSD) should be <10%.

2) Method detection limit—Determine the method detection limit (MDL) by preparing a standard at 2 to 5 times the expected MDL in reagent water. (NOTE: MDLs determined using standards prepared in reagent water may not be representative of the MDL for complex sample matrices, so consider developing matrix-specific MDLs when matrix effects indicate that a reagent-water-based MDL is inappropriate.) Perform at least seven non-consecutive injections of the standard over a minimum of 3 d. The MDL is calculated as follows:

$$\text{Organic Carbon Detection Limit} = St_{(n-1,1-\alpha=0.99)}$$

where:

S = standard deviation of replicate analyses.
$t(n-1,1-\alpha=0.99)$ = Student's t value for the 99% confidence level with n – 1 degrees of freedom (t = 3.14 for 7 replicates), and
n = number of replicates.

3) Demonstration of IC-removal efficiency—Purged samples may absorb organics or CO_2 from the atmosphere, so analyze samples immediately after purging or cap them tightly until an aliquot is injected. Use the procedure in 5310A.5*b*10) to demonstrate IC removal efficiency. The necessary sparging time may be determined by sparging for various times and selecting the time required to obtain a value <1/2 of the MRL.

4) TOC carryover check—Immediately following the analysis of the highest calibration standard, analyze a blank. The concentration of the blank must be <1/2 of the MRL. If the blank has a value >1/2 of the MRL, then the highest calibration point must be lowered until the blank immediately following the point has a value <1/2 of the MRL. Analyze the high calibration point/blank pair at the beginning of every analytical day to verify that carryover is not occurring.

b. Ongoing quality control:

1) Holding time—For DOC, an unpreserved sample must be filtered and then preserved within 48 hours of collection. For NPOC analysis, adjust the original sample or filtrate to pH ≤2. Samples must be stored at ≤6°C. Preserve samples with HCl, H_2SO_4, or H_3PO_4, depending on instrument recommendations. High chloride content in a sample may cause a bias to the reported results on some instruments. Analyze all samples within 28 d of collection.

When determining IC and then TOC via difference and high-temperature combustion, refrigerate sample, do NOT adjust its pH, and analyze it as soon as possible because TOC may biodegrade significantly before the regulatory 28-d holding time expires. Alternatively, determine IC on an unacidified sample and then determine TOC by difference using a second, acidified sample. Keep in mind that the IC result for the acidified sample used to determine TOC does not represent the IC concentration of the unacidified sample.

2) Collect samples in glass bottles—Amber glass is preferred, but clear glass may be used if the sample is protected from light. Alternative containers may be used if it is demonstrated that the container does not remove or add TOC at a level greater than 1/2 MRL.

3) Calibration check sample—At least once per analytical day, analyze a mid-range calibration check sample prepared from a different source than that used for the initial calibration. The results should be within 10% of the theoretical.

4) Initial instrument blank—Analyze a blank consisting of recycled water or low TOC water. The purpose is to determine if there is any TOC present in the instrument that may contaminate the system. Instrument blank results should be <1/2 MRL and not affect the linearity of the calibration curve. If the result is higher, analyze several blanks to clear the system.

5) Method blank—After the initial calibration or after an existing calibration has been verified, and prior to sample analysis, a method blank must be analyzed. The method blank consists of low TOC water as well as any reagents that have been added to samples. The value of the blank must be <1/2 of the MRL.

6) Continuing blank check—After every 10 samples, analyze a blank preferably consisting of recycled combusted water or low-TOC water. The blank must contain any reagents that have been added to the samples. With some instruments, this is difficult to do regularly. If so, monitor a given lot of reagent water throughout the day's run. The results for subsequent reagent-water blanks must be <1/2 MRL.

7) Continuing calibration check—After every 10 samples, analyze one of three calibration check samples—low-range, mid-range, and high-range standard concentrations—on a rotating basis. The low-range standard should be at or below the minimum reporting level. The low-range sample should agree within 50% of the true value, and the mid- and high-range samples should agree within 15%.

8) Duplicates—Perform a duplicate analysis for every ten samples (or part thereof) analyzed. The RPD (relative percent difference) should be less than 15%. The duplicate analysis can be a duplicated fortified sample.

9) Fortified sample—Spike one sample per every ten samples analyzed or part thereof. Spike at 50 to 200% of the expected concentration. The spike level should be greater than 5 times the MRL and generally within 50 to 200% of the expected concentration. The recovery should be between 85 and 115%.

10) Inorganic carbon check—Confirm on a frequency that corresponds to the laboratories' routine periodic maintenance schedule, or when maintenance has been conducted on an instrument that may affect the tool/technique for inorganic carbon removal, that inorganic carbon is sufficiently removed from the samples. Prepare a 102.5 mg/L IC test solution (based on bicarbonate calculations and impurities) and analyze the solution to confirm that the result is <1/2 MRL. The IC removal check may be performed on a different matrix, but the IC level in the check sample should be higher than those in the unknown samples.

c. Corrective actions:

1) High blank values—Consult instrument manual. Check for contamination of reagents, sample containers, and equipment. Both high inorganic-matrix samples and high-TOC samples may cause an ongoing elevated blank. Diluting samples may help. Higher MRLs may be required.

2) Bad calibration check samples—Consult instrument manual. The instrument may need to be recalibrated.

3) Poor spike recovery or poor duplicate precision—This suggests a matrix problem, or it may be caused by non-homogenous suspended particulates in the sample. Consult instruction manual. Diluting samples may help.

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5310 B. High-Temperature Combustion Method

1. General Discussion

The high-temperature combustion method has been used for a wide variety of samples, but when applied to particle-laden samples, particle-size reduction may be necessary because the method uses small-orifice syringes and/or sampling tubing.

a. Principle: The sample is homogenized and diluted as necessary, and a portion is injected into a heated reaction chamber packed with an oxidative catalyst (e.g., cobalt oxide, platinum group metals, or barium chromate). The water is vaporized, and organic carbon oxidizes to CO_2 and H_2O. The CO_2 from IC release and organic carbon oxidation is transported in the carrier-gas stream and measured via a nondispersive infrared analyzer, or titrated coulometrically.

Because total carbon is measured, remove IC via acidification and sparging, or measure it separately and calculate the difference to obtain TOC.

Measure IC by injecting the sample into a reaction chamber, where it is acidified. Under acidic conditions, all IC converts to CO_2, which is transferred to the detector and measured. Under these conditions, organic carbon is not oxidized so only IC is measured.

Alternatively, convert IC to CO_2 with acid and remove the CO_2 by purging before sample injection. The sample then contains only the NPOC fraction of total carbon: to determine TOC, purgeable organic carbon also must be measured. (NOTE: NPOC is reported as TOC when analyzing samples for drinking water regulatory purposes.)

b. Interference: Removing carbonate and bicarbonate via acidification and purging with purified gas results in the loss of volatile organics. Volatiles also can be lost during storage or sample blending, particularly if the temperature is allowed to rise. Another important loss can occur if large carbon-containing particles fail to enter the needle or sampling tube used for

injection. Although filtration eliminates particulate carbon when determining DOC only, it can result in DOC loss or gain, depending on the physical properties of carbonaceous compounds and the adsorption or desorption of carbonaceous material on the filter (see 5310A.4 for sample filtering requirements). Check the filters contribution to DOC by analyzing a filtered blank. (NOTE: Any contact with organic material may contaminate a sample. Avoid contaminated glassware, plastic containers, and rubber tubing.) Analyze sample treatment, system, and reagent blanks. Some carbonates require combustion temperatures above 650°C to decompose, so lower-temperature systems must destroy carbonates via acidification. Elemental carbon may not be oxidized at lower temperatures, but it generally is not present in water samples or formed during combustion of dilute samples. However, at lower temperatures (680°C), dissolved salt fusion is minimized, resulting in lower blank values.

Combustion gases (e.g., water, halide compounds, and nitrogen oxides) may interfere with the detection system. Consult manufacturers' recommendations regarding proper selection and maintenance of scrubber materials and check for any matrix interferences.

The major limitation to high-temperature techniques is the blank's magnitude and variability. Instrument manufacturers have developed new catalysts and procedures that yield lower blanks, resulting in lower detection levels.

c. Minimum detectable concentration: Although instrument performance varies, most high-temperature combustion analyzers can detect 1 mg/L of carbon or less. To reduce the minimum detectable concentration, it may be possible to increase the portion injected for analysis.

d. Sampling and storage: If possible, rinse bottles with sample before filling. Field blanks can be used to check for atmospheric contamination during sampling by exposing the field blank to the same atmospheric conditions as samples during the sampling procedure.

Collect and store samples in glass bottles protected from sunlight and seal with TFE-backed septa (preferably thick silicone rubber-backed TFE septa with open ring caps to produce a positive seal). Alternative containers may be used if it is demonstrated that the container does not remove or add TOC at a level >1/2 MRL. Check bottle blanks with each set of sample bottles to determine the effectiveness of or need for cleaning. Wash bottles with detergent, rinse copiously with reagent water, seal with aluminum foil, and bake at 400°C for at least 1 h. Wash uncleaned TFE septa with detergent, rinse repeatedly with organic-free water, wrap in aluminum foil, and bake at 100°C for 1 h. Alternatively, soak septa in 10% sodium persulfate for 1 hour at 60°C and rinse thoroughly with organic-free water. Check performance of new or cleaned septa by running appropriate blanks. Less rigorous cleaning may be acceptable if a carbon concentration range more than ten times the blank will be determined.

If samples cannot be examined immediately, refrigerate and hold them with minimal exposure to light and atmosphere. If NPOC will be determined, use appropriate acid during collection to adjust pH to ≤2. Keep in mind, however, that acid preservation invalidates any IC determination. The choice of acid depends on the instrument manufacturer; consult the instruction manual. Also, acidification may cause both inorganic and organic materials to precipitate; if this happens, try another acid. In general, changing acid will not affect precipitation of organics but may prevent precipitation of inorganics that may adsorb organics and the particles may cause clogging of the sample-introduction system. Any precipitate should be well suspended before the sample is injected into the analyzer. Precipitated organic material often can be re-dissolved by adding NaOH free of CO_2 and organic matter. Be sure to treat blanks and samples identically. Maintain acidified samples at <6°C during shipment and store them at ≤6°C until analysis (maximum holding time is 28 d). Sample stability depends on the type of organics and sample matrix involved; analyze samples as soon as possible. When determining IC and then TOC, refrigerate sample but do NOT adjust pH; then analyze it as soon as possible (because TOC may biodegrade significantly before the regulatory 28-d holding time expires). Alternatively, determine IC independently on an unacidified sample, and then determine TOC via difference using a second, acidified sample (recognizing that the IC result for the second sample, used to determine TOC, will not represent the IC concentration in the first sample).

e. *Quality control (QC):* The QC practices considered to be an integral part of this method are summarized in Table 5020:I. Additionally, follow all QC described in the method itself.

2. Apparatus

a. Total organic carbon analyzer, using high-temperature combustion techniques.

b. Sampling, injection, and sample preparation accessories, as prescribed by instrument manufacturer.

c. Sample blender or homogenizer.

d. Magnetic stirrer and TFE-coated stirring bars.

e. Filtering apparatus and filters: See 5310A.4.

3. Reagents

a. Reagent water: Prepare reagents, blanks, and standard solutions from Type I water whose TOC content is <1/2 MRL.

b. Acid: Refer to instrument manufacturer's specifications. Check the acid for TOC content.

c. Organic carbon stock solution: Dissolve 2.1254 g anhydrous primary-standard-grade potassium biphthalate, $C_8H_5KO_4$, in carbon-free water and dilute to 1000 mL (1.00 mL = 1.00 mg carbon). Prepare laboratory control standards using any other appropriate organic-carbon-containing compound of adequate purity, stability, and water solubility. Commercially prepared standards may be used. To preserve, use appropriate acid to adjust pH to ≤2, and store sample at ≤6°C. Use the same acid in standards, samples, and blanks.

d. IC stock solution: Dissolve 4.4122 g anhydrous sodium carbonate, Na_2CO_3, in water; add 3.497 g anhydrous sodium bicarbonate, $NaHCO_3$; and dilute to 1000 mL (1.00 mL = 1.00 mg carbon). Alternatively, use another IC compound of adequate purity, stability, and water solubility. Keep tightly stoppered. Do not acidify. (NOTE: This solution is used to prepare standards for the analyzer's IC channel.)

e. IC-removal check solution: Because inorganic salts are not soluble in a single concentrated solution, prepare four separate stock solutions (A–D). Prepare Solution A by adding 2.565 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$ to 1 L of low-TOC water. Prepare Solution B by adding 0.594 g ammonium

chloride (NH_4Cl), 2.050 g calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), 0.248 g calcium nitrate tetrahydrate [$Ca(NO_3)_2 \cdot 4H_2O$], 0.283 g potassium chloride (KCl), and 0.281 g sodium chloride (NaCl) to 1 L of low-TOC water. Prepare Solution C by adding 2.806 g sodium bicarbonate ($NaHCO_3$) and 0.705 g sodium phosphate dibasic heptahydrate ($NaHPO_4 \cdot 7H_2O$) to 1 L of low-TOC water. Prepare Solution D by adding 1.862 g sodium-meta silicate nonahydrate ($Na_2SiO_3 \cdot 9H_2O$) to 1 L of low-TOC water. Take a 10-mL aliquot from each solution (A–D) and add it to a 40-mL vial. Acidify with 40 μL of an appropriate acid; use the same acid used for sample preservation.

f. Carrier gas: Purified oxygen or air (as specified by instrument manufacturer), CO_2-free and containing less than 1 ppm hydrocarbon (as methane). Contaminated gases or gas lines will result in elevated background levels. Commercial gas purifiers are available.

g. Purging gas: Any appropriate gas that meets the carrier-gas specifications.

4. Procedure

a. Instrument operation: Follow manufacturer's instructions for assembling, testing, calibrating, and operating analyzer. Adjust to optimum combustion temperature before using instrument; monitor temperature to ensure stability.

b. Sample treatment: If a sample contains gross solids or insoluble matter, homogenize until satisfactory replication is obtained. Sometimes dilution will help dissolve and/or suspend solids. If the sample solids cannot be dispersed so they can be representatively inserted into the analyzer, then consider filtering sample through a quartz filter and analyzing the retentate for carbon. When determining DOC, filter samples (see 5310A.4). Analyze a homogenizing blank (reagent water carried through the homogenizing treatment).

When measuring NPOC, first remove IC in accordance with instrument manufacturer's instructions. Check IC-removal efficiency [see 5310A.5*b*10)]. Alternatively, transfer a representative portion (10 to 15 mL) to an appropriate tube or container, add acid to reduce pH to $\leq$2, and purge with gas for the time determined in 5310A.5. When samples are purged externally before being injected into the oxidation zone, acidifying them to pH $\leq$2 may cause most hydrophobic molecules to be lost to the sample vessel walls. Acidifying to pH 4 minimizes such losses, but check IC-removal efficiency. Some instruments automatically purge samples as part of the TOC analysis cycle. Volatile organic carbon will be lost when the acidified solution is purged, so report organic carbon as total NPOC. If the instrument separately determines IC (carbonate, bicarbonate, free CO_2) and total carbon, verify sparging efficiency for the TOC determination. If the instrument determines TOC by calculating the difference between TC and IC, IC still may need to be sparged from the sample to avoid excessive error. To verify that the instrument is operating appropriately, analyze standards with IC levels higher than those in samples and TOC levels near the MRL to show proper recoveries.

c. Sample injection: Withdraw a portion of prepared sample using a syringe fitted with a blunt-tipped needle. Select sample volume according to manufacturer's directions. Stir particle-laden samples with a magnetic stirrer. Select needle size consistent with sample particle size. Other sample-injection techniques (e.g., sample loops) may be used. Inject samples and standards into analyzer according to manufacturer's directions and record response. Repeat injection until at least three replicate measurements are reproducible to within $\pm$10% RSD.

d. Preparation of standard curve: Prepare standard organic and IC series by diluting stock solutions to create a range of samples that spans the instrument's linear range. At a minimum, a reagent water blank and four calibration levels may be required for regulatory compliance purposes. Use reagent water to dilute samples above the instrument's linear range. Inject each standard and dilution water blank and record its peak height or area. Plot carbon concentration in milligrams per liter against corrected peak height or area (unnecessary for instruments that digitally compute concentration). Instruments with coulometric detectors and computerized IR detectors do not require calibration curves. For instruments with these detectors, demonstrate linearity and verify that the instrument is operating correctly by evaluating blanks and standards covering the analytical range.

In many laboratories, reagent water is the major contributor to the blank's results. However, correcting only the instrument response to standards (which contain reagent water, reagents, and system blank) creates a positive error; correcting the instrument response to reagent-water blanks (which contain only reagents and system blank contributions) creates a negative error. Many analyzers allow combustion water to be recycled through the analyzer and analyzed as a blank. Others allow the trapped water to be sampled and re-injected. This should be done with and without purging to determine the TOC contribution of the purging step.

Inject samples and determine organic carbon concentrations directly from the readout or from measurements by comparing corrected instrument response to the calibration curve. Regularly analyze laboratory-control samples to confirm instrument performance (see 5310A.5). Monitor the system blank regularly.

5. Calculations

Calculate the corrected instrument response to standards and samples by subtracting the instrument-blank results from those of the standard and sample. Prepare a standard curve of corrected instrument response vs. TOC concentration. Only subtract reagent water blank from standards prepared in reagent water or from the portion of diluted samples containing reagent water. The instrument response is compared to the standard curve to determine carbon content. Apply appropriate dilution factor when necessary. Subtract IC from total carbon when TOC is determined by calculating the difference.

NOTE: The reagent water blank may include an instrument contribution not dependent on reagent-water carbon, and a true response due to reagent-water carbon. When reagent-water carbon is a significant fraction of reagent-water blank, a negative error no larger than the reagent-water blank is introduced in sample values. Using recycled water permits isolation of each contribution to the total blank. Apply appropriate blank corrections to the instrument response to standards (reagent blank, reagent water blank, system blank) and sample (reagent blank and system blank).

6. Quality Control

See 5310A.5.

7. Precision

The difficulty of sampling particulates can limit the method's precision.

Interlaboratory studies of high-temperature combustion methods have been conducted in the range above 2 mg/L of carbon.[1] The resulting equation for single-operator precision on matrix water is:

$$S_o = 0.027x + 0.29$$

Overall precision is:

$$S_t = 0.044x + 1.49$$

where:

S_o = single-operator precision,
x = TOC concentration, mg/L of carbon, and
S_t = overall precision

8. Reference

1. American Society for Testing & Materials. 1994. Standard Test Method for Total and Organic Carbon in Water by High Temperature Oxidation and by Coulometric Detection; D4129-88. *In* Annual Book of ASTM Standards. Philadelphia, Pa.

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5310 C. Persulfate–Ultraviolet or Heated-Persulfate Oxidation Method

1. General Discussion

Many instruments that use persulfate to oxidize organic carbon are available. They depend either on catalyzed heat or ultraviolet (UV) irradiation to activate the reagents. These oxidation methods measure trace levels of organic carbon in water rapidly and precisely.

a. Principle: Persulfate oxidizes organic carbon to CO_2 in the presence of heat and a catalyst and/or UV light. The CO_2 produced may be purged from the sample, dried, and transferred with a carrier gas to a nondispersive infrared (NDIR) analyzer, be coulometrically titrated, or be separated from the liquid stream by a membrane that specifically allows CO_2 to pass into high-purity water. The change in the high-purity water's conductivity corresponds to the amount of CO_2 passing the membrane.

Some instruments use a UV lamp submerged in a continuously gas-purged reactor filled with a constant-feed persulfate solution. The samples are either introduced serially into the reactor by an autosampler or injected manually. The CO_2 produced is sparged continuously from the solution and is carried in the gas stream to an infrared analyzer specifically tuned to the absorptive wavelength of CO_2. The instrument's microprocessor calculates the area of the peaks produced by the analyzer, compares them to the peak area of the calibration standard stored in its memory, and prints out a calculated organic carbon value in milligrams per liter.

Other UV–persulfate instruments use continuous-flow sample injection and have an option for IC removal via vacuum degassing. The sample is acidified, persulfate is added, and then the sample flow is split. One channel passes to a delay coil, while the other passes through the UV reactor. CO_2 is separated from each sample stream by CO_2-selective membranes into high-purity water, whose change in conductivity is measured. CO_2 from the non-UV-irradiated stream represents IC. CO_2 from the irradiated stream represents TC. The instrument automatically converts the detector signals to units of concentration (mg/L or μg/L of carbon). TOC is calculated as the difference between the TC and IC channels.

Heated-persulfate instruments use a catalyst in a digestion vessel heated to 95 to 100°C. Samples are added by direct injection, loop injection, line injection, or autosampler. After IC is removed via acidification and sparging, a measured amount of persulfate solution is added to the sample. After an oxidation period, the resulting CO_2 is sparged from the solution and carried to an infrared analyzer specifically tuned to the absorptive wavelength of CO_2. The instrument's microprocessor converts the detector signal into organic carbon concentrations in mg/L of carbon, based on stored calibration data.

b. Interferences: Chloride interferes with persulfate oxidation, so **HCL must not be used to acidify samples for this method** (see 5310B.1). Insufficient acidification or sparging will result in incomplete removal of IC [see 5310A.5*b*10)].

The intensity of the UV light reaching the sample matrix may be reduced by highly turbid samples or aging lamps, resulting in sluggish or incomplete oxidation. Large organic particles or large or complex organic molecules (e.g., tannins, lignins, and humic acid) may oxidize slowly or incompletely because persulfate oxidation is rate-limited. However, many large biological molecules (e.g., proteins and monoclonal antibodies) oxidize rapidly. Because the organic carbon-to-CO_2 conversion efficiency may be affected by many factors, check oxidation efficiency with selected model compounds representative of the compounds of interest in a matrix representative of the sample.

Some instruments give low results for difficult-to-oxidize compounds under certain conditions. The following compounds are difficult to oxidize, are sufficiently water-soluble, and can be mixed and measured accurately at trace levels: urea; nicotinic acid; pyridine; *n*-butanol; acetic acid; leucine; acetonitrile; octoxynol-9; tartaric acid; 1,10-phenanthroline; 1-glutonic acid; 2-propanol; caffeine; and sodium dodecylbenzenesulfonate. Use these compounds as matrix additions to evaluate oxidation efficiency.

In samples containing significant concentrations of chloride, persulfate oxidation of organic molecules is slowed by the preferential oxidation of chloride. When chloride concentrations exceed 0.05%, organic matter oxidation may be inhibited. To remove this interference, extend reaction time and/or increase the amount of persulfate solution in heated-persulfate instruments.

With any organic carbon measurement, contamination during sample handling and treatment is a likely source of interference. Take extreme care when sampling, handling, and analyzing samples <1 mg TOC/L.

c. Minimum detectable concentration: Concentrations <0.1 mg TOC/L can be measured by some instruments if scrupulous attention is given to minimizing sample contamination and method background.

d. Sampling and storage: See 5310B.l*d*.

e. Quality control (QC): See 5310A.5. The QC practices considered to be an integral part of this method are summarized in Table 5020:I. Also, follow all QC described in the method itself.

2. Apparatus

a. Total organic carbon analyzer using persulfate oxidation principle.

b. Sampling and injection accessories, as specified by the instrument manufacturer.

c. Filtering apparatus: See 5310A.4.

3. Reagents

a. Reagents listed in 5310B.3.

b. Persulfate solution: Different instrument manufacturers recommend different forms and concentrations of peroxydisulfate. Typical preparations are as follows:

1) *Sodium peroxydisulfate*, 10%—Dissolve 100 g reagent in water; bring volume to 1 L.

2) *Ammonium peroxydisulfate*, 15%—Dissolve 150 g reagent in water; bring volume to 1 L.

3) *Potassium peroxydisulfate*, 2%—Dissolve 20 g reagent in water; bring volume to 1 L.

Check blank values from reagents and, if values are high, purify reagent or use a higher-purity source.

4. Procedure

a. Instrument operation: Follow manufacturer's instructions for assembling, testing, calibrating, and operating analyzer.

b. Sample preparation: If a sample contains particulates, homogenize until a representative portion can be withdrawn through the syringe needle, autosampler tubing, or sample inlet system of continuous on-line monitor.

When determining DOC, filter sample and a reagent water blank through a filter consistent with the specification contained in 5310A.4.

When determining NPOC, transfer an appropriate volume of sample to an appropriate container and acidify to pH 2. Purge according to the IC-removal procedure in 5310A.5. Some instruments include the IC-removal steps, but they must be evaluated as specified in 5310A.5.

c. Sample injection: See 5310B.4*c*.

d. Standard curve preparation: Prepare an organic carbon standard series that spans the range of organic carbon concentrations in samples. Run standards and blanks, and record the analyzer's response. Determine instrument response for each standard and blank. Unless CO_2 is trapped and desorbed, producing consistent peak heights, determinations based on peak height may be inadequate because of differences in the oxidation rates of standards and samples. Correct the instrument response to standards by subtracting reagent water blank and plotting organic carbon concentration in milligrams per liter against corrected instrument response. (This is unnecessary for instruments that digitally compute concentration.) Be sure that the instrument's algorithm includes blank correction and linearity of response. To confirm proper instrument operation, analyze standards with concentrations above and below those determined in the samples (preferably prepared in a similar matrix).

5. Calculation

See 5310B.5 or use instrument manufacturer's procedure.

6. Quality Control

See 5310A.5.

7. Precision and Bias

Interlaboratory studies of persulfate and/or UV with NDIR detection methods have been conducted in the range of 0.1 to 4000 mg/L of carbon.[1] The resulting equation for organic carbon (single-operator precision) is:

$$S_o = 0.04x + 0.1$$

Overall precision is expressed as:

$$S_t = 0.08x + 0.1$$

where:

S_o = single-operator precision,
x = TOC concentration, mg/L of carbon, and
S_t = overall precision.

An interlaboratory study was conducted for the membrane conductivity method,* covering samples with organic carbon concentrations between 1 and 25 mg/L of carbon. The resulting equation for single-operator precision is:

$$S_o = 0.012x - 0.022$$

Overall precision is expressed as:

$$S_t = 0.027x + 0.09$$

where terms are defined as above.

8. Reference

1. American Society for Testing & Materials. 1994. Standard Test Method for Total Carbon in Water by Ultraviolet, or Persulfate Oxidation, or Both, and Infrared Detection; D4839-88. *In* Annual Book of ASTM Standards. Philadelphia, Pa.

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* Data may be obtained from *Standard Methods* manager, American Water Works Association.

5310 D. (Reserved)

5320 DISSOLVED ORGANIC HALOGEN*

5320 A. Introduction

Dissolved organic halogen (DOX) is a measurement used to estimate the total quantity of dissolved halogenated organic material in a water sample. This is similar to literature references to "total organic halogen" (TOX), "adsorbable organic halogen" (AOX), and carbon-adsorbable organic halogen (CAOX). The presence of halogenated organic molecules is indicative of disinfection byproducts and other synthetic chemical contamination. Halogenated compounds that contribute to a DOX result include, but are not limited to: the trihalomethanes (THMs); organic solvents such as trichloroethene, tetrachloroethene, and other halogenated alkanes and alkenes; chlorinated and brominated pesticides and herbicides; polychlorinated biphenyls (PCBs); chlorinated aromatics such as hexachlorobenzene and 2,4-dichlorophenol; and high-molecular-weight, partially chlorinated aquatic humic substances. Compound-specific methods, such as gas chromatography, typically are more sensitive than DOX measurements.

The adsorption-pyrolysis-titrimetric method for DOX measures only the total molar amount of dissolved organically bound halogen retained on the activated carbon adsorbent; it yields no information about the structure or nature of the organic compounds to which the halogens are bound or about the individualhalogens present. It is sensitive to organic chloride, bromide, and iodide, but does not detect fluorinated organics.

DOX measurement is an inexpensive and useful method for screening large numbers of samples before specific (and often more complex) analyses; for extensive field surveying for pollution by certain classes of synthetic organic compounds in natural waters; for mapping the extent of organohalide contamination in groundwater; for monitoring the breakthrough of some synthetic organic compounds in water treatment processes; and for estimating the level of formation of chlorinated organic byproducts after disinfection. When used as a screening tool, a large positive (i.e., above background measurements) DOX test result indicates the need for identifying and quantifying specific substances. In saline or brackish waters the high inorganic halogen concentrations interfere. The possibility of overestimating DOX concentration because of inorganic halide interference always should be considered when interpreting results.

* Approved by Standard Methods Committee, 2010.
Joint Task Group: 20th Edition—James M. Symons (chair), Jarmila Banovic, Karl O. Brinkmann.

5320 B. Adsorption-Pyrolysis-Titrimetric Method

1. General Discussion

a. Principle: The method consists of four processes. First, dissolved organic material is separated from inorganic halides and concentrated from aqueous solution by adsorption onto activated carbon. Second, inorganic halides present on the activated carbon are removed by competitive displacement by nitrate ions. Third, the activated carbon with adsorbed organic material is introduced into a furnace that pyrolyzes organic carbon to carbon dioxide (CO_2) and the bound halogens to hydrogen halide (HX). Fourth, the HX is transported in a carrier gas stream to a microcoulometric titration cell where the amount of halide is quantified by measuring the current produced by silver-ion precipitation of the halides. The microcoulometric detector operates by maintaining a constant silver-ion concentration in a titration cell. An electric potential is applied to a solid silver electrode to produce silver ions in the cell solution. As hydrogen halide from the pyrolysis furnace enters the cell in the carrier gas, it is partitioned into the acetic acid solution where it precipitates as silver halide. The current that is produced is integrated over the period of the pyrolysis. The integrated area under the curve is proportional to the number of moles of halogen recovered. The mass concentration of organic halides is reported as an equivalent concentration of organically bound chloride in micrograms per liter. Because this DOX procedure relies on activated carbon to adsorb organic halides, it also has been referred to as carbon-adsorbable organic halogen (CAOX). Because of the poor adsorption efficiency of some organic compounds containing halogen and the desorption of some halogen-containing compounds during the removal of adsorbed inorganic halogen, this method does not measure total organic halogen.

When a sample is purged with inert gas before activated carbon adsorption, analysis of that sample determines the nonpurgeable dissolved organic halogen (NPDOX) fraction of DOX. The purgeable organic halogen concentration (POX) may be estimated by subtracting the NPDOX value from the DOX value. Alternatively, the POX fraction may be determined directly by purging the sample with carrier gas and introducing that gas stream and the volatilized organics directly into the pyrolysis furnace. Thus, depending on approach, the analysis of POX, DOX, and NPDOX may be determined directly or by difference.

Finally, because the POX often is dominated by the THMs, they may be determined as directed in Section 6200 and used to estimate POX. However, this approach is not included here as a standardized procedure.

b. Interferences: The method is applicable only to aqueous samples free of visible particulate matter. Different instruments vary in tolerance of small amounts of suspended matter. Inorganic substances such as chloride, chlorite, chlorate, bromate, bromide, and iodide will adsorb on activated carbon to an extent dependent on their original concentration in the aqueous solution and the volume of sample adsorbed.[1] Positive interference will result if inorganic halides are not removed. Treating the activated carbon with a concentrated aqueous solution of nitrate ion causes competitive desorption from the activated carbon of inorganic halide species and washes inorganic halides from other surfaces. However, if the inorganic halide concentration is greater than 10 000 times[2] the concentration of organic halides, the DOX results may be affected significantly. In general, this procedure may not be applicable to samples with inorganic halide concentrations above 500 mg Cl^-/L, based on activated carbon quality testing results. Therefore, consider both the results of mineral analysis for inorganic halides and the results of the activated carbon quality test (see 5320B.5) when interpreting results.

Halogenated organic compounds that are weakly adsorbed on activated carbon are recovered only partially. These include certain alcohols and acids (e.g., chloroethanol), and such compounds as chloroacetic acid, that can be removed from activated carbon by the nitrate ion wash. However, for most halogenated organic molecules, recovery is very good; the activated carbon adsorbable organic halide (CAOX) therefore is a good estimate of true DOX.

Failure to acidify samples with nitric acid or sulfuric acid may result in reduced adsorption efficiency for some halogenated organic compounds and may intensify the inorganic halide interference. However, acidification may result in precipitation loss of humic acids and any DOX associated with that fraction. Further, if the water contains residual chlorine, reduce it before adsorption to eliminate positive interference resulting from continued chlorination reactions with organic compounds adsorbed on the activated carbon surface or with the activated carbon surface itself. The sulfite dechlorinating agent may cause decomposition of a small fraction of the DOX if nitric acid is used; this decomposition is avoided if sulfuric acid is used. Do not add acid in great excess.

Highly volatile components of the POX fraction may be lost during sampling, shipment, sample storage, sample handling, and sample preparation, or during sample adsorption. A laboratory quality-control program to ensure sample integrity from time of sampling until analysis is vital. During sample filtration for the analysis of samples containing undissolved solids, major losses of POX can be expected. Syringe-type filtration systems can minimize losses. Analyze for POX before sample filtration and analyze for NPDOX after filtration; the sum of POX and NPDOX is the total DOX. In preparing samples for DOX analysis, process a blank and a standard solution to determine effect of this procedure on DOX measurement. If an insignificant loss of POX occurs during the removal of particulate matter by filtration, DOX may be measured directly.

Granular activated carbon used to concentrate organic material from the sample can be a major source of variability in the analysis and has a dramatic effect on the minimum detectable concentration. Ideally, activated carbon should have a low halide content, readily release adsorbed inorganic halides on nitrate washing, be homogeneous, and readily adsorb *all* organic halide compounds even in the presence of large excesses of other organic material. An essential element of quality control for DOX requires testing and monitoring of activated carbon (see 5320B.5*a*). Nonhomogeneous activated carbon or activated carbon with a high background value affects the method reliability at low concentrations of DOX. A high and/or variable blank value raises the minimum detectable concentration. Random positive bias, in part because of the ease of activated carbon contamination during use, may necessitate analyzing duplicates of each sample. Because activated carbon from different sources may vary widely in the ease of releasing inorganic halides, test for this quality before using activated carbon. Proper quantification also may be affected by the adsorptive capacity of the activated carbon. If excessive organic loading occurs, some DOX may break through and not be recovered. For this reason, make serial adsorptions of each sample portion and individual analyses.

c. Sampling and storage: Collect and store samples in amber glass bottles with TFE-lined caps. If amber bottles are not available, store samples in the dark. To prepare sample bottles, acid wash, rinse with deionized water, seal with aluminum foil, and bake at 400°C for at least 1 h. If bottle blanks without baking show no detectable DOX, baking may be omitted. Wash septa with detergent, rinse repeatedly in organic-free, deionized water, wrap in aluminum foil, and bake for 1 h at 100°C. Preferably use thick silicone rubber-backed TFE septa and open ring caps to produce a positive seal that prevents loss of POX and contamination. Store sealed sample bottles in a clean environment until use. Completely fill sample bottles but take care not to volatilize any organic halogen compounds. Preserve samples that cannot be analyzed promptly by acidifying with concentrated nitric acid or sulfuric acid to pH 2. Refrigerate samples at 4°C with minimal exposure to light. Reduce any residual chlorine by adding sodium sulfite crystals (minimum: 5 mg/L). Add 4 drops conc H_2SO_4 plus sodium sulfite crystals to bottles shipped to the field. NOTE: Some organic chloramines are not completely dechlorinated by sodium sulfite, particularly at pH >7. This may affect reported concentrations.[1] Analyze all samples within 14 d.

d. Minimum detectable concentration: For nonsaline waters free of particulate matter, 5 to 10 μg organic Cl^-/L is considered a typical range for detection levels. The minimum detectable concentration may be influenced by the analytical repeatability, equipment used, activated carbon quality, and the analyst. Determine the detection level for each procedure, instrument, and analyst.

2. Apparatus

a. Adsorption assembly, including gas-tight sample reservoir, activated carbon-packed adsorption columns, column housings, and nitrate solution reservoir. In particular, note the following:

1) *Noncombustible insulating material (microcolumn method only)*—Form into plugs to hold activated carbon in columns. NOTE: Do not touch with fingers.

2) *Activated carbon columns (microcolumn method only)*—Pack 40 ± 5 mg activated carbon (5320B.3*m*) into dry glass tubing approximately 2 to 3 mm ID × 6 mm OD × 40 to 50 mm long. NOTE: Protect these columns from all sources of halogenated organic vapors. Clean glass tubes before use with a small-diameter pipe cleaner to remove residual carbon, then soak in

chromate cleaning solution for 15 min and dry at 400°C. Rinse between steps with deionized water. NOTE: Use prepacked columns with caution, because of occasional reported contamination.

b. Analyzer assembly, including carrier gas source, boat sampler, and pyrolysis furnace, that can oxidatively pyrolyze halogenated organics at a temperature of 800 to 900°C to produce hydrogen halides and deliver them to the titration cell with a minimum overall efficiency of 90% for 2,4,6-trichlorophenol; including a microcoulometric titration system with integrator, digital display, and data system or chart recorder connection; including (optional) purging apparatus.

c. Chart recorder or microprocessor, controlled data system.

d. Batch adsorption equipment: Use instrument manufacturer's purge vessel or similar purging flask, Erlenmeyer flasks (100 to 250 mL), and high-speed stirrers.

e. Filtering apparatus and filters: Use 0.45-μm-pore-diam filters, preferably HPLC syringe filters or similar, with no detectable DOX blank. Rinsed glass-fiber filters are satisfactory for sample filtration. Preferably use membrane filters for separating activated carbon from aqueous phase.

3. Reagents and Materials

Use chemicals of ACS reagent grade or other grades if it can be demonstrated that the reagent is of sufficiently high purity to permit its use without lessening accuracy of the determination.

a. Carbon dioxide, argon, or nitrogen, as recommended by the equipment manufacturer, purity 99.99%.

b. Oxygen, purity 99.99%.

c. Aqueous acetic acid, 70 to 85%, as recommended by the equipment manufacturer.

d. Sodium chloride standard (NaCl): Dissolve 0.1648 g NaCl and dilute to 100 mL with reagent water; 1 μL = 1 μg Cl^-.

e. Ammonium chloride standard (NH_4Cl): Dissolve 0.1509 g NH_4Cl and dilute to 100 mL with reagent water; 1 μL = 1 μg Cl^-.

f. Trichlorophenol stock solution: Dissolve 1.856 g trichlorophenol and dilute to 100 mL with methanol; 1 μL = 10 μg Cl^-. Alternatively, purchase stock solution of known concentration and dilute as noted.

g. Trichlorophenol standard solution: Make a 1:20 dilution of the trichlorophenol stock solution with methanol; 1 μL = 0.5 μg Cl^-. Alternatively, purchase stock solution of known concentration and dilute as noted.

h. Trichloroacetic acid stock solution: Dilute 199.44 mg trichloroacetic acid in 1000 mL reagent water; 1 mL = 130 μg Cl^-.

i. Trichloroacetic acid standard solution: Dilute 2.0 mL trichloroacetic acid stock solution into 1000 mL with reagent water; 1 mL = 0.260 μg Cl^-.

j. Chloroform standard solution ($CHCl_3$): Dilute 100 mg $CHCl_3$ to 100 mL with methanol; 1 μL = 1 μg $CHCl_3$. Alternatively, purchase stock solution of known concentration and dilute as noted.

k. Blank standard: Use reagent water. Reagent water preferably is carbon-filtered, deionized water that has been heated and purged.

l. Nitrate wash solution, 0.08*M:* Dilute 8.2 g KNO_3 to 1000 mL with reagent water. Adjust to pH 2 with HNO_3. 1 L = 6000 mg NO_3^-, depending on amount of acid used for pH adjustment.

m. Activated carbon, 100 to 200 mesh: Ideally use activated carbon having a very low apparent halide background that readily releases adsorbed inorganic halides on nitrate washing, and reliably adsorbs organic halides in the presence of a large excess of other organic compounds.* See 5320B.5 for preparation and evaluation of activated carbon. **CAUTION: Protect activated carbon from contact with halogenated organic vapors.**

n. Sodium sulfite (Na_2SO_3), crystals.

o. Nitric acid (HNO_3), conc, or *sulfuric acid*, H_2SO_4, conc.

4. Procedure

Use either the microcolumn (¶ *a* below) or batch adsorption (¶ *b* below) method to determine DOX (as CAOX). If present, determine POX separately (¶ *c* below). The microcolumn method utilizes small glass columns packed with activated carbon through which the sample is passed under positive pressure to adsorb the organic halogen compounds. The batch adsorption method uses a small quantity of activated carbon that is added to the sample. After stirring, activated carbon is removed by filtration, washed with nitrate, and analyzed. The batch adsorption procedure typically is run on samples that have had POX analyzed directly (¶ *c* below), yielding NPDOX directly as well.

a. Microcolumn procedure:

1) Apparatus setup—Adjust equipment in accordance with the manufacturer's instructions. Make several injections of NaCl solution directly into the titration cell [5320B.5*c*1)] as a microcoulometer/titration cell check at the start of each day.

2) Sample pretreatment for DOX analysis—If the sample has not been acidified during collection, adjust pH to 2 with HNO_3 or H_2SO_4. If the samples contain undissolved solids, filter through a glass-fiber filter (other means of removing particulate matter may be used, if it can be demonstrated that they do not cause significant interferences). Also filter a blank and standard. Analyze these to determine the contribution of filtration to the organic halogen measurement. Vacuum filtration will cause some loss of volatile organic halogen. Analyze for POX (¶ *c* below) before filtration and NPDOX after filtration, unless it is shown that POX losses during filtration are insignificant for a specific water type.

3) Sample adsorption—Transfer a representative portion of sample to the cleaned sample reservoir with two activated carbon adsorption columns in series attached by the column housings to the reservoir outlet. Seal the reservoir. Adjust to produce a flow rate of about 3 mL/min. When the desired volume has been processed, stop the flow, detach the activated carbon housings and columns, and rinse the sample reservoir twice with reagent-grade water. Vary volume processed to produce optimum quantities of adsorbed DOX on the columns. Suggested volumes are as follows:

Volume Processed *mL*	Instrument Optimum Range *μg Cl^-*	Conc of DOX in Waters *μg/L*
100	0.5–50	5–50
50	12.5–50	250–1000
25	12.5–50	500–2000

* Westvaco or Calgon Filtrasorb 400, or equivalent.

If possible, avoid using volumes greater than 100 mL because the maximum adsorptive capacity of the activated carbon may be exceeded, leading to adsorbate breakthrough and loss of DOX. Larger sample volumes processed lead to an increased quantity of inorganic halide accumulated on the activated carbon and may result in a positive interference. Do not use a sample less than 25 mL to minimize volumetric errors. For samples exceeding 2000 μg DOX/L dilute before adsorption. Protect columns from the atmosphere until DOX is determined.

4) Inorganic halide removal—Attach columns through which sample has been processed in series to the nitrate wash reservoir and pass 2 to 5 mL NO_3^- solution through the columns at a rate of approximately 1 mL/min.

5) DOX determination—After concentrating sample on activated carbon and removing inorganic halogens by nitrate washing, pyrolyze contents of each microcolumn and determine organic halogen content. Remove top glass microcolumn from the column housing, taking care not to contaminate the sample with inorganic halides. Using a clean ejector rod, eject the activated carbon and noncombustible insulating material plugs into the sample boat. Prepare sample boat during the preceding 4 h by heating at 400 to 800°C for at least 4 min in an oxygen-rich atmosphere (i.e., in the pyrolysis furnace). Remove residual ash. Place ejector rod on the plug of the effluent end of the carbon microcolumn and place the influent end of the carbon microcolumn in the quartz boat first. Seal sample inlet tube and let instrument stabilize. After NO_3^- wash avoid contact with inorganic halides. Wear latex gloves while carrying out this procedure. Preferably clean work area frequently with deionized water.

Pyrolyze the activated carbon and determine halide content. Repeat for each microcolumn. Check for excess breakthrough (5320B.5*b*) and repeat analysis as necessary.

6) Replicates—When DOX determination is used strictly as a screening tool, total replication is not necessary. Single-operator precision (% CV) is expected to be less than 15% for tap water and wastewater (Table 5320:I). If system performance is consistently worse as demonstrated by routine QA duplicates, or if quality objectives dictate, run replicates of each sample by repeating ¶s *a*3), 4), and 5) above.

7) Blanks—Analyze one method blank [5320B.5*e*2)] with each set of ten samples. Analyze the method blank before starting each sample set and run a blank after the last set of the day.

8) Preparation and analysis of calibration standard—Run daily calibration standards in accordance with 5320B.5*c*3) for POX analysis or 5320B.5*c*5) for microcolumn-adsorption DOX analysis. Accompany by a suitable blank (5320B.5*e*). Be certain that analytical conditions and procedures (e.g., purging temperature) are the same for the analysis of calibration standards as for the analysis of samples.

b. Batch adsorption procedure:

1) Apparatus setup—Adjust equipment in accordance with the manufacturer's instructions.

2) Sample pretreatment—Adjust sample pH to 2 with conc HNO_3 or H_2SO_4 [see ¶ *a*2) above].

3) Sample adsorption—Prepare carbon suspension by adding high-quality activated carbon to high-purity, deionized, granular activated carbon (GAC)-treated water to produce a uniform suspension of 10 mg carbon/mL. To an Erlenmeyer flask, transfer prepurged sample of optimum size from a purging flask standardized in the same manner as the instrument's purging vessel. Add 20 mg activated carbon (2 mL carbon suspension). Using a high-speed mixer (20 000 rpm), stir for 45 min in an organohalide vapor-free environment. Filter through a membrane filter under vacuum or pressure, and collect filtrate. Remove flask containing filtrate. Wash carbon cake and filter with 10 mL NO_3^- wash solution. Add portions of wash solution serially to keep activated carbon and NO_3^- solution in contact for 15 min. Using clean instruments, transfer carbon cake and membrane filter to pyrolysis unit sample boat. Let instrument stabilize, pyrolyze, and determine the halide content of the first serial filter.

Add 20 mg more activated carbon to filtrate in Erlenmeyer flask. Repeat carbon mixing, filtering, and washing procedures. Pyrolyze and determine halide content of second serial filter. If the second value is greater than 10% of the total value (first plus second), perform the NPDOX determination on an additional sample portion.

c. POX procedure (optional) (direct purge): Adjust apparatus [¶ *a*1) above]. Select sample volume by comparing expected POX value (if known) with optimum instrument range. Using a gastight syringe, inject sample through septum into purge vessel, and purge as recommended by equipment manufacturer. Carefully control gas flow rate, sample temperature, and purging time. The maximum POX that can be determined is:

$$\text{POX}_{\text{max}},\ \mu\text{m/L} = \frac{0.5 \times 1000}{\text{mL sample} \times 35.5}$$

If replicates are analyzed, sampling from replicate sample bottles may minimize variability due to volatilization losses.

5. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 5020:I.

a. Activated carbon quality: Purchase activated carbon ready for use or prepare activated carbon by milling and sieving high-quality activated carbon. Use only 100- to 200-mesh carbon in the microcolumn method. During preparation, take care not to expose the activated carbon to organic vapors. Use of a clean room is helpful. Prepare only small quantities (a month's supply or less) at one time. Discard the activated carbon if its DOX background concentration has increased significantly from the time of preparation or if the background is greater than 1 μg apparent organic Cl^-/40 mg activated carbon. Uniformity of activated carbon is important; therefore, after sieving small portions, combine and mix thoroughly. Transfer representative portions to clean glass bottles with ground-glass stoppers or with rubber-backed TFE septa and open ring caps. Store bottles in a gas-purged, evacuated, sealed desiccator.

Test each newly prepared batch of activated carbon to ensure adequate quality before use. Use only activated carbon meeting the guidelines outlined below.

1) Check activated carbon particle size by applying deionized water to two 40-mg activated carbon microcolumns. If flow rate is significantly less than 3 mL/min, resieve activated carbon to remove excess fines.
2) Analyze a pair of method blanks, ¶ *e*2) below. Reject carbon if the apparent organic halogen exceeds 1.2 μg/40 mg activated carbon.

TABLE 5320:I. INTRALABORATORY, SINGLE-OPERATOR, DISSOLVED ORGANIC HALOGEN (MICROCOLUMN PROCEDURE)—PRECISION AND BIAS DATA

Characteristic of Analysis	Tap Water	Tap Water + 43.5 μg Organic Chloride	Ground Water (50:1)	Wastewater	Wastewater + 1000 μg Organic Chloride
Concentration determined, μg Cl^-/L:					
Replicate 1	38.5	89.0	123.6	186.0	1178.0
Replicate 2	36.7	90.9	124.8	195.0	1183.0
Replicate 3	43.1	88.4	125.2	195.0	1185.5
Replicate 4	35.9	90.1	123.3	204.0	1196.5
Replicate 5	41.1	91.7	125.3	185.0	1183.0
Replicate 6	48.5	93.0	127.0	236.5	1204.0
Replicate 7	52.8	97.0	123.5	204.0	1138.0
Mean, μg Cl^-/L	42.37	91.5	124.7	200.8	1181.1
Standard deviation:					
μg Cl^-/L	±6.29	±3.0	±1.3	±17.47	±21.04
%	15	3	1	9	2
Value of blank + standard addition, μg Cl^-/L	—	85.87	—	—	1200.8
Recovery, %	—	107	—	—	98
Error, %	—	7	—	—	2

If the activated carbon originated from a previously untested batch from a commercial supplier, test it for adsorption efficiency and inorganic halide rejection.

3) Adsorb replicate 100-mL portions of solutions containing 100, 500, and 1000 mg inorganic Cl^-/L deionized water. Wash with nitrate solution and analyze. The apparent organic halogen yield should not increase by more than 0.50 μg over the value determined in ¶ *a*2) above. A greater increase indicates significant interference at that concentration.

b. Serial adsorption: Each aqueous standard and sample is serially adsorbed on activated carbon in both procedures given above. Of the net organic halide, 90% or more should be adsorbed on the first activated carbon portion and the remaining 10% or less on the second. If, upon separate analysis of the two serial activated carbon portions, the second shows more than 10% of the net (after subtracting the method blank), reanalyze sample. Inorganic halogen interference or organic breakthrough are the most common reasons for a high second activated carbon value. Sample dilution before adsorption may improve recovery on the first activated carbon in series, but the minimum detectable concentration will be affected.

c. Standards: The standards used in routine analysis, quality control testing, and isolating specific causes during corrective maintenance include:

1) Sodium chloride standard (5320B.3*d*)—Use to check functioning of the titration cell and microcoulometer by injecting directly into the acetic acid solution of the titration cell. By examining the height and shape of the peak produced on the chart recorder and from the integrated value, problems associated with the cell and coulometer may be isolated. Use this standard at startup each day and after cell cleaning throughout the day. At daily startup consecutive duplicates should be within 3% of the historical mean. Depending on sample loading and number of analyses performed, it may be necessary to clean the titration cell several times per day. After cleaning, cell performance may be very unstable; therefore, inject a single NaCl standard before analyzing an instrument calibration standard [see ¶ *c*4) below]. Do *not* introduce NaCl standards into the pyrolysis furnace by application to the sample boat.

2) Ammonium chloride standard (5320B.3*e*)—Apply this standard to the sample boat to check for loss of halide in the pyrolysis furnace and entrance of the titration cell. Typically, this may be necessary when injection of a NaCl standard indicates proper titration cell and microcoulometer function but the recovery of the calibration standard is poor: suspect either poor conversion of organic chloride to hydrogen chloride or loss of hydrogen halide after conversion but before partitioning into the cell solution. To isolate the possible loss of hydrogen halides inject NH_4Cl standard directly onto the quartz sample boat. Recovery should be better than 95%, with a single peak of uniform shape produced. Use only a new quartz sample boat free of any residue; an encrusted boat dramatically reduces recovery. Use this standard for corrective maintenance problem isolation but not for routine analyses.

3) Purgeable organic halide calibration standards—For the POX analysis use aqueous chloroform solutions for instrument calibration. Also for POX analysis an aqueous bromoform standard can be used initially to ensure acceptable purging conditions. Develop a standard curve over the dynamic range of the microcoulometer and check daily as in ¶ *c*5) below. Recovery of chloroform and bromoform should exceed 90 and 80%, respectively.

4) Instrument calibration standard—Direct injection of trichlorophenol working standard onto the nitrate-washed method blank in concentrations over the working range of the instrument determines linearity and calibration of the analyzer module. After checking for proper microcoulometer function by injecting NaCl standard, pyrolyze duplicate instrument calibration standards and then duplicate method blanks. The net response to the

calibration standards should be within 3% of the calibration curve value. If not, check for loss of halide in the pyrolysis furnace using the ammonium chloride standard [¶ *c*2) above].

5) Nonvolatile organic halide calibration standards—Develop an initial standard curve by analyzing aqueous solutions of 2,4,6-trichlorophenol, trichloroacetic acid (commonly formed during chlorination), or another appropriate halogenated organic compound over the dynamic range of the microcoulometer. This dynamic range typically is from 0.5 to 50 μg chloride, but will vary between microcoulometers and titration cells. Construct an initial calibration curve using five calibration standards in range of 0.5 to 50 μg organic chloride; recheck calibration curve after changes in an instrument's configuration, such as replacement of a titration cell or major instrument maintenance. Daily, analyze a calibration standard to check proper function of the instrumentation and procedures. Select check standard in the concentration range of samples to be analyzed that day. When sample filtration is used to remove particulate matter, also use this pretreatment with the calibration standard. If DOX recovery is less than 90%, analyze a set of instrument calibration standards [¶ *c*4) above].

d. Standard addition recovery: During routine analyses, ideally make standard additions to every tenth sample. Where the compounds constituting the DOX are known, use standards of these compounds. Where the compounds constituting the DOX are wholly or partially unknown, use standards reflecting the relative abundance of the halogens, the molecular size, and the volatility of the halogenated compounds presumed to be present. Recovery of 90% or more of the added amount indicates that the analyses are in control. Do not base acceptance of data on standard addition recoveries.

e. Blanks: High precision and accuracy of the background or blank value is important to the accurate measurement of DOX. Make blank measurements daily. Blanks that may be required are:

1) Reagent water blank—Analyze each batch of organic-free reagent water. The blank should have less than the minimum detectable concentration. Use this blank to ensure that the standards, equipment, and procedures are not contributing to the DOX. Once reagent water blank is demonstrated, it can be used to determine method blank and POX blank as described below.

2) Method blank—Analyze activated carbon that has been nitrate-washed. Analyze method blanks daily before sample analysis and after at least each 10 to 14 sample pyrolyses.

3) Purgeable organic halogen blank—Analyze organic-free, pre-purged, reagent water to determine the POX blank.

6. Calculation

Calculate the net organic halide content as chloride (C_4) of each replicate of each sample and standard:

$$C_4 = \frac{C_1 - C_3 + C_2 - C_3}{V}$$

where:

C_4 = uncorrected net organic halide as Cl^- of absorbed sample, μg organic halide as Cl^-/L,
C_1 = organic halide as Cl^- on the first activated carbon column or activated carbon cake, μg,
C_3 = mean of method blanks on the same day and same instrument, μg X as Cl^-,
C_2 = organic halide as Cl^- on the second activated carbon column or activated carbon cake, μg, and
V = volume of sample absorbed, L.

If $C_2 \leq C_3$, then use:

$$C_4 = \frac{C_1 - C_3}{V}$$

If applicable, calculate net purgeable organic halide as Cl^- content (P_3):

$$P_3 = \frac{P_1 - P_2}{V}$$

where:

P_3 = uncorrected net purgeable organic halide as Cl^-, μg X as Cl^-/L,
P_1 = sample purgeable organic halide as Cl^-, μg,
P_2 = blank purgeable organic halide as Cl^-, μg, and
V = volume of sample or standard purged, L.

Report sample results and percent recovery of the corresponding calibration standards [5320B.5*c*3) or *c*5)]. Also report the calibration standard curve if it is significantly nonlinear.

7. Precision and Bias

Precision and bias depend on specific procedures, equipment, and analyst. Develop and routinely update precision and bias data for each procedure, each instrument configuration, and each analyst. Table 5320:I shows sample calculations of precision expressed as the standard deviation among replicates and bias in the recovery of 2,4,6-trichlorophenol.

The precision and bias (accuracy) of analyses of TOX by this method were determined under the Information Collection Rule.[3] Precision was determined as relative percent difference for duplicate analyses and was calculated only when both analyses in the duplicate pair showed concentrations at, or greater than, the ICR minimum reporting level (MRL). Accuracy was calculated as percent recovery for cases in which the fortified concentration of organic halide from trichlorophenol was at least half the background concentration. Fortifying concentrations ranged from MRL (50 μg Cl^-/L) to 500 μg Cl^-/L. Results were as follows:[4]

Data Quality Variable	N	Percentile				
		10	25	50 (median)	75	90
Precision (RPD)	13911*	0.0	1.2	3.8	7.6	14
Accuracy (% rec.)	1109	82	93	100	108	126

* 6003 samples excluded—both samples < MRL.

NOTE: For precision study, median sample concentration was 140 μg Cl^-/L; for accuracy study, median background concentration was 75 μg Cl^-/L.

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5510 AQUATIC HUMIC SUBSTANCES*

5510 A. Introduction

1. General Discussion

Aquatic humic substances (AHS) are heterogeneous, yellow to black, organic materials that include most of the naturally occurring dissolved organic matter in water. Aquatic humic substances have been shown to produce trihalomethanes (THMs) on chlorination and to affect the transport and fate of other organic and inorganic species through partition/adsorption, catalytic, and photolytic reactions.

Humic substances, the major fraction of soil organic matter, are mixtures; their chemical composition is poorly understood. They have been classified into three fractions based on water "solubility"†: humin is the fraction not soluble in water at any pH value; humic acid is not soluble under acidic conditions (pH <2) but becomes soluble at higher pH; and fulvic acid is soluble at all pH conditions.

AHS have the solubility characteristics of fulvic acids but they should not be referred to as such unless they have been fractionated by precipitation at pH <2. Avoid using the terms "humic acid" and "tannic acid" to describe AHS because they represent other classifications of natural organic materials.

The heterogeneity of AHS requires an operational definition. Isolation by the methods included herein most likely will be incomplete and compounds that are not AHS may be isolated incidentally. Users of these methods are cautioned in the interpretation of results; the bibliography suggests several sources for more information.

Measurement of AHS begins by separation of the sample into dissolved (containing AHS) and particulate organic carbon fractions. Although there is no distinct size that separates these two groups, 0.45 μm is used as the compromise between acceptable flow rate and rejection of small colloidal materials. Low-pressure liquid chromatography serves to concentrate these materials and to isolate them from interfering substances. AHS are quantified by measuring dissolved organic carbon (DOC), Section 5310.

2. Selection of Method

Concentration/isolation of AHS may be achieved by sorption on the nonpolar polyacrylate macroporous resin (such as XAD-7 or XAD-8‡) (5510C) or by anion-exchange on diethylaminoethyl (DEAE) cellulose (5510B). In a collaborative study with seven laboratories using deionized water fortified with about 10 mg AHS/L (previously isolated with XAD), the DEAE method gave better recoveries. Nevertheless, the XAD method has been used extensively; refer to the discussions of interferences and minimum detectable concentrations to assist in method selection. Both methods require further quality control development.

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* Approved by Standard Methods Committee, 2010.

† "Solubility" is here used as a general description of whether or not the material can be uniformly dispersed in an aqueous phase rather than as an expression of equilibrium between a pure solute and its aqueous solution.

‡ Rohm and Haas Co., Philadelphia, PA.

5510 B. Diethylaminoethyl (DEAE) Method

1. General Discussion

a. Principle: AHS are concentrated by column chromatography on diethylaminoethyl (DEAE) cellulose and measured as dissolved organic carbon (DOC). AHS are weak organic acids that bind to anion-exchange materials, such as DEAE cellulose, at neutral pH values. The method is based on the assumption that AHS are the major dissolved organic acids present.

b. Interferences: Any carbonaceous nonhumic materials that are concentrated and isolated by the chromatographic method will interfere (false positive response). Substances that have been shown to interfere include fatty acids, phenols, surfactants, proteinaceous materials, and DOC leached from cellulose.

c. Minimum detectable concentration: Estimated limit of detection is 1.1 mg/L using a 50-mL water sample. The detection limit can be decreased by increasing sample volume. The major limitation is blank contamination.

d. Standard substance: Eliminate documentation of false negatives by analyses of a sample of known humic concentration at regular intervals (at least once per batch of samples).

e. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Membrane filtration apparatus: Use an all-glass filtering device and a filter than does not contribute total organic carbon (see Section 5910B.2.*b*). Consult manufacturer's specifications for filter details. Do not use filters that sorb AHS or are contaminated with detergents and other organic material.

b. Glass column, approximately 1 × 20 cm with silanized glass wool.

c. Dye-impregnated paper or strips for approximate pH measurements.

d. Organic carbon analyzer capable of measuring concentrations as low as 0.1 mg/L (see Section 5310).

*e. Buchner funnel and filter paper.**

3. Reagents

a. Water, DOC-free: Preferably use activated-carbon-filtered, redistilled water.

b. DEAE cellulose, exchange capacity 0.22–1.0 meq/g.† Do not use high-exchange-capacity cellulose, which may decrease recovery of AHS. Take care not to overload low-exchange-capacity cellulose.

c. Hydrochloric acid (HCl), 0.1*N:* Add 8.3 mL conc HCl to 1000 mL water.

d. Hydrochloric acid (HCl), 0.5*N:* Add 41.5 mL conc HCl to 1000 mL water.

e. Sodium hydroxide (NaOH), 0.1*N:* Dissolve 4.0 g NaOH in 1000 mL water.

f. Sodium hydroxide (NaOH), 0.5*N:* Dissolve 20 g NaOH in 1000 mL water.

g. DOC standards: See Section 5310.

h. Potassium chloride (KCl), 0.01*N:* Add 0.75 g KCl to 1000 mL water.

i. Phosphoric acid (H_3PO_4), conc.

4. Procedure

a. Sample concentration and preservation: AHS are sensitive to biodegradation and photodegradation. Collect and store samples in organic-free glass containers. Filter at least duplicate portions through a 0.45-μm silver membrane filter as soon after collection as possible. Store samples in the dark at 4°C.

Use care to avoid overloading chromatographic columns and losing AHS. A rough guideline for sample volume selection is as follows:

Sample DOC *mg/L*	Sample Volume *mL*
0–2	250
2–10	50
10–50	25

b. Preparation of DEAE cellulose: Add 70 g DEAE cellulose to 1000 mL 0.5*N* HCl and stir gently for 1 h. Rinse cellulose with water in a Buchner funnel until funnel effluent pH is about 4. Resuspend DEAE in 1000 mL 0.5*N* NaOH and stir for 1 h. Rinse in a Buchner funnel with water until pH is about 6. Remove fines by suspending the treated DEAE in a 1000-mL graduated cylinder filled with water. Let mixture stand undisturbed for 1 h, then decant and discard the supernatant. Repeat removal of fines. Filter remaining DEAE using a Buchner funnel and store in a refrigerated glass container. Avoid prolonged storage, which may lead to microbial contamination.

c. Chromatography: Add 10 mL water to about 1 g DEAE to make a slurry. Carefully pipet enough into a 1- × 20-cm column fitted with a small (0.5-cm) glass-wool plug to make a 1-cm-deep column bed. Avoid getting DEAE on the sides of the column. Carefully place another 0.5-cm glass-wool plug on top of the bed. Rinse column with 50 mL 0.01*N* KCl (adjusted to pH 6 with 0.1*N* HCl or NaOH) just before sample concentration.

Adjust sample to pH 6 and pass it through the column at a flow rate of about 2 mL/min. Rinse with 5 mL water (pH 6). Elute AHS by adding about 3 mL 0.1*N* NaOH to the top of the column. Start collecting column effluent when it appears colored. (This will occur after about 1 mL has passed out of the column). Collect eluate in a graduated, conical test tube until it becomes colorless (about 2 mL). Acidify with conc H_3PO_4 to a pH of 2 or less (about 2 to 3 drops) and remove dissolved carbon dioxide (inorganic carbon) by purging with nitrogen for 10 min. Avoid exposure of alkaline samples to air (i.e. acidify immediately) to minimize contamination with CO_2. Determine volume and DOC of acidified eluate.

Process two portions of water and a second portion of sample by the same procedure. Pack a fresh column of DEAE for each sample and each control (DEAE cannot be reused).

5. Calculation

Calculate the concentration of AHS as:

$$\text{AHS, mg DOC/L} = [(A - B) \times C]/D$$

where:

A = average DOC concentration of the two sample NaOH eluates, mg C/L,
B = average DOC concentration of the two control NaOH eluates, mg C/L,
C = volume of eluate, L, and
D = volume of sample, L.

Multiplication of AHS, mg DOC/L, by 2 converts concentration to AHS, mg/L, if it is assumed that AHS contain 50% carbon. This will be the minimum concentration of AHS because recoveries are less than 100%.

6. Precision and Bias

For seven single-operator analyses, the relative standard deviation of triplicate samples (about 10 mg/L as AHS) ranged from 2.5 to 14.4% with an average of 4.9% (n = 7).

For seven single-operator analyses, recoveries ranged from 59.3 to 97.3% with an average of 77.4% and a relative standard deviation of 18.1%.

* Whatman No. 1, or equivalent.
† Whatman pre-swollen microgranular DE 52 or DE 51, or equivalent.

5510 C. XAD Method

1. General Discussion

a. Principle: AHS are concentrated by column chromatography on XAD resin and measured as dissolved organic carbon (DOC). Acidification of AHS decreases polarity, allowing partition into the nonpolar XAD matrix. The method is based on the assumption that AHS are the major dissolved organic acids present.

b. Interferences: Any carbonaceous nonhumic materials that are concentrated and isolated by the chromatographic method will interfere. This includes fatty acids, phenols, surfactants, proteinaceous materials, and DOC leached from the resin, chromatography pump, or tubing.

c. Minimum detectable concentration: Estimated limit of detection is 1.4 mg/L using a 50-mL water sample. The detection limit can be decreased by increasing sample volume. The major limitation is blank contaminations.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

See 5510B.2*a*, *c*, and *d*. In addition, the following are required:

a. Glass column, 0.2 × 25 cm with silanized glass wool.

b. Pump, with inert internal parts and tubing, capable of flow rates of 0.2 to 1.0 mL/min.*

c. TFE tubing, 0.2 cm ID.

d. Extraction apparatus, Soxhlet.

3. Reagents

In addition to reagents 5510B.3*a*, *c*, *e*, *g*, and *i*:

a. XAD resin,† approximately 250-μm size.

b. Hexane.

c. Methanol.

d. Acetonitrile.

4. Procedure

a. Sample collection and preservation: See 5510B.4*a*.

b. Preparation of XAD resin: Clean resin by successive washing with 0.1*N* NaOH for 5 d. Extract resin sequentially in a Soxhlet extractor with hexane, methanol, acetonitrile, and methanol, for 24 h each. Pack clean resin into a 0.2- × 25-cm glass column that has a 2-mm length of glass wool in one end. After filling, cap column with another 2-mm length of glass wool.

Wet dry column with methanol. When the air has been displaced, pump distilled water through the column until the effluent concentration of DOC decreases to 0.5 mg/L (approximately 20 bed volumes). An alternative procedure can also be used.[1]

c. Chromatography: Preclean column with three cycles of 0.1*N* NaOH and 0.1*N* HCl just before pumping sample into column. Leave column saturated with 0.1*N* HCl. Acidify sample to pH 2.0 with concentrated HCl, and pump it onto the column at rate of 1.0 mL/min. Save column effluent for DOC analysis. Significant concentrations of DOC in the effluent can indicate that the column was overloaded and that a smaller sample volume should be used. Colored organic acids adsorb to the top of the column. Back-elute (reverse flow) the column with 0.1*N* NaOH at 0.2 mL/min and collect eluate in a graduated, conical test tube until it becomes colorless (about 2 mL). Acidify with conc H_3PO_4 to a pH of 2 or less (about 2 to 3 drops) and remove dissolved carbon dioxide (inorganic carbon) by purging with nitrogen for 10 min. Avoid exposure of alkaline samples to air (i.e. acidify immediately) to minimize contamination with CO_2. Determine volume and DOC of acidified column effluent.

After eluting and collecting AHS from the column with back-elution using 0.1*N* NaOH, continue rinsing with about 20 bed-volumes of the basic solution. Rinse with water for about 20 bed volumes. Repeat the triplicate acid/base column precleaning procedure described above, then reuse the column to analyze a replicate sample. Process two portions of water by the same procedure to serve as controls.

The XAD column may be reused to analyze subsequent samples and controls if the triplicate acid/base precleaning procedure is repeated immediately before analysis of each replicate. Replace the column if recovery is poor or the resin becomes discolored.

5. Calculation

Calculate the concentration of AHS as given in 5510B.5.

6. Precision and Bias

For seven single-operator analyses, the relative standard deviation of triplicate samples (about 10 mg/L as AHS) ranged from 0.9 to 20.7% with an average of 5.4% ($n = 7$).

For seven single-operator analyses, recoveries ranged from 15.1 to 71.0% with an average of 51.6% and a relative standard deviation of 35.1%.

7. Reference

1. Standley, L.J. & L.A. Kaplan. 1998. Isolation and analysis of lignin-derived phenols in aquatic humic substances: Improvements on the procedures. *Org. Geochem.* 28(11):689.

* Pump parts may be of stainless steel or TFE.

† XAD-8. XAD is a trademark of Rohm and Haas Co., Philadelphia, PA.

5520 OIL AND GREASE*

5520 A. Introduction

In the determination of oil and grease, an absolute quantity of a specific substance is not measured. Rather, groups of substances with similar physical characteristics are determined quantitatively on the basis of their common solubility in an organic extracting solvent. "Oil and grease" is defined as any material recovered as a substance soluble in the solvent. It includes other material extracted by the solvent from an acidified sample (such as sulfur compounds, certain organic dyes, and chlorophyll) and not volatilized during the test. The 12th Edition of *Standard Methods* prescribed the use of petroleum ether as the solvent for natural and treated waters and *n*-hexane for polluted waters. The 13th Edition added trichlorotrifluoroethane as an optional solvent for all sample types. In the 14th through the 17th Editions, only trichlorotrifluoroethane was specified. However, because of environmental problems associated with chlorofluorocarbons, an alternative solvent (80% *n*-hexane and 20% methyl-*tert*-butyl ether) was included for gravimetric methods in the 19th Edition. In the 20th Edition, trichlorotrifluoroethane was dropped from all gravimetric procedures (retained for 5520C, an infrared method), and replaced with *n*-hexane. Solvent-recovery techniques were included and solvent recycling was strongly recommended. In the methods given below, the 80% *n*-hexane and 20% methyl-*tert*-butyl ether solvent mix has been dropped from 5520B, D, and E, and an alternative to the liquid/liquid extraction procedure using solid-phase adsorbent disks is included.

It is important to understand that, unlike some constituents that represent distinct chemical elements, ions, compounds, or groups of compounds, oils and greases are defined by the method used for their determination. In detailed studies involving wastewaters and solid matrices, it was shown that *n*-hexane produced results statistically different from results produced by trichlorotrifluoroethane.[1,2] Trichlorotrifluoroethane may at some point be prohibited entirely by regulatory authorities. EPA currently recommends use of *n*-hexane in place of trichlorotrifluoroethane.[3]

The methods presented here are suitable for biological lipids and mineral hydrocarbons. They also may be suitable for most industrial wastewaters or treated effluents containing these materials, although sample complexity may result in either low or high results because of lack of analytical specificity. The method is not applicable to measurement of low-boiling fractions that volatilize at temperatures below 85°C.

1. Significance

Certain constituents measured by the oil and grease analysis may influence wastewater treatment systems. If present in excessive amounts, they may interfere with aerobic and anaerobic biological processes and lead to decreased wastewater treatment efficiency. When discharged in wastewater or treated effluents, they may cause surface films and shoreline deposits leading to environmental degradation.

A knowledge of the quantity of oil and grease present is helpful in proper design and operation of wastewater treatment systems and also may call attention to certain treatment difficulties.

In the absence of specially modified industrial products, oil and grease has two primary components: fatty matter from animal and vegetable sources and hydrocarbons of petroleum origin. The portion of oil and grease from each of these two major components can be determined with 5520F. A knowledge of the relative composition of a sample minimizes the difficulty in determining the major source of the material and simplifies the correction of oil and grease problems in wastewater treatment plant operation and stream pollution abatement.

2. Selection of Method

For liquid samples, four methods are presented: the liquid/liquid partition-gravimetric method (5520B), the partition-infrared method (5520C), the Soxhlet method (5520D), and the solid-phase, partition-gravimetric method (5520G). Method 5520C is designed for samples that might contain volatile hydrocarbons that otherwise would be lost in the solvent-removal operations of the gravimetric procedure. Method 5520D is the method of choice when relatively polar, heavy petroleum fractions are present, or when the levels of nonvolatile greases may challenge the solubility limit of the solvent. For low levels of oil and grease (<10 mg/L), 5520C is the method of choice because gravimetric methods do not provide the needed sensitivity. Method 5520G can be used as an alternate to 5520B to reduce solvent volumes and matrix problems.

Method 5520E is a modification of the Soxhlet method and is suitable for sludges and similar materials. Method 5520F can be used with 5520B, C, D, or G to obtain a hydrocarbon measurement in addition to, or instead of, the oil and grease measurement. This method makes use of silica gel to separate petroleum hydrocarbons from the total oil and grease on the basis of polarity.

3. Sample Collection, Preservation, and Storage

Collect a representative grab sample in a wide-mouth glass bottle that has been washed with soap, rinsed with water, and finally rinsed with solvent to remove any residues that might interfere with the analysis. As an alternative to solvent rinsing, cap bottle with aluminum foil and bake at 200 to 250°C for at least 1 h. Use PTFE-lined caps for sample bottles; clean liners as above, but limit temperature to 110 to 200°C. Collect a separate sample for an oil and grease determination. Do not overfill the sample container and do not subdivide the sample in the laboratory. Collect replicate samples for replicate analyses or known-addition QA checks. Collect replicates either in rapid succession, in parallel, or in one large container with mechanical stirring (in the latter case, siphon individual portions). Typically, collect wastewater samples of approx-

* Approved by Standard Methods Committee, 2001. Editorial revisions, 2011. Joint Task Group: 21st Edition—John Gute (chair), Jennifer R. Calles, Lavern V. Tamoria.

https://doi.org/10.2105/SMWW.2882.107

imately 1 L. If sample concentration is expected to be greater than 1000 mg extractable material/L, collect proportionately smaller volumes. If analysis is to be delayed for more than 2 h, acidify to pH 2 or lower with either 1:1 HCl or 1:1 H_2SO_4 and refrigerate. When information is required about average grease concentration over an extended period, examine individual portions collected at prescribed time intervals to eliminate losses of grease on sampling equipment during collection of a composite sample.

In sampling sludges, take every possible precaution to obtain a representative sample. When analysis cannot be made within 2 h, preserve samples with 1 mL conc HCl/80 g sample and refrigerate. Never preserve samples with $CHCl_3$ or sodium benzoate.

4. Interferences

a. Organic solvents have the ability to dissolve not only oil and grease but also other organic substances. Any filterable solvent-soluble substances (e.g., elemental sulfur, complex aromatic compounds, hydrocarbon derivatives of chlorine, sulfur, and nitrogen, and certain organic dyes) that are extracted and recovered are defined as oil and grease. No known solvent will dissolve selectively only oil and grease. Heavier residuals of petroleum may contain a significant portion of materials that are not solvent-extractable. The method is entirely empirical; duplicate results with a high degree of precision can be obtained only by strict adherence to all details.

b. For 5520B, D, E, F, and G, solvent removal results in the loss of short-chain hydrocarbons and simple aromatics by volatilization. Significant portions of petroleum distillates from gasoline through No. 2 fuel oil are lost in this process. Adhere strictly to sample drying time, to standardize gradual loss of weight due to volatilization. For 5520B, D, E, F, and G, during the cooling of the distillation flask and extracted material, a gradual increase in weight may be observed, presumably due to the absorption of water if a desiccator is not used. For 5520C use of an infrared detector offers a degree of selectivity to overcome some coextracted interferences (¶ *a* above). For 5520D and E, use exactly the specified rate and time of extraction in the Soxhlet apparatus because of varying solubilities of different greases. For 5520F, the more polar hydrocarbons, such as complex aromatic compounds and hydrocarbon derivatives of chlorine, sulfur, and nitrogen, may be adsorbed by the silica gel. Extracted compounds other than hydrocarbons and fatty matter also interfere.

c. Alternative techniques may be needed for some samples if intractable emulsions form that cannot be broken by centrifugation. Such samples may include effluents from pulp/paper processing and zeolite manufacturing. Determine such modifications on a case-by-case basis.

d. Some sample matrices can increase the amount of water partitioned into the organic extraction fluid. When the extraction solvent from this type of sample is dried with sodium sulfate, the drying capacity of the sodium sulfate can be exceeded, thus allowing sodium sulfate to dissolve and pass into the tared flask. After drying, sodium sulfate crystals will be visible in the flask. The sodium sulfate that passes into the flask becomes a positive interference in gravimetric methods. If crystals are observed in the tared flask after drying, redissolve any oil and grease with 30 mL of extraction solvent and drain the solvent through a funnel containing a solvent-rinsed filter paper into a clean, tared flask. Rinse the first flask twice more, combining all solvent in the new flask, and treat as an extracted sample.

e. Silica gel fines may give positive interferences in 5520F if they pass through the filter. Use filters with smaller pores if this occurs with a particular batch of silica gel.

f. Analyte recovery may be compromised unless efforts are made to ensure uniform sample passage through the solid-phase extraction disks used in 5520G.

5. References

1. U.S. Environmental Protection Agency. 1993. Preliminary Report of EPA Efforts to Replace Freon for the Determination of Oil and Grease, Rev. 1; EPA-821-R-93-001. Washington, D.C.
2. U.S. Environmental Protection Agency. 1995. Report of EPA Efforts to Replace Freon for the Determination of Oil and Grease and Total Petroleum Hydrocarbons: Phase II; EPA-821-R-95-003. Washington, D.C.
3. U.S. Environmental Protection Agency. 1998. Method 1664, Revision A: *n*-Hexane Extractable Material (HEM; Oil and Grease) and Silica Gel Treated *n*-Hexane Extractable Material (HEM; Non-polar material) by Extraction and Gravimetry; EPA-821-R-98-002. Washington, D.C.; 40 CFR Part 136, Table IB, Item 41 (July 1, 2000); *Fed. Reg.* 64:93, 26315.

5520 B. Liquid-Liquid, Partition-Gravimetric Method

1. General Discussion

Dissolved or emulsified oil and grease is extracted from water by intimate contact with an extracting solvent. Some extractables, especially unsaturated fats and fatty acids, oxidize readily; hence, special precautions regarding temperature and solvent vapor displacement are included to minimize this effect. Organic solvents shaken with some samples may form an emulsion that is very difficult to break. This method includes a means for handling such emulsions. Recovery of solvents is discussed. Solvent recovery can reduce both vapor emissions to the atmosphere and costs.

The quality control practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Separatory funnel, 2-L, with TFE* stopcock.
b. Distilling flask, 125-mL.
c. Liquid funnel, glass.
d. Filter paper, 11-cm diam.†

* Teflon, or equivalent.
† Whatman No. 40, or equivalent

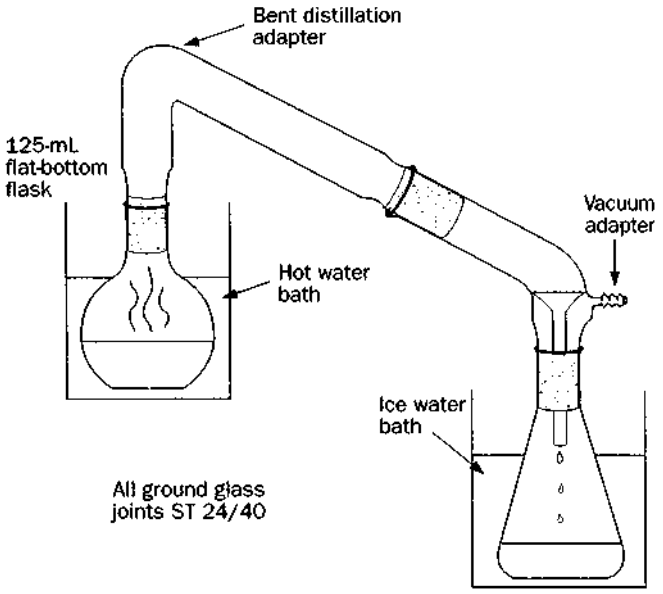


Figure 5520:1. Distillate recovery apparatus.

e. Centrifuge, capable of spinning at least four 100-mL glass centrifuge tubes at 2400 rpm or more.

f. Centrifuge tubes, 100-mL, glass.

g. Water bath, capable of maintaining 85°C.

h. Vacuum pump or other source of vacuum.

i. Distilling adapter with drip tip. Setup of distillate recovery apparatus is shown in Figure 5520:1. Alternatively, use commercially available solvent recovery equipment.

j. Ice bath.

k. Waste receptacle, for used solvent.

l. Desiccator.

3. Reagents

a. Hydrochloric or sulfuric acid, 1:1: Mix equal volumes of either acid and reagent water.

b. n-*Hexane*, 85% minimum purity, 99% minimum saturated C_6 isomers, residue less than 1 mg/L; distill if necessary. Do not use any plastic tubing to transfer solvent between containers.

c. Sodium sulfate (Na_2SO_4), anhydrous crystal. Dry at 200–250°C for 24 h.

d. Acetone, residue less than 1 mg/L.

e. Hexadecane, 98% minimum purity.

f. Stearic acid, 98% minimum purity.

g. Standard mixture, hexadecane/stearic acid 1:1 w/w, in acetone at 2 mg/mL each. Purchase prepared standard mixture, or prepare as follows.[1]

Place 200 ± 2 mg stearic acid and 200 ± 2 mg hexadecane in a 100-mL volumetric flask and fill to mark with acetone. NOTE: The solution may require warming for complete dissolution of stearic acid.

After the hexadecane and stearic acid have dissolved, transfer solution to a 100- to 150-mL vial with TFE-lined cap. Mark solution level on the vial and store in dark at room temperature.

Immediately before use, verify level on vial and bring to volume with acetone, if required. Warm to redissolve all visible precipitate. NOTE: If there is doubt of the concentration, remove 10.0 ± 0.1 mL with a volumetric pipet, place in a tared weighing pan, and evaporate to dryness in a fume hood. The weight must be 40 ± 1 mg. If not, prepare a fresh solution.

4. Procedure

When a sample is brought into the laboratory, either mark sample bottle at the water meniscus or weigh the bottle, for later determination of sample volume. If sample has not been acidified previously (see 5520A.3), acidify with either 1:1 HCl or 1:1 H_2SO_4 to pH 2 or lower (generally, 5 mL is sufficient for 1 L sample). Using liquid funnel, transfer sample to a separatory funnel. Carefully rinse sample bottle with 30 mL extracting solvent and add solvent washings to separatory funnel. Shake vigorously for 2 min. Let layers separate. Drain aqueous layer and small amount of organic layer into original sample container. Drain solvent layer through a funnel containing a filter paper and 10 g Na_2SO_4, both of which have been solvent-rinsed, into a clean, tared distilling flask containing boiling chips. If a clear solvent layer cannot be obtained and an emulsion of more than about 5 mL exists, drain emulsion and solvent layers into a glass centrifuge tube and centrifuge for 5 min at approximately 2400 rpm. Transfer centrifuged material to an appropriate separatory funnel and drain solvent layer through a funnel with a filter paper and 10 g Na_2SO_4, both of which have been prerinsed, into a clean, tared distilling flask. Recombine aqueous layers and any remaining emulsion or solids in separatory funnel. For samples with <5 mL of emulsion, drain only the clear solvent through a funnel with pre-moistened filter paper and 10 g Na_2SO_4. Recombine aqueous layers and any remaining emulsion or solids in separatory funnel. Extract twice more with 30 mL solvent each time, but first rinse sample container with each solvent portion. Repeat centrifugation step if emulsion persists in subsequent extraction steps. Combine extracts in tared distilling flask, and include in flask a final rinsing of filter and Na_2SO_4 with an additional 10 to 20 mL solvent. Distill solvent from flask in a water bath at 85°C. To maximize solvent recovery, fit distillation flask with a distillation adapter equipped with a drip tip and collect solvent in an ice-bath-cooled receiver (Figure 5520:1). When visible solvent condensation stops, replace bent distillation apparatus with vacuum/air adapter connected to vacuum source. Immediately draw air through flask with an applied vacuum for the final 1 min. Remove flask from bath and wipe outside surface to remove moisture. Cool in desiccator until a constant weight is obtained. To determine initial sample volume, either fill sample bottle to mark with water and then pour water into a 1-L graduated cylinder, or weigh empty container and cap and calculate the sample volume by difference from the initial weight (assuming a sample density of 1.00).

5. Calculation

Calculate oil and grease in sample as follows:

$$\text{mg oil and grease/L} = \frac{W_r}{V_s}$$

where:

W_r = total weight of flask and residue, minus tare weight of flask, mg, and
V_s = initial sample volume, L.

6. Precision and Bias

Method 5520B was tested in a single laboratory study and an interlaboratory method validation study.[1] Combined data from these studies yielded an average recovery of 93% and precision (as relative standard deviation) of 8.7%.

7. Reference

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1998. Method 1664, Revision A: *n*-Hexane Extractable Material (HEM; Oil and Grease) and Silica Gel Treated *n*-Hexane Extractable Material by Extraction and Gravimetry; EPA-821-R-98-002. 40 CFR Part 136 (July 1, 2000); *Fed. Reg.* 64(93):26315. Washington, D.C.

8. Bibliography

KIRSCHMAN, H.D. & R. POMEROY. 1949. Determination of oil in oil field waste waters. *Anal. Chem.* 21:793.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1995. Report of the Method 1664 Validation Studies; EPA-821-R-95-036. Washington, D.C.

5520 C. Partition-Infrared Method

1. General Discussion

a. Principle: The use of trichlorotrifluoroethane as extraction solvent allows absorbance of the carbon-hydrogen bond in the infrared to be used to measure oil and grease. Elimination of the evaporation step permits infrared detection of many relatively volatile hydrocarbons. Thus, the lighter petroleum distillates, with the exception of gasoline, may be measured accurately. With adequate instrumentation, as little as 0.2 mg oil and grease/L can be measured.

b. Terminology: A "known oil" is defined as a sample of oil and/or grease that represents the only material of that type used or manufactured in the processes represented by a wastewater. An "unknown oil" is defined as one for which a representative sample of the oil or grease is not available for preparation of a standard.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Separatory funnel, 2-L, with TFE* stopcock.
b. Volumetric flask, 100-mL.
c. Liquid funnel, glass.
d. Filter paper, 11-cm diam.†
e. Centrifuge, capable of spinning at least four 100-mL glass centrifuge tubes at 2400 rpm or more.
f. Centrifuge tubes, 100-mL, glass.
g. Infrared spectrophotometer, double-beam, recording.
h. Cells, near-infrared silica.

3. Reagents

a. Hydrochloric acid (HCl), 1 + 1.

b. Trichlorotrifluoroethane (1,1,2-trichloro-1,2,2-trifluoroethane), boiling point 47°C. The solvent should leave no measurable residue on evaporation; distill if necessary. Do not use any plastic tubing to transfer solvent between containers.

c. Sodium sulfate (Na_2SO_4), anhydrous, crystal. Dry at 200 to 250°C for 24 h.

d. Reference oil: Prepare a mixture, by volume, of 37.5% isooctane, 37.5% hexadecane, and 25.0% benzene. Store in sealed container to prevent evaporation.

4. Procedure

Refer to 5520B.4 for sample handling and for method of dealing with sample emulsions. After carefully transferring sample to a separatory funnel, rinse sample bottle with 30 mL trichlorotrifluoroethane and add solvent washings to funnel. Shake vigorously for 2 min. Let layers separate. Drain all but a very small portion of the lower trichlorotrifluoroethane layer through a funnel containing a filter paper and 10 g Na_2SO_4, both of which have been solvent-rinsed, into a clean, 100-mL volumetric flask. If a clear solvent layer cannot be obtained and an emulsion of more than about 5 mL exists, see 5520B.4. Extract twice more with 30 mL solvent each time, but first rinse sample container with each solvent portion. Repeat centrifugation step if emulsion persists in subsequent extraction steps. Combine extracts in volumetric flask, and include in flask a final rinsing of filter and Na_2SO_4 with an additional 10 to 20 mL solvent. Adjust final volume to 100 mL with solvent.

Prepare a stock solution of known oil by rapidly transferring about 1 mL (0.5 to 1.0 g) of the oil or grease to a tared 100-mL volumetric flask. Stopper flask and weigh to nearest milligram. Add solvent to dissolve and dilute to mark. If the oil identity is unknown (5520C.1*b*) use the reference oil (5520C.3*d*) as the standard. Using volumetric techniques, prepare a series of standards over the range of interest. Select a pair of matched near-infrared silica cells. A 1-cm-path-length cell is appropriate for a working range of about 4 to 40 mg. Scan standards and samples from 3200 cm^{-1} to 2700 cm^{-1} with solvent in the reference beam and record results on absorbance paper. Measure absorbances of samples and standards by constructing a straight base line over the scan range and measuring absorbance of the peak maximum at 2930 cm^{-1} and subtracting baseline absorbance at that point. If the absorbance exceeds 0.8 for a sample, select a shorter path length or dilute as required. Use scans of standards to prepare a calibration curve.

* Teflon, or equivalent.
† Whatman No. 40, or equivalent.

5. Calculation

$$\text{mg oil and grease/L} = \frac{A \times 1000}{\text{mL sample}}$$

where:

A = mg of oil or grease in extract as determined from calibration curve.

6. Precision and Bias

Method C was used by a single laboratory to test a wastewater sample. By this method the oil and grease concentration was 17.5 mg/L. When 1-L sample portions were dosed with 14.0 mg of a mixture of No. 2 fuel oil and Wesson oil, the recovery of added oils was 99% with a standard deviation of 1.4 mg.

7. Bibliography

GRUENFELD, M. 1973. Extraction of dispersed oils from water for quantitative analysis by infrared spectrophotometry. *Environ. Sci. Technol.* 7:636.

5520 D. Soxhlet Extraction Method

1. General Discussion

Soluble metallic soaps are hydrolyzed by acidification. Any oils and solid or viscous grease present are separated from the liquid samples by filtration. After extraction in a Soxhlet apparatus with solvent, the residue remaining after solvent evaporation is weighed to determine the oil and grease content. Compounds volatilized at or below 103°C will be lost when the filter is dried.

The quality control practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Extraction apparatus, Soxhlet, with 125-mL extraction flask.
b. Extraction thimble, paper, solvent-extracted.
c. Electric heating mantle.
d. Vacuum pump or other source of vacuum.
e. Vacuum filtration apparatus.
f. Buchner funnel, 12-cm.
g. Filter paper, 11-cm diam.*
h. Muslin cloth disks, 11-cm diam, solvent-extracted.
i. Glass beads or glass wool, solvent-extracted.
j. Water bath, capable of maintaining 85°C.
k. Distilling adapter with drip tip. See 5520B.2*i* and Figure 5520:1.
l. Ice bath.
m. Waste receptacle, for used solvent.
n. Desiccator.

3. Reagents

a. Hydrochloric acid (HCl), 1 + 1.
b. n-*Hexane:* See 5520B.3*b*.
c. Diatomaceous-silica filter aid suspension,† 10 g/L distilled water.

4. Procedure

When sample is brought into the laboratory, either mark sample bottle at the meniscus or weigh bottle for later determination of volume. If sample has not been acidified previously (see 5520A.3), acidify with 1:1 HCl or 1:1 H_2SO_4 to pH 2 or lower (generally, 5 mL is sufficient). Prepare filter consisting of a muslin cloth disk overlaid with filter paper. Wet paper and muslin and press down edges of paper. Using vacuum, pass 100 mL filter aid suspension through prepared filter and wash with 1 L distilled water. Apply vacuum until no more water passes filter. Filter acidified sample. Apply vacuum until no more water passes through filter. Using forceps, transfer entire filter to a watch glass. Add material adhering to edges of muslin cloth disk. Wipe sides and bottom of collecting vessel and Buchner funnel with pieces of filter paper soaked in extraction solvent, taking care to remove all films caused by grease and to collect all solid material. Add pieces of filter paper to material on watch glass. Roll all filter material containing sample and fit into an extraction thimble. Add any pieces of material remaining on watch glass. Wipe watch glass with a filter paper soaked in extraction solvent and place in extraction thimble. Dry filled thimble in a hot-air oven at 103°C for 30 min. Fill thimble with glass wool or small glass beads. Weigh extraction flask and add 100 mL extraction solvent (*n*-hexane, 5520B.3*b*). Extract oil and grease in a Soxhlet apparatus, at a rate of 20 cycles/h for 4 h. Time from first cycle. For stripping and recovery of solvent, cooling extraction flask before weighing, and determining initial sample volume, see 5520B.4.

5. Calculation

See 5520B.5.

6. Precision and Bias

In analyses of synthetic samples containing various amounts of Crisco and Shell S.A.E. No. 20 oil, an average recovery of 98.7% was obtained, with a standard deviation of 1.86%. Ten replicates each of two wastewater samples yielded standard deviations of 0.76 mg and 0.48 mg.

7. Bibliography

HATFIELD, W.D. & G.E. SYMONS. 1945. The determination of grease in sewage. *Sewage Works J.* 17:16.

* Whatman No. 40, or equivalent.
† Hyflo Super-Cel, Manville Corp., or equivalent.

GILCREAS, F.W., W.W. SANDERSON & R.P. ELMER. 1953. Two new methods for the determination of grease in sewage. *Sewage Ind. Wastes* 25:1379.

ULLMANN, W.W. & W.W. SANDERSON. 1959. A further study of methods for the determination of grease in sewage. *Sewage Ind. Wastes* 31:8.

5520 E. Extraction Method for Sludge Samples

1. General Discussion

Drying acidified sludge by heating leads to low results. Magnesium sulfate monohydrate is capable of combining with 75% of its own weight in water in forming $MgSO_4 \cdot 7H_2O$ and is used to dry sludge. After drying, the oil and grease can be extracted with *n*-hexane.

The quality control practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Beaker, 150-mL, glass.
b. Mortar and pestle, porcelain.
c. Extraction apparatus, Soxhlet.
d. Extraction thimble, paper, solvent-extracted.
e. Glass beads or glass wool, solvent-extracted.
f. Electric heating mantle.
g. Vacuum pump or other source of vacuum.
h. Liquid funnel, glass.
i. Grease-free cotton: Extract nonabsorbent cotton with solvent.
j. Water bath, capable of maintaining 85°C.
k. Distilling adapter with drip tip. See 5520B.2*i* and Figure 5520:1.
l. Ice bath.
m. Waste receptacle, for used solvent.
n. Desiccator.

3. Reagents

a. Hydrochloric acid (HCl), conc.
b. n-*Hexane:* See 5520B.3*b*.
c. Magnesium sulfate monohydrate: Prepare $MgSO_4 \cdot H_2O$ by drying a thin layer overnight in an oven at 150°C.

4. Procedure

When sample is brought into the laboratory, if it has not been acidified previously (5520A.3), add 1 mL conc HCl/80 g sample. In a 150-mL beaker weigh out a sample of wet sludge, 20 ± 0.5 g, for which the dry-solids content is known. Acidify to pH 2.0 or lower (generally, 0.3 mL conc HCl is sufficient). Add 25 g $MgSO_4 \cdot H_2O$. Stir to a smooth paste and spread on sides of beaker to facilitate subsequent sample removal. Let stand until solidified, 15 to 30 min. Remove solids and grind in a porcelain mortar. Add powder to a paper extraction thimble. Wipe beaker and mortar with small pieces of filter paper moistened with solvent and add to thimble. Fill thimble with glass wool or small glass beads. Tare extraction flask, and add 100 mL *n*-hexane. Extract in a Soxhlet apparatus at a rate of 20 cycles/h for 4 h. If any turbidity or suspended matter is present in the extraction flask, remove by filtering through grease-free cotton into another weighed flask. Rinse flask and cotton with solvent. For solvent stripping and recovery, and cooling the extraction flask before weighing, see 5520B.4.

5. Calculation

$$\text{Oil and grease as \% of dry solids} = \frac{\text{gain in weight of flask, g} \times 100}{\text{weight of wet solids, g} \times \text{dry solids fraction}}$$

6. Precision

The examination of six replicate samples of sludge yielded a standard deviation of 4.6%.

5520 F. Hydrocarbons

1. General Discussion

Silica gel has the ability to adsorb polar materials. If a solution of hydrocarbons and fatty materials in a nonpolar solvent is mixed with silica gel, the fatty acids are removed selectively from solution. The materials not eliminated by silica gel adsorption are designated hydrocarbons by this test.

The quality control practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Magnetic stirrer.
b. Magnetic stirring bars, TFE-coated.
c. Liquid funnel, glass.
d. Filter paper, 11-cm diam.*
e. Desiccator.

* Whatman No. 40, or equivalent.

3. Reagents

a. n-*Hexane:* See 5520B.3*b*.
b. Trichlorotrifluoroethane: See 5520C.3*b*.
c. Silica gel, 100 to 200 mesh.† Dry at 110°C for 24 h and store in a tightly sealed container.

4. Procedure

Use the oil and grease extracted by 5520B, C, D, E, or G for this test. When only hydrocarbons are of interest, introduce this procedure in any of the methods before final measurement. When hydrocarbons are to be determined after total oil and grease has been measured, redissolve the extracted oil and grease in trichlorotrifluoroethane (5520C) or 100 mL *n*-hexane (5520B, D, E, or G). To 100 mL solvent add 3.0 g silica gel/100 mg total oil and grease, up to a total of 30.0 g silica gel (1000 mg total oil and grease). For samples with more than 1000 mg total oil and grease use a measured volume of the 100 mL solvent dissolved sample, add appropriate amount of silica gel for amount of total oil and grease in the sample portion, and bring volume to 100 mL. Stopper container and stir on a magnetic stirrer for 5 min. For infrared measurement of hydrocarbons no further treatment is required before measurement as described in 5520C. For gravimetric determinations, filter solution through filter paper pre-moistened with solvent, wash silica gel and filter paper with 10 mL solvent, and combine with filtrate. For solvent stripping and recovery, and for cooling extraction flask before weighing, see 5520B.4.

† Davidson Grade 923, or equivalent.

5. Calculation

Calculate hydrocarbon concentration, in milligrams per liter, as in oil and grease (5520B, C, D, or G).

6. Precision and Bias

The following data, obtained on synthetic samples, are indicative for natural animal, vegetable, and mineral products, but cannot be applied to the specialized industrial products previously discussed.

For hydrocarbon determinations on 10 synthetic solvent extracts containing known amounts of a wide variety of petroleum products, average recovery was 97.2%. Similar synthetic extracts of Wesson oil, olive oil, Crisco, and butter gave 0.0% recovery as hydrocarbons measured by infrared analysis.

Using reagent water fortified with approximately 20 mg/L each of hexadecane and stearic acid, combined data from single-laboratory studies and an interlaboratory method validation study yielded an average recovery of 89% and precision (as relative standard deviation) of 13%.

7. Bibliography

U.S. Environmental Protection Agency. 1995. Report of the Method 1664 Validation Studies; EPA-821-R-95-036. Washington, D.C.

5520 G. Solid-Phase, Partition-Gravimetric Method

1. General Discussion

Dissolved or emulsified oil and grease is extracted from water by passing a sample through a solid-phase extraction (SPE) disk where the oil and grease are adsorbed by the disk and subsequently eluted with *n*-hexane. Some extractables, especially unsaturated fats and fatty acids, oxidize readily; hence, special precautions regarding temperature and solvent vapor displacement are provided. This method is not applicable to materials that volatilize at temperatures below 85°C, or crude and heavy fuel oils containing a significant percentage of material not soluble in *n*-hexane. This method may be a satisfactory alternative to liquid-liquid extraction techniques, especially for samples that tend to form difficult emulsions during the extraction step.[1]

The quality control practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. SPE extraction apparatus, 90 mm.*
b. Oil and grease SPE extraction disk, 90 mm.†
c. Distilling flask, 125 mL.
d. Water bath, capable of maintaining 85°C.
e. Vacuum pump or other source of vacuum.
f. Distilling adapter with drip tip. Setup of distillate recovery apparatus is shown in Figure 5520:1. Alternatively, use commercially available solvent recovery equipment.
g. Ice bath.
h. Waste receptacle, for used solvent.
i. Desiccator.

* SPE extraction apparatus is available from multiple vendors. Currently available equipment includes manual, semi-automated, and automated.
† SPE extraction disks are available from multiple vendors.

3. Reagents

a. Hydrochloric or sulfuric acid, 1:1: Mix equal volumes of either acid and reagent water.
b. n-*Hexane,* boiling point 69°C. The solvent should leave no measurable residue on evaporation; distill if necessary. Do not use any plastic tubing to transfer solvent between containers.
c. Sodium sulfate (Na_2SO_4), anhydrous crystal.
d. Acetone, residue less than 1 mg/L.
e. Methanol, ACS grade.
f. Hexadecane, 98% minimum purity.
g. Stearic acid, 98% minimum purity.
h. Standard mixture, hexadecane/stearic acid 1:1 w/w, in acetone at 2 mg/mL each.

4. Procedure

When a sample is brought into the laboratory, either mark sample bottle at water meniscus or weigh bottle, for later determination of sample volume. If sample has not been acidified previously (see 5520A.3), acidify with either 1:1 HCl or 1:1 H_2SO_4 to pH 2 or lower (generally, 5 mL is sufficient for 1 L sample). Assemble SPE extraction apparatus with oil and grease SPE disk installed and condition disk according to apparatus and disk manufacturers' instructions. Do not let disk dry before introducing sample for extraction. Conditioning is a critical step for uniform flow and good recovery. If the sample is high in suspended solids, decant liquid portion after allowing solids to settle. Extract sample at an optimum flow rate of 100 mL/min; faster extraction rates will reduce recoveries. After extraction, ensure that the disk dries fully before elution; however, use of an acetone rinse to aid in drying also will result in low recoveries. Carefully control vacuum during elution to let solvent fully penetrate the disk. A good-quality pump capable of controlling the vacuum from 20.3 cm (8 in.) Hg for elution to 63.5 cm (25 in.) Hg for disk drying will reduce variability of results. Pour sample into reservoir. If sample is high in suspended solids or sediment that may clog the disk, decant supernatant into reservoir first. Apply vacuum at an optimum flow rate of 100 mL/min and add sediment-laden residual from decanted sample to reservoir before allowing disk to go dry. After sample extraction is complete, apply full vacuum for 10 min to remove as much residual water as possible from disk and then change receivers. Rinse sample container with a portion of *n*-hexane and add solvent to reservoir, making sure that reservoir walls are rinsed in the process. Apply a slight vacuum to draw a few drops of *n*-hexane through disk, then release. Let solvent soak disk for 2 min. Collect eluent by applying enough vacuum to cause a slow stream of hexane to drip through disk. Let disk dry before repeating elution with a second portion of *n*-hexane. Pour combined eluent through a funnel containing a filter paper and 10 g Na_2SO_4, both of which have been solvent-rinsed, into a clean, tared distilling flask containing boiling chips. Rinse collection vial and filter containing Na_2SO_4 with *n*-hexane, and add to boiling flask. Distill solvent from flask in a water bath at 85°C. Fit distillation flask with a distillation adaptor equipped with a drip tip and collect solvent in an ice-bath-cooled receiver (Figure 5520:1); reduce volume, cool, and weigh to constant weight as described in 5520B.4.

5. Calculations

Calculate oil and grease in sample as follows:

$$\text{mg oil and grease/L} = \frac{W_r}{V_s}$$

where:

W_r = total weight of flask and residue, minus tare weight of flask, mg, and
V_s = initial sample volume, L.

6. Precision and Bias

In a comparison of laboratory-fortified reagent water samples (n = 46) with additions of approximately 20 mg/L each of hexadecane and stearic acid and analyzed by both automated and manual SPE systems, recoveries of 87.1 ± 7.8% and 92.5 ± 4.4% recovery on 10% fortified reagent water samples.‡

Precision for wastewaters may vary by matrix. In a single-laboratory testing replicate (n=4–6) domestic wastewater samples (8 different samples) in the 18- to 60-mg/L range, the CV averaged 9.0%. Another laboratory testing replicates (n = 4) of 16 different types of wastewater in the 4- to 3000-mg/L range reported CVs ranging from 1.1 to 57.1% for 19 wastewater types; pooled results from four laboratories showed an average CV of 17.0% ± 13.8%.‡

Single-laboratory control charts for matrix known additions analysis showed an average relative percent difference of 2.2% and an average percent recovery of 87.2% with a standard deviation of 5.5%.

Two laboratories were able to achieve an MDL of 1.4 mg/L, using either manual or semi-automated SPE apparatus.

7. Reference

1. U.S. Environmental Protection Agency. 1999. Method 1664, Revision A; EPA-821-R-98-002. Washington, D.C.

‡ Data provided by USEPA/DynCorp Systems from the public comment document on EPA Method 1664. Proposed Rule and Notice of Availability (62 *Fed. Reg.* 51621; FRL-5901-5).

5530 PHENOLS*

5530 A. Introduction

Phenols, defined as hydroxy derivatives of benzene and its condensed nuclei, may occur in domestic and industrial wastewaters, natural waters, and potable water supplies. Chlorination of such waters may produce odorous and objectionable-tasting chlorophenols. Phenol removal processes in water treatment include superchlorination, chlorine dioxide or chloramine treatment, ozonation, and activated carbon adsorption.

1. Selection of Method

The analytical procedures offered here use the 4-aminoantipyrine colorimetric method that determines phenol, ortho- and meta-substituted phenols, and, under proper pH conditions, those para-substituted phenols in which the substitution is a carboxyl, halogen, methoxyl, or sulfonic acid group. The 4-aminoantipyrine method does not determine those para-substituted phenols where the substitution is an alkyl, aryl, nitro, benzoyl, nitroso, or aldehyde group. A typical example of these latter groups is para-cresol, which may be present in certain industrial wastewaters and in polluted surface waters.

The 4-aminoantipyrine method is given in two forms: 5530C, for extreme sensitivity, is adaptable for use in water samples containing less than 1 mg phenol/L. It concentrates the color in a nonaqueous solution. Method 5530D retains the color in the aqueous solution. Because the relative amounts of various phenolic compounds in a given sample are unpredictable, it is not possible to provide a universal standard containing a mixture of phenols. For this reason, phenol (C_6H_5OH) itself has been selected as a standard for colorimetric procedures and any color produced by the reaction of other phenolic compounds is reported as phenol. Because substitution generally reduces response, this value represents the minimum concentration of phenolic compounds. A gas-liquid chromatographic procedure is included in Section 6420B and may be applied to samples or concentrates to quantify individual phenolic compounds.

2. Interferences

Interferences such as phenol-decomposing bacteria, oxidizing and reducing substances, and alkaline pH values are dealt with by acidification. Some highly contaminated wastewaters may require specialized techniques for eliminating interferences and for quantitative recovery of phenolic compounds.

Eliminate major interferences as follows (see 5530B for reagents):

Oxidizing agents, such as chlorine and those detected by the liberation of iodine on acidification in the presence of potassium iodide (KI)—Remove immediately after sampling by adding excess ferrous sulfate ($FeSO_4$). If oxidizing agents are not removed, the phenolic compounds will be oxidized partially.

Sulfur compounds—Remove by acidifying to pH 4.0 with H_3PO_4 and aerating briefly by stirring. This eliminates the interference of hydrogen sulfide (H_2S) and sulfur dioxide (SO_2).

Oils and tars—Make an alkaline extraction by adjusting to pH 12 to 12.5 with NaOH pellets. Extract oil and tar from aqueous solution with 50 mL chloroform ($CHCl_3$). Discard oil- or tar-containing layer. Remove excess $CHCl_3$ in aqueous layer by warming on a water bath before proceeding with the distillation step.

3. Sampling

Use glass containers for collecting phenol samples.

Sample in accordance with the instructions of Section 1060.

4. Preservation and Storage of Samples

Phenols in concentrations usually encountered in wastewaters are subject to biological and chemical oxidation. Preserve and store samples at 4°C or lower unless analyzed within 4 h after collection.

Acidify with 2 mL conc H_2SO_4/L.

Analyze preserved and stored samples within 28 d after collection.

5. Bibliography

ETTINGER, M.B., S. SCHOTT & C.C. RUCHHOFT. 1943. Preservation of phenol content in polluted river water samples previous to analysis. *J. Amer. Water Works Assoc.* 35:299.

CARTER, M.J. & M.T. HUSTON. 1978. Preservation of phenolic compounds in wastewaters. *Environ. Sci. Technol.* 12:309.

NEUFELD, R.D. & S.B. POLADINO. 1985. Comparison of 4-aminoantipyrine and gas-liquid chromatography techniques for analysis of phenolic compounds. *J. Water Pollut. Control Fed.* 57:1040.

* Approved by Standard Methods Committee, 2010.

5530 B. Cleanup Procedure

1. Principle

Phenols are distilled from nonvolatile impurities. Because the volatilization of phenols is gradual, the distillate volume must ultimately equal that of the original sample.

2. Apparatus

a. Distillation apparatus, all-glass, consisting of a 1-L borosilicate glass distilling apparatus with Graham condenser.*

b. pH meter.

* Corning No. 3360, or equivalent.

3. Reagents

Prepare all reagents with distilled water free of phenols and chlorine.

a. Phosphoric acid solution (H_3PO_4), 1 + 9: Dilute 10 mL 85% H_3PO_4 to 100 mL with water.

b. Methyl orange indicator solution.

c. Special reagents for turbid distillates:

1) *Sulfuric acid* (H_2SO_4), 1*N*.

2) *Sodium chloride* (NaCl).

3) *Chloroform* ($CHCl_3$) or *methylene chloride* (CH_2Cl_2).

4) *Sodium hydroxide* (NaOH), 2.5*N*—Dilute 41.7 mL 6*N* NaOH to 100 mL or dissolve 10 g NaOH pellets in 100 mL water.

4. Procedure

a. Measure 500 mL sample into a beaker, adjust pH to approximately 4.0 with H_3PO_4 solution using methyl orange indicator or a pH meter, and transfer to distillation apparatus. Use a 500-mL graduated cylinder as a receiver. Omit adding H_3PO_4 and adjust pH to 4.0 with 2.5*N* NaOH if sample was preserved as described in 5530A.4.

b. Distill 450 mL, stop distillation and, when boiling ceases, add 50 mL warm water to distilling flask. Continue distillation until a total of 500 mL has been collected.

c. One distillation should purify the sample adequately. Occasionally, however, the distillate is turbid. If so, acidify with H_3PO_4 solution and distill as described in ¶ *b* above. If second distillate is still turbid, use extraction process described in ¶ *d* below before distilling sample.

d. Treatment when second distillate is turbid: Extract a 500-mL portion of original sample as follows: Add 4 drops methyl orange indicator and make acidic to methyl orange with 1*N* H_2SO_4. Transfer to a separatory funnel and add 150 g NaCl. Shake with five successive portions of $CHCl_3$, using 40 mL in the first portion and 25 mL in each successive portion. Transfer $CHCl_3$ layer to a second separatory funnel and shake with three successive portions of 2.5*N* NaOH solution, using 4.0 mL in the first portion and 3.0 mL in each of the next two portions. Combine alkaline extracts, heat on a water bath until $CHCl_3$ has been removed, cool, and dilute to 500 mL with distilled water. Proceed with distillation as described in ¶s *a* and *b* above.

NOTE: CH_2Cl_2 may be used instead of $CHCl_3$, especially if an emulsion forms when the $CHCl_3$ solution is extracted with NaOH.

5530 C. Chloroform Extraction Method

1. General Discussion

a. Principle: Steam-distillable phenols react with 4-aminoantipyrine at pH 7.9 ± 0.1 in the presence of potassium ferricyanide to form a colored antipyrine dye. This dye is extracted from aqueous solution with $CHCl_3$ and the absorbance is measured at 460 nm. This method covers the phenol concentration range from 1.0 μg/L to over 250 μg/L with a sensitivity of 1 μg/L.

b. Interference: All interferences are eliminated or reduced to a minimum if the sample is preserved, stored, and distilled in accordance with the foregoing instructions.

c. Minimum detectable quantity: The minimum detectable quantity for clean samples containing no interferences is 0.5 μg phenol when a 25-mL $CHCl_3$ extraction with a 5-cm cell or a 50-mL $CHCl_3$ extraction with a 10-cm cell is used in the photometric measurement. This quantity is equivalent to 1 μg phenol/L in 500 mL distillate.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Photometric equipment: A spectrophotometer for use at 460 nm equipped with absorption cells providing light paths of 1 to 10 cm, depending on the absorbances of the colored solutions and the individual characteristics of the photometer.

b. Filter funnels: Buchner type with fritted disk.*

c. Filter paper: Alternatively use an appropriate 11-cm filter paper for filtering $CHCl_3$ extracts instead of the Buchner-type funnels and anhydrous Na_2SO_4.

d. pH meter.

e. Separatory funnels, 1000-mL, Squibb form, with ground-glass stoppers and TFE stopcocks. At least eight are required.

3. Reagents

Prepare all reagents with distilled water free of phenols and chlorine.

a. Stock phenol solution: Dissolve 100 mg phenol in freshly boiled and cooled distilled water and dilute to 100 mL. **CAUTION—Toxic; handle with extreme care.** Ordinarily this direct weighing yields a standard solution; if extreme accuracy is required, standardize as follows:

1) To 100 mL water in a 500-mL glass-stoppered conical flask, add 50.0 mL stock phenol solution and 10.0 mL bromate-bromide solution. Immediately add 5 mL conc HCl and swirl gently. If brown color of free bromine does not persist, add 10.0-mL portions of bromate-bromide solution until it does. Keep flask stoppered and let stand for 10 min; then add approximately 1 g KI. Usually four 10-mL portions of bromate-bromide solution are required if the stock phenol solution contains 1000 mg phenol/L.

2) Prepare a blank in exactly the same manner, using distilled water and 10.0 mL bromate-bromide solution. Titrate blank and sample with 0.025*M* sodium thiosulfate, using starch solution indicator.

* 15-mL Corning No. 36060, or equivalent.

3) Calculate the concentration of phenol solution as follows:

$$\text{mg phenol/L} = 7.842\,[(A \times B) - C]$$

where:

A = mL thiosulfate for blank,
B = mL bromate-bromide solution used for sample divided by 10, and
C = mL thiosulfate used for sample.

b. Intermediate phenol solution: Dilute 1.00 mL stock phenol solution in freshly boiled and cooled distilled water to 100 mL; 1 mL = 10.0 μg phenol. Prepare daily.

c. Standard phenol solution: Dilute 50.0 mL intermediate phenol solution to 500 mL with freshly boiled and cooled distilled water; 1 mL = 1.0 μg phenol. Prepare within 2 h of use.

d. Bromate-bromide solution: Dissolve 2.784 g anhydrous $KBrO_3$ in water, add 10 g KBr crystals, dissolve, and dilute to 1000 mL.

e. Hydrochloric acid (HCl), conc.

f. Standard sodium thiosulfate titrant, 0.025*M:* See Section 4500-O.C.2*e*.

g. Starch solution: See Section 4500-O.C.2*d*.

h. Ammonium hydroxide (NH_4OH), 0.5*N:* Dilute 35 mL fresh, conc NH_4OH to 1 L with water.

i. Phosphate buffer solution: Dissolve 104.5 g K_2HPO_4 and 72.3 g KH_2PO_4 in water and dilute to 1 L. The pH should be 6.8.

j. 4-Aminoantipyrine solution: Dissolve 2.0 g 4-aminoantipyrine in water and dilute to 100 mL. Prepare daily.

k. Potassium ferricyanide solution: Dissolve 8.0 g $K_3Fe(CN)_6$ in water and dilute to 100 mL. Filter if necessary. Store in a brown glass bottle. Prepare fresh weekly.

l. Chloroform ($CHCl_3$).

m. Sodium sulfate, anhydrous Na_2SO_4, granular.

n. Potassium iodide (KI), crystals.

4. Procedure

Ordinarily, use Procedure *a* below; however, Procedure *b* below may be used for infrequent non-regulatory analyses.

a. Place 500 mL distillate, or a suitable portion containing not more than 50 μg phenol, diluted to 500 mL, in a 1-L beaker. Prepare a 500-mL distilled water blank and a series of 500-mL phenol standards containing 5, 10, 20, 30, 40, and 50 μg phenol.

Treat sample, blank, and standards as follows: Add 12.0 mL 0.5*N* NH_4OH and *immediately* adjust pH to 7.9 ± 0.1 with phosphate buffer. Under some circumstances, a higher pH may be required.† About 10 mL phosphate buffer are required. Transfer to a 1-L separatory funnel, add 3.0 mL aminoantipyrine solution, mix well, add 3.0 mL $K_3Fe(CN)_6$ solution, mix well, and let color develop for 15 min. The solution should be clear and light yellow.

Extract immediately with $CHCl_3$, using 25 mL for 1- to 5-cm cells and 50 mL for a 10-cm cell. Shake separatory funnel at least 10 times, let $CHCl_3$ settle, shake again 10 times, and let $CHCl_3$ settle again. Filter each $CHCl_3$ extract through filter paper or fritted glass funnels containing a 5-g layer of anhydrous Na_2SO_4. Collect dried extracts in clean cells for absorbance measurements; do not add more $CHCl_3$ or wash filter papers or funnels with $CHCl_3$.

Read absorbance of sample and standards against the blank at 460 nm. Plot absorbance against micrograms phenol concentration. Construct a separate calibration curve for each photometer and check each curve periodically to ensure reproducibility.

b. For infrequent non-regulatory analyses prepare only one standard phenol solution. Prepare 500 mL standard phenol solution of a strength approximately equal to the phenolic content of that portion of original sample used for final analysis. Also prepare a 500-mL distilled water blank.

Continue as described in ¶ *a* above, but measure absorbances of sample and standard phenol solution against the blank at 460 nm.

5. Calculation

a. For 5530C.4*a:*

$$\mu\text{g phenol/L} = \frac{A}{B} \times 1000$$

where:

A = μg phenol in sample, from calibration curve, and
B = mL original sample.

b. For 5530C.4*b,* calculate the phenol content of the original sample:

$$\mu\text{g phenol/L} = \frac{C \times D \times 1000}{E \times B}$$

where:

C = μg standard phenol solution,
D = absorbance reading of sample,
E = absorbance of standard phenol solution, and
B = mL original sample.

6. Precision and Bias

Because the "phenol" value is based on C_6H_5OH, this method yields only an approximation and represents the minimum amount of phenols present. This is true because the phenolic reactivity to 4-aminoantipyrine varies with the types of phenols present.

In a study of 40 refinery wastewaters analyzed in duplicate at concentrations from 0.02 to 6.4 mg/L the average relative standard deviation was ±12%. Data are not available for precision at lower concentrations.

7. Bibliography

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† For NPDES permit analyses, pH 10 ± 0.1 is required.

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5530 D. Direct Photometric Method

1. General Discussion

a. Principle: Steam-distillable phenolic compounds react with 4-aminoantipyrine at pH 7.9 ± 0.1 in the presence of potassium ferricyanide to form a colored antipyrine dye. This dye is kept in aqueous solution and the absorbance is measured at 500 nm.

b. Interference: Interferences are eliminated or reduced to a minimum by using the distillate from the preliminary distillation procedure.

c. Minimum detectable quantity: This method has less sensitivity than 5530C. The minimum detectable quantity is 10 μg phenol when a 5-cm cell and 100 mL distillate are used.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Photometric equipment: Spectrophotometer equipped with absorption cells providing light paths of 1 to 5 cm for use at 500 nm.

b. pH meter.

3. Reagents

See 5530C.3.

4. Procedure

Place 100 mL distillate, or a portion containing not more than 0.5 mg phenol diluted to 100 mL, in a 250-mL beaker. Prepare a 100-mL distilled water blank and a series of 100-mL phenol standards containing 0.1, 0.2, 0.3, 0.4, and 0.5 mg phenol. Treat sample, blank, and standards as follows: Add 2.5 mL 0.5*N* NH_4OH solution and immediately adjust to pH 7.9 ± 0.1 with phosphate buffer. Add 1.0 mL 4-aminoantipyrine solution, mix well, add 1.0 mL $K_3Fe(CN)_6$ solution, and mix well.

After 15 min, transfer to cells and read absorbance of sample and standards against the blank at 500 nm.

5. Calculation

a. Use of calibration curve: Estimate sample phenol content from photometric readings by using a calibration curve constructed as directed in 5530C.4*a*.

$$\text{mg phenol/L} = \frac{A}{B} \times 1000$$

where:

A = mg phenol in sample, from calibration curve, and
B = mL original sample.

b. Use of single phenol standard:

$$\text{mg phenol/L} = \frac{C \times D \times 1000}{E \times B}$$

where:

C = mg standard phenol solution,
D = absorbance of sample, and
E = absorbance of standard phenol solution.

6. Precision and Bias

Precision and bias data are not available.

5540 SURFACTANTS*

5540 A. Introduction

1. Occurrence and Significance

Surfactants enter waters and wastewaters mainly by discharge of aqueous wastes from household and industrial laundering and other cleansing operations. A surfactant combines in a single molecule a strongly hydrophobic group with a strongly hydrophilic one. Such molecules tend to congregate at the interfaces between the aqueous medium and the other phases of the system such as air, oily liquids, and particles, thus imparting properties such as foaming, emulsification, and particle suspension.

The surfactant hydrophobic group generally is a hydrocarbon radical (R) containing about 10 to 20 carbon atoms. The hydrophilic groups are of two types, those that ionize in water and those that do not. Ionic surfactants are subdivided into two categories, differentiated by the charge. An anionic surfactant ion is negatively charged [e.g., $(RSO_3)^-Na^+$], and a cationic one is positively charged [e.g., $(RMe_3N)^+Cl^-$]. Nonionizing (nonionic) surfactants commonly contain a polyoxyethylene hydrophilic group ($ROCH_2CH_2OCH_2CH_2.....OCH_2CH_2OH$, often abbreviated RE_n, where n is the average number of $-OCH_2CH_2-$ units in the hydrophilic group). Hybrids of these types exist also.

In the United States, ionic surfactants amount to about two thirds of the total surfactants used and nonionics to about one third. Cationic surfactants amount to less than one tenth of the ionics and are used generally for disinfecting, fabric softening, and various cosmetic purposes rather than for their detersive properties. At current detergent and water usage levels the surfactant content of raw domestic wastewater is in the range of about 1 to 20 mg/L. Most domestic wastewater surfactants are dissolved in equilibrium with proportional amounts adsorbed on particulates. Primary sludge concentrations range from 1 to 20 mg adsorbed anionic surfactant per gram dry weight.[1] In environmental waters the surfactant concentration generally is below 0.1 mg/L except in the vicinity of an outfall or other point source of entry.[2]

2. Analytical Precautions

Because of inherent properties of surfactants, special analytical precautions are necessary. Avoid foam formation because the surfactant concentration is higher in the foam phase than in the associated bulk aqueous phase and the latter may be significantly depleted. If foam is formed, let it subside by standing, or collapse it by other appropriate means, and remix the liquid phase before sampling. Adsorption of surfactant from aqueous solutions onto the walls of containers, when concentrations below about 1 mg/L are present, may seriously deplete the bulk aqueous phase. Minimize adsorption errors, if necessary, by rinsing container with sample, and for anionic surfactants by adding alkali phosphate (e.g., 0.03*N* KH_2PO_4).[3]

3. References

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* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011.

5540 B. Surfactant Separation by Sublation

1. General Discussion

a. Principle: The sublation process isolates the surfactant, regardless of type, from dilute aqueous solution, and yields a dried residue relatively free of nonsurfactant substances. It is accomplished by bubbling a stream of nitrogen up through a column containing the sample and an overlying layer of ethyl acetate. The surfactant is adsorbed at the water-gas interfaces of the bubbles and is carried into the ethyl acetate layer. The bubbles escape into the atmosphere leaving behind the surfactant dissolved in ethyl acetate. The solvent is separated, dehydrated, and evaporated, leaving the surfactant as a residue suitable for analysis. This procedure is the same as that used by the Organization for Economic Co-operation and Development (OECD),[1] following the development by Wickbold.[2,3]

b. Interferences: The sublation method is specific for surfactants, because any substance preferentially adsorbed at the water-gas interface is by definition a surfactant. Although nonsurfactant substances largely are rejected in this separation process, some amounts will be carried over mechanically into the ethyl acetate.

c. Limitations: The sublation process separates only dissolved surfactants. If particulate matter is present it holds back an equilibrium amount of adsorbed surfactant. As sublation removes the initially dissolved surfactant, the particulates tend to reequilibrate and their adsorbed surfactants redissolve. Thus, continued sublation eventually should remove substantially all adsorbed surfactant. However, if the particulates adsorb the surfactant tightly, as sewage particulates usually do, complete removal may take a very long time. The procedure given herein calls for preliminary filtration and measures only dissolved surfactant. Determine adsorbed surfactant

content by analyzing particulates removed by filtration; no standard method is available now.

d. Operating conditions: Make successive 5-min sublations from 1 L of sample containing 5 g $NaHCO_3$ and 100 g NaCl. Under the conditions specified, extensive transfer of surfactant occurs in the first sublation and is substantially complete in the second.[2–4]

e. Quantitation: Quantitate the surfactant residue by the procedures in 5540C or D. Direct weighing of the residue is not useful because the weight of surfactant isolated generally is too low, less than a milligram, and varied amounts of mechanically entrained nonsurfactants may be present. The procedure is applicable to water and wastewater samples.

f. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Sublator: A glass column with dimensions as shown in Figure 5540:1.* For the sintered glass disk use a coarse-porosity frit (designation "c"–nominal maximum pore diam 40 to 60 μm as measured by ASTM E-128) of the same diameter as the column internal diameter. Volume between disk and upper stopcock should be approximately 1 L.

F1

b. Gas washing bottle, as indicated in Figure 5540:1, working volume 100 mL or more.

c. Separatory funnel, working volume 250 mL, preferably with inert TFE stopcock.

d. Filtration equipment, suitable for 1-L samples, using medium-porosity qualitative-grade filter paper.

e. Gas flowmeter, for measuring flows up to 1 L/min.

3. Reagents

a. Nitrogen, standard commercial grade.

b. Ethyl acetate: **Caution: Ethyl acetate is flammable and its vapors can form explosive mixtures with air.**

c. Sodium bicarbonate ($NaHCO_3$).

d. Sodium chloride (NaCl).

e. Water, surfactant-free.

4. Procedure

a. Sample size: Select a sample to contain not more than 1 to 2 mg surfactant.[4] For most waters the sample volume will be about 1 L; for wastewater use a smaller volume.

b. Filtration: Filter sample through medium-porosity qualitative filter paper. Wash filter paper by discarding the first few hundred milliliters of filtrate.

c. Assembly: Refer to Figure 5540:1.

Connect nitrogen cylinder through flowmeter to inlet of gas washing bottle. Connect gas outlet at top of sublator to a gas scrubber or other means for disposing of ethyl acetate vapor (e.g., vent to a hood or directly outdoors). In the absence of a flowmeter, ensure proper gas flow rate by measuring volume of gas leaving the sublator, with a water-displacement system.

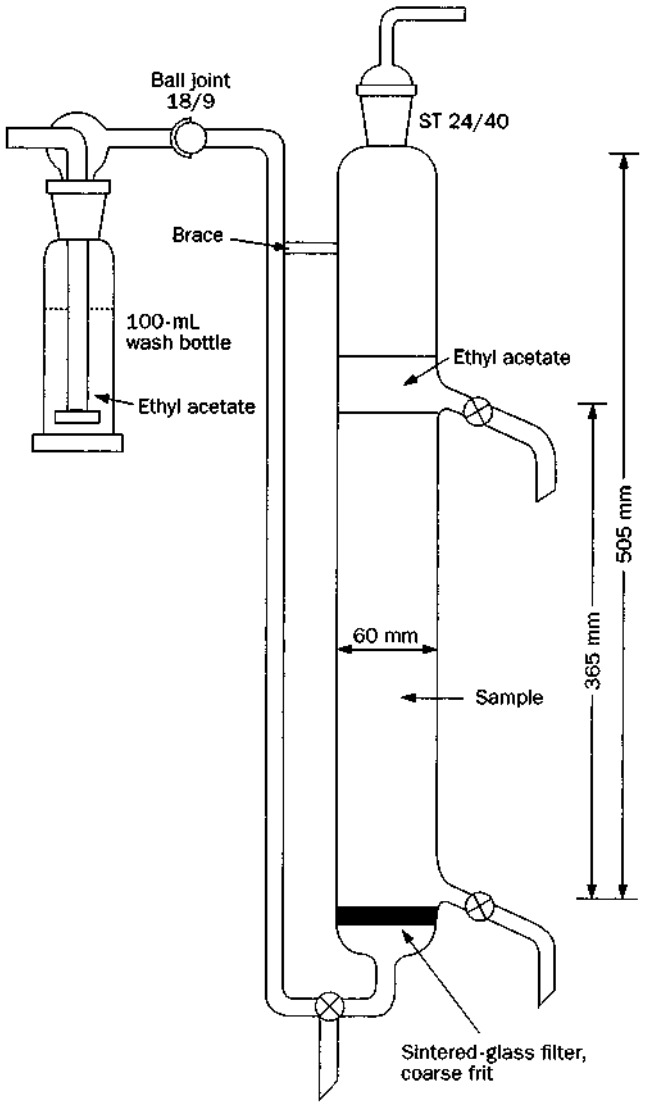


Figure 5540:1. Sublation apparatus.[1] See 5540B.2*a* and *b* and 4*c*. Bottom stopcock: TFE plug, 4-mm bore; side stopcocks: TFE plug, 2-mm bore.

d. Charging: Fill gas washing bottle about two-thirds full with ethyl acetate. Rinse sublation column with ethyl acetate and discard rinse. Place measured filtered sample in sublator and add 5 g $NaHCO_3$, 100 g NaCl, and sufficient water to bring the level up to or slightly above the upper stopcock (about 1 L total volume). If sample volume permits, add salts as a solution in 400 mL water or dissolve them in the sample and quantitatively transfer to the sublator. Add 100 mL ethyl acetate by running it carefully down the wall of the sublator to form a layer on top of the sample.

e. Sublation: Start the nitrogen flow, increasing the rate carefully to 1 L/min initially but do not exceed a rate at which the liquid phases begin vigorous intermixing at their interface. Avoid overly vigorous intermixing, which will lead to back-extraction of the surfactant into the aqueous phase and to dissolution of ethyl acetate. Continue sublation for 5 min at

* This apparatus is available from Kontes at kontes.com; Cal Glass, Costa Mesa, CA; or similar suppliers.

1 L/min. If a lower flow rate is necessary to avoid phase intermixing, prolong sublation time proportionally. If the volume of the upper phase has decreased by more than about 20%, repeat the operation on a new sample but avoid excessive intermixing at the interface. Draw off entire ethyl acetate layer through upper stopcock into the separatory funnel; return any transferred water layer to the sublator. Filter ethyl acetate layer into a 250-mL beaker through a dry, medium-porosity, qualitative filter paper (prewashed with ethyl acetate to remove any adventitious surfactant) to remove any remaining aqueous phase.

Repeat process of preceding paragraph with a second 100-mL layer of ethyl acetate, using the same separatory funnel and filter, and finally rinse sublator wall with another 20 mL, all into the original beaker.

Evaporate ethyl acetate from the beaker on a steam bath in a hood, blowing a gentle stream of nitrogen or air over the liquid surface to speed evaporation and to minimize active boiling. Evaporate the first 100 mL during the second sublation to avoid overfilling the beaker. To avoid possible solute volatilization, discontinue heating after removing the ethyl acetate. The sublated surfactant remains in the beaker as a film of residue.

Draw off aqueous layer in the sublator and discard, using the stopcock just above the sintered disk to minimize disk fouling.

5. Precision and Bias

Estimates of the efficiency of surfactant transfer and recovery in the sublation process include the uncertainties of the analytical methods used in quantitating the surfactant. At present the analytical methods are semiquantitative for surfactant at levels below 1 mg/L in environmental samples.

With various known surfactants at 0.2 to 2 mg/L and appropriate analytical methods, over 90% of added surfactant was recovered in one 5-min sublation from 10% NaCl. Without NaCl, recovery of nonionics was over 90% but recovery of anionics and cationics was only 2 to 25%.[4]

Five laboratories studied the recovery of five anionic surfactant types from concentrations of 0.05, 0.2, 1.0, and 5.0 mg/L in aqueous solutions.[5] The amount in each solution was determined directly by methylene blue analysis and compared with the amount recovered in the sublation process, also analyzed by methylene blue. The overall average recovery was 95.9% with a standard deviation of ±7.4 (n = 100). The extreme individual values for recovery were 65% and 115% and the other 98 values ranged from 75% to 109%. Recovery did not depend on surfactant concentration (average recoveries ranging from 94.7% at 5.0 mg/L to 96.8% at 1.0 mg/L) nor on the surfactant type (average recoveries ranging from 94.7% to 96.6%). Average recoveries at the five laboratories ranged from 90.0% to 98.0%.

Table 5540:I. Surfactant Recovery by Sublation

Variable	MBAS	CTAS
Sample volume, mL	200–300	500
Concentration without sublation, mg/L	2.2–4.7	—
Concentration found in sublate,* mg/L	1.8–4.4	0.3–0.6
Recovery in sublate, %	87 ± 16†	—
Amount in second sublate,‡ mg	0.02 ± 0.02†	0.08 ± 0.01†
Amount added, mg	0.05–0.10§	0.50–0.67‖
Recovery in sublation,# %	94 ± 17†	92 ± 6†

* Two 5-min sublations.
† Average ± SD (n = 8).
‡ Two more 5-min sublations.
§ Reference LAS.
‖ Linear alcohol ethoxylate $C_{12-18}E_{11}$.
Fifth and sixth 5-min sublations.

Application of the sublation method in three laboratories to eight different samples of raw wastewater in duplicate gave the results shown in Table 5540:I. Methylene blue active substances (MBAS) recovery in double sublation averaged 87 ± 16% of that determined directly on the filtered wastewater; these results would have been influenced by any nonsurfactant MBAS that might have been present. Repeating double sublation on the spent aqueous phase yielded another 0.02 mg MBAS and another 0.08 mg cobalt thiocyanate active substances (CTAS). Adding 0.05 to 0.10 mg of known linear alkylbenzene sulfonate (LAS) or 0.50 to 0.67 mg of known linear alcohol-based $C_{12-18}E_{11}$ to the same sublator contents and again running double sublation resulted in over 90% recovery of the amount added.

6. References

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5540 C. Anionic Surfactants as MBAS

1. General Discussion

a. Definition and principle: Methylene blue active substances (MBAS) bring about the transfer of methylene blue, a cationic dye, from an aqueous solution into an immiscible organic liquid upon equilibration. This occurs through ion pair formation by the MBAS anion and the methylene blue cation. The intensity of the resulting blue color in the organic phase is a measure of

MBAS. Anionic surfactants are among the most prominent of many substances, natural and synthetic, showing methylene blue activity. The MBAS method is useful for estimating the anionic surfactant content of waters and wastewaters, but the possible presence of other types of MBAS always must be kept in mind.

This method is relatively simple and precise. It comprises three successive extractions from acid aqueous medium containing excess methylene blue into chloroform ($CHCl_3$), followed by an aqueous backwash and measurement of the blue color in the $CHCl_3$ by spectrophotometry at 652 nm. The method is applicable at MBAS concentrations down to about 0.025 mg/L.

b. Anionic surfactant responses: Soaps do not respond in the MBAS method. Those used in or as detergents are alkali salts of C_{10-20} fatty acids $[RCO_2]^-Na^+$, and though anionic in nature they are so weakly ionized that an extractable ion pair is not formed under the conditions of the test. Nonsoap anionic surfactants commonly used in detergent formulations are strongly responsive. These include principally surfactants of the sulfonate type $[RSO_3]^-Na^+$, the sulfate ester type $[ROSO_3]^-Na^+$, and sulfated nonionics $[RE_nOSO_3]^-Na^+$. They are recovered almost completely by a single $CHCl_3$ extraction.

Linear alkylbenzene sulfonate (LAS) is the most widely used anionic surfactant and is used to standardize the MBAS method. LAS is not a single compound, but may comprise any or all of 26 isomers and homologs with structure $[R'C_6H_4SO_3]^-Na^+$, where R′ is a linear secondary alkyl group ranging from 10 to 14 carbon atoms in length. The manufacturing process defines the mixture, which may be modified further by the wastewater treatment process.

Sulfonate- and sulfate-type surfactants respond together in MBAS analysis, but they can be differentiated by other means. The sulfate type decomposes upon acid hydrolysis; the resulting decrease in MBAS corresponds to the original sulfate surfactant content while the MBAS remaining corresponds to the sulfonate surfactants. Alkylbenzene sulfonate can be identified and quantified by infrared spectrometry after purification.[1] LAS can be distinguished from other alkylbenzene sulfonate surfactants by infrared methods.[2] LAS can be identified unequivocally and its detailed isomer-homolog composition determined by desulfonation-gas chromatography.[3]

c. Interferences: Positive interferences result from all other MBAS species present; if a direct determination of any individual MBAS species, such as LAS, is sought, all others interfere. Substances such as organic sulfonates, sulfates, carboxylates and phenols, and inorganic thiocyanates, cyanates, nitrates, and chlorides also may transfer more or less methylene blue into the chloroform phase. The poorer the extractability of their ion pairs, the more effective is the aqueous backwash step in removing these positive interferences; interference from chloride is eliminated almost entirely and from nitrate largely so by the backwash. Because of the varied extractability of nonsurfactant MBAS, deviations in $CHCl_3$ ratio and backwashing procedure may lead to significant differences in the total MBAS observed, although the recovery of sulfonate- and sulfate-type surfactants will be substantially complete in all cases.

Negative interferences can result from the presence of cationic surfactants and other cationic materials, such as amines, because they compete with the methylene blue in the formation of ion pairs. Particulate matter may give negative interference through adsorption of MBAS. Although some of the adsorbed MBAS may be desorbed and paired during the $CHCl_3$ extractions, recovery may be incomplete and variable.

Minimize interferences by nonsurfactant materials by sublation if necessary (5540B). Other countermeasures are nonstandard. Remove interfering cationic surfactants and other cationic materials by using a cation-exchange resin under suitable conditions.[3] Handle adsorption of MBAS by particulates preferably by filtering and analyzing the insolubles. With or without filtration, adsorbed MBAS can be desorbed by acid hydrolysis; however, MBAS originating in any sulfate ester-type surfactant present is destroyed simultaneously.[1] Sulfides, often present in raw or primary treated wastewater, may react with methylene blue to form a colorless reduction product, making the analysis impossible. Eliminate this interference by prior oxidation with hydrogen peroxide.

d. Molecular weight: Test results will appear to differ if expressed in terms of weight rather than in molar quantities. Equimolar amounts of two anionic surfactants with different molecular weights should give substantially equal colors in the $CHCl_3$ layer, although the amounts by weight may differ significantly. If results are to be expressed by weight, as generally is desirable, the average molecular weight of the surfactant measured must be known or a calibration curve made with that particular compound must be used. Because such detailed information generally is lacking, report results in terms of a suitable standard calibration curve, for example "0.65 mg MBAS/L (calculated as LAS, mol wt 318)."

e. Minimum detectable quantity: About 10 μg MBAS (calculated as LAS).

f. Application: The MBAS method has been applied successfully to drinking water samples. In wastewater, industrial wastes, and sludge, numerous materials normally present can interfere seriously if direct determination of MBAS is attempted. Most nonsurfactant aqueous-phase interferences can be removed by sublation. The method is linear over an approximate range of 10 to 200 μg of MBAS standard. This may vary somewhat, depending on source of standard material.

g. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Colorimetric equipment: One of the following is required:

1) *Spectrophotometer,* for use at 652 nm, providing a light path of 1 cm or longer.

2) *Filter photometer,* providing a light path of 1 cm or longer and equipped with a red color filter exhibiting maximum transmittance near 652 nm.

b. Separatory funnels: 500-mL, preferably with inert TFE stopcocks and stoppers.

3. Reagents

a. Stock LAS solution: Weigh an amount of the reference material* equal to 1.00 g LAS on a 100% active basis. Dissolve

* For sources of suitable reference material, contact *Standard Methods* technical information manager at www.standardmethods.org/contact/.

in water and dilute to 1000 mL; 1.00 mL = 1.00 mg LAS. Store in a refrigerator to minimize biodegradation. If necessary, prepare weekly.

b. Standard LAS solution: Dilute 10.00 mL stock LAS solution to 1000 mL with water; 1.00 mL = 10.0 μg LAS. Prepare daily.

c. Phenolphthalein indicator solution, alcoholic.

d. Sodium hydroxide (NaOH), 1*N*.

e. Sulfuric acid (H_2SO_4), 1*N* and 6*N*.

f. Chloroform ($CHCl_3$): **Caution: Chloroform is toxic and a suspected carcinogen. Take appropriate precautions against inhalation and skin exposure**.

g. Methylene blue reagent: Dissolve 100 mg methylene blue† in 100 mL water. Transfer 30 mL to a 1000-mL flask. Add 500 mL water, 41 mL 6*N* H_2SO_4, and 50 g sodium phosphate, mono- basic, monohydrate, $NaH_2PO_4 \cdot H_2O$. Shake until dissolved. Dilute to 1000 mL.

h. Wash solution: Add 41 mL 6*N* H_2SO_4 to 500 mL water in a 1000-mL flask. Add 50 g $NaH_2PO_4 \cdot H_2O$ and shake until dissolved. Dilute to 1000 mL.

i. Methanol (CH_3OH). **Caution: Methanol vapors are flammable and toxic; take appropriate precautions.**

j. Hydrogen peroxide (H_2O_2), 30%.

k. Glass wool: Pre-extract with $CHCl_3$ to remove interferences.

l. Water, reagent-grade, MBAS-free. Use for making all reagents and dilutions.

4. Procedure

a. Preparation of calibration curve: Prepare an initial calibration curve consisting of at least five standards covering the referenced (5540C.1*f*) or desired concentration range. Provided that linearity is demonstrated over the range of interest (r = 0.995 or better) run daily check standards at the reporting limit and a concentration above the expected samples' concentration. Check standard results should be within 25% of original value at the reporting limit and 10% of original value for all others. Otherwise, prepare a new calibration curve.

Prepare a series of separatory funnels for a reagent blank and selected standards. Pipet portions of standard LAS solution (5540C.3*b*) into funnels. Add sufficient water to make the total volume 100 mL in each separatory funnel. Treat each standard as described in ¶s *d* and *e* below, and plot a calibration curve of absorbance vs. micrograms LAS taken, specifying the molecular weight of the LAS used.

b. Sample size: For direct analysis of waters and wastewaters, select sample volume on the basis of expected MBAS concentration:

Expected MBAS Concentration *mg/L*	Sample Taken *mL*
0.025–0.080	400
0.08–0.40	250
0.4–2.0	100

† Eastman No. P573, or equivalent.

If expected MBAS concentration is above 2 mg/L, dilute sample containing 40 to 200 μg MBAS to 100 mL with water.

For analysis of samples purified by sublation, dissolve sublate residue (5540B.4*e*) in 10 to 20 mL methanol, quantitatively transfer the entire amount (or a suitable portion if more than 200 μg MBAS is expected) to 25 to 50 mL water, evaporate without boiling until methanol is gone, adding water as necessary to avoid going to dryness, and dilute to about 100 mL with water.

c. Peroxide treatment: If necessary to avoid decolorization of methylene blue by sulfides, add a few drops of 30% H_2O_2.

d. Ion pairing and extraction:

1) Add sample to a separatory funnel. Make alkaline by dropwise addition of 1*N* NaOH, using phenolphthalein indicator. Discharge pink color by dropwise addition of 1*N* H_2SO_4.

2) Add 10 mL $CHCl_3$ and 25 mL methylene blue reagent. Rock funnel vigorously for 30 s and let phases separate. Alternatively, place a magnetic stirring bar in the separatory funnel; lay funnel on its side on a magnetic mixer and adjust speed of stirring to produce a rocking motion. Excessive agitation may cause emulsion formation. To break persistent emulsions add a small volume of isopropyl alcohol (<10 mL); add same volume of isopropyl alcohol to all standards. Some samples require a longer period of phase separation than others. Before draining $CHCl_3$ layer, swirl gently, then let settle.

3) Draw off $CHCl_3$ layer into a second separatory funnel. Rinse delivery tube of first separatory funnel with a small amount of $CHCl_3$. Repeat extraction two additional times, using 10 mL $CHCl_3$ each time. If blue color in water phase becomes faint or disappears, discard and repeat, using a smaller sample.

4) Combine all $CHCl_3$ extracts in the second separatory funnel. Add 50 mL wash solution and shake vigorously for 30 s. Emulsions do not form at this stage. Let settle, swirl, and draw off $CHCl_3$ layer through a funnel containing a plug of glass wool into a 100-mL volumetric flask; filtrate must be clear. Extract wash solution twice with 10 mL $CHCl_3$ each and add to flask through the glass wool. Rinse glass wool and funnel with $CHCl_3$. Collect washings in volumetric flask, dilute to mark with $CHCl_3$, and mix well.

e. Measurement: Determine absorbance at 652 nm against a blank of $CHCl_3$.

5. Calculation

From the calibration curve (5540C.4*a*) read micrograms of apparent LAS (mol wt ___) corresponding to the measured absorbance.

$$\text{mg MBAS/L} = \frac{\mu\text{g apparent LAS}}{\text{mL original sample}}$$

Report as "MBAS, calculated as LAS, mol wt ___."

6. Precision and Bias

A synthetic sample containing 270 μg LAS/L in distilled water was analyzed in 110 laboratories with a relative standard deviation of 14.8% and a relative error of 10.6%.

A tap water sample to which was added 480 μg LAS/L was analyzed in 110 laboratories with a relative standard deviation of 9.9% and a relative error of 1.3%.

A river water sample with 2.94 mg LAS/L added was analyzed in 110 laboratories with a relative standard deviation of 9.1% and a relative error of 1.4%.[4]

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5540 D. Nonionic Surfactants as CTAS

1. General Discussion

a. Definition and principle: Cobalt thiocyanate active substances (CTAS) are those that react with aqueous cobalt thiocyanate solution to give a cobalt-containing product extractable into an organic liquid in which it can be measured. Nonionic surfactants exhibit such activity, as may other natural and synthetic materials; thus, estimation of nonionic surfactants as CTAS is possible only if substantial freedom from interfering CTAS species can be assured.

The method requires sublation to remove nonsurfactant interferences and ion exchange to remove cationic and anionic surfactants, partition of CTAS into methylene chloride from excess aqueous cobalt thiocyanate by a single extraction, and measurement of CTAS in the methylene chloride by spectrophotometry at 620 nm. Lower limit of detectability is around 0.1 mg CTAS, calculated as $C_{12-18}E_{11}$. Beyond the sublation step the procedure is substantially identical to that of the Soap and Detergent Association (SDA).[1]

b. Nonionic surfactant responses: For pure individual molecular species the CTAS response is negligible up to about RE_5, where it increases sharply and continues to increase more gradually for longer polyether chains.[2,3] Fewer than about six oxygens in the molecule do not supply enough cumulative coordinate bond strength to hold the complex together. Commercial nonionic surfactants generally range from about RE_7 to RE_{15}; however, each such product, because of synthesis process constraints, is actually a mixture of many individual species ranging from perhaps RE_0 to RE_{2n} in a Poisson distribution averaging RE_n.

The hydrophobes used for nonionic surfactants in the U.S. household detergent industry are mainly linear primary and linear secondary alcohols with chain lengths ranging from about 12 to about 18 carbon atoms. Nonionics used in industrial operations include some based on branched octyl- and nonylphenols. These products give strong CTAS responses that may differ from each other, on a weight basis, by as much as a factor of 2. Specifically, eight such products showed responses from 0.20 to 0.36 absorbance units/mg by the SDA procedure.[1]

As with anionic surfactants measured as MBAS, the nonionic surfactants found in water and wastewater might have CTAS responses at least as varied as their commercial precursors because the proportions of the individual molecular species will have been changed by biochemical and physicochemical removal at varied rates, and further because their original molecular structures may have been changed by biodegradation processes.

c. Reference nonionic surfactant: Until it is practical to determine the nature and molecular composition of an unknown mixed CTAS, and to calculate or determine the CTAS responses of its component species, exact quantitation of uncharacterized CTAS in a sample in terms of weight is not possible. Instead, express the analytical result in terms of some arbitrarily chosen reference nonionic surfactant (i.e., as the weight of the reference that gives the same amount of CTAS response). The reference is the nonionic surfactant $C_{12-18}E_{11}$, derived from a mixture of linear primary alcohols ranging from 12 to 18 carbon atoms in chain length by reaction with ethylene oxide in a molar ratio of 1:11. $C_{12-18}E_{11}$ is reasonably representative of nonionic surfactants in commercial use; its CTAS response is about 0.21 absorbance units/mg.

If the identity of the nonionic surfactant in the sample is known, use that same material in preparing the calibration curve.

d. Interferences: Both anionic and cationic surfactants may show positive CTAS response[1,4] but both are removed in the ion-exchange step. Sublation removes nonsurfactant interferences. Physical interferences occur if some of the CTAS is adsorbed on particulate matter. Avoid such interference by filtering out the particulates for the sublation step; this will measure only dissolved CTAS.

e. Minimum detectable quantity: About 0.1 mg CTAS, calculated as $C_{12-18}E_{11}$, which corresponds to 0.1 mg/L in a 1-L sample.

f. Application: The method is suitable for determining dissolved nonionic surfactants of the ethoxylate type in most aqueous systems.

g. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Sublation apparatus: See 5540B.2.

b. Ion-exchange column, glass, about 1- × 30-cm. Slurry anion-exchange resin in methanol and pour into column to give a bed about 10 cm deep. Insert plug of glass wool and then add a 10-cm bed of cation-exchange resin on top in the same manner. One column may be used for treating up to six sublated samples before repacking.

c. Spectrophotometer and 2.0-cm stoppered cells, suitable for measuring absorbance at 620 nm.

d. Separatory funnels, 125-mL, preferably with TFE stopcock and stopper.

e. Extraction flasks, Soxhlet type, 150-mL.

3. Reagents

a. Sublation reagents: See 5540B.3.

b. Anion-exchange resin, polystyrene-quaternary ammonium-type,* 50- to 100-mesh, hydroxide form. To convert chloride form to hydroxide, elute with 20 bed volumes of 1*N* NaOH and wash with methanol until free alkali is displaced.

c. Cation-exchange resin, polystyrene-sulfonate type,† 50- to 100-mesh, hydrogen form.

d. Cobaltothiocyanate reagent: Dissolve 30 g $Co(NO_3)_2 \cdot 6H_2O$ and 200 g NH_4SCN in water and dilute to 1 L. This reagent is stable for at least 1 month at room temperature.

e. Reference nonionic surfactant, $C_{12-18}E_{11}$: Reaction product of C_{12-18} linear primary alcohol with ethylene oxide in 1:11 molar ratio.‡

f. Reference nonionic surfactant stock solution, methanolic, approximately 2 mg nonionic/mL methanol: Quantitatively transfer entire contents (approximately 1 g nonionic) from preweighed ampule into 500-mL volumetric flask, thoroughly rinse ampule with methanol, make up to volume with methanol, and reweigh dried ampule. Calculate concentration in milligrams per milliter as in 5540D.5*a*. Because of possible phase separation, use all material in the ampule.

g. Reference nonionic surfactant standard solution, methanolic, approximately 0.1 mg nonionic/mL methanol: Dilute 10.00 mL stock solution to 200 mL with methanol. Exact concentration is 1/20 that of the stock solution.

h. Sodium hydroxide, NaOH: 1*N*.

i. Glass wool: Pre-extract with chloroform or methylene chloride.

j. Methanol, CH_3OH: **Caution: Methanol vapors are flammable and toxic; take appropriate precautions.**

k. Methylene chloride, CH_2Cl_2: **Caution: Methylene chloride vapors are toxic; take adequate precautions.**

l. Water: Use distilled or deionized, CTAS-free water for making reagents and dilutions.

4. Procedure

a. Purification by sublation: Proceed according to 5540B.4, using sample containing no more than 2 mg CTAS. (Note: For samples of known character containing no interfering materials, omit this step.)

b. Ion-exchange removal of anionic and cationic surfactants: Dissolve sublation residue in 5 to 10 mL methanol and transfer quantitatively to ion-exchange column. Elute with methanol at 1 drop/s into a clean, dry 150-mL extraction flask until about 125 mL is collected. Evaporate methanol on a steam bath aided by a gentle stream of clean, dry nitrogen or air, taking care to avoid loss by entrainment; remove from heat as soon as the methanol is completely evaporated. (Note: With samples of known character containing no anionic or cationic materials, omit Step *b*.)

c. CTAS calibration curve: Into a series of 150-mL extraction flasks containing 10 to 20 mL methanol place 0.00, 5.00, 10.00, 20.00, and 30.00 mL reference nonionic surfactant standard solution and evaporate just to dryness. Continue as in ¶s *d* and *e* below, and plot a calibration curve of absorbance against milligrams of reference nonionic taken, specifying its identity (e.g., $C_{12-18}E_{11}$ and lot number).

d. Cobalt complexing and extraction: Charge a 125-mL separatory funnel with 5 mL cobaltothiocyanate reagent. With precautions against excessive and variable evaporation of the methylene chloride, dissolve residue from ion-exchange operation (¶ *b* above) by adding 10.00 mL methylene chloride and swirling for a few seconds. Immediately transfer by pouring into the separatory funnel. *Do not rinse flask.* (Note: Because of the

* Bio-Rad, AGl-X2, or equivalent.
† Bio-Rad AG 50W-X8, or equivalent.

‡ For sources of suitable reference material, contact *Standard Methods* technical information manager at www.standardmethods.org/contact/.

volatility of methylene chloride, rigidly standardize these operations with respect to handling and elapsed time; alternatively, evaporate the methanol in 200-mL Erlenmeyer flasks to be stoppered with glass or TFE stoppers during dissolution. Transfer as directed here is incomplete, but in this case it will not introduce error because the loss of nonionics is exactly compensated for by the diminished volume of the organic layer in the extraction.) Shake separatory funnel vigorously for 60 s and let layers separate. Run lower layer into a 2.0-cm cell through a funnel containing a plug of pre-extracted glass wool and stopper. Be sure filtrate is absolutely clear. (NOTE: If desired, clarify by running the lower layer into a 12-mL centrifuge tube, stopper, spin at or above $1000 \times g$ for 3 min, and transfer to the cell by a Pasteur pipet; use same procedure for both calibration and samples.)

e. Measurement: Determine absorbance at 620 nm against a blank of methylene chloride. (NOTE: If haze develops in the cell, warm slightly with a hot air gun or heat lamp to clarify.)

5. Calculations

a. Nonionic surfactant in reference nonionic stock solution 5540D.3*f:*

$$\text{mg nonionic/mL methanol} = \text{mg reference sample/500 mL}$$

b. Nonionic surfactant in sample: From the calibration curve read milligrams of reference nonionic corresponding to the measured absorbance:

$$\text{mg CTAS/L} = \text{mg apparent nonionic/L sample}$$

Report as "CTAS, calculated as nonionic surfactant $C_{12-18}E_{11}$."

6. Precision and Bias

Twenty-four samples of 6.22% w/v solution of reference nonionic surfactant $C_{12-18}E_{11}$ were analyzed in three laboratories by CTAS alone, without sublation or ion exchange. The overall relative standard deviation was about 3%. Results of the three laboratories individually were:

Laboratory	% w/w ± SD
A	6.08 ± 0.14 (n = 36)
B	6.56 ± 0.17 (n = 6)
C	6.25 ± 0.14 (n = 36)
Overall	6.20 ± 0.19 (n = 78)

Samples of raw wastewater were freed of surfactants by four successive sublations, then 0.50 or 0.67 mg reference nonionic surfactant $C_{12-18}E_{11}$ was added and carried through the entire sequence of sublation, ion exchange, and CTAS extraction. Recoveries averaged 92% with overall standard deviation around 6%:

Laboratory	% Recovery ± SD
A	87 ± 4 (n = 4)
B	97 ± 1 (n = 4)
Overall	92 ± 6 (n = 8)

The above data relate to the bias and precision of the method when applied to a known nonionic surfactant. When the nature of the nonionic surfactant is unknown, there is greater uncertainty. The response of the reference $C_{12-18}E_{11}$ is about 0.21 absorbance units/mg, while that of the eight nonionic types mentioned under 5540D.1*b* ranged from 0.20 to 0.36, and environmental nonionics might differ still more. If the nonionic surfactant in the sample has a response of 0.42, the result calculated in terms of milligrams $C_{12-18}E_{11}$ would be double the actual milligrams of the unknown nonionic.

7. References

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5550 TANNIN AND LIGNIN*

5550 A. Introduction

Lignin is a plant constituent that often is discharged as a waste during the manufacture of paper pulp. Another plant constituent, tannin, may enter the water supply through the process of vegetable matter degradation or through the wastes of the tanning industry. Tannin also is applied in the so-called internal treatment of boiler waters, where it reduces scale formation by causing the production of a more easily handled sludge.

* Approved by Standard Methods Committee, 2010.

5550 B. Colorimetric Method

1. General Discussion

a. Principle: Both lignin and tannin contain aromatic hydroxyl groups that react with Folin phenol reagent (tungstophosphoric and molybdophosphoric acids) to form a blue color suitable for estimation of concentrations up to at least 9 mg/L. However, the reaction is not specific for lignin or tannin, nor for compounds containing aromatic hydroxyl groups, inasmuch as many other reducing materials, both organic and inorganic, respond similarly.

b. Applicability: This method is generally suitable for the analysis of any organic chemical that will react with Folin phenol reagent to form measurable blue color at the concentration of interest. However, many compounds are reactive (see ¶ *c* below) and each yields a different molar extinction coefficient (color intensity). Hence, the analyst must demonstrate conclusively the absence of interfering substances.

c. Interferences: Any substance able to reduce Folin phenol reagent will produce a false positive response. Organic chemicals known to interfere include hydroxylated aromatics, proteins, humic substances, nucleic acid bases, fructose, and amines. Inorganic substances known to interfere include iron (II), manganese (II), nitrite, cyanide, bisulfite, sulfite, sulfide, hydrazine, and hydroxylamine hydrochloride. Both 2 mg ferrous iron/L and 125 mg sodium sulfite/L individually produce a color equivalent to 1 mg tannic acid/L.

d. Minimum detectable concentrations: Approximately 0.025 mg/L for phenol and tannic acid and 0.1 mg/L for lignin with a 1-cm-path-length spectrophotometer.

e. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

Colorimetric equipment: One of the following is required:

a. Spectrophotometer, for use at 700 nm. A light path of 1 cm or longer yields satisfactory results.

b. Filter photometer, provided with a red filter exhibiting maximum transmittance in the wavelength range of 600 to 700 nm. Sensitivity improves with increasing wavelength. A light path of 1 cm or longer yields satisfactory results.

c. Nessler tubes, matched, 100-mL, tall form, marked at 50-mL volume.

3. Reagents

a. Folin phenol reagent: Transfer 100 g sodium tungstate, $Na_2WO_4 \cdot 2H_2O$, and 25 g sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$, together with 700 mL distilled water, to a 2000-mL flat-bottom boiling flask. Add 50 mL 85% H_3PO_4 and 100 mL conc HCl. Connect to a reflux condenser and boil gently for 10 h. Add 150 g Li_2SO_4, 50 mL distilled water, and a few drops of liquid bromine. Boil without condenser for 15 min to remove excess bromine. Cool to 25°C, dilute to 1 L, and filter. Store finished reagent, which should have no greenish tint, in a tightly stoppered bottle to protect against reduction by air-borne dust and organic materials.

Alternatively, purchase commercially prepared Folin phenol reagent and use before the recommended expiration date.

b. Carbonate-tartrate reagent: Dissolve 200 g Na_2CO_3 and 12 g sodium tartrate, $Na_2C_4H_4O_6 \cdot 2H_2O$, in 750 mL hot distilled water, cool to 20°C, and dilute to 1 L.

c. Stock solution: The nature of the substance present in the sample dictates the choice of chemical used to prepare the standard, because each substance produces a different color intensity. Weigh 1.000 g tannic acid, tannin, lignin, or other compound being used for boiler water treatment or known to be a contaminant of the water sample. Dissolve in distilled water and dilute to 1000 mL. If the identity of the compound in the water sample is not known, use phenol and report results as "substances reducing Folin phenol reagent" in mg phenol/L. Interpret such results with caution.

Note that tannin and lignin are not individual chemical species of known molecular weight and structure; rather, they are substances containing a spectrum of chemicals of different molecular weights. Their chemical properties depend on source and method of isolation. If a particular substance is being added to the water, use it to prepare the stock solution.

d. Standard solution: Dilute stock solution with distilled water to desired range. Prepare a minimum of 3 standards bracketing expected sample concentration range.

4. Procedure

Bring 50-mL portions of clear sample and standards to a temperature above 20°C and maintain within a ±2°C range. Add in rapid

succession 1 mL Folin phenol reagent and 10 mL carbonate-tartrate reagent. Allow 30 min for color development. Compare visually against simultaneously prepared standards in matched Nessler tubes or make photometric readings against a reagent blank prepared at the same time. When using this method for regulatory compliance, use photometric detection. Use the following guide for instrumental measurement at a wavelength of 700 nm:

Tannic Acid in 61-mL Final Volume μg	Lignin in 61-mL Final Volume μg	Light Path *cm*
50–600	100–1500	1
10–150	30–400	5

Report results in mg/L of the compound known to be present or as "substances reducing Folin phenol reagent" in mg phenol/L.

5. Precision and Bias

In a single laboratory analyzing seven replicates for phenol at 0.1 mg/L the precision was ±7% and recovery was 107%.

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5560 ORGANIC AND VOLATILE ACIDS*

5560 A. Introduction

The measurement of organic acids, by adsorption and elution from a chromatographic column, by gas chromatography, or by distillation, can be used as a control test for anaerobic digestion. The chromatographic separation method is presented for organic acids (5560B), while a method using distillation (5560C) and a gas chromatographic method (5560D) are presented for volatile acids.

Volatile fatty acids are classified as water-soluble fatty acids that can be distilled at atmospheric pressure. These volatile acids can be removed from aqueous solutions by distillation, despite their high boiling points, because of co-distillation with water. This group includes water-soluble fatty acids with up to six carbon atoms.

The distillation method is empirical and gives incomplete and somewhat variable recovery. Factors such as heating rate and proportion of sample recovered as distillate affect the result, requiring the determination of a recovery factor for each apparatus and set of operating conditions. However, it is suitable for routine control purposes. Removing sludge solids from the sample reduces the possibility of hydrolysis of complex materials to volatile acids.

The gas chromatographic method determines individual concentrations of many of the fatty acids, giving additional information about the sample.

* Approved by Standard Methods Committee, 2006. Editorial revisions, 2011. Joint Task Group: 22nd Edition—Theadore C. Heesen (chair), Carlos De Leon, Peter J. Morrissey.

5560 B. Chromatographic Separation Method for Organic Acids

1. General Discussion

a. Principle: An acidified aqueous sample containing organic acids is adsorbed on a column of silicic acid and the acids are eluted with *n*-butanol in chloroform ($CHCl_3$). The eluate is collected and titrated with standard base. All short-chain (C_1 to C_6) organic acids are eluted by this solvent system and are reported collectively as total organic acids.

b. Interference: The $CHCl_3$-butanol solvent system is capable of eluting organic acids other than the volatile acids and also some synthetic detergents. Besides the so-called volatile acids, crotonic, adipic, pyruvic, phthalic, fumaric, lactic, succinic, malonic, gallic, aconitic, and oxalic acids; alkyl sulfates; and alkyl-aryl sulfonates are adsorbed by silicic acid and eluted.

c. Precautions: Basic alcohol solutions decrease in strength with time, particularly when exposed repeatedly to the atmosphere. These decreases usually are accompanied by the appearance of a white precipitate. The magnitude of such changes normally is not significant in process control if tests are made within a few days of standardization. To minimize this effect, store standard sodium hydroxide (NaOH) titrant in a tightly stoppered borosilicate glass bottle and protect from atmospheric carbon dioxide (CO_2) by attaching a tube of CO_2-absorbing material, as described in the inside front cover. For more precise analyses, standardize titrant or prepare before each analysis.

Although the procedure is adequate for routine analysis of most sludge samples, volatile-acids concentrations above 5000 mg/L may require an increased amount of organic solvent for quantitative recovery. Elute with a second portion of solvent and titrate to reveal possible incomplete recoveries.

d. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Centrifuge or filtering assembly.

b. Crucibles, Gooch or medium-porosity fritted-glass, with filtering flask and vacuum source. Use crucibles of sufficient size (30 to 35 mL) to hold 12 g silicic acid.

c. Separatory funnel, 1000-mL.

3. Reagents

a. Silicic acid, specially prepared for chromatography, 50 to 200 mesh: Remove fines by slurrying in distilled water and decanting supernatant after settling for 15 min. Repeat several times. Dry washed acid in an oven at 103°C until *absolutely dry,* then store in a desiccator.

b. Chloroform-butanol reagent: Mix 300 mL reagent-grade $CHCl_3$, 100 mL *n*-butanol, and 80 mL 0.5*N* H_2SO_4 in a separatory funnel. Let water and organic layers separate. Drain off lower organic layer through a fluted filter paper into a dry bottle. **CAUTION: Chloroform has been classified as a cancer suspect agent. Use hood for preparation of reagent and conduct of test.**

c. Thymol blue indicator solution: Dissolve 80 mg thymol blue in 100 mL absolute methanol.

d. Phenolphthalein indicator solution: Dissolve 80 mg phenolphthalein in 100 mL absolute methanol.

e. Sulfuric acid (H_2SO_4), conc.

f. Standard sodium hydroxide (NaOH), 0.02*N:* Dilute 20 mL 1.0*N* NaOH stock solution to 1 L with absolute methanol.

Prepare stock in water and standardize in accordance with the methods outlined in Section 2310B.3*d*.

4. Procedure

a. Pretreatment of sample: Centrifuge or vacuum-filter enough sludge to obtain 10 to 15 mL clear sample in a small test tube or beaker. Add a few drops of thymol blue indicator solution, then conc H_2SO_4 dropwise, until definitely red to thymol blue (pH = 1.0 to 1.2).

b. Column chromatography: Place 12 g silicic acid in a Gooch or fritted-glass crucible and apply suction to pack column. Tamp column while applying suction to reduce channeling when the sample is applied. With a pipet, distribute 5.0 mL acidified sample as uniformly as possible over column surface. Apply suction momentarily to draw sample into silicic acid. Release vacuum as soon as last portion of sample has entered column. Quickly add 65 mL $CHCl_3$-butanol reagent and apply suction. Discontinue suction just before the last of reagent enters column. Do not reuse columns.

c. Titration: Remove filter flask and purge eluted sample with N_2 gas or CO_2-free air immediately before titrating. (Obtain CO_2-free air by passing air through a CO_2 absorbant.*)

Titrate sample with standard 0.02*N* NaOH to phenolphthalein endpoint, using a fine-tip buret and taking care to avoid aeration. The fine-tip buret aids in improving accuracy and precision of the titration. Use N_2 gas or CO_2-free air delivered through a small glass tube to purge and mix sample and to prevent contact with atmospheric CO_2 during titration.

d. Blank: Carry a distilled water blank through steps ¶s *a–c* above.

* Ascarite, or equivalent.

5. Calculation

$$\text{Total organic acids (mg as acetic acid/L)} = \frac{(a - b) \times N \times 60\,000}{\text{mL sample}}$$

where:

a = mL NaOH used for sample,
b = mL NaOH used for blank, and
N = normality of NaOH.

6. Precision

Average recoveries of about 95% are obtained for organic acid concentrations above 200 mg as acetic acid/L. Individual tests generally vary from the average by approximately 3%. A greater variation results when lower concentrations of organic acids are present. Titration precision expressed as the standard deviation is about ±0.1 mL (approximately ±24 mg as acetic acid/L).

7. Bibliography

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5560 C. Distillation Method

1. General Discussion

a. Principle: This technique recovers acids containing up to six carbon atoms. Fractional recovery of each acid increases with increasing molecular weight. Calculations and reporting are on the basis of acetic acid. The method often is applicable for control purposes. Because it is empirical, carry it out exactly as described. Because the still-heating rate, presence of sludge solids, and final distillate volume affect recovery, determine a recovery factor.

b. Interference: Hydrogen sulfide (H_2S) and CO_2 are liberated during distillation and will be titrated to give a positive error. Eliminate this error by discarding the first 15 mL of distillate and account for this in the recovery factor. Residues on glassware from some synthetic detergents have been reported to interfere; use water and dilute acid rinse cycles to prevent this problem.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Centrifuge, with head to carry four 50-mL tubes or 250-mL bottles.

b. Distillation flask, 500-mL capacity.

c. Condenser, about 76 cm long.

d. Adapter tube.

e. pH meter or recording titrator: See Section 2310B.2*a*.

f. Distillation assembly: Use a conventional distilling apparatus. To minimize fluctuations in distillation rate, supply heat with a variable-wattage electrical heater.

3. Reagents

a. Sulfuric acid (H_2SO_4), 1 + 1.

b. Standard sodium hydroxide titrant, 0.1*N:* See Section 2310B.3*c*.

c. Phenolphthalein indicator solution.

d. Acetic acid stock solution, 2000 mg/L: Dilute 1.9 mL conc CH_3COOH to 1000 mL with deionized water. Standardize against 0.1*N* NaOH.

4. Procedure

a. Recovery factor: To determine the recovery factor, *f*, for a given apparatus, dilute an appropriate volume of acetic acid stock solution to 250 mL in a volumetric flask to approximate the expected sample concentration and distill as for a sample. Calculate the recovery factor

$$f = \frac{a}{b}$$

where:

a = volatile acid concentration recovered in distillate, mg/L, and
b = volatile acid concentration in standard solution used, mg/L.

b. Sample analysis: Centrifuge 200 mL sample for 5 min. Pour off and combine supernatant liquors. Place 100 mL supernatant liquor, or smaller portion diluted to 100 mL, in a 500-mL distillation flask. Add 100 mL distilled water, four to five clay chips or similar material to prevent bumping, and 5 mL H_2SO_4. Mix so that acid does not remain on bottom of flask. Connect flask to a condenser and adapter tube and distill at the rate of about 5 mL/min. Discard the first 15 mL and collect exactly 150 mL distillate in a 250-mL graduated cylinder. Titrate with 0.1*N* NaOH, using phenolphthalein indicator, a pH meter, or an automatic titrator. The endpoints of these three methods are, respectively, the first pink coloration that persists on standing a short time, pH 8.3, and the inflection point of the titration curve (see Section 2310B). Titration at 95°C produces a stable endpoint.

Distill and analyze a blank and reference standard with each sample batch to ensure system performance.

5. Calculation

$$\text{mg volatile acids as acetic acid/L} = \frac{\text{mL NaOH} \times N \times 60\,000}{\text{mL sample} \times f}$$

where:

N = normality of NaOH, and
f = recovery factor.

6. Bibliography

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5560 D. Gas Chromatographic Method

1. General Discussion

a. Principle: This gas chromatographic procedure may be used to determine the individual concentrations of the following fatty acids: acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids in digester sludge samples. Caproic and heptanoic acids also may be determined, but with less certainty because of their reduced solubility in water. Volatile fatty acids are generally soluble in digester sludge and exist in either the protonated or unprotonated form, depending on the pH of the sludge sample. Acetic and propionic acids usually are in higher concentrations than the other fatty acids in digester sludge. All acids must be converted to the protonated (volatile) form before injection into the gas chromatograph (GC) so vaporization may occur. The fatty acids must also be separated from the solids material in the sample before introduction into the GC to minimize degradation of the GC column. Digester sludge samples are prepared by acidification, centrifugation, and filtration. The sample is analyzed by direct injection into a gas chromatograph equipped with a flame ionization detector after both centrifugation and filtration.

b. Interference: The blank amount may be high relative to sample analyte. High blanks may be caused by a buildup of contaminants in the injector and guard column or by sample carry-over. The analyst must be aware of the blank levels and correct unacceptably high blank levels that are above the reporting limits.

c. Minimum detectable concentration: The method detection level has been determined by the USEPA method[1] in reagent water to be about 3 mg/L for acetic acid and about 1 mg/L for all other target compounds.

2. Apparatus

a. Gas chromatograph, with a flame ionization detector (FID) and programmable column oven. An on-column capillary injector, a detector make-up tee, and a gas chromatographic data system are recommended.

b. Chromatographic columns, preferably fused silica, bonded polyethylene glycol capillary columns 30 m long × 0.53 mm ID with a film thickness of 0.10 to 0.25 μm. An example of a chromatogram is presented in Figure 5560:1.*

* Several manufacturers of suitable bonded-phase capillary columns may be

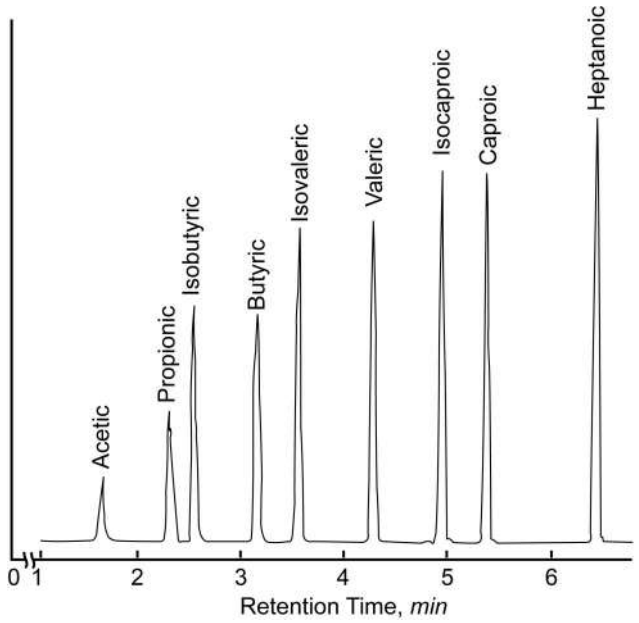


Figure 5560:1. Gas chromatogram of a fatty acid standard. Column DB-FFAP, 0.53-mm-ID, 30-m, 0.50-μm film thickness, temperature-programmed as described in 5560D.2*d*.

c. Guard column: Preferably use a 2- to 5-m 0.53-mm-ID deactivated fused silica guard column if an on-column injector is used. Attach guard column to analytical column with a press-fit connector. Service guard column periodically by breaking off the front section or replace guard column when system performance is degraded.

d. Gas chromatograph operating conditions: Use temperature programming to achieve optimal separation and desirably short run times. Optimize temperatures and flow rates for the particular gas chromatograph and chromatographic column. Optimal injector temperature will allow vaporization of the prepared sample and provide good peak shape. Incorrect injector temperature may cause splitting of the acetic acid peak. Typical conditions are as follows:

Injector temperature: 150°C

Oven temperature program: 95°C hold for 2 min, ramp to 140°C at 10°C/min, no hold. Ramp to 200°C at 40°C/min, 5 min hold. Cool to initial temperature.

Detector conditions: temperature 240°C, hydrogen flow rate 30 mL/min, and air flow rate 300 mL/min.

Carrier gas flow rate: 18 mL/min.

Detector make-up gas flow rate: 12 mL/min, for a total of 30 mL/min from the column into the detector (or as recommended by the manufacturer).

e. Syringe, for sample introduction into GC. Syringe may be for manual injection or used with an autosampler.

f. Disposable syringes, 10-mL, plastic with detachable tip,† for use with in-line syringe filter.

g. Disposable in-line syringe filters, 0.8/0.2μm (or equivalent), for filtering sample supernatant before introduction into the instrument.

h. Centrifuge, with polycarbonate centrifuge tubes, capable of separating sample solids from aqueous portion.

i. Large glass vials, 40-mL, with TFE-lined septa and caps.

j. Small glass vials, 3.5-mL, with TFE-lined septa and caps.

3. Reagents

a. Hydrogen, to fuel the FID.

b. Air, hydrocarbon-free-grade or better, for the FID.

c. Helium, carrier-grade or better, for use as GC carrier gas and detector makeup gas. Preferably use gas purification devices on carrier-gas line to remove oxygen and organic compounds.

d. Phosphoric acid, 85%.

e. Volatile fatty acid stock standard mixture, approximately 600 to 1000 mg/L for each of acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids. The stock standard may be prepared from pure fatty acid standard and reagent water or may be purchased in solution. Caproic and heptanoic acids also may be added if desired. Store at 4°C. Replace as needed, or every 6 months.

f. Volatile fatty acid calibration standard mixture, diluted from the stock volatile acid standard mixture to four levels of dilution to span a broad range of concentrations. Typical calibration standard concentrations are 350 mg/L, 140 mg/L, 35 mg/L, and 3.5 mg/L. Prepare fresh monthly or as needed by diluting volatile acid standard mixture with reagent water acidified with phosphoric acid. Store at 4°C.

g. Reagent water: Use any water that is free of volatile fatty acids. Prepare by passing house deionized water through an activated carbon column. Analyze to ensure an acceptable blank level well below method reporting limits.

4. Procedure

a. Sample preparation: Collect digester sludge samples on the day of analysis and refrigerate at 4°C immediately. Transfer a sample portion (10 to 30 mL) to a 40-mL glass vial and acidify to approximately pH 2 with phosphoric acid while stirring with a glass rod. Check pH with pH paper after mixing. Transfer acidified sludge sample into a polycarbonate centrifuge tube and centrifuge until centrate is separated from supernatant. Withdraw supernatant from centrifuge tube with a disposable syringe, filter through a disposable in-line filter, and place in a 3.5-mL glass vial (5560D.2*j*).

Perform one duplicate sample analysis for each set of 10 samples. Also fortify, and analyze, samples analyzed in duplicate as follows: Place 10-mL portion of digester sludge in a 40-mL vial (5560D.2*i*) and add an amount of volatile fatty acid stock standard mixture approximately equal to the largest amount of any single analyte expected in the sample. Process duplicate and fortified samples in the same way as other samples.

b. Calibration: Inject 1 μL of each of the volatile fatty acid calibration standard mixture concentrations (5560D.3*f*) into the GC for calibration. Preferably use the solvent flush technique for all standard and sample injections. Construct a calibration curve from the four calibration standard injections using the best fit through zero. The calibration curve may be used for up to 1 month if it is proven to be valid. Validate calibration curve daily by injection of a mid-point calibration curve validation

located on the Internet. For example, a tabulation of several brands may be found at www.restekcorp.com.

† Luerlok, or equivalent.

standard. Inject a check standard (mid-point calibration curve standard) at end of each day.

c. Sample analysis: Use 1-μL injection volume for all analyses, including blank, duplicate, and laboratory-fortified samples. Analyze at least one blank sample (reagent water adjusted to pH 2 with phosphoric acid) for each set of 10 samples. Also analyze blank samples after high-level samples or standards to check system cleanliness.

5. Calculations

a. Data collection: Collect and process all data with chromatographic data system software.

b. Calibration curve: Use area and concentration of each fatty acid component in each of the calibration standards to construct calibration curve by the external standard method. Use first-order linear regression through zero and compute calibration factor. The correlation coefficient should be >0.995. Recalculate each calibration point by comparison to the curve. The calculated values should be ±20% of the true concentration. If the correlation coefficient is <0.995 or any of the recalculated values are more than 20% from the true value, correct the problem before proceeding.

c. Continuing calibration and check standards: Validate calibration curve daily (5560D.4*b*). The curve is valid if the concentration of the calibration curve validation standard is within 20% of the expected value for all components. If the concentration of the calibration curve validation standard is outside of 20% of the expected value for any component, construct a new curve. Analyze at least one check standard on each day of instrument use. "Bracket" the samples with check standards, that is, analyze check standards before and after the samples. The analysis is valid if concentration of the check standard components is within 10% of the expected values. Correct problem and repeat analysis of any samples analyzed after last acceptable check standard, if a check standard is not within 10%.

d. Fatty acid concentration: Identify volatile fatty acids in the samples by comparing sample retention times with those of the standards. Determine concentration of each of the fatty acids in each sample by multiplying area of each sample component with the calibration factor for that component. The volume of phosphoric acid used to adjust the pH may need to be factored to correct the results if that volume is significant when compared to sample volume. Similarly, consider volume of fortifying solution when it is a significant portion of the sample volume.

6. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 5020:I.

a. Blank samples: For each sample set of 10 samples or less, analyze at least one blank sample. Also analyze a blank sample after high standard or sample injections to ensure the cleanliness of the system. An acceptable blank sample has no volatile fatty acids above the MDL.

b. Duplicate samples: Analyze at least one sample in duplicate for each set of 10 or fewer samples. Calculate percent difference; acceptable difference is less than 10% for major components.

c. Laboratory-fortified samples (LFS): Analyze at least one LFS for each set of 10 or fewer samples. Calculate percent recovery; acceptable value is ±20%.

d. Minimum quantitation level: Establish method detection level (MDL)[1] either in matrix or in reagent water. The MDL is defined as three times the standard deviation of the replicate measurements. The minimum quantitation level (MQL) is defined as four times the MDL. MQL must be at or above lowest standard on calibration curve.

e. Reporting protocol: For sample analyses to be reportable, ensure that all quality assurance samples (including blank, duplicate, LFS, and check standards), as well as the calibration curve or calibration curve validation standard, are acceptable, and that the sample response lies between the highest and lowest points on the calibration curve. Annotate results below the MQL but greater than the MDL as "less than the MQL." Report results less than the MDL as "less than nominal value of the MDL." Report results with response higher than the highest point on the calibration curve as "greater than x mg/L," where x = concentration corresponding to the highest point on the calibration curve. Report results to two significant figures with units of mg/L. Refer to Sections 1020B and 5020B for more information.

7. Precision and Bias

The single-laboratory LFS recovery and precision data in Table 5560:I were generated by adding a fortifying solution to digester sludge. The sample recovery was over 87% and relative standard deviation less than 7% for acids from acetic through valeric. Data for caproic and heptanoic acids were added to illustrate how the recovery values worsen as the molecular weight of the fatty acids increases. The single-laboratory duplicate sample precision is presented in Table 5560:II. Data for only

Table 5560:I. Single-Laboratory Laboratory-Fortified Sample Recovery and Precision*

Component	Concentration Added *mg/L*	Mean Recovery %	Relative Standard Deviation %
Acetic acid	120	95.2	6.8
Propionic acid	148	93.6	6.4
Isobutyric acid	176	90.3	6.5
Butyric acid	176	89.8	6.6
Isovaleric acid	204	88.9	6.4
Valeric acid	204	87.5	6.4
Isocaproic acid	232	83.2	6.0
Caproic acid	232	81.1	5.9
Heptanoic acid	260	63.9	6.2

* n = 34

Sample source is thermophilic and mesophilic digester sludge from the Los Angeles County Sanitation Districts, Joint Water Pollution Control Plant, Carson, Calif.

Table 5560:II. Single-Laboratory Duplicate Sample Precision*

Component	Mean Percent Difference %	Relative Standard Deviation %
Acetic acid	4.7	5.8
Propionic acid	3.6	4.6

* n = 30 for acetic acid and 25 for propionic acid.

Sample source is thermophilic and mesophilic digester sludge from the Los Angeles County Sanitation Districts, Joint Water Pollution Control Plant, Carson, Calif.

acetic and propionic acids are included because reportable data for other sample components are limited.

To check agreement between this gas chromatographic method and the distillation method, 18 thermophilic digester sludge samples were analyzed by 5710C and D. The results of the gas chromatographic method were converted to acetic acid equivalents for the purpose of comparison. The gas chromatographic method ranged from 118 to 593 mg volatile acids as acetic acid/L and the distillation method ranged from 128 to 610 mg volatile acids as acetic acid/L. The distillation method results ranged from 92 to 123% of the gas chromatographic method results, with a mean of 106% and a standard deviation of 7.7%.

8. Reference

1. U.S. Environmental Protection Agency. 1984. Definition and procedure for the determination of the method detection limit. 40 CFR Part 136, Appendix B. *Fed. Reg.* 49(209).

9. Bibliography

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Chu, A., D.S. Mavinic, W.D. Ramey & H.G. Kelly. 1996. A biochemical model describing volatile fatty acid metabolism in thermophilic aerobic digestion of wastewater sludge. *Water Res.* 30:1759.

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5710 FORMATION OF TRIHALOMETHANES AND OTHER DISINFECTION BYPRODUCTS*

5710 A. Introduction

Trihalomethanes (THMs) are produced during chlorination of water. The four regulated and most commonly occurring THMs are chloroform ($CHCl_3$), bromodichloromethane ($CHBrCl_2$), dibromochloromethane ($CHBr_2Cl$), and bromoform ($CHBr_3$). Additional chlorination byproducts can be formed (including haloacetic acids and halonitriles; for example, see 5710D) during the relatively slow organic reactions that occur between free chlorine and naturally occurring organic precursors such as humic and fulvic acids. The formation potentials of these additional byproducts also can be determined, but different quenching agents and different analytical procedures may be needed. Predictive models for estimating/calculating THM formation exist, but because eventual THM concentrations cannot be calculated precisely from conventional analyses, methods to determine the potential for forming THMs are useful in evaluating water treatment processes or water sources or for predicting THM concentrations in a distribution system.

To obtain reproducible and meaningful results, control such variables as temperature, reaction time, chlorine dose and residual, and pH. THM formation is enhanced by elevated temperatures and alkaline pH and by increasing concentrations of free chlorine residuals, although THM formation tends to level off at free chlorine residuals of 3 mg/L and above; a longer reaction time generally increases THM formation.[1,2]

Low concentrations of bromide exist in most natural waters and are responsible for the formation of brominated organic compounds. Figure 5710:1 shows that an oxidant ratio of about 40 times more chlorine than bromine (on a molar basis, = 40 on the *x* axis) is required to form equimolar amounts of substituted organic chloride and bromide (= 1 on the *y* axis); small amounts of bromide also can increase the molar yield of THMs.[3]

The possible addition of organic precursors contained in reagent solutions cannot be accounted for accurately without a great deal of extra work; therefore, sample dilutions resulting from reagent additions (approximately 2%) are ignored in the final calculations. However, sample dilution may need to be taken into account if other volumes are used. Sample dilution also changes the concentrations of bromide and organic matter, potentially leading to speciation changes.

1. Terminology

See Figures 5710:2a and b for the relationship among the following definitions.

Simulated distribution system trihalomethane (*SDS-THM*) (5710C)—the concentration of TTHMs in a sample that has been disinfected comparably to finished drinking water and under the same conditions and time as in a water distribution system. It includes pre-existing THMs plus those produced during storage. This method can be used with laboratory, pilot, or full-scale studies of treatment processes to estimate expected concentration of THMs in a distribution system. Do not use SDS-THM to estimate the precursor removal efficiency of a treatment process, because THM yields are highly variable at low chlorine residual concentrations. For SDS-type testing, low chlorine residuals (<1 mg Cl_2/L) are often encountered, thereby resulting in lower THM formation than would be obtained for higher chlorine residuals. THM yields at higher chlorine concentrations (>3 mg/L) tend to level off and become relatively independent of variations in free chlorine residuals.

Standard reaction conditions (5710B) are as follows: free chlorine residual at least 3 mg/L and not more than 5 mg/L at the end of a 7-d reaction (incubation) period, with sample incubation temperature of 25 ± 2°C, and pH controlled at 7.0 ± 0.2 with phosphate buffer. Standard conditions are not intended to simulate water treatment processes but are most useful for estimating the concentration of THM precursors, as well as for measuring the effectiveness of water-treatment options for reducing levels of THM precursors in the raw water.

Special applications permit different test conditions, but they must be stated explicitly when reporting results.

Total trihalomethane (*$TTHM_T$*)—the sum of all four THM compound concentrations (5710B.5) produced at any time *T* (usually days). $TTHM_0$ is the total THM concentration at the time of sampling. $TTHM_0$ concentrations can range from nondetectable, which usually means the sample has not been chlorinated, to several hundred micrograms per liter if the sample has been chlorinated. $TTHM_7$ is the sum of all four THM compound concentrations produced during reactions of sample precursors with excess free chlorine over a 7-d reaction time.

Trihalomethane formation potential (*THMFP or Δ THMFP*)—the difference between the final $TTHM_T$ concentration and the initial $TTHM_0$ concentration. If sample does not contain chlorine at the time of collection, $TTHM_0$ will be close to zero and the term THMFP may be used. If sample does contain chlorine at the time of collection, because of formation of THMs, use the term Δ THMFP (the increase of THM concentration during storage) when reporting the difference between TTHM concentrations.

The term "THMFP" often has been equated to the final TTHM concentration, even if the sample had contained chlorine when collected. To use this definition, explicitly define the term when reporting data.

* Approved by Standard Methods Committee, 2010.
Joint Task Group: 20th Edition—Leown A. Moore (chair), James K. Edzwald, Robert C. Hoehn, Bart Koch, Neil M. Ram, James M. Symons.

https://doi.org/10.2105/SMWW.2882.112

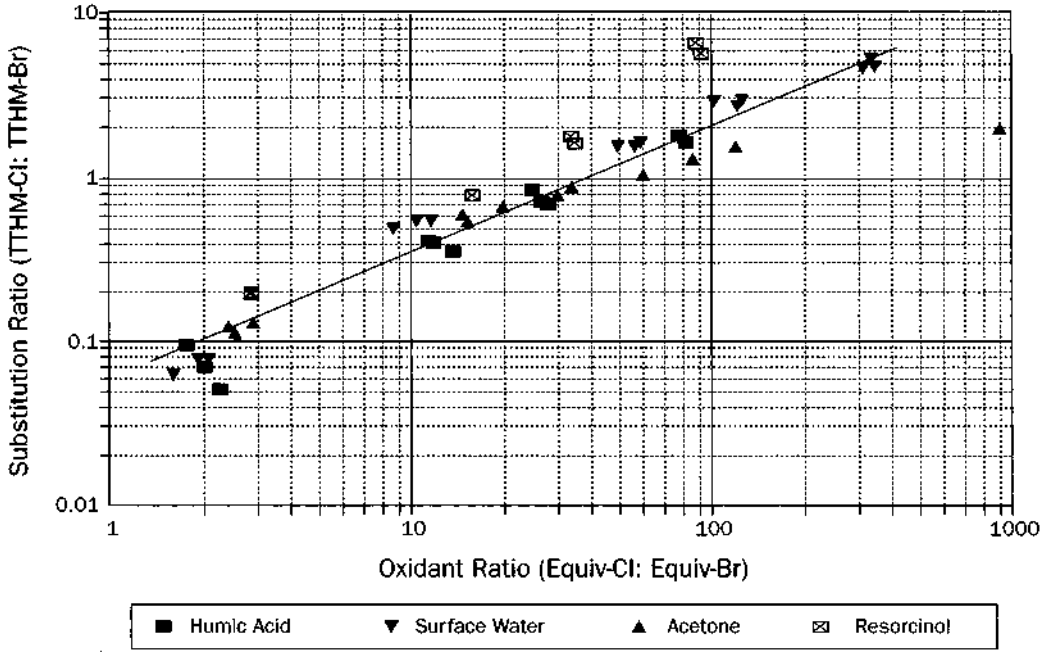


Figure 5710:1. Effect of changing molar oxidant ratios of free chlorine:free bromine on molar ratios of substituted organic chloride:organic bromide, using four different precursor substrates. Reaction times varied between 1 and 7 d. Standard conditions were used at pH=7.0, except that the free chlorine residual after 7 d storage for the surface water was 17 mg/L instead of the 3 to 5 mg/L range for the other three substrates.

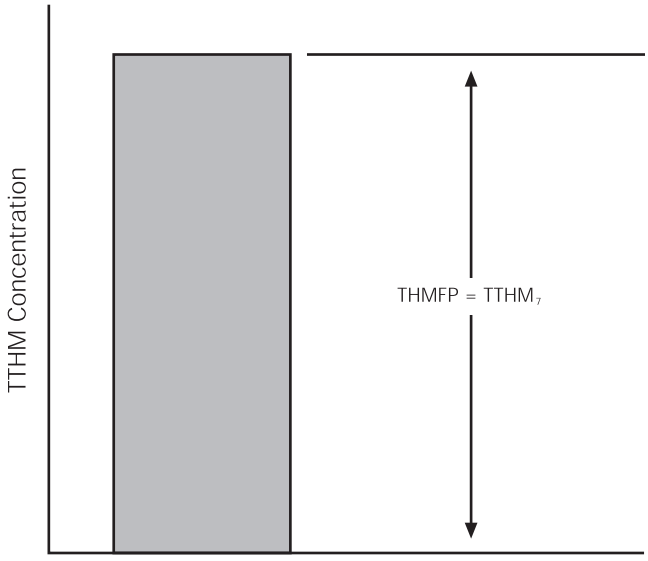


Figure 5710:2a. Relationships between definitions used in the formation potential test, for a sample that did not contain free chlorine at the time of sampling. Total THM concentration at the time of sampling ($TTHM_0$) was very close to or equal to zero; therefore, the THM formation potential for the 7-d reaction time (THMFP, with a free chlorine residual of at least 3 mg/L) was essentially equal to the total THM concentration in the sample at the end of the reaction storage time ($TTHM_7$).

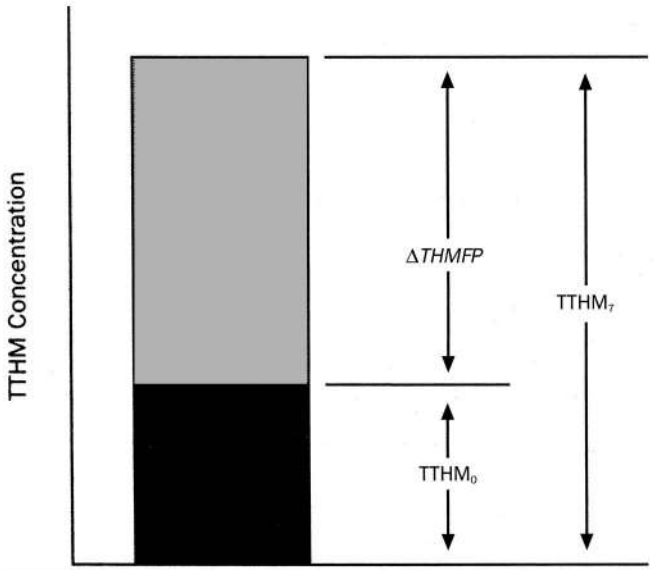


Figure 5710:2b. Relationships between definitions used in the formation potential test, for a sample that already contained free chlorine at the time of sampling. Total THM concentration at the time of sampling ($TTHM_0$) was a significant fraction of the final value obtained after 7-d storage ($TTHM_7$) with an excess of at least 3 mg/L free chlorine. Δ THMFP is the difference between these two values.

2. Sampling and Storage

Collect samples in 1-L glass bottles sealed with TFE-lined screw caps. If multiple tests will be performed for each sample, or if many different analyses will be performed for each sample (see 5710D), collect 4 L instead. Further, if multiple reaction time periods will be used to study reaction rates, process a separate sample bottle (taken from one large sample) for each time period. One liter is enough sample to determine chlorine demand and duplicate THM analyses. Use only freshly collected samples and process immediately. If this is not possible, store samples at 4°C and analyze as soon as possible. Significant sample degradation can occur in unpreserved samples within 24 h.

If the sample has been chlorinated previously, collect the sample with minimum turbulence and fill the sample bottle completely to avoid loss of THMs already present. Determine the zero-time THM concentration ($TTHM_0$), if desired, on another sample collected at the same time and dechlorinated immediately with fresh sodium sulfite solution, crystals, or sodium thiosulfate.

3. References

1. Stevens, A.A. & J.M. Symons. 1977. Measurement of trihalomethanes and precursor concentration changes. *J. Amer. Water Works Assoc.* 69:546.
2. Symons, J.M., A.A. Stevens, R.M. Clark, E.E. Geldreich, O.T. Love, Jr. & J. DeMarco. 1981. Treatment Techniques for Controlling Trihalomethanes in Drinking Water; EPA-600/2-81-56. U.S. Environmental Protection Agency, Cincinnati, Ohio.
3. Symons, J.M., S.W. Krasner, L. Simms & M. Sclimenti. 1993. Measurement of trihalomethane and precursor concentrations revisited: The impact of bromide ion. *J. Amer. Water Works Assoc.* 85(1):51.

5710 B. Trihalomethane Formation Potential (THMFP)

1. General Discussion

a. Principle: Under standard conditions, samples are buffered at pH 7.0 ± 0.2, chlorinated with an excess of free chlorine, and stored at 25 ± 2°C for 7 d to allow the reaction to approach completion. As a minimum, pH is buffered at a defined value and a free chlorine residual of 3 to 5 mg Cl_2/L exists at the end of the reaction time. THM concentration is determined by using liquid–liquid extraction (see Section 6232B) or purge and trap (see Section 6200).

b. Interference: If the water was exposed to free chlorine before sample collection (e.g., in a water treatment plant), a fraction of precursor material may have been converted to THM. Take special precautions to avoid loss of volatile THMs by minimizing turbulence and filling sample bottle completely.

Interference will be caused by any organic THM-precursor materials present in the reagents or adsorbed on glassware. Heat nonvolumetric glassware to 400°C for 1 h, unless routine analysis of blanks demonstrates that this precaution is unnecessary. Reagent impurity is difficult to control. It usually is traceable to reagent water containing bromide ion or organic impurities. Use high-grade reagent water as free of organic contamination and chlorine demand as possible. If anion exchange is used to remove bromide or organic ions, follow such treatment by activated carbon adsorption (see Section 1080).

Other interferences include volatile organic compounds (VOCs), including THMs and chlorine-demanding substances. VOCs may co-elute with THMs during analysis. THMs or other interfering substances that are present as the result of a chemical spill, etc., will bias the results.

Nitrogenous species and other constituents may interfere in the determination of free residual chlorine. Add enough free chlorine to oxidize chorine-demanding substances and leave a free chlorine residual of at least 3 mg/L, but not more than 5 mg/L, at the end of the incubation period. A free chlorine residual of at least 3 mg/L decreases the likelihood that a combined residual will be mistaken for a free residual and assures that THM formation occurs under conditions that are reasonably independent of variations in chlorine residual concentrations.

c. Minimum detectable quantity: The sensitivity of the method is determined by the analytical procedure used for THM.

2. Apparatus

a. Incubator, to maintain temperature of 25 ± 2°C.

b. Bottles, glass, with TFE-lined screw caps to contain 245 to 255 mL, 1-L, 4-L.

c. Vials, glass, 25- or 40-mL with TFE-lined screw caps.

d. pH meter, accurate to within ± 0.1 unit.

3. Reagents

Prepare aqueous reagents in organic-free water (¶ *e* below) unless chlorine-demand-free water (¶ *f* below) is specified.

a. Standardized stock hypochlorite solution: Dilute 1 mL, using a 1-mL volumetric pipet, 5% aqueous sodium hypochlorite (NaOCl, to be referred to as stock hypochlorite) solution to approximately 25 mL with chlorine-demand-free water (see ¶ *f* below), mix well, and titrate to a starch-iodide endpoint using 0.100*N* sodium thiosulfate titrant (see Section 4500-Cl.B). Calculate chlorine concentration of the stock hypochlorite solution as:

$$\text{Stock hypochlorite, mg } Cl_2\text{/mL} = \frac{N \times 35.45 \times \text{mL titrant}}{\text{mL stock hypochlorite added}}$$

where N is the normality of the titrant (= 0.100). Use at least 10 mL titrant; if less is required, standardize 2 mL stock hypochlorite solution. Measure chlorine concentration each time a dosing solution (¶ *b* below) is made; discard stock hypochlorite solution if its chlorine concentration is less than 20 mg Cl_2/mL.

b. Chlorine dosing solution, 5 mg Cl_2/mL: Calculate volume of stock hypochlorite solution required to produce a chlorine concentration of 5 mg Cl_2/mL:

$$\text{mL required} = \frac{1250}{\text{stock hypochlorite conc, mg } Cl_2\text{/mL}}$$

Dilute this volume of stock hypochlorite solution in a 250-mL volumetric flask to the mark with chlorine-demand-free water. Mix and transfer to an amber bottle, seal with a TFE-lined screw cap, and refrigerate. Keep away from sunlight. Discard if the chlorine concentration drops below 4.7 mg Cl_2/mL; this will occur if the "initial chlorine concentration" (as determined in 5710B.4*a*) drops below 94 mg Cl_2/L.

c. Phosphate buffer: Dissolve 68.1 g potassium dihydrogen phosphate (anhydrous), KH_2PO_4, and 11.7 g sodium hydroxide, NaOH, in 1 L water. Refrigerate when not in use. If a precipitate develops, filter through a glass fiber filter. After buffer is added to a sample, a pH of 7.0 should result. Check before use with a sample portion that can be discarded.

d. Sodium sulfite solution: Dissolve 10 g sodium sulfite, Na_2SO_3, in 100 mL water. Use for dechlorination: 0.1 mL will destroy about 5 mg residual chlorine. Make fresh every 2 weeks. NOTE: More dilute solutions oxidize readily.

e. Organic-free water: Pass distilled or deionized water through granular-activated-carbon columns. A commercial system may be used.* Special techniques such as preoxidation, activated carbon adsorption (perhaps accompanied by acidification and subsequent reneutralization), or purging with an inert gas to remove THMs may be necessary.

f. Chlorine-demand-free water: Follow the procedure outlined in 4500-Cl.C.3*m*, starting with organic-free water. After residual chlorine has been destroyed completely, purge by passing a clean, inert gas through the water until all THMs have been removed.

g. DHBA solution: Dissolve 0.078 g anhydrous 3,5-dihydroxy-benzoic acid (DHBA) in 2 L chlorine-demand-free water. This solution is not stable; make fresh before each use.

h. Nitric or hydrochloric acid (HNO_3 or HCl), concentrations of 1:1, 1.0*N*, and 0.1*N*.

i. Sodium hydroxide (NaOH), 1.0*N* and 0.1*N*.

j. Borate buffer (optional): Dissolve 30.9 g anhydrous boric acid, H_3BO_3, and 10.8 g sodium hydroxide, NaOH, in 1 L water. Filter any precipitate that may form with a glass fiber filter. This solution will keep sample pH at 9.2; check before using. NOTE: Waters containing significant amounts of calcium may precipitate calcium phosphate (or carbonate) at higher pH values.

k. Mixed buffer (optional): Mix equal amounts of phosphate and borate buffer solutions, then adjust pH as desired before using with samples. Determine the amount of acid or base needed on a separate sample that can be discarded. This mixed buffer is reasonably effective in the pH range of 6 to 11.

4. Procedure

a. Chlorine demand determination: Determine or accurately estimate the 7-d sample chlorine demand. A high chlorine dose is specified below to drive the reaction close to completion quickly. The following procedure yields only a rough estimate of chlorine demand; other techniques may be used.

Pipet 5 mL chlorine dosing solution into a 250-mL bottle, fill completely with chlorine-demand-free water, and cap with a TFE-lined screw cap. Shake well. Titrate 100 mL with 0.025*N* sodium thiosulfate to determine the initial chlorine concentration (C_I). This should be about 100 mg Cl_2/L. Pipet 5 mL phosphate buffer and 5 mL chlorine dosing solution into a second 250-mL bottle, fill completely with sample, and seal with a TFE-lined screw cap. Store in the dark for at least 4 h at 25°C. After storage, determine chlorine residual (C_R). Calculate chlorine demand (D_{Cl}) as follows:

$$D_{Cl} = C_I - C_R$$

where:

D_{Cl} = chlorine demand, mg Cl_2/L,
C_I = initial (dosed) chlorine concentration, mg Cl_2/L, and
C_R = chlorine residual of sample after at least 4 h storage, mg Cl_2/L.

b. Sample chlorination: If sample contains more than 200 mg/L alkalinity or acidity, adjust pH to 7.0 ± 0.2 using 0.1 or 1.0*N* HNO_3, HCl, or NaOH and a pH meter. With a graduated pipet, transfer appropriate volume of the 5 mg Cl_2/mL chlorine dosing solution, V_D, into sample bottle:

$$V_D = \frac{D_{Cl} + 3}{5} \times \frac{V_S}{1000}$$

where:

V_D = volume of dosing solution required, mL, and
V_S = volume of sample bottle, mL.

Add 5 mL phosphate buffer solution if using a 250-mL sample bottle (or 1 mL buffer/50 mL sample) and fill completely with sample. Immediately seal with a TFE-lined screw cap, shake well, and store in the dark at 25 ± 2°C for 7 d. Analyze a reagent blank (¶ *d* below) with each batch of samples. To increase the likelihood of achieving the desired chlorine residual concentration (3 to 5 mg/L) at the end of the 7-d reaction period, dose several sample portions to provide a range of chlorine concentrations, with each chlorine dose differing in increments of 2 mg Cl_2/L.

c. Sample analysis: After the 7-d reaction period, place 0.1 mL sulfite reducing solution in a 25-mL vial and gently and completely fill vial with sample. If free chlorine residual has not been determined previously, measure it using a method accurate to 0.1 mg/L and able to distinguish free and combined chlorine (see Section 4500-Cl). Adjust pH to the value required by the method chosen for chlorine analysis. [NOTE: If other byproducts are to be measured, a different quenching agent may be needed (see 5710D.4). Also, if sample portions have been dosed with different chlorine concentrations, first determine the free chlorine residual and select only that portion having the required chlorine residual concentration of 3 to 5 mg/L for further processing.] If THMs will not be analyzed immediately, lower the pH to <2 by adding 1 or 2 drops of 1:1 HCl to the reduced sample in the

* Milli-Q, Millipore Corp., or equivalent.

TABLE 5710:I. SINGLE-OPERATOR PRECISION AND BIAS DATA FOR THMFP*

Sample	THM $\mu g/L$				THMFP $\mu g/L$ as $CHCl_3$	Recovery %
	$CHCl_3$	$CHCl_2Br$	$CHClBr_2$	$CHBr_3$		
Blank 1	0.8	—	—	—	0.8	—
Blank 2	1.9	—	—	—	1.9	—
Blank 3	0.1	0.1	—	—	0.2	—
Blank 4	0.7	—	—	—	0.7	—
Blank 5	0.5	—	—	—	0.5	—
Blank 6	0.7	—	—	—	0.7	—
Average					0.8	—
Standard deviation					±0.6	—
DHBA 1	114.1	0.1	—	—	114.2	97.8
DHBA 2	113.2	—	—	—	113.2	96.9
DHBA 3	107.8	—	—	—	107.8	92.2
DHBA 4	108.3	—	—	—	108.3	92.7
DHBA 5	109.6	0.1	—	—	109.7	93.9
DHBA 6	111.8	0.1	—	—	111.9	95.8
DHBA 7	112.6	—	—	—	112.6	96.4
Average					111.1†	95.1
Standard deviation					±2.5	2.2

* Source: MOORE, L., Unpublished data. U.S. Environmental Protection Agency, Cincinnati, Ohio.
† Expected value = 116.

vial. Seal vial with TFE-lined screw cap. Store samples at 4°C until ready for THM analysis (preferably no longer than 7 d). Let sample reach room temperature before beginning analysis.

d. Reagent blank: Add 1 mL chlorine dosing solution to 50 mL phosphate buffer, mix, and completely fill a 25-mL vial, seal with a TFE-lined screw cap, and store with samples. (NOTE: This reagent blank is for quality control of reagent solutions only and is not a true blank, because the reagent concentrations in this blank are considerably higher than those in samples. THM concentrations in the reagent blank will be biased high and cannot be subtracted from sample values. Make no further dilutions before the reaction because the reagent water itself might contribute to THM formation.) After reaction for 7 d, pipet 1 mL sulfite reducing solution into a 250-mL bottle and add, without stirring, 5.0 mL reacted reagent mixture. Immediately fill bottle with organic-free water that has been purged free of THMs and seal with a TFE-lined screw cap. Mix. Analyze a portion of this reagent blank for THMs using the same method used for samples. The sum of all THM compounds in the reagent blank should be less than 5 μg TTHM as $CHCl_3$/L.

The reagent blank is a rough measure of THMs contributed by reagents added to the samples, but it cannot be used as a correction factor. If the reagent blank is greater than 5% of the sample value or greater than 5 μg TTHM/L, whichever is larger, additional treatment for reagent water is necessary. See Section 1080. It also may be necessary to obtain reagents of higher purity. Analyze a reagent blank each time samples are analyzed and each time fresh reagents are prepared.

5. Calculation

Report concentration of each of the four common THM compounds separately because it is desirable to know their relative concentrations. Larger amounts of bromine-substituted compounds, relative to chlorine-substituted compounds, indicate a higher concentration of dissolved bromide in the water (see Figure 5710:1). Also report free chlorine concentration at end of reaction time along with the incubation time, temperature, and pH.

THM concentrations may be reported as a single value as micrograms $CHCl_3$ per liter (μg $CHCl_3$/L), or micromoles per liter (μM). Do not use the simple sum of mass units micrograms per liter except when required for regulatory reporting. Compute TTHM concentration using one of the following equations:

To report TTHM in units of μg $CHCl_3$/L:

$$TTHM = A + 0.728B + 0.574C + 0.472D$$

where:

A = μg $CHCl_3$/L,
B = μg $CHBrCl_2$/L,
C = μg $CHBr_2Cl$/L, and
D = μg $CHBr_3$/L.

To report TTHM in units of μM as $CHCl_3$:

$$TTHM = \frac{TTHM, \mu g\ CHCl_3/L}{119}$$

To report TTHM on a weight basis as μg/L (not used except for regulatory purposes):

$$TTHM = A + B + C + D$$

To report a change of TTHM concentration over 7 d:

$$\Delta\ THMFP = TTHM_7 - TTHM_0$$

TABLE 5710:II. SINGLE-OPERATOR PRECISION AND BIAS DATA FOR TTHM (pH = 9.2)*

Sample	THM $\mu g/L$				TTHM $\mu g\ CHCl_3/L$	Recovery %
	$CHCl_3$	$CHCl_2Br$	$CHClBr_2$	$CHBr_3$		
Blank 1	3.0	0.3	—	—	3.2	—
Blank 2	1.7	0.1	—	—	1.8	—
Blank 3	1.3	0.1	—	—	1.4	—
Blank 4	1.6	0.1	—	—	1.7	—
Blank 5	2.3	0.2	—	—	2.4	—
Blank 6	2.6	0.1	—	—	2.7	—
Blank 7	2.5	0.2	—	—	2.6	—
Average					2.3	—
Standard deviation (relative standard deviation)					± 0.6 (± 26.1%)	
DHBA 1	45.4	3.3	0.1	—	47.9	98.3
DHBA 2	51.0	3.9	0.1	—	53.9	111.2
DHBA 3	39.2	3.0	0.1	—	41.4	84.3
DHBA 4	48.3	3.6	0.1	—	51.0	105.0
DHBA 5	47.6	3.7	0.1	—	50.4	103.7
DHBA 6	43.4	3.2	0.1	—	45.8	93.8
DHBA 7	46.0	3.6	0.1	—	48.7	100.0
Average					48.4	99.5
Standard deviation (relative standard deviation)					± 3.7 (± 7.6%)	
RWS 1†	33.1	17.2	11.3	0.5	52.3	—
RWS 2	31.7	16.1	10.6	0.5	49.7	—
RWS 3	38.7	18.4	11.7	0.6	59.1	—
RWS 4	35.1	18.0	11.7	0.8	55.3	—
RWS 5	36.0	17.9	11.7	0.6	56.0	—
RWS 6	38.7	18.7	11.7	0.6	59.3	—
RWS 7	37.7	18.1	11.2	0.6	57.6	—
Average					55.6	
Standard deviation (relative standard deviation)					± 3.3 (± 5.9%)	

* Source: MOORE, L., Unpublished data. U.S. Environmental Protection Agency, Cincinnati, Ohio.
† RWS = raw water sample, filtered and diluted 1 part filtrate, 2 parts organic-free water.

Finally, if $TTHM_0 = 0$, then:

$$THMFP = TTHM_7 = \Delta\, THMFP$$

Do not make blank correction or a correction for sample dilution resulting from addition of reagents. If conditions differ from pH of 7, 25°C, 7-d reaction time, and 3 to 5 mg/L chlorine residual, report these nonstandard test conditions with the results. Nonstandard test conditions may mimic water quality conditions in a specific distribution system or may be relevant to other investigations (see 5710C).

6. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 5020:I.

a. Use dihydroxy-benzoic acid solution (DHBA) as a quality-control check, especially for the presence of interfering bromides in reagents or reagent water.

Dilute 1.0 mL chlorine dosing solution to 1000 mL with chlorine-demand-free water (diluted chlorine dosing solution). Pipet 5 mL phosphate buffer solution (pH = 7.0) into each of two 250-mL bottles; add 1.00 mL DHBA solution to one bottle and fill both bottles completely with diluted chlorine dosing solution; seal with TFE-lined screw caps. Store in the dark for 7 d at 25 ± 2°C, and analyze as directed in 5710B.4*c*.

b. The THM concentration of the solution containing the added DHBA minus the THM concentration of the blank (i.e., the bottle that does not contain added DHBA, which is a true blank for this application only and differs from the reagent blank discussed in 5710B.4*d*) should be about 119 μg/L THM as $CHCl_3$, with essentially no contribution from bromide-containing THMs. If there is a significant contribution from brominated THMs, 10% or more of the total THM, it may be necessary to remove bromide from the reagent water or to obtain higher-purity reagents containing less bromide. Determine source of bromide and correct the problem. If the THM concentration of the water blank exceeds 20 μg/L, treat reagent water to reduce contamination.

7. Precision and Bias

The precision of this method is determined by the analytical precision and bias of the method used for measuring THM as well as the control of variables such as pH, chlorine residual,

temperature, sample homogeneity, etc. Method bias can be determined only for synthetic solutions (e.g., the DHBA solution), because THM formation potential is not an intrinsic property of the sample but rather a quantity defined by this method.

Table 5710:I presents single-operator precision and bias data for samples processed under standard conditions. The values were obtained by analyzing DHBA solutions and blanks. The expected value for the samples listed is 116 μg/L TTHM (as $CHCl_3$), rather than 119 μg/L, because the DHBA reagent used was only 97% pure. Percent recovery was calculated by the formula:

$$\% \text{ recovery} = \frac{\text{DHBA sample} - \text{average blank}}{116} \times 100$$

Table 5710:II presents the same data set, except that the pH of samples and blanks was adjusted to 9.2 with borate buffer. Also included are results for single-operator precision with filtered river-water samples that had been diluted with 2 parts organic-free water to 1 part filtrate, again using borate-buffered samples at a pH of 9.2.

5710 C. Simulated Distribution System Trihalomethanes (SDS-THM)

1. General Discussion

Principle: The SDS-THM testing method uses bench-scale techniques to provide an estimate of the THMs formed in a distribution system after disinfection.[1] It may be used to estimate the THM concentration at any point in a distribution system or to evaluate the formation of other disinfectant byproducts (5710D). However, to measure efficiency of any unit treatment process for precursor removal, see 5710B.

The term "disinfection," rather than "chlorination," is used because free chlorine residuals are not necessarily provided in all distribution systems. For example, monochloramine residuals may be used.

SDS-THM concentrations measured by this procedure generally will be lower than THM concentrations measured by procedures in 5710B because disinfectant concentrations used in SDS-type samples are intended to mimic conditions in a distribution system and are almost always lower than disinfectant concentrations used with standardized formation potential procedures.

Two types of SDS procedures may be used:

(1) a simple storage method that requires only the addition, at the end of the desired storage time, of a quenching agent, sodium sulfite, to a sample collected from the entry to a distribution system; and
(2) a comprehensive method that involves one or more steps described in 5710B, with appropriate modifications.

SDS procedures are not standard procedures in the traditional sense. Test variables are modified to mimic local distribution-system conditions with bench-top procedures. These conditions include temperature, pH, disinfectant dose and residual, bromide ion concentration, and reaction time (corresponding to the residence time of water within the distribution system). However, the method used to simulate a distribution system can be standardized according to specific needs.

2. Apparatus

See 5710B.2.

Temperature control: Appropriate equipment, such as a water bath or incubator, to control sample storage temperature, capable of a range of temperature adjustments required.

3. Reagents

See 5710B.3, and also 5710D.3, if applicable.

4. Procedure

a. Simple storage procedure: Measure and report both initial and final values for all variables, especially pH, temperature, $TTHM_0$ (if desired), and residual disinfectant concentration using a method accurate to 0.1 mg/L and able to distinguish among the various disinfectant forms—see Section 4500-Cl for chlorine analyses.

Collect treated, disinfected water from the clearwell of a treatment plant or other suitable location in either a 1-L or a 250-mL completely filled bottle. Seal with a TFE-lined screw cap and store at selected temperature for selected length of time. The storage time frequently chosen is the maximum residence time within the distribution system, and the temperature is representative of the distribution system.

A second sample for immediate quenching provides an initial THM concentration if desired ($TTHM_0$). Quench by adding 2 drops (0.1 mL) sodium sulfite solution to a 25-mL glass vial, and gently and completely fill vial with sample. [NOTE: If other byproducts will be analyzed, a different quenching agent may be needed (see 5710D.4).]

At end of storage period, quench a portion of stored sample with sodium sulfite solution. Report all values (pH, temperature, and residual disinfectant concentration) together with THM results. Ideally, residual disinfectant concentration after storage equals residual disinfectant concentration found in the distribution system.

b. Bench-top procedure: Use any or all steps given in 5710B, except that all variables, such as disinfectant dose, residual concentrations, temperature, pH, and storage time are adjusted to simulate distribution system conditions. For better sample pH control, use a buffer such as the mixed-buffer solution (5710B.3*k*). If a buffer is used, adjust buffer pH to the appropriate value before adding to the sample. Add buffer to a sample portion to be discarded after titrating to the desired pH with either NaOH or HCl solution; add this determined amount of acid or base to the buffer solution before adding to actual samples. When measuring disinfectant residuals, adjust sample

pH to that required by the analytical method for the residual disinfectant, because the buffer capacity of the sample may be greater than the amount of buffer required by the method. For example, DPD Methods 4500-Cl.F and 4500-Cl.G require control of the pH within the range from 6.2 to 6.5 for stable color development, but the sample pH might be buffered to a value of 8.3, requiring adjustment of the sample pH to the appropriate range by addition of mineral acid before color development with DPD reagents.

Also add disinfectant to sample if it does not already contain enough to provide the desired disinfectant residual for the chosen storage time and temperature. Use the chlorine demand procedure (5710B.4*a*) as a guide, or develop correlations between disinfectant use and TOC or other variables.

Process sample generally following procedures in 5710B. Measure and report both initial and final values of all test variables, especially temperature, pH, and residual disinfectant concentration, as well as all THM results.

5. Calculation

See 5710B.5. Report $TTHM_T$ values for SDS-type samples in any appropriate units, except that the concept of "formation potential" is not applicable. Use prefix "SDS-" to distinguish between "SDS-TTHM" and "THMFP" results. Also report each compound concentration (e.g., "μg/L SDS_T-$CHCl_3$").

6. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 5020:I.

See 5710B.6 for check on reagent purity or as a check on analytical precision and control by using a reaction with a pure, organic compound under more stable, standardized conditions. (This applies only to the formation of THM, using free chlorine.)

7. Precision and Bias

No data are available. More variability of results is expected for SDS-type testing than for samples tested with the standard conditions of 5710B. A larger number of replicates will be required for SDS procedures, as opposed to standard conditions, to obtain reliable estimates of effects of treatment changes and techniques.

8. Reference

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5710 D. Formation of Other Disinfection Byproducts (DBPs)

1. General Discussion

Principle: The techniques and principles discussed in 5710A–C may be applied to other disinfection byproducts (DBPs) and total/dissolved organic halogen (TOX/DOX) as well as for trihalomethanes (THMs). Although all the DBPs listed in this method may result from chlorination reactions, some may be formed by disinfectants other than free chlorine.

This method may be extended to cover formation potentials (5710B) and distribution system simulations (5710C) for additional byproducts formed by reactions between other disinfectants (ozone, chlorine dioxide, chloramines, etc.) with dissolved organic matter. Some of the commonly found DBPs are listed in this method, but many cannot be determined because well-defined analytical methods are not yet available.

The procedures by which formation potentials and behavior in distribution systems of other DBPs can be measured are exactly as described in 5710A–C, except that different quenching agents may be required for different compounds.

In general, byproduct concentrations increase with reaction time, but exceptions exist and different variables may produce different results. For example, at high pH values, THM concentrations increase with time, but if the pH is high enough, trichloroacetic acid will not form at all; at high pH, however, the concentrations of dihaloacetonitriles (DHANs) quickly reach a maximum value in a relatively short time and then decrease because of hydrolysis reactions. Further, some compounds, such as brominated haloacetic acids, are not stable and can degrade during storage—either during a long reaction time (7 d may be too long for some compounds) or after the reaction has been quenched (even if stored at 4°C).

Small concentrations of bromide ion may have significant effects. If a compound contains more than one halogen atom in its molecular formula, compounds containing all the possible combinations of mixed chloro-/bromo- formulas might also be formed.

The most common other disinfection byproducts are: trihaloacetic acids (THAAs), including trichloroacetic acid (TCAA), bromodichloroacetic acid (BDCAA), dibromochloroacetic acid (DBCAA), and tribromoacetic acid (TBAA); dihaloacetic acids (DHAAs), including dichloroacetic acid (DCAA), bromochloroacetic acid (BCAA), and dibromoacetic acid (DBAA); monohaloacetic acids (MHAAs), including monochloroacetic acid (MCAA) and monobromoacetic acid (MBAA); chloral hydrate (CH); dihaloacetonitriles (DHANs), including dichloroacetonitrile (DCAN), bromochloroacetonitrile (BCAN), and dibromoacetonitrile (DBAN); 1,1,1-trichloropropanone (111-TCP); chloropicrin (CP), which may be produced either with free or combined chlorine; cyanogen chloride, formed either with free chlorine or monochloramine (and is more stable in solutions containing monochloramine); and dissolved organic halide (DOX) (see Section 5320). Total organic halide (TOX) also may be determined if the sample is not filtered.

The present method differs from those described in 5710B and C in the means by which reactions are quenched: the different byproducts formed require different quenching agents to stop reactions. Sodium sulfite is used to quench the THM reaction, but it may react with and degrade other compounds formed, such as DHANs. The procedure below lists the recommended quenching steps. Several portions of the same sample may require different quenching agents, depending upon the byproducts to be determined. Use appropriate analytical methods to determine the different types of byproducts formed.

2. Apparatus

See 5710B.2.

Vials: 40-mL glass vials with TFE-lined screw caps.

3. Reagents

In addition to the reagents listed in 5710B.3, the following reagents also may be needed, depending upon the byproducts to be measured:

a. Ammonium chloride solution: Weigh 5 g NH_4Cl and dissolve in 100 mL organic-free water.

b. Nitric acid solution (approx. 3.5*N*): Dilute 80 mL conc HNO_3 (**CAUTION: strong oxidant**) to 250 mL with organic-free water.

c. Methyl tert*-butyl ether* (*MtBE*), highest purity.

d. Other reagents: Those required by analytical methods for determination of specific byproduct concentrations.

4. Procedure

Procedures for the formation of byproducts during reactions between disinfectants and dissolved organic matter have been described in 5710A–C, but the quenching agents needed to stop these reactions depend on the specific compounds to be analyzed. For THMs, the quenching agent is sodium sulfite, and its use has already been described (5710B.4*c*). For the other byproducts listed above, substitute as described below. Store all samples headspace-free and sealed with TFE-lined screw caps.

a. Chloral hydrate (CH): This compound may be analyzed with THMs. Use sulfite reducing solution to quench. Adjust sample pH 6 to 7 with (1.0*N* or 0.1*N*) HCl. Determine amount of acid to be added with a separate portion containing the same amount of sulfite reducing solution added to sample. Analyze by liquid-liquid extraction, capillary column, GC/electron capture analysis.[1]

b. DHANs, CP, and 111-TCP: Add 4 drops (0.2 mL) ammonium chloride solution to a 40-mL vial and nearly fill with sample. Add a predetermined amount of HCl that will adjust sample pH to 6 to 7 (see ¶ *a* above) and fill completely. (Add 3 drops of phosphate buffer solution for more control of sample pH, if desired, before determining amount of acid solution needed for pH adjustment.) NH_4Cl quenches the reaction by converting free chlorine to monochloramine. Analyze by liquid-liquid extraction, capillary column, GC/electron capture analysis.[1]

c. Haloacetic acids (HAAs), mono-, di-, and trihaloacetic acids: Add 4 drops (0.2 mL) NH_4Cl solution to a 250-mL bottle, and fill bottle completely with sample. Before acidifying and extracting sample, add 1 mL sodium sulfite solution to the 250-mL sample, mix well, and analyze by liquid-liquid extraction, capillary column, GC/electron capture analysis (see Section 6251 or equivalent methods[2]). Sodium sulfite can slowly degrade some of the brominated haloacetic acids during storage; do not add until just before acidification. If a GC/MS method is used, remove water in the sample by drying with sodium sulfate crystals before methylation.

d. Cyanogen chloride: Analyze by GC/MS purge and trap method,[3] which uses ascorbic acid to dechlorinate samples. Also see Section 4500-CN.J. Hydrolysis of cyanogen chloride to cyanate occurs rapidly in the pH range of 8.5 to 9.0 (within 30 min), but the reaction is much slower at pH values of 7.0 and below.

e. DOX: Add 1 mL sodium sulfite solution to a 250-mL bottle and nearly fill with sample. Add sufficient 3.5*N* HNO_3 to lower pH to 2.0 (approximately 12 drops, or 0.6 mL) and fill completely. Analyze according to Section 5320. Total organic halogen (TOX) also may be determined by the same method if the sample is not filtered.

f. Other disinfection byproducts not mentioned above: Disinfectants, such as chlorine, ozone, monochloramine, chlorine dioxide, etc., may form other disinfection byproducts. Formation potentials or SDS-type data also can be determined for these additional compounds.

5. Calculation

Report concentrations of each compound separately in μg/L. Compare concentrations of different compounds on a micromolar basis (micromoles/L, or μM):

$$\text{Compound, } \mu M = \frac{\text{compound concentration, } \mu\text{g/L}}{MW}$$

where:

MW = molecular weight of the compound.

Concentrations of a series of compounds that can be grouped together may sometimes be reported as one value. For example, the dihaloacetic acid group (DHAAs) includes DCAA, BCAA and DBAA and may be reported in terms of a group concentration, obtained by adding the molar concentrations of the separate compounds within the class:*

$$\text{DHAA, } \mu M = \frac{\text{DCAA, } \mu\text{g/L}}{129} + \frac{\text{BCAA, } \mu\text{g/L}}{173} + \frac{\text{DBAA, } \mu\text{g/L}}{219}$$

Alternatively, report in terms of μg/L as DCAA by multiplying the molar concentration by the molecular weight of DCAA:

$$\text{DHAA, } \mu\text{g/L as DCAA} = \text{DHAA, } \mu M \times 129$$

or, for SDS testing:

$$\text{SDS-DHAA, } \mu\text{g/L as DCAA} = \text{SDS-DHAA, } \mu M \times 129$$

The definitions given in 5710A–C also are valid. For example, if the initial concentration of disinfectant byproducts (DHAAs, for instance) is zero or insignificant, then:

* Federal regulations may require a simple sum in terms of mass units/L.

$$DHAAFP = DHAA_7.$$

Alternatively, if there is a significant concentration of initial byproduct, then:

$$\Delta DHAAFP = DHAA_7 - DHAA_0.$$

6. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 5020:I.

See 5710B.6 for check on reagent purity or as a check on analytical precision and control by using a reaction with a pure, organic compound under more stable, standardized conditions. The test detailed in 5710B.6 applies only to THM formation using excess free chlorination conditions.

7. Precision and Bias

Precision and bias measurements depend, in part, on the analytical procedure used to measure each specific disinfectant byproduct concentration. These measurements also depend upon compound properties such as stability toward oxidation and biodegradation. In general, however, formation potential reactions should be reproducible to the extent indicated in 5710B for chlorination reactions. SDS-type reactions (5710C) would not, however, be expected to be as accurate or as precise, although such reactions should predict distribution system concentrations reasonably well.

8. References

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3. Flesch, J. & P. Fair. 1988. The analysis of cyanogen chloride in drinking water. Proc. 16th Annu. AWWA Water Quality Technology Conf., Nov. 13–17, 1988, St. Louis, Mo., p. 465. American Water Works Assoc., Denver, Colo.

5910 UV-ABSORBING ORGANIC CONSTITUENTS*

5910 A. Introduction

1. Applications

Because some organic compounds commonly found in water and wastewater (e.g., lignin, tannin, humic substances, and various aromatic compounds) strongly absorb ultraviolet (UV) radiation, UV absorption is a useful surrogate measure of such constituents in fresh waters,[1–3] salt waters,[4–6] and wastewater.[7,8] Strong correlations may exist between UV absorption and organic carbon content, color, and precursors of trihalomethanes (THMs) and other disinfection byproducts.[9,10] UV absorption also has been used to monitor industrial wastewater effluents[11] and to evaluate the effectiveness of coagulation,[10] carbon adsorption,[12–14] and other water treatment processes that remove organics.[10,15] Specific absorption (the ratio of UV absorption to organic carbon concentration) has been used to characterize natural organic matter.[10,16–18] Some regulatory programs include specific ultraviolet absorbance (SUVA)—a ratio of UV absorption to dissolved organic carbon concentration—to help control disinfection byproducts.

Although UV absorption can be used to detect certain individual organic contaminants after separation (e.g., by HPLC), as described in Part 6000, the method described here is not suitable for detecting trace concentrations of individual chemicals. Rather, it provides an *indication* of the aggregate concentration of UV-absorbing organic constituents.

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* Approved by Standard Methods Committee, 2013.
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5910 B. Ultraviolet Absorption Method

1. General Discussion

a. Principle: UV-absorbing organics in a sample absorb UV light in proportion to their concentration. Filter sample to control particle-related variations in UV absorption. The sample's pH can be adjusted before filtration, but certain organic constituents (e.g., humic substances) may be lost to filters, suspended particles, or vessel walls at lower pH values.[1] When filtering samples for SUVA determination, do not adjust pH before filtration.

UV absorption historically has been measured at 253.7 nm (often rounded to 254 nm), but the choice of wavelength is arbitrary. Experienced analysts may choose a wavelength that minimizes interferences while maximizing absorption by the compound(s) of interest. If using a wavelength other than 253.7 nm, state that wavelength when reporting results.

b. Interferences: The primary interferences in UV-absorption measurements are from colloidal particles and UV-absorbing inorganics—notably iron, nitrate, nitrite, and bromide. Certain oxidants and reducing agents (e.g., ozone, chlorate, chlorite, chloramines, and thiosulfate) also absorb ultraviolet light at 253.7 nm. Many natural waters and waters processed in drinking water treatment plants have been shown to be free of these interferences.

Evaluate and correct for UV absorption contributed by specific interfering substances. If cumulative corrections exceed 10% of the total absorption, select another wavelength and/or method. Also, UV absorption by organic matter may vary when pH is below 4 or above 10, so avoid these ranges.[2]

A UV absorption scan from 200 to 400 nm can be used to determine the presence of interferences. Typical absorption scans of natural organic matter are featureless curves of increasing absorption with decreasing wavelength. Sharp peaks or irregularities in the scan may indicate inorganic interferences. Because many organic compounds in water and wastewater (e.g., carboxylic acids and carbohydrates) do not absorb significantly in UV wavelengths, correlate UV absorption to dissolved organic carbon (DOC) or soluble chemical oxygen demand (COD). However, use such correlations with care because they may vary from water to water, seasonally or during storms on the same water, and between raw and treated waters. In addition, chemical oxidation of the organic material (e.g., ozonation, chlorination) may reduce UV absorption without removing organics and thus may change correlations. Because UV absorption and correlations with UV absorption are site-specific, they may not be comparable from one water source to another.

c. Minimum detectable concentration: The minimum detectable concentration cannot be determined rigorously because this is a nonspecific measurement. Also, the minimum detectable concentration of a particular constituent depends on the relationship among UV absorption, the desired characteristic (e.g., trihalomethane formation potential or DOC), and any interfering substances. For precise measurement, select cell path length to provide a minimum absorbance of approximately 0.005 and a maximum absorbance of approximately 0.950 cm^{-1}. Dilute high-strength samples to fall within the determined range.

d. Sampling and storage: Collect samples in amber glass bottles that have been washed, thoroughly rinsed with organic-free water, and baked at 400°C for at least 1 hour. Seal with TFE-lined caps. If individual TFE septa are used, wash and rinse thoroughly with organic-free water, wrap in foil, and bake at 100°C for 1 hour or soak the septa in 10% sodium persulfate for 1 hour at 60°C and rinse thoroughly with organic-free water.[3] Pre-cleaned containers from commercial vendors may also be available. Store samples at <6°C; samples requiring shipping should be transported on ice. Adjust pH (if needed) and analyze within 48 h.

Samples may be filtered in the field. If these samples require pH adjustment, make the adjustment before filtration.

2. Apparatus

a. Spectrophotometer, for use between 200 and 400 nm with matched quartz cells providing a light path of 1 cm. For low-absorbance samples, use a path length of 5 or 10 cm. A scanning spectrophotometer is useful.

b. Filter: Use a glass fiber filter* without organic binder, or use TFE, polycarbonate, or silver filters. Polyethersulfone (PES) or hydrophilic polypropylene (HPP) filters are also recommended.[4] Other filters that neither sorb UV-absorbing organics of interest nor leach interfering substances (e.g., nitrate or organics) into the water may be used, especially if colloidal matter must be removed. The specific filter used should be reported with the data. Practical filter diameters are 2.2 to 4.7 cm, although this may vary. Pre-rinse filter with a sample of organic-free water to remove soluble impurities. As an option, glass fiber filters may be combusted at high temperatures (450 to 500°C for at least 6 h) before rinsing. If alternate separation techniques, filters, or filter preparations are used, demonstrate that equivalent results are produced.

Filter pore size will influence test results, especially in raw waters. When filtering samples for SUVA determination, check the applicable regulatory constraints to see if a filter with an absolute pore size of 0.45 μm is required, because glass fiber filters generally do not meet this requirement. For highly turbid samples, pre-filters (including glass fiber) may be used to remove interferences and minimize clogging of 0.45-μm filters.[5]

*c. Filter assembly, g*lass, TFE, stainless steel or other material that neither sorbs UV-absorbing organics nor leaches interfering substances, capable of holding the selected filters. Polypropylene syringes with syringe-type filter holders or peristaltic pumps with in-line filter holders may be used as an alternative to vacuum filtration.[3]

3. Reagents

a. Organic-free water: Reagent water containing less than 0.010 cm^{-1} of UV-absorbing compounds. For optimum performance, reagent water of less than 0.0045 cm^{-1} is recommended.

b. Hydrochloric acid (HCl) (optional), 0.1*N*.

c. Sodium hydroxide (NaOH) (optional), 0.1*N*.

d. Phosphate buffer (optional): Dissolve 2.5 g dried anhydrous KH_2PO_4 and 4.5 g dried anhydrous Na_2HPO_4 in 800 mL organic-free water. The pH should be close to 7; verify that it is between 6 and 8 and then dilute to 1 L with organic-free water. Store in a brown glass bottle at 4°C and re-prepare when the pH falls out of the 6 to 8 range or if microbial growth is observed. Commercially prepared reagent is also available.

e. Organic carbon stock solution: Dissolve 2.1254 g anhydrous primary-standard grade potassium biphthalate, $C_8H_5KO_4$ [also known as potassium hydrogen phthalate (KHP)], in organic-free water and dilute to 1000 mL (1.00 mL = 1.00 mg carbon). Commercially prepared standards are also available.

* Whatman grade 934AH; Gelman type A/E; Millipore type AP40; ED Scientific Specialties grade 161; or other products that give demonstrably equivalent results.

Table 5910:I. Precision of UV Analyses and Correlation to KHP Samples

Analysis	UV_{254} Result for Given KHP Sample Concentration* cm^{-1}							
	0.54	0.93	1.79	4.87	9.61	25.0	50.0	100.0
Laboratory 1	0.008	0.015	0.034	0.079	0.158	0.323	0.638	1.282
Laboratory 2	0.009	0.016	0.026	0.070	0.134	0.401	0.803	1.612
Laboratory 3	0.010	0.017	0.027	0.081	0.161	0.353	0.695	1.343
Laboratory 4	0.007	0.020	0.033	0.070	0.132	0.319	0.750	1.590
Laboratory 5	0.009	0.018	0.030	0.087	0.140	0.394	0.643	1.447
Mean	0.0086	0.0172	0.0300	0.0774	0.1450	0.3580	0.7058	1.4548
Standard deviation	0.0011	0.0019	0.0035	0.0074	0.0136	0.0384	0.0708	0.1461
Relative standard deviation, %†	12.8	11.1	11.7	9.56	9.38	10.7	10.0	10.0

* KHP sample concentration mg/L as C, measured as in Section 5310C.
† The percent relative standard deviation is given by:

$$\%RSD = \left[\frac{\text{standard deviation (S)}}{\text{mean } (\overline{X})}\right] \times 100$$

4. Procedure

a. Sample volume: Select sample volume based on cell path length, volume needed for filter rinsing, and/or dilution required to produce a UV absorbance between 0.005 and 0.950 cm^{-1}. For most applications, a 50- to 125-mL sample is adequate.

b. Sample preparation: Wash filter and filter assembly by passing a sufficient volume of organic-free water through the filter (to waste) to yield a filter blank of <0.010 cm^{-1}. For specific applications and correlations, sample pH may be adjusted with HCl or NaOH. In poorly buffered samples, an appropriate non-UV absorbing buffer system (e.g., a phosphate buffer) may be used. Add enough buffer such that a constant pH is maintained during analysis. Take care to avoid precipitate formation during pH adjustment. Ideally, the volume of buffer added should be <5% of the sample volume. If 5% is exceeded, apply an appropriate dilution correction factor. UV absorbance of fulvic acid solutions apparently remains constant between pH 4 and 10.[2] Samples analyzed for SUVA determination should be run without adjusting the pH of the original sample. Record sample pH value used with recorded absorbance. Once sample pH has been adjusted and/or measured, rinse filter with sample and discard filtrate. If using 4.7-cm PES or HPP filters, they can be adequately rinsed with 25 mL sample to bind sites that may adsorb UV-absorbing constituents.[4] This volume is also adequate for glass fiber filters. Smaller filters may require proportionately less. If other filter materials are used, confirm whether these volumes are adequate or if more is needed. Analyze the subsequent filtrate as a sample. Prepare an organic-free water filter blank and the sample in an identical manner.

c. Spectrophotometric measurement: Let spectrophotometer equilibrate according to manufacturer's instructions. Set wavelength to 253.7 nm (or 254 nm) and adjust spectrophotometer to read zero absorbance with organic-free water. Measure UV absorbance of at least two filtered portions of sample at room temperature.

5. Calculation

Report mean UV absorption in units of cm^{-1} using the following notation. To report units in m^{-1}, multiply the equation by one hundred.

$$UV_{\lambda}^{pH} = \left[\frac{\overline{A}}{b}\right] D$$

where:

UV_{λ}^{pH} = mean UV absorption, cm^{-1} (subscript denotes wavelength used, nm, and superscript denotes pH used if other than 7.0),
$\bar{A}$ = mean absorbance measured,
b = cell path length, cm, and
D = dilution factor resulting from pH adjustment and/or dilution with organic-free water.

$$D = \frac{\text{final sample volume}}{\text{initial sample volume}}$$

Correct results for absorption contributed by known interfering substances. If UV absorption contributed by interfering substances exceeds 10% of total UV absorption, do not use UV absorption at 253.7 nm as an indicator of organics. Record pH if other than 7.

6. Quality Control

a. Replicate measurements: Use at least two portions of filtered sample.

b. Duplicate analyses: Analyze every tenth sample in duplicate (i.e., duplicating the entire procedure) to assess method precision. Calculate relative percent difference (RPD) for duplicate analyses (D_1 and D_2, where each duplicate is the average of replicate readings) using the formula below:

$$RPD = \frac{|D_1 - D_2|}{(D_1 + D_2)/2} \times 100$$

For UV values >0.045 cm^{-1}, the RPD limit is ≤10%. For UV values <0.045 cm^{-1}, the RPD limit is ≤20%.

c. Baseline absorbance: Check system baseline UV absorbance after every 10 samples by measuring the absorbance of an organic-free water blank. A non-zero absorbance reading for the blank may indicate the need for cell cleaning, problems with the reference cell if a dual-beam instrument is being used, or a variation in spectrophotometer response caused by heating or power fluctuations over time.

d. Filter blanks: With each batch of filtered samples, analyze a portion of organic-free water that has been passed through a rinsed filter. The volume of organic-free water should be equal to the volume of a sample. Filter blanks should read <0.010 cm^{-1}; if not, larger rinsing volumes may be required. Also, the appropriateness of the filter and/or the filtering apparatus may need to be evaluated.

e. Filtrate turbidity check (optional): If a UV value or calculated SUVA value seems unusually high for a specific sample source, check filtrate turbidity. Turbidities greater than 0.5 NTU indicate that the sample may require pretreatment steps, such as centrifugation.[5]

f. Spectrophotometer check: Difficulties in comparing UV absorption data from different spectrophotometers have been reported. KHP standards were prepared in pH 7, phosphate-buffered (5910B.3*d*) reagent water without acidification and analyzed in five laboratories. Results suggest acceptable precision (Table 5910:I). These data also are useful for checking spectrophotometer results with KHP standards commonly used for TOC and/or COD analysis. A correlation equation for this 40-sample data set is:

$$UV_{254} = 0.0144\ KHP + 0.0018$$

with correlation coefficient (r^2) = 0.987, UV_{254} expressed in cm^{-1}, and KHP expressed as mg/L as C.

This equation can help verify spectrophotometer performance. For example, if a set of UV_{254} analyses is performed and results are in the 0.010 range, prepare a KHP standard of 0.5 mg/L as C. The projected UV_{254} of this KHP standard would be 0.009 cm^{-1}. If measured UV_{254} is outside the 13% relative standard deviation (RSD) of 0.009 cm^{-1} (using the RSD in Table 5910:1 as a guide), the spectrophotometer may be suspect and require maintenance. The correlation between UV_{254} and KHP standards is presented solely as a useful means of verifying spectrophotometer performance. Prepare at least one KHP standard in the range of interest and measure it with each analytical run. Commercially prepared standards may also be available for this purpose.

7. Precision and Bias

Table 5910:I shows interlaboratory precision data for 40 KHP samples. The RSD ranged from 9.38 to 12.8%.

Single-operator precision data are presented in Table 5910:II for fulvic acid solutions.[6] The RSD ranged from 0.9 to 6%. Because UV absorption is an aggregate measure of organic carbon, true standards are unavailable and bias cannot be determined.

Table 5910:II. Single-Operator Precision for UV Absorption Measurements of Fulvic Acid Solutions

	Result cm^{-1}		
	DOC Concentration *mg/L*		
Replicate No.	2.5	4.9	10.0
1	0.110	0.240	0.480
2	0.120	0.230	0.480
3	0.110	0.240	0.470
4	0.100	0.230	0.480
5	0.110	0.240	0.480
6	0.100	0.240	0.470
7	0.110	0.240	0.480
8	0.110	0.230	0.480
9	0.120	0.240	0.480
10	0.110	0.240	0.480
Mean	0.110	0.237	0.478
Standard deviation	0.0067	0.00483	0.00422
Relative standard deviation, %	6.06	2.05	0.882

The precision of analyses by this method was determined under the Information Collection Rule.[7] Precision was determined as RPD for duplicate analyses and was calculated only when both analyses in the duplicate pair showed concentrations at, or greater than, the ICR minimum reporting level (MRL= 0.009 cm^{-1}). Results were as follows:[8]

Data Quality Variable	N	Percentile				
		10	25	50 (median)	75	90
Precision (RPD)	33 306*	0.0	0.0	0.0	2.2	4.9

* 2744 excluded—both samples less than MRL.
NOTE: Median sample result was 0.040 cm^{-1}.

8. References

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PART 6000

INDIVIDUAL ORGANIC COMPOUNDS

Part Coordinator
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Joint Task Group Chairs for the 23rd Edition

6020 Quality Assurance/Quality Control.....John R. Gumpper
6810 Pharmaceuticals and Personal Care Products .....Eric D. Nelson

Summary of Major Changes Since 2012

Quality Assurance/Quality Control (6020) was revised to be consistent with Sections 1020, 2020, 3020, 4020, and 5020.

A new method for determining Pharmaceuticals and Personal Care Products (6810) in drinking water was added.

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6010 INTRODUCTION*

6010 A. General Discussion

The methods presented in Part 6000 are intended for the determination of individual organic compounds. Methods for determination of aggregate concentrations of groups of organic compounds are presented in Part 5000.

Most of the methods presented herein are highly sophisticated instrumental methods for determining very low concentrations of the organic constituents. Stringent quality control requirements are given with each method and require careful attention.

Many compounds are determinable by two or more of the methods presented in Part 6000. Table 6010:I[2] shows the specific analytical methods applicable to each compound. Guidance on method selection is provided in the introduction to each section.

* Joint Task Group: 21st Edition—Edward M. Glick.

Table 6010:I. Analysis Methods for Specific Organic Compounds*

Compound	Analysis Methods (section number)	Compound	Analysis Methods (section number)
Acenaphthene	6040B; 6410B; 6440B	Captan	6630B
Acenaphthylene	6410B; 6440B	Carbaryl	6610B
Acetaldehyde	6252B	Carbofuran	6610B
Aldicarb	6610B	Carbon tetrachloride	6200B, C
Aldicarb sulfone	6610B	Chlordane	6410B; 6630B, C
Aldicarb sulfoxide	6610B	Chlorobenzene	6040B; 6200B, C
Aldrin	6410B; 6630B, C	Chloroethane	6200B, C
Aminomethylphosphonic acid (AMPA)	6651B	Chloroethoxy methane	6040B; 6410B
Anthracene	6040B; 6410B; 6440B	Chloroethyl ether	6040B; 6410B
Baygon	6610B	Chloroethylvinyl ether	6200B, C
Bentazon	6640B	Chloroform	6200B, C; 6232B
Benzaldehyde	6252B	Chloroisopropyl ether	6410B
Benzene	6200B, C	Chloromethane	6200B, C
Benzidine	6410B	Chloromethyl benzene	6040B
Benzo(a)anthracene	6040B; 6410B; 6440B	Chloromethylphenol	6410B; 6420B
Benzo(a)pyrene	6410B; 6440B	Chloronaphthalene(s)	6040B; 6410B
Benzo(b)fluoranthene	6410B; 6440B	Chlorophenol(s)	6410B; 6420B
Benzo(ghi)perylene	6410B; 6440B	Chlorophenoxy benzene	6040B
Benzo(k)fluoranthene	6410B; 6440B	Chlorophenyl phenyl ether	6410B
BHC(s)	6410B; 6630C	Chlorotoluene	6200B, C
Bromobenzene	6040B; 6200B, C	Chrysene	6040B; 6410B; 6440B
Bromochloroacetic acid	6251B	Crotonaldehyde	6252B
Bromochloromethane	6200B, C	Cyclohexanone	6252B
Bromodichloromethane	6040B; 6200B, C; 6232B	2,4-D (dichlorophenoxyacetic acid)	6640B
Bromoform	6040B; 6200B, C; 6232B	Dalapon	6640B
Bromomethane	6200B, C	DDD	6410B; 6630B, C
Bromophenoxybenzene	6040B	DDE	6410B; 6630B, C
Bromophenyl phenyl ether	6410B	DDT	6410B; 6630B, C
Butanal	6252B	Decanal	6252B
Butyl benzyl phthalate	6410B	Dibenzo(a,h)anthracene	6410B; 6440B
Butylbenzene(s)	6200B, C	Dibromoacetic acid (DBAA)	6251B

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TABLE 6010:I. CONT.

COMPOUND	ANALYSIS METHODS (SECTION NUMBER)	COMPOUND	ANALYSIS METHODS (SECTION NUMBER)
Dibromochloromethane	6040B; 6200B, C; 6232B	Methylene chloride	6200B, C
Dibromochloropropane	6200B, C; 6231B	Methyl glyoxal	6252B
Dibromoethane	6040B; 6200B, C; 6231B	Methylisoborneol	6040B, D
Dibromomethane	6200B, C	Methyl parathion	6630B
Dibutyl phthalate	6410B	Methomyl	6610B
Dicamba	6640B	Mirex	6630B
Dichloran	6630B	Monobromoacetic acid (MBAA)	6251B
Dichloroacetic acid (DCAA)	6251B	Monochloroacetic acid (MCAA)	6251B
Dichlorobenzene(s)	6040B; 6200B, C; 6410B	Naphthalene	6040B; 6200B, C; 6410B; 6440B
Dichlorobenzidine	6410B	Nitrobenzene	6410B
Dichlorodifluoromethane	6200B, C	Nitrophenol(s)	6410B; 6420B
Dichloroethane	6200B, C	Nitrosodi-*n*-propylamine	6410B
Dichloroethene(s)	6200B, C	Nitrosodimethylamine	6410B
Dichlorophenol(s)	6410B; 6420B	Nitrosodiphenylamine	6410B
Dichloropropane(s)	6200B, C	Nonanal	6252B
Dichloropropene	6040B; 6200B, C	Octanal	6252B
Dieldrin	6410B; 6630B, C	Oxamyl	6610B
Diethyl phthalate	6040B; 6410B	Parathion	6630B
Dimethyl phthalate	6410B	PCB-1016, 1221, 1232, 1242, 1248, 1254, 1260	6410B, 6630B
Dimethylphenol(s)	6410B; 6420B	Pentachloronitrobenzene	6630B
Dinitrophenol(s)	6410B; 6420B	Pentachlorophenol	6410B; 6420B; 6640B
Dinitrotoluene(s)	6410B	Pentanal	6252B
Dinoseb	6640B	Phenanthrene	6040B; 6410B; 6440B
Di-*n*-octyl phthalate	6410B	Phenol	6410B; 6420B
Diphenyl hydrazine	6040B	Phenylbenzamine	6040B
Endosulfan	6410B; 6630B, C	Picloram	6640B
Endosulfan sulfate	6410B; 6630C	Propanal	6252B
Endrin	6410B; 6630B, C	Propylbenzene	6040B; 6200B, C
Endrin aldehyde	6410B; 6630C	Pyrene	6040B; 6410B; 6440B
Ethenyl benzene (styrene)	6040B	Silvex (trichlorophenoxy propionic acid)	6640B
Ethylbenzene	6040B; 6200B, C	Strobane	6630B
Ethylhexyl phthalate	6410B	Styrene (ethenyl benzene)	6200B, C
Fluoranthene	6040B; 6410B; 6440B	2,4,5-T (trichlorophenoxy acetic acid)	6640B
Fluorene	6040B; 6410B; 6440B	2,4,5-TP	6640B
Formaldehyde	6252B	Tetrachloroethane(s)	6040B; 6200B, C
Geosmin	6040B, D	Tetrachloroethene	6040B; 6200B, C
Glyoxal	6252B	Toluene	6200B, C
Glyphosate	6651B	Toxaphene	6410B; 6630B, C
Heptachlor	6410B; 6630B, C	Trichloroanisole	6040B
Heptachlor epoxide	6410B; 6630B, C	Trichloroacetic acid (TCAA)	6251B
Heptaldehyde	6252B	Trichlorobenzene(s)	6040B; 6200B, C; 6410B
Heptanal	6252B	Trichloroethane(s)	6040B; 6200B, C
Hexachlorobenzene	6040B; 6410B	Trichloroethene	6040B; 6200B, C
Hexachlorobutadiene	6040B; 6200B, C; 6410B	Trichlorofluoromethane	6200B, C
Hexachlorocyclopentadiene	6410B	Trichlorophenol	6251B; 6410B; 6420B
Hexachloroethane	6040B; 6410B	Trichloropropane	6200B, C
Hexanal	6252B	Trifluralin	6630B
3-Hydroxycarbofuran	6610B	Trimethylbenzene(s)	6200B, C
Indeno(1,2,3-cd))pyrene	6410B; 6440B	Vinyl chloride	6200B, C
Isobutylmethoxy pyrazine	6040B	Xylene(s)	6040B; 6200B, C
Isophorone	6410B		
Isopropylbenzene	6200B, C		
Isopropyl methoxy pyrazine	6040B		
Isopropyltoluene	6200B, C		
Lindane (γ-BHC)	6630B		
Malathion	6630B		
Methane	6211		
Methiocarb	6610B		
Methoxychlor	6630B		
Methyldinitrophenol(s)	6410B; 6420B		

* Compounds are listed under the names by which they are most commonly known and called in specific methods.

6010 B. Sample Collection and Preservation

1. Volatile Organic Compounds

Use 25- or 40-mL vial equipped with a screw cap with a hole in the center* and TFE-faced silicone septum.† Wash vials, caps, and septa with detergent, rinse with tap and distilled water, and dry at 105°C for 1 h before use in an area free of organic vapors. NOTE: Do not heat seals for extended periods of time (>1 h) because the silicone layer slowly degrades at 105°C. When bottles are cool, seal with TFE seals. Alternatively purchase precleaned vials free from volatile organic compounds.

Collect all samples in duplicate and prepare replicate field reagent blanks with each sample set. A sample set is all samples collected from the same general sampling site at approximately the same time. Prepare field reagent blanks in the laboratory by filling a minimum of two sample bottles with reagent water, sealing, and shipping to the sampling site along with empty sample bottles.

Fill sample bottle just to overflowing without passing air bubbles through sample or trapping air bubbles in sealed bottle. When sampling from a water tap, open tap and flush until water temperature has stabilized (usually about 10 min). Adjust flow rate to about 500 mL/min and collect duplicate samples from flowing stream. When sampling from an open body of water, fill a 1-L, wide-mouth bottle or beaker with a representative sample and carefully fill duplicate sample bottles from the container.

Preservation of samples is highly dependent on target constituents and sample matrix. Ongoing research indicates the following areas of concern: rapid biodegradation of aromatic compounds, even at low temperatures;[1] dehydrohalogenation reactions such as conversion of pentachloroethane to tetrachloroethane;[2] reactions of alkylbenzenes in chlorinated samples, even after acidification; and possible interactions among preservatives and reductants when dechlorination is used to prevent artifact formation, especially in samples potentially containing many target compounds.

There is as yet no single preservative that can be recommended. Ideally, maintain samples chilled (preferably at 4°C) and analyze immediately. In practice, delays between sampling and analysis often necessitate preservation. The recommended preservation techniques are summarized in Table 6010:II.

1) For samples and field blanks that contain volatile constituents but do not contain residual chlorine, add HCl (4 drops 6*N* HCl/40 mL) to prevent biodegradation and dehydrohalogenation. NOTE: HCl may contain traces of organic solvents. Verify freedom from contamination before using a specific lot for preservation.
2) For samples that contain residual chlorine and for field blanks, also add a reducing agent. In general, ascorbic acid (25 mg/40 mL) is the agent of choice for GC/MS determinations, while sodium thiosulfate (3 mg/40 mL) is more appropriate with conventional GC detectors. Alternative reducing agents may be specified or permitted in particular methods.

TABLE 6010:II. RECOMMENDED PRESERVATION FOR VOLATILE ORGANIC COMPOUNDS

Constituents	Chlorinated Matrix	Non-Chlorinated Matrix
Halocarbons	Reducing agent, then HCl	HCl
Aromatics	Reducing agent, then HCl	HCl
THMs	Reducing agent (HCl optional)*	None required
EDB/DBCP	None required	None required

* See Section 6232B.2.

In all cases run reagent blanks with reducing agent and acid to ensure absence of interferences. Always add the reducing agent before adding the HCl to the sample. The reducing agent can be added to the sample bottle as a powder before the bottle is shipped into the field. Do not add HCl to a chlorinated sample because formation of chlorinated volatiles can occur. When both preservatives are being added in the field, add reducing agent, fill bottle, and then, after at least 1 min to reduce residual chlorine, add 4 drops 1:1 HCl (or other amount sufficient to lower sample pH to 2.0).

Tightly seal sample bottle, TFE face down. After sampling and preservation invert several times to mix. Chill samples to 4°C immediately after collection and hold chilled in an atmosphere free of organic solvent vapors until analysis. Normally analyze all samples within 14 d of collection. Shorter or longer holding times may be appropriate, depending on constituents and sample matrix. Develop data to show that alternate holding times are appropriate.

2. Other Organic Compounds

See individual methods for sampling and preservation requirements.

3. References

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2. BELLAR, T. & J. LICHTENBERG. 1985. The Determination of Synthetic Organic Compounds in Water by Purge and Sequential Trapping Capillary Column Gas Chromatography. U.S. Environmental Protection Agency, Cincinnati, Ohio.

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* Pierce 13075, or equivalent.
† Pierce 12722, or equivalent.

6010 C. Analytical Methods

1. General Discussion

The methods presented in Part 6000 for identification and quantitation of trace organic constituents in water generally involve isolation and concentration of the organic compounds from a sample by solvent or gas extraction (see Section 6040 and individual methods), separation of the components, and identification and quantitation of the compounds with a detector.

2. Gas Chromatographic Methods

Gas chromatographic (GC) methods are highly sophisticated microanalytical procedures. They should be used only by analysts experienced in the techniques required and competent to evaluate and interpret the data.

a. Gas chromatograph:

1) Principle—In gas chromatography a mobile phase (a carrier gas) and a stationary phase (column packing or capillary column coating) are used to separate individual compounds. The carrier gas is nitrogen, argon-methane, helium, or hydrogen. For packed columns, the stationary phase is a liquid that has been coated on an inert granular solid, called the column packing, that is held in borosilicate glass tubing. The column is installed in an oven with the inlet attached to a heated injector block and the outlet attached to a detector. Precise and constant temperature control of the injector block, oven, and detector is maintained. Stationary-phase material and concentration, column length and diameter, oven temperature, carrier-gas flow, and detector type are the controlled variables.

When the sample solution is introduced into the column, the organic compounds are vaporized and moved through the column by the carrier gas. They travel through the column at different rates, depending on differences in partition coefficients between the mobile and stationary phases.

2) Interferences—Some interferences in GC analyses occur as a result of sample, solvent, or carrier gas contamination, or because large amounts of a compound may be injected into the GC and linger in the detector. Methylene chloride, chloroform, and other halocarbon and hydrocarbon solvents are ubiquitous contaminants in environmental laboratories. Make strenuous efforts to isolate the analytical system from laboratory areas where these or other solvents are in use. An important sample contaminant is sulfur, which is encountered generally only in base/neutral extracts of water, although anaerobic groundwaters and certain wastewaters and sediment/sludge extracts may contain reduced sulfur compounds, elemental sulfur, or polymeric sulfur. Eliminate this interference by adding a small amount of mercury or copper filings to precipitate the sulfur as metallic sulfide. Sources of interference originating in the chromatograph, and countermeasures, are as follows:

a) Septum bleed—This occurs when compounds used to make the septum on the injection port of the GC bleed from the heated septum. These high-molecular-weight silicon compounds can be distinguished readily from compounds normally encountered in environmental samples by their peak shape or baseline rise. Nevertheless, minimize septum bleed by using septum sweep, in which clean carrier gas passes over the septum to flush out the "bleed" compounds.

b) Column bleed—This term refers to loss of column coating or breakdown products when the column is heated. This interference is more prevalent in packed columns, but also occurs to a much lesser extent in capillary columns. It occurs when the column temperature is high or when water or oxygen is introduced into the system. Solvent injection can damage the stationary phase by displacing it. Certain organic compounds acting as powerful solvents, acids, or bases can degrade the column coating. Injection of large amounts of certain surface-active agents may destroy GC columns. Signs of column bleed are a rising baseline or a large peak at end of run.

c) Ghost peaks—These peaks occur when an injected sample contains either a large amount of a given compound, or a compound that adsorbs to the column coating or injector parts (e.g., septum). When a subsequent sample is injected, peaks can appear as a result of the previous injection. Eliminate ghost peaks by injecting a more dilute sample, by producing less reactive derivatives of a compound that may interact strongly with the column material, by selecting a column coating that precludes these interactions, by injecting solvent blanks between samples, or by heating the column for a longer time or to a higher temperature at the end of the run.

b. Detectors: Various detectors are available for use with gas chromatographic systems. See individual methods for recommendations on appropriate detectors.

1) Electrolytic conductivity detector—The electrolytic conductivity detector is a sensitive and element-specific detector that has gained considerable attention because of its applicability to the gas chromatographic analysis of environmentally significant compounds. It is used in the analysis of purgeable halocarbons, pesticides, herbicides, pharmaceuticals, and nitrosamines. This detector is capable of operation in each of four specific modes: halogen (X), nitrogen (N), sulfur (S), and nitrosamine (NO). Only organic compounds containing these elements will be detected.

Compounds eluting from a gas chromatographic column enter a reactor tube heated to 800°C. They are mixed with a reaction gas, hydrogen for X, N, or NO modes, and air for the S mode. The hydrogen catalytically reduces the compounds while the air oxidizes them. The gaseous products are transferred to the detector through a conditioned ion exchange resin or scrubber. In the halogen mode, only HX is detected, while NH_3 or H_2S are eliminated on the resin. In the nitrogen or nitrosamine mode, the NH_3 formed is ionized while HX and H_2S, if present, are eliminated with KOH/quality wool scrubber. The sulfur mode produces SO_2 or SO_3, which is ionized while HX is removed with a silver wire scrubber. All other products either are not ionizable or are produced in such low yield that they are not detectable.

The electrolytic conductivity detector contains reference and analytical electrodes, a gas–liquid contactor, and a gas–liquid separator. The conductivity solvent enters the cell and flows by the reference electrode. It combines with the gaseous reaction products in the gas–liquid phases in the gas–liquid contactor. This heterogeneous mixture is separated into gas and liquid phases in the gas–liquid separator, with the liquid phase flowing past the analytical electrode. The electrometer monitors the

difference in conductivity at the reference electrode (solvent) and the analytical electrode (solvent + carrier + reaction products).

2) Electron capture detector—The electron capture detector (ECD) usually is used for the analysis of compounds that have high electron affinities, such as chlorinated pesticides, drugs, and their metabolites. This detector is somewhat selective in its response, being highly sensitive toward molecules containing electronegative groups: halogens, peroxides, quinones, and nitrogen groups. It is insensitive toward functional groups, such as amines, alcohols, and hydrocarbons.

The detector is operated by passing the effluent from the gas chromatographic column over a radioactive beta particle emitter, usually nickel-63 or tritium adsorbed on platinum or titanium foil. An electron from the emitter ionizes the carrier gas, preferably nitrogen, and produces a burst of electrons. About 100 secondary electrons are produced for each initial beta particle. After further collisions, the energy of these electrons is reduced to the thermal level and they can be captured by electrophilic sample molecules.

The electron population in the ECD cell is collected periodically by applying a short voltage pulse to the cell electrodes and the resulting current is compared with a reference current. The pulse interval is adjusted automatically to keep the cell current constant, even when some of the electrons are being captured by the sample. The change in the pulse rate when a sample enters the ECD is then related to the sample concentration. The ECD offers linearity in the range of 10^4 and subpicogram detection limits for compounds with high electron affinities.

3) Flame ionization detector—The flame ionization detector (FID) is widely used because of its high sensitivity to organic carbon-containing compounds. The detector consists of a small hydrogen/air diffusion flame burning at the end of a jet. When organic compounds enter the flame from the column, electrically charged intermediates are formed. These are collected by applying a voltage across the flame. The resulting current is amplified by an electrometer and measured. The response of the detector is directly proportional to the total mass entering the detector per unit time and is independent of the concentration in the carrier gas.

The FID is perhaps the most widely used detector for gas chromatography because of several advantages:

- it responds to virtually all organic carbon-containing compounds with high sensitivity (approximately 10^{-13} g/mL);
- it does not respond to common carrier gas impurities such as water and carbon dioxide;
- it has a large linear response range (approximately 10^7) and excellent baseline stability;
- it is relatively insensitive to small column flow-rate changes during temperature programming;
- it is highly reliable, rugged, and easy to use; and
- it has low detector dead volume effects and fast response.

Its limitations include:

- it gives little or no response to noncombustible gases and all noble gases; and
- it is a destructive detector that changes the physical and chemical properties of the sample irreversibly.

4) Photoionization detector—Photoionization occurs when a molecular species absorbs a photon of light energy and dissociates into a parent ion and an electron. The photoionization detector (PID) detects organic and some inorganic species in the effluent of a gas chromatograph with detection limits as low as the picogram range. The PID is equipped with a sealed ultraviolet light source that emits photons which pass through an optically transparent window (made of LiF, MgF_2, NaF, or sapphire) into an ionization chamber where photons are absorbed by the eluted species. Compounds having ionization potential less than the UV source energy are ionized. A positively biased high-voltage electrode accelerates the resulting ions to a collecting electrode and the resulting current is measured by an electrometer. This current is proportional to the concentration.

The PID has high sensitivity, low noise (approximately 10^{-14} A), and excellent linearity (10^7), is nondestructive, and can be used in series with a second detector for more selective detection. The PID can be operated as a universal detector or a selective detector by simply changing the photon energy of the ionization source. Tables of ionization potentials are used to select the appropriate UV source for a given measurement.

5) Mass spectrometer—The mass spectrometer (MS) has the ability to detect a wide variety of compounds, coupled with a capacity to deduce compound structures from fragmentation patterns. Among the different types of mass spectrometers, the quadrupole has become the most widely used in water and wastewater analysis.

The mass spectrometer detects compounds by ionizing molecules into charged species with a 70-eV beam. The ions are accelerated toward the quadrupole mass filter through a series of lenses held at 0 to 200 V. The differently sized, charged fragments are separated according to mass-to-charge ratio (related to molecular weight) by means of the quadrupole, which uses varying electric and radiofrequency (rf) fields. The quadrupole is connected to a computer, which varies these fields so only fragments of one particular mass-to-charge ratio (± 0.5) can traverse the quadrupole at any one time. As the ions leave the quadrupole they are attracted to the electron multiplier through an electrical potential of several thousand volts. The charge fragments, in turn, are detected by the electron multiplier. Because the electric and the rf fields are cycled every few seconds, a fragmentation pattern is obtained. Each cycle is called a mass scan. Most chemicals have unique fragmentation patterns, called mass spectra. The computer contains, and can search, a library of known mass spectra to identify tentatively an unknown compound exhibiting a particular spectrum. Use authentic compounds for confirmation after tentative identifications are made.

Background mass interference can result from the ability of the mass spectrometer to detect any ions created in its ion volume (up to a specified mass). Any compounds continuously present in the source will be detected. Some mass ions always present are due to air components that leak into the system, such as oxygen (masses 16 and 32), nitrogen (masses 14 and 28), carbon dioxide (mass 44), argon (mass 40), and water (mass 18), or to helium carrier gas (masses 4 and 8), or to diffusion pump oil vapors.

3. High-Performance Liquid Chromatographic (HPLC) Methods

a. Principle: HPLC is an analytical technique in which a liquid mobile phase transports a sample through a column containing a liquid stationary phase. The interaction of the sample with the stationary phase selectively retains individual compounds and permits separation of sample components. Detection of the sep-

arated sample compounds is achieved mainly through the use of absorbance detectors for organic compounds and through conductivity and electrochemical detectors for metal and inorganic components.

b. Detectors:

1) Photodiode array detector (PDAD)—The PDAD measures the absorbance of a sample from an incident light source (UV-VIS). After passing through the sample cell, the light is directed through a holographic grating that separates the beam into its component wavelengths reflected on a linear array of photodiodes. This permits the complete absorbance spectrum to be obtained in ≤1 s and simultaneous multiwavelength analysis.

The PDAD is subject to the interference encountered with all absorbance detectors. Of special concern for HPLC is the masking of the absorbance region of the HPLC mobile phase and its additives. This may reduce the range and sensitivity of the detector to the sample components. Most interferences occur in monitoring the shorter wavelengths (200–230 nm). In this region, many organic compounds absorb light energy and can be sources of interference.

2) Post column reactor (PCR)—The PCR consists of in-line sample derivatizing/reacting equipment that permits chemical alteration of certain organic compounds. This equipment is used to enhance detection by attaching a chromophore to the compound(s) of interest. Sensitivity and selectivity of compounds that were initially undetectable are altered to make them detectable.

Interferences from this technique usually arise from the impurities in the reagents used in the reaction. When this technique is coupled with a selective detector such as fluorescence, these interferences are minimized. Generally, only compounds of the same class as the compounds of interest will cause interference.

3) Fluorescence detector—The fluorescence detector is an absorbance detector in which the sample is energized by a monochromatic light source. Compounds capable of absorbing the light energy do so and release it as fluorescence emission. Filters permit the detector to respond only to the fluorescent energy. The fluorescence detector is the most sensitive of the current HPLC detectors available and often is used with a post column reactor.

Because of instrument sensitivity, minute quantities of contaminants can cause interferences to fluorescence detectors. Contamination can happen from glassware, mobile phase solvents, postcolumn reagents, etc. These sources will raise the background signal and thus narrow the range of the detector. Interference from individual compounds is minimal because of detector specificity (i.e., all interferences must fluoresce).

6020 QUALITY ASSURANCE/QUALITY CONTROL*

6020 A. Introduction

Without quality control (QC) results, there is no confidence in the results of analytical tests. As described in Part 1000, essential QC measures include method calibration, reagent standardization, assessment of each analyst's capabilities, analysis of blind check samples, determination of the method's sensitivity (method detection level or quantification limit), and daily evaluation of bias, precision, and the presence of laboratory contamination or other analytical interference. The details of these procedures, their performance frequency, and expected ranges of results should be formalized in a written Quality Assurance Manual and standard operating procedures (SOPs).

Some of the methods in Part 6000 include specific QC procedures, frequencies, and acceptance criteria. These are considered the minimum quality controls needed to perform the method successfully; additional QC procedures can and should be used. If the QC criteria listed in this section exceed those listed in the individual methods, the criteria in this section must also be included. Some regulatory programs may require further QC or have alternative acceptance limits.

Each method typically includes acceptance-criteria guidance for precision and bias of test results. If not, the laboratory should determine its own criteria via control-charting techniques. Evaluate bias using recoveries from laboratory-fortified blanks (LFBs). Evaluate precision by analyzing duplicate or spiked duplicate samples. Additional acceptance criteria guidance may be provided by program- or project-specific requirements.

To help verify the accuracy of calibration standards and overall method performance, participate in an annual or preferably semi-annual analytical program of single-blind QC check samples (QCS), ideally provided by an external entity. Such programs are sometimes called *proficiency testing (PT)/performance evaluation (PE) studies*. An unacceptable result on a PT sample is often a strong indication that a test protocol is not being followed successfully. Investigate circumstances fully to find the cause. In many jurisdictions, participation in PT studies is a required part of laboratory certification/accreditation.

* Reviewed by Standard Methods Committee, 2011.
John R. Gumpper, Andrew D. Eaton.

6020 B. Quality Control Practices

1. Initial Quality Control

a. Initial demonstration of capability (IDC): Before new analysts run any samples, verify their capability with the method (see Section 1020B.1 for specifics). Run at least four LFBs (6020B.2*e*) and compare results to the limits listed in the method. All instrument performance checks and calibration requirements must be met before analysis. (NOTE: Analysis and evaluation of a method blank is required.) If no limit is specified, use the following procedure to establish initial limits:

Calculate the standard deviation of the four samples. The LFB's recovery limits are

$$\text{LFB's initial recovery limits} = \text{Mean} \pm (5.84 \times \text{Standard Deviation})$$

where:

5.84 = the two-sided Student's *t* factor for 99% confidence limit for three degrees of freedom.[1]

While this process will provide initial limits, they should be considered temporary. Limits developed from more replicates (e.g., at least 20) will give a better determination of accuracy and precision. (For basic guidance on demonstrating capability, see Sections 1020B.1 and 3.)

b. Method detection level (MDL): If data will be reported below the calibrated range, then before analyzing samples, determine the MDL for each analyte via Section 1020B.4 or other applicable procedures.[2] MDL determination and verification are not required if 1) data are not reported below the instrument's calibrated range, and 2) the ability to provide quantitative data at the reporting limit is verified. Determine MDL for each analyte in a method and matrix category. The laboratory should define all matrix categories in its QA plan. Perform a new MDL determination whenever changes in the method's instruments or operating conditions may affect sensitivity. Ideally, samples for MDL determinations should be analyzed over at least a 3-d period to generate a more realistic value. Include all sample-preparation steps in the MDL determination.

Ideally, use pooled data from several analysts rather than data from one analyst to determine overall lab MDLs. (For specific information on MDLs and pooling, see Section 1020B.4.)

Verify the MDL on each instrument used in the laboratory by analyzing a QC sample (subjected to all sample-preparation steps) spiked at a level 1 to 4 times the MDL. A successful verification is one that meets all the method's detection criteria. Repeat the verification at least annually.

c. Operational range: Before using a new method or instrument, determine its operational range (upper and lower limits), or at least verify that the intended range of use is within the operational range. For each analyte, use standard concentrations that provide increasing instrument response. The minimum reporting level (MRL) is set to a concentration at or above the lowest standard used in the analysis. Quantitation at the MRL

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must be verified initially and at least quarterly (preferably daily) by analyzing a QC sample (subjected to all sample-preparation steps) spiked at a level 1 to 2 times the MRL. A successful verification meets the method's or laboratory's accuracy requirements at the MRL. Laboratories must define acceptance criteria for the operational range, including the MRL, in their QA documentation.

2. Ongoing Quality Control

a. Calibration: Initially calibrate with at least five non-zero calibration standards of the analyte(s) of interest. If using second-order fits, include at least six non-zero standards.

Select calibration standards that bracket the sample's expected concentration and are within the method's operational range. The number of calibration points depends on the width of the operational range and the shape of the calibration curve. One calibration standard must be at or below the method's reporting limit.

As a general rule, the range of standard concentrations should not be greater than three orders of magnitude, and may be much less. For example, concentration variables of 1, 5, 10, 50, and 100 can be used if the operational range is two orders of magnitude.

Apply response-factor, linear, or quadratic curve-fitting statistics, as appropriate, to analyze the concentration–instrument response relationship. If the relative standard deviation of the response factors is ≤15%, then the average response factor may be used. Otherwise, use a regression equation. The appropriate linear or nonlinear correlation coefficient for standard concentration-to-instrument response should be greater than or equal to 0.995 for linear calibrations and 0.990 for quadratic calibrations. Weighting factors (e.g., $1/x$ or $1/x^2$) may be used to give more weight to the lower concentration points of the calibration.

Back calculate each calibration point's concentration. The back-calculated and true concentrations should agree within ±30% for points above the MRL and ±50% at ≤MRL, unless different criteria are specified in an individual method.

Use initial calibration to quantify analyte concentrations in samples. Use calibration verification only to check the initial calibration, not to quantify samples. Repeat initial calibration at least annually or when calibration verification criteria cannot be met. (For basic calibration guidance, see Section 1020B.11.)

b. Calibration verification: Verify calibration by periodically analyzing a continuing calibration standard during a run. If not specified otherwise in the individual method, analyze after each 20 samples and at the end of the run. Analyses employing internal standards may omit the verification at the end of the run. The calibration verification standard's analyte concentration may be varied over the calibration range to determine detector response. Some methods may also require the analysis of an instrument blank after the continuing calibration standard.

For the calibration verification to be valid, check standard results must not exceed the limits specified in the method or in Table 6020:I (if not specified in the method).

If a calibration verification fails, immediately cease analyzing samples and take corrective action. Often, the problem can be fixed by performing injector maintenance or trimming a few cm from the front of the column. Then, re-analyze the calibration verification. If the calibration verification passes, continue the analysis. Otherwise, repeat initial calibration and re-analyze samples run since the last acceptable calibration verification.

If the LFB is not prepared from a second source to confirm method accuracy, the laboratory must also verify the accuracy of its standard preparation by analyzing a mid-level second-source calibration standard whenever a new initial calibration curve is prepared. Results must agree within 25%, unless otherwise specified in a method. (A *second source* is either from another vendor or a completely different lot from the same vendor. If neither option is feasible, then the second-source calibration standard must be prepared from primary stock materials by a different analyst.)

c. Quality control sample (QCS): Analyze an externally generated, blind QCS (unknown concentration) at least annually (preferably semi-annually or quarterly). Obtain this sample from a source external to the laboratory, and compare results to that laboratory's acceptance results. If testing results do not pass acceptance criteria, investigate why, take corrective action, and analyze a new QCS. Repeat this process until results meet acceptance criteria. Record all attempts to meet criteria. Multiple failures indicate problems with method operation. External proficiency test (PT) samples meet this criterion.

d. Method blank (MB): Include at least one MB daily or with each batch of 20 or fewer samples, whichever is more frequent. Prepare and analyze the MB in exactly the same manner as field samples, including all preparation and cleanup steps, and all preservatives used in samples. Any constituent(s) recovered must generally be less than or equal to one-half the reporting level (unless the method specifies otherwise). If any MB measurements are at or above one-half the reporting level (if reporting to MRL) or greater than the MDL (if reporting to the MDL), take immediate corrective action (as outlined in Section 1020B.5). This may include re-analyzing the sample batch or qualifying the reported data. Sample results that are below the MRL are considered valid even if the MB has a detection above the MRL, but should be qualified for information purposes. For common lab contaminants, such as methylene chloride, a lab may need to use a higher MRL to meet the MB criteria.

e. Laboratory-fortified blank (LFB): The LFB and LFM may be made from the same source standard as the initial calibration or from a second source. If the LFB and LFM are from the same source as the ICAL, the ICAL must be verified using a second source standard (see 6020B.2*b*).

Using stock solutions, prepare fortified concentrations so they are within the calibration curve. Prepare at least one LFB each day samples are prepared or with each preparation batch of 20 or fewer samples, whichever is more frequent. Prepare and analyze the LFB in exactly the same manner as the field samples, including all preparation and cleanup steps and all preservatives.

Calculate percent recovery and determine control limits (Section 1020B) for these measurements. Some methods may have specific limits to use in lieu of plotting control charts. In those cases, control charts may still be useful in identifying potential problems but are not required. Ensure that the LFB meets the method's performance criteria when such criteria are specified. If the LFB does not meet the acceptance criteria, the method is out of control; take corrective action. Re-prepare and re-analyze as samples with an acceptable LFB. If impossible, qualify the reported data.

TABLE 6020:I. MINIMUM QUALITY CONTROL FOR METHODS IN PART 6000

Section		MB*	LFB†	LFM‡, LFMD§	Surrogate	ISTD‖	Other
6040B	Constituent Concentration by Gas Extraction	×	×	×	×	×	#
6040C		×	×	×	×	×	
6040D		×	×	×	×	×	2
6040E		×	×	×	×	×	2
6200B	Volatile Organic Compounds	×	×	×	×	×	2
6200C		×	×	×	×	×	3
6211B	Methane	×	×	–	–	–	
6211C		×	×	–	–	–	
6231B	EDB & DBCP	×	×	×	–	–	4
6231C		×	×	×	–	–	
6231D		×	×	×	–	–	4
6232B	THMs and Chlorinated Organic Solvents	×	×	×	–	**	1
6232C		×	×	×	–	–	
6232D		×	×	×	–	–	
6251B	DBPs: HAAs and Trichlorophenol	×	×	×	×	×	
6252B	DBP: Aldehydes	×	×	×	×	×	††
6410B	Extractable Base/Neutrals and Acids	×	×	×	×	×	2,3
6420B	Phenols	×	×	×	–	×	
6420C		×	×	×	×	×	2,3
6431B	PCBs	×	×	×	×	–	
6431C		×	×	×	×	×	
6440B	Polynuclear Aromatic Hydrocarbons	×	×	×	–	–	
6440C		×	×	×	×	×	
6450B	Nitrosamines	×	×	×	×	×	1
6450C		×	×	×	×	×	1
6610B	Carbamate Pesticides	×	×	×	–	**	
6630B	Organochlorine Pesticides	×	×	×	×	–	
6630C		×	×	×	×	–	4
6630D		×	×	×	×	×	
6640B	Acidic Herbicide Compounds	×	×	×	×	×	
6651B	Glyphosate Herbicide	×	×	×	–	–	
6710B	Tributyl Tin	×	×	×	×	×	
6710C		×	×	×	×	×	4

* Method blank.
† Laboratory-fortified blank.
‡ Laboratory-fortified matrix.
§ Laboratory-fortified matrix duplicate.
‖
LFM +Dup OK
** Optional
†† Confirm optional
1. Additional QC guidelines in method.
2. Gas chromatograph/mass spectrometer (GC/MS) tuning required.
3. Chromatography checks required.
4. Second-column confirmation or GC/MS confirmation required.

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f. Laboratory-fortified matrix (LFM)/Laboratory-fortified matrix duplicate (LFMD): Prepare at least one LFM/LFMD each day samples are prepared or with each preparation batch of 20 or fewer samples. (For basic guidance on LFMs and LFMDs, see Sections 1020B.7 and 8.) Some regulatory programs require more frequent use of LFMs. When analytes of interest are expected to be present, the laboratory may substitute a duplicate analysis for the LFMD. If the client does not provide enough sample volume for the LFM/LFMD analyses, the laboratory may perform duplicate LFB analyses to generate precision data for the analysis.

To prepare an LFM, add a known concentration of analytes to a randomly selected routine sample without increasing its volume by more than 1%. Otherwise, account for the dilution mathematically. Ideally, the new concentration should be at or below the midpoint of the calibration curve. Spike all analytes of interest to the client. Process the LFM and LFMD as separate samples through entire sample preparation and analysis. If necessary, dilute the spiked sample at analysis to bring the measurement within the calibration curve.

Calculate percent recovery and relative percent difference, plot control charts (unless the method specifies acceptance criteria), and determine control limits for spikes (Section 1020B). Ensure that the method's performance criteria are satisfied. If the LFB met acceptance criteria, failures usually indicate problems created by the sample matrix. If the native analyte concentration is more than four times (4×) greater than the spike concentration, spike recoveries may be unreliable. Precision data may still be usable based on the total analyte concentration (native + spike).

g. Duplicates: Using duplicates is appropriate when there is a high likelihood that the compounds of interest will be present in the sample, particularly at high concentrations that make spiking difficult. If not, use LFMDs instead. Methods in this section routinely use LFMDs. Process duplicate samples independently through the entire sample preparation and analysis. Include at least one duplicate for each matrix type each day samples are prepared or with each preparation batch of 20 or fewer samples. Calculate control limits for duplicates when method-specific limits are not provided. (For basic guidance on duplicates, see Section 1020B.8.) Some regulatory programs require more frequent use of duplicates.

h. Surrogate standards: Where indicated in methods, surrogate standard recoveries are used to monitor for matrix effects in field samples and analytical problems in all samples. Before preparation, spike all QC and field samples using a concentrated solution of surrogate standards. Ideally, target a concentration at or below the midpoint of the calibration range.

Calculate percent recovery and determine control limits (Section 1020B) for these measurements. Some methods may have specific limits to use in lieu of calculating control limits. If so, control charts may still be useful in identifying potential problems but are not required. Ensure that surrogate recoveries meet the method's performance criteria (when such criteria are specified) or the laboratory-generated limits. Failures may indicate analytical problems or problems tied to the sample matrix. Establish actions to be taken if surrogates do not satisfy acceptance criteria.

i. Internal standards: Internal standards are used in some methods to normalize instrument responses and provide retention time references. Where used, track internal standard response(s) and retention time(s) and compare to the criteria stated in the method. Establish actions to be taken if internal standards do not satisfy acceptance criteria.

j. Retention times: The laboratory must have procedures to develop retention time windows and monitor retention times. Although advances in chromatographic instrumentation controls mean that minor shifts in retention times may not be noted in some analyses, the laboratory must make at least initial determinations of retention-time windows on each type of analytical system for each analyte. Follow the criteria in the method. If there are none, then the laboratory must follow its own procedure for determining retention-time windows and analyte identification criteria.

k. Second column confirmation: If a method requires that analyte identification be confirmed via a dissimilar second column, ensure that the phases are dissimilar enough to invert the elution order of some compounds in the analysis or—if the method only involves a few target analytes—significantly change the pattern of elution. One column may be used solely to quantitate analytes and the other just to confirm analyte identification. If so, the confirmation column need not meet all of the method's calibration and QC criteria; however, demonstrate daily that the confirmation column is sensitive enough to identify all compounds at the level being reported. This may be accomplished by analyzing the lowest calibration standard showing adequate signal for all analytes on both columns. Some methods or programs may require quantitative analysis on both columns. If so, the laboratory must meet all QC criteria on both columns.

l. Additional instrument checks: Certain methods may require additional QC checks on analytical performance (e.g., endrin/DDT breakdown checks in the analysis of chlorinated pesticides or mass spectrometer tuning). If noted in a method, they are required and must be performed as indicated. However, instrument parameters relating to chromatography (e.g., temperature or gradient ramps and profiles and even column choices) may be optimized as long as all QC and compound identification criteria can be met. All calibration standards, QC samples, and field samples must be analyzed using identical conditions.

m. Demonstration of ongoing proficiency: Each laboratory analyst must demonstrate ongoing proficiency with the method according to criteria established in the laboratory's Quality Assurance Manual and SOPs. The demonstration may be accomplished be repeating the IDC or by an evaluation of ongoing QC data. Analysts who have not performed the analysis in more than a year should repeat the IDC to verify their proficiency.

3. Calculations

a. LFM recovery:

$$LFM\ \%\ Recovery\ = \left[\frac{LFM\ conc \times (spike\ vol\ +\ sample\ vol) - (sample\ conc\ \times\ sample\ vol)}{spike\ solution\ conc\ \times\ spike\ vol}\right] \times 100$$

b. LFB and surrogate recovery:

$$\frac{C_b}{I} \times 100 = \%\ \text{Recovery LFB}$$

where:

C_b = LFB or surrogate concentration determined experimentally, and
I = initial concentration of analytes (or surrogate) added to LFB or sample.

c. Relative percent difference:

$$\left[\frac{|LFM - LFMD|}{\left(\frac{LFM + LFMD}{2}\right)}\right] \times 100 = \%RPD$$

or

$$\left[\frac{|D_1 - D_2|}{\left(\frac{D_1 + D_2}{2}\right)}\right] \times 100 = \%RPD$$

where:

LFM = concentration determined for LFM,
$LFMD$ = concentration determined for LFMD,
D_1 = concentration determined for first duplicate, and
D_2 = concentration determined for second duplicate.

4. References

1. Meier, P.C. & E.E. Zünd. 2000. Statistical Methods in Analytical Chemistry, 2nd ed. Wiley Interscience, New York, N.Y.
2. U.S. Environmental Protection Agency. 1995. Definition and procedure for the determination of the method detection limit, revision 1.11. 40 CFR Part 136, Appendix B. *Fed. Reg.* 5:23703.

6040 CONSTITUENT CONCENTRATION BY GAS EXTRACTION*

6040 A. Introduction

The ability to analyze ultratrace levels of organic pollutants in water has been limited, in part, by the concentration technique. With the development of closed-loop stripping analysis (CLSA) (6040B), organic compounds of intermediate volatility and molecular weight (from heavier volatiles to lighter polynuclear aromatic hydrocarbons) can be extracted from water and concentrated to allow quantitative and semiquantitative analysis (depending on compound) at nanogram-per-liter levels. This extract can be analyzed on a gas chromatograph (GC) connected to one of several detectors. A CLSA technique coupled with gas chromatographic/mass spectrometric (GC/MS) analysis for trace organic compounds is presented here. It is applicable to both treated and natural waters.

The purge and trap technique (6040C) can concentrate volatile organic compounds by bubbling an inert gas through a sample, collecting it in a sorbent trap, and then desorbing the compound for analysis. The extract may be analyzed by GC or GC/MS methods. The technique is applicable to both water and wastewater.

Solid-phase microextraction (SPME) techniques for taste- and odor-causing compounds (6040D and E) are more rapid than 6040B and use much smaller samples, while achieving similar sensitivity. SPME techniques can be performed using two MS modes. SPME coupled with GC/MS using electron impact in the selected ion monitoring mode is presented in 6040D. SPME coupled with GC/MS in the chemical ionization (CI) mode with methanol as the reagent gas is presented in 6040E.

* Approved by Standard Methods Committee, 2011.
Joint Task Group: 22nd Edition—Jacob L. Nikonchuk and Melissa S. Dale (co-chairs), Chih-Fen Tiffany Lee, Cecilia O. Lei, Peggy Singley Moylan, Karen Muroi.

6040 B. Closed-Loop Stripping, Gas Chromatographic/Mass Spectrometric Analysis

1. General Discussion

a. Principle: CLSA–GC/MS is suitable for analyzing a broad spectrum of organic compounds in water. It can be used to identify and quantitate specific compounds, such as earthy- or musty-smelling compounds [e.g., 2-methylisoborneol (MIB) and geosmin][1–3] or U.S. Environmental Protection Agency (EPA) priority pollutants.[4,5] The method is also suitable for other taste- and odor-causing compounds (e.g., geranyl acetone 6-methylhept-5-en-2-one, β-ionone, and β-cyclocitral).[6]

In closed-loop stripping, volatile organic compounds of intermediate molecular weight are stripped from water by a recirculating stream of air. The organics are removed from the gas phase by an activated carbon filter. They are extracted from the filter with carbon disulfide (CS_2) or methylene chloride. A portion of the extract is injected into a capillary-column GC/MS so organic compounds can be identified by retention time and spectrum matching; they are quantified via single-ion current integration. Alternatively, the extract may be analyzed via a capillary-column GC equipped with a flame ionization detector; organic compounds can be identified by retention times on primary and secondary capillary columns.

b. Interference: Organic compounds that are stripped during this procedure may co-elute with the compounds of interest. The uniqueness of each target compound's mass spectrum makes it possible to confirm compound identity with a high probability when co-eluting components are present. Problems may arise if several isomers of a compound are present that are not resolvable chromatographically.

Table 6040:I. Method Detection Levels for Earthy-Musty Smelling Compounds by CLSA-GC/MS

Compound	Detection Limit *ng/L**	
	Unsalted Method[1]	Salting-Out Technique[3]
Geosmin	2	0.8
2-Methylisoborneol	2	0.8
2-Isopropyl-3-methoxy	2	0.8
2-Isobutyl-3-methoxy	2	0.8
2,3,6-Trichloroanisole	5	0.8

* Stripping at 25°C. Selective ion monitoring.

c. Detection limits: Trace organics can be detected at low nanogram-per-liter levels. The CLSA-GC/MS detection limits are affected by many factors, especially stripping efficiency and GC/MS condition. Stripping efficiencies can be improved by using an elevated stripping temperature and/or the salting-out technique. The stripping and extraction portion of the method can be evaluated independently of the instrumentation portion. As an option, add internal standard after stripping and extraction, and transfer extract quantitatively.

The method detection levels for five earthy-musty smelling compounds are shown in Table 6040:I. For each compound, detection limits for the salted CLSA method are less than half

TABLE 6040:II. METHOD DETECTION LEVELS FOR SELECTED ORGANIC COMPOUNDS BY CLSA-GC/MS[8]

Compound	Detection Limit *ng/L**†	Compound	Detection Limit *ng/L**†
1,1,1-Trichloroethane	2.0	1,3,5-Trichlorobenzene	0.1
Trichloroethene	100	1,2,4-Trichlorobenzene	10
Dichlorobromomethane	5.0	1,2,3-Trichlorobenzene	2.0
1,3-Dichloropropene	2.0	*bis*(2-Chloro-ethoxy)methane	10
1,1,2-Trichloroethane	2.0	Methylisoborneol (MIB)	0.5
Chlorodibromomethane	1.0	Geosmin	0.2
1,2-Dibromoethane	2.0	Naphthalene	100
Tetrachloroethene	100	1,1,2,3,4,4-Hexachloro-1,3-butadiene	2.0
Chlorobenzene	10	1-Chloronaphthalene	0.5
Ethylbenzene	50	2-Chloronaphthalene	0.5
m,p-Xylene	100	Acenaphthene	0.5
Bromoform	1.0	Fluorene	2.0
Ethylbenzene	5.0	Diethylphthalate	100
o-Xylene	50	1-Chloro-4-phenoxybenzene	0.5
1,1,2,2-Tetrachloroethane	50	*N*-Phenylbenzamine	20
Bromobenzene	0.5	1,2-Diphenylhydrazine (as azobenzene)	1.0
Propylbenzene	0.5	1-Bromo-4-phenoxybenzene	0.5
1-Chloro-3-methylbenzene	0.5	Hexachlorobenzene	1.0
bis(2-Chloroethyl)ether	1.0	Phenanthrene	10
o-Dichlorobenzene	0.1	Anthracene	50
m-Dichlorobenzene	10	Fluoranthene	20
p-Dichlorobenzene	10	Pyrene	20
Hexachloroethane	20	Chrysene	50
N-Nitrosodi-*n*-propylamine	5.0	Benzo(a)anthracene	50

* Elevated stripping temperature and salting-out both used.
† Instrument detection level based on a 2:1 signal:noise ratio (where a background interference existed, the target compound was required to be at least twice background).

those for the unsalted method. Using the elevated stripping temperature rather than the salting-out technique produces comparable recoveries[7] and similar detection limits. Detection limits for various organic compounds of interest—obtained with an elevated stripping temperature/salting-out technique—ranged from 0.1 to 100 ng/L (Table 6040:II).[8]

2. Apparatus

Use clean glassware when collecting samples and preparing calibration standards. Wash with soapy water and rinse with tap water, then demineralized water, and finally with reagent-grade acetone. Air-dry and bake at 180°C for 6 to 12 h. As an alternative to acetone rinse and bake, bake glassware in an annealing oven for 1 h at 400°C. Do not bake sample bottle caps or volumetric ware. After drying and baking, store inverted or cover mouths with aluminum foil to prevent accumulation of dust or other contaminants.

a. Sample bottles, 1-L capacity or larger, glass, with tetrafluoroethylene (TFE)-lined screw caps.

b. CLSA apparatus, equipped with the following components (Figure 6040:1) or their equivalents.[4]*

1) *Stripping bottle*—with mark at 1-L level and stainless-steel quick-connect stems (Figure 6040:2) or unpolished spherical glass joints sealed with TFE-covered silicone rubber O-rings and secured with metal clamps.† Immediately after use, clean stripping bottle by rinsing twice with demineralized water and once with organic-free water. For particularly adherent impurities, clean with acetone and bake at 180°C for at least 2 h. Turbid samples may cause a film to deposit on the stripping bottle and frit and may require washing with acid detergent.

2) *Gas heater*—with aluminum heating cylinder and soldering iron (25 W) controlled by a variable transformer (Figure 6040:3). Alternatively, use a temperature-controlled heater block to maintain a fixed temperature at the filter that is 10 to 20°C above the temperature of the thermostatic water bath.

3) *Filter holder*—stainless steel or glass.‡ If glass is used, also use an auxiliary heating device (e.g., an infrared light) to maintain proper filter temperature.

4) *Pump*—with stainless-steel bellows,§ providing air flow in the range of 1 to 1.5 L/min. When using the salting-out technique, periodically disassemble the pump and clean. Salt will leave deposits on the pump bellows. If there is a noticeable drop in pump performance, clean valve assembly with acetone or replace.

5) *Automatic timer* (optional)—connected to pump.

6) *Circuit,* with stainless-steel parts—1/8-in.-outer diameter (OD) (0.3-cm-OD) tubing, 4-in. (10.2-cm) × 1/4-in.-OD (0.6-cm-OD) flexible tubing, tube fittings, and quick-connect

* Model CLS 1, Tekmar, Cincinnati, OH; Brechbühler AG, 8952 Schlieren ZH, Switzerland, available from Chromapon, Whittier, CA; or equivalent.

† Rotulex Sovirel, Brechbühler AG, or equivalent.
‡ Brechbühler AG, or equivalent.
§ Metal Bellows Model MB-21, Sharon, MA, or equivalent.

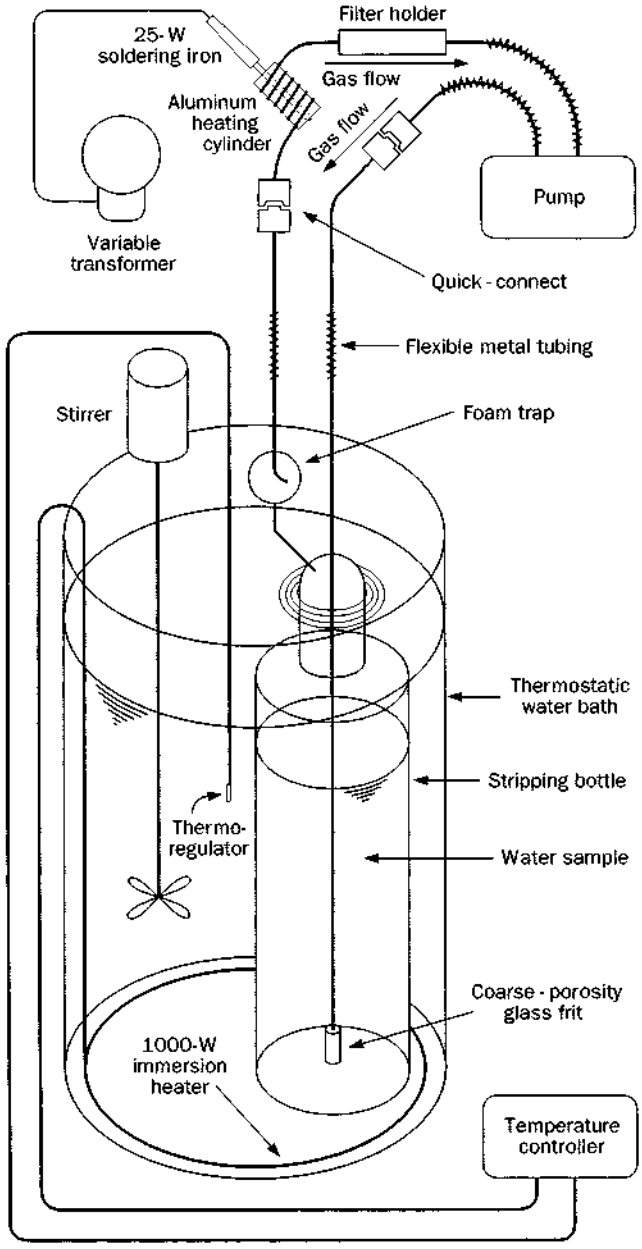


Figure 6040:1. Schematic of closed-loop stripping apparatus (not to scale). SOURCE: KRASNER, S.W., C.J. HWANG & M.J. MCGUIRE. 1983. A standard method for quantification of earthy-musty odorants in water. *Water Sci. Technol.* 15(6/7):127.

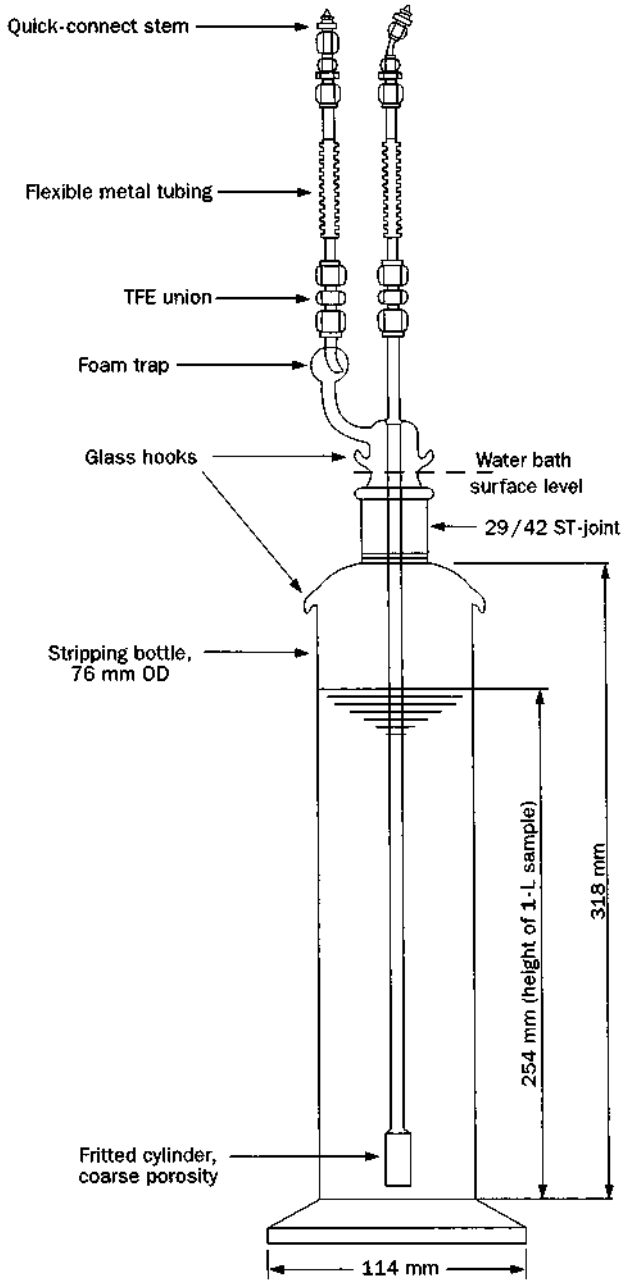


Figure 6040:2. One-liter "tall form" stripping bottle. SOURCE: KRASNER, S.W., C.J. HWANG & M.J. MCGUIRE. 1983. A standard method for quantification of earthy-musty odorants in water. *Water Sci. Technol.* 15(6/7):127. (To obtain dimensions in inches, divide dimensions in millimeters by 25.4.)

bodies‖; or glass joints [described in ¶ *b*1) above]. Glass sample lines can be used except where circuit enters and exits pump. Use TFE ferrules in making connections to glass and flexible metal tubing. Whenever sample carryover is observed, clean circuit and pump as follows: Connect fittings to the quick-connect bodies on both ends of the circuit to open system. Turn on pump and flush with approximately 100 mL each organic-free water, acetone, and methanol. After last rinse, dry with a heat gun with pump still running (or flush with nitrogen) until there is no residual methanol. (NOTE: Overheating quick-connect units can cause internal O-rings to deteriorate.)

7) *Thermostatic water bath*—with 222-mm-OD × 457-mm chromatography jar and thermoregulating system accurate to at least ±0.5°C. When the laboratory's ambient temperature is >25°C, maintain water bath at 25°C by inserting a coil of copper tubing connected to a cold water tap to circulate cold water.

‖ Swagelok fittings, or equivalent.

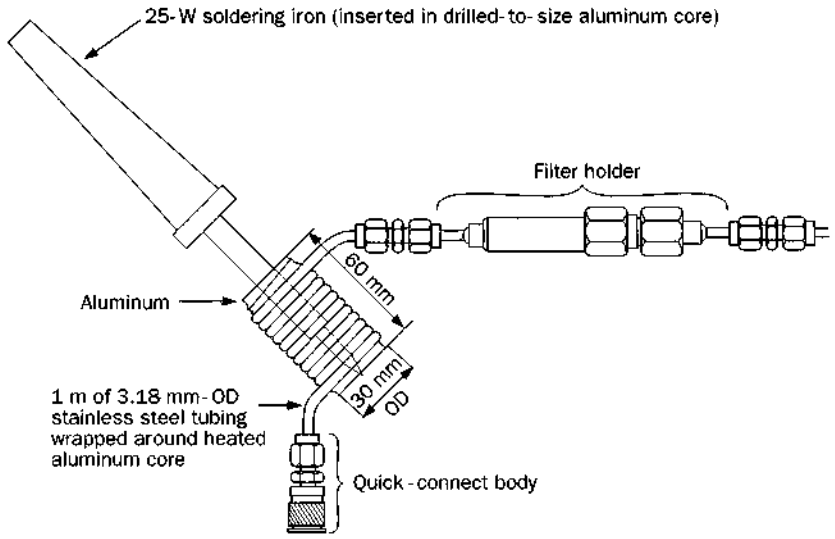


Figure 6040:3. Gas Heater.

8) *Filters*—with 1.5 mg activated carbon# (Figure 6040:4). Use a set of filters matched in solvent flow resistance and performance for each group of samples and calibration standards. Determine filter resistance by measuring solvent flow rate through a cleaned, solvent-wetted filter. First, fill the longer glass tube above the charcoal with organic-free water and let flow via gravity through filter disk. Rinse once with acetone, rinse twice with elution solvent, and rinse twice with acetone; on the final rinse, measure the time necessary to empty solvent (0.3-mL volume) from top of filter tube to carbon surface. Rates for new, commercially prepared filters vary significantly and decrease with use. Flow rates also depend on the solvent used. Determine optimal flow rate range from analyte recoveries. Preferably, verify filter performance by preparing check standards. Figure 6040:5 shows the air-flow reductions due to a "slow" filter. Figure 6040:6 shows how filter resistance affects the recovery of earthy-musty odorants and one internal standard. Clean filter as soon as possible after use. Fill glass tube with organic-free water and let flow through filter. Repeat once with acetone, twice with elution solvent, and twice with acetone. Measure flow on final acetone rinse. If solvent flow is slow due to salt deposits, pull 1*N* HNO_3 through filter, using a vacuum connection. After acid washing, rinse with distilled water and acetone, and continue with cleaning as above. After final rinse, remove residual solvent by connecting filter to a vacuum for approximately 5 min. Clean auxiliary filter after 40 uses or 2 weeks, whichever comes first.

If the salting-out technique is used, Na_2SO_4 may be carried over and could clog the filter. The initial water rinse removes deposited salts; it may be avoided if salt is not used.

c. Stirrer (optional), with 5-cm-long TFE stirring bar.

d. Microsyringes, 5-, 10-, 25-, and 50-mL capacity. Use a 25-mL gastight syringe with electrotapered (blunt-end) tip** for transferring extract.

e. Receivers, 50-mL capacity (Figure 6040:4): Receivers can be produced by a custom glass-blowing company. Have glass

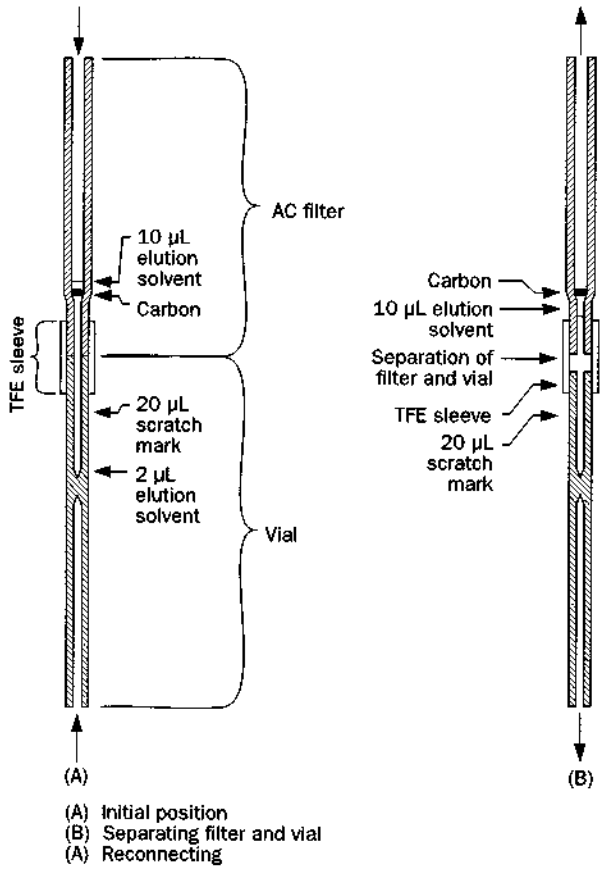


Figure 6040:4. Extraction of filter. SOURCE: KRASNER, S.W., C.J. HWANG & M.J. MCGUIRE. 1981. Development of a closed-loop stripping technique for the analysis of taste- and odor-causing substances in drinking water. *In* L.H. Keith, ed. Advances in the Identification and Analysis of Organic Pollutants in Water, Vol. 2. Ann Arbor Science Publishers, Ann Arbor, Mich.

Brechbühler AG, Chromapon, Inc., or equivalent.
** Hamilton Model 1805N electrotapered tip, Reno, NV.

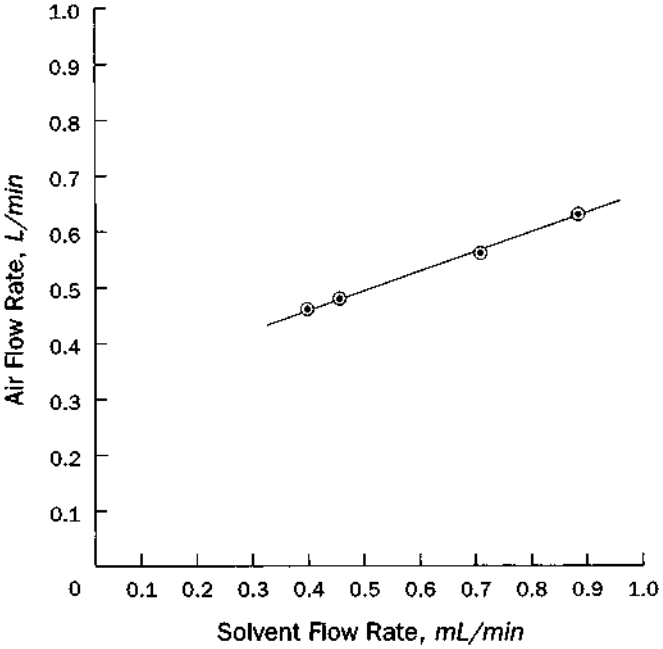


Figure 6040:5. Flow rate through 1.5-mg carbon filter. Air flow rate with no filter is 0.86 L/min.

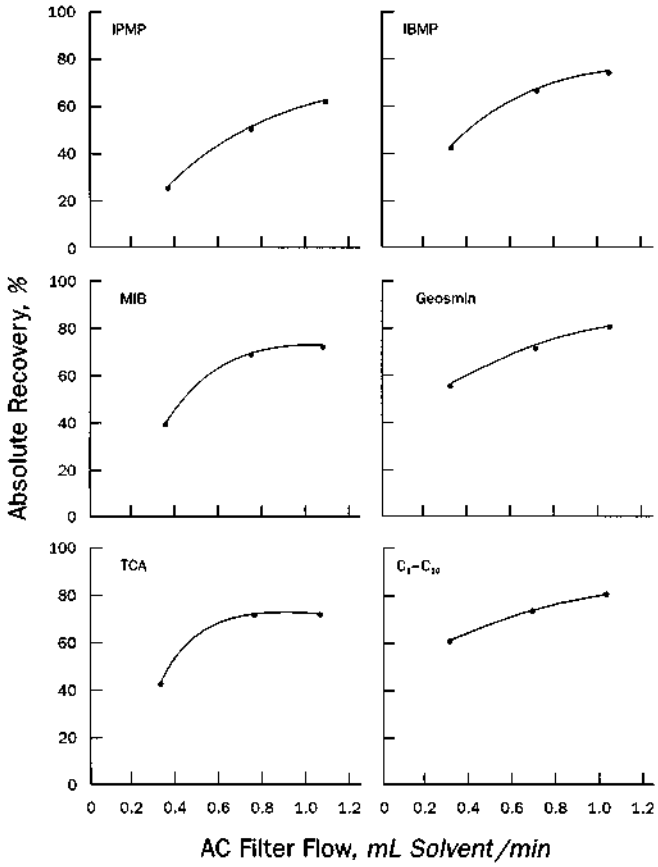


Figure 6040:6. Effect of filter resistance, measured as flow, on recovery of earthy-musty odorants and C_1–C_{10} internal standard. Reprinted with permission from: HWANG, C.J., S.W. KRASNER, M.J. MCGUIRE, M.S. MOYLAN & M.S. DALE. 1984. Determination of subnanogram per liter levels of earthy-musty odorants in water by the closed-loop stripping methods. *Environ. Sci. Technol.* 18:535. (Copyright 1984, American Chemical Society.)

blowers use a 1.6-mm-inner diameter (ID) precision-bore capillary glass and grind it to 5 mm OD, then heat-constrict to close off bore at approximately 29 mm from top. Mark at 20-μL level with glass scribe. To clean receivers and extract storage vials (¶ *g* below), rinse seven times with elution solvent and bake at 180°C overnight or rinse with acetone and bake in annealing oven at 400°C for 1 h. Rinse receivers several times with elution solvent before using.

f. TFE sleeve, 5-mm-ID TFE flexible tubing approximately 19 mm long. If a 5-mm-OD receiver is not prepared as described above, then connect filter and receiver with a piece of heat-shrink TFE tubing that is custom-shrunk to the dimensions of the filter and receiver. Rinse sleeve with acetone after each use and store in acetone until ready to use.

g. Extract storage vials, 100-μL capacity conical-shaped vials with TFE-lined septum screw cap or crimp cap.†† Transfer sample extracts from receiver to storage vial for extract storage. The storage vials are compatible with various autosamplers.

h. GC/MS/data system, equipped with:

1) *Capillary injector*—Grob-designed split–splitless injector or equivalent with 2.5-mm-ID glass insert or nonvaporizing, septumless, cold on-column injector.

2) *Capillary column*—30-m or 60-m × 0.25-mm-ID DB-1 or DB-5 fused silica or other capillary column capable of producing adequate and reproducible resolution. If using a Grob on-column injector, use a 0.32-mm-ID column for injection when a stainless-steel needle is used, or use a 0.25-mm-ID column with a fused-silica needle.

For other injector types, a 1-m pre-column of uncoated, deactivated 0.53-mm-ID fused silica is recommended. Connect pre-column to analytical column with a zero-dead-volume union.

†† Pierce Chemical Company #13100, or equivalent.

3) *Microsyringes*—5- and 10-mL capacity, with 75-mm-long needles. Use 0.23-mm-OD stainless-steel or 0.17-mm-OD fused-silica needle for on-column injection.

4) *Mass spectrometer analyzer*—See Section 6200B.2*d* for suggested specifications.

5) *Data system*—with software capable of performing reverse-library searches (optional).

6) *Autosampler injector* (recommended for improved precision of analysis).

3. Reagents

Use reagent-grade solvents or better and obtain purest standards available.

a. Carbon disulfide (CS_2): Use only after GC verification of purity to ensure that the solvent does not contain components that coelute with the compounds of interest. (**CAUTION: Use proper safety procedures; explosive, toxic, and occasionally allergenic.**)

TABLE 6040:III. 7-DAY HOLDING TIME STUDY FOR MIB AND GEOSMIN

	Deionized Water					Raw Water Source			
	MIB (N = 3)		Geosmin (N = 3)			MIB (N = 3)		Geosmin (N = 3)	
Days	Without preservation	With omadine	Without preservation	With omadine	Days	Without preservation	With omadine	Without preservation	With omadine
0	41	40	32	32	0	38	41	32	32
1	39	40	32	33	1	39	40	32	33
3	42	39	34	34	3	38	37	29	30
7	37	36	29	32	7	32	37	18	33

b. Acetone.

c. Methylene chloride, high-resolution grade. Preferably use for analyses conducted at high altitudes. MIB has been shown to be unstable in methylene chloride; extract with CS_2 whenever possible.[9]

d. Carrier gas: Helium gas, ultrapurified grade, moisture- and oxygen-free.

e. Internal standards and surrogates:

1) *1-Chlorooctane.*

2) *1-Chlorodecane.*

3) *1-Chlorododecane.*

4) *1-Chlorohexane, 1-chlorohexadecane,* and *1-chlorooctadecane* can be added for broad-spectrum analysis.

f. Stock internal standard and surrogate solutions: Dissolve 1 mL of each internal standard‡‡ in acetone and dilute to 25 mL with acetone in a volumetric flask; 1 μL = 35 μg.

g. Combination internal standard and surrogate solution: Combine 7.2 μL of each stock solution and dilute to 25 mL with acetone; 1 μL = 10 ng each.

h. Reference standards: Compounds of interest may be available commercially.§§ Deuterated geosmin and methylisoborneol can be synthesized.[10]

i. Stock reference solutions: Dissolve 20 mg of each target compound in acetone and dilute to 10 mL with acetone in a volumetric flask; 1 μL = 2 μg.

j. Combination reference standards solution: Combine 10 μL of each stock reference solution and dilute to 5 mL with acetone; 1 μL = 4 ng each.

k. Organic-free water: Prepare water by treating it with activated carbon, mixed-bed deionization, and filtration through a membrane filter.‖ ‖ Either irradiate under ultraviolet light (185 and 254 nm) for 1 h (optional) and prestrip in the CLSA apparatus for 1 h (optional if laboratory blanks are consistently free of interferences) using a clean activated carbon filter, or prestrip large quantities of water with nitrogen (ultra-high-purity grade) just before use. Store in a closed bottle tightly capped with TFE-lined screw cap, under nitrogen (optional), in a refrigerator, and away from solvent contamination for no longer than 1 week.

l. Sodium sulfate, Na_2SO_4 (optional), granular, anhydrous: Bake at 400°C for 2 h before use; store at room temperature in desiccator.

m. Sodium omadine, 3.2% solution: Prepare by weighing 3.2 g of sodium omadine into 100-mL volumetric flask. Dilute to mark with deionized water. This solution is stable for 1 month.

4. Procedure

a. Sample collection and storage: Rinse sample bottle with sample, fill to overflowing (with no air bubbles), and cap tightly. Collect duplicate samples whenever possible. Begin cooling samples immediately. Store samples at 4°C in the laboratory until analyzed. Samples must be analyzed within 3 d of collection or else preserved. When holding longer than 3 d, add 80 μL sodium omadine solution per 40 mL sample to inhibit biological activity. Analyze preserved samples within 7 d of collection. Results from the 7-d holding study are shown in Table 6040:III. Adding a dechlorinating agent is optional, because disinfection byproducts may be affected.

b. Treatment of samples:

1) Stripping—Rinse stripping bottle with sample and fill to the 1-L mark, wetting the glass joint with sample. Fill stripping bottle slowly, with minimal aeration, to prevent loss of volatile compounds. Add 10 μL combination internal standard and surrogate solution with the syringe needle tip immersed. Stopper tightly and attach springs. Place in thermostatic water bath at 25°C with glass joint below water level, and connect bottle to the circuit. Operate gas heater at 45 to 50°C. Put an "auxiliary" carbon filter in the holder and prestrip for 10 s to flush air contaminants from system. If the room's air quality is demonstrated to be free of interference (via method blank analysis), eliminate prestrip step. Exchange auxiliary filter for a clean one and strip for 2 h (pump flow rate of 1 to 1.5 L/min). Auxiliary filter may be reused many times before cleaning. If stripping bottle has a smaller height-to-diameter ratio than shown in Figure 6040:2, >2 h may be required for stripping. Optionally, use an automatic timer to terminate each stripping run. Strip time is adjustable, but strip calibration standards and samples under the same conditions.

If sample contains a large amount of algae or turbidity or foaming agents, use only 900 mL sample and 9 μL combination internal standard solution. Because this additional headspace can result in different stripping efficiencies, comparably analyze a calibration standard. Alternatively, dilute concentrated or foaming samples with organic-free water.

‡‡ 1-Chlorohexadene and 1-chlorooctadecane solidify upon refrigeration. Warm before removing a portion.

§§ Geosmin and 2-methylisoborneol are available from Wako Bioproducts, Wako Chemicals USA, Inc., 1600 Bellwood Rd., Richmond, VA 23237, or Dalton Chemical Lab, Inc., 4700 Keele St., Suite 119, FARQ, North York, Ontario, Canada M3J 1P3. NOTE: This synthetic geosmin is racemic and includes (+) geosmin, which has an odor intensity different from that of the natural (–) compound. This precludes its use in quantitative sensory analysis; however, its GC/MS characteristics (i.e., retention time and spectrum) are the same as those of natural geosmin.

‖ ‖ Millipore Milli-QUV Plus, or equivalent.

Table 6040:IV. Comparison of Monitoring and Quantitation Ions for Chlorodecane and Deuterated MIB and Geosmin Internal Standards[11]

Compound	Ions (*m/z*) Used for Quantitation		Ions (*m/z*) Monitored	
	A	B	A	B
1-Chlorodecane	91	—	43, 91, 93	—
MIB	95	168	93, 107, 135	168
Geosmin	112	112, 182	111, 112, 125	112, 182
MIB-d_3	—	171	—	171
Geosmin-d_3	—	115, 185	—	115, 185

A = 1-Chlorodecane as internal standard.
B = MIB-d_3 and geosmin-d_3 as internal standard.

2) Alternate stripping techniques—To improve stripping recovery, use a combination of ¶s *b*2)a), b), and c) below to reduce stripping time. Optimize combination, depending on compounds to be analyzed.

a) Elevated stripping temperature—Increase temperature of thermostatic water bath to 45°C to increase recovery of many organic compounds.[5,7] Raise temperature of gas heater to at least 55°C (for a 45°C stripping temperature) to avoid condensation of water vapor on the activated carbon filter. Further increases in stripping temperature reduce recovery.[5]

b) Salting-out technique—Raising the sample ionic strength with Na_2SO_4 before stripping increases the stripping rate of many organic compounds.[3] Bring sample to room temperature before analysis by immersing it in a water bath at 25°C for approximately 15 min. Transfer 800 mL to the stripping bottle and add stirring bar. With the stirring bar in motion (at intermediate setting), use a glass funnel to add 72 g Na_2SO_4. Replace funnel with a glass stopper. Continue stirring until Na_2SO_4 has dissolved (not more than 1 min). Remove stopper and stirring bar, then add remaining 100 mL of sample,## rinsing and wetting inside neck of bottle. Add 9 µL combination internal standard solution and strip at 25°C as described in standard stripping procedure above. If analysis is only for MIB and geosmin, a 1-h strip time is adequate. If additional target compounds are being analyzed, verify strip time needed for adequate recoveries of each target compound. Alternatively, combine salt and sample by pouring salt directly into 900 mL sample, stopper tightly, shake vigorously, let stand for several minutes, and add internal standards.

c) Alternative analysis—Use deuterium-labeled geosmin and MIB as internal standards for determining geosmin and MIB.[11] Table 6040:IV compares quantitation and monitoring ions between the 1-chlorodecane internal standard and the MIB-d_3 and geosmin-d_3 internal standards. Stripping or water-temperature variations do not affect the analysis' accuracy when labeled internal standards are used because MIB and geosmin will strip at the same rate as MIB-d_3 and geosmin-d_3.[11] In addition, labeled standards, if added in the field, will document the target compound's degradation within a 3- to 4-week period. The labeled standards compensate for analyte losses via physical, chemical, and biological processes*** during sample storage.[11]

3) Extracting the filter—Remove activated carbon filter from holder. In a fume hood, extract with CS_2, as indicated in Figure 6040:4. Keep solvents well within hood to avoid inhalation by analyst or contamination of stripping apparatus. Add 2 µL elution solvent to a clean receiver and connect filter and receiver with a TFE sleeve so there is no dead space between glass parts. Place 10 µL elution solvent above the carbon, taking care not to touch carbon with the needle. Warm receiver with hands and alternately pass elution solvent across carbon 10 times. Cool receiver with ice—taking care not to freeze elution solvent—to draw elution solvent below the carbon. Gently tap filter/vial assembly on a hard surface to complete transfer of elution solvent to bottom of vial.

Repeat filter extraction with a 10-µL and a 5-µL portion of elution solvent. Separate vial from filter, adjust volume precisely to the 20-µL mark,††† and, using a gastight electrotapered tip syringe, transfer extract to a clean, conical-shaped storage vial. Label and store at –20°C until analysis.

Filter may be extracted while maintaining tight seal between filter and vial during the procedure. Using ice chips, cool closed volume in the vial; solvent accumulates on lower side of the filter disk. Push solvent back to upper side by warming the closed vial between two fingers. Repeat and then extract with more solvent as above.

c. Gas chromatography/mass spectrometry:

1) "Hot-needle" injection technique—To reduce discrimination against higher-boiling compounds by distillation from the needle, use a hot-needle injection technique when the injector is a hot vaporizing type. (Do not use the following procedure for cold on-column injection.) Wet syringe needle and barrel with solvent and expel as much as possible. Pull syringe plunger back, leaving an air gap. Pull up approximately 1.5 µL sample and pull sample totally into syringe barrel. Close the split on the GC injector, wait 10 s, insert syringe needle into the injector, and let needle warm up for 1 to 2 s (optimize time by experience). Rapidly push plunger to bottom of syringe barrel to inject sample. Remove syringe and rinse well with solvent. Open split valve consistently at same time (suggested time 30 s) after the injection.

2) On-column injection technique—To more fully reduce discrimination against higher-boiling compounds, use an on-column injector. A cold on-column injector also can be used to avoid decomposition of thermally labile compounds (e.g., dimethyl polysulfides).[12] Determine thermally labile compounds quantitatively by using a cold on-column injector or an inactive, vaporizing injector.

With an on-column injector, increase sensitivity by injecting large sample volumes (up to 8 µL). To prevent problems from a heavy condensation of solvent with such large-volume injections, use a 2-m retention gap (an empty, deactivated piece of 0.53-mm-ID fused-silica tubing connected to the head of the column with a zero-dead-volume connector).[13] To preclude backpressure from large-volume injections, inject slowly at about 1 µL/5 s. Keep initial column temperature at 10°C above

A total sample volume of 900 mL is preferred to minimize foaming-over due to salt addition.

*** Note: Because synthetic labeled geosmin is racemic, best results for the compensation of prolonged biological processes are achieved by monitoring the degradation of natural (–)geosmin using (–) geosmin-d_3.

††† Calibration mark needs to be verified periodically.

Table 6040:V. Typical Operating Conditions for GC/MS Analysis of CLSA Extracts

Variable	Description or Value
Column	30- or 60-m × 0.25-mm-ID DB-I or DB-5 fused silica capillary column*
Column temperature program	35°C, 1 min; 35 to 130°C @ 4°C/min; 130–220 @ 10°C/min; 1 min
Carrier gas	Helium
Carrier gas flow rate	1 mL/min
Sample size	About 1.5 mL (splitless injection)
Injector temperature	Cold, on-column
Transfer line temperature	280°C
Ionizer temperature	280°C
Electron energy	70 eV
Mass range scanned	41 to 453 amu
Scan time	1 s

* J&W Scientific, Inc., or equivalent.

solvent's boiling point for a full solvent effect and to produce sharp peaks (narrow peak widths).[13] Because the entire injection is deposited directly into the head of the column, the column can develop active sites after only 50 to 80 injections. Check activity by injecting a polarity test mixture at least weekly. Breaking approximately 30 cm off the head of the column can restore inertness.

3) Operating conditions for GC/MS—After initial installation of the capillary column, condition it according to the manufacturer's instruction. Daily, make a conditioning run with a CS_2 injection or method blank extraction injection before injecting any samples (optional). Typical instrument conditions are given in Table 6040:V.

d. Calibration standard: The method is semiquantitative for a large number of compounds, but has been shown to be quantitative for many of the compounds listed in Table 6040:I. Prepare a 20-ng/L target-compound calibration standard by dosing 1 L organic-free water in the stripping bottle with 10 μL combination internal standard solution plus 5 μL combination reference standards solution. (Internal standards concentration is 100 ng/L each.) If the salting-out technique is used, add 72 g Na_2SO_4 to a total volume of 900 mL organic-free water before dosing with 9 μL combination internal standard solution plus 4.5 μL combination reference standards solution. Analyze as directed above. Inject the calibration standard extract, preferably daily, to determine GC/MS response factors and verify spectra.

Verify working linear range by analyzing standards and representative samples with added organics at different concentrations. Calibrate at least every 2 weeks. Use calibration levels that bracket the levels found in samples. At minimum, use a three-level calibration curve.

e. Blanks: Run a procedural blank daily to assess contamination from reagents, apparatus, and other sources.

Run a blank after any sample that is more than ten times the calibration range or after installing new parts in the system. Alternatively, verify absence of carryover from such high-level samples by initially analyzing a sample spiked at the highest expected concentration followed by a blank. If results are nondetect for the blank, there is no need to routinely analyze a blank after high-level samples.

Analyze organic-free water with internal standards under the same conditions as samples.

5. Calculations

a. Identification: Identify a compound by matching both retention time and spectra of sample and standard. If available, use both a reverse-search computer program with a target-compound library and a forward-search program with the National Institute of Standards and Technology library to tentatively identify other compounds present.

1) Retention times—Use each internal standard to calculate relative retention times for all compounds in the same part of the chromatogram. For compounds eluting on the solvent tail, use an early-eluting internal standard (e.g., 1-chlorohexane). Sample retention times should match predicted retention times within ±15 s.

$$\text{Predicted } T_{z,x} = \frac{T_{z,s}}{T_{I,s}} \times T_{I,x}$$

where:

$T_{z,x}$ = target compound's retention time in sample analysis,
$T_{z,s}$ = target compound's retention time in calibration standard analysis,
$T_{I,s}$ = internal standard's retention time in calibration standard analysis, and
$T_{I,x}$ = internal standard's retention time in sample analysis.

2) Spectra—Peaks of at least three characteristic ions should all maximize at the same retention time and have standard intensity ratios (spectra) within ±20% of those of the calibration-standard compounds. Characteristic ions and their typical relative intensities for three internal standards and two earthy–musty smelling compounds are given in Table 6040:VI . Preferably, use reference spectra of 10 to 14 key masses. Determine reference spectra by analyzing standards; verify these frequently. The spectra of MIB are particularly dependent on instrument condition; both 107 and 95 amu have been reported as base peaks (Figure 6040:7). Figure 6040:8 shows the mass spectrum for geosmin.

b. Quantitation: Determine concentrations by comparing peak areas of specific quantitation ions. A quantitation ion should be relatively intense in the mass spectrum, yet free from interferences due to closely eluting compounds (Table 6040:VI). Calculate a response factor for each compound from CLSA of a calibration standard as follows:

$$R_z = \frac{A_z \times C_I}{C_z \times A_I}$$

where:

R_z = response factor for target compound z,
A_z = peak area of target compound z,
C_I = concentration of internal standard,
C_z = concentration of target compound z, and
A_I = peak area of internal standard.

Alternatively, a calibration fit can be used.

TABLE 6040:VI. GC/MS DATA FOR THREE INTERNAL STANDARDS AND TWO EARTHY-MUSTY SMELLING COMPOUNDS

Compound	Retention Time* *min*	Quantification Mass *amu*	Characteristic Ions (with relative intensities)
1-Chlorooctane	30.8	91	43 (100), 91 (86), 93 (27)
2-Methylisoborneol	36.4	95, 107†	95 (100), 107 (26), 135 (9)
1-Chlorodecane	39.8	91	43 (100), 91 (87), 93 (28)
Geosmin	45.1	112	112 (100), 111 (28), 125 (18)
1-Chlorododecane	47.2	91	43 (100), 91 (61), 93 (19), 85 (12)

* See Table 6040:IV for GC conditions. Data accumulated using 30-m DB-5 capillary column.
† Quantify using two different masses and obtain an average value.

The compound's concentration in the sample (x) is:

$$C_{z,x}, \text{ng/L} = \frac{A_{z,x} \times C_{I,x}}{R_z \times A_{I,x}}$$

where:

$C_{z,x}$ = concentration of target compound in sample,
$A_{z,x}$ = peak area of target compound in sample,
$C_{I,x}$ = concentration of internal standard in sample, and
$A_{I,x}$ = peak area of internal standard in sample.

Use the internal standard 1-chlorodecane to determine response factors. Use other internal standards as a system check; calculated values should be up to ±20%. Computerized reverse-search spectral matching and automatic quantitation are recommended to improve identification in complex matrices and to facilitate data processing.

If calibration standards are unavailable, estimate concentrations by comparing the compound's total ion current to that of 1-chlorodecane.

6. Quality Assurance/Quality Control

The CLSA method is semiquantitative for some compounds because of the variability of stripping efficiencies. However, quantitative data are obtainable for compounds that are reproducibly stripped (e.g., MIB and geosmin).[1–3,7] The QC practices considered to be an integral part of this method are summarized in Table 6020:I. Also, follow all QC described in the method itself.

Analyze a replicate sample at least once per 10 samples to check precision. If unusual or unexpected results are obtained, analyze a replicate to confirm. Typically, single-analyst determinations for a relatively simple matrix have a coefficient of variation less than or equal to 10%. Otherwise, precision is usually within 20%. For compounds that are poorly stripped, the coefficient of variation may be higher.

Analyze a sample with a known addition at least once per 10 samples to check accuracy and recovery. If matrix problems exist, this will confirm the results' accuracy. Adjust these recoveries against the calibration standards' results. Even when absolute recoveries are <50%, standard adjusted recoveries, which correct for stripping efficiencies, are usually between 80 and 120%.

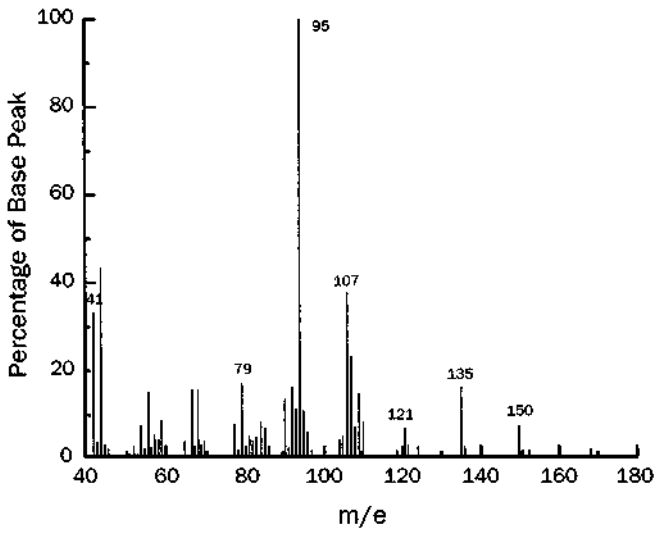


Figure 6040:7. Mass spectrum of 2-methylisoborneol.

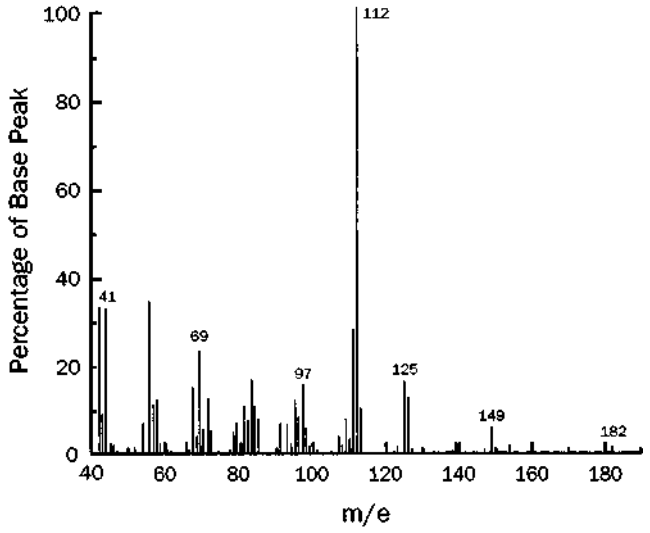


Figure 6040:8. Mass spectrum of geosmin.

TABLE 6040:VII. SINGLE-LABORATORY BIAS FOR SELECTED ORGANIC COMPOUNDS CAUSING TASTE AND ODOR

Stripping Technique Compound	Dose Level *ng/L*	Number of Samples*	Mean Recovery† %	Standard Deviation %
Unsalted method‡				
2-Isobutyl-3-methoxy pyrazine	4-20	23	89	21
2-Isobutyl-3-methoxy pyrazine	4-20	22	101	28
2-Methylisoborneol	4-120	30	101	21
2,3,6-Trichloroanisole	4-20	22	88	27
Geosmin	4-120	28	109	20
Salting-out method‡				
2-Isobutyl-3-methoxy pyrazine	4-20	44	120	24
2-Isobutyl-3-methoxy pyrazine	4-20	48	106	18
2-Methylisoborneol	4-20	48	106	15
2,3,6-Trichloroanisole	4-20	45	99	22
Geosmin	4-20	48	105	15

* Finished and natural surface waters.
† Standard-adjusted recovery.
‡ Stripping at 25°C.
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Internal standard response should equal ±40% of the daily standard. An unacceptable internal standard response requires extract reinjection. If the reinjection is still unacceptable, investigate cause, restrip sample, and re-analyze. If consecutive samples fail the internal standard acceptance criterion, immediately analyze a calibration standard. If the calibration standard internal standard response also is unacceptable, recalibrate the instrument.

Ideally prepare and analyze an intralaboratory check sample monthly. Prepare from a stock solution that is independent of the standards.

7. Precision and Bias

Precision and bias data for the analysis of earthy–musty smelling compounds are given in Tables 6040:VII and VIII. Table 6040:IX shows recovery and precision data for selected pollutants.

8. References

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TABLE 6040:VIII. PRECISION DATA FOR SELECTED ORGANIC COMPOUNDS CAUSING TASTE AND ODOR*

Compound	Dose Level *ng/L*	Multiple Laboratories† Mean *ng/L*	Multiple Laboratories† Standard Deviation *ng/L*	Multiple Laboratories† Coefficient of Variation %	Single Laboratory‡ Mean *ng/L*	Single Laboratory‡ Standard Deviation *ng/L*	Single Laboratory‡ Coefficient of Variation %
Sample A§							
2-Isopropyl-3-methoxy pyrazine	5.9	5.6	1.6	28	6.6	0.6	9
2-Isobutyl-3-methoxy pyrazine	3.0	3.0	0.7	24	3.2	0.3	11
2-Methylisoborneol	4.3	4.8	1.0	20	5.1	0.1	2
2,3,6-Trichloroanisole	8.7	7.3	3.1	43	9.3	2.0	22
Geosmin	2.9	3.3	0.9	27	3.6	0.5	14
Sample B§							
2-Isopropyl-3-methoxy pyrazine	25	22	8.0	36	27	2.8	10
2-Isobutyl-3-methoxy pyrazine	15	14	4.2	30	17	2.5	14
2-Methylisoborneol	20	18	6.2	34	23	2.0	9
2,3,6-Trichloroanisole	35	32	9.7	30	37	3.3	9
Geosmin	16	16	5.9	37	20	2.8	14

* Stripping at 25°C, unsalted method.
† Five analysts at three laboratories.
‡ Three analysts at one laboratory.
§ Organic-free water dosed with taste and odor compounds.

TABLE 6040:IX. RECOVERY AND PRECISION DATA FOR SELECTED PRIORITY POLLUTANTS*

Compound	Amount *ng*	Mean Recovered Amount† *ng*	Range	Recovery Efficiency %	RSD
Thiophene	25	9	7–11	35	16
Dibromochloromethane	29	17	13–213	57	13
Styrene	22	17	16–20	80	7
Isopropylbenzene	24	26	24–29	107	8
2-Chlorotoluene	26	23	22–27	90	8
bis(2-Chloroethyl)ether	24	3	3–4	12	11
α-Methylstyrene	22	19	18–22	90	6
1,4-Dichlorobenzene	20	18	16–21	93	8
2-Ethyl-1,3-dimethylbenzene	24	22	21–25	92	6
4-Chloro-*o*-xylene	26	22	20–26	85	9
1,1-Dimethylindan	22	24	22–26	110	7
p-Methylphenol	27	ND‡			
Tetrahydronaphthalene	23	23	20–26	99	10
1,2,4-Trichlorobenzene	23	19	18–21	83	7
Hexachloro-1,3-butadiene	27	31	28–34	114	9
2-Methylbiphenyl	24	25	22–27	101	8
1,6-Dimethylnaphthalene	24	10	8–11	39	12
2-Isopropylnaphthalene	23	21	19–22	91	6
Pentachlorobenzene	26	12	11–14	48	11
Hexachlorobenzene	20	6	5–7	31	13
2,2′,4,4′,6,6′-Hexachlorobiphenyl	27	28	26–32	104	7
2,2′,4,5,5′-Pentachlorobiphenyl	28	23	18–28	82	14

* Stripping at 40°C, unsalted method.
† Based on six purging analyses using single ion quantification.
‡ ND = not detected.
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6040 C. Purge and Trap Technique

For applications of this technique to volatile organics, volatile aromatic organics, and volatile halocarbons analyses, see Section 6200. NOTE: Such items as sample volume and purge conditions may need to be modified from those stated in Section 6200 to meet a particular client's or situation's criteria for taste and odor compounds.

6040 D. Solid-Phase Microextraction (SPME)

1. General Discussion

a. Principle: SPME of taste- and odor-causing compounds relies on target compounds adsorbing on a SPME fiber (a fiber coated with divinylbenzene-carboxen-polydimethylsiloxane cross-link). The fiber is placed in the headspace above the sample in a closed vessel and allowed to equilibrate with the aqueous sample. After a predetermined equilibration time, the fiber is removed and inserted directly into the injection port of a GC or GC/MS system. The port is heated and the analytes desorbed into the instrument. Perform analysis using GC/MS in the selected ion monitoring mode, monitoring the multiple major masses (Table 6040:II) for each target analyte, if possible, to ensure more accurate identification and quantitation. The method has been validated for three taste and odor compounds [MIB, geosmin, and isopropyl methoxy pyrazine (IPMP)] and may be applicable to others. SPME is much faster than closed-loop stripping and uses a much smaller sample size. However, it is critical that samples and standards be processed in *exactly* the same manner because extraction conditions determine the response factor.

b. Interference: Any organic compounds in the sample may be adsorbed onto the fiber and desorbed into the GC/MS. The mass spectrum identifies the compound of interest and eliminates possible interferences. For matrices containing intracellular geosmin or MIB, demonstrate recovery by using matrices with known additions of these compounds.

c. Sampling and storage: Collect samples in duplicate 60-mL glass vials with Teflon-lined caps, with no headspace. Minimize sample aeration. Begin cooling samples immediately and keep cold during transport to lab. Store samples at 2 to 6°C in the laboratory until analyzed. Samples must be analyzed within 3 d of collection or else preserved. If holding longer than 3 d, add 80 μL sodium omadine solution per 40 mL sample to inhibit biological activity. Preserved samples must be analyzed within 7 d of collection. Holding study results are shown in Table 6040:III.

d. Minimum detectable concentration: With 45 mL sample in a 60-mL vial for 30 min equilibration, concentrations as low as 1 to 5 ng/L of most target compounds can be determined. Table 6040:X shows two laboratories' minimum detection limit (MDL) results: one performed over 8 d with 8 replicates and 3 ng/L known addition, and the other over 1 d at 5 ng/L known addition. Under these conditions, the response for MIB, geosmin, and IPMP is linear from 10 to at least 100 ng/L (<20% RSD) with internal standard [3-isobutyl-2-methoxypyrazine (IBMP)] calibration (Table 6040:XI) and can be measured with a calibration curve to higher levels. Some laboratories have demonstrated linearity to 100 ng/L or higher without using an internal standard (Table 6040:XII).

TABLE 6040:X. METHOD DETECTION LEVEL (MDL) IN REAGENT WATER FOR MIB, GEOSMIN, AND IPMP USING METHOD 6040D

	MDL for Given Compound and Laboratory *ng/L*				
	MIB		Geosmin		IPMP
Replicate	Laboratory A*	Laboratory B	Laboratory A*	Laboratory B	Laboratory A
1	3.4	5.3	4.2	5.4	5.3
2	2.5	5.1	3.3	4.7	6.1
3	1.8	4.9	3.3	4.7	5.0
4	1.7	5.7	3.8	4.6	4.9
5	2.2	4.9	3.0	5.5	4.2
6	2.3	5.0	2.9	5.0	5.3
7	2.6	5.0	2.2	4.7	5.0
8	2.0	4.4	1.9	5.0	5.3
9	—	5.2	—	5.3	4.7
10	—	5.4	—	5.5	4.5
Average	2.3	5.1	3.1	5.0	5.0
Std deviation	0.5	0.4	0.8	0.4	0.52
MDL	1.6	1.0	2.3	1.0	1.6

* 3 ng/L known-addition level on 8 separate days.

e. Precautions: IPMP has been shown to cause allergic reactions when inhaled; use in a fume hood as much as possible. 2,3,6-Trichloroanisole (TCA) is a suspected carcinogen, so use proper precautions when working with this chemical.

2. Apparatus

a. Gas-chromatograph/mass spectrometer: Any GC/MS system capable of meeting the specifications in Section 6200B.2 is acceptable.* Several GC columns have been tested;† others may be acceptable but have not yet been tested.

b. Solid-phase microextraction apparatus:‡ This method was developed using a manual SPME holder. Automated units may be used if they can accommodate the required sample size.

c. Water-bath capable of maintaining at least 50°C. Higher temperatures (65°C) typically produce more precise results. A covered water bath is preferable. Sonication or stirring shortens equilibration times but is not necessary.

* Data reported herein were obtained with Finnigan-Mat 4000 MS, Varian 3400 GC, and HP 5890/5972 MSD.
† Supelcowax-10 (Supelco) and DB5-MS (Restek).
‡ Supelco catalog No. 5-7330, or equivalent.

https://doi.org/10.2105/SMWW.2882.117

Table 6040:XI. Internal Standard Corrected Response Factor for 5–100 ng/L Taste- and Odor-Causing Compounds in Reagent Water Using Method 6040D

Temperature °C	Concentration ng/L	Response Factor for Given Compound		
		MIB	Geosmin	IPMP
50	5	1.87	1.06	0.77
	20	1.27	0.92	0.87
	25	1.32	0.93	0.94
	40	1.13	0.96	0.87
	50	1.11	0.95	0.86
	75	1.05	1.10	0.81
	100	1.04	1.25	0.75
	Avg.	1.25	1.02	0.84
	SD	0.29	0.12	0.07
	% RSD	23.2	11.8	7.8
65	5	1.02	0.98	0.75
	10	0.71	1.31	0.86
	20	0.83	1.31	0.92
	50	0.61	1.35	0.75
	70	0.66	1.37	0.86
	100	0.75	1.85	0.75
	Avg.	0.71	1.44	0.83
	SD	0.06	0.23	0.07
	% RSD	11.6	16.1	9.2

d. Graduated cylinder, glass, 50 mL (Class A).

e. Block heater or hot plate (if using aluminum block).

f. Vial with septum, 60-mL.§

3. Reagents

Use reagent-grade solvents or better and American Chemical Society (ACS)-grade standards if available.

a. SPME fiber (divinylbenzene-carboxen-polydimethylsiloxane cross-link coating):‖ Other fiber chemistries may be applicable, but must be validated by the user.

b. Stock standard solutions, 100 mg/L MIB, geosmin, IBMP, and IPMP, or other relevant concentrations. Prepare stock standards from neat compounds weighed gravimetrically and dissolved in acetone or methanol using procedures described in 6040B.3, or purchase from commercial sources. Standards may be prepared as a mixture or as single solutions.

c. Working standard solutions, 10 mg/L MIB, geosmin, and/or IPMP. Prepare working standard by diluting stock standard 1:10 in acetone or methanol. Standard is stable for at least 30 d.

d. Calibration standard, 45 μg/L MIB, geosmin, and/or IPMP. Prepare a 45-μg/L calibration standard by diluting 45 μL of the 10-mg/L solution in 10 mL acetone or methanol. Store the standard at 4°C. Replace after 3 d. Prepare calibration curve by adding appropriate amounts of calibration standard to 45 mL reagent water (10 μL = 10 ng/L, 20 μL = 20 ng/L, etc.). Include at least five calibration standards if using a linear evaluation and at least six calibration standards if using a quadratic evaluation.

§ I-Chem RC346-0060, or equivalent.

‖ Supelco catalog No. 5-7348U, or equivalent.

Table 6040:XII. Calibration for 1–100 ng/L Taste- and Odor-Causing Compounds Without an Internal Standard at 65°C.

Concentration ng/L	Primary Ion Count		
	MIB	Geosmin	IPMP
1	612	1483	513
5	3994	7363	3076
10	6944	13 979	6126
20	11 134	29 225	11 254
50	29 834	75 165	25 251
70	36 935	9763	34 456
100	58 540	138 987	48 652
R^2	0.995	0.999	0.999

e. Laborotory-fortified blank (LFB) and laboratory-fortified matrix (LFM) standard, 15 to 20 ng/L MIB and geosmin.

f. Internal standard, 45 μg IBMP/L: Prepare internal standard by diluting from 100-mg/L stock standards and dissolving in acetone or methanol. IBMP's response factor is similar to those of MIB and geosmin, and is not generally found in samples. Final concentration in samples will be 10 ng/L.

g. Surrogate, 45 μg/L IPMP: IPMP is an effective surrogate to allow monitoring of fiber performance for each sample. Prepare surrogate by dilution from calibration standard as in ¶ *f* above. Final concentration in samples will be 10 ng/L. Higher levels (20 to 50 ng/L final concentration) also may be used if desired. Surrogate can be prepared as a mix with the internal standard if desired.

h. Sodium chloride (NaCl), reagent-grade: Check salt for contamination. Bake if necessary to eliminate contaminants. If baking does not work, use another lot of salt.

i. Sodium omadine, 3.2% solution: Prepare by weighing 3.2 g sodium omadine into 100-mL volumetric flask. Dilute to mark with deionized water. This solution is stable for 1 month.

4. Procedure

Load SPME fiber in SPME holder. Condition fiber according to manufacturer specifications before first use.

To each 60-mL vial, add at least 13.5 g NaCl. Use same amount of salt in samples and standards. Pour 45 mL sample into a NaCl-prepared vial. If using internal standard method, add 10 μL of 45-μg/L internal standard and surrogate to each sample. Otherwise, add only surrogate. Swirl gently for 15 s to mix well. Secure vial in a water-bath set at 65 ± 2°C. Lower temperatures (50°C) are acceptable but may lead to nonlinear calibration and poorer precision. Alternatively, use aluminum heating block to extract multiple samples at once, being very careful to control temperatures. Adjust SPME holder to a depth setting that places the SPME fiber in the headspace. (A depth setting of 1 is appropriate for the referenced equipment. Depth setting may vary by model and manufacturer.) Insert holder into vial and secure in place. Depress plunger to expose fiber to vial's headspace at the premarked level.

Extract for 30 to 35 min. Longer times are acceptable, as long as all samples and standards are extracted for exactly the same period. Shorter times do not give consistent recoveries. Fully retract fiber into holder before its removal. Check holder's needle for water; if present, blot water with clean tissue.

Adjust holder depth setting so when fiber is extended, it is several millimeters above the injector.#

Insert holder's needle into GC injector port. Depress plunger to expose fiber in injector and immediately start the run. Allow fiber to remain in injector port for 10 min to clean up fiber for the next extraction.

Analyze samples using conditions recommended for the apparatus used. The analyst may optimize conditions for the specific analysis as long as all QC requirements are met.**

Monitor primary masses for quantitation. Monitor secondary masses for confirmation if instrumentation has such capability.

MIB – 95 (primary), 93, 107, 108, 135 (secondary)
Geosmin – 112 (primary), 126 (secondary)
IPMP – 137 (primary), 152, 124 (secondary)
IBMP – 124 (primary), 151, 94 (secondary)

Inconsistent recoveries may be due to variations in exposure time, temperature, or condition of fiber. Confirm that the fiber is in good condition and that both time and temperature are identical to those for the calibration curve. Low recovery usually is due to water at the needle tip after extraction. Be sure to blot any residual water before injection. Using a surrogate in each sample monitors this problem. High, low, or variable water-bath temperatures may lead to lower recoveries and greater variability. Ensure that the bath or heating block temperature is between 50 and 65°C. If using a heating block, temperatures up to 60°C will not greatly affect headspace temperature. Be sure the temperature for both standards and samples is exactly the same.

If sample is suspected to contain IPMP or IBMP, or if internal standard or surrogate recoveries are >130% (indication of possible interference), re-analyze sample without adding internal standard and surrogate to verify absence of interference.

5. Calculations

a. Identification: Identify a compound by matching both retention time and spectra of sample to a calibration standard.

1) Retention times—Use the internal standard to calculate relative retention times for all compounds. Sample retention times should match predicted retention times within ±15 s.

$$\text{Predicted } T_{z,x} = \frac{T_{z,x}}{T_{I,s}} \times T_{I,x}$$

where:

$T_{z,x}$ = target compound's retention time in sample analysis,
$T_{z,s}$ = target compound's retention time in calibration standard analysis,
$T_{I,s}$ = internal standard's retention time in calibration standard analysis, and
$T_{I,x}$ = internal standard's retention time in sample analysis.

2) Spectra—Peaks of at least three characteristic ions should all maximize at the same retention time, plus or minus one scan, and have standard intensity ratios (spectra) within ±20% of those of the calibration-standard compounds. Determine reference spectra via analysis of standards; verify these frequently.

b. Quantitation: Determine concentrations by comparing peak areas of specific quantitation ions. A quantitation ion should be relatively intense in the mass spectrum, yet free from interferences caused by closely eluting compounds. Calculate a response factor for each compound from a calibration standard as follows:

$$R_z = \frac{A_Z \times C_I}{C_Z \times A_I}$$

where:

R_z = response factor for target compound z,
A_z = peak area of target compound z,
C_I = concentration of internal standard,
C_z = concentration of target compound z, and
A_I = peak area of internal standard,

Alternatively, a calibration fit can be used.

The compound concentration in the sample (x) is:

$$C_{z,x}, \text{ ng/L} = \frac{A_{z,x} \times C_{I,x}}{R_z \times A_{I,x}}$$

where:

$C_{z,x}$ = concentration of target compound in sample,
$A_{z,x}$ = peak area of target compound in sample,
$C_{I,x}$ = concentration of internal standard in sample, and
$A_{I,x}$ = peak area of internal standard in sample.

6. Quality Control

Prepare a calibration curve when necessary to achieve linearity with relative standard deviation (RSD) <20% over the desired calibration range. If regressed, the correlation coefficient (R^2) must be 0.995 (or 0.990 for quadratic fit). The QC practices considered to be an integral part of this method are summarized in Table 6020:I. Also, follow all QC described in the method itself.

Each day or when a new fiber is first used (whichever is more frequent), validate method by analyzing a midrange LFB. LFB recovery must be between 70 and 130% of nominal value. Recalibrate if QC criteria are not met. There is no limit to the number of samples that a fiber can analyze.

For each fiber, at a minimum, analyze a check sample at the desired minimum reporting level (MRL) with each 20 samples or weekly (whichever is more frequent); ideally, analyze a check sample daily to demonstrate that fiber is maintaining extraction efficiency. When fiber can no longer extract samples reliably down to the MRL, discard it. The acceptance criterion for the MRL check sample is ±50% from its nominal value.

Analyze an LFM with each 20 samples and with each new matrix or weekly (whichever is more frequent). The LFM should be near the midpoint of the calibration range or near the level of interest. The LFM acceptance criterion is 70 to 130%. Flag values outside these limits as a matrix effect as long as LFM addition at least doubles the ambient concentration.

For Varian 3400 and Hewlett Packard GCs, preferably use a depth setting of 3.
** For example:
Finnigan 4000 MS; Restek DB-5 column; detector: quadrupole, SIM mode; injector: splitless @ 250°C, split vent at 0.70 min @40 mL/min; temperature program: 50°C for 1 min, ramp 12°C/min, 250°C for 6 min.
HP 5972; Supelcowax 10 column; detector: quadrupole, SIM mode; injector: splitless@ 250°C, split vent at 2.0 min @ 33 mL/min, 0.75 mm liner; temperature program: 90°C for 8 min, ramp 15°C/min, 180°C for 6 min.

Table 6040:XIII. Comparison of Methods 6040B and D in a Single Laboratory

Water Analyzed*	Concentration† ng/L			
	MIB		Geosmin	
	6040B	6040D	6040B	6040D
RWS	13	9	8.9	9
LFM	41	41	43	43
RWS	6.5	5.8	6.5	3.3
RWS	32	40	22	28
RWS	19	16	4.1	2.5
LFM	39	39	24	24
RWS	77	91	2.8	2.9
RWS	3.8	3.5	ND(<3)	0.3
RWS	ND(<3)	1.3	5.6	5.2
RWS	ND	ND	ND	ND
RWS	ND(<3)	1.7	8.3	7.7
RWS	ND	ND	12.5	11.3
RWS	16/10	11	13.1	13.1
RWS	11	8	5.2/3.9	2.7
RWS	3.7	1.3	3.7	3.1
RWS	10.1	8.7	9	7.1
RWS	11	5.7/5.2	4.4	3.2
RWS	22	24	4.3	1.4

* RWS = raw water sample; LFM = laboratory-fortified matrix.
† ND = not determined.
Note: Closed-loop stripping (B) performed within 3 d of collection; solid-phase microextraction (D) performed after storage of up to 2 months. Extraction performed at 50°C. Results with value/value represent duplicate analyses.

Table 6040:XIV. Comparison of Results for MIB and Geosmin in Two Different Laboratories

Analyte	Laboratory A ng/L	Laboratory B ng/L
MIB	ND*	ND
	ND*	Interference
	Interference*	ND
	13*	16
	9	11
	5.8	3.2
	40†	77†
	16 (19 by CLS)	23
Geosmin	ND(<3)*	5.3
	ND*	5.3
	3.5*	2.3
	48*	64
	9	10
	3.3	3
	28 (22 by CLS)	20
	2.5	2.2

* Analyzed by 6040B.
† Re-analysis by CLS in Laboratory A gave a result of 32 and did not resolve the difference between the laboratories. Method 6040D extraction at 50°C in Laboratory A and 65°C in Laboratory B.

Analyze a blank daily. Analytes should not be detected in the blank. Run a blank after any sample that is more than ten times the calibration range or after installing new parts in the system. Alternatively, verify absence of carryover from such high-level samples by initially analyzing a sample spiked at the highest expected concentration followed by a blank. If results are nondetect for the blank, there is no need to routinely analyze a blank after high-level samples.

Preferably include a surrogate with each sample. The surrogate acceptance criterion is 70 to 130%.

The laboratory may wish to monitor internal standard recoveries as another indicator of fiber efficiency.

7. Precision and Bias

The MDL for this method was determined to be <5 ng/L for MIB, geosmin, and IPMP (Table 6040:X) under the conditions described above.

One laboratory analyzed a number of raw water samples for MIB and geosmin using both 6040B and 6040D, and two laboratories analyzed a series of samples via 6040D using the different extraction and analysis options included in the method. Comparison results are shown in Tables 6040:XIII and XIV. Recoveries for LFB at 20 ng/L in one laboratory are 95 ± 10% for MIB and 97 ± 12% for geosmin for a set of 30 points collected over 2 months.

8. Bibliography

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6040 E. Solid-Phase Microextraction (SPME) with CI GC/MS/MS

1. General Discussion

a. Principle: SPME of taste- and odor-causing compounds relies on target compounds adsorbing to an SPME fiber, which consists of a fiber coated with divinylbenzene-carboxen-polydimethylsiloxane cross-link. The fiber is placed in the headspace above a heated sample in a closed vessel and allowed to equilibrate with the aqueous sample. After a predetermined equilibra-

Table 6040:XV. Analytes with Parent and Quantitation Ions for Method 6040E

Analyte	Quantitation Ion	Parent Ion
2-Isopropyl-3-Methoxpyrazine (IPMP)	121	153
2-Isobutyl-3-Methoxypyrazine (IBMP)	125	167
2-Methylisoborneol (MIB)	95	151
2,3,6-Trichloroanisole (TCA)	176	211
Geosmin	109	165

tion time, the fiber is removed and inserted directly into the injection port of a GC/MS system. The port is heated and the analytes desorbed into the instrument. Analysis is performed using GC with methanol CI/MS/MS, monitoring the major product ion masses (Table 6040:XV) for each target analyte to ensure more accurate identification and quantitation. The method has been validated for at least three taste and odor compounds—MIB, geosmin, and IPMP—and may be applicable to others. SPME is faster than closed-loop stripping and uses a smaller sample. However, it is critical that samples and standards be processed in *exactly* the same manner because extraction conditions determine the response factor.

b. Interference: Any organic compounds in the sample may be adsorbed onto the fiber and desorbed into the GC/MS. The mass spectrum identifies the compound of interest and eliminates possible interferences. For matrices containing intracellular geosmin or MIB, demonstrate recovery by using matrices with known additions of these compounds.

c. Sampling and storage: Collect duplicate samples in 40- or 60-mL glass vials with Teflon-lined caps, with no headspace. Minimize sample aeration. Begin cooling samples immediately. Store samples at 4°C in the laboratory until analyzed. Samples must be analyzed within 3 d of collection or else preserved. If holding longer than 3 d, add 80 μL sodium omadine solution per 40 mL sample (120 μL per 60 mL sample) to inhibit biological activity. Preserved samples must be analyzed within 7 d of collection. Holding study results are shown in Table 6040:III.

d. Minimum detectable concentration: With a 10 mL sample in a 20-mL vial and 30-min equilibration, concentrations as low as 1 to 5 ng/L of most target compounds can be determined. Table 6040:XVI shows the results of MDL determinations at two laboratories: one performed over 7 d with seven replicates and a known addition of 3 ng/L, and the other performed in 1 d with a known addition of 5 ng/L.

e. Precautions: IPMP has been shown to cause allergic reactions when inhaled; use in a fume hood as much as possible. TCA is a suspected carcinogen, so use proper precautions when working with this chemical.

2. Apparatus

a. Gas chromatograph/mass spectrometer: The GC/MS system must be capable of CI and tandem MS.* Several GC columns have been tested;† others may be acceptable but have not yet been tested.

b. Solid-phase microextraction apparatus: A programmable autosampler system capable of conducting the entire SPME procedure—from fiber conditioning, heated/agitated extraction through sample injection—provides optimum reproducibility.‡ A manual SPME holder can be substituted.§

c. Water-bath capable of maintaining 65°C (required only if performing manual SPME).

d. Disposable pipet, glass, 10-mL.

e. Block heater or hot plate (if using manual SPME).

f. Vial with septum, 40-mL, for sampling.‖

g. Crimp-cap vial with septum or screw cap vial, 20-mL, for analysis.

h. 50-mL glass graduated cylinder.

i. Glass syringes (10-, 25-, 50-, and 100-μL).

* Data reported herein were obtained with Varian Saturn 2200 MS, Varian 3800 GC.
† VF5MS 60 m, 0.25-mm ID, 0.25μm film (Varian) and CP Sil8 CB-MS 30 m, 0.25-mm ID, 0.25μm film (Varian).
‡ Data reported herein were obtained using CTC Analytics Combi-Pal.
§ Supelco catalog No. 5-7330, or equivalent.
‖ I-Chem Series 200, or equivalent.

Table 6040:XVI. Method Detection Level (MDL) in Reagent Water for MIB and Geosmin by Method 6040E

	MDL for Given Compound and Laboratory *ng/L*			
	MIB		Geosmin	
Replicate	Laboratory A*	Laboratory B	Laboratory A*	Laboratory B
1	3.8.	4.9	3.6	5.3
2	3.1	4.2	3.4	5.4
3	2.5	6.4	2.8	5.3
4	3.3	4.8	3.0	4.7
5	2.9	5.7	2.9	3.9
6	2.3	5.0	2.4	4.7
7	3.8	3.8	3.1	5.1
Average	3.0	4.97	3.0	4.90
Std deviation	0.5	0.86	0.4	0.53
MDL	1.5	2.7	1.3	1.67

* 3 ng/L known-addition level on 7 separate days.

Table 6040:XVII. RSD and Mean Areas for IPMP, IBMP, and TCA

	IPMP (area counts)	IBMP (area counts)	TCA (area counts)
	35 052	62 908	94 124
	40 152	92 734	80 940
	29 141	90 623	73 271
	26 807	63 377	70 956
	28 053	69 057	80 245
	24 635	69 564	83 676
	19 147	54 225	76 331
Mean	28 998	71 784	79 935
RSD	24%	20%	10%

Table 6040:XVIII. Combipal Conditions/Parameters

Injection Mode:	SPME Mode
Sample Temperature:	65°C
Pre-Incubation Agitator Speed (rpm):	250
Agitator Cycle:	30 s on, 2 s off
Extraction Time:	25 min
Fiber Depth from Bottom:	13 mm
Desorb Time:	4 min
Use Fiber Bakeout Station:	Yes
Fiber Bakeout Time:	30 s
GC Cycle Time:	15 min

3. Reagents

Use reagent-grade solvents or better and ACS-grade standards if available.

a. SPME fiber (divinylbenzene-carboxen-polydimethylsiloxane cross-link coating):# Other fiber chemistries may be applicable but must be validated by the user.

b. Stock standard solutions, 100 mg/L MIB, geosmin, IBMP, and IPMP; 1000 mg/L TCA, or other relevant concentrations. Prepare stock standards from neat compounds weighed gravimetrically and dissolved in acetone or methanol using procedures described in 6040B.3, or purchase from commercial sources. Standards may be purchased as a mixture or as single solutions.

c. Working standard solutions, 5 mg/L MIB and geosmin and 10 mg/L IBMP, IPMP, and TCA. Prepare working standard solution for MIB and geosmin by diluting stock standard 1:20 in acetone. Prepare working standard solution for IBMP and IPMP by diluting stock standard 1:10 in acetone. Prepare standard working solution for TCA by diluting 1:1000 in acetone. Standard is stable for at least 30 d.

d. Calibration standard solution, 10 μg/L MIB and geosmin. Prepare a 10-μg/L calibration standard by diluting 2 μL of 5-mg/L solution in 1 mL acetone. Store at 4°C. Replace after 3 d. Prepare calibration curve by adding appropriate amounts of calibration standard to 10 mL reagent water (10 μL = 10 ng/L, 20 μL = 20 ng/L, etc.).

e. Laboratory-fortified blank (LFB) and laboratory-fortified matrix (LFM) standard, 15 to 20 ng/L MIB and geosmin.

f. Internal standard, 40 μg/L IBMP or 40 μg/L TCA: Prepare internal standards via dilution from 10-mg/L stock standards and dissolution in acetone or methanol. IBMP has a response factor similar to those of MIB and geosmin and is not generally found in samples. Final concentration in samples will be 20 ng/L. TCA has area counts similar to IBMP with a better RSD; however, some samples may contain TCA even if it was not added as an internal standard (Table 6040:XVII).

g. Surrogate, 4-μg/L IPMP: IPMP is an effective surrogate for monitoring fiber performance for each sample. Prepare surrogate by dilution from 10-mg/L stock standard. Final concentration in samples will be 20 ng/L. Higher levels (20- to 50-ng/L final concentration) also may be used if desired. Surrogate can be prepared as a mix with the internal standard if desired.

h. Sodium chloride (NaCl), reagent-grade: Check salt for contamination. Bake if necessary to eliminate contaminants. If baking does not work, use a different lot of salt.

i. Sodium omadine, 3.2%. Prepare by weighing 3.2 g sodium omadine into 100-mL volumetric flask. Dilute to mark with deionized water. This solution is stable for 1 month.

4. Procedure

Load SPME fiber in SPME holder. Condition the fiber according to manufacturer specifications before first use.

Pipet 10 mL sample into a 20-mL vial. To each sample, add 5 μL of 40-μg/L internal standard and surrogate (optional). To each 20-mL vial, add 2.5 g NaCl before adding sample. Use same amount of salt in samples and standards. Seal vial with cap. Shake gently for 15 s to mix well. Place vial in autosampler tray, and process using the conditions in Table 6040:XVIII (or alternate conditions if all QC requirements can be met). Alternatively, use the manual method described in 6040D.

Analyze samples using the GC/MS conditions listed in Table 6040:XIX. The quantitation and secondary ions are listed in Table 6040:XV.

Inconsistent recoveries may be due to variations in exposure time, temperature, or condition of fiber. Confirm that fiber is in good condition and that both time and temperature are identical to those for the calibration curve. Using a surrogate in each sample monitors analyte recoveries.

If the sample is suspected to contain IPMP or IBMP, or if internal standard or surrogate recoveries are >130% (indication of possible interference), re-analyze sample without internal standard and surrogate additions to verify absence of interference.

5. Calculations

a. Identification: Identify a compound by matching both retention time and spectra of sample to a calibration standard. Figures 6040:9 –13 are examples of mass spectra when methanol is the CI reagent.

1) Retention times—Use the internal standard to calculate relative retention times for all compounds. Sample retention times should match predicted retention times within ±15 s.

Supelco catalog No. 5-7348U, or equivalent.

$$\text{Predicted } T_{z,x} = \frac{T_{z,s}}{T_{i,s}} \times T_{i,x}$$

where:

$T_{z,x}$ = target compound's retention time in sample analysis,
$T_{z,s}$ = target compound's retention time in calibration standard analysis,
$T_{I,s}$ = internal standard's retention time in calibration standard analysis, and
$T_{I,x}$ = internal standard's retention time in sample analysis.

2) Spectra—Peaks of at least three characteristic ions should all maximize at the same retention time, plus or minus one scan, and have standard intensity ratios (spectra) within ±20% of those of the calibration-standard compounds. Determine reference spectra by analyzing standards; verify these frequently.

b. Quantitation: Determine concentrations by comparing peak areas of specific quantitation ions. A quantitation ion should be relatively intense in the mass spectrum, yet free from interferences caused by closely eluting compounds. Calculate a response factor for each compound from a calibration standard as follows:

$$R_z = \frac{A_z \times C_I}{C_z \times A_I}$$

where:

R_z = response factor for target compound z,
A_z = peak area of target compound z,
C_I = concentration of internal standard,
C_z = concentration of target compound z, and
A_I = peak area of internal standard,

Alternatively, a calibration fit can be used.

The compound concentration in the sample (x) is:

$$C_{z,x}, \text{ ng/L} = \frac{A_{z,x} \times C_{I,x}}{R_z \times A_{I,x}}$$

where:

$C_{z,x}$ = target compound's concentration in sample,
$A_{z,x}$ = peak area of target compound in sample,
$C_{I,x}$ = internal standard's concentration in sample, and
$A_{I,x}$ = peak area of internal standard in sample.

6. Quality Control

Prepare a calibration curve when necessary to achieve linearity with RSD<20% over the desired calibration range. If regressed, the correlation coefficient (R^2) must be at least 0.995 (or 0.990 for quadratic fit). The QC practices considered to be an integral part of this method are summarized in Table 6020:I. Also, follow all QC described in the method itself.

Each day or when a new fiber is first used (whichever is more frequent), validate method by analyzing a midrange LFB. LFB recovery must be between 70 and 130% of nominal value. Recalibrate if QC criteria are not met. There is no limit to the number of samples that a fiber can analyze.

TABLE 6040:XIX. GC/MS PARAMETERS FOR METHOD 6040E

Varian GC 3800 Operating conditions/parameters
Injector temperature: 250°C

Time *min*	Split State	Split ratio
Initial	On	20
0.01	Off	Off
2.00	On	20

Column Temperature Program

Temperature *°C*	Rate *°C/min*	Hold *min*	Total min
50	0.00	1	1
200	10	—	15
200	0.00	2	2

Column flow: 1.5 mL/min

Varian Saturn 2200 MS conditions/parameters

Segment Description	Start	End	Low Mass	High Mass	Ionization Mode	Ionization Preparation
Fil/mult.						
Delay	0.00	6.70	40	650	None	MS/MS
IPMP	6.70	7.50	75	155	CI Auto	MS/MS
IBMP	7.50	8.50	60	170	CI Auto	MS/MS
MIB	8.50	10.5	91	155	CI Auto	MS/MS
TCA	10.5	11.50	150	220	CI Auto	MS/MS
Geosmin	11.50	12.15	95	185	CI Auto	MS/MS

Segment Set Points, All Compounds

Scan time:	0.60
Mult. Offset:	300+
Emission Current:	30
Count Threshold:	1
Mass Defect, mmu/100u:	0
Cal Gas:	Off
Ionization Mode:	CI Auto
Reagent Gas:	Methanol
CI Storage Load, m/z:	19.0
Ejection Amplitude:	15.0 V
Background Mass:	55
Target TIC:	25 000
Maximum Ionization Time:	2500
Maximum Reaction:	60 m/z
Prescan Ionization:	200
Ion Preparation, MS/MS	

Isolation

Compound	Parent Ion *m/z*	Isolation Window *m/z*
IPMP	153	3.0
IBMP	167	3.0
MIB	151	3.0
TCA	211	3.0
Geosmin	165	3.0

Dissociation

Compound	Waveform Type	Excitation Storage Level	Excitation Amplitude *volts*
IPMP	Resonant	48	0.61
IBMP	Resonant	48	0.49
MIB	Resonant	48	0.38
TCA	Resonant	48	0.39
Geosmin	Resonant	62.6	0.51

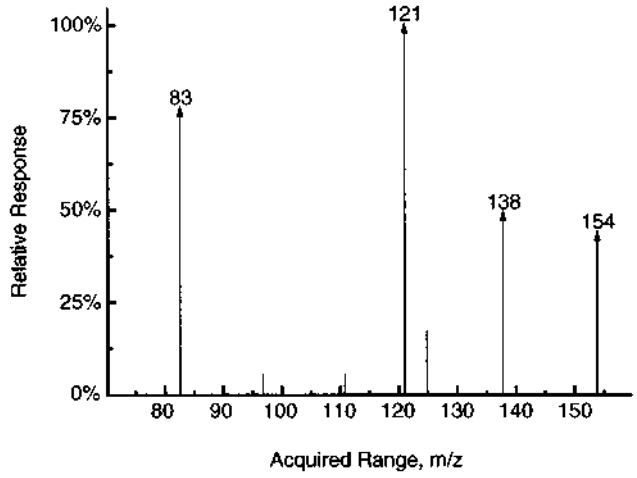


Figure 6040:9. Mass spectrum of IPMP with methanol as the chemical ionization reagent.**

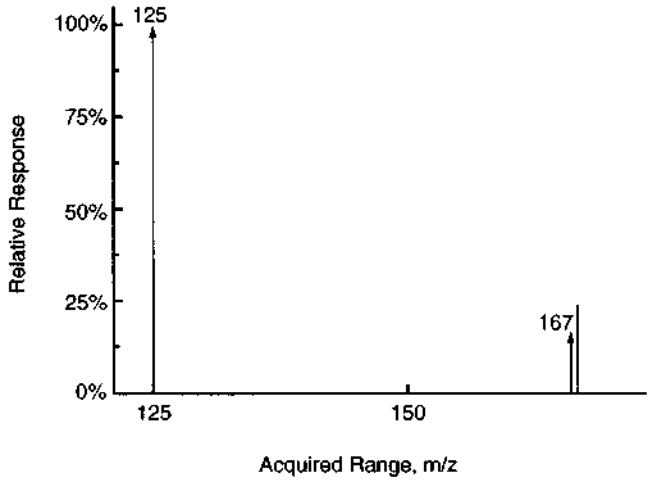


Figure 6040:12. Mass spectrum of geosmin with methanol as the chemical ionization reagent.**

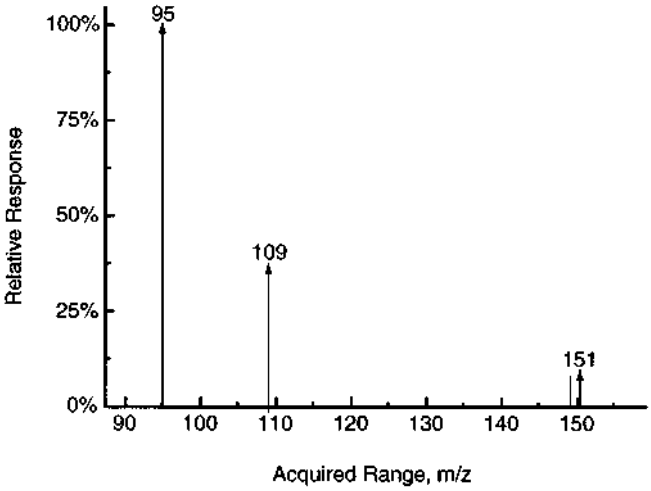


Figure 6040:10. Mass spectrum of IBMP with methanol as the chemical ionization reagent.**

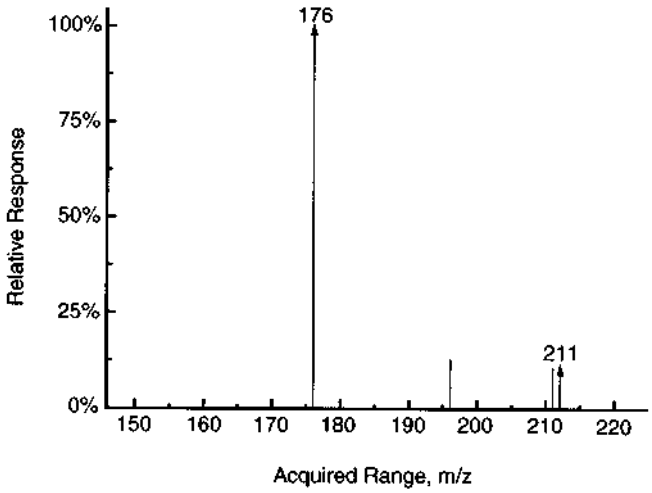


Figure 6040:13. Mass spectrum of TCA with methanol as the chemical ionization reagent.**

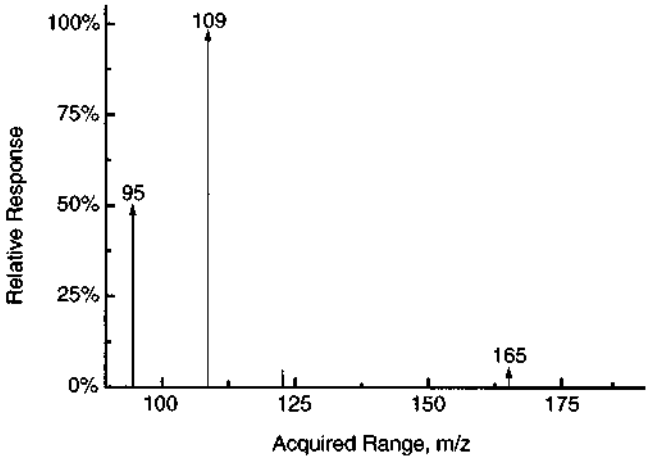


Figure 6040:11. Mass spectrum of MIB with methanol as the chemical ionization reagent.**

TABLE 6040:XX. COMPARISON OF RESULTS FOR MIB AND GEOSMIN IN TWO DIFFERENT LABORATORIES USING METHOD 6040E

Analyte	Actual Concentration	Laboratory A *ng/L*	Laboratory B *ng/L*
MIB	10	8	11
	40	43	44
	23	18	23
	ND	ND	ND
	50	52	52
	15	18	22
Geosmin	5	5	6
	27	25	23
	35	32	30
	ND	ND	1
	30	34	34
	7	8	8

For each fiber, at a minimum, analyze a check sample at the desired MRL with each 20 samples or weekly (whichever is more frequent); ideally, analyze a check sample daily to demonstrate that the fiber is maintaining extraction efficiency. When the fiber can no longer extract samples reliably down to the MRL, discard it. The acceptance criterion for the MRL check sample is ±50% from its nominal value.

**Figures 6040:9–13 courtesy of Metropolitan Water District of Southern California.

Analyze an LFM with each 20 samples and with each new matrix or weekly (whichever is more frequent). The LFM should be near the midpoint of the calibration range or near the level of interest. The LFM acceptance criterion is 70 to 130%. Flag values outside these limits as a matrix effect as long as the LFM addition at least doubles the ambient concentration.

Analyze a blank daily. Analytes should not be detected in the blank at or above the MRL. Run a blank after any sample that is more than ten times the calibration range or after installing new parts in the system. Alternatively, verify absence of carryover from such high-level samples by initially analyzing a sample spiked at the highest expected concentration followed by a blank. If results are nondetect for the blank, there is no need to routinely analyze a blank after high-level samples.

Preferably include a surrogate with each sample. The surrogate acceptance criterion is 70 to 130%.

7. Precision and Bias

This method's MDL was determined to be <5 ng/L for MIB and geosmin (Table 6040:XVI) under the conditions described above.

Two laboratories analyzed a series of blind samples for MIB and geosmin using 6040E but different extraction and analysis options. A comparison of results is shown in Table 6040:XX.

8. Bibliography

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6200 VOLATILE ORGANIC COMPOUNDS*

6200 A. Introduction

1. Source and Significance

Many organic compounds have been detected in ground and surface waters. While most groundwater contamination episodes are traceable to leaking underground fuel or solvent storage vessels, landfills, agriculture practices, and wastewater disposal, the most probable cause for contamination of some aquifers and surface waters has never been firmly established. Contamination may be due to past practices of onsite (leach field) disposal of domestic and industrial wastes or to illegal discharges. Organohalides, particularly the trihalomethanes, are present in most chlorinated water systems, especially those using surface waters as a source of supply. Toxicological studies on animal models have shown that some of these organics have the potential for teratogenesis or carcinogenesis in human beings. To minimize these health risks, sensitive detection and accurate and reproducible quantitation of organics is of paramount importance.

2. Selection of Method

Two capillary gas chromatographic methods for purgeable organic compounds are presented. The scope of analytes is detector-dependent. Method 6200B is a gas chromatographic/mass spectrometric (GC/MS) technique. Method 6200C combines GC with photoionization detection (PID)/electrolytic conductivity detection (ELCD) in series. Methods 6200B and C are applicable to a wide range of purgeable organics. Both methods can be applied to finished drinking water, drinking water in any stage of treatment, source water, or wastewater.

The methods presented are highly sophisticated micro-analytical procedures that should be used only by analysts experienced in chromatography and data evaluation and interpretation. While the methods are similar, they are not interchangeable from a regulatory point of view.[1,2]

3. Scope

Table 6200:I lists the compounds that can be determined by these methods. All are determinable by both Methods B and C. Other compounds may be amenable to these methods.

4. Sampling and Storage

See Section 6010B.1

5. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 6020:I.

a. Initial quality control:

1) Initial demonstration of capability—Conduct initial demonstration of capability study at least once, before analysis of any sample, by each analyst, to demonstrate proficiency with the method of choice. Include at least analysis of a reagent blank and four reagent blank samples fortified at a concentration between 10 times the minimum reporting level and the midpoint of the calibration curve. The blank should not contain any compound of interest at a concentration greater than minimum reporting level. Mean percent recovery for each compound calculated from the four fortified samples should be 80 to 120%, and the relative standard deviation (RSD) should be $<20\%$.

2) Method detection level (MDL)—The MDL is a statistical determination of the minimum concentration that can be measured by the method with a confidence level of 99% that the analyte concentration is greater than zero. Determine MDL before any samples are analyzed, using the procedure described in Section 1030C or other appropriate procedure[3] as required for each matrix to be analyzed. For MDL calculation, start with a concentration about five times the estimated instrument detection level. Perform MDL determination as an iterative process. (The values listed in Table 6200:III were generated using a concentration of 0.5 μg/L.) Conduct MDL determination at least annually. Analyze samples for MDL determination over a 3- to 5-d period to generate a more realistic value.

3) Quality-control sample—Analyze an externally generated quality-control sample as a laboratory fortified blank at least quarterly or whenever new stock solutions are generated. Obtain this sample from sources external to the immediate laboratory, and use it to validate the laboratory's standards both qualitatively and quantitatively. Acceptance criteria are supplied by the manufacturer. If all criteria are not met, determine cause of error, and correct it before continuing.

4) Minimum quantitation level (MQL)—The MQL is the lowest level that can be quantified accurately. The MQL is defined as four times the MDL.

b. Calibration:

1) Initial calibration—Perform initial calibration with a minimum of five concentrations of analytical calibration standards (CALs) for the compound(s) of interest. The lowest concentration should be at the working reporting level; the highest concentration should be at the upper end of the calibration range. Do not report values that are outside of the defined calibration range. For the calibration concentrations, there should be no more than one order of magnitude between concentrations.

Use any of the following calibration functions, as appropriate: response factor for internal standard calibration, calibration factor for external standard calibration, or calibration curve. Calibration curves may be linear through the origin, linear not through the origin, or quadratic through or not through the origin. Use the following recommended acceptance criteria for the various calibration functions.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011.
Joint Task Group: 20th Edition—Melissa S. Dale (chair), Anthony Bucciferro, Melly L. Fabro, T.J. Richards, William A. Saner, Robert Slater.

TABLE 6200:I. COMPOUNDS DETERMINABLE BY GAS CHROMATOGRAPHIC METHODS FOR PURGEABLE ORGANIC COMPOUNDS

Analyte	Chemical Abstract Services Registry Number	Analyte	Chemical Abstract Services Registry Number
Benzene	71-43-2	2,2-Dichloropropane	590-20-7
Bromobenzene*	108-86-1	1,1-Dichloropropene*	563-58-6
Bromochloromethane	74-97-5	*cis*-1,3-Dichloropropene*	10061-01-5
Bromodichloromethane	75-27-4	*trans*-1,3-Dichloropropene*	10061-02-6
Bromoform	75-25-2	Ethylbenzene*	100-41-4
Bromomethane	74-83-9	Hexachlorobutadiene*	87-68-3
n-Butylbenzene*	104-51-8	Isopropylbenzene*	98-82-8
sec-Butylbenzene*	135-98-8	*p*-Isopropyltoluene*	99-87-6
tert-Butylbenzene*	98-06-6	Methyl *t*-butyl ether*	1634-04-4
Carbon tetrachloride	56-23-5	Methylene chloride	75-09-2
Chlorobenzene*	108-90-7	Naphthalene*	91-20-3
Chloroethane	75-00-3	*n*-Propylbenzene*	103-65-1
Chloroform	67-66-3	Styrene*	100-42-5
Chloromethane	74-87-3	1,1,1,2-Tetrachloroethane	630-20-6
2-Chlorotoluene*	95-49-8	1,1,2,2-Tetrachloroethane	79-34-5
4-Chlorotoluene*	106-43-4	Tetrachloroethene*	127-18-4
Dibromochloromethane	124-48-1	Toluene*	108-88-3
1,2-Dibromo-3-chloropropane	96-12-8	1,2,3-Trichlorobenzene*	87-61-6
1,2-Dibromoethane	106-93-4	1,2,4-Trichlorobenzene*	120-82-1
Dibromomethane	74-95-3	1,1,1-Trichloroethane	71-55-6
1,2-Dichlorobenzene*	95-50-1	1,1,2-Trichloroethane	79-00-5
1,3-Dichlorobenzene*	541-73-1	Trichloroethene*	79-01-6
1,4-Dichlorobenzene*	106-46-7	Trichlorofluoromethane	75-69-4
Dichlorodifluoromethane	75-71-8	1,2,3-Trichloropropane	96-18-4
1,1-Dichloroethane	75-34-3	1,2,4-Trimethylbenzene*	95-63-6
1,2-Dichloroethane	107-06-2	1,3,5-Trimethylbenzene*	108-67-8
1,1-Dichloroethene*	75-35-4	Vinyl chloride*	75-01-4
cis-1,2-Dichloroethene*	156-59-4	*o*-Xylene*	95-47-6
trans-1,2-Dichloroethene*	156-60-5	*m*-Xylene*	108-38-3
1,2-Dichloropropane	78-87-5	*p*-Xylene*	106-42-3
1,3-Dichloropropane	142-28-9		

* Compound can be determined using 6200C with PID only.

If using response factors or calibration factors, relative standard deviation (RSD) for each compound of interest should be less than 20%. If the RSD is not <20% for any compound of interest, then identify and correct source of lack of linearity before sample quantitation. When using response factors (i.e., for GC/MS analysis), check performance or sensitivity of the instrument for the compound of interest against minimum acceptance values for the response factors. See specific analytical method for the acceptance criteria for the response factors for each compound.

For a linear regression, the correlation coefficient should be >0.994. Recalculate each calibration point compared to curve. Resulting values should be within ±20%. If any of the recalculated values are not within ±20%, identify and correct source of outlier(s) before sample quantitation.

Use initial calibration, with any of the above functions (response factor, calibration factor, or calibration curve) for quantitation of the analytes of interest in samples. Use continuing calibration, described in ¶ *b*2) below, only for checks on initial calibration and not for sample quantitation. Perform initial calibration when instrument is set up and whenever continuing calibration criteria are not met.

2) Continuing calibration—Continuing calibration (CCAL) is the periodic analysis of a calibration standard used to verify that the instrument response has not changed significantly from the initial calibration. Perform continuing calibration every 10 samples for GC analysis, every 20 samples for GC/MS analysis, or every 12 h, whichever is more frequent. Perform continuing calibration with one or more of the concentrations of analytical standards in the initial calibration. Vary actual concentration of continuing calibration standard over calibration range, with a minimum concentration greater than two times the reporting limit. The acceptance criterion for continuing calibration is 70 to 130% recovery compared to the known or expected value of the calibration standard (at the analyst's discretion, the acceptance criterion for the gases may be extended to 60 to 140% recovery). If the acceptance criteria are not met, re-analyze continuing calibration standard or repeat initial calibration. When using response factors, check performance or sensitivity of instrument for analytes of interest against minimum acceptance values for response factors.

3) Closing standard—Finish all sample sets with a closing standard to demonstrate that performance was still acceptable for

the last sample analyzed. Use acceptance criteria as for the CCAL.

c. Batch quality control:

1) Analytical day—An analytical day is defined as a 12-h analytical period.

2) Sample set (batch)—A sample set (batch) is defined as those samples extracted in an analytical day, not to exceed 20 samples.

3) Laboratory reagent blank (LRB)—A LRB is a blank sample consisting of all reagents that normally contact a sample when carried through the entire analytical procedure. Use reagent blank to determine contribution of reagents and preparative analytical steps to observed value. No compound of interest should be present in reagent blank at a level greater than the MQL. Include a minimum of one reagent blank with each sample set (batch).

4) Laboratory-fortified blank (LFB)—See ¶ *b*2) above. NOTE: For this method, the LFB and CCAL are the same.

5) Internal standard (IS)—An internal standard is a compound of known concentration added to each standard and sample just before sample analysis. Because of the nature of purge and trap analysis, the IS is taken through the entire analytical process, just as is the surrogate standard [see ¶ *c*6) below]. However, the IS is used for quantitation, whereas the surrogate standard is used to monitor ongoing purge recovery. Use IS to monitor retention time, relative response, and concentration of analytes in each sample. When quantifying by the internal standard method, measure all compound responses relative to this standard. Internal standard area response should be in the range of ±30% compared to the mean calibration curve area response. The IS compound should mimic the chromatographic conditions of the analytes of interest. The retention time of this compound should separate from all analytes of interest and elute in a representative area of the chromatogram. If a specific compound cannot be found to meet these criteria, use additional compounds to satisfy analytical needs.

6) Surrogate standard (SS)—A surrogate standard is a compound added to each standard and sample at a known concentration before extraction. Choose a compound(s) that is chemically similar to the analytes and that is unlikely to be found in environmental samples. Carry surrogate standard through entire sample extraction and analytical process to monitor extraction recovery for each sample. Surrogate recovery should remain reasonably constant over time. Recovery should not vary more than 30% from the known value. Refer to method of choice for specific surrogates.

7) Laboratory-fortified sample (LFS)—A LFS is an additional portion of a sample to which the analytes of interest have been added at a concentration at least two times the MRL or around the middle of the calibration range. Include a minimum of one LFS with each sample set (batch). Make LFSs at sufficient concentrations that sample background levels do not adversely affect recovery calculations. (If this is a known sample, adjust addition concentrations to be about five times background level). Base sample batch acceptance on results of CCALs and LFBs rather than on LFSs, because the matrix of the sample may interfere with method performance. Prepare fortifying solution for blanks and samples from a different primary mix than that used to develop working standard mix.

8) LFS duplicates—A LFS duplicate is a second LFS used to evaluate the precision of the method in a matrix sample. If sufficient sample volume is collected, fortify a large enough volume to yield two sample portions for analysis. If sufficient sample volume is not collected, use a second bottle of the same sample fortified to the same concentration as the first. Include a minimum of one LFS duplicate with each sample set (batch). Compare precision and bias to those listed in the method. Base sample batch acceptance on results of CCAL and LFB additions rather than LFS duplicates.

6. References

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1987. National Primary Drinking Water Regulations—synthetic organic chemicals; monitoring for unregulated contaminants; final rule. 40 CFR 141 & 142; *Fed. Reg.* 52, No. 130.
2. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1986. Guidelines establishing test procedures for the analysis of pollutants under the Clean Water Act. 40 CFR Part 136; *Fed. Reg.* 51, No. 125.
3. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1995. Definition and procedure for the determination of the method detection limit, rev. 1.11. 40 CFR Part 136, Appendix B.

6200 B. Purge and Trap Capillary-Column Gas Chromatographic/Mass Spectrometric Method

This method[1] is applicable to the determination of a wide range of purgeable organic compounds (see Table 6200:I). The method can be extended to include other volatile organic compounds, provided that all performance criteria are met. It should be used only by analysts experienced in the operation of GC/MS systems and in evaluation and interpretation of mass spectra.

1. General Discussion

a. Principle: Volatile organic compounds are transferred efficiently from the aqueous to the gaseous phase by bubbling an inert gas (e.g., helium) through a water sample contained in a specially designed purging chamber at ambient temperature. The vapor is swept through a sorbent trap that adsorbs the analytes of interest. After purging is complete, the trap is heated and back-flushed with the same inert gas to desorb the compounds onto a gas chromatographic column. The gas chromatograph is temperature-programmed to separate the compounds. The detector is a mass spectrometer. See Section 6010C for discussion of gas chromatographic and mass spectrometric principles.

b. Interferences: Impurities in the purge gas and organic compounds outgassing from the plumbing upstream of the trap account for most contamination problems. Demonstrate

that the system is free from contamination under operational conditions by analyzing laboratory reagent blanks daily. (NOTE: Use blanks for monitoring only; corrections for blank values are unacceptable.) Avoid using non-TFE plastic tubing, non-TFE thread sealants, or flow controllers with rubber components in the purge and trap system. Ensure that the analytical area is not subject to contamination from laboratory solvents, particularly methylene chloride and methyl *tert*-butyl ether (MtBE).

Samples can be contaminated by diffusion of volatile organics (particularly fluorocarbons and methylene chloride) through the septum seal during shipment and storage. Use a field reagent blank prepared from reagent water and carried through the sampling, handling, and shipping procedures as a check on such contamination.

Contamination by carryover can occur whenever high-level and low-level concentration samples are analyzed sequentially. To reduce carryover, rinse purging device and sample syringe with reagent water between samples. Follow analysis of an unusually high concentration sample with a LRB to check for carryover contamination. For samples containing large amounts of water-soluble materials, suspended solids, high boiling compounds, or high levels of volatile compounds, wash purging device with a detergent solution, rinse it with distilled water, and dry it in an oven at 105°C between analyses. The trap and other parts of the system also are subject to contamination; therefore, frequently bake and purge entire system.

c. Detection levels: Method detection levels (MDLs) are compound-dependent and vary with purging efficiency and instrument response. In a single laboratory using reagent water and known-addition concentrations of 0.5 μg/L, observed MDLs were in the range of 0.025 to 0.450 μg/L. The applicable calibration range of this method is compound- and instrument-dependent, but is approximately 0.2 to 200 μg/L. Compounds that are inefficiently purged from water will not be detected when present at low concentrations, but they can be measured with acceptable bias and precision when present at sufficient concentration. Determination of some geometrical isomers (e.g., xylenes) may be hampered by co-elution.

d. Safety: The toxicity or carcinogenicity of each analyte has not been precisely defined. Benzene, carbon tetrachloride, bis(1-chloroisopropyl)ether, 1,4-dichlorobenzene, 1,2-dichloroethane, hexachlorobutadiene, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane, chloroform, 1,2-dibromoethane, tetrachloroethene, trichloroethene, and vinyl chloride have been classified tentatively as known or suspect carcinogens. Handle pure standard materials and stock standard solutions of these compounds in a hood and wear a NIOSH/MESA-approved toxic gas respirator when handling high concentrations.

2. Apparatus

a. Purge and trap system: The purge and trap system consists of purging device, trap, and desorber. Several complete systems are available commercially.

1) *Purging device,* designed to accept 25-mL samples with a water column at least 5 cm deep. A smaller 5-mL purging device is acceptable if required method detection levels and performance criteria are met. Keep gaseous headspace between water column and trap to a total volume of <15 mL. Pass purge gas through water column as finely divided bubbles with a diameter <3 mm at the origin. Introduce purge gas no more than 5 mm from base of water column. The purging device illustrated in Figure 6200:1 meets these criteria.

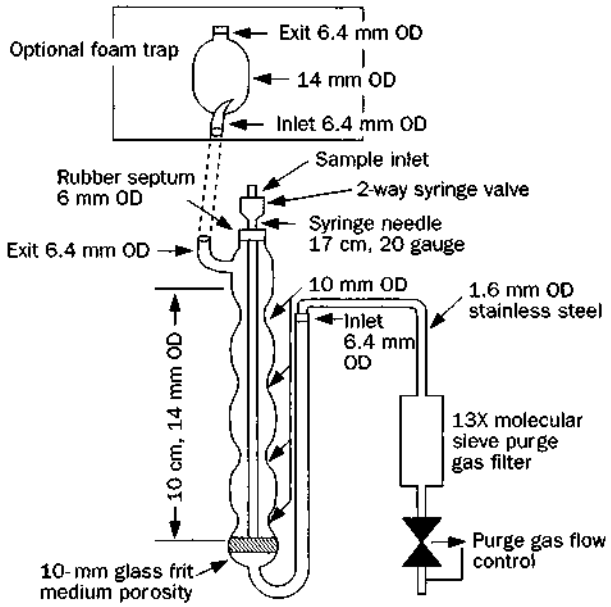


Figure 6200:1. Purging device.

Needle spargers may be used instead of the glass frit shown in Figure 6200:1; however, in either case, introduce purge gas at a point <5 mm from base of water column.

2) *Trap,* at least 25 cm long and with an inside diameter of at least 3 mm, packed with the following minimum lengths of adsorbents: 1.0 cm methyl silicone coated packing, 7.7 cm 2,6-diphenylene oxide polymer, 7.7 cm silica gel, and 7.7 cm coconut charcoal. If analysis is not to be made for dichlorodifluoromethane, the charcoal may be eliminated and the polymer section lengthened to 15 cm. Alternative sorbents may be used provided that all quality control criteria are met. Various sorbent traps are available commercially*; ensure that trap keeps total purge gas volume and purge time constant (i.e., 40 mL/min for 11 min) and that performance will meet all quality control criteria. The minimum specifications for the trap are illustrated in Figure 6200:2.

Methyl silicone coated packing is recommended, but not mandatory. The packing protects the diphenylene oxide polymer adsorbent from aerosols, re-coating any active site that may develop during the heating process, and ensures that the polymer is fully enclosed within the heated zone of the trap, thus eliminating potential cold spots. Alternatively, silanized glass wool may be used as a spacer in the trap inlet.

Before initial use, condition trap overnight following manufacturer's instructions. Vent trap effluent to the room, not to analytical column. Before daily use, condition trap for 10 min with back-flushing. Optimally, vent trap to analytical column

* Tekmar VOCARB 4000, or equivalent.

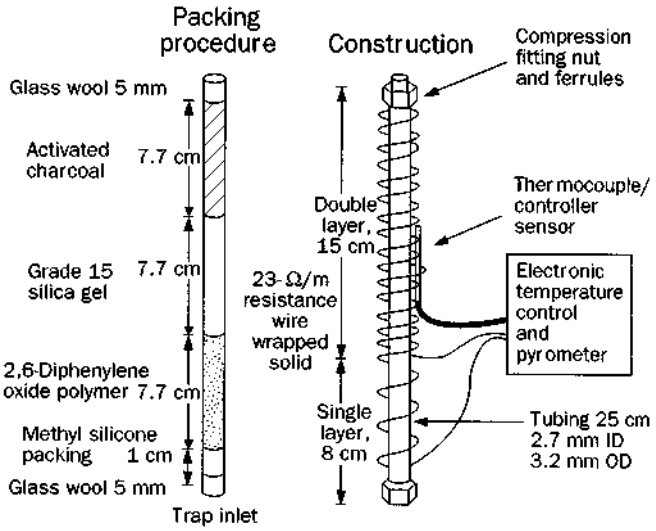


Figure 6200:2. Trap packings and construction to include desorb capability.

during daily conditioning; however, run column through temperature program before sample analysis.

b. Gas chromatograph (GC):† Use a temperature-programmable GC, suitable for on-column injection. Deactivate all glass components (e.g., injector liners) with a silanizing agent.

c. Capillary GC columns: Use any capillary GC column that meets all performance criteria. Ensure that desorb flow rate is compatible with the column of choice. Four examples of acceptable columns are listed below.

1) *Column 1*—60-m-long × 0.75-mm-ID VOCOL‡ wide-bore capillary column with 1.5-μm film thickness.

2) *Column 2*—30-m-long × 0.53-mm-ID DB-624§ megabore capillary column with 3-μm film thickness.

3) *Column 3*—30-m-long × 0.32-mm-ID DB-5§ capillary column with 1-μm film thickness.

4) *Column 4*—30-m-long × 0.25-mm-ID DB-624§ capillary column with 1.4-μm film thickness.

d. Mass spectrometer, capable of scanning from 35 to 300 amu every 2 s or less, using 70 eV (nominal) electron energy in the electron impact ionization mode, and producing a mass spectrum that meets all criteria in Table 6200:II when ≤25 ng of 4-bromofluorobenzene is introduced into GC. To ensure sufficient precision, the desired scan rate permits acquisition of at least five spectra while a sample component elutes from the GC.

e. Purge and trap—GC/MS interface: Use an open-split or direct-split interface, depending on which column is used. Alternatively, if the narrow-bore column (4) is used, a capillary concentrator preceding the GC may be necessary. This interface condenses desorbed materials onto an uncoated fused silica pre-column and when flash-heated transfers compounds onto the capillary column. The uncoated section of column is cooled to −150°C during desorption and heated to 250°C to transfer condensed materials.

f. Data system: To the mass spectrometer, attach a computer that allows continuous acquisition and storage of all mass spectra obtained throughout the chromatographic program. Computer software should allow for a search of all acquired spectra for specific *m/z* (masses) and the plot of such *m/z* abundances versus time or scan number. This type of plot is an extracted ion current profile (EICP). Software also should allow the integration of the abundances in any EICP over a specified time or scan limit.

g. Syringes, 0.5-, 1.0-, 5-, and 25-mL glass hypodermic with detachable tip.‖

h. Syringe valves, two-way, with detachable tip.‖

i. Microsyringes, 10-, 25-, and 100-μL with a 5-cm × 0.15-mm-ID and 220 bevel needle.#

j. Bottles, 40-mL with TFE-lined screw cap.

Table 6200:II. BFB Key *m/z* Abundance Criteria

Mass	*m/z* Abundance Criteria
50	15 to 40% of mass 95
75	30 to 60% of mass 95
95	Base peak, 100% relative abundance
96	5 to 9% of mass 95
173	<2% of mass 174
174	>50% of mass 95
175	5 to 9% of mass 174
176	95 to 101% of mass 174
177	5 to 9% of mass 176

3. Reagents

a. Reagent water, in which no interferent is observed at or above the MDL of the constituents of interest. Prepare by passing tap water through a carbon filter bed containing about 0.5 kg activated carbon, by distillation, or by using a water purification system.**

b. Trap packing materials:

1) *2,6-Diphenylene oxide polymer,* 60/80 mesh, chromatographic grade.

2) *Methyl silicone packing,* 3 OV-1.

3) *Silica gel,* 35/60 mesh.

c. Methanol, purge-and-trap grade.

d. Hydrochloric acid (HCl): 1 + 1.

e. Vinyl chloride, 99.9% pure.

f. Ascorbic acid.

g. Stock standard solutions: Prepare from pure standard materials or purchase as certified solutions. Prepare stock standard solutions in methanol using assayed liquids or gases as appropriate. **Caution: Toxic substances.** See 6200B.1*d*.

Place about 9.8 mL methanol in a 10-mL ground-glass-stoppered volumetric flask. Let stand unstoppered for about

† Gas chromatographic methods are extremely sensitive to the materials used. Mention of trade names by *Standard Methods* does not preclude the use of other existing or as-yet-undeveloped products that give *demonstrably* equivalent results.
‡ Supelco, Inc., or equivalent.
§ J&W, or equivalent.
‖ Luerlok, or equivalent.
Hamilton No. 702, or equivalent.
** Millipore Super Q, or equivalent.

10 min or until all alcohol-wetted surfaces have dried. Weigh flask to nearest 0.1 mg.

Add assayed reference materials as follows: For liquids, using a 100-μL syringe or disposable capillary-tip glass pipet, immediately add two or more drops of assayed reference material to flask, then reweigh. Ensure that the drops fall directly into the alcohol without contacting flask neck. For halocarbon gases that boil below 30°C (bromomethane, chloroethane, chloromethane, dichlorofluoromethane, trichlorofluoromethane, vinyl chloride), attach a vinyl plastic†† tube to port of gas bottle containing reference material, with open end bubbling into a beaker of methanol showing flow through the tubing; insert needle of 5-mL valved gastight syringe into tube and pull gas into syringe slowly to 5.0-mL mark. Lower syringe needle to within 5 mm of methanol surface and slowly force gas onto surface. The gas will dissolve into the methanol and will be seen as a vortex as it dissolves into the solvent. Reweigh flask (difference is amount of gas dissolved into methanol), dilute to volume, stopper, and mix by inverting several times. Calculate concentration in micrograms per microliter from net gain in weight. When compound purity is assayed to be ≥96%, calculate concentration of stock standard from uncorrected weight. Preferably use commercially prepared stock standards at any concentration if they are certified by the manufacturer or an independent source. Transfer stock standard solution into a TFE-sealed screw-cap bottle. Store with minimum headspace at −10 to −20°C away from light.

h. Secondary dilution standards: Using stock standard solutions, prepare in methanol secondary dilution standards that contain the compounds of interest, either singly or mixed together. Prepare secondary dilution standards at concentrations that will permit aqueous calibration standards (¶ *j* below) to bracket working range of the analytical system. Store secondary dilution standards with minimal headspace in a freezer and check frequently for signs of evaporation (which would indicate need for regeneration). Always bring to room temperature before preparing calibration standards. Prepare standards fresh weekly for gases. Replace all other standards monthly, or sooner if comparison with check standards indicates a problem.

i. Internal standard/surrogate standard known addition: Prepare a solution containing fluorobenzene (internal standard) and 1,2-dichlorobenzene-d_4 (surrogate) in methanol. Alternate internal standard and surrogate compounds may be used, provided that they meet method criteria and do not interfere with any method analyte(s). Prepare secondary dilution standard at a concentration of 5 μg/mL of each compound. Adding 5.0 μL standard to 25.0 mL sample or calibration standard yields a concentration equivalent to 1.0 μg/L. Alternate secondary standard concentrations can be used if addition volume is adjusted accordingly and all internal standard criteria are met. Add this mixture to each sample, standard, and blank.

j. Calibration standards: Prepare at least five concentration levels for each compound by adding appropriate amounts of secondary standard solution to reagent water and inverting water sample twice. Prepare one standard at a concentration near, but above, the MDL (i.e., 4 × MDL for potable-water-type samples) or a level that defines the low end of the working range and the others to correspond to the expected range of sample concentrations or to define the detector working range. Aqueous calibration standards can be stored up to 24 h if held in sealed vials with zero headspace. Otherwise, discard within 1 h. Alternatively, prepare calibration standards by injecting, with a solvent flush, an appropriate amount of a standard mix dilution and internal standard/surrogate mix, directly into a 25-mL syringe filled with reagent water; immediately inject water standard into purge vessel.

†† Tygon, or equivalent.

4. Procedure

a. Operating conditions: Table 6200:III provides recommended operating conditions for the gas chromatograph and gives estimated retention times and MDLs that can be achieved under these conditions. An example of the separations obtained with the specified column is shown in Figure 6200:3. Other chromatographic columns or conditions can be used if the quality control criteria are met.

b. GC/MS performance tests: At the beginning of each 12-h period during which analyses are to be performed, check GC/MS system by a performance test with BFB before any samples, blanks, or standards are analyzed. Performance tests require the following instrument parameters:

Electron energy: 70 eV (nominal)
Mass range: 35 to 300 amu
Scan time: at least 5 scans/peak but not more than 2 s/scan

Inject 25 ng BFB directly on GC column. If direct injection is not easily performed, add 1 μL 25-μg/mL BFB solution to 25 mL reagent water in syringe used for sample transfer to purge device and analyze as a sample. Obtain a background-corrected mass spectrum of BFB and confirm that all key *m/z* criteria in Table 6200:II are achieved. If all criteria are not achieved, re-tune mass spectrometer and repeat test until all criteria are met.

c. Calibration: Calibrate system as follows:

1) System setup—Condition trap initially overnight at 180°C by back-flushing with inert gas at 20 mL/min. Condition trap daily for 10 min at manufacturer's suggested temperature. Connect purge and trap system to GC using recommended temperature program and flow-rate conditions. Calibrate system by either the internal or the external standard technique.

2) Internal standard calibration technique—Select one or more internal standards similar in analytical behavior to the compounds of interest. Fluorobenzene is a recommended internal standard compound. Demonstrate that measurement of internal standard is not affected by method or matrix interference. Because of such limitations, no one internal standard may be applicable to all samples. The compounds used as surrogates (e.g., 1,2-dichlorobenzene-d_4) for quality control also can be used successfully as internal standards. Prepare calibration standards at a minimum of five concentration levels for each compound as described in 6200B.3*j*. Prepare a secondary dilution standard containing each of the internal standards (6200B.3*i*). Analyze each calibration standard according to procedure for samples, adding internal standard solution directly to syringe. Tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound in each calibration standard as follows:

TABLE 6200:III. PRIMARY QUANTITATION ION, RETENTION TIMES, AND METHOD DETECTION LEVELS

Analyte	Retention Time *min*	MDL *μg/L*	Primary *m/z*
Dichlorodifluoromethane	1.49	0.190	85
Chloromethane	1.71	0.150	50
Vinyl chloride	1.79	0.120	62
Bromomethane	2.16	0.220	94
Chloroethane	2.28	0.230	64
Trichlorofluoromethane	2.57	0.059	101
1,1-Dichloroethene	3.22	0.130	96
1,1,2-Trichloro-1,2,2-trifluoroethane	3.25	0.065	101
Methylene chloride	3.96	0.099	49
trans-1,2-Dichloroethene	4.40	0.200	96
Methyl *t*-butyl ether	4.45	0.450	73
1,1-Dichloroethane	5.14	0.047	63
cis-1,2-Dichloroethene	6.30	0.130	96
2,2-Dichloropropane	6.24	0.041	77
Bromochloromethane	6.77	0.032	128
Chloroform	7.00	0.126	83
1,1,1-Trichloroethane	7.24	0.043	97
1,1-Dichloropropene	7.67	0.040	75
Carbon tetrachloride	7.65	0.042	117
Benzene	8.07	0.036	78
1,2-Dichloroethane	8.14	0.055	62
Trichlorethene	9.44	0.045	95
1,2-Dichloropropane	9.85	0.053	63
Dibromomethane	10.07	0.035	93
Bromodichloromethane	10.47	0.112	83
cis-1,3-Dichloropropene	11.29	0.048	75
Toluene	11.81	0.047	91
trans-1,3-Dichloropropene	12.27	0.051	75
1,1,2-Trichloroethane	12.56	0.043	83
1,3-Dichloropropane	12.83	0.090	76
Tetrachloroethene	12.77	0.047	166
Dibromochloromethane	13.24	0.133	129
1,2-Dibromoethane	13.35	0.133	107
Chlorobenzene	14.21	0.052	112
1,1,1,2-Tetrachloroethane	14.37	0.048	131
Ethylbenzene	14.42	0.032	91
m,p-Xylene	14.63	0.038	91
o-Xylene	15.27	0.038	91
Styrene	15.30	0.031	104
Bromoform	15.60	0.131	173
Isopropylbenzene	15.90	0.074	105
Bromobenzene	16.34	0.140	156
1,1,2,2-Tetrachloroethane	16.41	0.066	83
1,2,3-Trichloropropane	16.44	0.072	75
n-Propylbenzene	16.57	0.260	91
2-Chlorotoluene	16.68	0.042	126
4-Chlorotoluene	16.86	0.040	126
1,3,5-Trimethylbenzene	16.88	0.035	105
tert-Butylbenzene	17.38	0.100	119
sec-Butylbenzene	17.46	0.025	105
1,2,4-Trimethylbenzene	17.74	0.046	105
4-Isopropyltoluene	17.99	0.037	119
1,3-Dichlorobenzene	17.89	0.045	146
1,4-Dichlorobenzene	18.04	0.033	146
1,2-Dichlorobenzene	18.64	0.031	146
n-Butylbenzene	18.65	0.028	91
Hexachlorobutadiene	21.69	0.033	225
1,2,4-Trichlorobenzene	21.34	0.043	180
Naphthalene	21.80	0.049	128
1,2,3-Trichlorobenzene	22.32	0.047	180

GC conditions: Column: J&W DB-624, 30 m, 0.25-mm ID, 1.4-μm film; Temperature program: 35°C, 4 min; 4°C/min; 50°C, 0 min; 10°C/min; 175°C, 4 min.

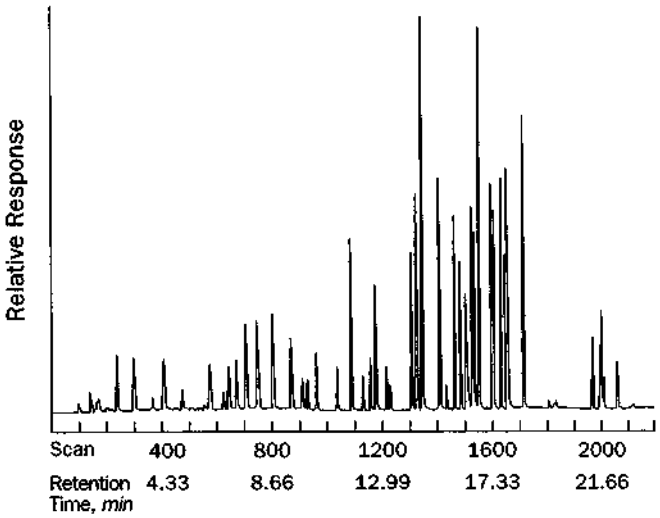


Figure 6200:3. GC/MS chromatogram. Column: J&W DB-624, 30 m, 0.25-mm ID, 1.4-μm film; temperature program: 35°C, 4 min; 4°C/min; 50°C, 0 min; 10°C/min; 175°C, 4 min.

$$RF = \frac{(A_s)(C_{is})}{(A_{is})(C_s)}$$

where:

A_s = response for compound to be measured,
C_{is} = concentration for internal standard,
A_{is} = response for internal standard, and
C_s = concentration of compound to be measured.

Calculate % RSD for the average RFs for each compound. Average RF can be used if RSD is <20%.

3) External standard calibration technique—Prepare standards as directed in 6200B.3*j*. Analyze each calibration standard and tabulate peak area responses versus concentration. Prepare calibration curve for each compound. Alternatively, if ratio of response to concentration (calibration factor) is a constant over the working range (<20% RSD), assume linearity through the origin and use average calibration factor in place of a calibration curve.

4) Calibration check—See 6200A.5*b*2).

d. Sample analysis: Bring sample to ambient temperature. Remove plunger from 25-mL syringe and close attached valve. Open sample bottle and carefully pour sample into syringe barrel to just short of overflowing. Replace syringe plunger, invert syringe, and open valve. Vent any air and adjust sample volume to 25.0 mL, in duplicate if sufficient sample is available (once sample cap has been removed, sample cannot be stored, because of headspace). Add an appropriate amount of surrogate/internal standard through valve bore, and close valve. Attach to purge device, open valves, and inject sample into purge vessel. Close valves and purge sample for 11.0 min at ambient temperature at a flow rate of 40 mL/min (helium or nitrogen). If water vapor causes problems in the mass spectrometer, use a 3-min dry purge and/or a moisture control module.

Desorb trapped materials onto head of chromatographic column at 180°C while back-flushing trap for 4 min with inert gas

TABLE 6200:IV. SINGLE-LABORATORY BIAS AND PRECISION DATA IN REAGENT WATER*

Analyte	Recovery %	Standard Deviation	Relative Standard Deviation %	Analyte	Recovery %	Standard Deviation	Relative Standard Deviation %
Benzene	107	0.046	9	1,1-Dichloropropene	110	0.044	8
Bromobenzene	111	0.034	6	*cis*-1,3-Dichloropropene	99	0.044	9
Bromochloromethane	88	0.052	12	*trans*-1,3-Dichloropropene	101	0.038	7
Bromodichloromethane	104	0.036	7	Ethylbenzene	109	0.049	9
Bromoform	107	0.042	8	Hexachlorobutadiene	112	0.053	9
Bromomethane	89	0.049	11	Isopropylbenzene	112	0.044	8
n-Butylbenzene	115	0.048	8	4-Isopropyltoluene	117	0.046	8
sec-Butylbenzene	113	0.043	8	Methylene chloride	85	0.050	12
tert-Butylbenzene	116	0.057	10	Methyl *t*-butyl ether	81	0.017	11
Carbon tetrachloride	119	0.048	8	Naphthalene	121	0.068	11
Chlorobenzene	108	0.033	6	*n*-Propylbenzene	107	0.048	9
Chloroethane	115	0.073	13	Styrene	101	0.039	8
Chloroform	108	0.043	8	1,1,1,2-Tetrachloroethane	113	0.037	7
Chloromethane	74	0.036	10	1,1,2,2-Tetrachloroethane	104	0.053	10
2-Chlorotoluene	111	0.045	8	Tetrachloroethene	106	0.046	9
4-Chlorotoluene	112	0.049	9	Toluene	106	0.045	8
Dibromochloromethane	108	0.042	8	1,2,3-Trichlorobenzene	118	0.054	9
1,2-Dibromoethane	102	0.042	8	1,2,4-Trichlorobenzene	109	0.049	9
Dibromomethane	132	0.113	17	1,1,1-Trichloroethane	106	0.040	8
1,2-Dichlorobenzene	106	0.043	8	1,1,2-Trichloroethane	97	0.041	9
1,3-Dichlorobenzene	108	0.052	10	Trichloroethene	105	0.041	8
1,4-Dichlorobenzene	106	0.045	8	Trichlorofluoromethane	105	0.045	9
Dichlorodifluoromethane	80	0.058	15	1,2,3-Trichloropropane	104	0.034	6
1,1-Dichloroethane	109	0.049	9	1,1,2-Trichloro-1,2,2-trifluoroethane	113	0.042	7
1,2-Dichloroethane	102	0.031	6				
1,1-Dichloroethene	99	0.059	12	1,2,4-Trimethylbenzene	116	0.044	8
cis-1,2-Dichloroethene	103	0.062	12	1,3,5-Trimethylbenzene	110	0.051	9
trans-1,2-Dichloroethene	113	0.045	8	Vinyl chloride	85	0.037	9
1,2-Dichloropropane	129	0.064	10	*m,p*-Xylene	110	0.057	10
1,3-Dichloropropane	107	0.046	9	*o*-Xylene	106	0.044	8
2,2-Dichloropropane	106	0.049	9				

* For all analytes, seven samples, each of 0.5 µg/L concentration, were analyzed.

at a flow rate compatible with the column of choice, and begin GC temperature program.

Set system auto-drain to empty purge chamber while trap is being desorbed into GC, or alternatively, use sample syringe to empty vessel. Washing chamber with two 25-mL flushes of reagent water is useful if highly contaminated samples are being analyzed. Be sure all areas wetted during purging are also wetted during rinsing to maximize flushing.

Recondition trap by baking at conditioning temperature for 5 to 7 min. Let trap cool to ambient before introduction of next sample into purge vessel. When all sample compounds have been eluted from chromatographic column, end data acquisition and store data files. Use data system software to display full range mass spectra and appropriate extracted ion current profiles (EICP). If any ion abundances exceed system working range, dilute sample in second syringe with reagent water and analyze. (NOTE: Take care with sample because compounds can be very volatile and can be lost if sample is reopened.) Estimate amount of dilution needed and expel excess sample from second syringe, inject that portion into purge vessel, and with a second syringe, add necessary reagent water to a total of 25.0 mL in purge vessel.

5. Calculation

When compounds have been identified, base quantitation on integrated area abundance from the EICP of the primary characteristic *m/z* given in Table 6200:III. If sample produces an interference for the primary *m/z*, calculate a response factor or calibration curve using a secondary characteristic *m/z*, and use secondary *m/z* to quantitate. Report results in micrograms per liter. Report all quality control data with sample results.

6. Quality Control

See 6200A.5.

7. Precision and Bias

Typical single-laboratory precision and bias data are shown in Table 6200:IV.

8. Reference

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1992. Methods for the Determination of Organic Compounds in Finished Drinking Water and Raw Source Water. Environmental Monitoring & Support Lab., Cincinnati, Ohio.

6200 C. Purge and Trap Capillary-Column Gas Chromatographic Method

This method[1] is applicable to the determination of purgeable halocarbons and aromatic organic compounds (Table 6200:I) in finished drinking water, raw source water, drinking water in any treatment stage, and wastewater.

1. General Discussion

a. Principle: See 6200B.1*a*.

b. Interferences: See 6200B.1*b*.

c. Detection levels: In a single laboratory using reagent water and known additions of 0.5 μg/L, calculated method detection levels (MDLs) for these compounds were in the range of 0.01 to 0.05 μg/L, depending on the compound. Some laboratories may not be able to achieve these detection levels because results depend on instrument sensitivity and matrix effects. Analysis of complex mixtures containing partially resolved compounds may be hampered by concentration differences larger than a factor of 10. This problem commonly occurs in analyses of finished drinking waters because of the relatively high trihalomethane content.

d. Safety: The toxicity or carcinogenicity of each reagent has not been defined precisely. Carbon tetrachloride, 1,2-dichloroethane, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane, chloroform, 1,2-dibromoethane, tetrachloroethene, trichloroethene, and vinyl chloride have been classified tentatively as known or suspected human or mammalian carcinogens. Prepare primary standards of these compounds in a hood and wear a NIOSH/MESA-approved toxic gas respirator when handling high concentrations.

2. Apparatus

a. Purge and trap system: The purge and trap system consists of three separate pieces of equipment: purging device, trap, and desorber. Several complete systems are commercially available.

1) *Purging device*—See 6200B.2*a*1).

2) *Trap*—See 6200B.2*a*2). If only compounds boiling above 35°C are to be analyzed, both silica gel and charcoal can be eliminated and polymer increased to fill entire trap. Trap failure is characterized by a pressure drop above 21 kPa across trap during purging or by poor bromoform sensitivities.

3) *Assembly*—See Figures 6200:1 and 2.

b. Gas chromatograph: See 6200B.2*b*.

1) *Column*—See 6200B.2*c*.

2) *Electrolytic conductivity or microcoulometric detector*—Halogen-specific systems eliminate misidentifications due to non-organohalides that may be coextracted during purging.

3) *Photoionization detector*—A high-temperature detector equipped with a 10.2-eV (nominal) lamp.* Insert between analytical column and halide detector to analyze simultaneously for aromatic and unsaturated volatile organic compounds (see Table 6200:I).

c. Syringes, 5-mL glass hypodermic with detachable tip.†

d. Other equipment: See 6200B.2*g–j*.

* Tracor Model 703, or equivalent.
† Luerlok, or equivalent.

3. Reagents

See 6200B.3*a–h*.

4. Procedure

a. Operating conditions: Table 6200:V summarizes recommended operating conditions for the gas chromatograph, estimated retention times, and method detection levels. Examples of separations obtained with the specified column are shown in Figures 6200:4 and 5.

b. Calibration: See 6200A.5*b*. Use either internal or external calibration technique. If using internal standard technique, prepare a dilution standard as described in 6200B.3*i*.

c. Instrument performance: See 6200A.5. Ensure that all peaks in standard chromatograms are sharp and symmetrical. Correct any peak tailing significantly in excess of that shown in method chromatograms. Tailing problems generally are traceable to active sites on the GC column or to detector operation. If only compounds eluting before chloroform give random responses or unusually wide peak widths, are poorly resolved, or are missing, the problem usually is traceable to the trap/desorber. If only brominated compounds show poor peak geometry or do not respond properly at low concentrations, replace trap. Excessive detector reactor temperatures also can cause low bromoform response. If negative peaks appear in the chromatogram, replace both ion-exchange column and electrolyte in detector. Check precision between replicate analyses. A properly operating system shows an average relative standard deviation of $<10\%$. Poor precision generally is traceable to pneumatic leaks, especially around sample purger and detector reactor inlet and exit, electronic problems, or sampling and storage problems. Monitor retention times for each compound using calibration standards and laboratory control standard. If individual retention times vary by $>10\%$ over an 8-h period or do not fall within 10% of an established norm, locate and correct source of retention data variance.

d. Sample analysis: See 6200B.4*d*.

5. Calculation

Identify each organohalide in sample chromatogram by comparing retention time of suspect peak to retention times generated by calibration standards and laboratory control standard. Determine concentrations of individual compounds. If external standard calibration procedure is used, calculate concentration of compound being measured from peak response using calibration curve or calibration factor previously determined.

If internal standard calibration procedure is used, calculate concentration using response factor [*RF*, 6200B.4*c*2)] by the following equation:

TABLE 6200:V. RETENTION TIMES AND METHOD DETECTION LEVELS

Analyte	Retention Time *min*	Method Detection Level: Electrolytic Conductivity Detector *μg/L*	Method Detection Level: Photo-ionization Detector *μg/L*
Dichlorodifluoromethane	6.22	0.037	—
Chloromethane	7.09	0.041	—
Vinyl chloride	7.68	0.025	0.088
Bromomethane	9.45	0.103	—
Chloroethane	9.76	0.025	—
Trichlorofluoromethane	11.04	0.042	—
1,1-Dichloroethene	13.59	0.018	0.035
1,1,2-Trichloro-1,2,2-trifluoroethane	13.07	0.047	—
Methylene chloride	15.83	0.068	—
Methyl *t*-butyl ether	16.49	—	0.411
trans-1,2-Dichloroethene	16.78	0.015	0.015
1,1-Dichloroethane	18.49	0.015	—
2,2-Dichloropropane	20.27	0.220	—
cis-1,2-Dichloroethene	20.54	0.012	0.032
Chloroform	21.04	0.017	—
Bromochloromethane	21.53	0.025	—
1,1,1-Trichloroethane	22.14	0.014	—
1,1-Dichloropropene	22.57	0.019	0.008
Carbon tetrachloride	22.80	0.022	—
Benzene	23.38	—	0.017
1,2-Dichloroethane	23.62	0.074	—
Trichloroethene	25.30	0.012	0.014
1,2-Dichloropropane	25.92	0.021	—
Bromodichloromethane	26.63	0.040	—
cis-1,3-Dichloropropene	28.38	0.067	0.041
Dibromomethane	28.40	0.057	—
Toluene	29.16	—	0.023
trans-1,3-Dichloropropene	30.00	0.029	0.046
1,1,2-Trichloroethane	30.39	0.042	—
Tetrachloroethene	31.04	0.013	0.014
1,3-Dichloropropane	31.18	0.020	—
Dibromochloromethane	31.86	0.039	—
1,2-Dibromoethane	32.36	0.070	—
Chlorobenzene	33.67	0.029	0.027
Ethylbenzene	33.89	—	0.028
1,1,1,2-Tetrachloroethane	33.91	0.020	—
m,p-Xylene	34.08	—	0.021
o-Xylene	35.44	—	0.024
Styrene	35.67	—	0.027
Isopropylbenzene	36.64	—	0.018
Bromoform	36.72	0.023	—
1,1,2,2-Tetrachloroethane	37.43	0.034	—
1,2,3-Trichloropropane	37.88	0.048	—
n-Propylbenzene	37.94	—	0.023
Bromobenzene	37.98	0.026	0.026
1,3,5-Trimethylbenzene	38.44	—	0.019
2-Chlorotoluene	38.48	0.017	0.017
4-Chlorotoluene	38.63	0.026	0.028
1,2,4-Trimethylbenzene	39.61	—	0.030
tert-Butylbenzene	39.76	—	0.018
sec-Butylbenzene	40.34	—	0.018
4-Isopropyltoluene	40.80	—	0.019
1,3-Dichlorobenzene	41.01	0.017	0.028
1,4-Dichlorobenzene	41.40	0.059	0.061
n-Butylbenzene	42.21	—	0.028
1,2-Dichlorobenzene	42.62	0.023	0.031
1,2,4-Trichlorobenzene	48.21	0.019	0.028
Hexachlorobutadiene	48.75	0.026	0.019
Naphthalene	49.05	—	0.043
1,2,3-Trichlorobenzene	49.92	0.018	0.032

GC conditions: Column—Supelco VOCOL, 60 m, 0.75-mm ID, 1.5-μm film; Temperature program–0°C, 8 min; 4°C/min; 185°C, 1.5 min.

$$\text{Concentration, } \mu\text{g/L} = \frac{A_s \times C_{is}}{A_{is} \times RF}$$

where:

A_s = response for compound to be measured,
C_{is} = concentration of internal standard, and
A_{is} = response for internal standard.

Report results in micrograms per liter without correction for recovery. Report quality control data with sample results.

6. Quality Control

See 6200A.5.

7. Precision and Bias

See Table 6200:VI.

8. Reference

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1991. Method 502.2: Volatile organic compounds in water by purge and trap capillary column gas chromatography with photoionization and electrolytic conductivity detectors in series. *In* Methods for the Determination of Organic Compounds in Finished Drinking Water and Raw Source Water. Environmental Monitoring & Support Lab., Cincinnati, Ohio.

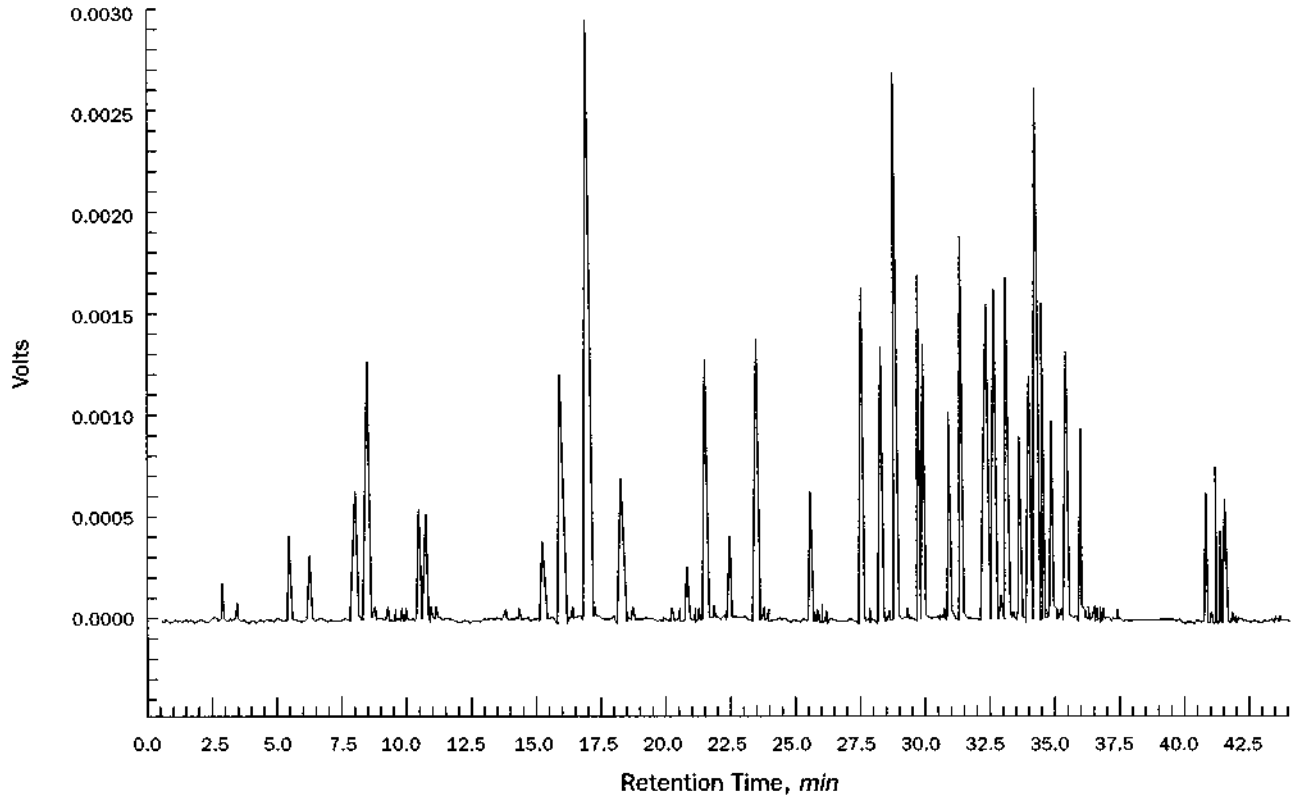


Figure 6200:4. PID chromatogram. GC conditions: Column: Supelco VOCOL, 60 m, 0.75-mm ID, 1.5-μm film; temperature program: 0°C, 8 min: 4°C/min; 185°C, 1.5 min.

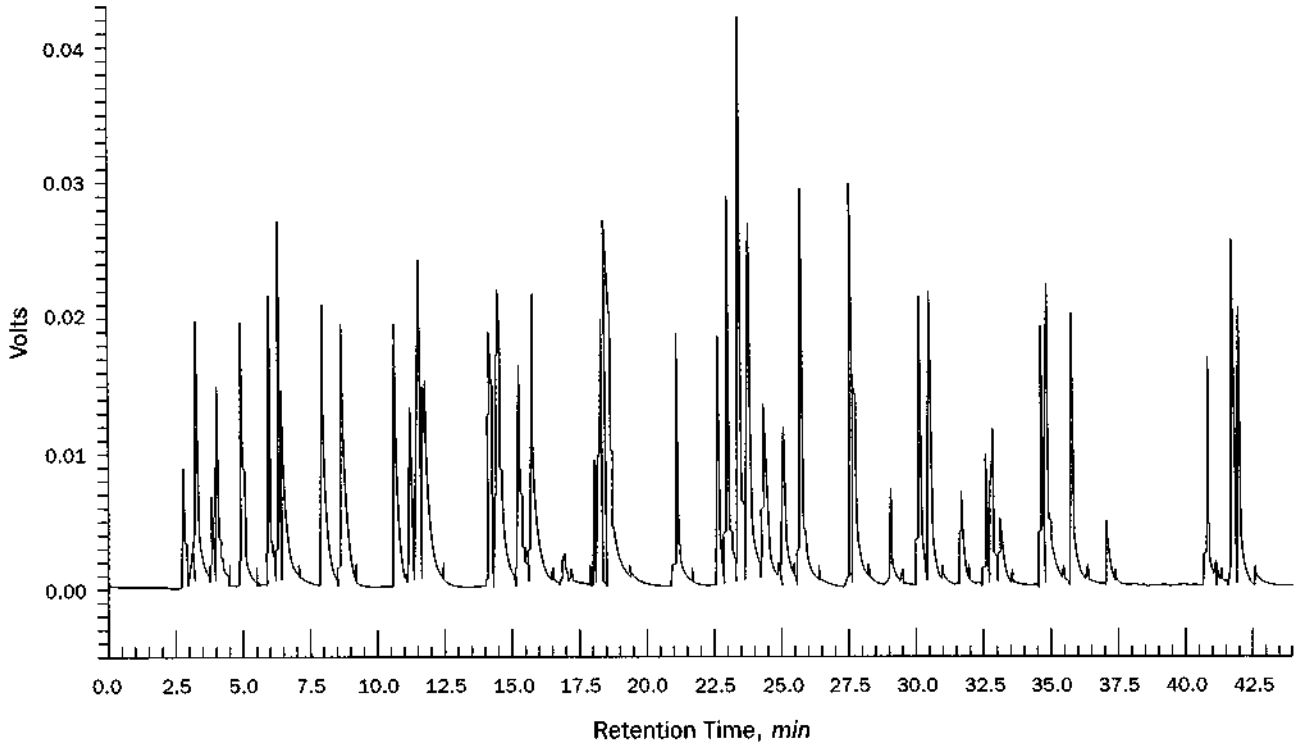


Figure 6200:5. ELCD chromatogram. GC conditions: Column: Supelco VOCOL, 60 m, 0.75-mm ID, 1.5-μm film; temperature program: 0°C, 8 min; 4°C/min; 185°C, 1.5 min.

TABLE 6200:VI. SINGLE-LABORATORY BIAS AND PRECISION DATA IN REAGENT WATER*

Analyte	Photoionization Detector			Electrolytic Conductivity Detector		
	Recovery %	Standard Deviation	Relative Standard Deviation %	Recovery %	Standard Deviation	Relative Standard Deviation %
Benzene	70	0.006	2	—	—	—
Bromobenzene	—	—	—	89	0.008	2
Bromochloromethane	—	—	—	83	0.008	2
Bromodichloromethane	—	—	—	135	0.021	3
Bromoform	—	—	—	81	0.007	2
Bromomethane	—	—	—	73	0.033	9
n-Butylbenzene	63	0.009	3	—	—	—
sec-Butylbenzene	65	0.009	3	—	—	—
tert-Butylbenzene	72	0.006	2	—	—	—
Carbon tetrachloride	—	—	—	79	0.007	2
Chlorobenzene	70	0.009	2	97	0.009	2
Chloroethane	—	—	—	64	0.008	2
Chloroform	—	—	—	83	0.006	1
Chloromethane	—	—	—	96	0.063	13
2-Chlorotoluene	—	—	—	91	0.005	1
4-Chlorotoluene	73	0.009	2	81	0.008	2
Dibromochloromethane	—	—	—	88	0.013	3
1,2-Dibromoethane	—	—	—	139	0.022	3
Dibromomethane	—	—	—	79	0.018	5
1,2-Dichlorobenzene	67	0.010	3	93	0.007	2
1,3-Dichlorobenzene	70	0.009	3	95	0.005	1
1,4-Dichlorobenzene	70	0.019	6	91	0.019	4
Dichlorodifluoromethane	—	—	—	71	0.027	8
1,1-Dichloroethane	—	—	—	82	0.005	1
1,2-Dichloroethane	—	—	—	78	0.024	6
1,1-Dichloroethene	61	0.011	4	81	0.006	1
cis-1,2-Dichloroethene	61	0.010	3	76	0.004	1
trans-1,2-Dichloroethene	79	0.005	1	77	0.005	1
1,2-Dichloropropane	—	—	—	85	0.007	2
1,3-Dichloropropane	—	—	—	148	0.018	2
2,2-Dichloropropane	—	—	—	74	0.045	12
1,1-Dichloropropene	54	0.003	1	74	0.006	2
cis-1,3-Dichloropropene	57	0.013	5	78	0.021	5
trans-1,3-Dichloropropene	63	0.015	5	78	0.009	2
Ethylbenzene	70	0.009	3	—	—	—
Hexachlorobutadiene	55	0.006	2	76	0.008	2
Isopropylbenzene	67	0.006	2	—	—	—
4-Isopropyltoluene	65	0.006	2	—	—	—
Methylene chloride	—	—	—	83	0.022	5
Methyl *t*-butyl ether†	75	0.130	3	—	—	—
Naphthalene	73	0.014	4	—	—	—
n-Propylbenzene	70	0.007	2	—	—	—
Styrene	70	0.009	3	—	—	—
1,1,1,2-Tetrachloroethane	—	—	—	83	0.001	0
1,1,2,2-Tetrachloroethane	—	—	—	88	0.011	2
Tetrachloroethene	54	0.005	2	79	0.004	1
Toluene	69	0.007	2	—	—	—
1,2,3-Trichlorobenzene	72	0.010	3	84	0.006	1
1,2,4-Trichlorobenzene	70	0.009	3	94	0.006	1
1,1,1-Trichloroethane	—	—	—	79	0.005	1
1,1,2-Trichloroethane	—	—	—	118	0.014	2
Trichloroethene	57	0.004	2	80	0.004	1
Trichlorofluoromethane	—	—	—	70	0.013	4
1,2,3-Trichloropropane	—	—	—	87	0.015	3
1,1,2-Trichloro-1,2,2-trifluoroethane	—	—	—	79	0.029	7
1,2,4-Trimethylbenzene	65	0.010	3	—	—	—
1,3,5-Trimethylbenzene	70	0.006	2	—	—	—
Vinyl chloride	73	0.022	6	67	0.008	2
m,p-Xylene	73	0.007	2	—	—	—
o-Xylene	68	0.008	2	—	—	—

* For all analytes, seven samples, each at a concentration of 0.5 μg/L (unless otherwise noted), were analyzed.
† Sample concentration 5 μg/L.

6231 1,2-DIBROMOETHANE (EDB) AND 1,2-DIBROMO-3-CHLOROPROPANE (DBCP)*

6231 A. Introduction

1. Sources and Significance

Dibromoethane and dibromochloropropane have been found in groundwater supplies in many areas of the United States; typically they are found in agricultural areas where these compounds have been applied in the past as fumigants. Toxicological studies suggest that they may have detrimental effects on human health, and therefore many states have established maximum contaminant levels for them.

* Approved by Standard Methods Committee, 2010.

2. Selection of Method

Method 6231B is a liquid–liquid extraction gas chromatographic (GC) method that uses microextraction and two capillary columns (one for primary quantitation and the second for confirmation). Another detector, such as MS, also may be used for confirmation (see 6231C). Method 6231B is the preferred method. In addition, these compounds can be detected by the purge-and-trap gas chromatographic/mass spectrometric (GC/MS) and GC methods (6200B and C), and dibromoethane by closed-loop stripping analysis (see Section 6040B). For additional information on applicability, sensitivity, precision, and bias, see specific methods.

6231 B. Liquid–Liquid Extraction Gas Chromatographic Method

This method[1–3] is applicable to the determination of 1,2-dibromoethane (EDB) and 1,2-dibromo-3-chloropropane (DBCP) in drinking water and untreated groundwater.

1. General Discussion

a. Principle: The sample is extracted with hexane and injected into a gas chromatograph equipped with a linearized electron capture detector for separation and analysis. Identification is confirmed by analyzing the sample with a dissimilar column. See Section 6010C.2 for discussion of gas chromatographic principles.

b. Interferences: Impurities in the extracting solvent usually account for most analytical problems. Analyze solvent blanks on each new bottle of solvent before use. Obtain indirect daily checks on the extracting solvent by monitoring sample blanks; whenever an interference is noted, re-analyze the extracting solvent. If necessary, remove interference by distillation or column chromatography[3] or, more simply, obtain a new source solvent. Interference-free solvent contains <0.1 μg/L individual compound interference. Store solvents in an area free of organochlorine solvents.

Accidental sample contamination can occur by diffusion of volatile organics through the septum seal into the sample bottle during shipment and storage. Trip blanks monitor this type of contamination.

EDB at low concentrations may be masked by high levels of dibromochloromethane (DBCM).

For further information on interferences in gas chromatographic methods, see Section 6010C.2.

c. Detection levels: The method detection levels (MDL)[4] for EDB and DBCP are 0.01 μg/L. The method is useful over a concentration range from approximately 0.03 to 200 μg/L. Actual detection levels are highly dependent on the characteristics of the gas chromatographic system used.

d. Safety: The toxicity or carcinogenicity of each reagent has not been defined precisely. EDB and DBCP have been classified tentatively as known or suspected human or mammalian carcinogens. Handle pure standard materials and stock standard solutions in a hood or glovebox and wear a NIOSH/MESA-approved toxic gas respirator when handling high concentrations.

2. Sampling and Storage

Collect all samples in duplicate and prepare replicate trip blanks with each sample set. A sample set is all of the samples collected from the same general sampling site at approximately the same time. Prepare the trip reagent blanks in the laboratory by filling a minimum of two sample bottles with reagent water, sealing, and shipping to the sampling site along with sample bottles.

Fill sample bottle to overflowing without air bubbles. When sampling from a water tap, open tap and flush until water temperature has stabilized (usually about 10 min). Adjust flow rate to about 500 mL/min and collect duplicate samples from the flowing stream. When sampling from a well, fill a wide-mouth bottle or beaker with sample, and carefully fill duplicate 40-mL sample bottles.

Keep samples chilled in an atmosphere free of organic solvent vapors, from day of collection until analysis. Do not acidify. Thiosulfate has no effect on EDB and DBCP. For regulatory compliance monitoring, thiosulfate ($\geq$0.008%) is required to prevent interferences from other disinfection byproducts.

Analyze all samples within 28 d of collection.

3. Apparatus

a. Sample containers, 40-mL screw-cap vials* each with a TFE-faced silicone septum.† Wash vials and septa with detergent and rinse with tap and distilled water before using. Let vials

* Pierce No. 13075, or equivalent.
† Pierce No. 12722, or equivalent.

Table 6231:I. Chromatographic Conditions for 1,2-Dibromoethane (EDB) and 1,2 Dibromo-3-Chloropropane (DBCP)

Compound	Retention Time *min* Column 1	Column 2
EDB	9.5	8.9
DBCP	17.3	15.0

Column 1 conditions: Durawax-DX 3 (0.25-μm film thickness) in a 30-m-long × 0.32-mm-ID fused silica capillary column with helium carrier gas at linear velocity of 25 cm/s. Column temperature held isothermal at 40°C for 4 min, then programmed at 8°C/min to 180°C for final hold.
Column 2 conditions: DB-1 (0.25-μm film thickness) in a 30-m-long × 0.32-mm-ID fused silica capillary column with helium carrier gas at linear velocity of 25 cm/s. Column temperature held isothermal at 40°C for 4 min, then programmed at 10°C/min to 270°C for final hold.

and septa air dry at room temperature, place in a 105°C oven for 1 h, then remove and let cool in an area free of organics.

b. Vials, auto sampler, screw cap with septa, 1.8 mL.‡

c. Microsyringes, 10- and 100-μL.

d. Microsyringe, 25-μL with a 51- by 0.15-mm needle.§

e. Pipets, 2.0- and 5.0-mL transfer.

f. Volumetric flasks, 10- and 100-mL, glass stoppered.

g. Standard solution storage containers, 15-mL bottles with TFE-lined screw caps.

h. Gas chromatograph:‖ See Section 6200B.2*b*. The system is equipped with a linearized electron capture detector and a capillary column splitless injector.

Two GC columns are recommended. Column 1 is a highly efficient column that provides separations for EDB and DBCP without interferences from trihalomethanes. Use Column 1 as the primary analytical column unless routinely occurring compounds are not adequately resolved. Use Column 2 as a confirmatory column when GC/MS confirmation is not available.

1) *Column 1*—30 m long × 0.32-mm ID fused silica capillary with dimethyl silicone mixed phase.# See Table 6231:I. Injector temperature: 200°C; detector temperature: 290°C. See Figure 6231:1 for a sample chromatogram.

2) *Column 2* (confirmation column)—30 m long × 0.32-mm ID fused silica capillary with methyl polysiloxane phase.** See Table 6231:I. Injector temperature: 200°C; detector temperature: 290°C.

4. Reagents

a. Reagent water: See Section 6200B.3*a*.

b. Hexane extraction solvent, UV grade.††

c. Methanol, pesticide quality or equivalent.

d. Sodium chloride (NaCl): Before using, pulverize and place in a muffle furnace at room temperature. Increase temperature to 400°C for 30 min. Store in capped bottle.

‡ Varian No. 96-000099-00 or equivalent.
§ Hamilton 702N, or equivalent.
‖ Gas chromatographic methods are extremely sensitive to the materials used. Mention of trade names by *Standard Methods* does not preclude the use of other existing or as-yet-undeveloped products that give *demonstrably* equivalent results.
Durawax-DX3, 0.25-μm film, or equivalent.
** DB-1, 0.25-μm film, or equivalent.
†† Burdick and Jackson No. 216 or equivalent.

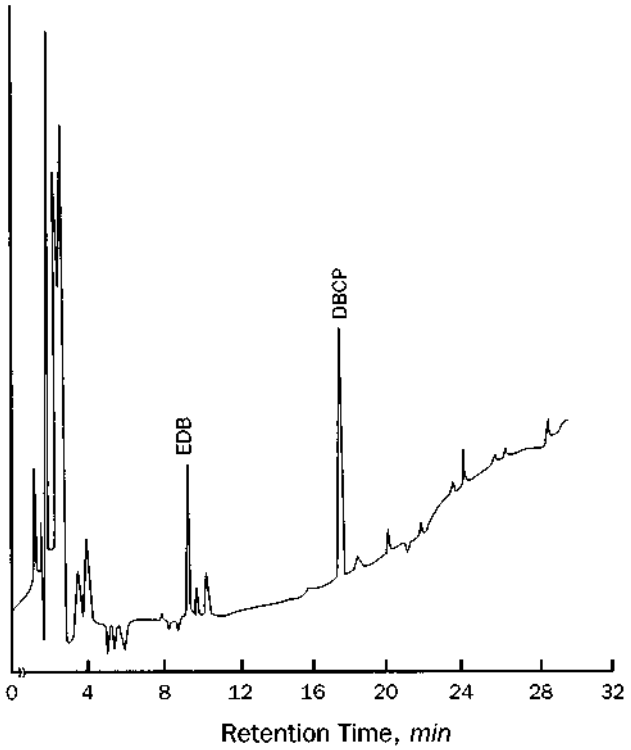


Figure 6231:1. Extract of reagent water with 0.114 μg/L added EDB and DBCP. Column: fused silica capillary; liquid phase: Durawax-DX3; film thickness: 0.25 μm; column dimensions: 30-m × 0.317-mm-ID.

e. 1,2-Dibromoethane, 99%.‡‡

f. 1,2-Dibromo-3-chloropropane, 99.4%.§§

g. Standard stock solutions: See Section 6200B.3*g*. Store in 15-mL bottles with TFE-lined screw caps. Methanol solutions prepared from liquid standard materials are stable for at least 4 weeks when stored at 4°C.

h. Secondary dilution standards: See Section 6200B.3*h*. Dilution standards are as stable as stock solutions.

5. Procedure

a. Operating conditions: Table 6231:I summarizes recommended operating conditions for the gas chromatograph and provides estimated retention times.

b. Calibration: Prepare calibration standards as directed in Section 6200B.3*j* and analyze according to ¶ *d* below. Follow rest of calibration procedure in Section 6200A.5*b*), but limit variations from predicted response to ±15% rather than ±20%.

c. Instrument performance: See Section 6200C.4*c*.

d. Sample analysis: Let samples and standards come to room temperature. For samples and trip blanks, open bottle, discard 5 mL using a 5-mL transfer pipet, and replace container cap. Weigh to nearest 0.1 g; record weight for subsequent volume determination. For calibration standards, QC check standards,

‡‡ Such as that available from Aldrich Chemical Company.
§§ Such as that available from AMVAC Chemical Corporation, Los Angeles, CA.

and reagent blank, measure 35 mL using a 50-mL graduated cylinder and transfer to a 40-mL sample container.

Remove container cap and add 7 g NaCl. Add 2.0 mL hexane with a transfer pipet. Recap and shake vigorously by hand for 1 min. Let water and hexane phases separate. (If sample is stored at this stage, keep container upside down). Carefully transfer 0.5 mL of hexane layer into an autosampler vial using a disposable glass pipet or other suitable device. Transfer remaining hexane phase, but not any of the water phase, into a second autosampler vial. Hold second vial at 4°C for reanalysis if necessary. Transfer first sample vial to an autosampler set up to inject 2.0-μL portions into the gas chromatograph. Alternatively, manually inject 2-μL portions. Begin analysis immediately, or hold the extract at 4°C and analyze within 24 h.

To determine sample volume for samples and field blanks, remove cap and discard remaining sample/hexane mixture. Shake off remaining drops using short, brisk wrist movements. Reweigh empty container with original cap and calculate net weight of sample by difference to the nearest 0.1 g. This net weight is equivalent to the volume of water (in mL) extracted. Alternatively, weigh vial before collection and reweigh full vial. Sample volume then equals gross weight (g) − [tare weight (g) + 5 g].

6. Calculation

Identify EDB and DBCP in sample chromatogram by comparing retention time of suspect peak to retention times generated by calibration and laboratory control standards. Retention times of samples should be within ±0.1 min of standard for positive identification.

Use calibration curve or calibration factor to calculate uncorrected concentration (C_i) of each compound (e.g., calibration factor × response). Calculate sample volume (V_s) as equal to the net sample weight:

$$V_s = \text{gross weight} - \text{bottle tare}$$

The corrected sample concentration is:

$$\text{Concentration, } \mu\text{g/L} = C_i \times \frac{35}{V_s}$$

Round off results to the nearest 0.1 μg/L or two significant figures.

Table 6231:II. Single-Laboratory Precision and Bias for EDB and DBCP in Tap Water

Compound	Number of Samples	Addition $\mu g/L$	Average Bias %	Relative Standard Deviation %
1,2-Dibromoethane	7	0.03	114	9.5
	7	0.24	98	11.8
	7	50.0	95	4.7
1,2-Dibromo-3-chloropropane	7	0.03	90	11.4
	7	0.24	102	8.3
	7	50.0	94	4.8

7. Quality Control

Follow procedures given in Section 6200A.5.

8. Precision and Bias

Single-laboratory precision and bias at several concentrations in tap water are presented in Table 6231:II. [5]

9. References

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2. Henderson, J.E., G.R. Peyton & W.H. Glaze. 1976. A convenient liquid-liquid extraction method for the determination of halomethanes in water at the parts-per-billion level. *In* L.H. Keith, ed. Identification and Analysis of Organic Pollutants in Water. Ann Arbor Science Publ., Ann Arbor, Mich.
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4. Glaser, J.A., D.L. Foerst, G.D. McKee, S.A. Quave & W.L. Budde. 1981. Trace analyses for wastewater. *Environ. Sci. Technol.* 15:1426.
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6231 C. Purge and Trap Gas Chromatographic/Mass Spectrometric Method

See Section 6200B for capillary-column method.

6231 D. Purge and Trap Gas Chromatographic Method

See Section 6200C for capillary-column method.

6232 TRIHALOMETHANES AND CHLORINATED ORGANIC SOLVENTS*

6232 A. Introduction

1. Sources and Significance

The trihalomethane (THM) compounds have been found in most chlorinated water supplies in the United States; typically they are produced in the treatment process as a result of chlorination. The formation of these compounds is a function of precursor concentration, contact time, chlorine dose, and pH. Toxicological studies suggest that chloroform is a potential human carcinogen. Consequently, total trihalomethanes are being regulated in potable waters. Chlorinated organic solvents are found in many raw waters because of industrial contamination.

2. Selection of Method

Several methods are available for measurement of the trihalomethanes and chlorinated organic solvents. Some of these are specific for these compounds and others have a much broader spectrum. Method 6232B is a simple liquid–liquid extraction gas chromatographic (GC) method that is highly sensitive and very precise for these compounds and certain other chlorinated solvents. Method 6232C refers to purge-and-trap gas chromatographic/mass spectrometric (GC/MS) methods that can detect not only THMs but also a wide variety of other compounds. Method 6232D refers to purge-and-trap GC methods with similar target compounds. All of these methods have approximately the same sensitivity for the trihalomethanes; method choice depends on availability of equipment, operator choice, and the list of desired target compounds. In addition, closed-loop stripping analysis can be used for several of these compounds (see Section 6040).

* Approved by Standard Methods Committee, 2010.
Joint Task Group: 20th Edition—Nancy E. Grams (chair), Bradford R. Fisher.

6232 B. Liquid–Liquid Extraction Gas Chromatographic Method

This method[1–3] is applicable to the determination of four trihalomethanes (THMs) (i.e., chloroform, bromodichloromethane, dibromochloromethane, and bromoform) and the selected chlorinated solvents in finished drinking water, drinking water during intermediate stages of treatment, and in both surface and ground water. For other compounds or sample matrices, collect precision and bias data on actual samples[4] and provide qualitative confirmation of results by gas chromatography/mass spectrometry (GC/MS) to demonstrate the usefulness of the method. Retain documentation to demonstrate method performance. This method is particularly useful when only a few compounds are being monitored.

1. General Discussion

a. Principle: Sample is extracted once with pentane and the extract is injected into a gas chromatograph equipped with a linearized electron capture detector (ECD) for separation and analysis. Extraction and analysis time is 10 to 30 min per sample, depending on analytical conditions.

Confirmatory evidence, where necessary, is obtained by using dissimilar columns, other selective detectors, or mass spectrometry. Component concentrations must be sufficiently high (i.e., >50 μg/L) for confirmatory analyses using a mass spectrometer. See other methods in this section for alternative means of confirming positive results.

Standards added to organic-free water and samples are extracted and analyzed in the same manner, under identical conditions. This step is essential to adjust for the $<100\%$ extraction efficiency of the simplified extraction technique. Extreme differences in ionic strength or organic content between standards and samples can result in different equilibria of sample constituents with the extracting solvent, and a method bias may result. Monitor known additions recoveries on various matrices for bias.

Where required, sum the concentrations of the four trihalomethanes and report as total trihalomethanes in micrograms per liter.

See Section 6010C.2 for discussion of gas chromatographic principles.

b. Interferences: Impurities contained in the extracting solvent account for many analytical problems. Maintain records of the reagent's manufacturer, lot number, purity, date bottle was opened, and expiration date. Analyze solvent blanks before using a new bottle of solvent. Make indirect daily checks on the extracting solvent by monitoring the method blanks. Whenever an interference is noted in the method blank, analyze a solvent blank. Discard (or use for another purpose) extraction solvent if a high level of interference is traced to it. Low-level interferences can be removed by distillation or column chromatography;[5] however, it usually is more economical to obtain new solvent or select an approved alternative solvent. Interference-free solvent is defined as a solvent containing less than the laboratory determined detection limit of interference for each constituent. Protect interference-free solvents by storing in an area known to be free of organohalogen solvents. *Do not subtract blank values from sample analysis results as a correction for contamination.*

Sample contamination has been attributed to diffusion of volatile organics through the septum seal on a sample bottle

during shipment and storage. Use the trip blank to monitor for this problem.

Contamination also may occur whenever equipment and materials used to store, extract, or analyze samples are inadequately cleaned, prepared, tested, or stored. There are many sources of contamination, including contamination of reagents during storage and contamination of equipment reused in the sequential extraction of samples and standards. Maintain records of dates of preparation and cleaning and inclusive dates of use of reagents, standards, bottles, and equipment. Test all reagents and standards before initial use. To reduce possibility of carryover contamination, always clean equipment thoroughly after each use. Where equipment contamination is a concern, processing and analysis of additional method blanks beyond the minimum requirements of this method may be useful. Start by placing reagent water in a sample vial of the same lot that was used for samples and add preservative as was done for samples. Process this method blank with samples using the same reagents, materials, and equipment. Where analysis of a method blank indicates contamination, investigate possible sources and isolate the cause. Take and document corrective action. Following analysis of a sample containing late-eluting interferences, or containing over-range concentrations of constituents of interest, analyze a solvent blank or method blank to demonstrate freedom from carry-over.

This liquid–liquid extraction technique efficiently extracts a wide boiling range of nonpolar organic compounds and also extracts polar organic components with varying efficiencies. To analyze rapidly for trihalomethanes and chlorinated solvents with sensitivities in the low microgram-per-liter range in the presence of these other organic compounds, use the semi-specific electron capture detector. Trihalomethanes are primarily products of the chlorination process and seldom appear in raw unchlorinated source water. The absence of peaks with retention times similar to the trihalomethanes in raw source water analysis is supporting evidence of an interference-free finished drinking water analysis. Because of possible interferences, analysis of a representative raw source water when analyzing finished drinking water provides evidence of freedom from this interference source. When potential interferences are noted in the raw source water, use the alternate chromatographic columns to re-analyze the sample set. If interferences still are noted, make confirmatory qualitative identifications as directed in ¶ *a* above. If the peaks are determined to be other than the constituents of interest and they add significantly to the constituents' value in the finished drinking water, analyze sample set by the purge-and-trap method.[6]

Where chlorinated solvents are present in finished drinking water, the most likely source is the raw water. Analyze individual raw water samples to isolate the source of contamination. Always consider the possibility of co-eluting interferences. Analysis using capillary chromatography minimizes this possibility. Analysis using dissimilar columns may confirm the presence of interferences through differences in retention time between the constituent of interest and the unknown compound in the sample; however, the most definitive confirmation routinely available is GC/MS.[7]

Because the simplified extraction technique depends on equilibria between solvent and water, because extraction is not 100% efficient, and because efficiency is dependent on concentration, it is important to:

- extract samples and standards in the same manner;
- monitor matrix recoveries to assess differences in equilibria; and,
- where the concentration is found to be above the linear range of the method, to either dilute samples carefully before extraction, or prepare standards in water at the estimated sample concentration and carefully dilute *both* sample and standard extracts.

Always process standards and samples together and hold constant such variables as water temperature, solvent temperature, room temperature, extraction time, and separation times.

For further information on interferences in gas chromatographic methods, see Section 6010C.2.

c. Detection levels: The method is useful for trihalomethane and selected chlorinated solvents at concentrations from approximately 0.1 to 200 μg/L. Actual detection levels are highly dependent on the characteristics of the gas chromatographic system used, the ratio of solvent to water, and interferences present in the solvent. See Section 1030C.

2. Sampling and Storage

See Sections 6010B.1 and 5710A.2.

If trihalomethane formation potential is to be measured, do not add any preservatives at the time of sample collection. If chemical stabilization is not used at time of sampling, add the reducing agent just before extracting the sample or add it at the time specified in the formation potential method for quenching the sample.[8,9]

The raw source water sample history should resemble that of the finished drinking water. Take into account the average retention time of the finished drinking water within the water plant when sampling the raw source water.

Store blanks and samples, collected at a given site (sample set), together in a protected area known to be free from contamination. At a water treatment plant, duplicate raw source water, duplicate finished water, and duplicate sample blanks comprise the minimum sample set. When samples are collected and stored under conditions specified in Section 6010B.1, no measurable loss has been detected over extended periods of time.[8] Analyze samples within 14 d of collection.

For samples collected soon after chlorination, quenching with reducing agent may not be sufficient to prevent further formation of THMs completely, because of hydrolysis of intermediates. In that case, acidification is necessary and consistent with the recommended preservation techniques.

3. Apparatus

a. Sample storage vials: Clean, baked 40-mL glass open screw-top TFE-faced septum VOA vials or equivalent. See Section 6010B for additional information on cleaning, storage, and preparation.

b. Microsyringes, 10, 25, and 100-μL: Microsyringes with extended barrels are suggested for proper injection of methanolic standards when preparing aqueous standards in volumetric flasks.

c. Volumetric flasks, glass-stoppered, 10, 25, 50, 100, 250, 500, and 1000 mL, Class A: Choose size according to final

volume of aqueous standard required and concentration of methanolic standards.

d. Extraction vessels: Use sample-storage vials. Optionally, where samples are transferred to smaller extraction vessels, select an extraction container on the basis of specific requirements for final extract volume, solvent-to-water volume ratio, and availability. If a separate vessel is used for extraction, place standards along with samples in sample-storage vials and follow identical procedures for preservation and transfer to the extraction vessel. Use the same lots of vessels for sample and standard extraction. Use of clean, oven-baked glass vessels and TFE-faced septa is critical.

e. Extract storage vials: 1.8-mL autosampler vials with open screw-top caps and TFE septa, or equivalent.

f. Gas chromatograph, preferably temperature-programmable with linearized electron-capture detector.

*g. Chromatographic columns:**

1) 0.32-mm ID × 30 m fused silica capillary, 1 μm DB-5,† or equivalent, at linear velocity of 20 cm/s. Temperature program: 35°C for 5 min, ramp 10°C/min to 70°C, then 20°C/min to 200°C. See Figure 6232:1 for a typical standard chromatogram.

2) 0.53-mm ID × 30 m, 1.5 μm DB-5† or equivalent, at 25 cm/s. Starting at 30°C for 1 min, ramp 6°C/min to 150°C.

3) 2-mm ID × 2 m long glass packed with 1% SP-1000‡ on Carbopack B (60/80) operated at 50°C with 60 mL/min flow, or, if temperature-programmable GC is available, 45°C for 1 min, ramp 8°C/min to 240°C.

4) 2-mm ID × 2 m long glass packed with 10% squalene on Chromosorb WAW (80/100 mesh) operated at 67°C with 25 mL/min flow.

5) 2-mm ID × 3 m long glass packed with 6% OV-11/4% SP-2100 on Supelcoport (100/120 mesh); temperature program 45°C for 12 min, then 1 to 70°C with 25-mL/min flow rate.

h. Mechanical shaker: Optionally, a rotary platform shaker.

i. Solvent pipettor: 2-mL transfer pipet, pipettor, or all-glass and TFE repipettor that attaches to the pentane storage bottle.

j. Transfer pipets, 5 mL. Preferably use a pipettor with disposable tips, cleaned and dried as recommended for TFE septa.

k. Analytical balance, capable of measuring to ±0.01 g.

4. Reagents

a. Extraction solvent: See 6232B.1*b*. For capillary column split injection technique, preferably use only pentane. For other techniques, recommended solvent is pentane; alternatively, use hexane, methylcyclohexane, methyl-*tert*-butyl ether (MtBE) or 2,2,4-trimethylpentane. For alternative solvents, collect and document precision and bias data, evaluate extraction efficiency and effect of constituent concentration on efficiency, and maintain documents demonstrating applicability. Demonstrate that solvent is free of compounds for which the analysis is being performed.

b. Methyl alcohol, demonstrated to be free of interferences.

c. Neat standard materials: Use materials of 96% purity or greater for:

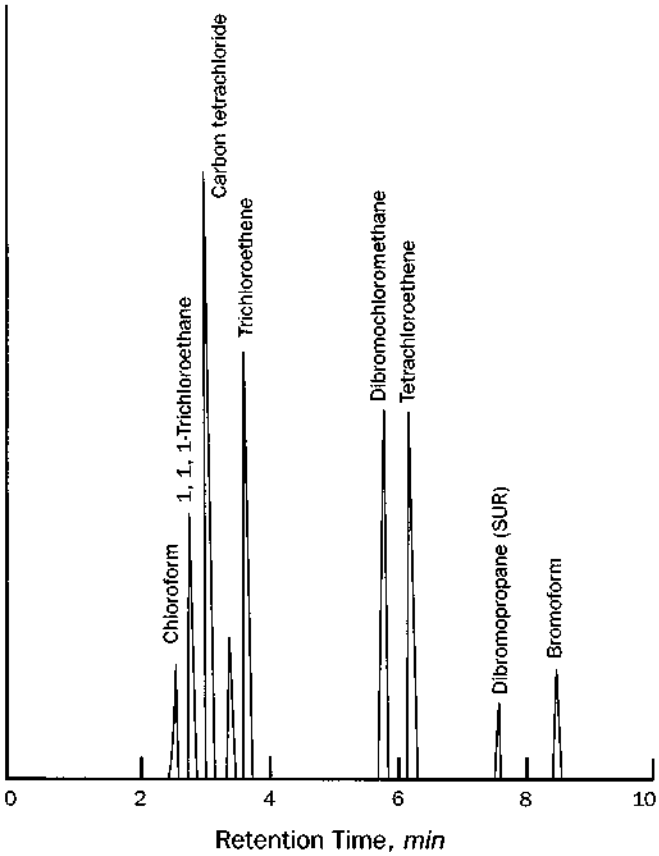


Figure 6232:1. Chromatogram for THMs and chlorinated organic solvents. Concentration was 50 μg/L for each compound; primary column DB-5.

1) *Calibration standards*—bromoform ($CHBr_3$), bromodichloromethane (BDCM), dibromochloromethane (DBCM), chloroform ($CHCl_3$), 1,1,1-trichloroethane (TCA), tetrachloroethene (PCE), trichloroethene (TCE), and carbon tetrachloride (CCl_4).

2) *Internal standard*—1,2 dibromopropane. A compound selected as an internal standard should have baseline resolution to separate it from constituents of interest and any interferences. Because this requirement is highly dependent on the samples and the analytical conditions and equipment used, no single internal standard is universally applicable. 1,2-dibromopropane has been found to be generally useful.

d. Reagent water: Generate VOC-free water, defined as water free of interference when used in the procedure described herein, by passing tap water through a carbon filter. Alternatively, prepare VOC-free water as follows: boil water for 15 min, then maintain at 90°C while bubbling a contaminant-free inert gas through water at 100 mL/min for 1 h. While water is still hot, transfer to a narrow-mouth screw-cap bottle with a TFE seal. Test VOC-free water each day before use by analyzing a method blank for constituents of interest. If any chlorine residual remains after such treatment, destroy it. See Section 1080 for additional information and general discussion of reagent-grade water.

e. Stock standard solutions: See Section 6200B.3*g*. Alternatively, purchase prepared standard solutions in methanol.

* Chromatographic methods are extremely sensitive to the materials used. Mention of trade names by *Standard Methods* does not preclude the use of other existing or as-yet-undeveloped products that give *demonstrably* equivalent results.
† J&W Scientific.
‡ Supelco.

CAUTION: Trihalomethanes and chlorinated solvents are toxic and may be carcinogenic: prepare primary stock solutions in a hood and wear appropriate personal protective equipment.

f. Secondary dilution standards: From standard stock solutions, prepare multi-component secondary standards in methyl alcohol so standards over the working range of the instrument can be prepared using no more than 20 μL methanolic standard solution /100 mL reagent water. See Section 6200B.*3h*.

g. Internal standard solution: Prepare stock solution from neat material in hexane. Make secondary dilution directly into storage container of pentane extracting solvent to produce a concentration of 30 μg/L internal standard in pentane.

h. Aqueous calibration standards: Construct a calibration curve for each constituent using a minimum of three different concentrations, but preferably use five to seven concentrations. Bracket each sample with two of the concentrations. Use one concentration near, but above, the laboratory-determined detection limit. Where a sample component exceeds the range bracketed by standards, dilute a fresh volume of sample and re-extract, or prepare new standards in reagent water to bracket the concentration and dilute sample and standard extracts to bring them into the linear range of the detector. To prepare calibration standards, rapidly inject the required volume of alcoholic standard into the expanded area of a reagent-water-filled volumetric flask. Using an extended barrel syringe, inject the methanol *well below* the reagent water surface. Preferably incline the volumetric flask at an approximately 45° angle while injecting the standard. Remove syringe and stopper flask. Mix aqueous standards by gently inverting flask three times only. Discard to waste the contents in the neck of flask before transferring standards to sample vials. Add any preservatives to both samples and standards before extraction. Process standards through extraction along with sample sets. Aqueous standards, when stored with a headspace, are not stable; discard after 1 h. When stored in headspace-free sample storage vials, aqueous standards may be used for 24 h.

Avoid standard preparation procedures that require delivery of <10 μL of methanolic standards into volumetric flasks. Instead, use a larger volumetric flask and a larger volume of methanolic standard.

i. Quality control (QC) check standards: Obtain concentrate in methanol from a vendor of proficiency-testing materials or NIST for each compound, or if not available, from a second source vendor. If no second source is available, prepare stock standards separately from neat materials used for calibration standards. Prepare a mixed secondary dilution standard containing each compound and then an aqueous QC check standard at a concentration approximating the midlevel calibration standard.

5. Procedure

a. Extraction: Let samples and standards come to room temperature. Open each sample vial and remove 5 mL of sample and discard to waste, preferably using a transfer pipettor with disposable tips. Replace cap, weigh vial to nearest 0.1 g, and record weight. Alternatively, use a 25-mL gastight syringe to measure sample volume (Section 6200B.4*d*) and perform subsequent extraction.

Using a clean, dedicated volumetric measurement device (6232B.3*i*) carefully measure 2.00 mL pentane and add to sample vial. *Vigorously* shake by hand for 1 min or use a rotary platform shaker set at 60 to 100 rpm.

Let phases separate for at least 2 min. Where emulsions do not separate on standing, centrifuge or transfer entire emulsion to a separate vial and cool extract below 4°C to promote separation. Using a disposable glass pipet, transfer at least 1 mL of upper pentane extract to extract storage vials. Optionally, transfer half of the pentane extract to each of two vials to provide for re-analysis where necessary. Protect pentane extracts from warm temperatures and minimize extract holding time at room temperature. Store extracts at 4°C.

Empty sample/extraction vial to waste, rinse, and shake dry. Reweigh empty container with original cap to nearest 0.1 g and record weight. Calculate weight of sample extracted to the nearest 0.1 g by subtraction of vial-only weight from sample-plus-vial weight. For an assumed density of 1 g/mL, weight of sample extracted is equal to volume of sample extracted, in milliliters. Convert volume in milliliters to liters and record.

b. Sample and standard analysis: Before extraction of samples or standards, prepare and analyze a method blank to verify freedom from interferences. Once extracts have been prepared, analyze standards and calculate a calibration curve or calibration factor as outlined in 6232B.6. Inject 1 to 5 μL of standard extract, depending on the instrument configuration and the required sensitivity. Inject exactly the same volume of extract each time, preferably using an autosampler. To test that injection volumes are repeatable, inject replicates of a single standard extract, and determine the standard deviation. Percent relative standard deviation (%RSD) should not be >5%. If this precision is not routinely achievable, use the internal standard calibration procedure.

After calibration, analyze the method blank, samples, and quality-control samples. Extract and analyze a quality-control check standard each twentieth analysis (5%) and at the end of the analytical sequence. The percent recovery for the QC check standard should be between 80 and 120%. Develop historical mean control charts of QC check standard recovery for each compound and use the 99% confidence about historical data as the control criteria for rejection of QC check standards validity. Where criteria are failed, repeat analysis of any samples analyzed since the last QC check standard was in control.

c. Internal standard analysis procedure: Add the internal standard to the pentane solvent in the storage container at the concentration specified, and proceed with extraction and analysis of samples and standards as outlined above.

d. Compound identification: Identification of compounds in samples is based on comparison of retention times (RT) of suspect peaks to the confidence limits for RT of the authentic compounds in standards. Using the retention times of the standards analyzed, determine the average retention time for each compound and the standard deviation of the retention time. Tentatively identify peaks in sample chromatograms as compounds on basis of the 99% confidence interval around the calculated mean value using the calculated standard deviation. Nominally, the retention time window would be expected to be no wider than 0.25 min (packed column) and 0.05 min (capillary) before and after the average retention time calculated for

the standards. When the 99% confidence limits for the data set are wider than the nominal value, institute corrective action.

Additional evidence of compound identity may be obtained by adding standard material to the suspect extract (standard addition) and reanalyzing. Presence of separate peaks in the extract with the known addition confirms that the suspect peak is not the compound of interest.

If chromatographic data systems are used to identify compounds, follow manufacturer's specifications. If the RT windows calculated by a computerized system are wider than the nominal values, investigate sources of retention time variability and take corrective action.

6. Calculations

a. External standard procedure: Use this procedure only if the volume of the injection can be held constant. Calculate individual response factors (RFs) for each standard analyzed as follows:

$$RF = \frac{\text{Nominal amount compound extracted, } \mu g}{\text{Response (peak area or peak height)}}$$

Calculate the amount of compound for each standard as:

$$W_s = V_s \times C_s$$

where:

W_s = amount of compound, μg,
V_s = volume of standard extracted, L, and
C_s = concentration of prepared standard, μg/L.

For each compound, determine average RF and standard deviation of the RFs using all calibration standards analyzed. If the percent relative standard deviation [%RSD = (SD/mean RF) × 100] is <10%, use average RF to calculate sample concentration.

If the %RSD is >10%, plot a calibration curve of amount injected versus response. Use the graph to determine the amount of compound present in each sample. Then determine the concentration by dividing amount, μg, by the volume, L, of sample extracted. Optionally use a data system to prepare a linear regression and use the linear-regression equation to calculate compound amounts in samples from response values.

Where average RF is used, determine sample concentration as follows:

$$C_x = \frac{RF \times R_x}{V_x}$$

where:

C_x = compound concentration, μg/L,
R_x = sample response (mm, area, etc.), and
V_x = volume of sample extracted, L.

Round all final sample results to two significant figures.

b. Internal standard procedure: For all analyses made in a given analytical sequence, determine average internal standard response and standard deviation of the internal standard response. Calculate percent relative standard deviation. If the %RSD is >25%, take corrective action to improve method precision. Establish the 99% confidence interval for the internal standard response using the calculated mean and standard deviation for the sample set. Reject analyses where the internal standard response is outside these confidence limits, and reanalyze. After analysis of calibration standards, calculate individual relative response factors (RRF) for each compound in each standard as follows:

$$RRF = \frac{R_s \times C_i}{R_i \times C_s}$$

where:

R_s, R_i = responses for calibration standard and internal standards, respectively, and
C_s, C_i = compound concentrations in calibration and internal standards, respectively.

Calculate average RRF for each compound, standard deviation of the RRFs, and %RSD. If %RSD is <10%, use the average RRF; if it is >10%, develop a calibration curve or a linear regression equation as outlined in the external standard procedure.

When using the average internal standard RRF, calculate concentration in samples as follows:

$$C_x = \frac{R_x \times C_i}{R_i \times RRF}$$

where:

C_x = compound concentration in sample, μg/L, and
R_x = sample response.

c. Total trihalomethane concentration: Calculate total trihalomethane concentration by summing the concentration of the four individual trihalomethanes in each sample. This is required for USEPA reporting purposes but it is preferable to report only individual THMs.

7. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 6020:I.

A minimum program of quality control consists of an initial demonstration of proficiency for each analyst and each instrument system and an ongoing program of quality-control analysis. Record initial quality by documenting initial performance relative to published performance criteria. Maintain records of performance by comparing ongoing quality-control checks to performance criteria and objectives for data quality. Document this performance as outlined in Sections 1020, 1030, and 6020.

a. Analyst proficiency: The analyst should be experienced in the operation of a GC/ECD and produce an initial demonstration of proficiency in accordance with the procedure outlined in Section 6200A.5*a*1).

b. Method blanks: Prepare and analyze method blanks on each shift after calibration and before first sample analysis. Concentrations of compounds in the method blank should not exceed the experimentally determined method detection level. If the method

Table 6232:I. Precision and Bias Data for THM-Chlorinated Organic Solvent Method, DB-5 Column

Compound	Added Amount	Amount Recovered μg/L								Bias % Recovery	Precision % RSD
		A	B	C	D	E	F	G	H		
Chloroform ($CHCl_3$)	20.0	18.7	18.6	19.4	19.5	19.2	18.5	19.5	19.7	95.6	2.4
Bromodichloromethane (BDCM)	20.0	19.8	20.2	20.7	20.8	20.3	19.7	20.6	20.5	101.6	2.1
Dibromochloromethane (DBCM)	20.0	18.7	19.4	20.0	20.2	19.7	18.7	20.1	20.6	98.3	3.6
Bromoform ($CHBr_3$)	20.0	17.4	18.5	18.7	19.2	19.3	17.9	18.8	19.8	93.5	4.1
Trichloroethane (TCA)	20.0	18.5	18.8	19.7	19.9	19.8	18.5	20.1	20.4	97.3	3.8
Carbon tetrachloride (CCl_4)	20.0	20.1	20.4	20.0	20.2	20.6	20.0	20.1	20.0	100.8	1.2
Trichloroethene (TCE)	20.0	17.9	18.3	18.9	19.2	19.1	17.9	19.2	19.6	93.8	3.4
Tetrachloroethene (PCE)	20.0	19.8	20.4	20.6	20.9	20.7	19.7	20.7	20.7	102.2	2.2
Internal standard	100.0	99.0	95.0	100.0	102.0	101.0	99.0	105.0	105.0	100.8	3.3

blank is out of control, isolate the source of contamination, apply corrective action, and process a new method blank. Under no circumstances subtract method blank values from the sample result.

c. Quality control (QC) check standards: Preferably obtain QC standards from a separate source and prepare independently from calibration standards. Analyze QC check standards as though they were samples at a frequency of 5% (every 20 samples) and at the end of the analytical sequence. Compare results to known concentration of the check standard and calculate percent recovery. Percent recovery nominally should be between 80 and 120%. Develop mean recovery control charts of QC check standards results and use historical 99% confidence limits to accept or reject the ongoing calibration. Where historical confidence limits are wider than the nominal limits, investigate standard materials, preparation and storage procedures, and other potential sources of error. Take and document corrective actions.

d. Detector sensitivity: Maintain a log of detector response, in area counts or peak height, using one standard that is analyzed each day, to monitor changes in detector sensitivity. Optionally, plot these data to observe trends in detector sensitivity. Note the sensitivity at which method detection level studies were performed and replace or repair detectors where minimum detectable quantities are significantly affected by declining detector sensitivity.

e. Laboratory-fortified samples with known additions: In a laboratory analyzing >10 samples daily, extract and analyze a known addition on each tenth sample. Be sure this is representative of different sample types because there is some evidence of matrix effects with liquid–liquid extraction methods. See Section 1020B.6. In a laboratory analyzing <10 samples daily, each time sample extractions are performed, extract and analyze at least one laboratory-generated known-addition sample. Chart percent recovery as outlined in Section 1020B.13 using a means chart. To evaluate method bias, see Section 1030.

f. Duplicate analysis: Randomly select, then extract and analyze in duplicate, 10% of all samples. Maintain an up-to-date log on bias and precision data collected on known-addition samples and duplicate samples. Evaluate results as outlined in Section 1030. If results are significantly different from those cited in 6232B.8, check entire analytical scheme to determine why the laboratory's precision and bias limits are excessive.

g. Laboratory control standards (performance evaluation standards): Quarterly, add an external reference laboratory evaluation standard to organic-free water, extract, and analyze. Obtain this standard from an authorized proficiency-testing provider. The results from this sample should agree within 20% of the true value for each compound. If not, check each step in preparation and analysis to isolate the problem. Document external reference standard results and any corrective action taken.

8. Precision and Bias

The single-laboratory precision and bias data in Table 6232:I were generated by adding known amounts of trihalomethanes and chlorinated organic solvents to organic-free water. The mixtures were analyzed as true unknowns.

9. References

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6232 C. Purge-and-Trap Gas Chromatographic/Mass Spectrometric Method

See Section 6200B for capillary-column method.

6232 D. Purge-and-Trap Gas Chromatographic Method

See Section 6200C for capillary-column method.

6251 DISINFECTION BYPRODUCTS: HALOACETIC ACIDS AND TRICHLOROPHENOL*

6251 A. Introduction

1. Sources and Significance

The haloacetic acids (HAAs) are[1] formed by the chlorination of natural organic (humic and fulvic) matter. Utilities using chlorine as a water disinfectant generate haloacetic acids, usually as the second most prevalent group of known disinfection byproducts[1]; the primary group formed is usually the trihalomethanes. Toxicological studies indicate that dichloroacetic acid and trichloroacetic acid are animal carcinogens.[2] The U.S. Environmental Protection Agency has promulgated a maximum contaminant level for the sum of five haloacetic acids.[3]

* Approved by Standard Methods Committee, 2007.
Joint Task Group: 21st Edition—Sanwat N. Chaudhuri (chair), Russell Chinn, Patricia Snyder Fair, Yuefeng Xie.

2. References

1. U.S. Environmental Protection Agency & Association of Metropolitan Water Agencies. 1989. Disinfection By-Products in U.S. Drinking Water, Vol. 1 Report. James M. Montgomery Consulting Engineers, Pasadena, Calif.
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3. U.S. Environmental Protection Agency. 1998. Disinfectants and Disinfection Byproducts Final Rule. *Fed. Reg.* 63(241):69390.

6251 B. Micro Liquid–Liquid Extraction Gas Chromatographic Method

This method[1] was developed to analyze simultaneously for monochloroacetic acid (MCAA), monobromoacetic acid (MBAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), bromochloroacetic acid (BCAA), dibromoacetic acid (DBAA), and 2,4,6-trichlorophenol (TCPh) in treated and untreated drinking water. Additional haloacetic acids may be present and analyzed by this method; however, standards for these compounds are not readily available and are less stable.

1. General Discussion

a. Principle: The sample is extracted with methyl *tertiary*-butyl ether (MtBE) at an acidic pH to extract the nondissociated acidic compounds to be determined. A salting agent is added to increase extraction efficiency. The extracted compounds are methylated with diazomethane solution to produce methyl ester or ether derivatives that can be separated chromatographically. A temperature-programmable gas chromatograph using a fused silica capillary column and an electron capture detector (ECD) is used for analysis. Simultaneous analysis and confirmation using a single injection can be effected by setting up both the analytical column and the confirmation column to share a common injection port. Alternatively, use separate analytical and confirmation columns. Alternative detectors may be used if QC criteria can be met. Aqueous calibration standards are extracted, methylated, and analyzed in the same manner to compensate for <100% extraction and derivatization efficiencies.

b. Interferences: Impurities in extraction solvent and on glassware and other equipment can interfere. Follow specifications and cleaning procedures carefully to minimize interference. As shown in Figure 6251:1, the analysis separates peaks of haloacetic acids from those of other common disinfection byproducts. Use of two columns is recommended because for waters with high carbonate contents, false-positive MCAA peaks have been observed on the column described in 6251B.3*h*4).

Table 6251:I. Method Detection Levels and Precision Data*

Compound	Added Conc. μg/L	Found Conc. μg/L	Standard Deviation μg/L	Relative Standard Deviation %	Method Detection Level μg/L
Monochloroacetic acid	0.50	0.54	0.026	4.8	0.082
Monobromoacetic acid	0.50	0.80	0.028	3.4	0.087
Dichloroacetic acid	0.50	0.5	0.017	3.5	0.054
Trichloroacetic acid	0.50	0.5	0.017	3.4	0.054
Bromochloroacetic acid	0.50	0.49	0.015	3.1	0.04
Dibromoacetic acid	0.50	0.47	0.021	4.4	0.065
2,4,6-Trichlorophenol	0.25	0.27	0.011	4.1	0.034

* Based on the analysis of seven portions of reagent water with known additions.[2]

c. Detection levels: Method detection levels (MDL) are given in Table 6251:I.[2] The method has been shown to be useful for haloacetic acids over a working range of 0.5 to 30 μg/L (1.0 to 30 μg/L for MCAA) and 0.25 to 15 μg/L for TCPh. The calibration range can be extended, depending on the compound and detector characteristics.

d. Safety: The toxicity and carcinogenicity of each reagent has not been defined precisely. Minimize exposure to these chemicals and use them only in a properly operating ventilation hood.

Avoid exposure to DCAA and TCAA because they are carcinogens.[3] Avoid contact with the other haloacetic acids and their solutions.

MNNG (1-methyl-3-nitro-1-nitrosoguanidine) is carcinogenic. Keep in properly labelled plastic containers, containing

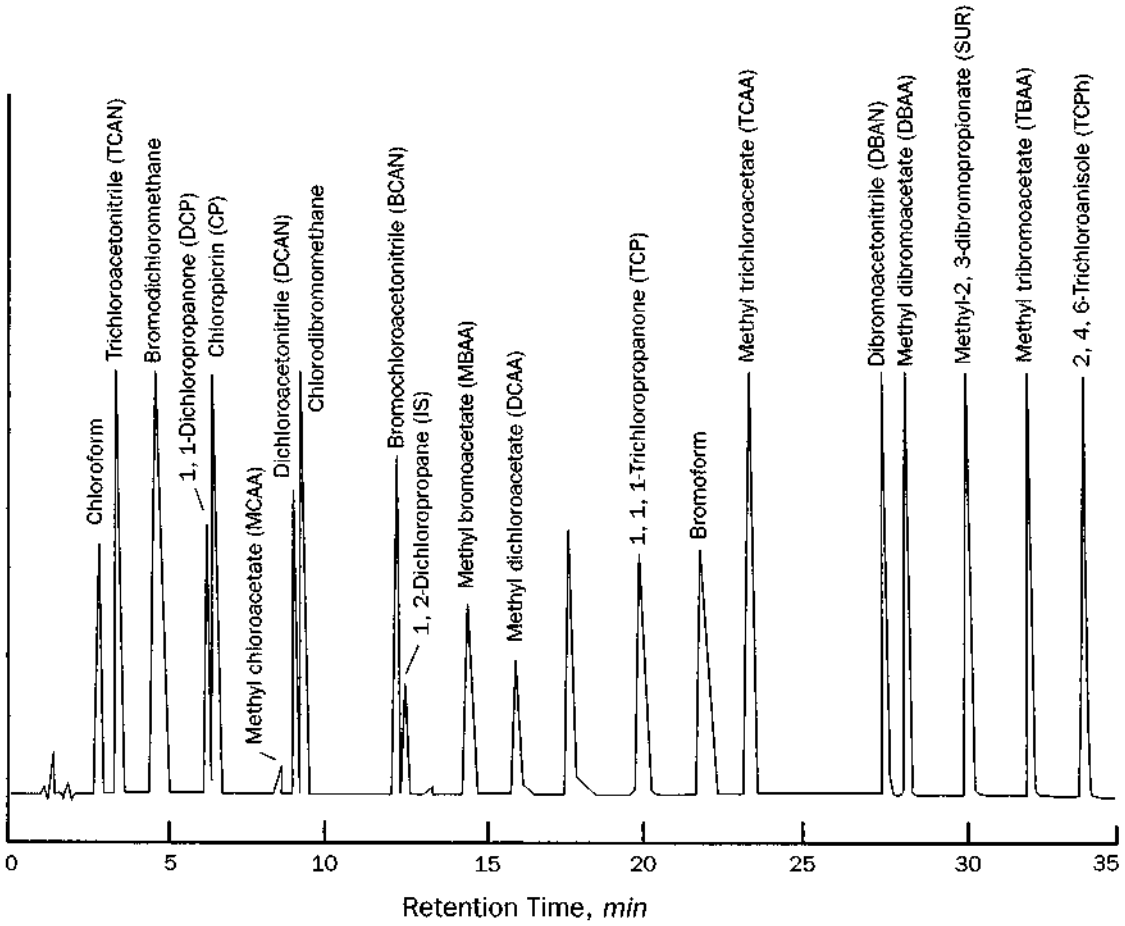


Figure 6251:1. Haloacetic acids separation from other commonly produced disinfection by-products on a DB-1701 column. Chromatogram produced by mixing the methyl esters of each haloacetic acid with other disinfection by-products in MtBE (disinfection by-products tentatively identified).

activated carbon, with tight-fitting lids and store in a refrigerator used only for chemical storage. Store spatulas and glassware for the handling of MNNG in specially labelled plastic containers and use only for MNNG.

Diazomethane is toxic, carcinogenic, and an explosion hazard.[4] Follow special precautions whenever handling this material. Use only in a properly operating fume hood; **CAUTION: Do not breathe vapors.** To avoid explosions, do not heat above 90°C and do not use glassware with ground-glass surfaces (e.g., ground-glass joints, sleeve bearings) or glass stirrers. Special glassware for diazomethane generation and handling, as well as screw-cap volumetric flasks, are available commercially. Always use a safety shield when generating diazomethane. Always quench excess diazomethane with silica gel. Do not store diazomethane/ether solutions; they are extremely hazardous and tend to become contaminated.

Store ether in tightly-closed amber bottles in an explosion-safe or -proof refrigerator. Store only with compatible chemicals. Eliminate all sources of ignition; keep away from heat, sparks, and flames. Handle ether only in a hood and avoid direct physical contact. Do not breathe vapors. If ether is spilled or leaks, evacuate area, ventilate, and absorb on vermiculite or similar material. Wear appropriate OSHA equipment before entering spill area. Also see Section 1090.

2. Sampling and Storage

See Section 6010B.1.

Preferably collect grab samples in quadruplicate to allow sufficient volume for replicates and known additions. Flush sampling tap until water temperature stabilizes and stagnant lines are cleared. Collect samples in nominal 40- or 60-mL vials containing approximately 65 mg crystalline NH_4Cl (bake overnight at >100°C to eliminate contaminants), which converts free chlorine to a combined chlorine residual, and sealed with TFE-faced septa and screw caps. To minimize aeration, fill vials so no air bubbles pass through the sample. Do not rinse with sample and do not let vial overfill. Seal sample vials with no headspace.

Analyze samples as soon as possible after collection. Store dechlorinated samples at 4°C, but for no more than 14 d;[5,6] check compound stability in any unknown sample matrix. Sample extracts can be held in a freezer at −11°C for 21 d.

3. Apparatus

Preferably dedicate all analytical glassware used in this method to this procedure.

a. Sample containers and extraction vials,* 40- or 60-mL screw-cap vials with TFE-faced silicone septa. Clean vials by washing with detergent, rinsing thoroughly with tap water, rinsing with 1:10 HCl, rinsing again with tap water, and finally rinsing with reagent water. Heat in an oven at 180° for at least 1 h. Clean caps and septa by rinsing with acetone, then hexane. Heat at 80°C for not more than 1 h in a clean, forced-air convection oven.

b. Microsyringes, 5, 10, 25, 50, 100, 250, 500, and 1000 μL.

c. Syringe, 30-mL glass hypodermic, metal luer-lok tip with 8.9-cm- (3.5-in.-) long × 17 gauge stainless steel pipetting needle (alternatively use a 30-mL volumetric pipet). See ¶ *a* above for glassware cleaning procedure.

d. Micro volumetric flasks,† TFE-lined screw-cap: 2-mL, 5-mL, and 10-mL. Immediately after use, rinse three times with methanol. Invert to drain. Let air-dry completely in a ventilation hood.

e. Mechanical shaker,‡ to automate MtBE extraction. Insert vials into a wooden holding block (20 vial capacity) made from laminated plywood with drilled holes to accept vials, dimensioned to fit snugly onto the shaker table.

f. Extract and standard solution storage container, 1.8-mL clear glass, 7- and 14-mL amber glass screw-cap vials with TFE-lined silicone septa. For cleaning procedures, see ¶ *a* above.

g. Transfer pipets, 14.6- and 23-cm (5.75- and 9-in.) disposable glass pasteur pipets. See ¶ *a* above for glassware cleaning procedure.

h. Gas chromatograph, temperature-programmable (preferably with multiple ramp capability) with injector. Optimally use an autosampler for sample injection and a computer data system for peak integration and quantitation. (A detector base that can mount two electron capture detectors is ideal.)

1) *Gas handling equipment*—Use carrier (helium) and makeup (nitrogen) gases of high purity (99.999%) grade that pass through indicating calcium sulfate, molecular sieve 5A, activated charcoal, and an oxygen purifying cartridge. Use two-stage metal diaphragm high-purity regulators at the compressed gas sources. Use flow controllers to regulate carrier gas flow. Make all gas lines 0.3-cm (1/8-in.) copper (or stainless steel) tubing; rinse with acetone and bake before use.

2) *Injector*—split/splitless (using straight open bore insert).

3) *Analytical column*§ —30 m long × 0.25 mm ID, fused silica capillary column with a 0.25-μm film thickness or equivalent.

4) *Confirmation column*—30 m long × 0.25 mm ID, fused silica capillary column‖ with a 0.25-μm film thickness or a 30 m long × 0.25 mm ID, fused silica capillary column# with a 0.5-μm film thickness.

5) *Detectors*—a constant-current pulse-modulated ^{63}Ni ECD with standard size cell (use two ECDs for simultaneous confirmation analysis).

i. Salt scoops for sodium sulfate, made from stainless steel 1.3-cm- (0.5-in.-) diam bar stock drilled out to a volume of 1.73 mL so that each level scoopful contains 3 g. Alternatively, weigh the salt.

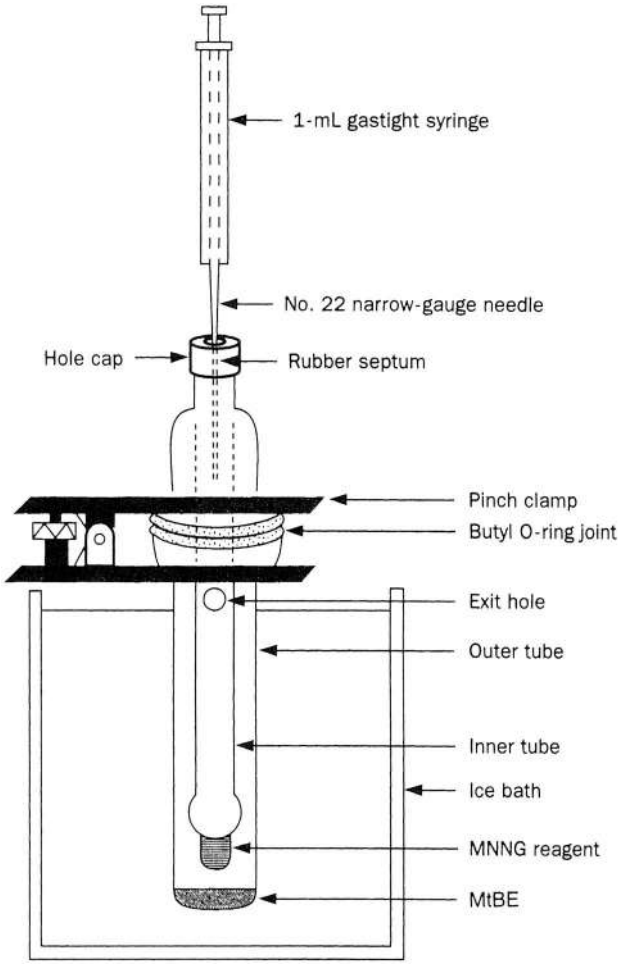


Figure 6251:2. Easy-to-use diazomethane generator apparatus for preparing small amounts of diazomethane in methyl *tertiary*-butyl ether (MtBE).

j. Pipetting dispensers, adjustable 5- and 2-mL sizes with TFE transfer lines, that can be mounted on the supplier's reagent bottles. Use for dispensing H_2SO_4 and MtBE. Alternatively, use a 3-mL volumetric pipet and a 5-mL graduated pipet with manual pipet bulbs.

k. Diazomethane generator: Use millimole-size generator with "O"-ring joint (Figure 6251:2).** Immediately after use, rinse inner tube twice with 20% NaOH, then rinse twice with tap water. Immediately add 1 g silica gel to the outside tube to quench any residual diazomethane solution, rinse twice with methanol, and twice with tap water. Rinse both inner and outer tubes with reagent water three times. Bake at 180°C until dry in a clean, forced-air convection oven.

Alternatively use the millimole-size diazomethane generator shown in Figure 6251:3.†† To clean, rinse with reagent water and then with methanol, invert, and let air-dry.

l. pH-strips, pH indicating strips, 0 to 2.5 range.

* Wheaton: Industrial Glassware, Millville, NJ, or equivalent.
† Kontes, or equivalent.
‡ Eberbach, or equivalent.
§ Durabond-1701, J&W Scientific, or equivalent.
‖ Durabond-5, J&W Scientific, or equivalent.
Durabond-210, J&W Scientific, or equivalent.

** Aldrich, or equivalent.
†† Paxton Woods Glass, Cincinnati, OH, or equivalent.

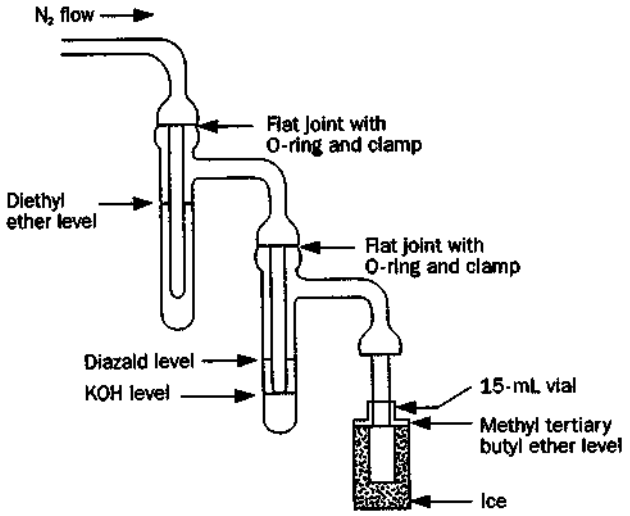


Figure 6251:3. Easy-to-use alternative diazomethane generator for preparing small amounts of diazomethane in MtBE.

4. Reagents

a. Extraction solvent, 99+ % MtBE, preservative-free.‡‡

b. Sodium sulfate reagents:

1) *Granular sodium sulfate* (Na_2SO_4), reagent grade, suitable for pesticide analysis. Heat at 400°C overnight in a shallow stainless steel pan covered with aluminum foil. Store in a 1-L glass bottle with TFE-lined polypropylene cap.

2) *Acidified sodium sulfate*—To 100 g anhydrous sodium sulfate, heated as above and cooled, add diethyl ether to just cover the solid; make a slurry. Add 0.1 mL conc sulfuric acid and mix thoroughly. Remove ether under low vacuum. Mix 1 g acidified sodium sulfate with 5 mL reagent water and check that pH is <4. Store remainder of reagent at 130°C.

c. Methanol, pesticide grade or equivalent.

d. Ammonium chloride (NH_4Cl).

e. Reagents for diazomethane generation with the apparatus shown in Figure 6251:2:

1) *Sodium hydroxide solution* (NaOH), 20%—Dissolve 200 g ACS low-carbonate-grade pellets in 800 mL reagent water.

2) *1-methyl-3-nitro-1-nitrosoguanidine* (MNNG).§§

f. Reagents for diazomethane generation with apparatus shown in Figure 6251:3:

1) *Diethylene glycol monoethyl ether.*‖‖

2) N-*methyl*-N-*nitroso*-p-*toluene sulfonamide.*##

3) *Ethyl ether,* absolute.

4) N-*methyl*-N-*nitroso*-p-*toluene sulfonamide solution*—Mix 10 g reagent [¶ *f* 2) above] **in 100 mL 1:1 (v:v) solution of ethyl ether and reagent** [¶ *f* 1) above]. Solution is stable for 1 month when stored at 4°C in an amber-colored bottle with a TFE-lined screw cap.

5) *Potassium hydroxide solution* (KOH)—Dissolve 37 g in 100 mL reagent water.

‡‡ Omnisolv, manufactured by EM Science, Gibbstown, NJ, or equivalent.
§§ Aldrich, or equivalent.
‖‖ Carbitol, (Aldrich), or equivalent.
Diazald (Aldrich), or equivalent.

TABLE 6251:II. ANALYTICAL STANDARDS

Compound*	Purity %	Molecular Weight	Boiling Point[7] °C @ mm†
MCAA	99	94.5	183
MBAA	99+	138.95	208
DCAA	99+	128.94	194
TCAA	98	163.39	198
BCAA	97	173.39	215‡
DBAA	99	217.86	195 @ 250
TCPh	95	197.45	246
IS-DBP	95	201.9	140–142
IS-TCP§	99	147.43	156
SUR-DBPA	99	231.88	160 @ 20
SUR-TFBA	99	194.09	—
MeCA	99+	108.52	130 @ 740
MeBA	98	152.98	144
MeDCA	99+	142.97	143
MeTCA	99	177.42	152–153
MeBCAA	98	187.42	155
MeDBA	—	—	182
TCAn	99	211.48	132 @ 28

* Sources: BCAA, Radian Corp., Austin, TX; DBAA, Fluka Chemika-Bio-Chemika, Switzerland; TCPh obtainable from Chem Service, Inc., Westchester, PA; MeDBA and SUR ester, derivatized acid at Metropolitan Water District of Southern California laboratory; other compounds from Aldrich Chemical Company, Inc., Milwaukee, WI.
† °C at reduced pressure in mm Hg.
‡ Decomposes.
§ Ensure that TCP is not a contaminant when it is used as IS.

g. Silica gel, 35/60 mesh activated at 180°C and stored in a desiccator.

h. Sulfuric acid (H_2SO_4), conc.

i. Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$).

j. Standard material, see Table 6251:II for source and physical information.

1) *Individual haloacetic acid standard stock solutions*—Prepare individual haloacetic acid and the trichlorophenol stock solutions as follows: Weigh 0.150 g of each acid. Dilute each standard in MtBE to 10 mL in a screw-top volumetric flask. (NOTE: Do not use methanol for dilution, because spontaneous methylation of the haloacetic acid may occur in methanolic solution.[8]) Transfer each to a separate clean 14-mL amber vial and store in a freezer at −11°C. Stock standards are usable for 6 months.

2) *Multicomponent haloacetic acid additive solution*—Prepare a six-component additive solution using individual haloacetic acid stock solutions. Dilute 16.7 μL of each stock standard into a 10-mL volumetric flask containing 9 mL MtBE, but use 8.4 μL of 2,4,6-trichlorophenol solution. After adding all stock solutions, dilute to volume with MtBE. This gives 25 μg/mL of each HAA and 12.5 μg/mL for 2,4,6-trichlorophenol. The additive solution is usable for 3 months when stored at less than −10°C. Alternatively, prepare known-addition solution monthly in methanol and store at 4°C.

Measure microliter volumes with a gastight syringe using the solvent flush delivery technique. Do solvent flush with a 25-μL syringe by first drawing up 2.5 μL of solvent and then drawing the syringe plunger to the 5-μL mark with air. From the 5-μL

mark, measure amount of stock solution desired and then deliver the entire contents to the volumetric flask.

3) *Individual haloester standard stock solutions*—Prepare individual methyl ester stock solutions as follows: Weigh 0.1 × (molecular weight of ester/molecular weight of acid) g of each methyl ester in a 10-mL volumetric flask and dilute to mark with MtBE. Prepare methyl ester for dibromoacetic acid by derivatizing 1 mL of a 20 000-μg/mL acid solution with 100 μL methanol (follow derivatization steps in 6251B.5*e*, but substitute dibromoacetic acid stock solution as the solution added to the outer tube for collection of the diazomethane). After derivatizing, transfer ester quantitatively to a 2-mL volumetric flask with a TFE-lined screw cap and dilute to mark with MtBE. Stock standards are usable for 6 months when stored at less than −10°C.

4) *Multicomponent haloester additive solution*—Prepare a multicomponent additive solution by diluting 10 μL of each haloester stock standard, but use 5 μL of 2,4,6-trichloroanisole (methyl ether of the phenol), in a 10-mL volumetric flask and bring to volume with MtBE. This will yield a mixture containing approximately 10 μg/mL each, except for 2,4,6-trichloroanisole, which will be approximately 5 μg/mL. Additive solution is usable for 3 months when stored at less than −10°C.

5) *Direct injection haloester standards*—Prepare direct injection standards using 10 μg/mL multicomponent haloester additive solution, a 30-μg/mL internal standard additive solution [see ¶ *k*2) below], and a 10-μg/mL methanol solution of methyl-2,3-dibromopropionate [surrogate ester, see ¶ *l*4) below]. Prepare direct injection standards by diluting appropriate volumes of multicomponent haloester additive mix, internal standard additive solution, and surrogate ester solution with enough MtBE to give a final volume of 1.0 mL.

k. Internal standard, 1,2,3-trichloropropane (IS-TCP), 98% pure [alternatively, use 1,2-dibromopropane (IS-DBP)].

1) *Internal standard stock solutions*—Weigh 50 mg into a 10-mL volumetric flask and bring to volume with methanol. This will yield a 5000-μg/mL stock solution. Stock standards are usable for 6 months when stored at less than −10°C.

2) *Internal standard additive solution,* 30 μg/mL—Deliver 60 μL internal standard stock solution into a 10-mL volumetric flask and dilute to volume with methanol. Divide evenly among six 1.8-mL vials and store at −11°C. Additive solution is usable for 3 months.

Add 20 μL of internal standard additive solution to each 2 mL extract, yielding internal standard of 300 ng/mL.

l. Surrogate, (DBPA-SUR) 2,3-dibromopropionic acid, 99% pure or 2,3,5,6-tetrafluorobenzoic acid (TFBA-SUR).

1) *Surrogate stock solution,* 20 000 μg/mL—Weigh 0.2000 g SUR acid into a 10-mL screw-cap volumetric flask and dilute to mark with MtBE. Stock solutions are usable for 6 months when stored at less than −10°C.

2) *Surrogate additive solution:*

a) *DBPA-SUR,* 10 μg/mL—Deliver 5 μL DBPA-SUR stock solution into a 10-mL volumetric flask and dilute to volume with methanol. Divide evenly among six 1.8-mL vials and store at −11°C. Additive solutions are usable for 3 months.

Add 30 μL DBPA-SUR additive solution to each 30-mL sample portion, yielding DBPA-SUR 10 μg/L.

b) *TFBA-SUR*, 20 μg/mL—Deliver 300 μL TFBA-SUR stock solution into 1-L volumetric flask and dilute to volume with reagent water. Add 0.5 mL TFBA-SUR additive solution to each 30-mL sample, yielding 100 μg/L.

3) *Esterified surrogate stock solution,* 10 000 μg/mL—Derivatize 1 mL SUR stock solution and 100 μL methanol, according to derivatization steps in 6251B.5*e*, but substitute SUR stock solution and 100 μL methanol for MtBE. After derivatizing, transfer quantitatively to a 2-mL volumetric flask with a TFE-lined screw cap and dilute to mark with MtBE. Stock solutions are usable for 6 months when stored at less than −10°C.

4) *Surrogate ester additive solution,* 10 μg/mL—Deliver 10 μL SUR ester stock solution into a 10-mL volumetric flask and dilute to volume with methanol. Additive solutions are usable for 3 months when stored at less than −10°C.

Add 10 μL DBPA-SUR or 100 μL TFBA-SUR ester additive solution to each 1 mL of direct injection standard yielding 100 ng/mL DBPA-SUR or 1000 ng/mL TFBA-SUR.

m. Reagent water: See Section 1080.

n. Calibration standards: Prepare aqueous calibration standards in reagent water by injecting a measured amount of the multicomponent haloacetic acid solution directly into water using the solvent flush technique. Prepare five different concentration levels from 0.5 to 30 μg/L for the HAAs and 0.25 to 15 μg/L for 2,4,6-trichlorophenol in 40-mL TFE-lined screw-top bottles containing 30 mL reagent water. Extract these standards and process the same way as samples, using the procedure given below.

5. Procedure

a. Sample preparation: Remove samples and standards from storage and let equilibrate to room temperature. Each time a new sample matrix is analyzed check that the amount of H_2SO_4 added will reduce pH to <0.5 before beginning microextraction. Test a separate 10-mL portion by adding 1 g $CuSO_4$, 4 g granular Na_2SO_4 [6251B.4*b*1)], and 0.5 mL conc H_2SO_4; mix until salt dissolves; and then test using pH indicating strips.

b. Microextraction: Transfer 30 mL from the sample container to a 40- or 60-mL vial with TFE-faced septum and screw cap.

Add surrogate additive solution as indicated in 6251B.4*l*2) to each sample, including standards and blanks. Add haloacetic acid additive solution at this step for known additions.

Take one vial at a time and add the following in sequence: 1.5 mL conc H_2SO_4, 3 g $CuSO_4$, 12 g baked Na_2SO_4 [6251B.4*b*1)], and 3 mL MtBE. Immediately cap and shake briefly by hand to break up any salt clumps.

When using automated extraction, place vials in mechanical shaker wooden holding block. Shake vials at fast speed (approximately 300 cycles/min) for 9 min; alternatively, shake manually for 2 min until salt is dissolved.

Remove vials, place upright, and let stand for at least 3 min until the phases separate.

c. Preparation of diazomethane[9]: Using the apparatus in Figure 6251:2, add approximately 130 mg MNNG to the inside tube of the generator. Add 0.5 mL reagent water to the MNNG and secure cap and septum. Add 2 mL MtBE to the outside tube. Place butyl O-ring in glass joint, place inside tube firmly on top of O-ring, and clamp securely with a screw-type pinch clamp.

Place generator in an ice-water bath containing enough ice to keep diazomethane MtBE solution at 0°C until used.

Add 600 μL 20% NaOH (1 drop/5 s) using a 1-mL gastight syringe (22-gauge needle) through the generator septum (check

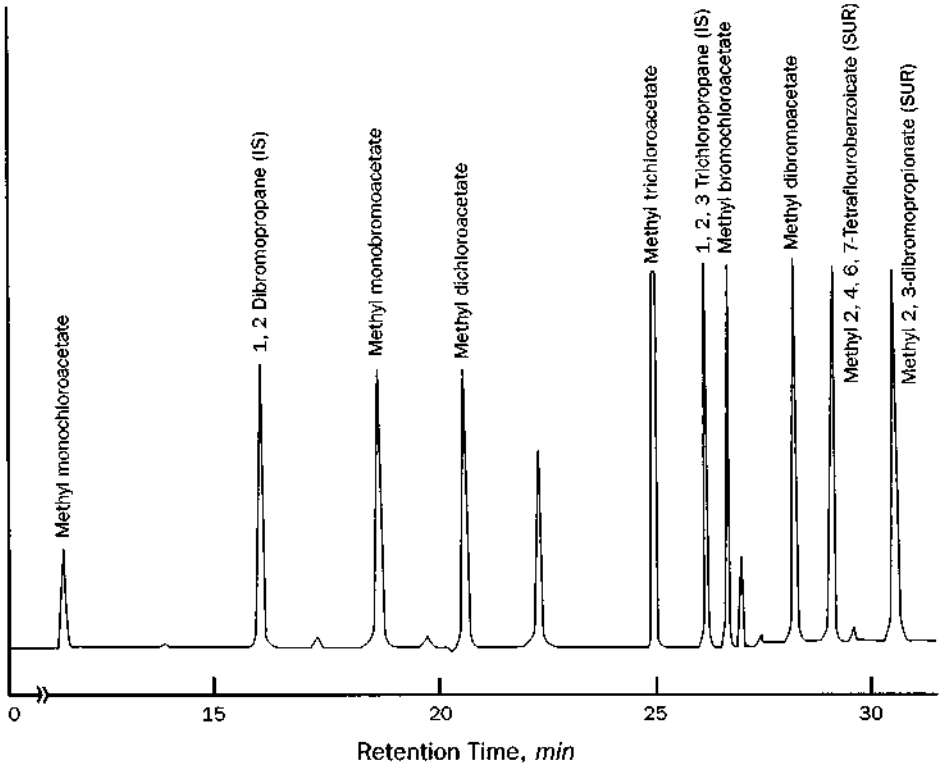


Figure 6251:4. Chromatogram produced by reagent water with known additions: 30 μg/L extracted standard on DB-1701 column.

that the syringe needle is on the opposite side of the vapor exit hole). Let derivatization continue for 30 min after adding NaOH; use product as soon as possible. Add more ice if necessary to maintain temperature.

If more diazomethane is needed, prepare two or more batches and combine before use.

Alternatively, use the apparatus in Figure 6251:3 to prepare diazomethane. Add enough ethyl ether to tube 1 to cover the first impinger. Add 10 mL MtBE to 15-mL collection vial. Set nitrogen flow at 5 to 10 mL/min. Add 4 mL sulfonamide solution and 3 mL 37% KOH solution to the second impinger. Connect tubing as shown and let nitrogen flow purge diazomethane from the reaction vessel into the collection vial for 30 min. Cap vial when collection is complete and hold at 0°C. When stored at 0°C, diazomethane solution may be used over 48 h.

d. Separation and concentration: NOTE: Ensure that all items that come into contact with the sample prior to methylation have been washed with a dilute solution of sulfuric acid.

The drying step included here may be used if excess diazomethane is required to maintain the persistent yellow color of the sample (¶ *e* below). It is not necessary in every case and may be used at the discretion of the analyst. Plug a small disposable pipet with a small amount of acid-washed glass wool. Add approximately 1 g acidified Na_2SO_4 [6251B.4*b*2)] to the pipet and pass exactly 2 mL of the top MtBE sample extract through the salt, being careful not to transfer any of the aqueous phase. Rinse the salt in the pipet with two 250-μL volumes of solvent and collect together in a receiver ampule (a 2-mL volumetric flask with TFE-lined screw cap) for subsequent concentration and methylation.

Concentrate MtBE extract to approximately 1.7 mL using a moderate stream of nitrogen blowing on the surface of the extract.

e. Derivatization: Add 20 μL of 30 μg/mL internal standard additive solution to each concentrated extract. (The internal standard is added at this time to minimize manipulation in the presence of diazomethane.) Cool in an explosion-safe freezer or in an ice bath for 7 min and add diazomethane (cooling extracts is unnecessary if diazomethane is generated by apparatus shown in Figure 6251:3).

Uncap one volumetric flask and add 250 μL of cold diazomethane/MtBE solution. Cap immediately with TFE-lined screw cap; mix gently by inverting once. Repeat for remaining extracts. A persistent yellow color after addition of diazomethane indicates that an excess is available for esterification. If necessary, add more diazomethane solution.

Hold for 15 min at 4°C in an explosion-safe or -proof refrigerator. Alternatively, keep extracts in an ice bath (cooling is unnecessary if diazomethane was generated by the apparatus shown in Figure 6251:3).

After holding 15 min, place extracts in a hood and let stand another 15 min until they reach room temperature. Dilute to mark with MtBE and invert flask to mix. If using an autosampler, transfer each extract evenly between two labeled autosampler vials containing approximately 0.01 g silica gel with a 23-cm (9-in.) disposable pasteur pipet to quench excess diazomethane. Keep each extract in contact with diazomethane for approximately the same amount of time before quenching. Store extra autosampler vial in freezer at −10°C as a backup extract. Alter-

TABLE 6251:III. RETENTION TIMES

Compound	Retention Time *min* DB-1701 Column	DB-5 Column	DB-210 Column
Methyl chloroacetate (MeCA)	11.11	5.80	10.97
Methyl bromoacetate (MeBA)	18.80	9.23	13.03
Methyl dichloroacetate (MeDCA)	20.53	10.11	12.72
Methyl trichloroacetate (MeTCA)	24.78	18.94	14.37
Methyl bromochloroacetate	26.23	19.19	15.11
Methyl dibromoacetate (MeDBA)	28.64	25.51	16.83
2, 4, 6-Trichloroanisole (TCAn)	~ 34.7	33.74	22.08
1,2-Dibromopropane (internal standard)	15.97	10.78	—
1,2,3-Trichloropropane (internal standard)	25.87	18.43	13.87
Methyl-2,3-dibromopropionate (surrogate ester)	30.74	28.60	—
Methyl-2,3,6,7- tetrafluoro-benzoicate (surrogate)	29.19	26.84	—

TABLE 6251:IV. RECOMMENDED QUANTITATION LIMITS

Compound	Recommended Quantitation Limit *μg/L*
Monochloroacetic acid (MCAA)	1.0
Monobromoacetic acid (MBAA)	0.5
Dichloroacetic acid (DCAA)	0.6
Trichloroacetic acid (TCAA)	0.6
Bromochloroacetic acid (BCAA)	0.8
Dibromoacetic acid (DBAA)	0.6
2,4,6-Trichlorophenol (TCPh)	0.4

natively, add silica gel to volumetric flask after derivatization is complete.

f. Gas chromatography: Typical operating conditions for the chromatograph are as follows:

Injector temperature 160°C; split valve opened at 0.5 min.

Temperature program: 37°C for 21 min, rising 11°C/min to 136°C, holding 3 min at 136°C, rising 20°C/min to 236°C, holding 3 min at 236°C.

Detector temperature: 300°C.

Carrier gas flow: 30 cm/s at 37°C.

Makeup gas flow: 23 mL/min.

At the beginning of each analytical run, inject two MtBE solvent blanks to condition the GC and to verify that interferences are absent. A 2-μL extract is injected in splitless mode. Always inject same sample volume and use sample dilution to obtain response in the calibration range. If levels greater than the highest standard are obtained, re-analyze diluted sample extract and readjust internal standard concentration. Calculate concentration only for those compounds that were at levels higher than the calibration curve; for other compounds use values obtained from the undiluted sample extract. See Figure 6251:4 for a chromatogram of an extracted and derivatized 30-μg/L standard on the analytical column. See Table 6251:III for retention times. A direct standard may be injected after the MtBE solvent blanks to verify continued system performance.

g. Calibration: Use five levels of calibration standards to define the quantitation range. The lowest standard should be near the limit of quantitation (LOQ) (see Table 6251:IV) for each compound. Use other standards to bracket the expected range of sample concentrations; do not exceed linear range of detector. Prepare standards by adding haloacetic acids and trichlorophenol to reagent water and then extract with the same solvent and derivatize with the same batch of diazomethane as used for the samples. Use the same extraction/esterification procedure for both standards and samples to correct for recovery characteristics. Analyze calibration standards under the same chromatographic conditions as samples.

Analyze calibration standard at one or more levels with each sample set to verify the working calibration curve. If the calibration standard is within ±15% of the expected value, a new five-level calibration curve is not needed.

6. Calculation

A 2-μL injection of each calibration level will provide peak area (A_a) data for each compound and an internal standard peak area (A_i) for each level; use these peak areas to calculate relative response for each compound.

$$\text{Relative response} = A_a/A_i$$

A calibration curve passing through zero is generated from the plotted points for each compound using the relative response versus standard concentration. Use the internal standard quantitation method to determine unknown concentrations by a linear, quadratic, or point-to-point curve fit.

7. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 6020:I.

a. Quality control program: Because sample preparation requires many manipulations, chances for errors are increased. Consequently, at least follow minimum quality control requirements to monitor and maintain method performance. Include method blanks, an initial demonstration of laboratory capability and detection levels, assessment of the internal standard recovery, determination of surrogate compound recoveries, evaluation of calibration data and curves, sample matrix additions, and precision of replicate sample analysis. Additional quality control measures may be used.

b. Method blanks: Process a method blank (30 mL reagent water) with each set of samples. If the blank produces any peak within the retention time window of a compound that would prevent its determination, seek out and eliminate the source of contamination and re-analyze samples.

c. Initial demonstration of capability: To demonstrate an adequate level of performance, conduct the following operations before analyzing samples and whenever any major analytical change, such as new analyst or switch in type of column, is made.

TABLE 6251:V. ADDITIVE RECOVERY IN REAGENT WATER*

Compound	Conc. Added μg/L	Mean Conc. Recovered μg/L	Standard Deviation μg/L	Relative Standard Deviation %	Mean Recovery %
Monochloroacetic acid	5.00	4.90	0.19	3.88	98.0
Monobromoacetic acid	5.00	4.95	0.13	2.67	99.0
Dichloroacetic acid	5.00	4.95	0.15	3.11	99.0
Trichloroacetic acid	5.00	5.06	0.16	3.06	101
Dibromoacetic acid	5.00	4.98	0.16	3.11	99.6
2,4,6-Trichlorophenol	2.50	2.51	0.075	2.99	100

* Based on the analysis of seven portions of reagent water with known additions in a single laboratory.

1) Accuracy as percent recovery—Establish a calibration curve as in 6251B.5*g* and select a representative additive concentration (5 μg/L is convenient) for each target compound. Using a syringe, add the appropriate amount of stock standard mix to each of a minimum of seven 30-mL portions of reagent water and analyze.

Calculate average percent recovery (P) and standard deviation of the recovery (S_r). Compare results to the single-laboratory recovery and precision data in Table 6251:V. Compare precision at similar concentrations (i.e., $P \pm 30\%$ of the additive level). Acceptable mean recovery values are within the interval $P \pm 30\%$. For compounds not meeting this criterion, repeat with another seven samples until satisfactory performance has been demonstrated.

2) Absolute recovery—Use direct-injection haloester standards [6251B.4*j*5)] to check absolute recoveries of extracted and derivatized haloacetic acids. Calculate absolute recoveries by comparing the ratio (area for compound/area for internal standard) for samples in ¶ *c*1) above with the area ratio for direct- injection standard at a tenfold concentration to account for the extraction (expressed as the haloacetic acid). Typical absolute recoveries are given in Table 6251:VI; acceptable absolute recoveries are within ±30%. Recoveries outside this range may indicate insufficient shaking or poor methylation, possibly due to water in the extracts. Correct problem and produce acceptable absolute recoveries before analyzing samples.

Because of rapid advances in chromatography, columns and conditions may be modified to improve separation or to lower cost. Repeat procedure in ¶ *c* for each modification.

d. Internal standard assessment: The internal standard corrects for any deviation in sample volume injected. A sample injection is acceptable if the area counts of the internal standard peak do not vary more than ±20% from the daily calibration standard(s) IS response.

Reinject an extract exceeding the ±20% range. If re-analysis does not produce acceptable results, re-extract and re-analyze. If the re-extracted sample results are not acceptable or if samples have exceeded holding time, resample and re-analyze or record results as suspect and out-of-control.

e. Evaluating surrogate recovery: The surrogate is added directly to all samples before acidification and extraction. If the surrogate area is low or absent, it is likely that there has been a derivatization problem (e.g., water in extract) or extraction problem (e.g., water insufficiently acidified).

An extract is acceptable if the area counts of the surrogate standard recovery are ±30% from the surrogate standard recovery for the daily calibration standard(s).

When surrogate recovery is not acceptable, check the following: locate possible errors in calculations or procedure, degradation of standard solution, contamination sources, and instrument performance. If these steps do not reveal the problem, re-analyze the extract. If re-analysis does not produce acceptable results, re-extract and re-analyze samples. If the re-extracted sample results are not acceptable or if samples have exceeded holding time, record results as suspect and out-of-control.

f. Extracted standard calibration: Quantitation is done by internal standard referencing with relative areas. Produce a minimum five-level extracted standard calibration curve for sample quantitation.

If the response for any compound falls outside the predicted response by >15% from a previous calibration, make a new calibration standard and analyze it until an acceptable curve is obtained.

Analyze calibration standards with each sample set after an acceptable five-level calibration curve is generated. If the continuing calibration standards are not within ±15%, check for errors or degradation of standards and construct a new calibration curve.

g. Matrix additions: Add each target compound into one sample per sample set (a sample set is all samples extracted within a 24-h period) or 10% of the samples, whichever is greater.

The added concentration should be near to or greater than the background. Take care, particularly with dichloroacetic acid, to ensure that the addition plus background concentration does not exceed calibration range (extract dilution may be needed).

Analyze one sample portion to determine the background concentration (B) of each compound. Add working standard mix to a second sample portion and analyze to determine the concentration of each compound (A). Calculate percent recovery (P_i) as $100\ (A - B)/T$, where T is the known concentration of the material added to the sample.

Compare percent recovery (P_i) for each compound with established QC acceptance criteria. Establish QC criteria by initially analyzing seven samples with additions and calculating the

Table 6251:VI. Absolute Recovery Data for Reagent Water with Known Additions

Compound	Conc. Added µg/L	Mean Conc. Recovered µg/L	Standard Deviation µg/L	Relative Standard Deviation %	Mean Recovery %
Monochloroacetic acid	1.00	0.789	0.047	5.92	78.9
Monobromoacetic acid	1.00	0.706	0.034	4.76	70.6
Dichloroacetic acid	1.00	1.10	0.048	4.38	110
Trichloroacetic acid	1.00	0.927	0.051	5.49	92.7
Bromochloroacetic acid	0.50	0.49	0.015	3.07	98
Dibromoacetic acid	1.00	1.16	0.032	2.75	116
2,4,6-Trichlorophenol	0.50	0.523	0.030	5.89	105

average percent recovery (P) and the standard deviation of the percent recovery (S_r).

Calculate QC acceptance criteria as follows:

$$\text{Upper control limit (UCL)} = P + 3S_r$$

$$\text{Lower control limit (LCL)} = P - 3S_r$$

The data generated during the initial demonstration of capability, ¶ *c*1) above, may be used to set the initial upper and lower control limits.

Monitor all data from dosed samples. Compound recoveries must fall within established control limits. After 10 new recovery measurements, recalculate P and S_r using all the data, and construct new control limits. When the total number of data points reaches 20, update control limits by calculating P and S_r using only the most recent 20 data points.

Compare percent recovery (P_i) for each compound with the QC acceptance criteria established by the control limits. If recovery of any compound falls outside the designated range, performance is judged to be out of control. Seek source of problem immediately and resolve before continuing the analysis.

However, if recovery of a compound meets calibration, blank, internal standard, surrogate, and replicate quality control, laboratory performance is in control, and the recovery problem is matrix-related. Label result for that compound in the sample as suspect/matrix.

h. Replicate analysis: Analyze sample duplicates to monitor precision. Analyze duplicates on at least 10% of all samples randomly selected.

Determine control limits by calculating the range as a function of the relative standard deviation. The range, R, is the absolute difference of the duplicate values, X_1 and X_2, as follows:

$$R = |X_1 - X_2|$$

The normalized range (R_n) is calculated by dividing the range by the average of the duplicate values (X_m):

$$R_n = \frac{R}{X_m}$$

$$X_m = \frac{X_1 + X_2}{2}$$

Calculate mean normalized range (R_m) for 20 pairs of duplicate data points initially and 20 pairs of points quarterly:

$$R_m = \frac{\Sigma R_n}{n}$$

where:

n = number of duplicate pairs.

$$\text{Variance} = S^2 = \frac{\Sigma(R_n - R_m)^2}{n - 1}$$

The standard deviation (s) is the square root of the variance. Upper and lower control limits are $R_m + 3s$ and zero, respectively. Acceptable duplicates fall within the control limits. The upper warning limit is $R_m + 2s$. If an R_n value is outside the warning limit, a potential problem is indicated and investigated before the analysis is out of control. Recalculate control limits quarterly using the most recent 20 points, not including any data points that are out of control. Recalculate control limits when any major analytical changes are made and after at least 20 points have been collected.

Analyze any problem and correct it. If the duplicate is not acceptable, re-extract only for those compounds out of control. If the duplicates are still unacceptable or the sample holding time has been exceeded, resample and re-analyze. If this is not possible, record results as suspect and out of control. Do not use such data in range calculations.

i. Additional quality control: Each quarter, analyze QC check standards from an external source. Independent confirmation may include interlaboratory split sampling for comparison. Analyze performance evaluation samples, preferably from EPA or appropriate state agency, at least once a year. Results for each target compound must be within established acceptance limits.

Shipping blanks are containers filled with reagent water containing appropriate amount of NH_4Cl (see 6251B.2), shipped to all sample locations with sample bottles, and returned with the samples. They are used to assess contamination during sampling and transit. Analyze a shipping blank with each sample set. If the shipping blank contains reportable levels, compare with the laboratory reagent blank. If contamination is not detected in the laboratory reagent blank, the sampling or transportation practices may have caused the contamination. Discard all samples in the set and resample.

Table 6251:VII. Sample Duplicate Data from Two Laboratories

Laboratory	Compound	Number of Pairs of Replicates	Average Difference Between Duplicates %	Standard Deviation of Difference Between Duplicates %
A	MCAA	5	7.6	10.6
	MBAA	3	1.9	1.5
	DCAA	7	1.5	0.8
	TCAA	6	1.4	1.0
	DBAA	5	6.0	6.0
	BCAA	11	1.8	1.3
B	MCAA	10	16.7	14.8
	MBAA	3	8.9	8.4
	DCAA	11	8.5	10.6
	TCAA	11	5.5	3.6
	DBAA	5	5.4	4.0
	BCAA	10	5.3	4.3

Table 6251:VIII. Field Sample Recovery with Known Additions to Drinking Water, in Two Laboratories

Laboratory	Compound	Added Conc. μg/L	Number of Samples	Mean Recovery %	Relative Standard Deviation %
A	MCAA	5.0	7	99	4
	MBAA	5.0	7	101	4
	DCAA	5.0	7	96	4
	TCAA	5.0	7	100	3
	BCAA	10.0	14	96	5
	DBAA	5.0	7	102	5
	TCPh	2.5	7	100	6
B	MCAA	5.0	13	101	8
	DCAA	4.0	14	103	7
	TCAA	4.0	14	103	6
	MBAA	5.0	14	97	8
	BCAA	2.0	14	106	8
	DBAA	4.0	14	102	7
	TCPh	0.4	14	104	15

Direct ester standards (concentration based on corresponding acid) may be injected at the start of each set to verify the sensitivity, chromatography, and retention times on the gas chromatograph.

Make an instrument check of the entire analytical system daily using data gathered from analyses of reagent blanks, standards, and replicate samples.

8. Precision and Bias

Single-laboratory method detection levels (MDL) and extracted recovery data from reagent water are presented in Table 6251:I. Data for absolute recoveries in reagent water are given in Table 6251:VI. Laboratory data from two laboratories showing duplicate precision and matrix additions recoveries are presented in Tables 6251:VII and VIII.

The precision and bias of analyses performed under the Information Collection Rule[10] (ICR) using this method are presented in Tables 6251:IX and X, respectively. Precision was determined as relative percent difference for duplicate analyses [(Rn)(100)] and was calculated only when both analyses in the duplicate pair showed concentrations at or greater than the ICR minimum reporting level (MRL). Bias was evaluated as percent recovery for cases in which the fortified amount was at least half the backgound concentration. The fortifying concentrations ranged from the ICR MRL (2 μg/L for MCAA, 1 μg/L for all other HAAs) to 40 μg/L.

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Table 6251:IX. Relative Percent Difference (RPD) Determinations from Duplicate Samples

Haloacetic Acid*	MRL μ/L	N	RPD in Given Percentile					Median Sample Concentration μgL
			10th	25th	Median	75th	90th	
HAA5		775	0.0	0.83	2.9	6.4	12	21
Monochloroacetic acid	2.0	232	0.0	1.6	4.9	12.	28	3.2
Dichloroacetic acid	1.0	774	0.0	0.0	2.9	6.3	11	10
Trichloroacetic acid	1.0	716	0.0	0.0	2.4	6.1	10	9.8
Monobromoacetic acid	1.0	98	0.0	0.0	6.9	15.	27	1.4
Dibromoacetic acid	1.0	294	0.0	0.0	3.6	7.4	11	2.4
HAA6		771	0.07	0.86	2.8	6.3	11	24
Bromochloroacetic acid	1.0	656	0.0	0.0	3.2	6.7	12	3.4

* HAA5 data are the sum of the individual concentrations of MCAA + DCAA + TCAA + MBAA + DBAA; HAA6 data are the sum of HAA5 + BCAA.

TABLE 6251:X. PERCENT RECOVERY DETERMINATIONS FROM FORTIFIED SAMPLES

Haloacetic Acid*	N	Percent Recovery in Given Percentile					Median Sample Concentration
		10th	25th	Median	75th	90th	μgL
HAA5	811	93	98	103	108	120	16.
Monochloroacetic acid	937	88	96	105	116	138	<2.0
Dichloroacetic acid	806	89	95	100	106	116	7.2
Trichloroacetic acid	793	90	97	102	110	120	4.9
Monobromoacetic acid	953	91	99	105	112	140	0.0
Dibromoacetic acid	925	92	98	103	114	127	0.0
HAA6	804	93	99	103	108	118	19
Bromochloroacetic acid	873	90	98	102	108	120	1.8

* HAA5 data are the sum of the individual concentrations of MCAA + DCAA + TCAA + MBAA + DBAA; HAA6 data are the sum of HAA5 + BCAA.

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6252 DISINFECTION BYPRODUCTS: ALDEHYDES (PROPOSED)*

6252 A. Introduction

1. Sources and Significance

Ozone reactions during water treatment are complex and often produce a wide range of unstable oxidation byproducts, usually oxygenated and polar. Among the intermediate products formed, when ozone attacks the organic matter present in raw waters, are low-molecular-weight byproducts, such as aldehydes. If oxidized further, these aldehydes can produce aldo-acids and carboxylic acids. Formaldehyde, a ubiquitous component of the environment, may be introduced into drinking water by ozone treatment, natural metabolism, and commercial processes.

There are two postulated mechanisms for aldehyde formation during ozone treatment. The first involves a two-step Criegee attack at unsaturated C–C bonds by molecular ozone with ozonides or epoxides formed as intermediates.[1] The second involves an indirect reaction of OH radicals.[2] Although the levels of aldehyde formation are usually a function of ozone dose, their concentrations are often controlled in water treatment by increasing the pH and thus the alkalinity of the water.

Aldehydes are unlikely to pose a serious health hazard to the consumer at microgram-per-liter concentrations as usually encountered in drinking water treatment. However, they react with nucleophiles even at these low levels and can therefore be a potential threat.[3] Thus, for example, formaldehyde, acetaldehyde, and crotonaldehyde are known animal carcinogens. Formaldehyde is a known human carcinogen.[4] Aldehydes also may serve as important components of assimilable organic carbon in promoting undesirable bioactivity.

2. Selection of Method

The most effective method for the determination of aldehydes in aqueous solutions involves the use of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA)† as a derivatizing agent. PFBHA reacts with low-molecular-weight carbonyl compounds, including aldehydes, to form the corresponding oximes. Unless the carbonyl compound is a symmetrical ketone or formaldehyde, two geometric isomers of the oxime derivatives are formed. These derivatives are extractable with organic solvents and are highly sensitive to analysis by gas chromatography with electron capture detection (GC/ECD) and gas chromatography with selective ion mass spectrometric detection (GC/SIM-MS).

3. References

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* Approved by Standard Methods Committee, 2005.
Joint Task Group: 21st Edition—Stephen D. Winslow (chair), Devon A. Cancilla, Patricia Snyder Fair, Shane S. Que Hee, Harumi Yamada.

† This reagent is known under various synonyms. The more common are *O*-(2,3,4,5,6-pentafluorophenyl) methylhydroxylamine hydrochloride with CAS RN 57981-02-9 and pentafluorobenzyloxylamine hydrochloride (PFBOA). It also has appeared with the acronym PFBHOX.

6252 B. PFBHA Liquid–Liquid Extraction Gas Chromatographic Method

This method measures straight-chain, low-molecular-weight aldehydes in raw and treated drinking water and simultaneously can analyze for C_1–C_{10} mono-carbonyl saturated aliphatic aldehydes, benzaldehyde, the dialdehyde glyoxal, and the keto-aldehyde methyl glyoxal.[1] The effectiveness of the derivatizing agent (PFBHA) in its reactions with these carbonyl compounds has been reviewed.[2]

1. General Discussion

a. Principle: Samples at room temperature are buffered to pH 4, PFBHA is added, and the samples are placed in a constant-temperature water bath. The carbonyl compounds are converted to their corresponding oximes during reaction with PFBHA. Sulfuric acid is used to protonate excess PFBHA and the oxime derivatives are extracted with hexane. After H_2SO_4 cleanup, the organic extract is analyzed by gas chromatography where the volatile derivatives are easily separated in a temperature-programmable gas chromatograph equipped with a fused-silica capillary column and either an electron-capture detector or selective ion mass spectrometer. Simultaneous analysis and confirmation with a single injection can be effected by setting up both the analytical column and the confirmation column to share a common injection port. Alternatively, use separate analytical and confirmation columns. Aqueous calibration standards similarly are derivatized, extracted, and analyzed. A surrogate recovery standard is added to the samples before derivatization to indicate any variation in derivatization and extraction efficiency.

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TABLE 6252:I. METHOD DETECTION LEVELS AND PRECISION DATA*

Compound	Added Concentration μg/L	Found Concentration μg/L	Standard Deviation μg/L	Relative Standard Deviation %	Method Detection Level† μg/L
Formaldehyde	0.450	1.49‡	0.0485	3.25	0.15
Acetaldehyde†	0.450	0.757‡	0.0242	3.20	0.076
Propanal	0.150	0.200	0.0083	4.15	0.026
Butanal	0.450	0.585	0.0504	8.62	0.16
Crotonaldehyde	0.250	0.242	0.0148	6.12	0.047
Pentanal	0.250	0.326	0.0153	4.70	0.048
Hexanal	0.250	0.307	0.0154	5.02	0.048
Cyclohexanone	0.150	0.199	0.0090	4.52	0.028
Heptanal	0.250	0.323	0.0170	5.25	0.053
Octanal	0.150	0.151	0.0136	8.99	0.043
Benzaldehyde	0.450	0.385	0.0405	10.5	0.13
Nonanal	0.450	0.602	0.0403	6.70	0.13
Decanal	0.450	0.669	0.0561	8.38	0.18
Glyoxal	0.250	0.133	0.220	16.5	0.069
Methyl glyoxal	0.150	0.133	0.0096	7.22	0.030

* Based on the analysis of seven portions of organic-free water with known additions.
† Method detection level for $n = 7$ found by multiplying the standard deviation by 3.14.
‡ Level of quantitation (LOQ) should take into consideration the level of target compound contamination rather than MDL.

With the exception of symmetrical ketones and formaldehyde, most carbonyl compounds form two geometrical isomers of oxime derivative. Methyl glyoxal, however, produces only one prominent isomer.

The method described may be used if appropriate quality control can be demonstrated for quantification of all C_1–C_{10} mono-carbonyl, saturated aliphatic aldehydes, benzaldehyde, glyoxal, and methyl glyoxal, but precision and quality control data are presented only for the most commonly found ozonation by-products, namely, formaldehyde, acetaldehyde, heptanal, benzaldehyde, glyoxal, and methyl glyoxal.

b. Interferences: Dissolved ozone, residual chlorine, and other oxidizing substances interfere with the PFBHA reaction. Quantitative addition of potassium iodide (KI) as a reducing agent before derivatization or the addition of ammonium chloride or sulfate at the time of sampling (KI if ozone is present) prevents this interference. Ketones and quinones or large quantities of aldehydes may deplete the PFBHA reagent excess necessary to ensure complete reaction. Waters with high sulfide content inhibit the derivatization of carbonyl compounds. The occurrence of artifacts by aldehyde formation from thermal decomposition of water components is a potential positive interference. Because formaldehyde is used as a preservative for membranes, purified water produced by reverse osmosis is also a potential positive interference. In addition, formaldehyde and acetaldehyde are air pollutants and some formaldehyde in the air can be traced to certain insulation materials. The use of reducing agents, such as sodium thiosulfate, sodium sulfite, and ascorbic acid, can cause the formation of aldehydes if the sample water contains the corresponding organic acids.

c. Detection level: The method detection level (MDL) and precision data for those aldehydes most commonly found in ozonated waters are given in Table 6252:I. These levels were evaluated for the extracted oximes in hexane from aldehyde-free water. The level of quantitation (LOQ) for these aldehydes usually is set at five times the MDL. In effect, the LOQ for all aldehydes analyzed by this method is 0.5 μg/L except for acetaldehyde and glyoxal, where the value is 1 μg/L. Background formaldehyde contamination may require elevating the LOQ to as high as 5 μg/L. The precision data presented may be matrix-sensitive. Use known standard additions to the matrix if oxime standards are not available.

This method is useful for detecting carbonyl compounds, such as short-chain aldehydes (C_1–C_{10}), benzaldehyde, glyoxal, and methyl glyoxal, in the range of 1 to 100 μg/L. A clean laboratory reagent water blank, free of these contaminants, is essential.

d. Safety: The toxicity or carcinogenicity of each reagent used in this method has not been defined precisely. Acetaldehyde contains material that can cause cancer in animals, formaldehyde contains material that can cause cancer in humans, and crotonaldehyde causes respiratory tract and eye burns if inhaled or swallowed. Observe proper ventilation and handling procedures. Wear quantitatively-fitted negative-pressure respirators with charcoal air-purifying filter canisters, gloves (such as butyl but not natural rubber, latex, or nitrile[2]), and protective garments resistant to the degrading effects and permeation of these chemicals. Glyoxal and methyl glyoxal are mutagenic in *in vitro tests* and the former has subchronic oral toxicity. Take care when handling high concentrations of aldehydes during preparation of primary standards. When handling hexane solutions of the oxime derivatives, wear nitrile gloves (not butyl or latex).

2. Sampling and Storage

See Section 6010B.1 and note the following additional requirements:

Seal sample vials with TFE-lined polypropylene screw caps.* Do not use bakelite black caps made from a formulation containing phenol and formaldehyde. Do not add HCl for this

* I-Chem Research, Hayward, CA, or equivalent.

method. Reduce residual free chlorine according to the following method. Additional reagents are required for ozonated samples as described below. If free chlorine is present in the samples, add ammonium chloride or sulfate (0.1 mL of a 20% solution /40 mL sample) before sample collection. Monochloramine may form but will not change the aldehyde concentration of samples subsequently stored at 4°C. If residual ozone is present, the levels of aldehydes may change as the ozone–natural organic matter reaction continues. To prevent this, quench residual ozone by adding 50 μL of a 6.40-g KI/L solution to each 40-mL vial before shipment to the field.

In chlorinated waters, addition of KI will eliminate an interferant that coelutes with the surrogate. The reduction of oxidizing compounds with KI may cause iodine to be extracted into the hexane extract, resulting in a pink-colored extract. Iodine in the extract also may interfere, chromatographically, with the internal standard. The formation of iodine can be suppressed by the addition of 50 μL of 8.52-g mercuric chloride/L solution to each 40-mL vial before shipment to the field. In most situations, an ozone residual is unstable and the use of KI and the toxic $HgCl_2$ is unnecessary.

Before sample collection, add 20 mg (for 40 mL sample) of copper sulfate pentahydrate to each bottle to prevent microbial decay of the method analytes. If mercuric chloride is added to suppress iodine formation, copper sulfate pentahydrate need not be added, because mercuric chloride is an effective biocide at the concentration given above. Without a biocide, aldehydes are extremely susceptible to microbial decay. Preservation of the sample is important for obtaining valid data. Ideally, derivatize aldehyde samples immediately after collection. If sample is preserved with a biocide and stored at 4°C, extract within 7 d.

Prepare field reagent blanks from organic-free reagent water (6252B.4*e*).

3. Apparatus

a. Sample containers and extraction vials: 40-mL screw-top, glass sample vials with aldehyde-free caps. (NOTE: Do not use thermoset, phenol-formaldehyde, or urea-formaldehyde caps.) Prepare these, together with the 14-mL amber vials for storing stock solutions, as follows: Wash with detergent, rinse with tap water, soak in 10% HNO_3 for at least 30 min, rinse with tap water, rinse with laboratory organic-free water (6252B.4*e*), and oven dry at 180°C for at least 1 h.

Clean caps and septa by rinsing with methanol, then with hexane, and dry at 80°C for no more than 1 h in a clean, forced-air convection oven.

b. Microsyringes or micropipets with glass tips, to measure the following volumes: 5, 10, 25, 50, 100, 250, 500, and 1000 μL.

c. Volumetric flasks, 5, 10, and 25 mL, borosilicate glass. Prepare initially by the method of ¶ *a* above, except that after rinsing with organic-free water, rinse with methanol and invert to drain. Air-dry only. *Do not dry in oven.*

d. Syringe: 20-mL glass hypodermic, metal luer lock tip with 8.9-cm- (3.5-in.-) long × 17 gauge stainless steel pipetting needle (alternatively, use a 20-mL volumetric pipet). Clean as above.

e. Automatic pipet dispensers: To simplify batch processing, add reagent by use of these dispensers. Preferably use adjustable 1-mL and 4-mL sizes with PTFE transfer lines that can be mounted on the suppliers' reagent bottles. If these are not available, use 1-mL and 4-mL volumetric pipets.

f. Constant-temperature water bath or incubator, capable of holding multiple 40-mL sample vials and maintaining [35 ± 0.5°C].

g. Pasteur pipets: Have a selection of short-tipped (14.6-cm or 5.75-in.) and long-tipped (23-cm or 9-in.) pipets.

h. Mechanical shaker,† to automate hexane extraction (see Section 6251B.3*e*). Alternatively, use a vortex mixer or manual shaking for 1 min.

i. Storage vials: 7-mL glass, screw-cap vials with PTFE-lined silicone septa cleaned as described above.

j. Gas chromatograph, with capillary columns, temperature programmable, and supplied with a temperature-controlled injector and electron-capture detector.

1) *Gas handling equipment*—Use carrier (helium) and make-up (nitrogen or 95% argon/5% methane) gases of high purity (99.999%) that pass through indicating calcium sulfate, molecular sieve 5A, activated charcoal, and an oxygen-purifying cartridge. Use two-stage metal diaphragm high-purity regulators at the compressed gas sources. Use flow controllers to regulate carrier gas flow. Ensure that all gas lines use 0.3-cm (0.125-in.) copper (or stainless steel) tubing; rinse with high-purity acetone, and bake before use.

2) *Injector*—split/splitless (using straight open-bore insert).

3) *Analytical column*‡ —30 m long × 0.25-mm ID, fused silica capillary column with a 0.25-μm film thickness.

4) *Confirmation column*§ —30 m long × 0.25-mm ID, fused silica capillary column with a 0.25-μm film thickness.

5) *Detectors*—a constant current pulse modulated ^{63}Ni ECD with standard size cell (use two ECDs for simultaneous confirmation analysis).

4. Reagents

a. Extraction solvent, UV-grade, glass-distilled hexane.‖

b. Solvent for standard preparation, reagent-grade acetonitrile, free of the target aldehydes.#

c. Preservation agents, ammonium chloride (NH_4Cl) or sulfate [$(NH_4)_2SO_4$], copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and mercuric chloride, anhydrous ($HgCl_2$).

d. Sulfuric acid (H_2SO_4), conc and 0.2*N*.

e. Organic-free reagent water: Treat water prepared in commercially available water systems to remove all traces of aldehydes. Two methods have been demonstrated: Either expose reagent water produced by a laboratory purification system** to UV irradiation for 1 h or distill reagent water from acidified potassium permanganate (500 mL water with 64 mg potassium permanganate and 1 mL conc H_2SO_4).

Alternative purification techniques, such as addition of another granular activated carbon filtration step, may be used if they can be shown to effectively eliminate background levels of aldehydes. *Do not use a reagent water with formaldehyde contamination to quantify formaldehyde in aqueous samples.*

† Eberbach Corp., or equivalent.
‡ Durabond-5, J&W Scientific, or equivalent.
§ Durabond-1701, J&W Scientific, or equivalent.
‖ Burdick & Jackson, Muskegon, MI, or equivalent.
Sigma Chemical Co., St. Louis, MO, or equivalent.
** Milli-Q, Millipore Corp., Bedford, MA, or equivalent.

Table 6252:II. Analytical Standards of Carbonyl Compounds Used in the PFBHA Method

Compound	Purity %	Molecular Weight mg/mmol	Boiling Point °C	Density g/mL
Formaldehyde*	†	33.03	96	1.083
Acetaldehyde‡	99.9	44.05	21	0.788
Propanal (propionaldehyde)*	97	58.08	46–50	0.805
Butanal (*n*-butyraldehyde)‡	99.9	72.11	75	0.800
Pentanal(*n*-valeraldehyde)*	99	86.13	103	0.810
Hexanal (caproaldehyde)*	98	100.16	131	0.834
Heptanal (heptaldehyde)*	95	114.19	153	0.818
Octanal (caprylic aldehyde)*	99	128.22	171	0.821
Nonanal (nonyl aldehyde)‡	99.9	142.24	93	0.827
Decanal (decyl aldehyde)‡	99.9	156.27	207–209	0.830
Benzaldehyde‡	99.9	106.12	178–179	1.044
Glyoxal (ethanedial)‡	§	58.04	50	1.14
Methyl glyoxal (pyruvic aldehyde or 2-oxopropionaldehyde)*	§	72.06	72	1.045

* Aldrich Chemical Company, Inc., Milwaukee, WI, or equivalent.
† Available in 37% solution (by weight) in water.
‡ ChemService, Inc., West Chester, PA, or equivalent.
§ Available in 40% solutions (by weight) in water.

f. Buffer pH 4 reagent: Add 200 mg potassium hydrogen phthalate per 20 mL aqueous sample.

g. Derivatizing agent (PFBHA):†† Weigh *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride into organic-free water to give a solution concentration of 15 mg/mL. Prepare fresh daily. Prepare enough to add 1 mL/20 mL sample.

h. Standard materials: See Table 6252:II for source and physical characteristics of the standards. Obtain purity assay of each purchased standard before use.

1) *Individual aldehyde standard stock solutions*—Prepare by weighing aldehyde standards in acetonitrile. Weigh between 20 and 70 mg of each standard into a 10-mL volumetric flask. Weigh solid standards directly into the empty flask, then fill with acetonitrile. Add liquid standards to the flask, which has been filled to the neck previously with acetonitrile. Place on a weighing balance and stabilize the weight. Inject liquid standard with a microsyringe directly into the bulk of the acetonitrile and determine exact weight after the addition. Because some aldehyde standards are supplied as aqueous solutions, evaluate weight of actual standard component and make an approximate determination of the required volume to be added from either the density (for pure liquids) or the percentage by weight (for solutions). Due to the high volatility of acetaldehyde, keep in the refrigerator at all times and place measurement syringe in a freezer for 10 min before preparing the stock solution. After diluting to 10 mL with acetonitrile, cap flask and invert three times to mix. Transfer stock solutions to separate 14-mL amber vials with screw caps and PTFE liners and store at 4°C bound with self-adhesive film.‡‡ Stock solutions (except formaldehyde), are usable for up to 3 months. Let them come to room temperature before pipetting. Overcome presence of turbidity or a precipitate by ultrasonication in warm water. If a precipitate persists, make a new stock solution. Prepare formaldehyde stock solutions each month.

Verify the aldehyde concentrations of the aqueous solutions after filtering through 0.45-μm PTFE filters by the sodium bisulfite-iodine titration method.[3]

2) *Multicomponent aldehyde additive standards*—Prepare additive standards solution using individual stock solutions of those aldehydes of interest. Make mixture weekly. The concentration of each component in this additive standard solution should be about 10 mg/L when added to 20-mL aqueous samples. When preparing a calibration curve in 100 mL of organic-free water, prepare two or more multicomponent additive standards from which a volume in the range 10 to 100 μL can be injected directly into the water. For example, if the stock solution concentration is 50 mg in 10 mL, add 20 μL in 10 mL acetonitrile to produce a 10-mg/L additive standard. This is best achieved by first filling the 10-mL volumetric flask to just above the neck with acetonitrile. Inject required volume of each of the stock solutions, using a clean microsyringe for each component, into the bulk of the acetonitrile. After adding all stock solutions, fill to the mark with acetonitrile. Cap and invert three times to mix.

i. Standards derivatives: To determine reaction and extraction efficiency of each aldehyde in different matrices compare the chromatographic response of the derivatized standard in the matrix to that of authentic standards of the oximes. The surrogate standard used to establish optimum conditions for derivatization and laboratory-synthesized oximes for six of the aldehydes have been used to verify recovery of derivatized aldehydes from organic-free water. See Table 6252:III. Unforeseen matrix effects can occur, and because PFBHA-derivatized aldehyde standards (oximes) are not available commercially, some representative syntheses of these derivatives are available.[4]

j. Internal standard, 1,2-dibromopropane and decafluorobiphenyl, 98% purity.§§

1) *Internal standard stock solution*—Weigh 50 mg into a 10-mL volumetric flask containing acetonitrile up to the neck.

†† Aldrich Chemical Co., or equivalent.
‡‡ Parafilm®, American Can Co., Greenwich, CT, or equivalent.

§§ Aldrich Chemical Company, Inc., Milwaukee, WI, or equivalent.

TABLE 6252:III. RECOVERY OF TRIPLICATE *IN SITU* DERIVATIZED ALDEHYDES COMPARED TO THE RECOVERY OF PURE OXIME DERIVATIVES FROM ORGANIC-FREE WATER

Derivatized Aldehyde	Concentration of Oxime Added* μg/L	Mean Concentration Recovered† μg/L	Standard Deviation μg/L	Relative Standard Deviation %	Mean Recovery‡ %
Formaldehyde	19.2	21.2	0.118	0.56	90.4
Acetaldehyde	19.3	19.3	0.095	0.49	100
Heptaldehyde	19.9	23.6	0.104	0.44	84.3
Benzaldehyde	19.8	18.6	0.138	0.74	107
Glyoxal	19.4	23.7	0.235	0.99	82.0
Methyl glyoxal	20.5	22.1	0.194	0.88	92.5

* Amount of standard oxime added to OPW.
† Evaluated by comparing the relative response of the extracted standard oximes to the calibration curve using *in situ* derivatized aldehydes.
‡ Calculated recovery of *in situ* derivatized aldehydes compared to standard oximes.

Fill to mark with acetonitrile. This 5-g/L stock solution can be used for up to 6 months when stored as described in ¶ *h*1) above.

2) *Internal standard working solvent*, 100 μg/L in hexane—Deliver 20 μL internal standard stock solution directly into 1 L hexane in the solvent bottle to be used in the extraction. Cap bottle and invert three times to ensure thorough mixing. This dilution can be used for 4 weeks. To ensure suitability for extraction, run a sample of this working solvent on the GC before extraction of aqueous samples. Before processing samples, provide enough working solvent to extract all calibration and aqueous samples to be analyzed. *Never make up fresh working solvent for use during sample processing*.

k. Surrogate (SUR), 2,3,5,6-tetrafluorobenzaldehyde, 98% pure. Alternative surrogate: 4′-(trifluoromethyl)acetophenone.

1) *Surrogate stock solution*, 20 g/L—Weigh 0.2 g SUR into a 10-mL volumetric flask containing acetonitrile up to the neck. After determining the weight difference, fill to mark with acetonitrile. Stock solutions can be used for up to 6 months if stored as described in ¶ *h*1) above.

2) *Surrogate additive solution*, 20 mg/L—Deliver 10 μL SUR stock solution into a 10-mL volumetric flask and dilute to volume with acetonitrile. This solution can be used for up to 3 months when stored at 4°C. At the beginning of sample processing, add 10-μL surrogate additive solution to each 20-mL sample portion, yielding a surrogate concentration of 10 μg/L.

l. Calibration standards: Prepare aqueous calibration standards in 100 mL organic-free water by injecting a measured amount of the multicomponent aldehyde additive standard solution directly into the water using the solvent flush technique. Prepare five different concentration levels within the expected sample range of approximately 2 to 40 μg/L. The lowest level of standard will depend on level of blank contamination for each analyte.

5. Procedure

a. Sample preparation: Remove samples and standard solutions from storage and let reach room temperature.

b. Derivatization: Withdraw 20 mL sample from sample vial using a 20-mL glass syringe or glass pipet. Discard remaining sample, shake the vial dry by hand, and return the syringe contents to the vial. Add 10 μL surrogate additive solution using either a microsyringe or automatic pipettor, to all samples and standards. Add 200 mg potassium hydrogen phthalate and dissolve. Add 1 mL freshly prepared 15-mg/mL PFBHA solution to each vial by automatic pipet, secure cap, and swirl to mix gently. Place all samples in a constant-temperature water bath set at 35 ± 0.5°C for 2 h. Remove vials and cool to room temperature for 10 min.

c. Microextraction: To each vial add 0.05 mL (approximately 2 drops) conc H_2SO_4 to quench the derivatization reaction and then add 4 mL hexane working solvent containing the internal standard. When using automated extraction, place vials in a mechanical wooden shaker box. Shake vials on fast setting (approximately 300 cycles) for 3.5 min. Alternatively, shake manually for approximately 3 min. Remove vials and place upright. Let stand for approximately 5 min to permit phases to separate.

d. Extraction cleanup: Draw off top hexane layer using a clean 14.6-cm (5.75-in.) disposable Pasteur pipet for each sample into a smaller 7-mL clear vial containing 3 mL 0.2*N* H_2SO_4. Shake for 30 s by hand and let stand for approximately 5 min for phase separation. This wash step reduces the amount of PFBHA reagent and other interferants in the hexane extract. Do not skip this step. Draw off top hexane layer using another clean 14.6-cm (5.75-in.) disposable Pasteur pipet for each sample and place in two 1.8-mL autosampler vials per sample. Store extra autosampler vials in a refrigerator at 4°C as a backup extract. Analyze extracts within 14 d of extraction.

e. Gas chromatography: Use the following operating conditions for the gas chromatograph: injector temperature 180°C; split valve open at 0.5 min; split flow at 50 mL/min; temperature program: 50°C for 1 min, rising at 4°C/min to 220°C and then at 20°C/min to 250°C; detector temperature: 300°C; carrier gas flow: 1.5 mL/min at 100°C; make-up gas flow: 27 mL/min.

At the beginning of each analysis, inject one hexane solvent blank to condition the GC and to verify that there are no interferences present. Inject 1 μL onto the splitless injector. See Figures 6252:1 and 2 for examples of chromatograms obtained with the above GC conditions for both the analytical and confirmation columns. If dual-column analysis is unavailable, use the column specified in 6252B.3*j*3), but be aware of possible interferences. Table 6252:IV lists retention times for both columns.

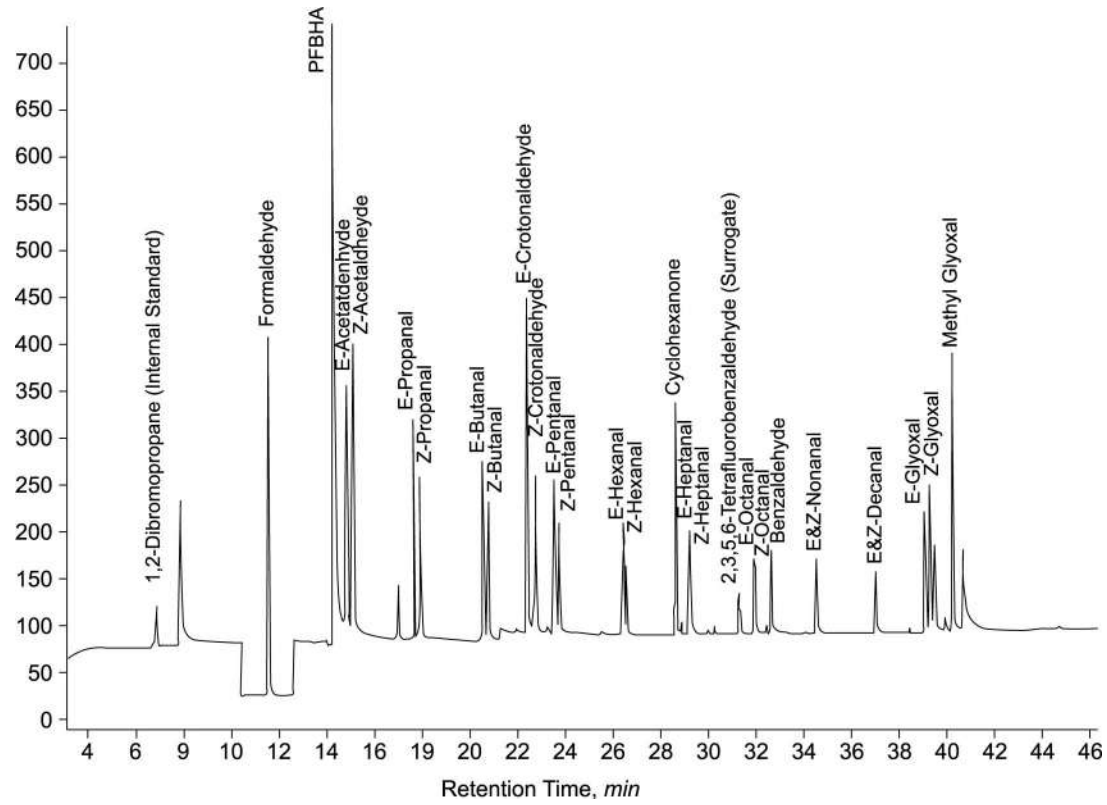


Figure 6252:1. Chromatogram for analytical (primary) column. The formaldehyde peak was attenuated by a factor of four.

Tabulate GC peak area response and concentration for each analyte and the internal standard. The formaldehyde peak will be much larger, for the same concentration, than the other analyte peaks. The formaldehyde peak may need to be attenuated on some instruments to avoid signal saturation.

For most asymmetrical carbonyl compounds derivatized with PFBHA, two isomers [referred to as (E) and (Z)] are formed. Chromatographic resolution is usually obtained with the columns suggested for acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, octanal, and crotonaldehyde. With dicarbonyl species, such as glyoxal and methyl glyoxal, (E) and (Z) isomerism occurs during oxime formation, increasing the number of isomers. Use one of the following methods for both calibration and quantification of each method analyte:

1) Use the sum of the isomer peak areas for each constituent for both calibration and quantification.
2) Use peak area of each individual isomer to calculate independently a concentration for each isomer. Then calculate average amount for the two isomers to report one value for the analyte.

f. Calibration: Demonstrate acceptable initial calibration before analyzing any samples. Carry standards (6252B.4*l*) through entire derivatization and extraction procedure as above. This corrects for any recovery characteristics inherent in the method. Analyze the calibration standards under the same GC conditions as the samples.

Generate a calibration curve for each analyte by plotting the area ratios (A_a/A_{is}) against the concentration ratios (C_a/C_{is}) of the five calibration standards where:

A_a = peak area of analyte (or analyte isomer pair),
A_{is} = peak area of internal standard,
C_a = concentration of analyte, and
C_{is} = concentration of internal standard.

This curve must be forced through zero and can be defined as either first or second order. Forcing through zero allows for a better estimate of the background level of method analytes.

6. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 6020:I.

a. General considerations: PFBHA is a highly reactive *O*-substituted hydroxylamine. Like hydroxylamine, PFBHA reacts readily with a variety of carbonyl functional groups to produce corresponding oximes. The ease with which PFBHA reacts with carbonyl-containing compounds makes the potential contamination of samples a serious concern. Lower-molecular-weight aldehydes are commonly found in laboratory and outside air and can ultimately contaminate water samples, leading to incorrect calculation of aldehyde concentrations. As a further concern, PFBHA, especially in moist laboratory environments, can react to form oximes when directly exposed to aldehydes in air. For these reasons, exercise care to reduce the sources and exposure of samples, standard solutions, and PFBHA reagents to aldehyde contaminants. Consider storing PFBHA in a desiccator under an inert atmosphere, drying laboratory solvents with molecular

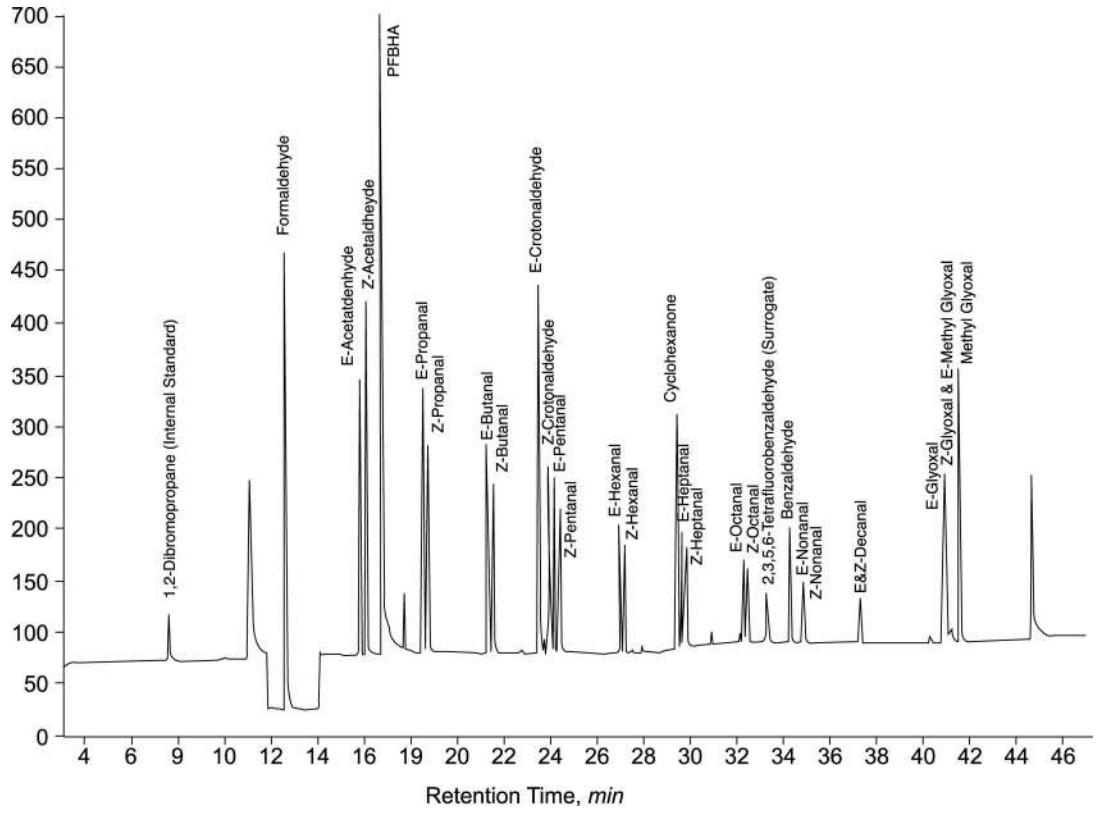


Figure 6252:2. Chromatogram for confirmation column. The formaldehyde peak was attenuated by a factor of four.

TABLE 6252:IV. RETENTION TIMES (RTS) FOR DERIVATIZED CARBONYLS, DERIVATIZED SURROGATE STANDARD, AND INTERNAL STANDARD ON ELECTRON-CAPTURE DETECTOR

Derivatized Carbonyls	Retention Time on Primary Column [6252B.3*j*3)] *min*	Retention on Confirmation Column [6252B.3*j*4)] *min*
1,2-Dibromopropane (internal standard)*	6.81	7.82
Formaldehyde	11.49	12.82
E- & Z-Acetaldehyde†	14.77 & 15.04	15.95 & 16.22
E- & Z-Propanal†	17.64 & 17.86	18.63 & 18.88
E- & Z-Butanal†	20.56 & 20.77	21.44 & 21.68
E- & Z-Crotonaldehyde†	22.41 & 22.73	23.56 & 24.08
E- & Z-Pentanal†	23.52 & 23.70	24.28 & 24.50
E- & Z-Hexanal†	26.44 & 26.59	27.10 & 27.28
Cyclohexanone	28.67	29.54
E- & Z-Heptanal†	29.25 & 29.34	29.78 & 29.92
2,3,5,6-Tetrafluorobenzaldehyde (surrogate)	31.34	33.34
E- & Z-Octanal†	31.96 & 32.04	32.40 & 32.51
Benzaldehyde	32.70	34.38
E- & Z-Nonanal†	34.61 (coelution)	34.93 & 35.01
E- & Z-Decanal†	37.12 (coelution)	37.36 (coelution)
E- & Z-Glyoxal†	39.16 & 39.40	40.92‡
Methyl glyoxal§	40.28	41.64

* 1,2-Dibromopropane, the internal standard, is not derivatized.
† These aldehydes form E- and Z-pentafluorobenzyloxime (PFBO) isomers in an assumed order of elution that has not been confirmed.
‡ On the confirmation column, only the E-isomer of glyoxal was used for quantitation, because Z-glyoxal co-eluted with an isomer of derivatized methyl glyoxal.
§ Derivatized methyl glyoxal had several conformational isomers, though only one peak was used for quantitation. The isomer peak used had significantly more peak area than two other isomer peaks and was free of co-elutions.

sieves, using purified water, and making fresh derivatizing stocks on a regular basis. If, after analysis of appropriate sample blanks, contamination remains a problem, the source of the problem may be in the PFBHA reagent and solutions. Recrystallization of PFBHA may be necessary to remove oximes formed as a result of reagent contamination.

The effects of chromatographic and analytical conditions on E/Z ratios of the oximes have not been fully explored. The possibility of changing E/Z ratios under differing analytical conditions, such as injection temperature, requires that analytical conditions be carefully controlled. E/Z ratios may change as a function of time; therefore analyze samples as soon as possible after preparation and within groups. Use the sum of the isomer peak areas for each constituent for both calibration and quantification. With dicarbonyl species, such as glyoxal, E/Z isomerism occurs from oxime formation with both carbonyl groups, increasing the number of possible isomers. Formation of the mono-derivatives from these di-carbonyl species may pose a problem if analytical conditions do not favor the complete derivatization of both carbonyl groups. Mono-derivatives have been shown to have similar retention and mass spectral characteristics as single carbonyl-containing oxime derivatives, potentially leading to incorrect identification and underestimation of the amounts of di-carbonyl species present in water samples. This method has been validated for the recovery of oxime derivatives of aldehydes from organic-free water; the recovery of the surrogate standard from this matrix appears to reflect method performance. Consequently, compare the *RRF* of surrogate standard extracted from aqueous samples to the value obtained when building the calibration curve. If these values are outside the range for accepted mean recovery values of 30% (see Section 6251B.7*c*), authentic oxime standards may have to be used to validate the method for the new matrix. In this case, if pure standards are unavailable, recognize that analyses of aldehydes are semi-quantitative; report as such.

b. Monitoring for interferences:

1) Solvent blank—Analyze each reagent bottle of hexane containing internal standard before it is used. If there are any spurious peaks in the chromatogram, solvent purity has been compromised. Remake the working solvent.

2) Method blank—See Section 6251B.7*b*.

3) Travel or shipping blanks—Prepare blanks for each sampling location in the laboratory by filling 40-mL vials, as described above, with organic-free water and containing the same reagents present (if any) in the sample vials. Ship to the sampling site and back to the laboratory with the sample bottles. Do not open these bottles in the field.

c. Internal standard assessment: Injections of the hexane extracts are acceptable if the area counts of the internal standard peak do not vary more than ±20% from the mean of all the samples analyzed with the same batch of PFBHA. Reanalyze samples that do not meet this precision. If, after reinjection, criteria are still not met, the sample holding time has not been exceeded, and the same working solvent used for constructing the calibration curve is still available, the second vial may be analyzed.

d. Verification of calibration standard materials: Analyze a laboratory-fortified blank from standard materials from a source other than those used to prepare the initial calibration curve. Calculate the concentration of this quality control sample (QCS) from the calibration curve. The calculated concentration of the QCS must be within 60 to 140% of its true value. This step validates the calibration standard materials before sample analysis.

e. Ongoing calibration: Time, temperature, pH, and PFBHA concentration affect the rate, efficiency, and reproducibility of the derivatization reaction. Calibration frequency depends on the laboratory's ability to control these parameters so continuing calibration check standard criteria can be met. After a successful initial calibration, maintain an ongoing calibration by either of the following options:

- Verify initial calibration daily using a minimum of two calibration standards. Prepare a minimum of one low-level calibration standard (suggested concentration 2 to 5 μg/L) and one mid-level calibration standard (suggested concentration 10 to 30 μg/L) with each batch of samples. Using these two standards, verify calibration before analyzing the sample extracts from the batch. In addition, re-analyze one of these two standard extracts after every tenth sample extract, and after the last sample in an analysis batch to ensure instrument stability throughout the analysis batch. Recovery must be within 70 to 130% of the true value for the mid-level standard, and within 50 to 150% of the true value for the low-level standard.
- Calibrate method daily with all five calibration standards. Some laboratories may find it more productive to prepare and analyze a calibration curve with each batch of samples.

f. Surrogate standard recovery: Add the surrogate (2,3,5,6-tetrafluorobenzaldehyde) directly to the 20-mL aqueous sample portions before reagent addition to monitor constituent recovery from the sample matrix. If the surrogate area is low or absent, there is likely to be a problem with derivatization or extraction that needs to be resolved before quantification can be undertaken (see Section 6251B.7*e*). A sample extract is acceptable if the area counts of the surrogate peak (or the *RRF* values compared to an acceptable internal standard area) do not vary more than 30% from other samples analyzed with the same batch of PFBHA.

g. Sample quantification: See Section 6251B.7*f*.

h. Matrix additions: See Section 6251B.7*g*.

i. Replicate analysis: See Section 6251B.7*h*.

7. References

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4. Cancilla, D.A., C.C. Chou, R. Barthel & S.S. Que Hee. 1992. Characterization of the *O*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride (PFBOA) derivatives of some aliphatic mono- and dialdehydes and quantitative water analysis of these aldehydes. *J. Assoc. Offic. Anal. Chem. Int.* 75:842.

6410 EXTRACTABLE BASE/NEUTRALS AND ACIDS*

6410 A. Introduction

1. Sources and Significance

The semivolatile compounds covered by this section include many classes of compounds, each characterized by different sources. The compounds include polynuclear aromatic hydrocarbons, often as byproducts of petroleum processing or combustion; phthalates, used as plasticizers; phenolics, found most often in wood preservatives; organochlorine pesticides, found most often in agricultural runoff or in wastewaters draining such areas; and PCBs (also see Section 6431A). Many of the listed compounds are toxic or carcinogenic. However, they generally are relatively insoluble in water so they do not occur frequently in potable waters or most wastewaters.

* Approved by Standard Methods Committee, 2000.

2. Selection of Method

Method 6410B is a broad-spectrum gas chromatographic/mass spectrometric (GC/MS) packed- or capillary-column method for detection of these compounds following liquid–liquid extraction. Although this method can be used to determine all the listed compounds, it is not the most sensitive method for individual classes of compounds, which are detected at lower concentrations by GC methods, such as those listed in Section 6420C (phenols), Sections 6440B and C (polynuclear aromatic hydrocarbons), and Sections 6630C and D (organochlorine pesticides and PCBs). In some cases, notably the pesticides, the GC method is substantially more sensitive than the GC/MS method. In other cases, such as the phenols, there is less difference between the methods.

6410 B. Liquid–Liquid Extraction Gas Chromatographic/Mass Spectrometric Method

This method[1] is applicable to the determination of organic compounds that are partitioned into an organic solvent and are amenable to gas chromatography,* in municipal and industrial discharges.

1. General Discussion

a. Principle: A measured volume of sample is extracted serially with methylene chloride at a pH >11 and again at a pH <2. The extract is dried, concentrated, and analyzed by GC/MS.[2,3] Qualitative compound identification is based on retention time and relative abundance of three characteristic masses (*m/z*). Quantitative analysis uses internal-standard techniques with a single characteristic *m/z*.

b. Interferences:

1) General precautions—See Section 6010C. Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample-processing hardware that lead to discrete artifacts and/or elevated base lines in detector output. Routinely demonstrate that all materials are free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 6200A.5*c*3).

Clean all glassware thoroughly[4] as soon as possible after use by rinsing with the last solvent used in it, followed by detergent washing with hot water and rinsing with tap water and distilled water. Drain glassware dry and heat in a muffle furnace at 400°C for 15 to 30 min. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide-quality hexane may be substituted for the baking. Thorough rinsing with such solvents usually eliminates PCB interference. Do not heat volumetric ware in a muffle furnace. After drying and cooling, seal and store glassware in a clean environment to prevent accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

Use high-purity reagents and solvents to minimize interference. Purification of solvents by distillation in all-glass systems may be required.

Matrix interferences may be caused by coextracted contaminants. The extent of matrix interferences will vary considerably depending on the sample.

2) Special precautions—Benzidine can be lost by oxidation during solvent concentration. Under the alkaline conditions of the extraction step, α-BHC, γ-BHC, endosulfan I and II, and

* *Base/neutral extractables*: acenaphthene, acenaphthylene, anthracene, aldrin, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, benzyl butyl phthalate, β-BHC, δ-BHC, bis(2-chloroethyl) ether, bis(2-chloroethoxy) methane, bis(2-ethylhexyl) phthalate, bis(2-chloroisopropyl) ether more correctly known as 2,2-oxybis (1-chloropropane), 4-bromophenyl phenyl ether, chlordane, 2-chloronaphthalene, 4-chlorophenyl phenyl ether, chrysene, 4,4′-DDD, 4,4′-DDE, 4,4′-DDT, dibenzo(a,h)anthracene, di-*n*-butylphthalate, 1,3-dichlorobenzene, 1,2-dichlorobenzene, 1,4-dichlorobenzene, 3,3′-dichlorobenzidine, dieldrin, diethyl phthalate, dimethyl phthalate, 2,4-dinitrotoluene, 2,6-dinitrotoluene, di-*n*-octylphthalate, endosulfan sulfate, endrin aldehyde, fluoranthene, fluorene, heptachlor, heptachlor epoxide, hexachlorobenzene, hexachlorobutadiene, hexachloroethane, indeno(1,2,3-cd)pyrene, isophorone, naphthalene, nitrobenzene, *N*-nitrosodi-*n*-propylamine, PCB-1016, PCB-1221, PCB-1232, PCB-1242, PCB-1248, PCB-1254, PCB-1260, phenanthrene, pyrene, toxaphene, 1,2,4-trichlorobenzene.

Acid extractables: 4-chloro-3-methylphenol, 2-chlorophenol, 2,4-dichlorophenol, 2,4-dimethylphenol, 2,4-dinitrophenol, 2-methyl-4,6-dinitrophenol, 2-nitrophenol, 4-nitrophenol, pentachlorophenol, phenol, 2,4,6-trichlorophenol.

The method may be extended to include the following compounds: benzidine, α-BHC, γ-BHC, endosulfan I, endosulfan II, endrin, hexachlorocyclopentadiene, *N*-nitrosodimethylamine, *N*-nitrosodiphenylamine.

https://doi.org/10.2105/SMWW.2882.124

endrin are subject to decomposition. Hexachlorocyclopentadiene is subject to thermal decomposition in the inlet of the gas chromatograph, chemical reaction in acetone solution, and photochemical decomposition. *N*-nitrosodimethylamine is difficult to separate from the solvent under the chromatographic conditions described. *N*-nitrosodiphenylamine decomposes in the gas chromatographic inlet and cannot be separated from diphenylamine. Other methods may be preferred for these compounds.[1]

The base-neutral extraction may cause significantly reduced recovery of phenol, 2-methylphenol, and 2,4-dimethylphenol. Results obtained under these conditions are minimum concentrations.

The packed gas chromatographic columns recommended for the basic fraction may not be able to resolve certain isomeric pairs [e.g., anthracene and phenanthrene; chrysene and benzo(a)anthracene; and benzo(b)fluoranthene and benzo(k)fluoranthene] because retention time and mass spectra for these pairs are not sufficiently different to make unambiguous identification possible. Use alternative techniques, such as the method for polynuclear aromatic hydrocarbons (Section 6440B), to identify and quantify these compounds.

In samples containing many interferences, use chemical ionization (CI) mass spectrometry to make identification easier. Tables 6410:I and II give characteristic CI ions for most compounds covered by this method. Use of CI mass spectrometry to support electron ionization (EI) mass spectrometry is encouraged but not required.

c. Detection levels: The method detection level (MDL) is the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero.[5] The MDL concentrations listed in Tables 6410:I and II were obtained with reagent water.[6] The MDL actually obtained in a given analysis will vary, depending on instrument sensitivity and matrix effects.

d. Safety: The toxicity or carcinogenicity of each reagent has not been defined precisely. Benzo(a)anthracene, benzidine, 3,3′-dichlorobenzidine, benzo(a)pyrene, α-BHC, β-BHC, δ-BHC, γ-BHC, dibenzo(a,h)anthracene, *n*-nitrosodimethylamine, 4,4′-DDT, and polychlorinated biphenyls (PCBs) have been tentatively classified as known or suspected, human or mammalian carcinogens. Prepare primary standards of these compounds in a hood and wear a NIOSH/MESA-approved toxic gas respirator when handling high concentrations.

2. Sampling and Storage

Collect grab samples in 1-L amber glass bottles fitted with a screw cap lined with TFE. Foil may be substituted for TFE if the sample is not corrosive. If amber bottles are not available, protect samples from light. Wash and rinse bottle and cap liner with acetone or methylene chloride, and dry before use. Follow conventional sampling practices[7] but do not rinse bottle with sample. Collect composite samples in refrigerated glass containers. Optionally, use automatic sampling equipment as free as possible of plastic tubing and other potential sources of contamination; incorporate glass sample containers for collecting a minimum of 250 mL. Refrigerate sample containers at 4°C and protect from light during compositing. If the sampler includes a peristaltic pump, use a minimum length of compressible silicone rubber tubing, but before use, thoroughly rinse it with methanol and rinse repeatedly with distilled water to minimize contamination. Use an integrating flow meter to collect flow-proportional composites.

Fill sample bottles and, if residual chlorine is present, add 80 mg sodium thiosulfate per liter of sample and mix well. Ice all samples or refrigerate at 4°C from time of collection until extraction.

Extract samples within 7 d of collection and analyze completely within 40 d of extraction.

3. Apparatus

a. Separatory funnel, 2-L, with TFE stopcock.

b. Drying column, chromatographic, 400 mm long × 19 mm ID, with coarse frit filter disk.

c. Concentrator tube, Kuderna-Danish, 10-mL, graduated.† Check calibration at volumes used. Use ground-glass stopper to prevent evaporation.

d. Evaporative flask, Kuderna-Danish, 500-mL.‡ Attach to concentrator tube with springs.

e. Snyder column, Kuderna-Danish, three-ball macro.§

f. Snyder column, Kuderna-Danish, two-ball micro.‖

g. Vials, 10- to 15-mL, amber glass, with TFE-lined screw cap.

h. Continuous liquid-liquid extractor, equipped with TFE or glass connecting joints and stopcocks requiring no lubrication.#

i. Boiling chips, approximately 10/40 mesh. Heat to 400°C for 30 min or extract in a Soxhlet extractor with methylene chloride.

j. Water bath, heated, with concentric ring cover and temperature control to ±2°C. Use bath in a hood.

k. Balance, analytical, capable of accurately weighing 0.0001 g.

*l. Gas chromatograph:*** An analytical system complete with a temperature-programmable gas chromatograph and all required accessories, including syringes, analytical columns, and gases. Use chromatograph with the injection port designed for on-column injection when packed columns are used and for splitless injection when capillary columns are used.

1) *Column for base/neutrals*—1.8 m long × 2-mm ID glass, packed with 3% SP-2250 on Supelcoport (100/200 mesh) or equivalent. This column was used to develop the detection level and precision and bias data presented herein. Guidelines for the use of alternate columns (e.g., DB-5 fused silica capillary) are provided in 6410B.5*b*.

2) *Column for acids*—1.8 m long × 2-mm ID glass, packed with 1% SP-1240DA on Supelcoport (100/120 mesh) or equivalent. The detection level and precision and bias data presented herein were developed with this column. For guidelines for the use of alternate columns (e.g., DB-5 fused silica capillary) see 6410B.5*b*.

m. Mass spectrometer, capable of scanning from 35 to 450 amu every 7 s or less, using 70-V (nominal) electron energy in the electron impact ionization mode, and producing a mass spectrum that meets all the criteria in Table 6410:III when 50 ng of decafluorotriphenyl phosphine [DFTPP; bis(perfluorophenyl) phenyl phosphine] is injected through the GC inlet.

† Kontes K-570050-1025, or equivalent.
‡ Kontes K-570001-0500, or equivalent.
§ Kontes K-503000-0121, or equivalent.
‖ Kontes K-569001-0219, or equivalent.
Hershberg-Wolf Extractor, Ace Glass Co., Vineland, NJ, P/N 6841-10, or equivalent.
** Gas chromatographic methods are extremely sensitive to the materials used. Mention of trade names by *Standard Methods* does not preclude the use of other existing or as-yet-undeveloped products that give *demonstrably* equivalent results.

TABLE 6410:I. CHROMATOGRAPHIC CONDITIONS, METHOD DETECTION LEVELS, AND CHARACTERISTIC MASSES FOR BASE/NEUTRAL EXTRACTABLES

Compound	Retention Time *min*	Method Detection Level *μg/L*	Characteristic Masses					
			Electron Impact			Chemical Ionization		
			Primary	Secondary	Secondary	Methane	Methane	Methane
1,3-Dichlorobenzene	7.4	1.9	146	148	113	146	148	150
1,4-Dichlorobenzene	7.8	4.4	146	148	113	146	148	150
Hexachloroethane	8.4	1.6	117	201	199	199	201	203
bis(2-Chloroethyl) ether	8.4	5.7	93	63	95	63	107	109
1,2-Dichlorobenzene	8.4	1.9	146	148	113	146	148	150
bis(2-Chloroisopropyl) ether*	9.3	5.7	45	77	79	77	135	137
N-Nitrosodi-*n*-propylamine			130	42	101			
Nitrobenzene	11.1	1.9	77	123	65	124	152	164
Hexachlorobutadiene	11.4	0.9	225	223	227	223	225	227
1,2,4-Trichlorobenzene	11.6	1.9	180	182	145	181	183	209
Isophorone	11.9	2.2	82	95	138	139	167	178
Naphthalene	12.1	1.6	128	129	127	129	157	169
bis(2-Chloroethoxy) methane	12.2	5.3	93	95	123	65	107	137
Hexachlorocyclopentadiene†	13.9		237	235	272	235	237	239
2-Chloronaphthalene	15.9	1.9	162	164	127	163	191	203
Acenaphthylene	17.4	3.5	152	151	153	152	153	181
Acenaphthene	17.8	1.9	154	153	152	154	155	183
Dimethyl phthalate	18.3	1.6	163	194	164	151	163	164
2,6-Dinitrotoluene	18.7	1.9	165	89	121	183	211	223
Fluorene	19.5	1.9	166	165	167	166	167	195
4-Chlorophenyl phenyl ether	19.5	4.2	204	206	141			
2,4-Dinitrotoluene	19.8	5.7	165	63	182	183	211	223
Diethyl phthalate	20.1	1.9	149	177	150	177	223	251
N-Nitrosodiphenylamine†	20.5	1.9	169	168	167	169	170	198
Hexachlorobenzene	21.0	1.9	284	142	249	284	286	288
α-BHC†	21.1		183	181	109			
4-Bromophenyl phenyl ether	21.2	1.9	248	250	141	249	251	277
γ-BHC†	22.4		183	181	109			
Phenanthrene	22.8	5.4	178	179	176	178	179	207
Anthracene	22.8	1.9	178	179	176	178	179	207
β-BHC	23.4	4.2	181	183	109			
Heptachlor	23.4	1.9	100	272	274			
δ-BHC	23.7	3.1	183	109	181			
Aldrin	24.0	1.9	66	263	220			
Dibutyl phthalate	24.7	2.5	149	150	104	149	205	279
Heptachlor epoxide	25.6	2.2	353	355	351			
Endosulfan I†	26.4		237	338	341			
Fluoranthene	26.5	2.2	202	101	100	203	231	243
Dieldrin	27.2	2.5	79	263	279			
4,4′-DDE	27.2	5.6	246	248	176			
Pyrene	27.3	1.9	202	101	100	203	231	243
Endrin†	27.9		81	263	82			
Endosulfan II†	28.6		237	339	341			
4,4′-DDD	28.6	2.8	235	237	165			
Benzidine†	28.8	44	184	92	185	185	213	225
4,4′-DDT	29.3	4.7	235	237	165			
Endosulfan sulfate	29.8	5.6	272	387	422			
Endrin aldehyde			67	345	250			
Butyl benzyl phthalate	29.9	2.5	149	91	206	149	299	327
bis(2-Ethylhexyl) phthalate	30.6	2.5	149	167	279	149		
Chrysene	31.5	2.5	228	226	229	228	229	257
Benzo(a)anthracene	31.5	7.8	228	229	226	228	229	257
3,3′-Dichlorobenzidine	32.2	16.5	252	254	126			
Di-*n*-octyl phthalate	32.5	2.5	149					
Benzo(b)fluoranthene	34.9	4.8	252	253	125	252	253	281
Benzo(k)fluoranthene	34.9	2.5	252	253	125	252	253	281
Benzo(a)pyrene	36.4	2.5	252	253	125	252	253	281
Indeno(1,2,3-cd)pyrene	42.7	3.7	276	138	277	276	277	305
Dibenzo(a,h)anthracene	43.2	2.5	278	139	279	278	279	307

TABLE 6410:I. CONT.

Compound	Retention Time *min*	Method Detection Level *μg/L*	Characteristic Masses					
			Electron Impact			Chemical Ionization		
			Primary	Secondary	Secondary	Methane	Methane	Methane
Benzo(ghi)perylene	45.1	4.1	276	138	277	276	277	305
N-Nitrosodimethylamine†			42	74	44			
Chlordane‡	19–30		373	375	377			
Toxaphene‡	25–34		159	231	233			
PCB 1016‡	18–30		224	260	294			
PCB 1221‡	15–30	30	190	224	260			
PCB 1232‡	15–32		190	224	260			
PCB 1242‡	15–32		224	260	294			
PCB 1248‡	12–34		294	330	262			
PCB 1254‡	22–34	36	294	330	362			
PCB 1260‡	23–32		330	362	394			

* The proper chemical name is 2,2′-oxybis(1-chloropropane).
† See introductory section of text.
‡ These compounds are mixtures of various isomers. (See Figures 6410:2–12.)
Column conditions: Supelcoport (100/120 mesh) coated with 3% SP-2250 packed in a 1.8-m-long × 2-mm-ID glass column with helium carrier gas at 30 mL/min flow rate. Column temperature held isothermal at 50°C for 4 min, then programmed at 8°C/min to 270°C and held for 30 min.

n. GC/MS interface: Any GC to MS interface that gives acceptable calibration points at 50 ng or less per injection for each compound of interest and achieves all acceptable performance criteria may be used. GC to MS interfaces constructed of all glass or glass-lined materials are recommended. Glass can be deactivated by silanizing with dichlorodimethylsilane.

o. Data system: See Section 6200B.2*f.*

4. Reagents

a. Reagent water: See Section 6200B.3*a*.

b. Sodium hydroxide solution (NaOH), 10*N:* Dissolve 40 g NaOH in reagent water and dilute to 100 mL.

c. Sodium sulfate (Na_2SO_4), granular, anhydrous. Purify by heating at 400°C for 4 h in a shallow tray.

d. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$), granular.

e. Sulfuric acid (H_2SO_4), 1 + 1: Slowly add 50 mL conc H_2SO_4 to 50 mL reagent water.

f. Acetone, methanol, methylene chloride, pesticide quality or equivalent.

g. Stock standard solutions: Prepare from pure standard materials or purchase as certified solutions. Prepare by accurately weighing about 0.0100 g of pure material, dissolve in pesticide-quality acetone or other suitable solvent, and dilute to volume in a 10-mL volumetric flask; 1 μL = 1.00 μg compound. When compound purity is assayed to be 96% or greater, use the weight without correction to calculate concentration of the stock standard. Use commercially prepared stock standards at any concentration if certified by the manufacturer or by an independent source.

Transfer stock standard solutions into TFE-sealed screw-cap bottles. Store at 4°C and protect from light. Check stock standard

TABLE 6410:II. CHROMATOGRAPHIC CONDITIONS, METHOD DETECTION LEVELS, AND CHARACTERISTIC MASSES FOR ACID EXTRACTABLES

Compound	Retention Time *min*	Method Detection Level *μg/L*	Characteristic Masses					
			Electron Impact			Chemical Ionization		
			Primary	Secondary	Secondary	Methane	Methane	Methane
2-Chlorophenol	5.9	3.3	128	64	130	129	131	157
2-Nitrophenol	6.5	3.6	139	65	109	140	168	122
Phenol	8.0	1.5	94	65	66	95	123	135
2,4-Dimethylphenol	9.4	2.7	122	107	121	123	151	163
2,4-Dichlorophenol	9.8	2.7	162	164	98	163	165	167
2,4,6-Trichlorophenol	11.8	2.7	196	198	200	197	199	201
4-Chloro-3-methylphenol	13.2	3.0	142	107	144	143	171	183
2,4-Dinitrophenol	15.9	42	184	63	154	185	213	225
2-Methyl-4,6-dinitrophenol	16.2	24	198	182	77	199	227	239
Pentachlorophenol	17.5	3.6	266	264	268	267	265	269
4-Nitrophenol	20.3	2.4	65	139	109	140	168	122

Column conditions: Supelcoport (100/120 mesh) coated with 1% SP-1240DA packed in a 1.8-m-long × 2-mm-ID glass column with helium carrier gas at 30 mL/min flow rate. Column temperature held isothermal at 70°C for 2 min then programmed at 8°C/min to 200°C.

Table 6410:III. DFTPP Key Masses and Abundance Criteria

Mass	*m/z* Abundance Criteria
51	30–60% of mass 198
68	<2% of mass 69
70	<2% of mass 69
127	40–60% of mass 198
197	<1% of mass 198
198	Base peak, 100% relative abundance
199	5–9% of mass 198
275	10–30% of mass 198
365	>1% of mass 198
441	Present but less than mass 443
442	>40% of mass 198
443	17–23% of mass 442

Table 6410:IV. Suggested Internal and Surrogate Standards

Base/Neutral Fraction	Acid Fraction
Aniline-d_5	2-Fluorophenol
Anthracene-d_{10}	Pentafluorophenol
Benzo(a)anthracene-d_{12}	Phenol-d_5
4,4′-Dibromobiphenyl	2-Perfluoromethyl phenol
4,4′-Dibromooctafluorobiphenyl	
Decafluorobiphenyl	
2,2′-Difluorobiphenyl	
4-Fluoroaniline	
1-Fluoronaphthylene	
2-Fluoronaphthylene	
Naphthalene-d_8	
Nitrobenzene-d_5	
2,3,4,5,6-Pentafluorobiphenyl	
Phenanthrene-d_{10}	
Pyridine-d_5	

solutions frequently for signs of degradation or evaporation, especially just before preparing calibration standards. Replace stock standard solutions after 6 months, or sooner if comparison with check standards indicates a problem.

h. Surrogate standard known-addition solution: Select a minimum of three surrogate compounds from Table 6410:IV. Prepare a surrogate standard solution containing each selected surrogate compound at a concentration of 100 μg/mL in acetone. Adding 1.00 mL to 1000 mL sample is equivalent to a concentration of 100 μg/L of each surrogate standard. Store at 4°C in TFE-sealed glass container. Check solution frequently for stability. Replace solution after 6 months, or sooner if comparison with quality-control check standards indicates a problem.

i. DFTPP standard: Prepare a 25-μg/mL solution of DFTPP in acetone.

j. Calibration standards: Prepare calibration standards at a minimum of three concentration levels for each compound by adding appropriate volumes of one or more stock standards to a volumetric flask. To each calibration standard or standard mixture, add a known constant amount of one or more internal standards (such as those listed in Table 6410:IV) and dilute to volume with acetone. Prepare one calibration standard at a concentration near, but above, the MDL and others corresponding to the expected range of sample concentrations or defining the working range of the GC/MS system.

k. Quality control (QC) check sample concentrate: Obtain a check sample concentrate containing each compound at a concentration of 100 μg/mL in acetone. Multiple solutions may be required. PCBs and multicomponent pesticides may be omitted. If such a sample is not available from an external source, prepare using stock standards prepared independently from those used for calibration.

5. Procedure

a. Extraction: Extraction by means of a separatory funnel, ¶ *a*1) below, is most common, but if emulsions will prevent acceptable solvent recovery, use continuous extraction, ¶ *a*2) below.

1) Separatory funnel extraction—Normally use a sample volume of 1 L. For sample volumes of 2 L, use 250-, 100-, and 100-mL volumes of methylene chloride for the serial extraction of the base/neutrals and 200-, 100-, and 100-mL volumes of methylene chloride for the acids.

Mark water meniscus on side of sample bottle for later determination of sample volume. Pour entire sample into a 2-L separatory funnel. Pipet 1.00 mL surrogate standard solution into separatory funnel and mix well. Check pH with wide-range pH paper and adjust to pH >11 with NaOH solution.

Add 60 mL methylene chloride to sample bottle, seal, and shake for 30 s to rinse inner surface. Transfer solvent to separatory funnel and extract sample by shaking for 2 min with periodic venting to release excess pressure. Let organic layer separate from water phase for a minimum of 10 min. If emulsion interface between layers is more than one-third the volume of the solvent layer, use mechanical techniques to complete phase separation. The optimum technique depends on the sample, but may include stirring, filtering emulsion through glass wool, centrifuging, or other physical methods. Collect methylene chloride extract in a 250-mL Erlenmeyer flask.

If the emulsion cannot be broken (recovery of <80% of the methylene chloride, corrected for water solubility of methylene chloride) in the first extraction, transfer sample, solvent, and emulsion into extraction chamber of a continuous extractor and proceed as described in ¶ *a*2) below.

Add a second 60-mL volume of methylene chloride to sample bottle and repeat extraction procedure, combining extracts in the Erlenmeyer flask. Perform a third extraction in the same manner.

After the third extraction, adjust pH of aqueous phase to <2 using H_2SO_4. Serially extract acidified aqueous phase three times with 60-mL portions of methylene chloride. Collect and combine extracts in a 250-mL Erlenmeyer flask and label combined extracts as the acid fraction.

For each fraction, assemble a Kuderna–Danish (K–D) concentrator by attaching a 10-mL concentrator tube to a 500-mL evaporative flask. Other concentration devices or techniques may be used if the requirements of 6410B.7 are met.

Pour combined extract through a solvent-rinsed drying column containing at least 10 cm anhydrous Na_2SO_4 or more and collect extract in concentrator. Rinse Erlenmeyer flask and column with 20 to 30 mL methylene chloride to complete transfer.

Add one or two clean boiling chips to the evaporative flask and attach a three-ball Snyder column. Prewet Snyder column by adding about 1 mL methylene chloride to the top. Place K–D apparatus on a hot water bath (60 to 65°C) in a hood so concentrator tube is partially immersed in the hot water and entire lower rounded surface of flask is bathed with hot vapor. Adjust vertical position of apparatus and water temperature as required to complete concentration in 15 to 20 min. At proper rate of distillation, the column balls actively chatter but the chambers are not flooded with condensed solvent. When the apparent volume of liquid reaches 1 mL, remove K–D apparatus and let drain and cool for at least 10 min.

Remove Snyder column and rinse flask and its lower joint into the concentrator tube with 1 to 2 mL methylene chloride, preferably using a 5-mL syringe.

Add another one or two clean boiling chips to concentrator tube for each fraction and attach a two-ball micro-Snyder column. Prewet Snyder column by adding about 0.5 mL of methylene chloride to the top. Place K–D apparatus on a hot-water bath (60 to 65°C) so concentrator tube is partially immersed in hot water and continue concentrating as directed above without further solvent addition until apparent volume of liquid reaches about 0.5 mL. After cooling, remove Snyder column and rinse flask and its lower joint into the concentrator tube with approximately 0.2 mL acetone or methylene chloride. Adjust final volume to 1.0 mL with solvent. Stopper concentrator tube and store refrigerated if further processing will not be done immediately. If extract is to be stored >2 d, transfer to a TFE-sealed screw-cap vial and label base/neutral or acid fraction as appropriate.

Determine original sample volume by refilling sample bottle to mark and transferring liquid to a 1000-mL graduated cylinder. Record sample volume to nearest 5 mL.

2) Continuous extraction—Mark water meniscus on side of sample bottle, and determine sample volume later as described in ¶ *a*1) above. Check pH with wide-range pH paper and adjust to pH >11 with NaOH solution. Transfer sample to continuous extractor and, using a pipet, add 1.00 mL surrogate standard solution and mix well. Add 60 mL methylene chloride to sample bottle, seal, and shake for 30 s to rinse inner surface. Transfer solvent to extractor. Repeat rinse with an additional 50- to 100-mL portion methylene chloride and add rinse to extractor.

Add 200 to 500 mL methylene chloride to distilling flask, add sufficient reagent water to ensure proper operation, and extract for 24 h. Let cool and detach distilling flask. Dry, concentrate, and seal extract as in ¶ *a*1) above.

Charge a clean distilling flask with 500 mL methylene chloride and attach it to continuous extractor. Carefully, while stirring, adjust pH of aqueous phase to <2 with H_2SO_4. Extract for 24 h. Dry, concentrate, and seal extract as in ¶ *a*1) above.

b. GC/MS operating conditions: Table 6410:I summarizes the recommended gas chromatographic operating conditions for the base/neutral fraction and Table 6410:II for the acid fraction. Included in these tables are retention times and MDLs that can be achieved under these conditions. Examples of the separations obtained with these columns are shown in Figures 6410:1–12. Other packed or capillary (open-tubular) columns or chromatographic conditions may be used if the requirements of 6410B.7 are met.

c. GC/MS performance tests: At the beginning of each day on which analyses are to be performed, check GC/MS system to see if acceptable performance criteria are achieved for DFTPP.[8] Each day that benzidine is to be determined, the tailing factor criterion described in ¶ *c*2) below must be achieved. Each day that the acids are to be determined, the tailing factor criterion described in ¶ *c*3) below must be achieved.

These performance tests have the requirements given in Section 6200B.4*b*, but use following conditions:

- Electron energy: 70 V (nominal)
- Mass range: 35 to 450 amu
- Scan time: To give at least 5 scans per peak but not to exceed 7 s per scan.

1) DFTPP performance test—At beginning of each day, inject 2 μL (50 ng) DFTPP standard solution. Obtain a background-corrected mass spectrum of DFTPP and confirm that all the key *m*/*z* criteria in Table 6410:III are achieved. If not, retune mass spectrometer and repeat test until all criteria are achieved. Meet performance criteria before any samples, blanks, or standards are analyzed. The tailing factor tests in ¶s *c*2) and 3) below may be performed simultaneously with the DFTPP test.

2) Column performance test for base/neutrals—At beginning of each day that base/neutral fraction is to be analyzed for benzidine, calculate benzidine tailing factor. Inject 100 ng benzidine either separately or as a part of a standard mixture that may contain DFTPP, and calculate tailing factor, which must be <3.0. Calculation of the tailing factor is illustrated in Figure 6410:13.[9] Replace column packing if tailing factor criterion cannot be met.

3) Column performance test for acids—At beginning of each day that acids are to be determined, inject 50 ng pentachlorophenol either separately or as a part of a standard mix that may contain DFTPP. The tailing factor for pentachlorophenol must be <5. Calculation of the tailing factor is illustrated in Figure 6410:13.[9] Replace column packing if tailing factor criterion cannot be met.

d. Calibration of GC/MS system: Calibrate system daily after performance tests.

Select three or more internal standards similar in analytical behavior to the compounds of interest. Demonstrate that the measurement of the internal standards is not affected by method or matrix interferences. Some recommended internal standards are listed in Table 6410:IV. Use base peak *m*/*z* as the primary *m*/*z* for quantification. If interferences are noted, use one of the next two most intense *m*/*z* quantities for quantification. Using injections of 2 to 5 μL, analyze each calibration standard according to ¶ *e* below and tabulate area of primary characteristic *m*/*z* (Tables 6410:I and II) against concentration for each compound and internal standard. Calculate response factors (RF) for each compound by the equation given in Section 6200B.4*c*2). If the RF value over the working range is a constant (<35% RSD), it can be assumed to be invariant; use the average RF for calculations. Alternatively, use the results to plot a calibration curve of response ratios, A_s/A_{is} vs. *RF*.

Verify working calibration curve or RF on each working day by measuring one or more calibration standards. If the response for any compound varies from the predicted response by >20%, repeat test using a fresh calibration standard. Alternatively, prepare a new calibration curve for that compound.

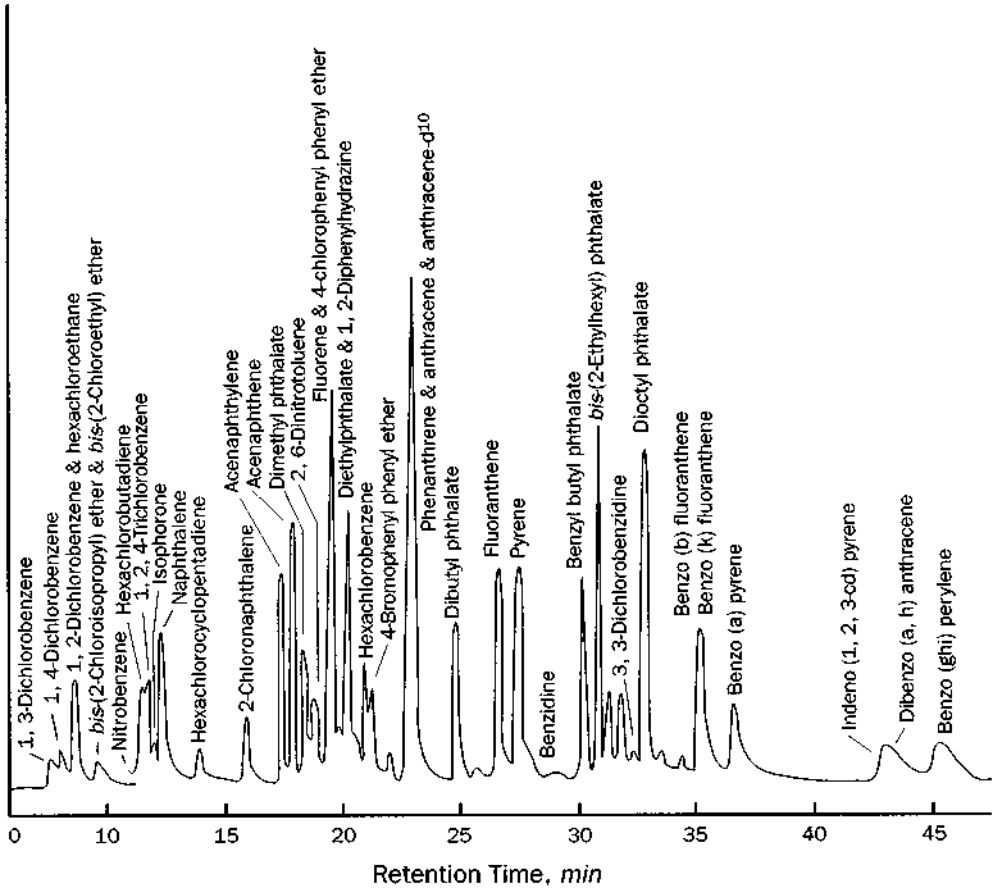


Figure 6410:1. Gas chromatogram of base/neutral fraction. Column: 3% SP-2250 on Supelcoport; program: 50°C for 4 min, 8°C/min to 270°C; detector: mass spectrometer.

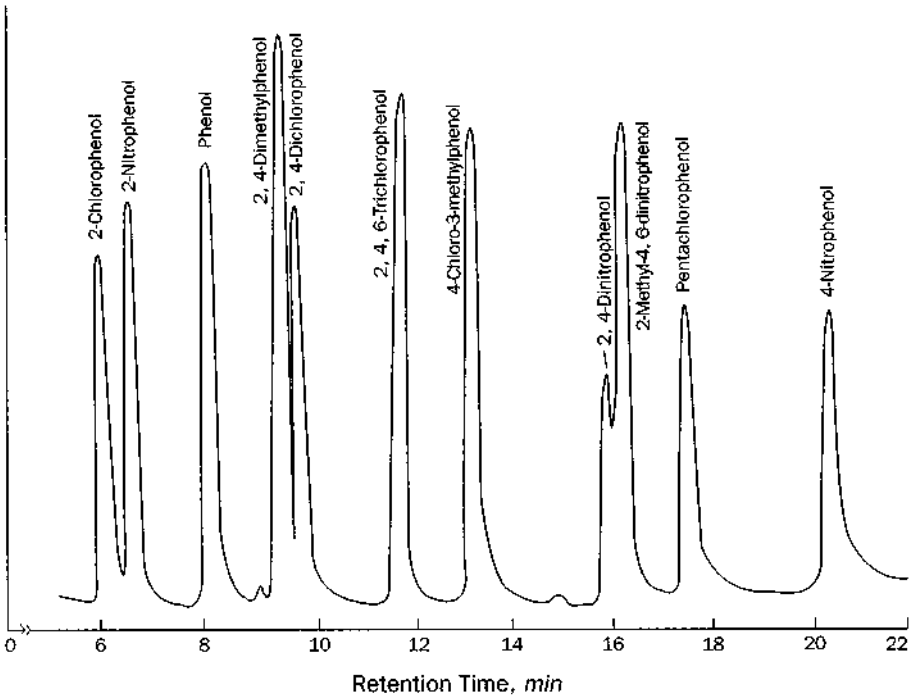


Figure 6410:2. Gas chromatogram of acid fraction. Column: 1% SP-1240DA on Supelcoport; program: 70°C for 2 min, 8°C/min to 200°C; detector: mass spectrometer.

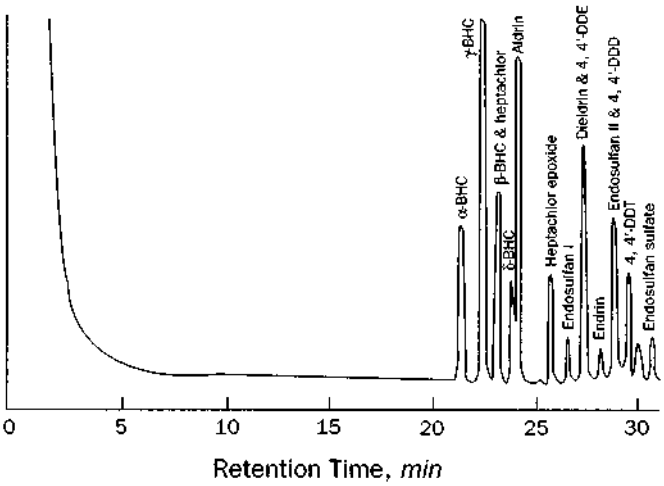


Figure 6410:3. Gas chromatogram of pesticide fraction. Column: 3% SP-2250 on Supelcoport; program: 50°C for 4 min, 8°C/min to 270°C; detector: mass spectrometer.

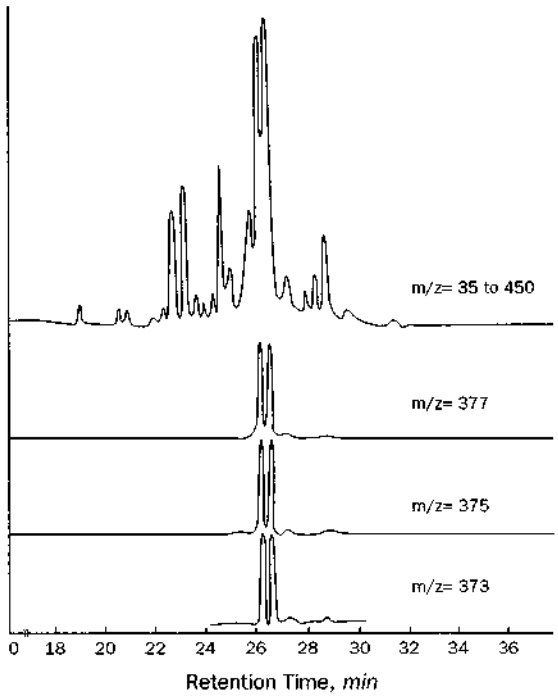


Figure 6410:4. Gas chromatogram of chlordane. Column: 3% SP-2250 on Supelcoport; program: 50°C for 4 min, 8°C/min to 270°C; detector: mass spectrometer.

e. Sample analysis: Add internal standard to sample extract, mix thoroughly, and immediately inject 2 to 5 μL of sample extract or standard into GC/MS system using solvent-flush technique[10] to minimize losses due to adsorption, chemical reaction, or evaporation. Smaller (1.0-μL) volumes may be injected if automatic devices are used. Record volume injected to nearest 0.05 μL. If response for any *m/z* exceeds the working range of the GC/MS system, dilute extract and re-analyze. Make all

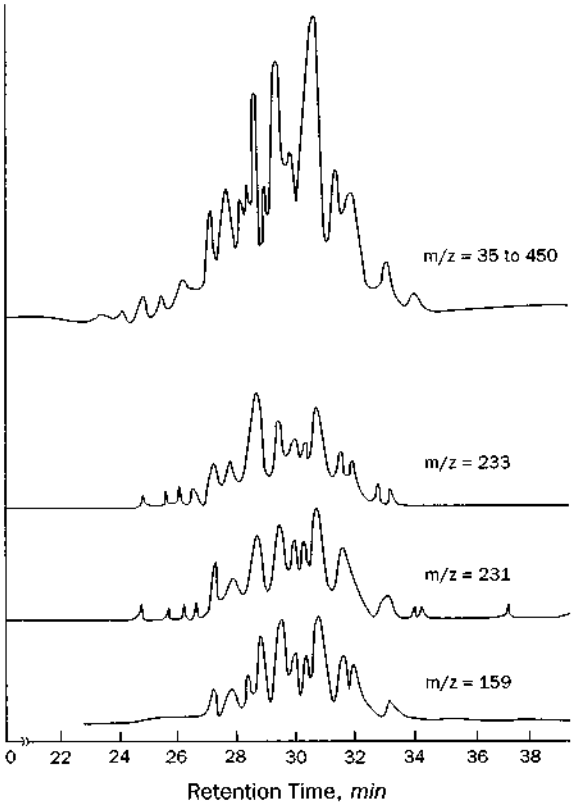


Figure 6410:5. Gas chromatogram of toxaphene. Column: 3% SP-2250 on Supelcoport; program: 50°C for 4 min, 8°C/min to 270°C; detector: mass spectrometer.

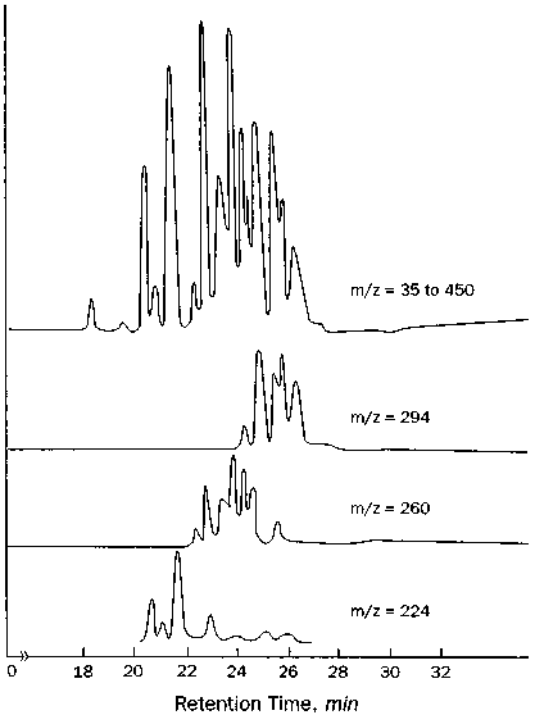


Figure 6410:6. Gas chromatogram of PCB-1016. Column: 3% SP-2250 on Supelcoport; program: 50°C for 4 min, 8°C/min to 270°C; detector: mass spectrometer.

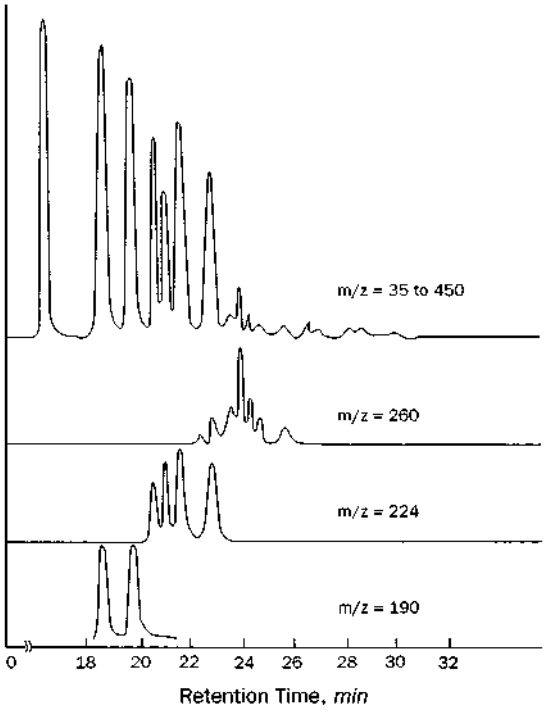


Figure 6410:7. Gas chromatogram of PCB-1221. Column: 3% SP-2250 on Supelcoport; program: 50°C for 4 min, 8°C/min to 270°C; detector: mass spectrometer.

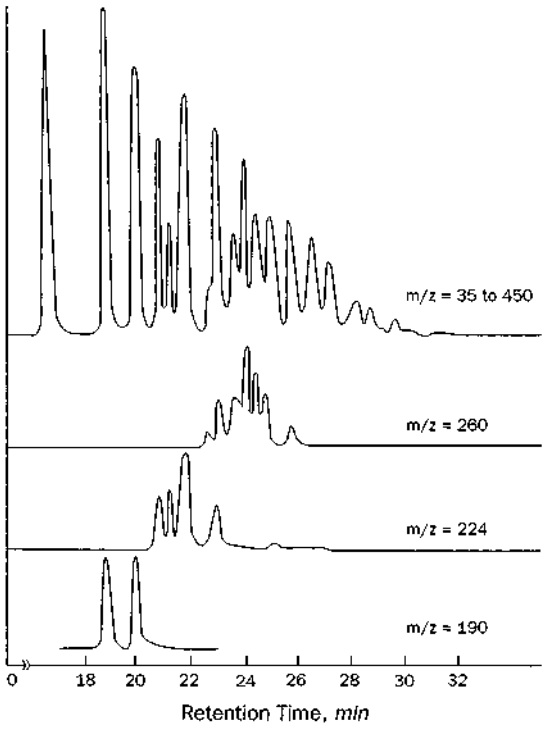


Figure 6410:8. Gas chromatogram of PCB-1232. Column: 3% SP-2250 on Supelcoport; program: 50°C for 4 min, 8°C/min to 270°C; detector: mass spectrometer.

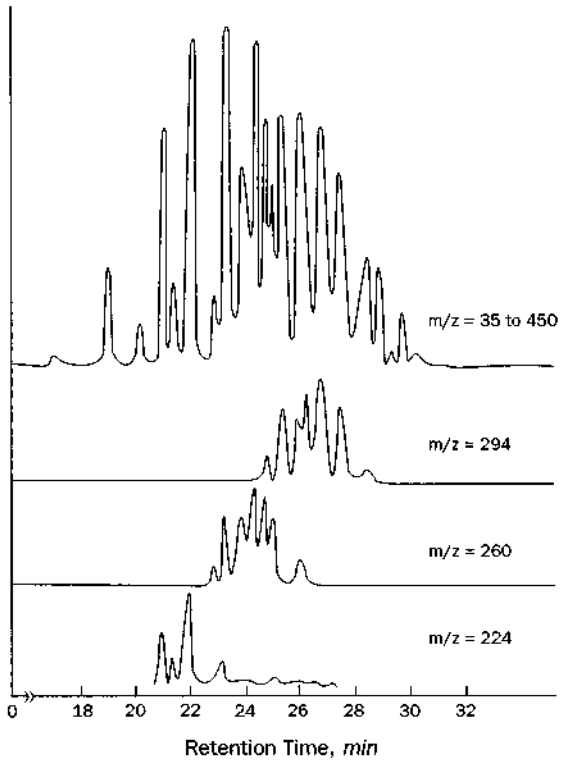


Figure 6410:9. Gas chromatogram of PCB-1242. Column: 3% SP-2250 on Supelcoport; program: 50°C for 4 min, 8°C/min to 270°C; detector: mass spectrometer.

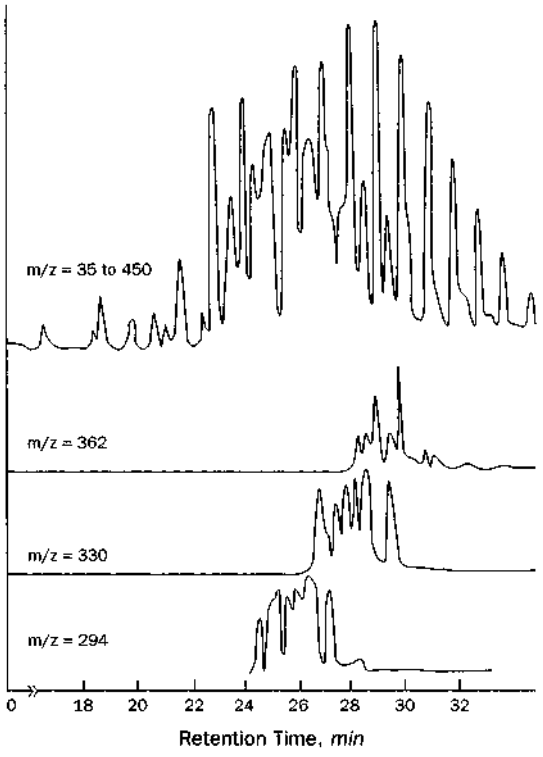


Figure 6410:10. Gas chromatogram of PCB-1248. Column: 3% SP-2250 on Supelcoport; program: 50°C for 4 min, 8°C/min to 270°C; detector: mass spectrometer.

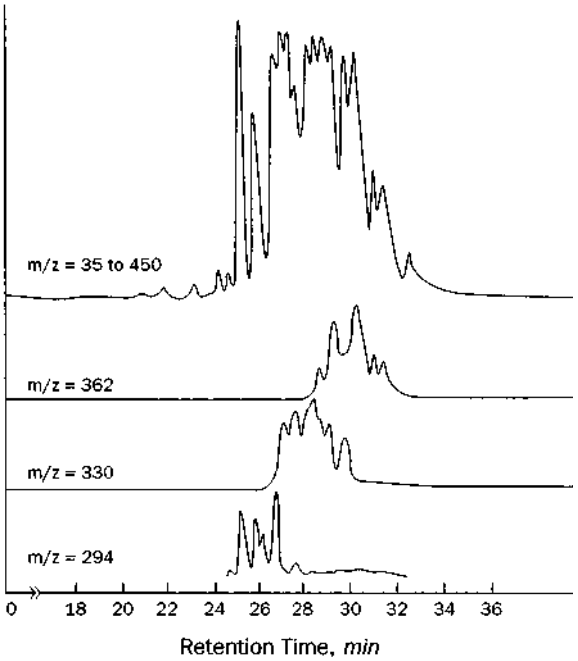


Figure 6410:11. Gas chromatogram of PCB-1254. Column: 3% SP-2250 on Supelcoport; program: 50°C for 4 min, 8°C/min to 270°C; detector: mass spectrometer.

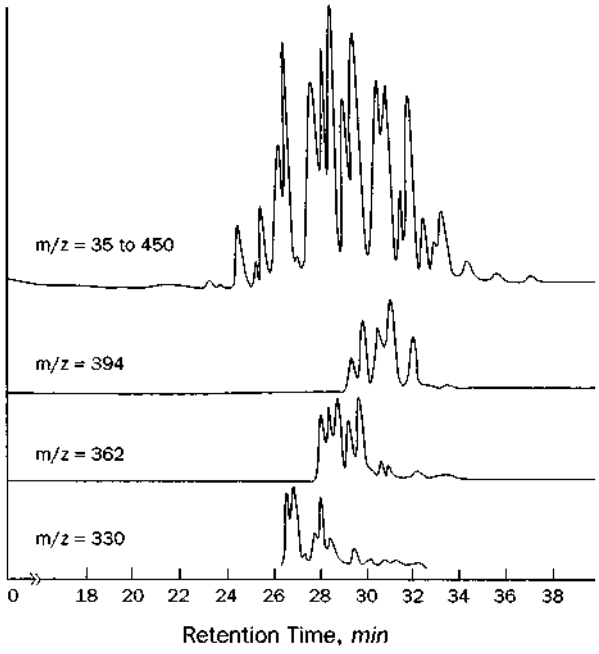


Figure 6410:12. Gas chromatogram of PCB-1260. Column: 3% SP-2250 on Supelcoport; program: 50°C for 4 min, 8°C/min to 270°C; detector: mass spectrometer.

qualitative and quantitative measurements as described below and in 6410B.6. When extract is not being used, store at 4°C, protected from light, in screw-cap vial equipped with unpierced TFE-lined septum.

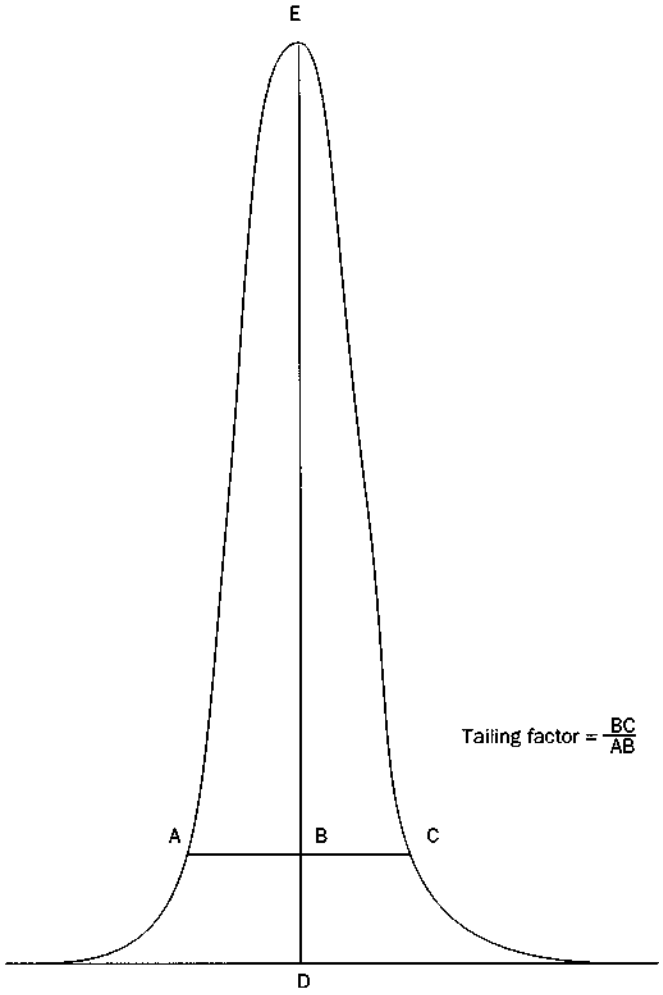


Figure 6410:13. Tailing factor calculation. Example calculation:
Peak height = DE = 100 mm 10% peak height = BD = 10 mm
Peak width at 10% peak height = AC = 23 mm
AB = 11 mm BC = 12 mm

$$\text{Tailing factor} = \frac{12}{11} = 1.1$$

Obtain EICPs for the primary *m/z* and the two other masses listed in Tables 6410:I and II. See ¶ *d* above for masses to be used with internal and surrogate standards. Use the following criteria to make a qualitative identification:

- The characteristic masses of each compound maximize in the same or within one scan of each other.
- The retention time falls within ±30 s of the retention time of the authentic compound.
- The relative peak heights of the three characteristic masses in the EICPs fall within ±20% of the relative intensities of these masses in a reference mass spectrum obtained from a standard analyzed in the GC/MS system or from a reference library.

Structural isomers that have very similar mass spectra and <30 s difference in retention time can be identified explicitly

TABLE 6410:V. QC ACCEPTANCE CRITERIA*

Compound	Test Concentration μg/L	Limits for s μg/L	Range for $\bar{X}$ μg/L	Range for P, P_s %
Acenaphthene	100	27.6	60.1–132.3	47–145
Acenaphthylene	100	40.2	53.5–126.0	33–145
Aldrin	100	39.0	7.2–152.2	D–166
Anthracene	100	32.0	43.4–118.0	27–133
Benzo(a)anthracene	100	27.6	41.8–133.0	33–143
Benzo(b)fluoranthene	100	38.8	42.0–140.4	24–159
Benzo(k)fluoranthene	100	32.3	25.2–145.7	11–162
Benzo(a)pyrene	100	39.0	31.7–148.0	17–163
Benzo(ghi)perylene	100	58.9	D–195.0	D–219
Benzyl butyl phthalate	100	23.4	D–139.9	D–152

only if the resolution between authentic isomers in a standard mix is acceptable. Acceptable resolution is achieved if the baseline to valley height between the isomers is <25% of the sum of the two peak heights. Otherwise, structural isomers are identified as isomeric pairs.

f. Screening procedure for 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD): **CAUTION: In screening a sample for 2,3,7,8-TCDD, do not handle reference material without taking extensive safety precautions.** It is sufficient to analyze the base/neutral extract by selected ion monitoring (SIM) GC/MS techniques, as follows:

Concentrate base/neutral extract to a final volume of 0.2 mL. Adjust temperature of base/neutral column to 220°C. Operate mass spectrometer to acquire data in the SIM mode using the ions at *m/z* 257, 320, and 322 and a dwell time no greater than 333 ms/mass. Inject 5 to 7 μL of base/neutral extract. Collect SIM data for a total of 10 min. The possible presence of 2,3,7,8-TCDD is indicated if all three masses exhibit simultaneous peaks at any point in the selected ion current profiles. For each occurrence where the possible presence of 2,3,7,8-TCDD is indicated, calculate and retain the relative abundances of each of the three masses. False positives may be caused by the presence of single or coeluting combinations of compounds whose mass spectra contain all of these masses. Conclusive results of the presence and concentration level of 2,3,7,8-TCDD can be obtained only from a properly equipped laboratory using a specialized test method.[11]

6. Calculation

When a compound has been identified, base quantitation on the integrated abundance from the EICP of the primary characteristic *m/z* given in Tables 6410:I and II. Use base peak *m/z* for internal and surrogate standards. If sample produces an interference for the primary *m/z*, use a secondary characteristic *m/z* to quantitate.

Calculate sample concentration using the response factor (RF) determined in 6410B.5*d* and the equation:

$$\text{Concentration, } \mu\text{g/L} = \frac{(A_s)\,(I_s)}{(A_{is})\,(RF)\,(V_e)}$$

where:

A_s = area of characteristic *m/z* for compound or surrogate standard to be measured,
I_s = amount of internal standard added to each extract, μg,
A_{is} = area of characteristic *m/z* for internal standard, and
V_e = volume of water extracted, L.

Report results in μg/L without correction for recovery. Report all QC data with sample results.

7. Quality Control

a. Quality control program: See Section 6200A.5.

b. Initial quality control: Proceed according to Sections 6200A.5*a*1) and 2). Use Table 6410:V for acceptance criteria.

c. Analyses of samples with known additions: Use quality acceptance criteria given in Table 6410:V.

d. Quality-control check standard analysis: Proceed as in Section 6200A.5*a*3); prepare QC check standard with 1.0 mL QC check standard concentrate and 1 L reagent water.

e. Bias assessment and records: Assess method bias and maintain records. For example, after the analysis of five wastewater samples, calculate the average percent recovery ($\bar{P}$) and the standard deviation of the percent recovery (s_p). Express bias assessment as a percent recovery interval from $\bar{P} - 2s_p$ to $\bar{P} + 2s_p$. If $\bar{P} = 90\%$ and $s_p = 10\%$, the recovery interval is expressed as 70–110%. Update bias assessment for each compound regularly (e.g., after each five to ten new accuracy measurements).

f. Use of surrogate compounds: As a quality control check, make known additions to all samples of surrogate standard solution as described in 6410B.5*a*1), and calculate percent recovery of each surrogate compound.

g. Additional quality-assurance practices: Other desirable practices depend on the needs of the laboratory and the nature of the samples. Analyze field duplicates to assess precision of environmental measurements. Whenever possible, analyze standard reference materials and participate in relevant performance evaluation studies. Certain compounds, such as phthalates, are common laboratory contaminants. When these are measured above the detection limits in sample blanks, locate their source and repeat the analysis after taking corrective action.

TABLE 6410:V. CONT.

Compound	Test Concentration µg/L	Limits for s µg/L	Range for $\bar{X}$ µg/L	Range for P, P_s %
δ-BHC	100	31.5	41.5–130.6	24–149
β-BHC	100	21.6	D–100.0	D–110
bis(2-Chloroethyl) ether	100	55.0	42.9–126.0	12–158
bis(2-Chloroethoxy) methane	100	34.5	49.2–164.7	33–184
bis(2-Chloroisopropyl) ether†	100	46.3	62.8–138.6	36–166
bis(2-Ethylhexyl) phthalate	100	41.1	28.9–136.8	8–158
4-Bromophenyl phenyl ether	100	23.0	64.9–114.4	53–127
2-Chloronaphthalene	100	13.0	64.5–113.5	60–118
4-Chlorophenyl phenyl ether	100	33.4	38.4–144.7	25–158
Chrysene	100	48.3	44.1–139.9	17–168
4,4′-DDD	100	31.0	D–134.5	D–145
4,4′-DDE	100	32.0	19.2–119.7	4–136
4,4′-DDT	100	61.6	D–170.6	D–203
Dibenzo(a,h)anthracene	100	70.0	D–199.7	D–227
Di-*n*-butyl phthalate	100	16.7	8.4–111.0	1–118
1,2-Dichlorobenzene	100	30.9	48.6–112.0	32–129
1,3-Dichlorobenzene	100	41.7	16.7–153.9	D–172
1,4-Dichlorobenzene	100	32.1	37.3–105.7	20–124
3,3′-Dichlorobenzidine	100	71.4	8.2–212.5	D–262
Dieldrin	100	30.7	44.3–119.3	29–136
Diethyl phthalate	100	26.5	D–100.0	D–114
Dimethyl phthalate	100	23.2	D–100.0	D–112
2,4-Dinitrotoluene	100	21.8	47.5–126.9	39–139
2,6-Dinitrotoluene	100	29.6	68.1–136.7	50–158
Di-*n*-octylphthalate	100	31.4	18.6–131.8	4–146
Endosulfan sulfate	100	16.7	D–103.5	D–107
Endrin aldehyde	100	32.5	D–188.8	D–209
Fluoranthene	100	32.8	42.9–121.3	26–137
Fluorene	100	20.7	71.6–108.4	59–121
Heptachlor	100	37.2	D–172.2	D–192
Heptachlor epoxide	100	54.7	70.9–109.4	26–155
Hexachlorobenzene	100	24.9	7.8–141.5	D–152
Hexachlorobutadiene	100	26.3	37.8–102.2	24–116
Hexachloroethane	100	24.5	55.2–100.0	40–113
Indeno(1,2,3-cd)pyrene	100	44.6	D–150.9	D–171
Isophorone	100	63.3	46.6–180.2	21–196
Naphthalene	100	30.1	35.6–119.6	21–133
Nitrobenzene	100	39.3	54.3–157.6	35–180
N-Nitrosodi-*n*-propylamine	100	55.4	13.6–197.9	D–230
PCB-1260	100	54.2	19.3–121.0	D–164
Phenanthrene	100	20.6	65.2–108.7	54–120
Pyrene	100	25.2	69.6–100.0	52–115
1,2,4-Trichlorobenzene	100	28.1	57.3–129.2	44–142
4-Chloro-3-methylphenol	100	37.2	40.8–127.9	22–147
2-Chlorophenol	100	28.7	36.2–120.4	23–134
2,4-Dichlorophenol	100	26.4	52.5–121.7	39–135
2,4-Dimethylphenol	100	26.1	41.8–109.0	32–119
2,4-Dinitrophenol	100	49.8	D–172.9	D–191
2-Methyl-4,6-dinitrophenol	100	93.2	53.0–100.0	D–181
2-Nitrophenol	100	35.2	45.0–166.7	29–182
4-Nitrophenol	100	47.2	13.0–106.5	D–132
Pentachlorophenol	100	48.9	38.1–151.8	14–176
Phenol	100	22.6	16.6–100.0	5–112
2,4,6-Trichlorophenol	100	31.7	52.4–129.2	37–144

* s = standard deviation for four recovery measurements,
$\bar{X}$ = average recovery for four recovery measurements,
P, P_s = percent recovery measured, and
D = detected; results must be >0.

† The proper chemical name is 2,2′-oxybis(1-chloropropane).

NOTE: These criteria are based directly on the method-performance data in Table 6410:VI. Where necessary, the limits for recovery were broadened to ensure the limits' applicability to concentrations below those used to develop Table 6410:VI.

TABLE 6410:VI. METHOD BIAS AND PRECISION AS FUNCTIONS OF CONCENTRATION*

Compound	Bias as Recovery, X' $\mu g/L$	Single-Analyst Precision, s_r' $\mu g/L$	Overall Precision, S' $\mu g/L$
Acenaphthene	$0.96C + 0.19$	$0.15\bar{X} - 0.12$	$0.21\bar{X} - 0.67$
Acenaphthylene	$0.89C + 0.74$	$0.24\bar{X} - 1.06$	$0.26\bar{X} - 0.54$
Aldrin	$0.78C + 1.66$	$0.27\bar{X} - 1.28$	$0.43\bar{X} + 1.13$
Anthracene	$0.80C + 0.68$	$0.21\bar{X} - 0.32$	$0.27\bar{X} - 0.64$
Benzo(a)anthracene	$0.88C - 0.60$	$0.15\bar{X} + 0.93$	$0.26\bar{X} - 0.28$
Benzo(b)fluoranthene	$0.93C - 1.80$	$0.22\bar{X} + 0.43$	$0.29\bar{X} + 0.96$
Benzo(k)fluoranthene	$0.87C - 1.56$	$0.19\bar{X} + 1.03$	$0.35\bar{X} + 0.40$
Benzo(a)pyrene	$0.90C - 0.13$	$0.22\bar{X} + 0.48$	$0.32\bar{X} + 1.35$
Benzo(ghi)perylene	$0.98C - 0.86$	$0.29\bar{X} + 2.40$	$0.51\bar{X} - 0.44$
Benzyl butyl phthalate	$0.66C - 1.68$	$0.18\bar{X} + 0.94$	$0.53\bar{X} + 0.92$
β-BHC	$0.87C - 0.94$	$0.20\bar{X} - 0.58$	$0.30\bar{X} - 1.94$
δ-BHC	$0.29C - 1.09$	$0.34\bar{X} + 0.86$	$0.93\bar{X} - 0.17$
bis(2-Chloroethyl) ether	$0.86C - 1.54$	$0.35\bar{X} - 0.99$	$0.35\bar{X} + 0.10$
bis(2-Chloroethoxy) methane	$1.12C - 5.04$	$0.16\bar{X} + 1.34$	$0.26\bar{X} + 2.01$
bis(2-Chloroisopropyl) ether†	$1.03C - 2.31$	$0.24\bar{X} + 0.28$	$0.25\bar{X} + 1.04$
bis(2-Ethylhexyl) phthalate	$0.84C - 1.18$	$0.26\bar{X} + 0.73$	$0.36\bar{X} + 0.67$
4-Bromophenyl phenyl ether	$0.91C - 1.34$	$0.13\bar{X} + 0.66$	$0.16\bar{X} + 0.66$
2-Chloronaphthalene	$0.89C + 0.01$	$0.07\bar{X} + 0.52$	$0.13\bar{X} + 0.34$
4-Chlorophenyl phenyl ether	$0.91C + 0.53$	$0.20\bar{X} - 0.94$	$0.30\bar{X} - 0.46$
Chrysene	$0.93C - 1.00$	$0.28\bar{X} + 0.13$	$0.33\bar{X} - 0.09$
4,4′-DDD	$0.56C - 0.40$	$0.29\bar{X} - 0.32$	$0.66\bar{X} - 0.96$
4,4′-DDE	$0.70C - 0.54$	$0.26\bar{X} - 1.17$	$0.39\bar{X} - 1.04$
4,4′-DDT	$0.79C - 3.28$	$0.42\bar{X} + 0.19$	$0.65\bar{X} - 0.58$
Dibenzo(a,h)anthracene	$0.88C + 4.72$	$0.30\bar{X} + 8.51$	$0.59\bar{X} + 0.25$
Di-*n*-butyl phthalate	$0.59C + 0.71$	$0.13\bar{X} + 1.16$	$0.39\bar{X} + 0.60$
1,2-Dichlorobenzene	$0.80C + 0.28$	$0.20\bar{X} + 0.47$	$0.24\bar{X} + 0.39$
1,3-Dichlorobenzene	$0.86C - 0.70$	$0.25\bar{X} + 0.68$	$0.41\bar{X} + 0.11$
1,4-Dichlorobenzene	$0.73C - 1.47$	$0.24\bar{X} + 0.23$	$0.29\bar{X} + 0.36$
3,3′-Dichlorobenzidine	$1.23C - 12.65$	$0.28\bar{X} + 7.33$	$0.47\bar{X} + 3.45$
Dieldrin	$0.82C - 0.16$	$0.20\bar{X} - 0.16$	$0.26\bar{X} - 0.07$
Diethyl phthalate	$0.43C + 1.00$	$0.28\bar{X} + 1.44$	$0.52\bar{X} + 0.22$
Dimethyl phthalate	$0.20C + 1.03$	$0.54\bar{X} + 0.19$	$1.05\bar{X} - 0.92$
2,4-Dinitrotoluene	$0.92C - 4.81$	$0.12\bar{X} + 1.06$	$0.21\bar{X} + 1.50$
2,6-Dinitrotoluene	$1.06C - 3.60$	$0.14\bar{X} + 1.26$	$0.19\bar{X} + 0.35$
Di-*n*-octylphthalate	$0.76C - 0.79$	$0.21\bar{X} + 1.19$	$0.37\bar{X} + 1.19$
Endosulfan sulfate	$0.39C + 0.41$	$0.12\bar{X} + 2.47$	$0.63\bar{X} - 1.03$
Endrin aldehyde	$0.76C - 3.86$	$0.18\bar{X} + 3.91$	$0.73\bar{X} - 0.62$
Fluoranthene	$0.81C + 1.10$	$0.22\bar{X} - 0.73$	$0.28\bar{X} - 0.60$
Fluorene	$0.90C - 0.00$	$0.12\bar{X} + 0.26$	$0.13\bar{X} + 0.61$
Heptachlor	$0.87C - 2.97$	$0.24\bar{X} - 0.56$	$0.50\bar{X} - 0.23$
Heptachlor epoxide	$0.92C - 1.87$	$0.33\bar{X} - 0.46$	$0.28\bar{X} + 0.64$
Hexachlorobenzene	$0.74C + 0.66$	$0.18\bar{X} - 0.10$	$0.43\bar{X} - 0.52$
Hexachlorobutadiene	$0.71C - 1.01$	$0.19\bar{X} + 0.92$	$0.26\bar{X} + 0.49$
Hexachloroethane	$0.73C - 0.83$	$0.17\bar{X} + 0.67$	$0.17\bar{X} + 0.80$
Indeno(1,2,3-cd)pyrene	$0.78C - 3.10$	$0.29\bar{X} + 1.46$	$0.50\bar{X} + 0.44$
Isophorone	$1.12C + 1.41$	$0.27\bar{X} + 0.77$	$0.33\bar{X} + 0.26$
Naphthalene	$0.76C + 1.58$	$0.21\bar{X} - 0.41$	$0.30\bar{X} - 0.68$
Nitrobenzene	$1.09C - 3.05$	$0.19\bar{X} + 0.92$	$0.27\bar{X} + 0.21$
N-Nitrosodi-*n*-propylamine	$1.12C - 6.22$	$0.27\bar{X} + 0.68$	$0.44\bar{X} + 0.47$
PCB-1260	$0.81C - 10.86$	$0.35\bar{X} + 3.61$	$0.43\bar{X} + 1.82$
Phenanthrene	$0.87C - 0.06$	$0.12\bar{X} + 0.57$	$0.15\bar{X} + 0.25$
Pyrene	$0.84C - 0.16$	$0.16\bar{X} + 0.06$	$0.15\bar{X} + 0.31$
1,2,4-Trichlorobenzene	$0.94C - 0.79$	$0.15\bar{X} + 0.85$	$0.21\bar{X} + 0.39$
4-Chloro-3-methylphenol	$0.84C + 0.35$	$0.23\bar{X} + 0.75$	$0.29\bar{X} + 1.31$
2-Chlorophenol	$0.78C + 0.29$	$0.18\bar{X} + 1.46$	$0.28\bar{X} + 0.97$
2,4-Dichlorophenol	$0.87C + 0.13$	$0.15\bar{X} + 1.25$	$0.21\bar{X} + 1.28$
2,4-Dimethylphenol	$0.71C + 4.41$	$0.16\bar{X} + 1.21$	$0.22\bar{X} + 1.31$
2,4-Dinitrophenol	$0.81C - 18.04$	$0.38\bar{X} + 2.36$	$0.42\bar{X} + 26.29$
2-Methyl-4,6-dinitrophenol	$1.04C - 28.04$	$0.10\bar{X} + 42.29$	$0.26\bar{X} + 23.10$

TABLE 6410:VI. CONT.

Compound	Bias as Recovery, X' $\mu g/L$	Single-Analyst Precision, s_r' $\mu g/L$	Overall Precision, S' $\mu g/L$
2-Nitrophenol	$1.07C - 1.15$	$0.16\bar{X} + 1.94$	$0.27\bar{X} + 2.60$
4-Nitrophenol	$0.61C - 1.22$	$0.38\bar{X} + 2.57$	$0.44\bar{X} + 3.24$
Pentachlorophenol	$0.93C + 1.99$	$0.24\bar{X} + 3.03$	$0.30\bar{X} + 4.33$
Phenol	$0.43C + 1.26$	$0.26\bar{X} + 0.73$	$0.35\bar{X} + 0.58$
2,4,6-Trichlorophenol	$0.91C - 0.18$	$0.16\bar{X} + 2.22$	$0.22\bar{X} + 1.81$

* X' = expected recovery for one or more measurements of a sample containing a concentration of C,
s_r' = expected single-analyst standard deviation of measurements at an average concentration found of $\bar{X}$,
S' = expected interlaboratory standard deviation of measurements at an average concentration found of $\bar{X}$,
C = true value for the concentration, and
$\bar{X}$ = average recovery found for measurements of samples containing a concentration of C.
† The proper chemical name is 2,2′-oxybis(1-chloropropane).

8. Precision and Bias

This method was tested by 15 laboratories using reagent water, drinking water, surface water, and industrial wastewaters with additions at six concentrations over the range 5 to 1300 $\mu g/L$.[3] Single-operator precision, overall precision, and method bias were found to be related directly to the compound concentration and essentially independent of the sample matrix. Linear equations describing these relationships are presented in Table 6410:VI.

9. References

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6420 PHENOLS*

6420 A. Introduction

1. Sources and Significance

Phenols are found in many wastewaters and some raw source waters in the United States. They generally are traceable to industrial effluents or landfills. These compounds have a low taste threshold in potable waters and also may have a detrimental effect on human health at higher levels.

2. Selection of Method

For methods of determining total phenols in water and wastewater, see Section 5530.

The methods presented in this section are intended for the determination of individual phenolic compounds. For specific compounds covered, see each method. Method 6420B is a gas chromatographic (GC) method using liquid-liquid extraction and either flame ionization detection (FID) or derivatization and electron capture detection (ECD) to determine a wide variety of phenols at relatively low concentrations. In addition, 6420C, a liquid-liquid extraction gas chromatographic/mass spectrometric (GC/MS) method, can be used to determine the phenols at slightly higher concentrations.

* Approved by Standard Methods Committee, 2000.

6420 B. Liquid–Liquid Extraction Gas Chromatographic Method

This method[1] is applicable to the determination of phenol and certain substituted phenols* in municipal and industrial discharges. When analyzing unfamiliar samples for any or all of these compounds, support the identifications by at least one additional qualitative technique. Alternatively, use the derivatization, cleanup, and electron capture detector gas chromatography (ECD/GC) procedure to confirm measurements made by the flame ionization detector gas chromatographic (FID/GC) procedure. The method for base/neutrals and acids (Section 6410B) provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for qualitative and quantitative confirmation of results using the extract produced.

1. General Discussion

a. Principle: See Section 6010C.2 for discussion of gas chromatographic principles. A measured volume of sample is acidified and extracted with methylene chloride. The extract is dried and exchanged to 2-propanol during concentration. The extract is separated by gas chromatography and phenols are measured with a flame ionization detector.[2]

The method provides for a derivatization and column chromatography cleanup procedure to aid in the elimination of interferences.[2,3] Derivatives are analyzed by an electron capture detector.

b. Interferences:

1) General precautions—See Section 6410B.1*b*1).

2) Other countermeasures—The cleanup procedure in 6420B.5*c*2) can be used to overcome many of these inteferences, but unique samples may require additional cleanup to achieve the method detection levels.

The basic sample wash (6420B.5*a*) may cause low recovery of phenol and 2,4-dimethylphenol. Results obtained under these conditions are minimum concentrations.

c. Detection levels: The method detection level (MDL) is the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero.[4] The MDL concentrations listed in Tables 6420:I and II were obtained by using reagent water.[5] Similar results were achieved with representative wastewaters. The MDL actually obtained in a given analysis will vary, depending on instrument sensitivity and matrix effects.

d. Safety: The toxicity or carcinogenicity of each reagent used in this method has not been defined precisely. Take special care in handling pentafluorobenzyl bromide, which is a lachrymator, and 18-crown-6-ether, which is highly toxic.

2. Sampling and Storage

See Section 6410B.2.

3. Apparatus

Use all the apparatus specified in Section 6410B.3*a–g* and *i–k*, and in addition:

a. Chromatographic column, 100 mm long × 10 mm ID, with TFE stopcock.

b. Reaction flask, 15- to 25-mL round-bottom, with standard tapered joint, fitted with a water-cooled condenser and U-shaped drying tube containing granular calcium chloride.

c. Gas chromatograph:† An analytical system complete with a temperature-programmable gas chromatograph suitable for on-

* 4-Chloro-3-methylphenol; 2-chlorophenol; 2,4-dichlorophenol; 2,4-dimethylphenol; 2,4-dinitrophenol; 2-methyl-4,6-dinitrophenol; 2-nitrophenol; 4-nitrophenol; pentachlorophenol; phenol; 2,4,6-trichlorophenol.

† Gas chromatographic methods are extremely sensitive to the materials used. Mention of trade names by *Standard Methods* does not preclude the use of other existing or as-yet-undeveloped products that give *demonstrably* equivalent results.

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Table 6420:I. Chromatographic Conditions and Method Detection Levels

Compound	Retention Time *min*	Method Detection Level *μg/L*
2-Chlorophenol	1.70	0.31
2-Nitrophenol	2.00	0.45
Phenol	3.01	0.14
2,4-Dimethylphenol	4.03	0.32
2,4-Dichlorophenol	4.30	0.39
2,4,6-Trichlorophenol	6.05	0.64
4-Chloro-3-methylphenol	7.50	0.36
2,4-Dinitrophenol	10.00	13.0
2-Methyl-4,6-dinitrophenol	10.24	16.0
Pentachlorophenol	12.42	7.4
4-Nitrophenol	24.25	2.8

Column conditions: Supelcoport (80/100 mesh) coated with 1% SP-1240DA packed in a 1.8-m-long × 2-mm-ID glass column with nitrogen carrier gas at 30-mL/min flow rate. Column temperature was 80°C at injection, programmed immediately at 8°C/min to 150°C final temperature. MDLs determined with an FID.

Table 6420:II. Silica Gel Fractionation and Electron Capture Gas Chromatography of PFBB Derivatives

Parent Compound	Percent Recovery by Fraction* 1	2	3	4	Retention Time *min*	Method Detection Level *μg/L*
2-Chlorophenol	—	90	1	—	3.3	0.58
2-Nitrophenol	—	—	9	90	9.1	0.77
Phenol	—	90	10	—	1.8	2.2
2,4-Dimethylphenol	—	95	7	—	2.9	0.63
2,4-Dichlorophenol	—	95	1	—	5.8	0.68
2,4,6-Trichlorophenol	50	50	—	—	7.0	0.58
4-Chloro-3-methylphenol	—	84	14	—	4.8	1.8
Pentachlorophenol	75	20	—	—	28.8	0.59
4-Nitrophenol	—	—	1	90	14.0	0.70

Column conditions: Chromosorb W-AW-DMCS (80/100 mesh) coated with 5% OV-17 packed in a 1.8-m-long × 2.0-mm-ID glass column with 5% methane/95% argon carrier gas at 30-mL/min flow rate. Column temperature held isothermal at 200°C. MDLs determined with an ECD.

* Eluent composition:
Fraction 1: 15% toluene in hexane.
Fraction 2: 40% toluene in hexane.
Fraction 3: 75% toluene in hexane.
Fraction 4: 15% 2-propanol in toluene.

column injection and all required accessories including syringes, analytical columns, gases, detector, and strip-chart recorder. Preferably use a data system for measuring peak areas.

1) *Column for underivatized phenols*—1.8 m long × 2-mm ID glass, packed with 1% SP1240DA on Supelcoport (80/100 mesh) or equivalent. The detection level and precision and bias data presented herein were developed with this column. For guidelines for the use of alternate columns (e.g., capillary or megabore), see 6420B.5*b*1).

2) *Column for derivatized phenols*—1.8 m long × 2-mm ID, glass, packed with 5% OV-17 on Chromosorb W-AW-DMCS (80/100 mesh) or equivalent. This column was used to develop the detection limit and precision and bias data presented herein. For guidelines for the use of alternate columns (e.g., capillary or megabore), see 6420B.5*b*1).

3) *Detectors*—flame ionization (FID) and electron capture (ECD). Use the FID to determine parent phenols. Use the ECD when determining derivatized phenols. For guidelines for use of alternative detectors, see 6420B.5*b*1).

4. Reagents

Use reagents listed in Section 6410B.4*a–f*, and in addition:

a. Sodium hydroxide solution (NaOH), 1*N:* Dissolve 4 g NaOH in reagent water and dilute to 100 mL.

b. Sulfuric acid (H_2SO_4), 1*N:* Slowly add 58 mL conc H_2SO_4 to 500 mL reagent water and dilute to 1 L.

c. Potassium carbonate (K_2CO_3), powdered.

d. Pentafluorobenzyl bromide (α-bromopentafluorotoluene), 97% minimum purity. (**Caution: This chemical is a lachrymator**.)

e. 18-Crown-6-ether (1,4,7,10,13,16-hexaoxacyclooctadecane), 98% minimum purity. (**Caution: This chemical is highly toxic**.)

f. Derivatization reagent: Add 1 mL pentafluorobenzyl bromide and 1 g 18-crown-6-ether to a 50-mL volumetric flask and dilute to volume with 2-propanol. Prepare fresh weekly. Prepare in a hood. Store at 4°C and protect from light.

g. Acetone, hexane, methanol, methylene chloride, 2-propanol, toluene, pesticide quality or equivalent.

h. Silica gel, 100/200 mesh.‡ Activate at 130°C overnight and store in a desiccator.

i. Stock standard solutions: Prepare from pure standard materials or purchase as certified solutions. Prepare as directed in Section 6410B.4*g*, but dissolve material in 2-propanol.

j. Calibration standards: Prepare standards appropriate to chosen means of calibration.

1) *External standards*—Prepare at a minimum of three concentration levels for each compound by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with 2-propanol. Prepare one standard at a concentration near, but above, the MDL (see Table 6420:I or II) and the others to correspond to the expected range of sample concentrations or to define the working range of the detector.

2) *Internal standards*—Prepare at a minimum of three concentration levels for each compound by adding volumes of one or more stock standards to a volumetric flask. To each calibration standard, add a known constant amount of one or more internal standards, and dilute to volume with 2-propanol. Prepare one standard at a concentration near, but above, the MDL and the others to correspond to the expected range of sample concentrations or to define the working range of the detector.

k. Quality control (QC) check sample concentrate: Obtain a check sample concentrate containing each compound at a concentration of 100 μg/mL in 2-propanol. If such a sample is not available from an external source, prepare using stock standards prepared independently from those used for calibration.

‡ Davison grade 923, or equivalent.

5. Procedure

a. Extraction: Mark water meniscus on side of sample bottle for later determination of volume. Pour entire sample into a 2-L separatory funnel. For samples high in organic content, solvent wash sample at basic pH (as prescribed in next paragraph) to remove potential interferences. During wash, avoid prolonged or exhaustive contact with solvent, which may result in low recovery of some phenols, notably phenol and 2,4-dimethylphenol. For relatively clean samples, omit wash and extract directly.

To wash, adjust pH to 12.0 or greater with NaOH solution. Add 60 mL methylene chloride and shake the funnel for 1 min with periodic venting to release excess pressure. Discard solvent layer. Repeat wash up to two additional times if significant color is being removed.

Before extraction, adjust to pH of 1 to 2 with H_2SO_4. Extract three times with methylene chloride as directed in Section 6410B.5*a*1). Assemble Kuderna–Danish apparatus, concentrate extract to 1 mL, and remove, drain, and cool K–D apparatus as directed in Section 6410B.5*a*1).

Increase temperature of hot water bath to 100°C. Remove Snyder column and rinse flask and its lower joint into concentrator tube with 1 to 2 mL 2-propanol. Preferably use a 5-mL syringe for this operation. Attach a two-ball micro-Snyder column to concentrator tube and prewet column by adding about 0.5 mL 2-propanol to the top. Place micro-K–D apparatus on water bath so concentrator tube is partially immersed in hot water. Adjust vertical position of apparatus and water temperature so as to complete concentration in 5 to 10 min. (**CAUTION: If temperature is raised too quickly, the sample may be blown out of the K–D apparatus**). At proper rate of distillation, the column balls actively chatter but the chambers are not flooded. When the apparent volume of liquid reaches 2.5 mL, remove K–D apparatus and let drain and cool for at least 10 min. Add 2 mL 2-propanol through top of micro-Snyder column and resume concentrating as before. When the apparent volume of liquid reaches 0.5 mL, remove K–D apparatus and let drain and cool for at least 10 min.

Remove micro-Snyder column and rinse lower joint into concentrator tube with a minimum amount of 2-propanol. Adjust extract volume to 1.0 mL. Stopper concentrator tube and store at 4°C if further processing will not be done immediately. If extract is to be stored >2 d, transfer to a TFE-sealed screw-cap vial. If sample extract requires no further cleanup, proceed with chromatographic analysis (¶ *b* below). If sample requires further cleanup, proceed to ¶ *c* below.

Determine original sample volume by refilling sample bottle to mark and transferring liquid to a 1000-mL graduated cylinder. Record sample volume to nearest 5 mL.

b. Flame ionization detector gas chromatography (FID/GC):

1) Operating conditions—Table 6420:I summarizes the recommended operating conditions for the gas chromatograph and gives retention times and MDLs that can be achieved under these conditions. An example of the separations obtained with this column is shown in Figure 6420:1. Other packed or capillary (open-tubular) columns, chromatographic conditions, or detectors may be used if the requirements of 6420B.7 are met.

2) Calibration—To calibrate the system for underivatized phenols, establish gas chromatographic operating conditions equivalent to those given in Table 6420:I. Calibrate using the external or the internal standard technique as follows:

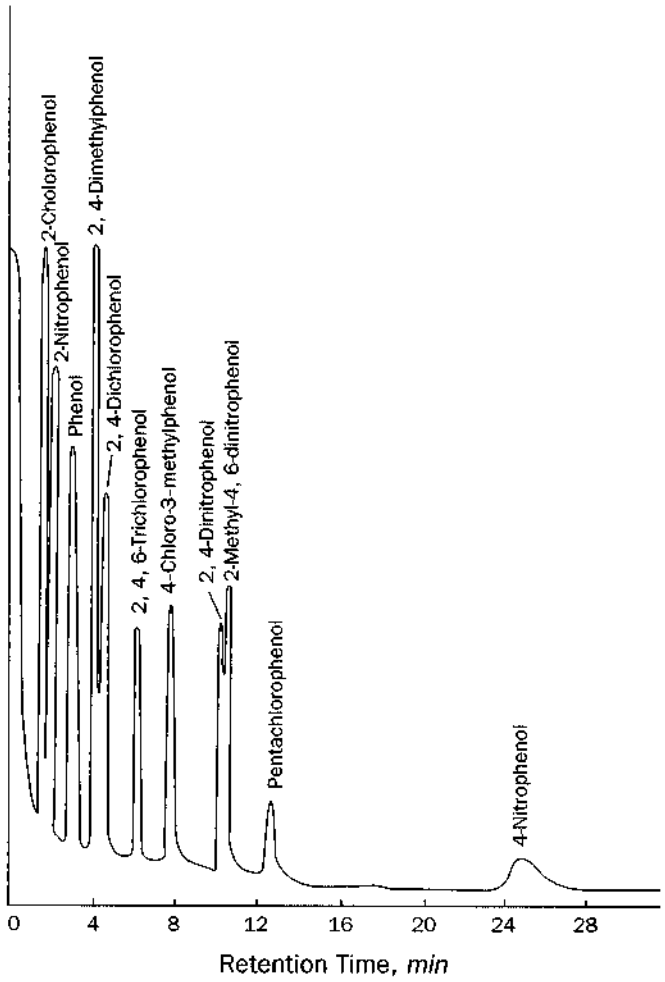


Figure 6420:1. Gas chromatogram of phenols. Column: 1% SP-1240DA on Supelcoport; program: 80°C at injection, immediate 8°C/min to 150°C; detector: flame ionization.

a) External standard calibration procedure—Prepare standards as directed in 6420B.4*j*1) and follow the procedure of ¶ *b*3) below. Tabulate data and obtain calibration curve or calibration factor as directed in Section 6200B.4*c*3).

b) Internal standard calibration procedure—Prepare samples as directed in 6420B.4*j*2) and follow the procedure of ¶ *b*3) below. Tabulate data and calculate response factors as directed in Section 6200B.4*c*2).

Verify working calibration curve, calibration factor, or RF on each working day by measuring one or more calibration standards. If the response for any compound varies from the predicted response by more than ±15%, prepare a new calibration curve for that compound.

3) Sample analysis—If the internal standard calibration procedure is used, add internal standard to sample extract and mix thoroughly immediately before injecting 2 to 5 μL sample extract or standard into gas chromatograph using the solvent-flush technique.[6] Smaller (1.0-μL) volumes may be injected if auto-

matic devices are used. Record volume injected to nearest 0.05 μL and resulting peak size in area or peak height units.

Identify compounds in sample by comparing peak retention times with peaks of standard chromatograms. Base width of retention time window used to make identifications on measurements of actual retention time variations of standards over the course of a day. To calculate a suggested window size, use three times the standard deviation of a retention time for a compound. Analyst's experience is important in interpreting chromatograms.

If the response for a peak exceeds the working range of the system, dilute extract and re-analyze.

If peak response cannot be measured because of interferences, use the alternative gas chromatographic procedure (¶ *c* below).

c. Derivatization and electron capture detector gas chromatography (ECD/GC):

1) Derivatization—Pipet 1.0 mL of the 2-propanol solution of standard or sample extract into a glass reaction vial. Add 1.0 mL derivatizing reagent (6420B.4*f*); this is sufficient to derivatize a solution having a total phenolic content not exceeding 0.3 mg/mL. Add about 3 mg K_2CO_3 and shake gently. Cap mixture and heat for 4 h at 80°C in a hot water bath. Remove from hot water bath and let cool. Add 10 mL hexane and shake vigorously for 1 min. Add 3.0 mL distilled, deionized water and shake for 2 min. Decant a portion of the organic layer into a concentrator tube and cap with a glass stopper.

2) Cleanup—Place 4.0 g silica gel in a chromatographic column. Tap column to settle silica gel and add about 2 g anhydrous Na_2SO_4 to the top. Pre-elute column with 6 mL hexane. Discard eluate and just before exposing Na_2SO_4 layer to air, pipet onto the column 2.0 mL hexane solution [¶ *c*1) above] that contains the derivatized sample or standard. Elute column with 10.0 mL hexane and discard eluate. Elute column, in order, with 10.0 mL 15% toluene in hexane (Fraction 1), 10.0 mL 40% toluene in hexane (Fraction 2), 10.0 mL 75% toluene in hexane (Fraction 3), and 10.0 mL 15% 2-propanol in toluene (Fraction 4). Prepare all elution mixtures on a volume:volume basis. Elution patterns for the phenolic derivatives are shown in Table 6420:II. Fractions may be combined as desired, depending on the specific phenols of interest or level of interferences.

3) Operating conditions—Table 6420:II summarizes the recommended operating conditions for the gas chromatograph and gives retention times and MDLs that can be achieved under these conditions. An example of the separations obtained with this column is shown in Figure 6420:2.

4) Calibration—Calibrate system daily by preparing a minimum of three 1-mL portions of calibration standards, 6420B.4*j*1), containing each of the phenols of interest and derivatized as above. Analyze 2 to 5 μL of each column eluate collected as in ¶ *c*5) below and tabulate peak height or area responses against calculated equivalent mass of underivatized phenol injected. Prepare a calibration curve for each compound.

Before using any cleanup procedure, process a series of calibration standards through the procedure to validate elution patterns and to assure absence of interferences from the reagents.

5) Sample analysis—Inject 2 to 5 μL column fractions into the gas chromatograph using the solvent-flush technique. Smaller (1.0-μL) volumes can be injected if automatic devices are used. Record volume injected to nearest 0.05 μL and resulting peak size in area or peak height units. If peak response exceeds linear range of system, dilute extract and re-analyze.

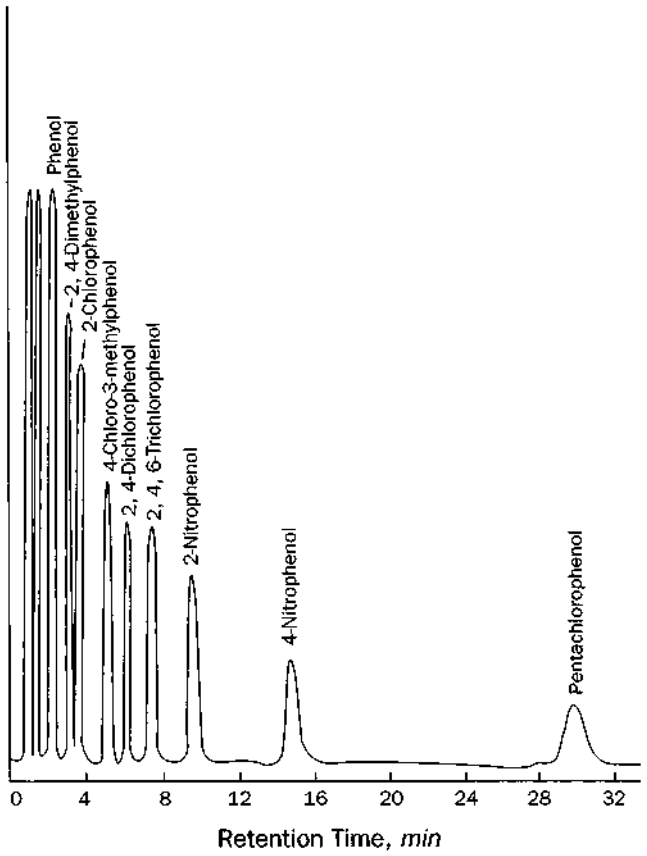


Figure 6420:2. Gas chromatogram of PFB derivatives of phenols. Column: 5% OV-17 on Chromosorb W-AW-DMCS; temperature: 200°C; detector: electron capture.

6. Calculation

a. FID/GC analysis: Determine concentration of individual compounds. If the external standard calibration procedure is used, calculate amount of material injected from peak response using calibration curve or calibration factor determined previously. Calculate sample concentration from the equation:

$$\text{Concentration, } \mu\text{g/L} = \frac{(A)\,(V_t)}{(V_i)\,(V_s)}$$

where:

A = amount of material injected, ng,
V_t = volume of total extract, μL,
V_i = volume of extract injected, μL, and
V_s = volume of water extracted, mL.

If the internal standard calibration procedure is used, calculate concentration in sample using the response factor (RF) determined above and the equation:

$$\text{Concentration, } \mu\text{g/L} = \frac{(A_s)\,(I_s)}{(A_{is})\,(RF)\,(V_o)}$$

where:

TABLE 6420:III. QC ACCEPTANCE CRITERIA*

Compound	Test Conc. µg/L	Limit for s µg/L	Range for $\bar{X}$ µg/L	Range for P, P_s %
4-Chloro-3-methylphenol	100	16.6	56.7–113.4	49–122
2-Chlorophenol	100	27.0	54.1–110.2	38–126
2,4-Dichlorophenol	100	25.1	59.7–103.3	44–119
2,4-Dimethylphenol	100	33.3	50.4–100.0	24–118
2-Methyl-4,6-dinitrophenol	100	25.0	42.4–123.6	30–136
2,4-Dinitrophenol	100	36.0	31.7–125.1	12–145
2-Nitrophenol	100	22.5	56.6–103.8	43–117
4-Nitrophenol	100	19.0	22.7–100.0	13–110
Pentachlorophenol	100	32.4	56.7–113.5	36–134
Phenol	100	14.1	32.4–100.0	23–108
2,4,6-Trichlorophenol	100	16.6	60.8–110.4	53–119

* s = standard deviation for four recovery measurements,
$\bar{X}$ = average recovery for four recovery measurements, and
P, P_s = percent recovery measured.

NOTE: These criteria are based directly on the method-performance data in Table 6420:IV. Where necessary, the recovery limits were broadened to ensure the limits' applicability to concentrations below those used to develop Table 6420:IV.

A_s = response for compound to be measured,
I_s = amount of internal standard added to each extract, µg,
A_{is} = response for internal standard, and
V_o = volume of water extracted, L.

b. Derivatization and ECD/GC analysis: To determine concentration of individual compounds in the sample, use the equation:

$$\text{Concentration, } \mu\text{g/L} = \frac{(A)\,(V_t)\,(B)\,(D)}{(V_i)\,(V_s)\,(C)\,(E)}$$

where:

A = mass of underivatized phenol represented by area of peak in sample chromatogram, determined from calibration curve in 6420B.5*c*4), ng,
V_t = total volume of column eluate or combined fractions from which V_i was taken, µL,
B = total volume of hexane added in 6420B.5*c*1), mL,
D = total volume of 2-propanol extract before derivatization, mL,
V_i = volume of eluate injected, µL,
V_s = volume of water extracted in 6420B.5*a*, mL,
C = volume of hexane sample solution added to cleanup column in 6420B.5*c*2), mL, and
E = volume of 2-propanol extract carried through derivatization in 6420B.5*c*1), mL.

Report results in µg/L without correction for recovery. Report QC data with sample results.

7. Quality Control

a. Quality control program: See Section 6200A.5.

b. Initial quality control: To establish the ability to generate data with acceptable bias and precision, perform the following operations:

Using a pipet, prepare QC check samples at a concentration of 100 µg/L by adding 1.00 mL of 100 µg/mL QC check sample concentrate to each of four 1-L portions reagent water. Analyze check samples according to the method of 6420B.5 and proceed with the check described in Sections 6200A.5*a*1) and 2). Use acceptance criteria given in Table 6420:III.

c. Analyses of laboratory-fortified samples: On an ongoing basis, make known additions to at least 10% of the samples from each sample site being monitored. For laboratories analyzing one to ten samples per month, analyze at least one such sample with a known addition per month. Use the procedure detailed in Sections 6200A.5*c*7) and 8), but use an addition of 100 µg/L rather than 20 µg/L and compare percent recovery for each compound with the corresponding QC acceptance criteria found in Table 6420:III. If the known addition was at a concentration <100 µg/L, use either the QC acceptance criteria in Table 6420:III or optional QC acceptance criteria calculated for the specific addition concentration based on the equations in Table 6420:IV.

d. Quality-control check standard analysis: If analysis of any compound fails to meet the acceptance criteria for recovery, prepare and analyze a QC check standard containing each compound that failed. NOTE: The frequency for the required analysis of a QC check standard will depend on the number of compounds being tested for simultaneously, the complexity of the sample matrix, and the performance of the laboratory.

Prepare the QC check standard by adding 1.0 mL of QC check sample concentrate to 1 L reagent water and proceed as in Section 6200A.5*a*3) using Table 6420:III.

e. Bias assessment and records: Assess method bias and maintain records as directed in Section 6410B.7*e*.

8. Precision and Bias

This method was tested by 20 laboratories using reagent water, drinking water, surface water, and three industrial

Table 6420:IV. Method Bias and Precision as Functions of Concentration*

Compound	Bias, as Recovery, X' $\mu g/L$	Single-Analyst Precision, s_r' $\mu g/L$	Overall Precision, S' $\mu g/L$
4-Chloro-3-methylphenol	$0.87C - 1.97$	$0.11\bar{X} - 0.21$	$0.16\bar{X} + 1.41$
2-Chlorophenol	$0.83C - 0.84$	$0.18\bar{X} + 0.20$	$0.21\bar{X} + 0.75$
2,4-Dichlorophenol	$0.81C + 0.48$	$0.17\bar{X} - 0.02$	$0.18\bar{X} + 0.62$
2,4-Dimethylphenol	$0.62C - 1.64$	$0.30\bar{X} - 0.89$	$0.25\bar{X} + 0.48$
2-Methyl-4,6-dinitrophenol	$0.84C - 1.01$	$0.15\bar{X} + 1.25$	$0.19\bar{X} + 5.85$
2,4-Dinitrophenol	$0.80C - 1.58$	$0.27\bar{X} - 1.15$	$0.29\bar{X} + 4.51$
2-Nitrophenol	$0.81C - 0.76$	$0.15\bar{X} + 0.44$	$0.14\bar{X} + 3.84$
4-Nitrophenol	$0.46C + 0.18$	$0.17\bar{X} + 2.43$	$0.19\bar{X} + 4.79$
Pentachlorophenol	$0.83C + 2.07$	$0.22\bar{X} - 0.58$	$0.23\bar{X} + 0.57$
Phenol	$0.43C + 0.11$	$0.20\bar{X} - 0.88$	$0.17\bar{X} + 0.77$
2,4,6-Trichlorophenol	$0.86C - 0.40$	$0.10\bar{X} + 0.53$	$0.13\bar{X} + 2.40$

* X' = expected recovery for one or more measurements of a sample containing a concentration of C,
s_r' = expected single-analyst standard deviation of measurements at an average concentration found of $\bar{X}$,
S' = expected interlaboratory standard deviation of measurements at an average concentration found of $\bar{X}$,
C = true value for the concentration, and,
$\bar{X}$ = average recovery found for measurements of samples containing a concentration of C.

wastewaters with known additions at six concentrations over the range 12 to 450 μg/L.[7] Single-operator precision, overall precision, and method bias were found to be related directly to compound concentration and essentially independent of sample matrix. Linear equations describing these relationships for a flame ionization detector are presented in Table 6420:IV.

9. References

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6420 C. Liquid–Liquid Extraction Gas Chromatographic/Mass Spectrometric Method

See Section 6410B.

6431 POLYCHLORINATED BIPHENYLS (PCBs)*

6431 A. Introduction

1. Sources and Significance

The polychlorinated biphenyls (PCBs) are found principally in water supplies contaminated by transformer oils in which PCBs were originally used as a heat-exchange medium. Although the use of these compounds has been banned, there are still numerous transformers in existence that contain PCBs, which results in their occasional discharge into potable water or wastewater. These compounds are toxic, bioaccumulative, and extremely stable, and thus there is a need to monitor them in wastewaters.

2. Selection of Method

The liquid–liquid extraction (LLE) gas chromatographic (GC) method is used to monitor both the PCBs and the organochlorine pesticides simultaneously. This method has excellent sensitivity. The LLE gas chromatographic/mass spectrometric (GC/MS) method also can be used to detect PCBs, but with substantially less sensitivity.

PCBs usually are measured as commercial mixtures of isomers rather than as individual isomers (congeners).

* Approved by Standard Methods Committee, 2005.

6431 B. Liquid–Liquid Extraction Gas Chromatographic Method

See Sections 6630B and C.

6431 C. Liquid–Liquid Extraction Gas Chromatographic/Mass Spectrometric Method

See Section 6410B.

https://doi.org/10.2105/SMWW.2882.126

6440 POLYNUCLEAR AROMATIC HYDROCARBONS*

6440 A. Introduction

1. Sources and Significance

The polynuclear aromatic hydrocarbons (PAHs) often are by-products of petroleum processing or combustion. Many of these compounds are highly carcinogenic at relatively low levels. Although they are relatively insoluble in water, their highly hazardous nature merits their monitoring in potable waters and wastewaters.

* Approved by Standard Methods Committee, 2005.

2. Selection of Method

Method 6440B encompasses both a high-performance liquid chromatographic (HPLC) method with UV and fluorescence detection and a gas chromatographic (GC) method using flame ionization detection. Method 6440C is a gas chromatographic/mass spectrometric (GC/MS) method that also can detect these compounds at somewhat higher concentrations. Certain of these compounds may also be measured by closed-loop stripping analysis (see Section 6040).

6440 B. Liquid–Liquid Extraction Chromatographic Method

This method[1] is applicable to the determination of certain polynuclear aromatic hydrocarbons (PAHs)* in municipal and industrial discharges. When analyzing unfamiliar samples for any or all of these compounds, support the identifications by at least one additional qualitative technique. The method for base/neutrals and acids (Section 6410B) provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for qualitative and quantitative confirmation of results using the extract produced.

1. General Discussion

a. Principle: A measured volume of sample is extracted with methylene chloride. The extract is dried, concentrated, and separated by the high-performance liquid chromatographic (HPLC) or gas chromatographic (GC) method. If other analyses having essentially the same extraction and concentration steps are to be performed, extraction of a single sample will be sufficient for all the determinations. Ultraviolet (UV) and fluorescence detectors are used with HPLC to identify and measure the PAHs. A flame ionization detector is used with GC.[2]

The method provides a silica gel column cleanup to aid in eliminating interferences. When cleanup is required, sample concentration levels must be high enough to permit separate treatment of subsamples before the solvent-exchange steps.

Chromatographic conditions (6440B.5*d*) appropriate for the simultaneous measurement of combinations of these compounds may be selected but they do not adequately resolve the following four pairs of compounds: anthracene and phenanthrene; chrysene and benzo(a)anthracene; benzo(b)fluoranthene and benzo(k)fluoranthene; and dibenzo(a,h)anthracene and indeno(1,2,3-cd)pyrene. Unless reporting the sum of an unresolved pair is acceptable, use the liquid chromatographic method, which does resolve all 16 listed PAHs.

b. Interferences: See Section 6410B.1*b* for precautions concerning glassware, reagent purity, and matrix interferences. Interferences in liquid chromatographic techniques have not been assessed fully. Although HPLC conditions described allow for unique resolution of specific PAHs, other PAH compounds may interfere. PAHs in water samples containing particulate matter may actually be absorbed onto the particulate matter. This may result in hidden coeluting peaks and consequently false fingerprint or erroneous quantitation. The use of capillary GC or MS detection can remedy this situation.

c. Detection levels: The method detection level (MDL) is the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero.[3] The MDL concentrations listed in Table 6440:I were obtained with reagent water.[4] Similar results were achieved with representative wastewaters. MDLs for the GC method were not determined. The MDL actually obtained in a given analysis will vary, depending on instrument sensitivity and matrix effects. This method has been tested for linearity of known-addition recovery from reagent water and has been demonstrated to be applicable over the concentration range from 8 × MDL to 800 × MDL,[4] with the following exception: benzo(ghi)perylene recovery at 80 × and 800 × MDL were low (35 and 45%, respectively).

d. Safety: The toxicity or carcinogenicity of each reagent has not been defined precisely. The following compounds have been classified tentatively as known or suspected, human or mammalian carcinogens: benzo(a)anthracene, benzo(a)pyrene, and dibenzo(a,h)anthracene. Prepare primary standards of these compounds in a hood and wear NIOSH/MESA-approved toxic gas respirator when handling high concentrations.

* Acenaphthene, acenaphthylene, anthracene, benzo-(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(ghi)perylene, benzo(k)fluoranthene, chrysene, dibenzo(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, and pyrene.

https://doi.org/10.2105/SMWW.2882.127

TABLE 6440:I. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY CONDITIONS AND METHOD DETECTION LEVELS

Compound	Retention Time *min*	Column Capacity Factor *k′*	Method Detection Level *μg/L**
Naphthalene	16.6	12.2	1.8
Acenaphthylene	18.5	13.7	2.3
Acenaphthene	20.5	15.2	1.8
Fluorene	21.2	15.8	0.21
Phenanthrene	22.1	16.6	0.64
Anthracene	23.4	17.6	0.66
Fluoranthene	24.5	18.5	0.21
Pyrene	25.4	19.1	0.27
Benzo(a)anthracene	28.5	21.6	0.013
Chrysene	29.3	22.2	0.15
Benzo(b)fluoranthene	31.6	24.0	0.018
Benzo(k)fluoranthene	32.9	25.1	0.017
Benzo(a)pyrene	33.9	25.9	0.023
Dibenzo(a,h)anthracene	35.7	27.4	0.030
Benzo(ghi)perylene	36.3	27.8	0.076
Indeno(1,2,3-cd)pyrene	37.4	28.7	0.043

HPLC column conditions: Reverse phase HC-ODS Sil-X, 5-μm particle size, in a 25-cm × 2.6-mm-ID stainless steel column. Isocratic elution for 5 min using acetonitrile/water (4 + 6), then linear gradient to 100% acetonitrile over 25 min at 0.5 mL/min flow rate. If columns with other internal diameters are used, adjust flow rate to maintain a linear velocity of 2 mm/s.

* The MDLs for naphthalene, acenaphthylene, acenaphthene, and fluorene were determined using a UV detector. All others were determined using a fluorescence detector.

TABLE 6440:II. GAS CHROMATOGRAPHIC CONDITIONS AND RETENTION TIMES

Compound	Retention Time *min*
Naphthalene	4.5
Acenaphthylene	10.4
Acenaphthene	10.8
Fluorene	12.6
Phenanthrene	15.9
Anthracene	15.9
Fluoranthene	19.8
Pyrene	20.6
Benzo(a)anthracene	24.7
Chrysene	24.7
Benzo(b)fluoranthene	28.0
Benzo(k)fluoranthene	28.0
Benzo(a)pyrene	29.4
Dibenzo(a,h)anthracene	36.2
Indeno(1,2,3-cd)pyrene	36.2
Benzo(ghi)perylene	38.6

GC column conditions: Chromosorb W-AW-DCMS (100/120 mesh) coated with 3% OV-17 packed in a 1.8-m × 2-mm-ID glass column with nitrogen carrier gas at 40 mL/min flow rate. Column temperature held at 100°C for 4 min, then programmed at 8°C/min to a final hold at 280°C.

2. Sampling and Storage

For collection and general storage requirements, see Section 6410B.2. Because PAHs are light-sensitive, store samples, extracts, and standards in amber or foil-wrapped bottles to minimize photolytic decomposition.

3. Apparatus

Use all the apparatus specified in Section 6410B.3*a–g* and *i–k*, and in addition:

a. Chromatographic column, 250 mm long × 10-mm ID with coarse frit filter disk at bottom and TFE stopcock.

b. High-performance liquid chromatograph (HPLC): An analytical system complete with column supplies, high-pressure syringes, detectors, and compatible strip-chart recorder. Preferably use a data system for measuring peak areas and retention times.

1) *Gradient pumping system*, constant flow.

2) *Reverse phase column*, HC-ODS Sil-X, 5-μm particle diam, in a 25-cm × 2.6-mm ID stainless steel column† —This column was used to develop MDL and precision and bias data presented herein. For guidelines for the use of alternate column packings see 6440B.5*d*1).

† Perkin Elmer No. 089-0716, or equivalent.

3) *Detectors*—fluorescence and/or UV. Use the fluorescence detector for excitation at 280 nm and emission >389 nm cutoff.‡ Use fluorometers with dispersive optics for excitation using either filter or dispersive optics at the emission detector. Operate the UV detector at 254 nm and couple it to the fluorescence detector. These detectors were used to develop MDL and precision and bias data presented herein. For guidelines for the use of alternate detectors, see 6440B.5*d*1).

c. Gas chromatograph:§ An analytical system complete with temperature-programmable gas chromatograph suitable for on-column or splitless injection and all required accessories including syringes, analytical columns, gases, detector, and strip-chart recorder. Preferably use a data system for measuring peak areas.

1) *Column*—1.8 m long × 2-mm ID glass, packed with 3% OV-17 on Chromosorb W-AW-DCMS (100/120 mesh) or equivalent. This column was used to develop the retention time data in Table 6440:II. For guidelines for the use of alternate columns (e.g. capillary or megabore), see 6440B.5*d*2).

2) *Detector*—flame ionization. This detector is effective except for resolving the four pairs of compounds listed in 6440B.1*a*. With the use of capillary columns, these pairs may be resolved with GC. For guidelines for the use of alternate detectors, see 6440B.5*d*2).

4. Reagents

a. Reagent water: See Section 6200B.3*a*.

b. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$), granular.

‡ Corning 3-75, or equivalent.

§ Gas chromatographic methods are extremely sensitive to the materials used. Mention of trade names by *Standard Methods* does not preclude the use of other existing or as-yet-undeveloped products that give *demonstrably* equivalent results.

c. Cyclohexane, methanol, acetone, methylene chloride, pentane, pesticide quality or equivalent.

d. Acetonitrile, HPLC quality, distilled in glass.

e. Sodium sulfate (Na_2SO_4), granular, anhydrous. Purify by heating at 400°C for 4 h in a shallow tray.

f. Silica gel, 100/200 mesh, desiccant.‖ Before use, activate for at least 16 h at 130°C in a shallow glass tray, loosely covered with foil.

g. Stock standard solutions: Prepare as directed in Section 6410B.4*g*, using acetonitrile as the solvent.

h. Calibration standards: Prepare standards appropriate to chosen means of calibration following directions in Section 6420B.4*j*, except that acetonitrile is the diluent instead of 2-propanol. See Table 6440:I for MDLs.

i. Quality control (QC) check sample concentrate: Obtain a check sample concentrate containing each compound at the following concentrations in acetonitrile: 100 μg/mL of any of the six early-eluting PAHs (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, and anthracene); 5 μg/mL of benzo(k)fluoranthene; and 10 μg/mL of any other PAH. If such a sample is not available from an external source, prepare using stock standards prepared independently from those used for calibration.

5. Procedure

a. Extraction: Mark water meniscus on side of sample bottle for later determination of volume. Pour entire sample into a 2-L separatory funnel and extract as directed in Section 6410B.5*a*1) without any pH adjustment.

After extraction, concentrate by adding one or two clean boiling chips to the evaporative flask and attach a three-ball Snyder column. Prewet Snyder column by adding about 1 mL methylene chloride to the top. Place K–D apparatus on a hot water bath (60 to 65°C) in a hood so the concentrator tube is partially immersed in the hot water, and the entire lower rounded surface of flask is bathed with hot vapor. Adjust vertical position of apparatus and water temperature as required to complete the concentration in 15 to 20 min. At proper rate of distillation, the column balls actively chatter but the chambers are not flooded with condensed solvent. When the apparent volume of liquid reaches 1 mL, remove K–D apparatus and let drain and cool for at least 10 min.

Remove Snyder column and rinse flask and its lower joint into concentrator tube with 1 to 2 mL methylene chloride. Preferably use a 5-mL syringe for this operation. Stopper concentrator tube and store refrigerated if further processing will not be done immediately. If extract is to be stored >2 d, transfer to a TFE-sealed screw-cap vial and protect from light. If sample extract requires no further cleanup, proceed with gas or liquid chromatographic analysis (¶s *c–f* below). If sample requires further cleanup, first follow procedure of ¶ *b* below before chromatographic analysis.

Determine original sample volume by refilling sample bottle to mark and transferring liquid to a 1000-mL graduated cylinder. Record sample volume to nearest 5 mL.

‖ Davison, grade 923, or equivalent.

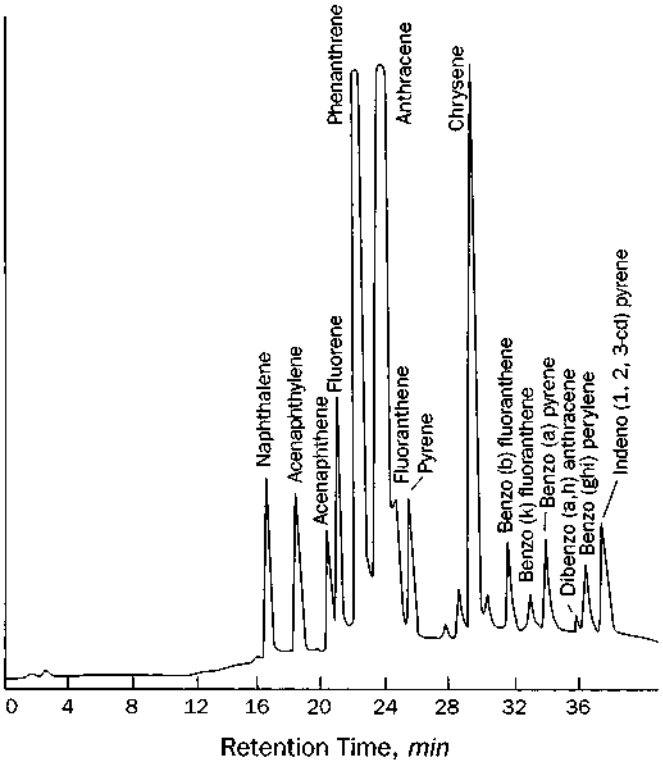


Figure 6440:1. Liquid chromatogram of polynuclear aromatic hydrocarbons. Column: HC—ODS SIL-X; mobile phase: 40 to 100% acetonitrile in water; detector: ultraviolet at 254 nm.

b. Cleanup and separation: Use procedure below or any other appropriate procedure; however, first demonstrate that the requirements of 6440B.7 can be met.

Before using silica-gel cleanup technique, exchange extract solvent to cyclohexane. Add 1 to 10 mL sample extract (in methylene chloride) and a boiling chip to a clean K–D concentrator tube. Add 4 mL cyclohexane and attach a two-ball micro-Snyder column. Prewet column by adding 0.5 mL methylene chloride to the top. Place micro-K–D apparatus on a boiling (100°C) water bath so concentrator tube is partially immersed in hot water. Adjust vertical position of apparatus and water temperature so as to complete concentration in 5 to 10 min. At proper rate of distillation, the column balls actively chatter but the chambers are not flooded. When apparent volume of liquid reaches 0.5 mL, remove K–D apparatus and let drain and cool for at least 10 min. Remove micro-Snyder column and rinse its lower joint into concentrator tube with a minimum amount of cyclohexane. Adjust extract volume to about 2 mL.

To perform silica-gel column cleanup, make a slurry of 10 g activated silica gel in methylene chloride and place in a 10-mm-ID chromatographic column. Tap column to settle silica gel and elute with methylene chloride. Add 1 to 2 cm anhydrous Na_2SO_4 to top of silica gel. Pre-elute with 40 mL pentane. Elute at rate of about 2 mL/min. Discard eluate and just before exposure of Na_2SO_4 layer to the air, transfer all the cyclohexane sample extract onto column using an additional 2 mL cyclohexane. Just before exposure of Na_2SO_4 layer to air, add 25 mL pentane and continue elution. Discard this pentane eluate. Next,

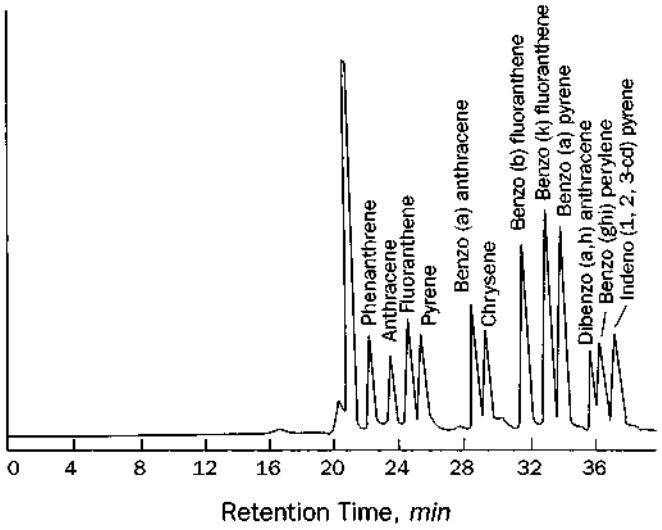


Figure 6440:2. Liquid chromatogram of polynuclear aromatic hydrocarbons. Column: HC—ODS SIL-X; mobile phase: 40 to 100% acetonitrile in water; detector: fluorescence.

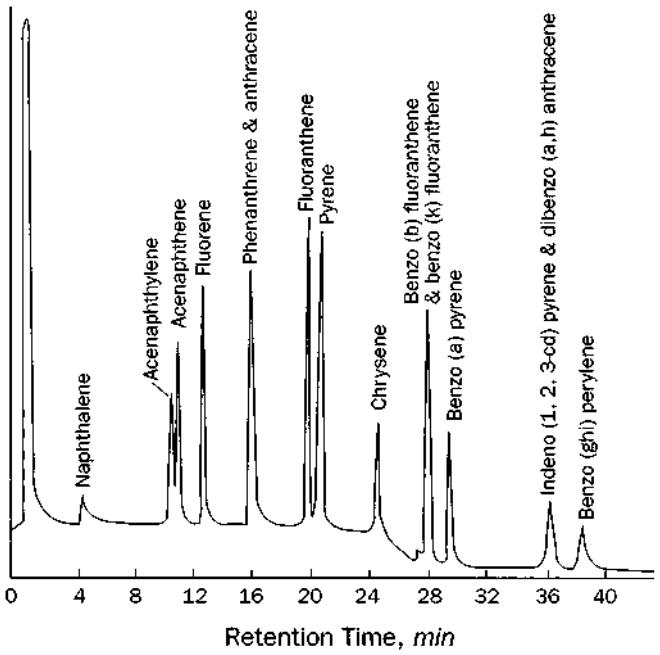


Figure 6440:3. Gas chromatogram of polynuclear aromatic hydrocarbons. Column: 3% OV-17 on Chromosorb W-AW-DCMS; program: 100°C for 4 min, 8°C/min to 280°C; detector: flame ionization.

elute column with 25 mL methylene chloride/pentane (4 + 6) (v/v) into a 500-mL K–D flask equipped with a 10-mL concentrator tube. Concentrate collected fraction to less than 10 mL as in ¶ *a* above. After cooling, remove Snyder column and rinse flask and its lower joint with pentane.

c. Reconcentration: Concentrate further as follows:

1) For high-performance liquid chromatography—To extract in a concentrator tube, add 4 mL acetonitrile and a new boiling chip. Attach a two-ball micro-Snyder column and concentrate solvent as in ¶ *a* above (but set water bath at 95 to 100°C.) After cooling, remove micro-Snyder column and rinse its lower joint into the concentrator tube with about 0.2 mL acetonitrile. Adjust extract volume to 1.0 mL.

2) For gas chromatography—To achieve maximum sensitivity with this method, concentrate extract to 1.0 mL. Add a clean boiling chip to methylene chloride extract in concentrator tube. Attach a two-ball micro-Snyder column. Prewet column by adding about 0.5 mL methylene chloride to the top. Place micro-K–D apparatus on a hot water bath (60 to 65°C) and continue concentration as in ¶ *b* above. Remove micro-Snyder column and rinse its lower joint into concentrator tube with a minimum amount of methylene chloride. Adjust final volume to 1.0 mL and stopper concentrator tube.

d. Operating conditions:

1) High-performance liquid chromatography—Table 6440:I summarizes the recommended operating conditions for HPLC and gives retention times, capacity factors, and MDLs that can be achieved under these conditions. Preferably use the UV detector for determining naphthalene, acenaphthylene, acenapthene, and fluorene, and the fluorescence detector for the remaining PAHs. Examples of separations obtained with this HPLC column are shown in Figures 6440:1 and 2. Other HPLC columns, chromatographic conditions, or detectors may be used if the requirements of 6440B.7 are met.

2) Gas chromatography—Table 6440:II summarizes the recommended operating conditions for the gas chromatograph and gives retention times that were obtained under these conditions. An example of the separations is shown in Figure 6440:3. Other packed or capillary (open-tubular) columns, chromatographic conditions, or detectors may be used if the requirements of 6440B.7 are met.

e. Calibration: Calibrate system daily using either external or internal standard procedure.

1) External standard calibration procedure—Prepare standards as directed in 6440B.4*h* and follow either procedure of ¶ *f* below. Tabulate data and obtain calibration curve or calibration factor as directed in Section 6200B.4*c*3).

2) Internal standard calibration procedure—Prepare standards as directed in 6440B.4*h* and follow either procedure of ¶ *f* below. Tabulate data and calculate response factors as directed in Section 6200B.4*c*2).

Verify working calibration curve, calibration factor, or RF on each working shift by measuring one or more calibration standards. If the response for any compound varies from the predicted response by more than ±15%, repeat test using a fresh calibration standard. Alternatively, prepare a new calibration curve for that compound.

Before using any cleanup procedure, process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

f. Sample analysis:

1) High-performance liquid chromatography—If the internal standard calibration procedure is being used, add internal standard to sample extract and mix thoroughly. Immediately inject 5 to 25 μL sample extract or standard into HPLC using a high-pressure syringe or a constant-volume sample injection loop. Record volume injected to nearest 0.1 μL and resulting peak size in area or peak height units. Re-equilibrate HPLC column at initial gradient conditions for at least 10 min between injections.

TABLE 6440:III. QC ACCEPTANCE CRITERIA*

Compound	Test Conc. μg/L	Limit for s μg/L	Range for $\bar{X}$ μg/L	Range for P, P_s %
Acenaphthene	100	40.3	D–105.7	D–124
Acenaphthylene	100	45.1	22.1–112.1	D–139
Anthracene	100	28.7	11.2–112.3	D–126
Benzo(a)anthracene	10	4.0	3.1–11.6	12–135
Benzo(a)pyrene	10	4.0	0.2–11.0	D–128
Benzo(b)fluoranthene	10	3.1	1.8–13.8	6–150
Benzo(ghi)perylene	10	2.3	D–10.7	D–116
Benzo(k)fluoranthene	5	2.5	D–7.0	D–159
Chrysene	10	4.2	D–17.5	D–199
Dibenzo(a,h)anthracene	10	2.0	0.3–10.0	D–110
Fluoranthene	10	3.0	2.7–11.1	14–123
Fluorene	100	43.0	D–119	D–142
Indeno(1,2,3-cd) pyrene	10	3.0	1.2–10.0	D–116
Naphthalene	100	40.7	21.5–100.0	D–122
Phenanthrene	100	37.7	8.4–133.7	D–155
Pyrene	10	3.4	1.4–12.1	D–140

* s = standard deviation of four recovery measurements,
$\bar{X}$ = average recovery for four recovery measurements,
P, P_s = percent recovery measured, and
D = detected; result must be >0.

NOTE: These criteria are based directly on the method performance data in Table 6440:IV. Where necessary, the recovery limits were broadened to ensure the limits' applicability to concentrations below those used to develop Table 6440:IV.

Identify compounds in sample by comparing peak retention times with peaks of standard chromatograms. Base width of retention time window used to make identifications on measurements of actual retention time variations of standards over the course of a day. To calculate a suggested window size use three times the standard deviation of a retention time for a compound. Analyst's experience is important in interpreting chromatograms.

If the response for a peak exceeds the working range of the system, dilute extract with acetonitrile and re-analyze.

If peak response cannot be measured because of interferences, further cleanup is required.

2) Gas chromatography—See Section 6420B.5*b*3). If peak response cannot be measured because of interferences, further cleanup is required.

6. Calculation

Determine concentration of individual compounds using the procedures given in Section 6420B.6*a*. Report results in μg/L without correction for recovery. Report QC data with sample results.

7. Quality Control

a. Quality-control program: See Section 6200A.5.

b. Initial quality control: To establish the ability to generate data with acceptable precision and bias, proceed as follows: Using a pipet, prepare QC check samples at test concentrations shown in Table 6440:III by adding 1.00 mL of QC check sample concentrate (6440B.4*i*) to each of four 1-L portions of reagent water. Analyze QC check samples according to the procedure in 6440B.5. Calculate average recovery and standard deviation of the recovery, compare with acceptance criteria, and evaluate and correct system performance as directed in Sections 6200A.5*a*1) and 2), using acceptance criteria given in Table 6440:III.

c. Analyses of samples with known additions: See Section 6420B.7*c*. Prepare QC check sample concentrate according to 6440B.4*i* and use Tables 6440:III and IV. On an ongoing basis, make known additions to at least 10% of the samples from each sample site being monitored. For laboratories analyzing one to ten samples per month, analyze at least one such sample with a known addition per month. Use the procedure described in Sections 6200A.5*c*7) and 8).

d. Quality-control check standard analysis: See Section 6420B.7*d*. Prepare QC check standard according to 6440B.4*i* and use Table 6440:III. If all compounds in Table 6440:III are to be measured in the sample in ¶ *c* above, it is probable that the analysis of a QC check standard will be required; therefore, routinely analyze the QC check standard with the known-addition sample.

e. Bias assessment and records: See Section 6410B.7*e*.

8. Precision and Bias

This method was tested by 16 laboratories using reagent water, drinking water, surface water, and three industrial wastewaters with known additions at six concentrations over the range 0.1 to 425 μg/L.[5] Single-operator precision, overall precision, and method bias were found to be related directly to compound concentration and essentially independent of sample matrix. Linear equations describing these relationships are presented in Table 6440:IV.

TABLE 6440:IV. METHOD BIAS AND PRECISION AS FUNCTIONS OF CONCENTRATION*

Compound	Bias as Recovery, X' $\mu g/L$	Single-Analyst Precision, s_r $\mu g/L$	Overall Precision, S' $\mu g/L$
Acenaphthene	$0.52C + 0.54$	$0.39\bar{X} + 0.76$	$0.53\bar{X} + 1.32$
Acenaphthylene	$0.69C - 1.89$	$0.36\bar{X} + 0.29$	$0.42\bar{X} + 0.52$
Anthracene	$0.63C - 1.26$	$0.23\bar{X} + 1.16$	$0.41\bar{X} + 0.45$
Benzo(a)anthracene	$0.73C + 0.05$	$0.28\bar{X} + 0.04$	$0.34\bar{X} + 0.02$
Benzo(a)pyrene	$0.56C + 0.01$	$0.38\bar{X} + 0.01$	$0.53\bar{X} - 0.01$
Benzo(b)fluoranthene	$0.78C + 0.01$	$0.21\bar{X} + 0.01$	$0.38\bar{X} - 0.00$
Benzo(ghi)perylene	$0.44C + 0.30$	$0.25\bar{X} + 0.04$	$0.58\bar{X} + 0.10$
Benzo(k)fluoranthene	$0.59C + 0.00$	$0.44\bar{X} - 0.00$	$0.69\bar{X} + 0.01$
Chrysene	$0.77C - 0.18$	$0.32\bar{X} - 0.18$	$0.66\bar{X} - 0.22$
Dibenzo(a,h)anthracene	$0.41C + 0.11$	$0.24\bar{X} + 0.02$	$0.45\bar{X} + 0.03$
Fluoranthene	$0.68C + 0.07$	$0.22\bar{X} + 0.06$	$0.32\bar{X} + 0.03$
Fluorene	$0.56C - 0.52$	$0.44\bar{X} - 1.12$	$0.63\bar{X} - 0.65$
Indeno(1,2,3-cd)pyrene	$0.54C + 0.06$	$0.29\bar{X} + 0.02$	$0.42\bar{X} + 0.01$
Naphthalene	$0.57C - 0.70$	$0.39\bar{X} - 0.18$	$0.41\bar{X} + 0.74$
Phenanthrene	$0.72C - 0.95$	$0.29\bar{X} + 0.05$	$0.47\bar{X} - 0.25$
Pyrene	$0.69C - 0.12$	$0.25\bar{X} + 0.14$	$0.42\bar{X} - 0.00$

* X' = expected recovery for one or more measurements of a sample containing a concentration of C,
s_r = expected single-analyst standard deviation of measurements at an average concentration found of $\bar{X}$,
S' = expected interlaboratory standard deviation of measurements at an average concentration found of $\bar{X}$,
C = true value for concentration, and
$\bar{X}$ = average recovery found for measurements of samples containing a concentration of C.

9. References

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1984. Method 610—Polynuclear aromatic hydrocarbons. 40 CFR Part 136, 43344; *Fed. Reg.* 49, No. 209.
2. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1982. Determination of polynuclear aromatic hydrocarbons in industrial and municipal wastewaters; EPA-600/4-82-025. National Technical Information Serv., PB82-258799, Springfield, Va.
3. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1984. Definition and procedure for the determination of the method detection limit. 40 CFR Part 136, Appendix B. *Fed. Reg.* 49, No. 209.
4. COLE, T., R. RIGGIN & J. GLASER. 1980. Evaluation of method detection limits and analytical curve for EPA Method 610 PNA's. International Symp. Polynuclear Aromatic Hydrocarbons, 5th, Battelle's Columbus Lab., Columbus, Ohio.
5. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1984. EPA Method Study 20, Method 610—PNA; EPA-600/4-84-063, National Technical Information Serv., PB84-211614. Springfield, Va.

6440 C. Liquid–Liquid Extraction Gas Chromatographic/Mass Spectrometric Method

See Section 6410B.

6450 NITROSAMINES*

6450 A. Introduction

1. Sources and Significance

The chemical *N*-nitrosodimethylamine (NDMA) has been identified as a probable human carcinogen by the U.S. Environmental Protection Agency. It is commonly found as a byproduct of industrial materials, such as liquid rocket fuel, lubricants, and pesticides. The general public can be exposed to NDMA by such sources as outdoor air, tobacco smoke, rubber products, cosmetics, and dietary consumption (e.g., cured meats, fish, cheese, and beer). The average concentrations of NDMA measured in various foods range from 90 to 100 ng/L for pasteurized milk, 600 to 1000 ng/kg for fried pork bacon, and 50 to 5900 ng/kg for beer. NDMA also has been detected in recycled water, wastewater, and potable water, and it may be formed by chlorine and chloramine disinfection processes.[1,2] The formation mechanisms that produce NDMA also may form other nitrosamines. Seven other nitrosamines (see Table 6450:I) that are structurally related to NDMA and in some instances known to cause cancer in animals have been included in this method.

2. Selection of Method

Two extraction methods are presented here. Method 6450B is a solid-phase extraction (SPE) method using a granular carbonaceous adsorbent resin. Method 6450C is a micro-liquid-liquid extraction (MLLE) method. The SPE method has a concentration factor of 500- to 1000-fold and can typically achieve method detection levels (MDL) in the 0.5- to 2.0-ng/L range, depending on the detection system. The MLLE method achieves a maximum concentration factor of 200-fold with MDL in the 2- to 4-ng/L range. Although the MLLE method generally has a higher MDL than the SPE method, it may be adequate for many wastewater samples and formation potential samples, and is especially useful when sample volume is limited.

A recent report[3] provides additional details on the methods presented here, as well as other methods potentially useful in nitrosamine analysis.

The methods presented here are applicable to the analysis of wastewater, recycled water, and potable water. The methods use extraction procedures followed by analysis with gas chromatography (GC) /chemical ionization (CI) tandem mass spectrometry (MS/MS) for the separation and detection of eight nitrosamine compounds. The GC/MS/MS conditions are discussed in detail in 6450B, but are also applicable to the extracts from 6450C. Either acetonitrile or methanol may be used as the CI reagents.

3. References

1. Choi, J. & R.L. Valentine. 2002. Formation of *N*-nitrosodimethylamine (NDMA) from reaction of monochloramine: A new disinfection by-product. *Water Res.* 36:817.
2. Mitch, W.A. & D.L. Sedlak. 2002. Formation of *N*-nitrosodimethylamine (NDMA) from dimethylamine during chlorination. *Environ. Sci. Technol.* 36:588.
3. Cheng, R.C., C. Andrews-Tate, C.J. Hwang, Y.C. Guo, S.J. Pastor, J.E. Grebel & I.H. Suffet. 2005. Alternative Methods for the Analysis of NDMA and Other Nitrosamines in Water and Wastewater. WateReuse Foundation, Alexandria, Va.

* Approved by Standard Methods Committee, 2007.
Joint Task Group: 22nd Edition—Robert C. Cheng (chair), Cordelia J. Hwang, Cecilia O. Lei, Irwin H. (Mel) Suffet, Vincent Y. Taguchi.

6450 B. Carbonaceous-Resin Solid-Phase Extraction GC/MS Method

This method is applicable to the determination of *N*-nitrosodimethylamine (NDMA) and seven other nitrosamines at concentrations of 2 to 300 ng/L, in raw source water, finished water, wastewater, and recycled water.

1. General Discussion

a. Principle: This method, based on a previously published procedure,[1] entails extraction of a sample to which isotopically labeled surrogates have been added through adsorption onto a carbonaceous resin, desorption of nitrosamines from the resin with dichloromethane (DCM or methylene chloride), and analysis of the resulting solution by gas chromatography (GC)/tandem mass spectrometry (MS/MS) (GC/MS/MS) with chemical ionization (CI).

Isotope dilution/internal standard quantitation is implemented with the addition of three isotopically labeled internal standards (d_6-NDMA, d_{14}-NDPA, and $^{15}N_2$-NDEA) before extraction to correct for extraction, as well as instrument variations. A large injection volume of sample extract (8 μL) is recommended for increased sensitivity, yielding detection levels in the low part-per-trillion levels.

In chemical ionization tandem mass spectrometry (MS/MS) a CI reagent (acetonitrile or methanol) is introduced into the ion trap, ionized, and allowed to react with sample molecules. Reactions between the ionized CI reagent and sample molecules result in various product ions, $(M+H)^+$. The ion corresponding to the analyte of interest is isolated from other matrix ions in the trap and is selected as the precursor ion for subsequent fragmentation. CI is a softer ionization technique than electron impact

Table 6450:I. Target Nitrosamine Analytes: Formula, Molecular Weight, Internal Standard, and Quantitation Ion

Nitrosamine	Abbreviation	Formula	Mol Wt	Internal Standard	Quantitation Ion* *amu*
N-nitrosodimethylamine	NDMA	$C_2H_6N_2O$	74	d_6-NDMA	44
N-nitrosomethylethylamine	NMEA	$C_3H_8N_2O$	88	$^{15}N_2$-NDEA	61
N-nitrosodiethylamine	NDEA	$C_4H_{10}N_2O$	102	$^{15}N_2$-NDEA	75
N-nitrosodi-*n*-propylamine	NDPA	$C_6H_{14}N_2O$	130	d_{14}-NDPA	89
N-nitrosomorpholine	NMOR	$C_4H_8N_2O_2$	116	d_{14}-NDPA	86 87†
N-nitrosopyrrolidine	NPYR	$C_4H_8N_2O$	100	d_{14}-NDPA	55
N-nitrosopiperidine	NPIP	$C_5H_{10}N_2O$	114	d_{14}-NDPA	69
N-nitrosodi-*n*-butylamine	NDBA	$C_8H_{18}N_2O$	158	d_{14}-NDPA	57 103†

* Methanol used as CI reagent; quantitation ions are also used for acetonitrile as CI reagent unless otherwise noted (see below).
† Acetonitrile used as CI reagent.

ionization, resulting in less sample molecule fragmentation and consequently a higher concentration of the desired precursor ion. The fragmentation process is induced by a waveform applied to the trap, which increases the energy of the isolated precursor ion. The amplitude of this waveform is called the collision induced dissociation (CID) excitation amplitude. As the energy of the precursor ion increases, chemical bonds are broken and product ions (daughter ions) of lower mass than the precursor ion are formed. Quantitation is performed by using the product ions shown in Table 6450:I. Use of the product ions is of particular importance in the analysis of wastewater samples or matrices with interfering compounds.

Identification of NDMA and the other nitrosamines is based on their retention times, parent ion isolation, and fragmentation patterns. Further information about this method is available elsewhere.[1,2]

b. Interferences: Method interferences may be caused by contaminants, especially from NDMA in solvents, reagents (including reagent water), sample bottles and caps, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in the chromatograms. The samples or analytical system also may be contaminated by rubber products in the work area.

Preserve samples properly to avoid inaccurate target analyte recovery due to formation of nitrosamines caused by the presence of residual chloramine.

Clean all glassware meticulously by washing with detergent and tap water, rinsing with tap water, and rinsing again with reagent water. A final solvent rinse may be needed. In place of a solvent rinse, nonvolumetric glassware can be heated in a muffle furnace at 400°C for 2 h. Rinse volumetric glassware with methanol and dry in a fume hood. Store glassware in an inverted position or capped with aluminum foil to prevent contamination.

Condition and store resin as in 6450B.4*d*. Proper resin storage is critical because evidence shows that nitrosamines can be transmitted to the resin through the gaseous phase.

Coeluting GC peaks with nominal masses equivalent to the target analytes and internal standards have been observed in potable water and wastewater samples, requiring MS/MS or high-resolution MS for quantitation. Surfactants and other organic contaminants in wastewater may cause emulsions during extraction. Analyze a reagent-water blank under the same conditions as the samples to demonstrate an absence of interferences. NDMA has been found in deionized (DI) water at levels up to 10 ng/L; therefore, use an ultraviolet water purification system to avoid contamination. The blank concentration must be equal to, or less than, one-half the minimum reporting level (MRL) or one and one-half the MDL value.

Carryover may be observed during GC analysis. To avoid contamination, a solvent injection may be required between high-concentration and low-concentration sample analyses.

c. Safety: Because DCM and most nitrosamines, including NDMA, have been identified as animal carcinogens and some as probable human carcinogens, minimize exposure to these compounds and their isotopically labeled analogs. Keep a reference file of current material safety data sheets (MSDS), and make it available to all personnel involved in analyses.

Pass effluents of GC sample splitters and GC/MS vacuum pumps through a column of activated carbon, bubble effluents through a trap, or use some other means to remove possible contamination.

The following precautions for safe handling of NDMA and other nitrosamines in the laboratory are presented as guidelines only. Additional information is available elsewhere.[3,4]

Use laboratory hood, safety glasses, disposable plastic gloves, and apron or lab coat. Thoroughly wash hands and forearms after each sample analysis. If contact of personnel with the chemicals occurs, scrub the affected area with any mild soap. Wash contaminated glassware, tools, and surfaces with detergent and water. Minimize solvent waste during the analytical process. Handling the dilute solutions normally used in analytical work presents no significant inhalation hazards except in case of an accident.

d. Detection levels: Method detection levels (MDL) (see Section 1030C) for the analytes (Table 6450:I) listed using the SPE method in deionized (DI) water are provided in Table 6450:II. Prepare at least seven replicates of laboratory-fortified blanks with nitrosamine standards at a concentration of 1 to 5 ng/L and extract over a 3-d period. Calculate mean recovery and standard deviation for each analyte. Multiply the Student *t* value at the 99% confidence level and n-1 degrees of freedom (e.g., 3.143 for seven replicates) by the standard deviation to yield a statistical estimate of the MDL.

Table 6450:II. Method Detection Levels for Nitrosamines in Reagent Water,* Solid-Phase Extraction

Nitrosamine	Fortification Level *ng/L*	MDL *ng/L*
NDMA	1.0	0.84
NMEA	1.0	0.45
NDEA	1.0	0.81
NDPA	1.0	1.08
NMOR	1.0	0.62
NPYR	1.0	0.83
NPIP	1.0	0.74
NDBA	1.0	0.71

* For $n=10$, $t=2.821$ at the 99% confidence level. SPE, HP-VOC column, methanol CI, Ambersorb resin.
(Courtesy of WateReuse Foundation.)

2. Sampling and Storage

This section describes sampling and storage protocols. More detailed information on sampling and storage can be found in other reports.[5,6]

a. Sample collection: Ensure that sample site is free of automotive exhaust, cigarette smoke, fresh paint, and any other possible source of contamination. Sample location should provide for collection of a representative grab sample or composite (maintained cold and with proper disinfectant residual quenching).

When sampling from a water tap, let tap run until the water temperature has stabilized (usually about 3 to 5 min). When sampling from an open body of water, collect sample in a clean stainless steel bucket with a clean rope. Avoid plastic and rubber tubing, gaskets, or other equipment that may leach interfering analytes into the water sample.

Collect samples in pre-cleaned amber glass bottles with TFE-lined polyethylene caps. Collect sufficient sample volume for the analysis of the sample, a laboratory-fortified matrix (LFM), and LFM duplicate for quality assurance purposes, preferably in two or more bottles. Fill sample bottles to the top, but take care not to flush out the preservative.

When sampling chlorinated or chloraminated water, quench the residual at time of sampling. Add solid sodium sulfite or sodium thiosulfate (approximately 500 mg for wastewater, or 40 to 100 mg for potable water) to a 1-L bottle to minimize additional nitrosamine formation. For potable water samples, 20 mg of ascorbic acid may be used. Use more quenching agent if the chloramine residual is >4 mg/L. Do not rinse sample bottle with sample before collection if bottle has been prepared with preservatives before sampling.

b. Storage: Ice or refrigerate samples at 6°C or lower (but not freezing) and maintain at these conditions, away from light, until extraction. To prevent photo-decomposition, protect samples from light from the time of collection until extraction; amber-colored, glass bottles work well for this. Avoid storing samples under low pH conditions because these conditions have been observed to produce elevated levels of NDMA in some wastewater effluents and potable waters. Degradation and formation of nitrosamines in wastewater matrices can be a complicated process and may vary from situation to situation. Therefore, select appropriate holding times on the basis of case-specific data. As a general guideline, extract potable water samples within 28 d and wastewater samples within 14 d.

Store extracts at ≤10°C and keep them away from light in amber glass vials with TFE-lined caps. Under the conditions used during the method development, archived extracts generally show minimal NDMA loss over 6 months.

3. Apparatus

a. Sample containers: 1-L amber glass bottles fitted with PTFE-lined screw caps.

b. Standard solution storage containers: 10- to 20-mL amber glass vials with PTFE-lined screw caps.

c. Vials: Screw-cap 2.0-mL amber glass autosampler vials with PTFE-faced septa.

d. Volumetric flasks: Class A, various sizes used for preparation of standards and samples.

e. Microsyringes, glass with stainless steel needle and plunger in various sizes for adding solutions and preparing intermediate solutions.

f. Filters, disposable, used to isolate resin from the water samples. Use either paper* or glass fiber† filters.

g. Balance, analytical, capable of accurately weighing to 0.0001 g.

h. Aluminum dishes, disposable.

i. Mixing apparatus‡ capable of shaking samples at 50 rpm and accepting 1-L bottles. Alternatively, an orbital shaker capable of 200 rpm may be used for the 2-h extraction.

j. Vacuum filtration apparatus.

k. Gas chromatograph: Capillary GC with split/splitless temperature-programmable injector capable of large-volume injections. GC oven and injector should be able to maintain 35°C.

l. Chromatographic column:§ Several types of capillary columns‖ have been used successfully.

m. Autosampler: The autosampler used during method development# was equipped with a side-port needle capable of slow injection (approximately 0.2 μL/s) of 8-μL volumes. Other autosamplers capable of injecting this volume may be used.

n. Detector: Ultra-trace mass spectrometer, capable of chemical ionization (CI), and tandem mass spectrometry with the sensitivity to detect low part-per-billion levels of NDMA. One such system,** using methanol or acetonitrile CI, was used for development of this method.

4. Reagents

Use chemicals that are reagent-grade or better and high resolution-gas chromatography (HR/GC)-grade solvents. Unless

* Whatman, 55 mm, No.1 (11-μm, Cat. No. 1001 055), No. 4 (20- to 25-μm, Cat. No. 1004 055), or equivalent.
† Whatman GF/F, 0.7-μm, No. 1825 047, or equivalent.
‡ LE 2002 Heavy Duty, Lab-Line Instruments, Melrose Park, IL, or equivalent.
§ Gas chromatographic methods are extremely sensitive to the materials used. Mention of trade names by *Standard Methods* does not preclude the use of other existing or as-yet-undeveloped products that give *demonstrably* equivalent results.
‖ HP-VOC (used in method development), 60 m long × 0.32-mm ID, 1.8-μm film thickness; DB1701, 30-m-long × 0.25-mm-ID, 1.0-μm film thickness; DB-VRX, 60-m-long × 0.32-mm-ID, 1.8-μm film thickness.
Varian 8200cx, Varian, Inc., Palo Alto, CA.
** Varian Saturn 2000, Varian, Inc., Palo Alto, CA.

otherwise indicated, ensure that all reagents conform to the specifications of the American Chemical Society's *Reagent Chemicals*,[7] where such specifications are available.

a. Reagent water: Use purified water that does not contain target analytes or interfering compounds at levels greater than one half the MRL for each compound of interest.

b. Methanol (CH_3OH), high-purity, demonstrated to be free from analytes or interferences.

c. Dichloromethane (DCM) (CH_2Cl_2), high-purity, demonstrated to be free from analytes or interferences. An alkene-stabilized reagent is preferable to a cyclohexene-stabilized reagent because of possible interferences in the chromatogram during analysis.

d. Carbonaceous adsorbent resin:†† Condition resin in a shallow tray at a temperature >250°C (300°C recommended) for 3 h before use and store in a capped amber glass bottle in a desiccator. To ensure a uniform size distribution of beads, sieve with a No. 50 ASTM mesh or let the finer particles settle in the storage container and take beads from the upper portion only.

e. Helium, ultra-high-purity (UHP), as GC carrier gas.

f. Carbon dioxide, completely dry, with siphon tube, for injector cryogenics.

g. Nitrogen, UHP grade, for autosampler pneumatics.

h. Sodium thiosulfate ($Na_2S_2O_3$) or *sodium sulfite* (Na_2SO_3) for use as dechlorinating agent.

i. Nitrosamine stock standard solutions: Prepare from pure standard materials or purchase as certified solutions, available at concentrations of 100 to 5000 μg/mL. To prepare a stock standard from a pure material, partially fill a volumetric flask with methanol. Allow flask to equilibrate, weigh to nearest 0.1 mg, add desired quantity of pure standard material with a microsyringe, and reweigh. Dilute to volume, stopper, and mix by inverting three times. Calculate concentration of stock standard from net gain in weight. When compound purity is assayed to be 96% or greater, use the weight without correction to calculate concentration of stock standard. Store stock standard solution in an amber glass container at a temperature below 0°C for up to 3 months.

j. Nitrosamine primary dilution standard (PDS): Prepare a nitrosamine mix PDS of a suitable concentration by accurately transferring the appropriate volume of stock standard solution into a volumetric flask partially filled with methanol. Dilute to volume, mix thoroughly, transfer to an amber glass vial, and store at ≤6°C. For example, a 1000 ng/mL PDS may be prepared in 25-mL volumetric flasks and used to make a 100-ng/mL solution through serial dilutions, which can then be used for low-level known additions.

k. Nitrosamine internal standard and surrogate primary dilution standard (ISPDS): Use d_6-NDMA, d_{14}-NDPA, and $^{15}N_2$-NDEA‡‡ as internal standards. Prepare an internal standard and surrogate mix PDS of suitable concentration by accurately transferring the appropriate volumes of internal standard and surrogate stock solutions into a volumetric flask containing methanol as described in ¶ *j* above. For example, 1000 ng/mL PDS of internal standard and surrogate were prepared in 25-mL volumetric flasks and stored in an amber glass vial at ≤6°C.

†† Ambersorb® 572, Supelco P/N: 10432-U.
‡‡ Cambridge Isotopes, Cambridge, MA, or equivalent.

TABLE 6450:III. PROCEDURAL CALIBRATION STANDARDS*

Calibration Standard Concentration *ng/L*	Volume of 100-ng/mL Nitrosamine Mix *μL*	Volume of 1000-ng/mL Nitrosamine Mix *μL*	Volume of 1000-ng/mL Internal Standard and Surrogate Mix *μL*
1	5	—	10
2	10	—	10
5	25	—	10
10	—	5	10
20	—	10	10
50	—	25	10
100	—	50	10
300	—	150	10

* Add tabulated volumes of analyte and internal standard/surrogate PDS into 500 mL reagent water to obtain the different concentrations of calibration standards.
(Courtesy of WateReuse Foundation.)

l. Nitrosamine calibration standards: This method uses a procedural standard calibration curve. Prepare and extract solution to produce calibration curve with at least five points, as outlined in 6450B.5*c*. Fortify a series of 500-mL reagent water samples contained in 1-L amber bottles with the PDS and the ISPDS to produce a calibration curve ranging from 2 to 300 ng/L with internal/surrogate standards at 20 ng/L, as is shown in Table 6450:III.

5. Procedure

a. Sample extraction by solid-phase extraction (SPE): Remove samples from storage and allow to equilibrate at room temperature. Transfer 500 mL sample with a clean Class A graduated cylinder into designated 1-L amber glass bottles. QC samples, including method blanks, calibration standards, continuing check standards, duplicates, and laboratory-fortified matrices, are prepared in the same manner. If sample is a method standard, laboratory-fortified blank (LFB), laboratory-fortified sample matrix (LFSM), or LFSM duplicate (LFSMD), add appropriate volume of intermediate nitrosamine mix (in methanol).

Add a portion of internal standard and surrogate ISPDS that results in a 20-ng/L final concentration (for example 10 μL of a 1000 ng/mL ISPDS). Place needle of syringe below water surface when adding standards to an aqueous sample. After injection, cap bottle and invert to allow for mixing. Wipe needle between samples and after standards to prevent cross-contamination of the ISPDS.

Add 200 mg resin (6450B.4*d*) to the sample, place bottles in mixing apparatus (6450B.3*i*), and rotate for 2 h at 50 rpm in a rotator apparatus or 200 rpm in an orbital-type shaker.

Rinse the filter paper and the filter holder with laboratory water before filtering the sample. Filter the sample through filter paper (6450B.3*f*) with the aid of a vacuum system under a hood to isolate the resin from the water.

To ensure complete transfer of resin, rinse walls of bottle thoroughly with reagent water (a squeeze bottle can be used for

Table 6450:IV. Gas Chromatograph Injection Program Temperature Conditions for Nitrosamine Analyses

Column	Temperature °C	Rate °C/min	Hold Time min	Total Time min
HP-VOC	37	0	0.7	0.7
	250	200	27.0	28.7
DB-1701	35	0	0.8	0.8
	260	200	2.1	4.0
	150	200	21.0	25.6

Note: Injection volume, 8 μL; plunger inject speed, 0.2 μL/s; post-injection delay, 99 s.

Table 6450:V. Gas Chromatograph Injection Program Split Conditions for Nitrosamine Analyses

Time min	Split State	Split Ratio
0.0	On	5
0.8	Off	—
2.2	On	100
20.0	On	30

(Courtesy of WateReuse Foundation.)

Table 6450:VI. Gas Chromatograph Column Conditions for Nitrosamine Analyses

Column	Temperature °C	Rate °C/min	Hold Time min	Total Time min
HP-VOC	32	0	1.7	1.7
	100	15	2	8.2
	190	5	0	26.2
	270	50	5	30.0*
DB-1701	35	0	4	4
	200	15	0	15
	240	40	10	26

* May be increased depending on sample content.

this purpose) and add to collected resin on the filter. Leave resin in the filtration apparatus under vacuum for approximately 5 min to remove as much water as possible.

With forceps, transfer the filter paper containing the resin to a disposable aluminum dish. Air-dry resin for at least 45 min under the hood or under a gentle stream of dry helium to minimize contamination. Transfer dry resin to a 2-mL autosampler vial and cap vial. Store vials containing resin in the refrigerator or freezer until time of analysis.

Just before analysis, remove vials from refrigerator, immediately uncap, and add 400 μL of DCM with a microsyringe. Heat is released when the solvent comes into contact with the resin. To minimize this exothermic reaction, slowly deliver DCM along the walls of the cold vial.

Cap the vial, gently shake to allow contact between resin and solvent, and tap vial to expel air bubbles from resin.

Allow a half-hour contact time between resin and DCM to ensure that desorption equilibrium has been reached.

Load vials onto autosampler tray for analysis, and set for 8-μL injection (suggested volume). The MS instrument conditions given in this method are set for an 8-μL injection volume. Adjust instrument parameters as necessary if other injection volumes are used.

b. Gas chromatography/mass spectrometry: Operating conditions for several capillary columns are shown in Tables 6450:IV and V (injection parameters), and Table 6450:VI (column parameters).

For the CI analysis, default ionization parameters and a list of typical ionized forms of various CI reagents and modified ionization parameters for enhanced sensitivity are shown in Table 6450:VII.

Various parameters, including collision–induced dissociation (CID) values, and precursor and product ions, are shown in Table 6450:VIII (methanol CI), and Table 6450:IX (acetonitrile CI). CID values were selected to maximize the transition from precursor ion to product ions while retaining a minimum of the precursor ion for confirmation. Because each instrument exhibits different sensitivity, the percentage of product ion required to achieve the desired sensitivity will vary. Therefore, follow the general guideline of maximizing the percentage of product ions while retaining a minimal amount of the precursor ion for confirmation. In some instances, the degree of fragmentation has also been observed to depend on analyte concentration. Other GC/MS/MS conditions may be used if QC requirements are met. For each target and surrogate analyte, establish an appropriate retention-time window and precursor to product ion mass ratio to facilitate detection and identification in all QC and field samples.

A representative nitrosamine chromatogram is presented in Figure 6450:1.

c. Calibration: Prepare standards as described in 6450B.4*i–l*. Extract and analyze each standard under the same conditions used for sample extracts, using internal standards designated in Table 6450:I for quantitation of each nitrosamine. A relative response factor (RRF) (equal to the relative signal area ratio of the nitrosamine of interest versus that of the corresponding nitrosamine internal standard) is used in the isotope dilution/internal standard quantitation technique. The concentration of a specific nitrosamine in a sample is determined by comparing the RRF for the nitrosamine in the sample with added internal standard to the average RRF value determined for the set of calibration standards. C/MS software can be used to generate a linear regression or quadratic calibration curve plotting absolute area ratios (nitrosamine of interest/nitrosamine internal standard) against known-addition concentration for each nitrosamine. The coefficient of determination (r^2) for the plot should round to 0.995 or higher, and curves may be linear from the MRL up to 500 ng/L. A representative NDMA calibration curve is shown in Figure 6450:2. Verify all initial calibrations with a standard from a second source.

d. Continuing calibration: To perform continuing calibration checks, verify the initial calibration by preparing and extracting a midpoint calibration standard with each batch of 10 samples or less. Analyze continuing calibration standards at the beginning and end of each batch of 10 samples or less. If the sample run contains more than 10 samples, analyze the continuing calibration standard after every 10 samples in the batch.

TABLE 6450:VII. CHEMICAL IONIZATION SETTINGS*

Variable	Default Settings		Modified CI Settings	
	Acetonitrile (liquid)	Methanol (liquid)	Acetonitrile (liquid)	Methanol (liquid)
Molecular mass, g/mol	41	32	41	32
Maximum ionization time,† μs	2000	2000	2000	2500
Maximum reaction time,‡ msec	40	40	120	120
CI storage level,§ m/z	25	15	19	15
CI background mass,‖ m/z	65	45	40	45
Reagent ion eject,# V	9.0	10.0	15.0	10.0
Typical reagent ions/radicals	CH_3CN^+, CH_2CN^+, CH_4CN^+, CH_3CHCN^+	CH_3OH^+, CH_2OH^+, CH_3^+, $OH^•$, $H^•CHO^+$		

* Pressure must be adjusted for different reagent gases, and changes in CI reagent may require GC system to be modified by the vendor.
† The time electrons are allowed to react with reagent gas molecules, set from 10 to 2500 μs, which should generally be 1/10 of the EI ionization time. Increasing maximum reaction time may result in a larger peak signal area and consequently better sensitivity.
‡ How long reagent gas ions can react with sample molecules, set from 1 to 120 ms. Increasing maximum reaction time may increase peak signal area, improving sensitivity.
§ The smallest mass stored in the trap during ionization, set from 0 to 50 m/z, usually much smaller than the reagent-ion mass.
‖ Greater than or equal to the largest reagent ion. Masses less than this are ejected from the trap after reaction. If too low, unwanted reagent ions can cause ionization time to be reduced by the automatic reaction control (ARC) scan function. Therefore, significant peaks at the low end of a spectrum should be eliminated.
In selected chemical ionization (SECI), a low mass eject ejection cutoff slightly higher than the mass of the largest reagent ion produced is set to remove unwanted radical cations between ionization and reaction periods without affecting reagent ions. This can be checked by lowering CI background mass and then lowering the ion eject amp to the point where reagent ions are diminished. Restore CI background mass and verify that reagent ions are no longer present.

TABLE 6450:VIII. METHANOL CI/MS/MS CONDITIONS

Segment *min*	Analyte	Retention Time *min*	Precursor Ion *amu*	CID *mV*	Mass Range *amu*	Select Product Ion *amu*	Internal Standard	Relative Response Factor (RRF) *unitless*
0–10.5	Fil/Mul delay	—	—	—	—	—	—	—
10.5–12.0	d_6-NDMA	11.0	81	0.60	40–85	50	—	—
	NDMA	11.1	75	0.68	—	44	d_6-NDMA	1.4
12.0–14.5	NMEA	13.6	89	0.70	50–95	61	$^{15}N_2$-NDEA	4.7
14.5–17.0	$^{15}N_2$-NDEA	16.0	105	0.65	65–110	77	—	—
	NDEA	16.0	103	0.77		75	$^{15}N_2$-NDEA	1.2
17.0–21.0	None							
21.0–23.2	d_{14}-NDPA	21.6	145	0.77	50–160	97	—	—
	NDPA	21.9	131	0.74		89	d_{14}-NDPA	0.4
	NMOR	22.3	117	0.70		86	d_{14}-NDPA	1.0
	NPYR	22.3	101	0.70		55	d_{14}-NDPA	0.8
23.2–25.0	NPIP	23.9	115	0.49	50–120	69	d_{14}-NDPA	1.6
25.0–28.8	NDBA	28.2	159	0.60	50–165	57	d_{14}-NDPA	0.2

NOTE: Ionization mode: CI auto; ion prep: MRM; isolation window: 2; waveform type: resonant; HP-VOC column. All other values set at instrument-recommended or default values.
(Courtesy of WateReuse Foundation.)

TABLE 6450:IX. ACETONITRILE CI/MS/MS CONDITIONS

Segment *min*	Analyte	Retention Time *min*	Precursor Ion *amu*	CID *mV*	Mass Range *amu*	Select Product Ion *amu*	Internal Standard	Relative Response Factor (RRF) *unitless*
0–8.5	Fil/Mul delay	—	—	—	—	—	—	—
8.5–10.5	d_6-NDMA	9.2	81	0.36	40–83	49	—	—
	NDMA	9.2	75	0.34		44	d_6-NDMA	0.5
10.5–12.5	NMEA	11.4	89	0.31	40–91	61	$^{15}N_2$-NDEA	1.4
12.5–15.5	$^{15}N_2$-NDEA	13.3	105	0.33	40–107	77	—	—
	NDEA	13.3	103	0.34		75	$^{15}N_2$-NDEA	0.7
15.5–18.8	d_{14}-NDPA	18.2	145	0.33	40–150	97	—	—
	NDPA	18.4	131	0.34		89	d_{14}-NDPA	0.6
18.8–19.6	NMOR	19.4	117	0.30	40–125	87	d_{14}-NDPA	1.1
19.6–20.2	NPYR	20.0	101	0.33	40–106	55	d_{14}-NDPA	0.9
20.2–21.5	NPIP	20.6	115	0.34	40–120	69	d_{14}-NDPA	2.0
21.5–24.5	NDBA	23.8	159	0.37	40–165	103	d_{14}-NDPA	0.7

NOTE: Ionization mode: CI auto; isolation window: 2; waveform type: resonant; DB-1701 column. All other values set at instrument-recommended or default values.

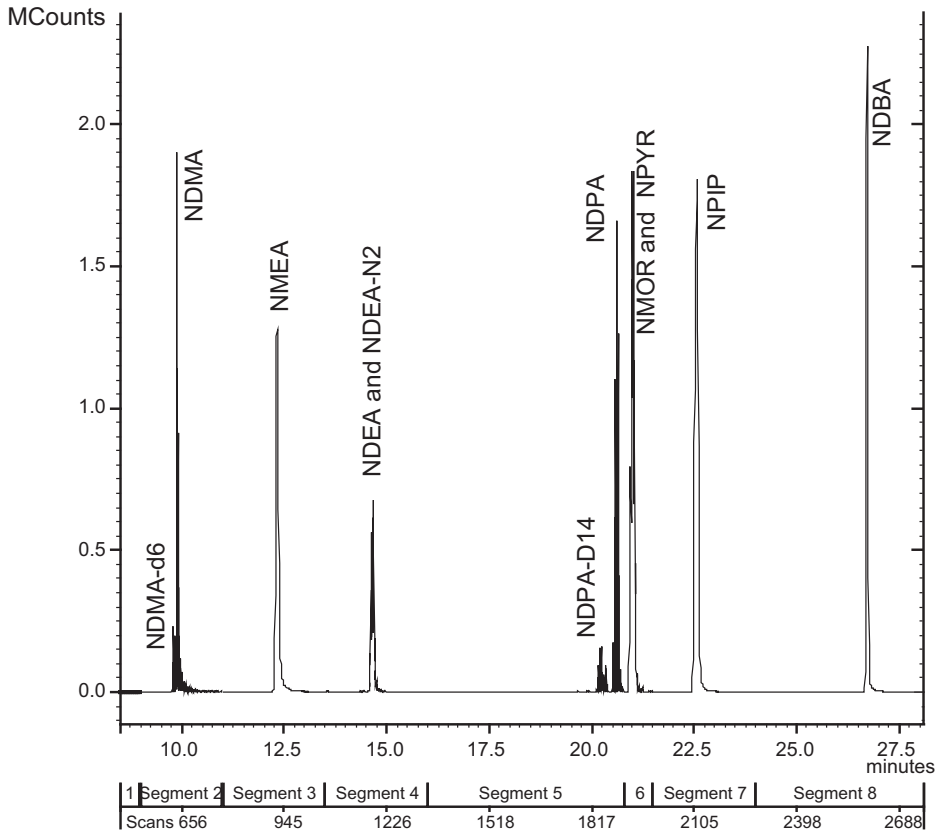


Figure 6450:1. Typical chromatogram of a nitrosamine mix (200 μg/L); solid-phase extraction, HP-VOC column, methanol CI. (Courtesy of WateReuse Foundation.)

6. Data Analysis and Calculations

a. Review: Check the chromatogram for any incorrect peak identification or poor integration.

b. Identification: Identify analytes in a sample by comparing retention times from the GC chromatogram and *m/z* ratios from the mass spectrum to those of the reference analyte in the calibration standard. The internal standard determination must meet the criteria specified in 6450B.7*g*. All ions present in the reference mass spectrum above 30% should be present in the sample mass spectrum at intensities within ±20% of the reference mass spectrum.

c. Quantitation: Calculate sample concentration with the best-fit calibration equation or the average RRF as explained in 6450B.5*c*. Quantitate samples that fall between the MRL and the highest calibration standard. If the analyte concentration determined exceeds the highest standard concentration, dilute the original sample into specified final volume, re-extract, and re-analyze. Never dilute the extract.

d. Correction: Adjust calculated concentrations of detected analytes to reflect any dilutions performed.

e. Reporting: Report analyte concentrations in nanograms per liter (ng/L) or as specified by the data user.

7. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 6020:I.

The following are minimum quality control measures: method detection level (MDL) study, acceptable calibration curve, second-source verification, continuing calibration checks, low-level calibration checks [at or near the minimum reporting level (MRL)], reagent method blanks, and laboratory-fortified sample matrices. Additional quality control parameters including field duplicate samples, are recommended for ongoing quality assurance for each batch of samples. A *sample batch* is defined as a maximum of 10 samples, excluding the samples used for quality control. If no analytes are detected, the retention times (RTs) and the responses of the internal standards (ISs) must be verified to ensure that analytes did not shift outside of their MS/MS window.

a. Laboratory reagent blank (LRB): Extract and analyze a laboratory reagent blank with each sample batch to demonstrate that the preparation procedure, glassware, reagents, and instrument system are free from nitrosamine contaminants. Analyze the LRB daily, before analyzing any samples or standards. The LRB results should be less than one-half the MRL; if results are above this criterion, examine the blank samples and take steps to meet this requirement.

b. Calibration checks: Initial demonstration consists of an acceptable calibration curve for which the relative standard deviation (RSD) is ≤20% (standard deviation/average value × 100%), or, if a regression analysis is used, $r^2 \geq 0.995$. Establish an initial demonstration of an acceptable calibration

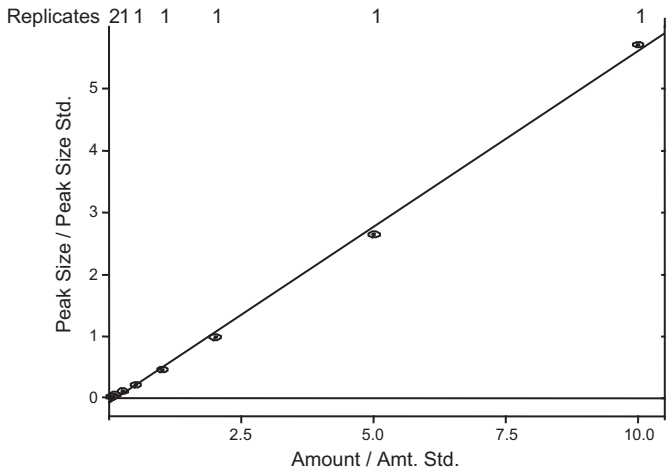


Figure 6450:2. Calibration curve for solid-phase extraction of NDMA (2–100 ng/L). (HP-VOC column, methanol CI.) (Courtesy of WateReuse Foundation.)

curve following the procedure outlined 6450B.4*j*, *k*, and *l*, and 5*c*. Verify the calibration with an extracted standard prepared from a source separate from that used to prepare the initial calibration standards. The results of the analysis must be within ±20% of the target value.

c. Continuing calibration: The acceptable response factor (RF) should be within 20% of the RF_{mean} from the initial calibration or within 20% of the true value if the calibration was performed by linear regression. No sample re-extraction or continuing calibration is needed if the RF or measured concentration is >20% above the target value and the associated samples show no detection for nitrosamines. In this instance, the laboratory may report "not detected" for these samples. However, if the measured concentration is >20% above the target value and nitrosamines are detected in these samples, prepare a fresh standard and re-extract together with the associated samples, being careful not to exceed permissible holding times.

d. MDL determination: Prepare and extract at least seven replicates of a laboratory fortified blank (LFB) containing the nitrosamines at or near the MRL over three or more days. The mean recovery should be within 50% of the true value and the relative standard deviation should be <20%. Process the LFB solution through the full method with each batch of samples as a MRL confirmation.

e. Laboratory-fortified sample matrix (LFSM) and laboratory-fortified sample matrix duplicate (LFSMD): The LFSM is used to determine that the sample matrix does not adversely affect method accuracy. Extract and analyze a LFSM with each batch of 10 samples or less. If more than 10 samples are extracted, also prepare a laboratory-fortified sample matrix duplicate (LFSMD). To prepare the LFSM, add an appropriate amount of the nitrosamines standards to the sample. If known historical samples are used, select a concentration that is greater than or equal to the matrix background concentration. Calculate percent recovery (*R*) for each analyte using the equation:

Table 6450:X. Absolute Recovery of Nitrosamines in Reagent Water Fortified at 100 ng/L, Solid-Phase Extraction

Nitrosamine	Recovery %
NDMA	56
NMEA	100
NDEA	118
NDPA	124
NPYR	99
NMOR	99
NPIP	108
NDBA	103

Note: SPE, HP-VOC column, methanol CI, Ambersorb resin. (Courtesy of WateReuse Foundation.)

$$R = \frac{(A - B)}{C} \times 100$$

where:

A = measured concentration in the fortified sample,
B = measured concentration in the unfortified sample, and
C = fortification concentration.

For samples fortified at or above their native concentration, recoveries should range between 70 and 130%.

Calculate the relative percent difference (RPD) for each analyte using the equation:

$$RPD = \frac{(LFSM - LFSMD)}{(LFSM + LFSMD)/2} \times 100$$

The RPD should be less than or equal to 50% if the measured concentrations in the FSM and LFSMD pair are ≤2 times the MRL. For higher concentrations, the RPDs should be less than or equal to 20%. Greater variability may be observed when LFSMs are fortified to a concentration less ≤2 times the MRL. The RPDs should fall in the range of ≤50% for samples fortified at the MRL level.

f. Sample duplicate (LD1, LD2): If historical trends are not available, prepare and analyze sample duplicates for each batch of 10 samples or less to assess method precision. Calculate relative percent difference (RPD) for the duplicate measurement, (LD1 and LD2) by the equation:

$$RPD = \frac{(LD1 - LD2)}{(LD1 + LD2)/2} \times 100$$

The RPD should be ≤50% if the measured concentrations in the LD1 and LD2 pair are less than 2 times the MRL. For higher concentrations, the RPDs should be ≤20%.

g. Internal standard: The absolute area of the quantitation ion of the internal standards in the samples should not deviate by more standard or >50% from the initial calibration standard. The retention window should be stable to within 0.2 min in a sample batch. Monitor the internal standard's area and retention time for each batch of samples. If the internal standard criterion is not met, take steps up to and including re-extraction.

TABLE 6450:XI. SINGLE-LABORATORY BIAS AND PRECISION DATA FOR NITROSAMINES ADDED TO POTABLE AND SECONDARY EFFLUENT WATERS, SOLID-PHASE EXTRACTION

Sample Type	Nitrosamine	Sample Concentration ng/L	Recovery by Run %				Mean Recovery %	RSD %
			Run 1	Run 2	Run 3	Run 4		
Chloraminated potable surface water	NDMA	<1	104	101	100	99	101	2.2
	NMEA	<1	108	102	102	101	103	2.5
	NDEA	<1	106	101	101	98	101	1.9
	NDPA	<1	103	95	104	101	101	4.6
	NPYR	<1	104	88	93	92	94	3.8
	NMOR	<1	99	101	100	98	100	1.1
	NPIP	<1	97	103	98	92	97	2.7
	NDBA	1.1	107	101	99	91	99	3.6
Secondary wastewater effluent	NDMA	93	104	106	102	107	105	0.9
	NMEA	<4	75	66	71	70	71	4.2
	NDEA	9.4	101	100	106	95	101	3.3
	NDPA	<4	102	102	109	110	106	3.0
	NPYR	<4	95	90	101	83	92	6.9
	NMOR	5.9	100	95	99	85	95	6.6
	NPIP	<4	98	99	105	104	102	2.9
	NDBA	<4	101	102	107	104	104	2.1

NOTE: Samples of each type (n=4), added concentration = 20 ng/L. Methanol used as CI reagent, Ambersorb resin.
(Courtesy of WateReuse Foundation.)

TABLE 6450:XII. INTERLABORATORY BIAS AND PRECISION DATA FOR NITROSAMINES ADDED TO POTABLE SURFACE WATER AND SECONDARY WASTEWATER EFFLUENT, SOLID-PHASE EXTRACTION

Sample Type	Nitrosamine	Sample Concentration ng/L	Recovery by Laboratory %			Mean Recovery %	RSD %
			1	2	3		
Chloraminated potable surface water	NDMA	4.4	101	91	91	94	5.8
	NMEA	<2	86	81	133	100	28.7
	NDEA	<2	95	93	98	96	2.8
	NDPA	<2	90	106	85	94	10.8
	NPYR	<2	94	93	113	100	11.1
	NMOR	<2	127	148	142	139	10.8
	NPIP	<2	90	80	101	90	10.6
	NDBA	<3	91	94	101	95	4.9
Secondary wastewater effluent	NDMA	156	121	114	83	106	20.1
	NMEA	<2	93	118	107	106	12.7
	NDEA	<11	113	88	91	97	13.6
	NDPA	<2	99	102	81	94	11.2
	NPYR	<2	107	112	88	102	12.9
	NMOR	<5	112	106	77	98	18.8
	NPIP	<2	98	107	91	99	8.2
	NDBA	<3	92	83	91	89	5.1

NOTE: Potable sample observed (n=3), added concentration = 12.7 ng/L; secondary effluent water samples observed (n=3), added concentration = 376 ng/L; Ambersorb resin.
(Courtesy of WateReuse Foundation.)

8. Precision and Bias

Absolute recovery values for nitrosamines recovered from 100-ng/L known-addition reagent water samples are shown in Table 6450:X.

Method precision and accuracy data for a single laboratory are shown in Table 6450:XI for potable water and treated wastewater samples. Interlaboratory precision and accuracy data are shown in Table 6450:XII for potable water and treated wastewater samples.

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6450 C. Micro Liquid–Liquid Extraction GC/MS Method

1. General Discussion

a. Principle: NDMA and other nitrosamines listed in Table 6450:I are analyzed by the micro liquid–liquid extraction (MLLE) method, followed by gas chromatography/tandem mass spectrometry (GC/MS/MS). The term "micro" is used as a comparative term with reference to the standard LLE method, because the micro method uses approximately one-fifth of the standard sample volume and one-ninth of its solvent volume. The advantages offered by this method are the ability to use lesser volumes when the larger amounts required for the standard LLE techniques are not available, the ability to analyze for higher concentrations of nitrosamines (e.g., >10 ng/L), and the use of this method as a screening method. The reduction in the required sample volume can be significant when the sample shipment is considered.

Isotope dilution/internal standard quantitation is implemented with the addition of three isotopically labeled internal standards (i.e., d_6-NDMA, d_{14}-NDPA, and $^{15}N_2$-NDEA) before extraction to correct for extraction, as well as instrument variations. A volume of sample with added sodium chloride is extracted with DCM; the DCM layer is removed, dried with sodium sulfate, and concentrated. A portion of sample extract is injected into a GC/MS operated in the CI (acetonitrile, methanol), MS/MS mode (see 6450B.1*a*). Identification of NDMA and the other nitrosamines is based on their retention times, precursor ion isolation, and fragmentation patterns.

b. Interferences: See 6450B.1*b*.

c. Safety: See 6450B.1*c*.

d. Detection levels: See 6450B.1*d*. Experimentally determined MDL values for the MLLE method are provided in Table 6450:XIII. The concentration range for target analytes in this method was evaluated between 5 and 500 ng/L.

Table 6450:XIII. Method Detection Levels in Reagent Water, Micro Liquid-Liquid Extraction

Nitrosamine	Fortification Level *ng/L*	MDL *ng/L*
NDMA	10.0	1.7
NMEA	10.0	1.7
NDEA	10.0	2.5
NDPA	10.0	2.1
NPYR	10.0	4.4
NMOR	10.0	2.7
NPIP	10.0	1.6
NDBA	10.0	2.2

Note: MDLs were determined by analysis of seven replicates. DB-1701 column, acetonitrile CI used.

2. Sampling and Storage

See 6450B.2.

3. Apparatus

a. Shaker: Reciprocal shaker capable of vigorous shaking (approximately 180 strokes/min)* with custom holder (e.g., wooden block with holes bored to the diameter of the bottles) keeping bottles secure and on their side during shaking.

b. Concentrator with glassware: Heated water bath with inert gas sample evaporation stations and accessories needed to concentrate extract from 20 to 0.5 mL.†

c. Syringe: 20-mL glass hypodermic (glass plunger) syringe with 17-gauge, 9-cm- (3.5-in.-) long stainless steel needle.

d. Transfer pipet‡ with plastic disposable tips.

* Eberbach No. 6010, or equivalent.
† TurboVap II concentration workstation, Zymark Corp., Hopkinton, MA., or equivalent.
‡ Eppendorf, or equivalent.

e. Inert gas: Ultra-high-purity nitrogen with purifying cartridge and low-pressure regulator.

f. Glass bottles, 125-mL, clear, with solid PTFE-lined screw caps.

See 6450B.3*a–e, g, k–n* for additional items.

4. Reagents

a. Sodium sulfate (Na_2SO_4), granular, anhydrous, ACS reagent-grade. Heat at 400°C for 3 h and store at 110°C. Cool in a desiccator before use.

b. Sodium chloride (NaCl), ACS grade. Heat at 400°C overnight and store at 110°C. Cool in a desiccator before use.

c. Acetonitrile (CH_3CN), high purity, demonstrated to be free from analytes or interferences.

See 6450B.4*a–c, e–l* for additional items.

5. Procedure

a. Sample extraction: Remove samples from storage and allow to equilibrate to room temperature. Transfer 100 mL sample or reagent water (for standards, etc.) to a clean 125-mL bottle, using a clean Class A graduated cylinder. If sample is a method standard, laboratory-fortified blank (LFB), laboratory-fortified sample matrix (LFSM), or LFSM duplicate (LFSMD), add appropriate volume of intermediate nitrosamine mix (in methanol).

Add the three isotopically labeled standards (d_6-NDMA, d_{14}-NDPA, and $^{15}N_2$-NDEA) to the sample so the final concentration of each compound is 50 ng/L (e.g., 25 µL of a 200-ng/mL ISPDS). Add 30 g sodium chloride and 20 mL DCM to the sample, cap, and shake immediately to dissolve as much salt as possible. Shake on reciprocal shaker for 10 min. Let sample bottles stand for approximately 15 min to allow layers to separate well.

Withdraw as much of DCM layer as possible from bottle with a 20-mL glass syringe inserted in the DCM layer at the bottom of the bottle and transfer to a 40-mL vial or other suitable glassware.

Dry DCM layer with approximately 0.4 g sodium sulfate for 3 min and transfer DCM fraction to a concentrator tube. Rinse the sodium sulfate twice with DCM and combine with original fraction. Concentrate the solution at 35°C under approximately 70 kPa (10 psi) nitrogen until final volume reaches 0.5 mL. Rinse tube walls with approximately 5 mL of DCM and concentrate again to 0.5 mL.

Transfer concentrate to a 2-mL autosampler vial, store at −11°C, or load onto GC/MS/MS autosampler and inject an 8-µL portion.

b. Gas chromatography/mass spectrometry: See 6450B.5*b*.

A representative nitrosamine chromatogram (displayed in two segments) is presented in Figure 6450:3.

c. Calibration: See 6450B.5*c*. A representative NDMA calibration curve is presented in Figure 6450:4.

6. Data Analysis and Calculations

See 6450B.6.

7. Quality Control

See 6450B.7.

8. Precision and Bias

Absolute recovery values for nitrosamines recovered from 100 ng/L known-addition reagent water samples are shown in Table 6450:XIV.

Method precision and bias data for a single laboratory are shown in Table 6450:XV for potable, recycled, and wastewater samples. Interlaboratory precision and bias data for potable water and secondary wastewater samples are shown in Table 6450:XVI.

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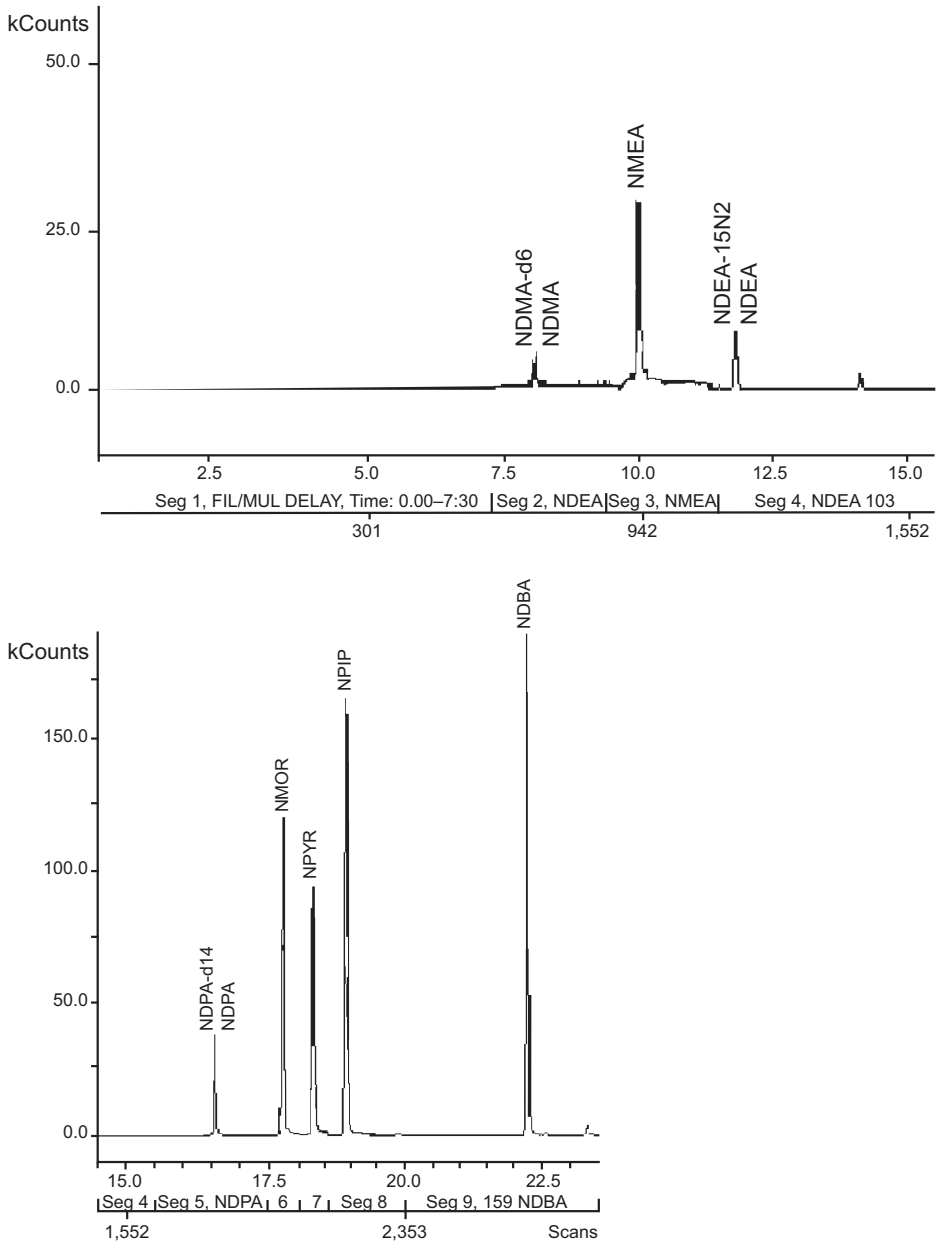


Figure 6450:3. Example chromatogram of 200 ng/L MLLE extracted nitrosamine standard. (above): 0–15 min; (below): 15–23 min, on DB-1701 column, acetonitrile CI.

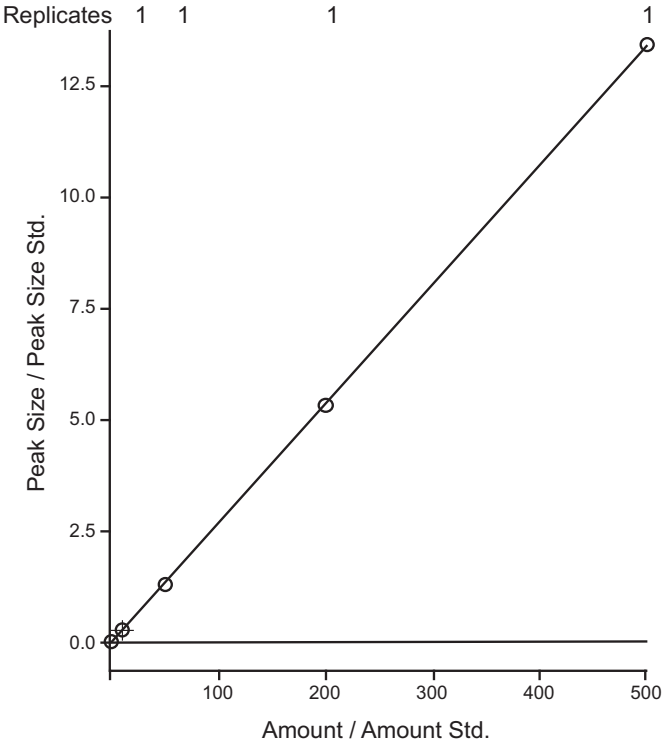


Figure 6450:4. Calibration curve for NDMA by micro liquid–liquid extraction (10–500 ng/L). (DB-1701 column, acetonitrile CI.) The + mark at the 10-ng/L level indicates that manual integration was performed.

TABLE 6450:XIV. ABSOLUTE RECOVERY OF NITROSAMINES IN REAGENT WATER FORTIFIED AT 100 NG/L, MICRO LIQUID-LIQUID EXTRACTION

Nitrosamine	Recovery %
NDMA	30
NMEA	40
NDEA	54
NDPA	51
NPYR	53
NMOR	57
NPIP	57
NDBA	71

NOTE: DB-1701 column, acetonitrile CI used.
(Courtesy of WateReuse Foundation.)

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TABLE 6450:XV. SINGLE-LABORATORY METHOD PRECISION AND BIAS FOR NITROSAMINES, MICRO LIQUID-LIQUID EXTRACTION

	Bias		Precision	
Nitrosamine	Mean Recovery *%*	Standard Deviation *ng/L*	Mean Relative Percent Difference (RPD) *%*	Standard Deviation *ng/L*
NDMA	98	12	10	8.9
NMEA	97	15	12	13
NDEA	92	16	15	15
NDPA	83	19	6.2	3.2
NPYR	93	16	8.3	6.0
NMOR	97	17	11	11
NPIP	87	15	6.2	4.8
NDBA	90	17	13	8.2

NOTE: Drinking water, wastewater, and recycled water, $n=19$, DB-1701 column, acetonitrile CI used.
(Courtesy of WateReuse Foundation.)

TABLE 6450:XVI. INTERLABORATORY BIAS AND PRECISION DATA FOR NITROSAMINES ADDED TO CHLORAMINATED POTABLE SURFACE WATER AND SECONDARY WASTEWATER EFFLUENT, MICRO LIQUID-LIQUID EXTRACTION

			Recovery by Laboratory No. *%*			Mean Recovery	RSD
Sample Type	Nitrosamine	Sample Concentration *ng/L*	1	2	3	*%*	*%*
Chloraminated potable surface water	NDMA	<10	74	117	87	93	24.0
	NMEA	<10	118	86	82	95	20.9
	NDEA	<10	87	80	92	86	6.8
	NDPA	<10	91	91	96	92	3.2
	NPYR	<10	88	92	100	94	6.7
	NMOR	<10	99	113	135	116	15.4
	NPIP	<10	95	86	92	91	5.3
	NDBA	<10	93	92	108	98	9.1
Secondary wastewater effluent	NDMA	142	101	115	90	102	12.0
	NMEA	<20	93	85	88	89	4.7
	NDEA	<20	92	93	118	101	14.8
	NDPA	<20	93	77	100	90	13.2
	NPYR	<14	101	98	132	110	17.4
	NMOR	<20	101	86	115	101	14.3
	NPIP	<20	92	90	94	92	2.0
	NDBA	<20	101	92	100	98	5.0

NOTE: DB-1701 column, acetonitrile CI used. Potable surface water samples observed ($n=3$), added concentration = 13 ng/L. Secondary effluent water samples observed ($n=3$), added concentration = 376 ng/L.
(Courtesy of WateReuse Foundation.)

6610 CARBAMATE PESTICIDES*

6610 A. Introduction

1. Sources and Significance

Carbamates are used as insecticides, nematicides, and aracides to control pests on agricultural crops, as well as to control lawn and garden insects. Their toxicity comes from their ability to act as cholinesterase inhibitors. Residues of several carbamates have been found in groundwater in a number of states.[1,2] Two of the target compounds in this method—carbofuran and oxamyl—are regulated by the U.S. Environmental Protection Agency, with aldicarb and its metabolites (aldicarb sulfoxide and aldicarb sulfone) under consideration for regulation.[3]

2. Selection of Method

High-performance liquid chromatography (HPLC) is the method of choice for analysis for carbamates, many of which are thermally labile. This HPLC analysis method is an updated version of a previous method developed using direct injection, post-column derivatization, and fluorescence detection to yield sensitivity and selectivity while keeping sample preparation to a minimum.[4–6] This method is suitable for the analysis of the following carbamate compounds and metabolites in drinking waters: aldicarb sulfoxide, aldicarb sulfone, oxamyl, methomyl, 3-hydroxycarbofuran, aldicarb, propoxur, carbofuran, carbaryl, 1-naphthol, and methiocarb.

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* Approved by Standard Methods Committee, 2004. Editorial revisions, 2011. Joint Task Group: 21st Edition Supplement—Steven C. Wendelken (chair), Margarita V. Bassett, Linda F. Henry, David J. Munch, Barry V. Pepich.

6610 B. High-Performance Liquid Chromatographic Method

1. General Discussion

a. Principle: After the addition of a surrogate compound and filtration, water samples are injected directly onto a HPLC and separated by use of a gradient and a C_{18} column. The carbamates analyzable by this method (Table 6610:I), which are generally classified as phenyl and oxime carbamates, all have an *N*-methyl group in common. After chromatographic separation, these compounds are hydrolyzed with 0.05*N* NaOH at 80 to 95°C, yielding a methyl amine which is then reacted with *o*-phthalaldehyde (OPA) and 2-mercaptoethanol (or *N,N*-dimethyl-2-mercaptoethylamine) to form a highly fluorescent isoindole that is detected instrumentally. The external standard technique is used for quantitation.

b. Interferences: Method interferences may be caused by contaminants, especially primary amines and ammonia, in solvents, reagents (including reagent water), sample bottles and caps, and other sample-processing hardware, that lead to discrete artifacts and/or elevated baselines in the chromatograms. The samples or analytical system may be contaminated from being handled with bare fingers. Routinely demonstrate that all items used in analysis are free from interferences under the conditions

Table 6610:I. Detection Levels in Reagent Water

Analyte	Fortification Level $\mu g/L$	Detection Level* $\mu g/L$	Signal-to-Noise Ratio
Aldicarb sulfoxide	0.20	0.059	8:1
Aldicarb sulfone	0.10	0.057	3:1
Oxamyl	0.20	0.065	10:1
Methomyl	0.20	0.050	10:1
3-Hydroxycarbofuran	0.20	0.029	18:1
Aldicarb	0.20	0.026	9:1
Propoxur	0.20	0.037	6:1
Carbofuran	0.20	0.043	9:1
Carbaryl	0.20	0.045	13:1
1-Naphthol	0.20	0.063	10:1
Methiocarb	0.20	0.061	11:1
BDMC (SUR)	2.00	N/A	N/A

* Detection levels were determined by analyzing 7 replicates over 3 d under the conditions outlined in Table 6610:IV with a 1000-μL injection.
N/A = not applicable

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TABLE 6610:II. SINGLE-ANALYST PRECISION AND ACCURACY OF COMPOUND DETECTION IN VARIOUS WATERS AT LOW (0.20 μG/L) AND HIGH (10 μG/L) FORTIFICATION LEVELS*

	Reagent Water				Drinking Water, Surface Water				Drinking Water, Groundwater			
	0.20 μg/L		10.0 μg/L		0.20 μg/L		10.0 μg/L		0.20 μg/L		10.0 μg/L	
Compound	MR%	%RSD	MR%	%RSD	MR%	%RSD	MR%	%RSD	MR%	%RSD	MR%	%RSD
Aldicarb sulfoxide	112	6.2	106	1.8	113	7.0	104	2.8	111	7.3	106	1.1
Aldicarb sulfone	92	9.5	106	2.6	104	5.5	106	1.4	98	9.2	106	0.9
Oxamyl	101	8.6	106	2.2	107	6.4	104	2.2	99	8.4	105	1.2
Methomyl	101	6.5	106	2.9	110	9.8	104	1.6	99	10.2	105	1.4
3-Hydroxycarbofuran	105	6.8	108	1.2	128	3.9	107	1.1	107	3.0	108	0.4
Aldicarb	95	7.4	106	1.3	123	2.7	105	1.5	100	6.3	105	0.6
Propoxur	109	5.9	109	2.0	128	6.0	106	2.1	112	6.1	107	0.8
Carbofuran	112	6.7	110	2.2	140	5.6	105	2.5	112	4.1	107	1.6
Carbaryl	112	7.0	107	2.1	112	9.7	106	0.9	119	5.1	108	1.3
1-Naphthol	113	12.6	108	3.1	113	12.1	101	1.3	109	8.2	109	1.2
Methiocarb	105	5.9	107	1.5	104	13.3	107	1.1	105	3.9	107	1.0
BDMC (SUR)†	108	4.3	101	2.3	108	2.1	96	3.9	109	2.0	97	4.3

* MR% = mean recovery expressed as % recovery; %RSD = percent relative standard deviation.
† Surrogate concentration in all samples was 2.0 μg/L; all data from $n=7$ replicates using a 1000-μL injection volume.

of the analysis by analyzing laboratory reagent blanks. Do not subtract blank values from sample results. Clean all glassware meticulously: wash glassware with detergent and tap water, rinse with tap water, and rinse again with reagent water. A final rinse with solvents may be needed. In place of a solvent rinse, nonvolumetric glassware can be heated in a muffle furnace at 400°C for 2 h. Do not heat volumetric glassware above 120°C.

Samples that are not properly preserved (6610B.2) may yield poor target analyte recovery due to degradation caused by chlorine residual and base-catalyzed hydrolysis at neutral and high pH.

c. Detection levels: Detection levels are compound, instrument, and matrix dependent. The detection level is defined as the statistically calculated minimum concentration that can be measured with 99% confidence that the reported value is greater than zero.[1] Experimentally determined detection levels for the target analytes are provided in Table 6610:I. The detection level differs from, and is lower than, the minimum reporting level (MRL). The concentration range for target analytes in this method was evaluated between 0.2 and 10 μg/L. Precision and bias data are presented in Table 6610:II.

d. Safety: The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; treat each chemical compound as a potential health hazard, and minimize exposure. Handle pure standard materials and stock standards of these compounds with suitable protection to skin and eyes. Take care not to breathe the vapors or ingest the materials. Maintain a current-awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. Make a reference file of MSDSs available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available.[2–4]

2. Sampling and Storage

a. Sample bottle preparation: Use amber glass bottles fitted with polytetrafluoroethylene (PTFE)-lined screw caps. Before shipping sample bottles to the field, add preservatives (as dry solids) to each bottle. To adjust sample pH to ~3.8 to prevent hydrolysis of oxamyl, 3-hydroxycarbofuran, carbaryl, and methiocarb and to guard against biodegradation, add a sufficient amount of potassium dihydrogen citrate ($C_6H_7KO_7$) to yield a concentration in the sample of 9.2 to 9.5 g/L. To eliminate the residual-free chlorine in the samples, which rapidly degrades aldicarb and methiocarb, add sodium thiosulfate ($Na_2S_2O_3$) to yield a sample concentration in the range of 80 to 320 mg/L.

b. Sample collection: Collect grab samples in accordance with conventional sampling practices. Do not pre-rinse sample bottles with sample before collection, because pre-rinsing will wash out the preservatives added to the bottles before shipment.

When sampling from a cold water tap, remove aerator so no air bubbles will be trapped in the sample. Open tap and let system flush until water temperature has stabilized (usually about 3 to 5 min). Collect samples from the flowing system.

Fill sample bottles, taking care not to flush out sample-preservation reagents. Samples do not need to be collected headspace-free. After collecting sample, cap carefully to avoid spillage and agitate by hand for 1 min. Keep samples sealed from collection time until analysis.

c. Sample storage and holding time: Keep all samples iced during shipment and do not let temperature exceed 10°C during the first 48 h after collection. Assuming that samples are in transit for 48 h or less, confirm that they are ≤10°C when they are received at the laboratory. In the laboratory, store samples ≤6°C and protect from light until analysis. Do not freeze samples. Results of the sample storage stability study of all method analytes indicated that all compounds are stable for 28 d in water samples that are collected, dechlorinated, preserved, shipped, and stored as described above.[5] Analyze samples within 28 d.

3. Apparatus

a. High-performance liquid chromatograph (HPLC): A system capable of reproducibly injecting up to 1000-μL portions,

and performing ternary linear gradients at a constant flow rate of approximately 1.5 mL/min. A column heater is desirable. Data included in this method were obtained with a heater set at 30°C.

1) *Analytical column*—Any column that provides adequate resolution, peak shape, capacity, accuracy, and precision (6610B.6) may be used. For development of this method, an HPLC "carbamate" column, 3.9 × 150 mm, packed with 4-μm d_p C_{18} solid-phase particles* was used.

2) *Postcolumn reaction system* capable of mixing reagents into the mobile phase—Use a reactor constructed of polyether-etherketone (PEEK) or PTFE tubing and equipped with two pumps capable of delivering up to 0.5 mL/min of each reagent; mixing tees; and two reaction coils. Various post-column system manufacturers recommend different reaction-coil temperatures for the carbamate hydrolysis reaction; therefore, the first reaction coil temperature may range from 80 to 95°C. Analyte signal can increase with temperature over this temperature range; however, baseline noise can also increase with increasing temperatures. The second reaction takes place at ambient temperature.

3) *Detector*—Use a fluorescence detector capable of excitation at approximately 340 nm and detection of emission energy at approximately 465 nm. Specific optimum excitation and emission wavelengths may vary slightly for each system.

4) *Data system*—Preferably use a computerized data system for data acquisition and processing.†

b. Vials: Screw-cap or crimp-top glass autosampler vials with PTFE-faced septa, amber or clear.

c. Volumetric flasks, Class A, various sizes, used for preparation of standards and samples.

d. Microsyringes, various sizes.

e. Disposable syringes, 5- to 30-mL size, used to filter sample extracts before analysis.‡

f. Filters, disposable, used to filter samples before analysis.§

g. Analytical balance, capable of weighing accurately to 0.0001 g.

4. Reagents

Use only reagent-grade or better chemicals and HPLC-grade solvents. Unless otherwise indicated, ensure that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available.

a. Reagent water: Use purified water that does not contain measurable quantities of any target analytes or interfering compounds greater than ⅓ the MRL for each compound of interest.

b. Buffered reagent water: To reagent water, add sample preservation reagents in the same concentrations present in the samples. To prepare 1 L buffered reagent water, add a sufficient amount of potassium dihydrogen citrate to yield a concentration of 9.2 to 9.5 g/L and sodium thiosulfate to yield a concentration in the range of 80 to 320 mg/L to a graduated bottle or volumetric flask. Fill to 1-L mark with reagent water.

c. Acetonitrile (CH_3CN), high-purity, demonstrated to be free of analytes and interferences (HPLC grade or better).

d. Methanol (CH_3OH), high-purity, demonstrated to be free of analytes and interferences (HPLC grade or better).

e. Hydrolysis solution (postcolumn reagent 1): Make sodium hydroxide, NaOH, 0.05*N*, by diluting 4 mL 50% (w/w) sodium hydroxide solution to 1 L with reagent water. Because hydrolysis solution concentration can dramatically affect analyte response, use extra care in preparing. Filter and degas (with helium or other appropriate gas) just before use.

f. Sodium borate solution, 0.05*N:* Dissolve 19.1 g sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$) in a 1-L volumetric flask. Bring volume up to 1.0 L with reagent water. The sodium borate will dissolve in <2 h if a stir bar is used. Filter and degas.

g. o-*Phthalaldehyde (OPA):* Dissolve 100 ± 10 mg of OPA in 5 to 10 mL methanol.

h. OPA derivatization solution (postcolumn reagent 2): Prepare by *either* ¶ *h*1) or 2) below. Both 2-mercaptoethanol and *N,N*-dimethyl-2-mercaptoethylamine hydrochloride react with OPA and the target methylamine to form the isoindole detected by the fluorescence detector. Both reagents have characteristic strong odors. Prepare solutions in a fume hood. This reagent, as prepared by either method, is stable for at least 36 h. However, individual laboratory conditions vary and daily preparation of this solution may be necessary.

1) Preparation with 2-mercaptoethanol—Add dissolved OPA (¶ *g* above) to 1 L filtered and degassed sodium borate solution (¶ *f* above). Add 1.0 mL 2-mercaptoethanol and mix.

2) Preparation with *N,N*-dimethyl-2-mercaptoethylamine hydrochloride—Dissolve 2.0 ± 0.2 g *N,N*-dimethyl-2-mercaptoethylamine hydrochloride in approximately 10 mL sodium borate solution (¶ *f* above). Add dissolved OPA (¶ *g* above) to 1 L filtered and degassed sodium borate solution. Add dissolved *N,N*-dimethyl-2-mercaptoethylamine-hydrochloride to the sodium borate solution and mix.

i. Calibration standards: Prepare standard solutions either from certified, commercially available solutions or from neat compounds. Compounds used to prepare solutions must be ≥96% pure; their weights may be used without correction for purity to calculate the concentration of the stock standard. Solution concentrations mentioned in this section were used to develop this method and are included as an example, not a requirement. Generally, prepare standards for sample fortification in the smallest volume that can be measured accurately, to minimize the addition of organic solvent to aqueous samples. NOTE: Although stability times for standard solutions are suggested below, use standard QC practices to determine when standard solutions need to be replaced.

1) *Surrogate analyte (SUR) standard solution, 4-bromo-3,5-dimethylphenyl* n-*methylcarbamate (BDMC)*

a) *SUR stock standard*—If preparing from neat material, accurately weigh approximately 25 to 35 mg neat SUR to nearest 0.1 mg into a tared, 5-mL volumetric flask. Dilute to the mark with methanol. Stock solutions have been shown to be stable for 12 months when stored at −10°C or less.

b) *SUR primary dilution standard (PDS)*—Prepare by adding enough of the SUR stock standard to a volumetric flask partially filled with methanol to make a final concentration of 10 μg/mL when filled to the mark with methanol. The PDS has been shown to be stable for 6 months when stored at −10°C or less.

* Waters catalog No. WAT035577.
† A Waters Millennium software system was used to generate all data contained in the tables provided in this method.
‡ B-D catalog No. 309603, 309650, or equivalent.
§ Millipore 0.22-μm PVDF membrane, catalog No. SLGV 013 NL, or equivalent.

Table 6610:III. Preparation of Calibration (CAL) Curve Solutions

CAL Level	Analyte PDS Conc. *μg/mL*	Volume of Analyte PDS *μL*	Volume of 10-μg/mL SUR PDS *μL*	Final Volume of CAL Solution *mL*	Final Conc. of CAL Solution *μg/L*
1	1.0	5.0	5.0	25	0.20
2	1.0	12.5	5.0	25	0.50
3	1.0	25.0	5.0	25	1.00
4	10.0	5.0	5.0	25	2.00
5	10.0	12.5	5.0	25	5.00
6	10.0	25.0	5.0	25	10.0

2) *Target analyte standard solution*

a) *Target analyte stock standard*—Obtain analytes listed in Table 6610:I as neat or solid standards or as commercially prepared ampulized solutions. If preparing from neat material, accurately weigh approximately 25 to 35 mg of pure material to the nearest 0.1 mg into a tared, 5-mL volumetric flask. Dilute to the mark with methanol. Repeat for each target analyte.

b) *Target analyte primary dilution standard (PDS)*—Prepare by dilution of the target analyte stock standards. Add enough of each target stock standard to a volumetric flask partially filled with methanol to make a final concentration near 10 μg/mL when filled to the mark with methanol. A serial dilution of this PDS, to make a 1.0-μg/mL solution, is useful for low-level fortification. The PDSs have been shown to be stable for 6 months when stored at −10°C or less.

3) *Calibration solutions (CAL)*—Prepare the initial calibration curve with at least 5 calibration concentrations. Prepare working calibration solutions over the concentration range of interest from dilutions of the analyte PDSs in buffered reagent water. Add SUR PDS to each CAL. Filter CAL solutions in same manner as samples (6610B.5*c*). The lowest concentration of calibration solution must be at or below the MRL, which may depend on system sensitivity. Prepare calibration standards using buffered reagent water (¶ *b* above). An example of the dilutions used to prepare the CALs used to collect the data in this method is shown in Table 6610:III. These standards also may be used as continuing calibration checks (6610B.6*d*).

5. Procedure

a. Chromatographic conditions: Establish operating conditions as recommended in Table 6610:IV for the HPLC system. Other HPLC conditions may be used as long as all QC requirements (6610B.6) are met. Establish an appropriate retention time window for each target and surrogate to identify them in the QC and field samples. Base this retention time window on measurements of actual retention time variation for each compound in standard solutions analyzed on the HPLC over the course of time. Plus or minus three times the standard deviation of the retention time for each compound during initial calibration and completion of IDC can be used to calculate a suggested window size; however, consider the experience of the analyst in determination of the appropriate retention window size. Confirm that retention times, compound separation, and resolution are similar to those listed in Table 6610:V and Figure 6610:1. Check reso-

Table 6610:IV. Instrument Gradient and Conditions

Time *min*	% Water	% Methanol	% Acetonitrile
Initial	88.0	12.0	0.0
5.30	88.0	12.0	0.0
5.40	68.0	16.0	16.0
14.00	68.0	16.0	16.0
16.10	50.0	25.0	25.0
20.00	50.0	25.0	25.0
22.00	88.0	12.0	0.0
30.00	88.0	12.0	0.0

Instrument conditions:
HPLC: A ternary gradient of water, methanol, and acetonitrile with a flow of 1.5 mL/min as shown above.
Injection volume: System-dependent; for the development of this method: 250 to 1000 μL.
Column: See 6610B.3*a*1).
Postcolumn reactor: Reactor coil: 80°C; reagent 1 and 2 flow rates are instrument-dependent; 0.3 to 0.5 mL/min.
Fluorescence detector: 340-nm excitation, 465-nm emission with a 18-nm band width; gain=100, attenuation=16; response=standard; 16-μL flow cell.

Table 6610:V. Retention Times for Analytes*

Analyte	Retention Time *min*	Standard Deviation	Relative Standard Deviation %
Aldicarb sulfoxide	4.369	0.0092	0.21
Aldicarb sulfone	5.072	0.0089	0.17
Oxamyl	5.744	0.0095	0.17
Methomyl	6.526	0.0077	0.12
3-Hydroxycarbofuran	9.824	0.0128	0.13
Aldicarb	11.461	0.0129	0.11
Propoxur	14.321	0.0195	0.14
Carbofuran	14.834	0.0244	0.16
Carbaryl	16.993	0.0264	0.16
1-Naphthol	18.579	0.0187	0.10
Methiocarb	21.826	0.015	0.07
BDMC (SUR)	22.341	0.015	0.07

* Retention-time data calculated from precision and accuracy data results presented in Table 6610:II reagent-water injections and calibration curve used to quantitate these data.

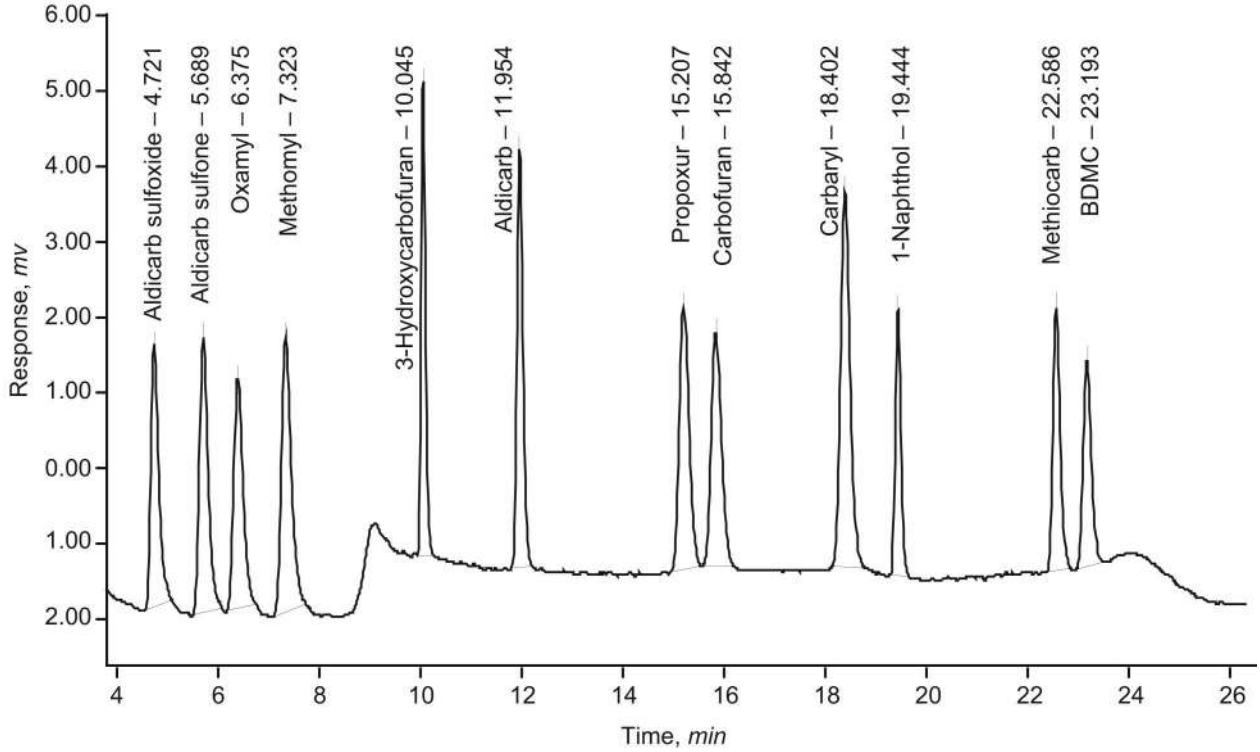


Figure 6610:1. Sample chromatogram of target analytes. Concentration 2.0 μg/L, 1000-μL injection volume, analyzed under conditions stated in Table 6610:IV.

lution for closely eluting compounds that are not baseline resolved according to 6610B.6*h*.

b. Calibration:

1) Initial calibration—Calibrate system by using the external standard technique. Prepare a set of at least five calibration standards as described in 6610B.4*i*3).

Generate a calibration curve for each analyte by plotting peak response (preferably area) against analyte concentration. The instrument used during method development yielded linear curves for the target analytes over the concentration range of interest. However, data may be fitted with either a linear regression (response vs. concentration) or quadratic fit (response vs. concentration). Alternatively, if the ratio of the analyte peak area to concentration (or response factor) is relatively constant (RSD <30%), an average response factor may be used to calculate analyte concentration.

When each calibration standard is calculated as an unknown using the calibration curve, the results must be 70 to 130% of the true value for all but the lowest standard, and the lowest standard must be 50 to 150% of the true value.

2) Daily calibration verification—Verify accuracy of initial calibration during each analysis batch (set of samples prepared and analyzed on the same instrument during a 24-h period). Begin an analysis batch with a continuing calibration check (CCC) at or below the MRL, analyze a CCC after every 10 field samples (alternate between medium and high concentrations), and end batch with a CCC. Limit analysis batch to 20 field samples. See 6610B.6 for the required QC samples for each analysis batch.

Inject a portion of the appropriate concentration calibration solution and analyze with the same conditions used during initial calibration.

Calculate concentration of each analyte and surrogate in the CCC standard. The calculated amount for each analyte for medium- and high-level CCCs must be ±30% of the true value, and the calculated amount for the lowest calibration point for each analyte must be within ±50% of the true value. If the values are outside these ranges, consider all data for the problem analyte invalid and take remedial action (possibly including recalibration). After adequate calibration has been restored, re-analyze any field or QC samples that have been analyzed since the last acceptable calibration verification.

c. Sample preparation: Preserve, collect, and store samples as directed in 6610B.2. Ensure that all field and QC samples contain the required preservatives. Measure a portion of sample into a volumetric flask (25-mL recommended). When the volumes of SUR and analyte PDS added to the field and QC samples are kept to a minimum as described below, no volume adjustment is necessary.

Add a portion of the SUR PDS [6610B.4*i*1)b)] to all samples and mix by capping and inverting sample. For the development of this method, the addition of 5.0 μL of a 10-μg/mL SUR PDS to a 25-mL sample resulted in a SUR concentration of 2.0 μg/L.

If sample is a CCC or other target-analyte-fortified QC sample 6610B.6*f* and *g*), add the necessary amount of analyte PDS [6610B.4*i*2)b)]. Cap and invert each sample to ensure that all components are properly mixed.

TABLE 6610:VI. SUMMARY OF REQUIREMENTS FOR INITIAL DEMONSTRATION OF CAPABILITY (IDC)

Method Reference	Requirement	Specification and Frequency	Acceptance Criteria
6610B.6*a*1)	Initial demonstration of low system background	Analyze laboratory reagent blank before any other IDC steps.	Target analytes below 1/3 intended MRL; possible interferences from reagents and glassware do not prevent analyte identification and quantitation.
6610B.6*a*2)	Quality control sample (QCS)	Use second-source standard to fortify buffered reagent water. Analyze as a CCC after initial calibration but before IDA sample analysis.	Verifies initial calibration accuracy; recovery within ±30% of true value.
6610B.6*a*2)	Initial demonstration of accuracy (IDA)	Analyze 4–7 replicate LFBs/CCCs fortified at mid-range concentration. Calculate average recovery for replicates used in IDP.	Mean recovery within ±20% of true value.
6610B.6*a*3)	Initial demonstration of precision (IDP)	Calculate average recovery for replicates used in IDA.	RSD ≤ 20%
6610B.6*a*4)	Detection level determination	Over 3-d period prepare at least 7 replicate LFBs fortified at concentration estimated to be near the detection level. Analyze replicates through all steps of analysis. Calculate detection level according to 6610B.6*a*4).	Data *not required* to meet method precision and accuracy criteria.[See note, 6610B.6*a*4)].

Filter samples before filling appropriate autosampler vials. Preferably use filters specified in 6610B.3*f*.

d. Analysis of samples: Inject and analyze samples, including CCCs and QC samples, using conditions identical with those used for initial calibration. Do not extrapolate beyond the established calibration range. If an analyte peak area exceeds the range of the initial calibration curve, the extract may be diluted with buffered reagent water. Determine acceptable surrogate performance (6610B.6*i*) from the undiluted sample extract. Any dilutions will also affect analyte MRL. For final calculations, see 6610B.7.

6. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 6020:I.

Quality control (QC) requirements include the initial demonstration of capability (IDC), determination of the detection level, and subsequent analysis in each analysis batch of a laboratory reagent blank (LRB), continuing calibration check (CCC) standards, a laboratory-fortified blank (LFB), a laboratory-fortified sample matrix (LFSM), either a laboratory-fortified sample matrix duplicate (LFSMD) or a field duplicate sample, and the periodic analysis of a quality control sample (QCS). This section details the specific requirements for each QC parameter. The QC criteria discussed in the following sections are summarized in Tables 6610:VI and VII. These criteria are considered the minimum acceptable QC criteria; institute additional QC practices to meet specific needs.

a. Initial demonstration of capability: Requirements for the IDC are described in ¶s *a*1)–4) below and summarized in Table 6610:VI.

1) Initial demonstration of low system background—Before any field samples are analyzed, and any time a new set of reagents is used, demonstrate that a laboratory reagent blank is reasonably free of contamination and that the criteria in 6610B.6*c* are met.

2) Initial demonstration of accuracy—Before analysis of the IDC samples, verify calibration accuracy with the preparation and analysis of a mid-level QCS as defined in ¶ *j* below. If the analyte recovery is not within ±30% of the true value, the accuracy of the method is unacceptable; identify and correct the source of the problem. After calibration accuracy has been verified, prepare and analyze four to seven replicate LFBs (or CCCs in this method) fortified at 2 μg/L, or near the mid-range of the initial calibration curve, according to the procedure described in 6610B.4*i*3). Also add sample preservatives (6610B.2*a*) to these samples. Average recovery of the replicate values must be within ±20% of the true value.

3) Initial demonstration of precision—Using the same set of replicate data generated for ¶ *a*2) above, calculate the standard deviation and percent relative standard deviation of the replicate recoveries. The percent relative standard deviation (% RSD) of the results of the replicate analyses must be <20%.

4) Detection level determination—Prepare and analyze at least seven replicate LFBs at a concentration estimated to be near the detection level over at least 3 d by the procedure described in 6610B.4*i*3). This fortification level may be estimated by selecting a concentration with a signal of 2 to 5 times the noise level. The appropriate concentration will depend on the sensitivity of the HPLC system being used. Also add sample preservatives (6610B.2*a*) to these samples. Calculate the detection level using the equation

TABLE 6610:VII. SUMMARY OF QUALITY CONTROL REQUIREMENTS

Method Reference	Requirement	Specification and Frequency	Acceptance Criteria
6610B.2*c*	Sample holding times	Ship preserved samples at or below 10°C; hold in lab at ≤6°C for up to 28 d. Do not freeze.	Do not report data for samples that have not been properly preserved or stored, or that have exceeded specified holding time.
6610B.6*c*	Laboratory reagent blank (LRB)	Include with each analysis batch (up to 20 samples). Analyze before samples and determine to be free from interferences.	All target analytes below 1/3 intended MRL; possible interferences from reagents and glassware do not prevent analyte identification and quantitation. If targets exceed 1/3 MRL, analytical batch results for that analyte are invalid.
6610B.5*b*2)	Continuing calibration check (CCC)	Verify initial calibration by analyzing CCC (at or below MRL) before analyzing samples. Inject CCCs after every 10 samples and after last sample, rotating concentrations to cover calibration range.	Recovery for each analyte within 70–130% of true value for all but lowest calibration level. Lowest calibration level CCC within 50–150% of true value. Results not bracketed by acceptable CCCs are invalid.
6610B.6*i*	Surrogate standards	Add surrogate, 6610B.4*i*1), to all field and QC samples.	Surrogate recovery within 70–130% of true value. Report samples that fail criteria as suspect.
6610B.6*f* & *g*	Laboratory-fortified sample matrix (LFSM) and laboratory-fortified sample matrix duplicate (LFSMD)	With each analysis batch, extract and analyze at least one LFSM. Extract LFSMD when occurrence of target analytes is low. Field duplicate analysis is not required for extraction batches containing a LFSMD.	Recoveries not within 70–130% (50–150% at MRL) of fortified amount may indicate matrix effect. If LFSMD is analyzed instead of a laboratory duplicate, accept if target RPDs within ±30%. If all CCCs meet acceptance criteria and LFSM or LFSMD do not, designate sample "suspect/matrix."
6610B.6*g*	Field duplicates (FD)	Analyze at least one duplicate with each extraction batch (20 samples or less). LFSMD may be substituted when analyte occurrence is low.	RPDs within ±30%. If all CCCs meet acceptance criteria and FDs do not, designate sample "suspect/matrix."
6610B.6*h*	Resolution check	Monitor once/24-h analysis period.	Resolution of ≥1.0 for closely eluting peaks that are not baseline resolved (see 6610B.6*h*).
6610B.6*j*	Quality control sample	Analyze at least quarterly or when preparing new standards, as well as during IDC.	Same acceptance criteria as CCC.
6610B.5*b*1)	Initial calibration	Use external standard calibration technique to generate calibration curve with at least 5 standards.	For each calibration standard, calculated as an unknown using calibration curve, results within 70–130% of true value for all but lowest standard; lowest standard (concentration ≤ MRL) results within 50–150% of true value.

$$\text{Detection level} = St_{(n-1,\,1-\alpha=0.99)}$$

where:

S = standard deviation of replicate analyses,
$t_{(n-1,1-\alpha=0.99)}$ = Student's t value for the 99% confidence level with $n-1$ degrees of freedom, and
n = number of replicates.

NOTE: Calculated detection levels need only be less than ⅓ of the laboratory's MRL to be considered acceptable. Do not subtract blank values when performing detection-level calculations. The detection level is a statistical determination of precision only.[1] If the detection-level replicates are fortified at a low enough concentration, it is likely that they will not meet precision and accuracy criteria, and may result in a calculated detection level higher than the fortified concentration.

b. Minimum reporting level (MRL): The MRL is the threshold concentration of an analyte that a laboratory can expect to quantitate accurately in an unknown sample. Do not establish the MRL at an analyte concentration lower than that of the lowest calibration standards. The MRL also should not be less than either three times the detection level or a concentration yielding a response less than a signal-to-noise ratio of five. Depending upon the study's data quality objectives, it may be set at a higher concentration.

c. Laboratory reagent blank (LRB): This is a direct-injection method without a conventional extraction. A LRB, prepared using buffered reagent water and filtering in the same manner as

the samples, is required with each analysis batch of samples to determine any background system contamination. If, within the retention time window of any analyte, the LRB produces a peak that would prevent determination of that analyte, determine source of contamination and eliminate the interference before processing samples. Reduce background contamination to an acceptable level before proceeding. Keep background from method analytes or contaminants that interfere with the measurement of method analytes below ⅓ the MRL. If target analytes are detected in the LRB at concentrations equal to or greater than this level, consider all data for the problem analyte(s) invalid for all samples in the analysis batch.

d. Continuing calibration check (CCC): Prepare a CCC in the same manner as the initial calibration solutions, using buffered reagent water and filtering in the same manner as the samples. Analyze during an analysis batch at a required frequency to confirm that the instrument meets initial calibration criteria. See 6610B.5*b*2) for concentration requirements, frequency requirements, and acceptance criteria.

e. Laboratory-fortified blank (LFB): For this direct injection method, a CCC is the same as an LFB. Consequently, the analysis of an LFB is not required.

f. Laboratory-fortified sample matrix (LFSM): Analyze an LFSM in each analysis batch to determine that the sample matrix does not adversely affect method accuracy. If the occurrence of target analytes in the samples is infrequent, or if historical trends are unavailable, prepare a second LFSM, or LFMSD, from a duplicate of the field sample used to prepare the LFSM, and analyze to assess method precision. Extraction batches that contain LFSMDs do not require the analysis of a field duplicate. If a variety of different sample matrices are analyzed regularly, for example, drinking water from groundwater and surface water sources, establish method performance for each. Over time, document LFSM data for all routine sample sources for the laboratory.

Within each analysis batch, fortify a minimum of one field sample as an LFSM for every 20 samples processed. Prepare LFSM by adding an appropriate amount of the analyte PDS [6610B.4*i*2)b)] to a sample. Select a fortifying concentration at least twice the matrix background concentration, if known. Use historical data or rotate through the designated concentrations when selecting a fortifying concentration. Selecting a duplicate bottle of a sample that has already been analyzed aids in the selection of appropriate fortifying levels.

Calculate the percent recovery (R) for each analyte with the equation

$$R = \frac{(A - B)}{C} \times 100$$

where:

A = measured concentration in the fortified sample,
B = measured concentration in the unfortified sample, and
C = fortification concentration.

Analyte recoveries may exhibit a matrix bias. For samples fortified at or above their original concentration, recoveries should be between 70 and 130%. For LFSM fortification at the MRL, 50 to 150% recoveries are acceptable. If the accuracy of any analyte falls outside the designated range, and the laboratory performance for that analyte is shown to be in control in the CCCs, the recovery is matrix-biased. Label the result for that analyte in the unfortified sample "suspect/matrix".

g. Field duplicate (FD) or laboratory-fortified sample matrix duplicate (LFSMD): Within each analysis batch, analyze a minimum of one FD or LFSMD. Duplicates check the precision associated with sample collection, preservation, storage, and laboratory procedures. If target analytes are not routinely observed in field samples, analyze a LFSMD rather than a FD.

Calculate relative percent difference (RPD) for duplicate measurements (FD1 and FD2) using the equation

$$RPD = \frac{(FD1 - FD2)}{(FD1 + FD2)/2} \times 100$$

If a LFSMD is analyzed instead of a FD, calculate relative percent RPD for duplicate LFSMs (LFSM and LFSMD) using the equation

$$RPD = \frac{(LFSM - LFSMD)}{(LFSM + LFSMD)/2} \times 100$$

RPDs for FDs and duplicate LFSMs should fall in the range of ±30% for samples fortified at or above their original concentration. Greater variability may be observed when LFSMs are fortified near the MRL. At the MRL, RPDs should fall in the range of ±50% for samples fortified at or above their original concentration.

h. Resolution check: In each analytical batch, monitor resolution of peaks in a calibration standard or CCC near the mid-level of calibration. During the development of this method, the 2-μg/L level was monitored. Check that closely eluting peaks that are not baseline-resolved have a resolution (R_s) of 1.0 or greater, calculated by the equation[6]

$$R_s = \frac{1.18(t_2 - t_1)}{W_{0.5,1} + W_{0.5,2}}$$

where:

t_1, t_2 = retention times of the first and second adjacent peaks, and
$W_{0.5,1}$, $W_{0.5,2}$ = widths of the adjacent peaks at half height.

Monitor resolution once for every 24-h analytical batch at any time during the 24-h period. Preferably check resolution before sample analysis, especially if the system in use has a history of resolution problems. If a resolution check fails, re-analyze all samples in the analytical batch, including the QC samples, after the problem is corrected.

i. Surrogate recovery: Fortify all samples, blanks, LRBs, and LFSMs and LFSMDs with surrogate standard before filtration and analysis. Also add it to the calibration curve and calibration check standards. The surrogate is a means of assessing method performance from preparation and filtration to final chromatographic measurement.

When surrogate recovery from a sample, blank, or CCC is <70% or >130%, check the following: calculations to locate possible errors, standard solutions for degradation, possible contamination,

and instrument performance. If those checks do not reveal the cause of the problem, reanalyze the sample.

If the re-analysis meets the surrogate recovery criterion, report only data for the re-analyzed sample.

If the sample re-analysis fails the 70–130% recovery criterion, check calibration by reinjecting the most recently acceptable calibration standard. If the calibration standard fails the criteria of 6610B.5*b*2), recalibrate. If the calibration standard is acceptable, repeat preparation (including fortifying with surrogate and filtration) and analysis of the sample if the sample is still within the holding time. If this sample re-analysis also fails the recovery criterion, report all data for that sample as suspect because of unsatisfactory surrogate recovery.

j. Quality control sample (QCS): During the analysis of the IDC (¶ *a* above), each time that new analyte standard solutions are prepared, or at least quarterly, analyze a QCS from a source different from the source of the calibration standards. Fortify QCS at the mid-level of calibration in buffered reagent water and analyze in same manner as a CCC. The acceptance criteria are the same as the CCC criteria at mid-level: the calculated amount for each analyte must be ±30% of the true value. If measured analyte concentrations are not of acceptable accuracy, check entire analytical procedure to locate and correct the problem source.

7. Data Analysis and Calculation

Identify method analytes in the sample chromatogram by comparing the retention time of the suspect peak to the retention time of an analyte peak in a calibration standard. Confirm that surrogate retention times are within acceptance limits (6610B.6*i*), even if no target compounds are detected.

Calculate analyte concentrations using the initial calibration curve generated as described in 6610B.5*b*1). Quantitate only those values that fall between the MRL and the highest calibration standard. Samples with target analyte responses that exceed the highest standard require dilution and reanalysis (6610B.5*d*).

Adjust calculated concentrations of detected analytes to reflect initial sample volume and any dilutions performed.

Before reporting the data, review the chromatogram for any incorrect peak identification or poor integration.

Report analyte concentrations in micrograms per liter, usually to two significant figures.

8. References

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6. Snyder, L.R., J.J. Kirkland & J.L. Glajch. 1997. Practical HPLC Method Development, 2nd ed. John Wiley & Sons, New York, N.Y.

6630 ORGANOCHLORINE PESTICIDES*

6630 A. Introduction

1. Sources and Significance

The organochlorine pesticides commonly occur in waters that have been affected by agricultural discharges. Some of the listed compounds are degradation products of other pesticides detected by this method. Several of the pesticides are bioaccumulative and relatively stable, as well as toxic or carcinogenic; thus they require close monitoring.

2. Selection of Method

Methods 6630B and C consist of gas chromatographic (GC) procedures following liquid–liquid extraction of water samples. They are relatively sensitive methods that can be used to detect numerous pesticides. Differences between the methods are minimal after extraction. Method 6630D is a gas chromatographic/mass spectrometric (GC/MS) method that can detect all of the target compounds, but at much higher concentrations. The use of extracted ion traces or time scheduled selective ion monitoring (SIM) can reduce the concentration level needed for detection.[1,2] All these methods also are useful for determination of polychlorinated biphenyls (PCBs) (also see Section 6431A).

3. References

1. Zaugg, S.D., M.W. Sandstrom, S.G. Smith & K.M. Fehlberg. 1995. Methods of Analysis by the U.S. Geological Survey National Water Quality Laboratory—Determination of Pesticides in Water by C-18 Solid Phase Extraction and Capillary Column Gas Chromatography/Mass Spectrometry with Selected-Ion Monitoring; USGS Open File Rep. 95-181. U.S. Geological Survey, Earth Science Information Center, Denver, Colo.
2. Stan H.-J & T. Heberer. 1995. Identification and confirming analysis based on gas chromatography-mass spectrometry. *In* H.J. Stan. Analysis of Pesticides in Ground and Surface Water 1. Chemistry of Plant Protection, Vol. 11, p. 141. Springer, Berlin, Germany.

* Approved by Standard Methods Committee, 2007.

6630 B. Liquid–Liquid Extraction Gas Chromatographic Method I

1. General Discussion

a. Application: This gas chromatographic procedure is suitable for quantitative determination of the following specific compounds: BHC, lindane (γ-BHC), heptachlor, aldrin, heptachlor epoxide, dieldrin, endrin, captan, DDE, DDD, DDT, methoxychlor, endosulfan, dichloran, mirex, and pentachloronitrobenzene. Under favorable circumstances, strobane, toxaphene, chlordane (tech.), and others also may be determined when relatively high concentrations of these complex mixtures are present and the chromatographic fingerprint is recognizable in packed or capillary column analysis. Trifluralin and certain organophosphorus pesticides (e.g., parathion, methylparathion, and malathion) that which respond to the electron-capture detector, also may be measured. However, the usefulness of the method for organophosphorus or other specific pesticides must be demonstrated before it is applied to sample analysis.

b. Principle: In this procedure, the pesticides are extracted with a mixed solvent, diethyl ether/hexane or methylene chloride/hexane by either liquid–liquid extraction using a separatory funnel or by continuous liquid–liquid extraction. The extract is concentrated by evaporation and, if necessary, is cleaned up by column adsorption-chromatography. The individual pesticides then are determined by gas chromatography. Although procedures detailed below refer primarily to packed columns, capillary column chromatography also may be used. See Section 6010C.2*a*1) for discussion of gas chromatographic principles and 6010C.2*b*2) for discussion of electron-capture detector.

As each component passes through the detector, a quantitatively proportional change in electrical signal is measured on a strip-chart recorder. Each component is observed as a peak on the recorder chart. The retention time is indicative of the particular pesticide and peak height/peak area is proportional to its concentration.

Variables may be manipulated to obtain important confirmatory data. For example, the detector system may be selected on the basis of the specificity and sensitivity needed. The detector used in this method is an electron-capture detector that is very sensitive to chlorinated compounds. Additional confirmatory identification can be made from retention data on two or more columns where the stationary phases are of different polarities. A two-column procedure that has been found particularly useful is specified. If sufficient pesticide is available for detection and measurement, confirmation by a more definitive technique, such as mass spectrometry, is desirable.

c. Interference: See Sections 6010C.2*a*2) and 2*b*2). Some compounds other than chlorinated compounds respond to the electron-capture detector. Among these are oxygenated and unsaturated compounds. Sometimes plant or animal extractives obscure pesticide peaks. These interfering substances often can be removed by auxiliary cleanup techniques. A magnesia-silica gel column cleanup and separation procedure is used for this purpose. Such cleanup usually is not required for potable waters.

1) Polychlorinated biphenyls (PCBs)—Industrial plasticizers, hydraulic fluids, and old transformer fluids that contain PCBs are a potential source of interference in pesticide analysis. The presence of PCBs is suggested by a large number of partially resolved or unresolved peaks that may occur throughout the entire chromatogram. Particularly severe PCB interference will require special separation procedures.

2) Phthalate esters—These compounds, widely used as plasticizers, cause electron-capture detector response and are a source of interferences. Water leaches these esters from plastics, such as polyethylene bottles and plastic tubing. Phthalate esters can be separated from many important pesticides by the magnesia-silica gel column cleanup. They do not cause response to halogen-specific detectors, such as microcoulometric or electrolytic conductivity detectors.

d. Detection limits: The ultimate detection limit of a substance is affected by many factors (e.g., detector sensitivity, extraction and cleanup efficiency, concentrations, and detector signal-to-noise level). Lindane (γ-BHC) usually can be determined at 10 ng/L in a sample of relatively unpolluted water; the DDT detection limit is somewhat higher, 20 to 25 ng/L. Increased sensitivity is likely to increase interference with all pesticides.

e. Sample preservation: Some pesticides are unstable. Transport under iced conditions, store at 4°C until extraction, and do not hold >7 d. When possible, extract upon receipt in the laboratory and store extracts at 4°C until analyzed. Analyze extracts within 40 d.

f. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 6020:I.

2. Apparatus

Clean thoroughly all glassware used in sample collection and pesticide residue analyses. Clean glassware as soon as possible after use. Rinse with water or the solvent that was last used in it, wash with soapy water, rinse with tap water, distilled water, redistilled acetone, and finally with pesticide-quality hexane. As a precaution, glassware may be rinsed with the extracting solvent just before use. Heat heavily contaminated glassware in a muffle furnace at 400°C for 15 to 30 min. High-boiling-point materials, such as PCBs, may require overnight heating at 500°C, but no borosilicate glassware can exceed this temperature without risk. Do not heat volumetric ware. Clean volumetric glassware with special reagents.* Rinse with water and pesticide-quality hexane. After drying, store glassware to prevent accumulation of dust or other contaminants. Store inverted or cover mouth with aluminum foil.

a. Sample bottles: 1-L capacity, glass, with TFE-lined screw cap. Bottle may be calibrated to minimize transfers and potential for contamination.

b. Evaporative concentrator, Kuderna–Danish, 500-mL flask and 10-mL graduated lower tube fitted with a 3-ball Snyder column, or equivalent.

c. Extractor device: Separatory funnels, 2-L capacity, with TFE stopcock, or continuous liquid–liquid extractor, 1- or 2-L capacity.

d. Graduated cylinders, 1-L capacity.

e. Funnels, 125-mL.

f. Glass wool, filter grade.

g. Chromatographic column, 20 mm in diam and 400 mm long, with coarse fritted disk at bottom.

h. Microsyringes, 10- and 25-μL capacity.

i. Hot water bath.

j. Gas chromatograph, equipped with:

1) *Glass-lined injection port.*

2) *Electron-capture detector.*

3) *Recorder*—Potentiometric strip chart, 25-cm, compatible with detector and associated electronics.

4) *Borosilicate glass column,* 1.8 m × 4-mm ID or 2-mm ID.

Variations in available gas chromatographic instrumentation necessitate different operating procedures for each. Therefore, refer to the manufacturer's operating manual, gas chromatography catalogs, and other references (see Bibliography). In general, use equipment with the following features:

- Carrier-gas line with a molecular sieve drying cartridge and a trap for removal of oxygen from the carrier gas. A special purifier† may be used. Use only dry carrier gas and ensure that there are no gas leaks.
- Oven temperature stable to ±0.5°C or better at desired setting.
- Chromatographic columns—A well-prepared column is essential to an acceptable gas chromatographic analysis. Obtain column packings and pre-packed columns from commercial sources or prepare column packing in the laboratory.

It is inappropriate to give rigid specifications on size or composition to be used because some instruments perform better with certain columns than do others. Columns with 4-mm ID are used most commonly. The carrier-gas flow is approximately 60 mL/min. When 2-mm-ID columns are used, reduce carrier-gas flow to about 25 mL/min. Adequate separations have been obtained by using 5% OV-210 on 100/120 mesh dimethyl-dichlorosilane-treated diatomaceous earth‡ in a 2-m column. The 1.5% OV-17 and 1.95% QF-1 column is recommended for confirmatory analysis. Two additional column options are included: 3% OV-1 and mixed-phase 6% QF-1 + 4% SE-30, each on dimethyl-dichlorosilane-treated diatomaceous earth, 100/120 mesh. OV-210, which is a refined form of QF-1, may be substituted for QF-1. A column is suitable when it effects adequate and reproducible resolution. Sample chromatograms are shown in Figures 6630:1–4.

Alternately, use fused silica capillary columns§ 30 m long with 0.32-mm ID or 0.25-mm ID and 0.25-μm film thickness, for primary and confirmatory analyses (see Figure 6630:5). Coelution of Alpha-chlordane and Endosulfan I on the recommended confirmatory column makes it less desirable for primary analysis. Another type of detector, such as a mass spectrometer, also can be used for confirmatory analysis. Use carrier gas flows recommended by manufacturer according to the ID of the column, along with nitrogen make-up gas for the proper operation of the electron capture detector.

* No Chromix, Godax, 6 Varick Place, New York, NY, or equivalent.

† Hydrox, Matheson Gas Products, P.O. Box E, Lyndhurst, NJ, or equivalent.

‡ Gas Chrom Q, Applied Science Labs., Inc., P.O. Box 440, State College, PA, or equivalent.

§ Primary column DB-1701, J&W Scientific, or equivalent; confirmatory column DB-5, or equivalent.

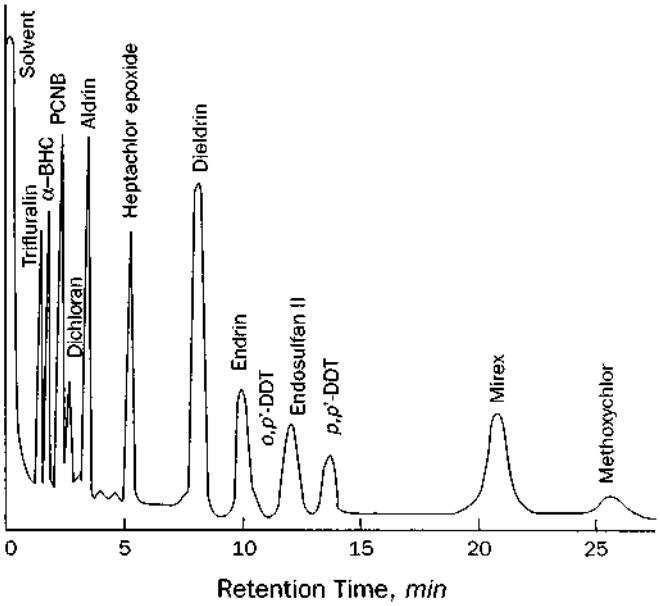


Figure 6630:1. Results of gas chromatographic procedure for organochlorine pesticides. Column packing: 1.5% OV-17 + 1.95% QF-1; carrier gas: argon/methane at 60 mL /min; column temperature: 200°C; detector: electron capture in pulse mode.

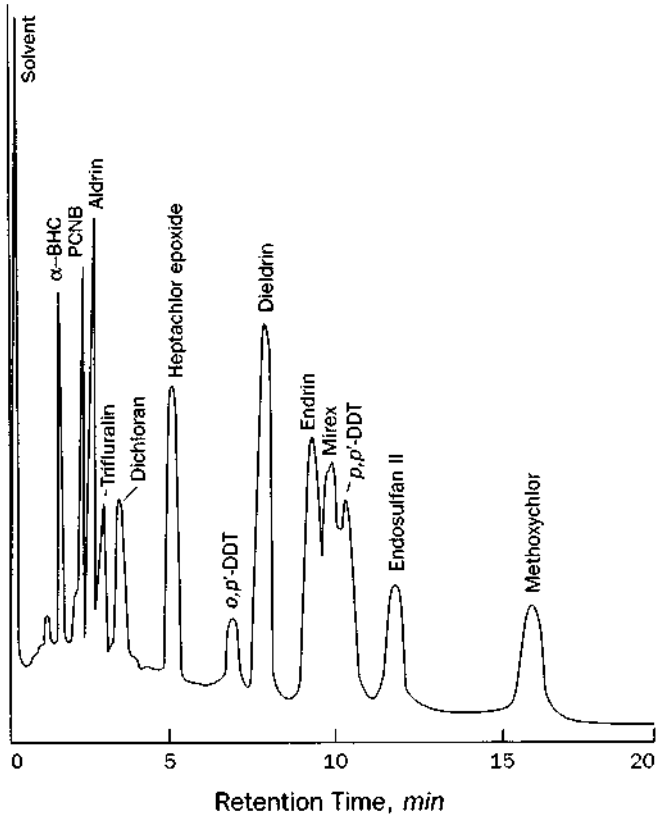


Figure 6630:2. Results of gas chromatographic procedure for organochlorine pesticides. Column packing: 5% OV-210; carrier gas: argon/methane at 70 mL /min; column temperature: 180°C; detector: electron capture.

3. Reagents‖

Use solvents, reagents, and other materials for pesticide analysis that are free from interferences under the condition of the analysis. Specific selection of reagents and distillation of solvents in an all-glass system may be required. "Pesticide quality" solvents usually do not require redistillation; however, always determine a blank before use.

a. Hexane.

b. Petroleum ether, boiling range 30 to 60°C.

c. Diethyl ether: **Caution: Explosive peroxides tend to form.** Test for presence of peroxides# and, if present, reflux over granulated sodium-lead alloy for 8 h, distill in a glass apparatus, and add 2% methanol. Use immediately or, if stored, test for peroxides before use.

d. Ethyl acetate.

e. Methylene chloride.

*f. Magnesia-silica gel,*** PR grade, 60 to 100 mesh. Purchase activated at 676°C and store in the dark in glass container with glass stopper or foil-lined screw cap; do not accept in plastic container. Before use, activate each batch overnight at 130°C in foil-covered glass container.

g. Sodium sulfate (Na_2SO_4), anhydrous, granular: Do not accept in plastic container. If necessary, bake in a muffle furnace to eliminate interferences.

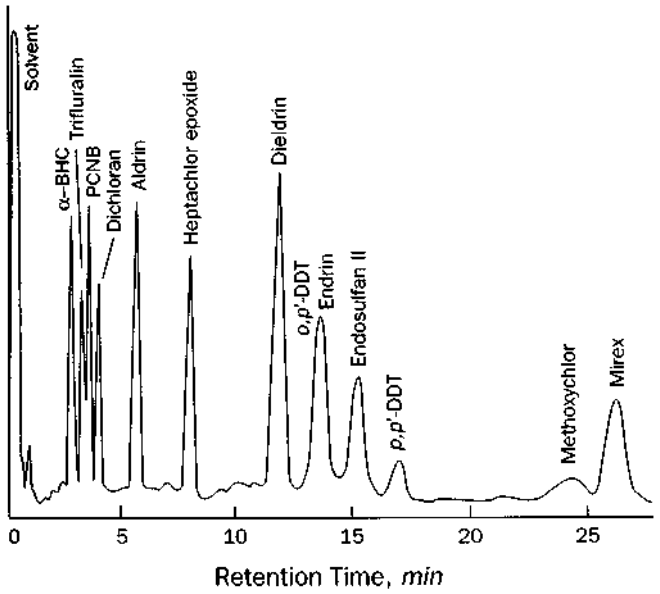


Figure 6630:3. Chromatogram of pesticide mixture. Column packing: 6% QF-1 + 4% SE-30; carrier gas; argon/methane at 60 mL/min; column temperature: 200°C; detector: electron capture.

‖ Gas chromatographic methods are extremely sensitive to the materials used. Mention of trade names by *Standard Methods* does not preclude the use of other existing or as-yet-undeveloped products that give *demonstrably* equal results.

Use E.M. Quant™, MCB Manufacturing Chemists, Inc., 2909 Highland Ave., Cincinnati, OH, or equivalent.

** Florisil™, or equivalent.

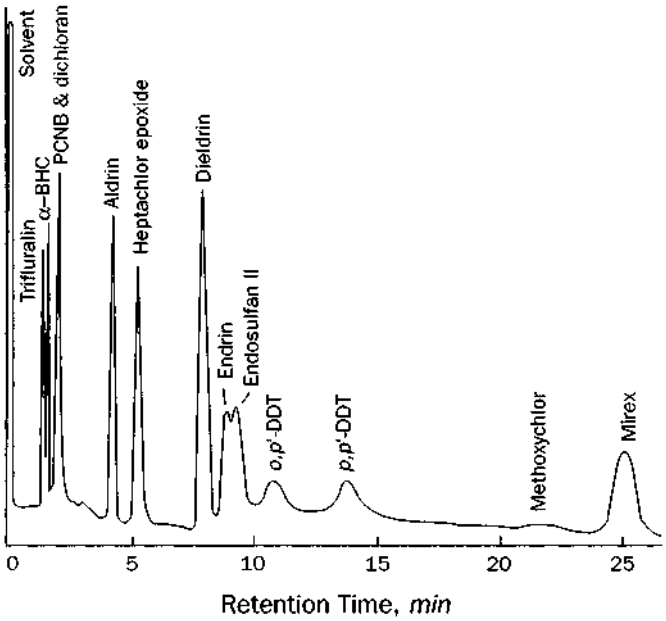


Figure 6630:4. Chromatogram of pesticide mixture. Column packing: 3% OV-1; carrier gas: argon/methane at 70 mL/min; column temperature: 180°C; detector: electron capture.

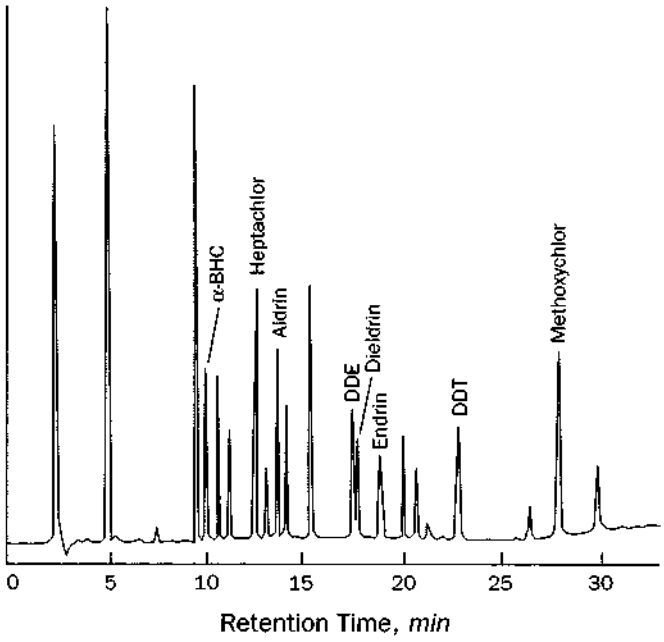


Figure 6630:5. Chromatogram of pesticide mixture. Column DB-5, 30 m long, multilevel program temperature, electron-capture detector.

h. Silanized glass wool.

i. Column packing:

1) Solid support—Dimethyl dichlorosilane-treated diatomaceous earth,†† 100 to 120 mesh.

2) Liquid phases—OV-1, OV-210, 1.5% OV-17 (SP 2250) + 1.95% QF-1 (SP 2401), and 6% QF-1 + 4% SE-30, or equivalent.

j. Carrier gas: One of the following is required:

1) *Nitrogen gas*—purified grade, moisture- and oxygen-free.

2) *Argon-methane* (95 + 5%) for use in pulse mode.

k. Pesticide reference standards: Obtain purest standards available (95 to 98%) from gas chromatographic and chemical supply houses.

l. Stock pesticide solutions: Dissolve 100 mg of each pesticide in ethyl acetate and dilute to 100 mL in a volumetric flask; 1.00 mL = 1.00 mg.

m. Intermediate pesticide solutions: Dilute 1.0 mL stock solution to 100 mL with ethyl acetate; 1.0 mL = 10 μg.

n. Working standard solutions for gas chromatography: Prepare final concentration of standards in hexane solution as required by detector sensitivity and linearity.

4. Procedure

a. Preparation of chromatograph

1) Packing the column‡‡— Use a column constructed of silanized borosilicate glass because other tubing materials may catalyze sample component decomposition. Before packing, rinse and dry column tubing with solvent (e.g., methylene chloride), then methanol. Pack column to a uniform density not so compact as to cause unnecessary back pressure and not so loose as to create voids during use. Do not crush packing. Fill column through a funnel connected by flexible tubing to one end. Plug other end of column with about 1.3 cm silanized glass wool and fill with aid of *gentle* vibration or tapping but do not use an electric vibrator because it tends to fracture packing. Optionally, apply a vacuum to plugged end. Plug open end with silanized glass wool.

2) Conditioning—For packed columns, proper thermal and pesticide conditioning are essential to eliminate column bleed and to provide acceptable gas chromatographic analysis. The following procedure provides excellent results: Connect packed column to the injection port. Do NOT connect column to detector; however, maintain gas flow through detector by using the purge-gas line, or in dual-column ovens, by connecting an unpacked column to the detector. Adjust carrier-gas flow to about 50 mL/min and slowly (over a 1-h period) raise oven temperature to 230°C. After 24 to 48 h at this temperature, the column is ready for pesticide conditioning.

Adjust oven temperature and carrier-gas flow rate to approximate operating levels. Make six consecutive 10-μL injections of a concentrated pesticide mixture through column at about 15-min intervals. Prepare this injection mixture from lindane (γ-BHC), heptachlor, aldrin, heptachlor epoxide, dieldrin, endrin, and *p,p′*-DDT, each compound at a concentration of 200 ng/μL. After pesticide conditioning, connect column to detector and let equilibrate for at least 1 h, preferably overnight. Column is then ready for use.

For capillary columns, conditioning is minimal. After installation, bake column for 1 h at 280 to 300°C. Do not exceed the

†† Gas-Chrom Q™, Supelcoport, or equivalent.

‡‡ Does not apply to fused silica capillary columns.

maximum temperature rating specified by the manufacturer for the column.

3) Injection technique

a) Develop an injection technique with constant rhythm and timing. The "solvent flush" technique described below has been used successfully and is recommended to prevent sample blow-back or distillation within the syringe needle. Flush syringe with solvent, then draw a small volume of clean solvent into syringe barrel (e.g., 1 μL in a 10-μL syringe). Remove needle from solvent and draw 1 μL of air into barrel. For packed columns, draw 3 to 4 μL of sample extract into barrel; for capillary columns, use 1 μL. Remove needle from sample extract and draw approximately 1 μL air into barrel. Record volume of sample extract between air pockets. Rapidly insert needle through inlet septum, depress plunger, withdraw syringe. After each injection, thoroughly clean syringe by rinsing several times with solvent.

b) Inject standard solutions of such concentration that the injection volume and peak height of the standard are approximately the same as those of the sample.

4) Calibration procedure—Prepare a stock calibration mix containing the target analytes, including surrogate compounds. Dilute to five calibration concentration levels. The lowest calibration standard is at the concentration equivalent to the MRL. Inject standards using same technique used to introduce actual samples. Use ratios of peak areas to amounts injected to calculate calibration factors for each analyte at each standard concentration.

Calculate percent relative standard deviation (% RSD) of the calibration factors. If % RSD is <20%, linearity through the origin is assumed and the average calibration factor may be used in place of the calibration curve.

After five-point calibration, analyze a quality control standard (QCS) containing the same target analytes obtained from a source different than that used for the primary calibration standards. The percent recovery must be 80 to 120% of expected value calculated from the calibration curve.

b. Treatment of samples:

1) Sample collection—Fill sample bottle to neck. Collect samples in duplicate.

2) Extraction of samples—Shake sample well and accurately measure all the sample in a 1-L graduated cylinder in two measuring operations if necessary (or use a precalibrated sample bottle to avoid transfer operation). Pour sample into a 2-L separatory funnel or 1- to 2-L continuous liquid–liquid extractor. Rinse sample bottle and cylinder with 60 mL 15% diethyl ether or methylene chloride in hexane, pour this solvent into extraction device. For separatory funnel, shake vigorously for 2 min. Let phases separate for at least 10 min. For continuous liquid–liquid extractors, see Section 6410B.5*a*2) but do not adjust pH.

Drain water phase from separatory funnel into sample bottle and carefully pour organic phase through a 2-cm-OD column containing 8 to 10 cm of Na_2SO_4 into a Kuderna–Danish apparatus fitted with a 10-mL concentrator tube. Pour sample back into separatory funnel.

Rinse sample bottle with 60 mL mixed solvent, use solvent to repeat sample extraction, and pass organic phase through Na_2SO_4. Complete a third extraction with 60 mL of mixed solvent that was used to rinse sample bottle again, and pass organic phase through Na_2SO_4. Wash Na_2SO_4 with several portions of hexane and drain well. Fit Kuderna–Danish apparatus with a three-ball Snyder column and reduce volume to about 7 mL in a hot water bath (90 to 95°C). At this point all methylene chloride present in the initial extracting solvent has been distilled off. Cool, remove concentrator tube from Kuderna–Danish apparatus, rinse ground-glass joint, and dilute to 10 mL with hexane. Make initial gas chromatographic analysis at this dilution.

3) Gas chromatography—Inject 3 to 4 μL of extract solution into a packed column or 1 μL on a fused silica capillary column (split or splitless). Always inject the same volume. Inspect resulting chromatogram for peaks corresponding to pesticides of concern and for presence of interferences.

a) If there are presumptive pesticide peaks and no significant interference, rechromatograph the extract solution on an alternate column.

b) Inject standards frequently to ensure optimum operating conditions. If necessary, concentrate or dilute (*do not use methylene chloride*) the extract so peak size of pesticide is very close to that of corresponding peaks in standard. (See dilution factor, 6630B.5*a*).

c) If significant interference is present, separate interfering substances from pesticide materials by using the cleanup procedure described in the following paragraph.

4) Magnesia-silica gel cleanup—Adjust sample extract volume to 10 mL with hexane. Place a charge of activated magnesia-silica gel§§ (weight determined by lauric-acid value, see 6630B.Appendix) in a chromatographic column. After settling gel by tapping column, add about 1.3 cm anhydrous granular Na_2SO_4 to the top. Pre-elute column, after cooling, with 50 to 60 mL petroleum ether. Discard eluate and just before exposing sulfate layer to air, quantitatively transfer sample extract into column by careful decantation and with subsequent petroleum ether washings (5 mL maximum). Adjust elution rate to about 5 mL/min and, separately, collect the eluates in 500-mL Kuderna–Danish flasks equipped with 10-mL receivers.

Make first elution with 200 mL 6% ethyl ether in petroleum ether, and the second with 200 mL 15% ethyl ether in petroleum ether. Make third elution with 200 mL 50% ethyl ether-petroleum ether and the fourth with 200 mL 100% ethyl ether. Follow with 50 to 100 mL petroleum ether to ensure removal of all ethyl ether from the column. Alternatively, to separate PCBs elute initially with 0% ethyl ether in petroleum ether and proceed as above to yield four fractions.

Concentrate eluates in Kuderna–Danish evaporator in a hot water bath as in ¶ *b*2) above, dilute to appropriate volume, and analyze by gas chromatography.

Eluate composition—By use of an equivalent quantity of any batch of magnesia-silica gel as determined by its lauric acid value (see 6630B.Appendix), the pesticides will be separated into the eluates indicated below:

6% Ethyl Ether Eluate

Aldrin	Heptachlor	Pentachloro-nitrobenzene
BHC	Heptachlor epoxide	
Chlordane	Lindane (γ-BHC)	Strobane

§§ Florisil™, or equivalent.

DDD	Methoxychlor	Toxaphene
DDE	Mirex	Trifluralin
DDT		PCBs

15% Ethyl Ether Eluate	*50% Ethyl Ether Eluate*
Endosulfan I	Endosulfan II
Endrin	Captan
Dieldrin	
Dichloran	
Phthalate esters	

If present, certain thiophosphate pesticides will occur in each of the above fractions as well as in the 100% ether fraction. For additional information regarding eluate composition and the procedure for determining the lauric acid value, refer to the FDA Pesticide Analytical Manual (see Bibliography). For elution pattern test procedure, see 6630B.Appendix, Section 4.

5) Determination of extraction efficiency—Add known amounts (at concentrations similar to those expected in samples) of pesticides in ethyl acetate solution to 1 L water sample and carry through the same procedure as for samples. Dilute an equal amount of intermediate pesticide solution (6630B.3*m*) to the same final volume. Call peak height from standard "*a*" and peak height from sample to which pesticide was added "*b*," whereupon the extraction efficiency equals *b/a*. Periodically determine extraction efficiency and a control blank to test the procedure. Also analyze one set of duplicates with each series of samples as a quality-control check.

5. Calculation

a. Dilution factor: If a portion of the extract solution was concentrated, the dilution factor, *D*, is a decimal; if it was diluted, the dilution factor exceeds 1.

b. Pesticide concentrations: Determine pesticide concentrations by direct comparison to a single standard when the injection volume and response are within 10% of those of the sample pesticide of interest (Table 6630:I). Calculate concentration of pesticide:

$$\mu g/L = \frac{A \times B \times C \times D}{E \times F \times G}$$

where:

A = ng standard pesticide,
B = peak height of sample, mm, or area count,
C = extract volume, μL,
D = dilution factor,
E = peak height of standard, mm, or area count,
F = volume of extract injected, μL, and
G = volume of sample extracted, mL.

Typical chromatograms of representative pesticide mixtures are shown in Figures 6630:1–5.

Report results in micrograms per liter without correction for efficiency.

6. Precision and Bias

Ten laboratories in an interlaboratory study selected their own water samples and added four representative pesticides to replicate samples, at two concentrations in acetone. The added pesticides came from a single source. Samples were analyzed with and without magnesia-silica gel cleanup. Precision and recovery data are given in Table 6630:II.

TABLE 6630:I. RETENTION RATIOS OF VARIOUS ORGANOCHLORINE PESTICIDES RELATIVE TO ALDRIN

Liquid phase*	1.5% OV-17 + 1.95% QF-1	5% OV-210	3% OV-1	6% QF-1 + 4% SE-30
Column temperature	200°C	180°C	180°C	200°C
Argon/methane carrier flow	60 mL/min	70 mL/min	70 mL/min	60 mL/min
Pesticide	RR	RR	RR	RR
∝-BHC	0.54	0.64	0.35	0.49
PCNB	0.68	0.85	0.49	0.63
Lindane (γ-BHC)	0.69	0.81	0.44	0.60
Dichloran	0.77	1.29	0.49	0.70
Heptachlor	0.82	0.87	0.78	0.83
Aldrin	1.00	1.00	1.00	1.00
Heptachlor epoxide	1.54	1.93	1.28	1.43
Endosulfan I	1.95	2.48	1.62	1.79
p,p′-DDE	2.23	2.10	2.00	1.82
Dieldrin	2.40	3.00	1.93	2.12
Captan	2.59	4.09	1.22	1.94
Endrin	2.93	3.56	2.18	2.42
o,p′-DDT	3.16	2.70	2.69	2.39
p,p′-DDD	3.48	3.75	2.61	2.55
Endosulfan II	3.59	4.59	2.25	2.72
p,p′-DDT	4.18	4.07	3.50	3.12
Mirex	6.1	3.78	6.6	4.79
Methoxychlor	7.6	6.5	5.7	4.60
Aldrin (Min absolute)	3.5	2.6	4.0	5.6

* All columns glass, 180-cm × 4-mm-ID, solid support Gas-Chrom Q (100/200 mesh).

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TABLE 6630:II. PRECISION AND BIAS DATA FOR SELECTED ORGANOCHLORINE PESTICIDES

Pesticide	Level Added *ng/L*	Pretreatment	Mean Recovery *ng/L*	Recovery %	Precision* *ng/L* S_T	Precision* *ng/L* S_o
Aldrin	15	No cleanup	10.42	69	4.86	2.59
	110		79.00	72	32.06	20.19
	25	Cleanup†	17.00	68	9.13	3.48‡
	100		64.54	65	27.16	8.02‡
Lindane (γ-BHC)	10	No cleanup	9.67	97	5.28	3.47
	100		72.91	73	26.23	11.49‡
	15	Cleanup†	14.04	94	8.73	5.20
	85		59.08	70	27.49	7.75‡
Dieldrin	20	No cleanup	21.54	108	18.16	17.92
	125		105.83	85	30.41	21.84
	25	Cleanup	17.52	70	10.44	5.10‡
	130		84.29	65	34.45	16.79‡
DDT	40	No cleanup	40.30	101	15.96	13.42
	200		154.87	77	38.80	24.02
	30	Cleanup†	35.54	118	22.62	22.50
	185		132.08	71	49.83	25.31

* S_T = overall precision and S_o = single-operator precision.
† Use of magnesia-silica gel column cleanup before analysis.
‡ $S_o < S_T/2$.

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Appendix—Standardization of Magnesia-Silica Gel* Column by Weight Adjustment Based on Adsorption of Lauric Acid

A rapid method for determining adsorptive capacity of magnesia-silica gel is based on adsorption of lauric acid from hexane solution. An excess of lauric acid is used and the amount not adsorbed is measured by alkali titration. The weight of lauric acid adsorbed is used to calculate, by simple proportion, equivalent quantities of gel for batches having different adsorptive capacities.

1. Reagents

a. Ethyl alcohol, USP or absolute, neutralized to phenolphthalein.

b. Hexane, distilled from all-glass apparatus.

c. Lauric acid solution: Transfer 10.000 g lauric acid to a 500-mL volumetric flask, dissolve in hexane, and dilute to 500 mL; 1.00 mL = 20 mg.

d. Phenolphthalein indicator: Dissolve 1 g in alcohol and dilute to 100 mL.

e. Sodium hydroxide, 0.05*N:* Dilute 25 mL 1*N* NaOH to 500 mL with distilled water. Standardize as follows: Weigh 100 to 200 mg lauric acid into 125-mL Erlenmeyer flask; add 50 mL neutralized ethyl alcohol and 3 drops phenolphthalein indicator; titrate to permanent endpoint; and calculate milligrams lauric acid per milliliter NaOH (about 10 mg/mL).

2. Procedure

Transfer 2.000 g magnesia-silica gel to a 25-mL glass-stoppered Erlenmeyer flask. Cover loosely with aluminum foil

* Florisil™, or equivalent.

and heat overnight at 130°C. Stopper, cool to room temperature, add 20.0 mL lauric acid solution (400 mg), stopper, and shake occasionally during 15 min. Let adsorbent settle and pipet 10.0 mL supernatant into a 125-mL Erlenmeyer flask. Avoid including any gel. Add 50 mL neutral alcohol and 3 drops phenolphthalein indicator solution; titrate with 0.05*N* NaOH to a permanent endpoint.

3. Calculation of Lauric Acid Value and Adjustment of Column Weight

Calculate amount of lauric acid adsorbed on gel as follows:

$$\text{Lauric acid value} = \text{mg lauric acid/g gel} \times 200\ (\text{mL required for titration} \times \text{mg lauric acid/mL } 0.05N \text{ NaOH})$$

To obtain an equivalent quantity of any batch of gel, divide 110 by lauric acid value for that batch and multiply by 20 g. Verify proper elution of pesticides by the procedure given below.

4. Test for Proper Elution Pattern and Recovery of Pesticides

Prepare a test mixture containing aldrin, heptachlor epoxide, *p,p′*-DDE, dieldrin, parathion, and malathion. Dieldrin and parathion should elute in the 15% eluate; all but a trace of malathion in the 50% eluate, and the others in the 6% eluate.

6630 C. Liquid–Liquid Extraction Gas Chromatographic Method II

This method[1] is applicable to the determination of organochlorine pesticides and PCBs*† in municipal and industrial discharges. When analyzing unfamiliar samples for any or all of these compounds, support the identifications by at least one additional qualitative technique. This method includes analytical conditions for a second, confirmatory gas chromatographic column. Alternatively, analyze by a gas chromatographic/mass spectrometric (GC/MS) method for base/neutrals and acids (Section 6410B) using the extract produced by this method.

Additional PCB congeners can be determined if standards are included.

1. General Discussion

a. Principle: A measured volume of sample is extracted with methylene chloride either by liquid–liquid extraction using separatory funnels or by continuous liquid–liquid extraction. The extract is dried and exchanged to hexane during concentration. If other determinations having essentially the same extraction and concentration steps are to be performed, a single sample extraction is sufficient. The extract is separated by gas chromatography and the compounds are measured with an electron capture detector.[2] See Section 6010C.2*a*1) for discussion of gas chromatographic principles.

The method provides procedures for magnesia-silica gel column cleanup and elemental sulfur removal to aid in the elimination of interferences. When cleanup is required, sample concentration levels must be high enough to permit separate treatment of subsamples. Chromatographic conditions appropriate for the simultaneous measurement of combinations of compounds may be selected.

b. Interferences: See Section 6410B.1*b*1) for precautions concerning glassware, reagent purity, and matrix interferences.

Phthalate esters may interfere in pesticide analysis with an electron capture detector. These compounds generally appear in the chromatogram as large, late-eluting peaks, especially in the 15 and 50% fractions from magnesia-silica gel. Common flexible plastics contain phthalates that are easily extracted during laboratory operations. Cross-contamination of clean glassware can occur when plastics are handled during extraction steps, especially when solvent-wetted surfaces are handled. Minimize interferences from phthalates by avoiding use of plastics. Exhaustive cleanup of reagents and glassware may be required to eliminate phthalate contamination.[3,4] Phthalate ester interference can be avoided by using a microcoulometric or electrolytic conductivity detector.

c. Detection levels: The method detection level (MDL) is the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero.[5] The MDL concentrations listed in Table 6630:III were obtained with reagent water.[6] Similar results were achieved with representative wastewaters. The MDL actually obtained in a given analysis will vary, depending on instrument sensitivity and matrix effects. This method has been tested for linearity of known-addition recovery from reagent water and is applicable over the concentration range from 4 × MDL to 1000 × MDL with the following exceptions: Chlordane recovery at 4 × MDL was low (60%); toxaphene recovery was linear over the range of 10 × MDL to 1000 × MDL.[6] It is difficult to determine MDLs for mixtures such as these. To calculate the MDLs given, a few of the GC peaks in each mixture were used. Depending on the particular peaks selected, these results may or may not be reproducible in other laboratories.

d. Safety: The toxicity or carcinogenicity of each reagent has not been defined precisely. The following compounds have been classified tentatively as known or suspected, human or mammalian carcinogens: 4,4′-DDT, 4,4′-DDD, the BHCs, and the PCBs. Prepare primary standards of these compounds in a hood and wear a NIOSH/MESA-approved toxic gas respirator when handling high concentrations. Treat and dispose of Hg used for sulfur removal as a hazardous waste.

* Aldrin, α-BHC, β-BHC, δ-BHC, γ-BHC, chlordane, 4,4′-DDD, 4,4′-DDE, 4,4′-DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, heptachlor, heptachlor epoxide, toxaphene, PCB-1016, PCB-1221, PCB-1232, PCB-1242, PCB-1248, PCB-1254, PCB-1260.

† The PCBs constitute a class of 209 compounds. This procedure is designed to determine nine commercial formulations known as the Aroclors, each of which is a mixture of PCBs.

TABLE 6630:III. CHROMATOGRAPHIC CONDITIONS AND METHOD DETECTION LEVELS*

Compound	Retention Time *min*		Method Detection Level
	Column 1	Column 2	*μg/L*
α-BHC	1.35	1.82	0.003
γ-BHC	1.70	2.13	nd
β-BHC	1.90	1.97	nd
Heptachlor	2.00	3.35	0.003
δ-BHC	2.15	2.20	0.009
Aldrin	2.40	4.10	0.004
Heptachlor epoxide	3.50	5.00	0.083
Endosulfan I	4.50	6.20	0.014
4,4′-DDE	5.13	7.15	0.004
Dieldrin	5.45	7.23	0.002
Endrin	6.55	8.10	0.006
4,4′-DDD	7.83	9.08	0.011
Endosulfan II	8.00	8.28	0.004
4,4′-DDT	9.40	11.75	0.012
Endrin aldehyde	11.82	9.30	0.023
Endosulfan sulfate	14.22	10.70	0.066
Chlordane	mr	mr	0.014
Toxaphene	mr	mr	0.24
PCB-1016	mr	mr	nd
PCB-1221	mr	mr	nd
PCB-1232	mr	mr	nd
PCB-1242	mr	mr	0.065
PCB-1248	mr	mr	nd
PCB-1254	mr	mr	nd
PCB-1260	mr	mr	nd

Column 1 conditions: Supelcoport (100/120 mesh) coated with 1.5% SP-2250/1.95% SP-2401 packed in a 1.8-m-long × 4-mm-ID glass column with 5% methane/95% argon carrier gas at 60-mL/min flow rate. Column temperature held isothermal at 200°C, except for PCB-1016 through PCB-1248 at 160°C.

Column 2 conditions: Supelcoport (100/120 mesh) coated with 3% OV-1 packed in a 1.8-m-long × 4-mm-ID glass column with 5% methane/95% argon carrier gas at 60-mL/min flow rate. Column temperature held isothermal at 200°C for the pesticides; at 140°C for PCB-1221 and 1232; and at 170°C for PCB-1016 and 1242 to 1268.

* mr = multiple peak response. See Figures 6630:2–10.
nd = not determined.

2. Sampling and Storage

For collection and storage requirements, see Section 6410B.2. If samples will not be extracted within 72 h of collection, adjust pH to the range 5.0 to 9.0 with NaOH or H_2SO_4. Record volume of acid or base used. If aldrin is to be determined, add sodium thiosulfate when residual chlorine is present.

3. Apparatus

Use apparatus specified in Section 6410B.3*a–e*, *g*, and *i–k*.

In addition:

a. Chromatographic column, 400 mm long × 22 mm ID, with TFE stopcock and coarse frit filter disk.‡

b. Gas chromatograph:§ An analytical system complete with gas chromatograph suitable for on-column injection and all required accessories, including syringes, analytical columns, gases, and strip-chart recorder. Preferably use a data system for measuring peak areas. See column specifications in 6630B.2*j* for both packed columns and fused silica capillary columns.

1) *Column 1*—1.8 m long × 4 mm ID, glass, packed with 1.5% SP-2250/1.95% SP-2401 on Supelcoport (100/120 mesh) or equivalent. This column was used to develop the detection level and precision and bias data presented herein. For guidelines for the use of alternate column packings, see 6630C.5*c*.

Although procedures detailed below refer primarily to packed columns, capillary columns may be used if equivalent results can be demonstrated.

2) *Column 2*—1.8 m long × 4 mm ID, glass, packed with 3% OV-1 on Supelcoport (100/120 mesh) or equivalent.

3) *Detector*—electron-capture. This detector was used to develop the detection level and precision and bias data presented herein. For use of alternate detectors, see 6630C.5*c*.

4. Reagents

This method requires reagents described in Section 6410B.4*a–e*, and in addition:

a. Acetone, hexane, isooctane, methylene chloride, pesticide quality or equivalent.

b. Ethyl ether, nanograde redistilled in glass if necessary. Demonstrate before use freedom from peroxides by means of test strips.‖ Remove peroxides by procedures provided with the test strips. After cleanup, add 20 mL ethyl alcohol preservative per liter of ether.

c. Magnesia-silica gel,# 60/100 mesh. Purchase activated at 1250°F and store in the dark in glass containers with ground-glass stoppers or foil-lined screw caps. Before use, activate each batch for at least 16 h at 130°C in a foil-covered glass container; let cool.

d. Mercury, triple-distilled.

e. Copper powder, activated.

f. Stock standard solutions: Prepare as directed in Section 6410B.4*g*, using isooctane as the solvent.

g. Calibration standards: See Section 6420B.4*j*. Dilute with isooctane and use MDL values from Table 6630:III.

h. Quality control (QC) check sample concentrate: Obtain a check sample concentrate containing each compound at the following concentrations in acetone: 4,4′-DDD, 10μg /mL; 4,4′-DDT, 10 μg /mL; endosulfan II, 10 μg /mL; endosulfan sulfate, 10 μg/mL; endrin, 10 μg/mL; any other single-component pesticide, 2 μg/mL. If this method will be used only to analyze for PCBs, chlordane, or toxaphene, the QC check sample concentrate should contain the most representative multicomponent compound at a concentration of 50 μg/mL in acetone. If such a sample is not available from an external source, prepare using stock standards prepared independently from those used for calibration.

5. Procedure

a. Extraction: Mark water meniscus on side of sample bottle for later determination of volume. Pour entire sample into a 2-L

‡ Kontes K-42054, or equivalent.
§ Gas chromatographic methods are extremely sensitive to the materials used. Mention of trade names by *Standard Methods* does not preclude the use of other existing or as-yet-undeveloped products that give *demonstrably* equivalent results.
‖ E. Merck, EM Science Quant, or equivalent.
Florisil, or equivalent.

separatory funnel or a 1- or 2-L continuous liquid–liquid extractor for a period of 18 h. Extract with methylene chloride as directed in Section 6410B.5*a*1), without any pH adjustment or solvent wash.

After extracting and concentrating with a three-ball Snyder column, increase temperature of hot water bath to about 80°C. Momentarily remove Snyder column, add 50 mL hexane and a new boiling chip, and reattach Snyder column. Concentrate extract as before, but use hexane to prewet column. Complete concentration in 5 to 10 min.

Remove Snyder column and rinse flask and its lower joint into the concentrator tube with 1 to 2 mL hexane. Preferably use a 5-mL syringe for this operation. Stopper concentrator tube and store refrigerated if further processing will not be done immediately. If extract is to be stored longer than 2 d, transfer to a TFE-sealed screw-cap vial. If extract requires no further cleanup, proceed with gas chromatographic analysis. If further cleanup is required, follow procedure of ¶ *b* below before chromatographic analysis.

Determine original sample volume by refilling sample bottle to mark and transferring liquid to a 1000-mL graduated cylinder. Record sample volume to nearest 5 mL.

b. Cleanup and separation: Use either procedure below or any other appropriate procedure; however, first demonstrate that the requirements of 6630C.7 can be met. The magnesia-silica gel column allows for a select fractionation of compounds and eliminates polar interferences. Elemental sulfur, which interferes with the electron-capture gas chromatography of certain pesticides, can be removed by the technique described below.

1) Magnesia-silica gel column cleanup—Place a weight of magnesia-silica gel (nominally 20 g) predetermined by calibration, ¶ *d*3) below, into a chromatographic column. Tap column to settle gel and add 1 to 2 cm anhydrous Na_2SO_4 to the top. Add 60 mL hexane to wet and rinse. Just before exposure of the Na_2SO_4 layer to air, stop elution of hexane by closing stopcock on column. Discard eluate. Adjust sample extract volume to 10 mL with hexane and transfer it from K–D concentrator tube onto column. Rinse tube twice with 1 to 2 mL hexane, adding each rinse to the column. Place a 500-mL K–D flask and clean concentrator tube under chromatographic column. Drain column into flask until Na_2SO_4 layer is nearly exposed. Elute column with 200 mL 6% ethyl ether in hexane (v/v) (Fraction 1) at a rate of about 5 mL /min. Remove K–D flask and set aside. Elute column again, using 200 mL 15% ethyl ether in hexane (v/v) (Fraction 2), into a second K–D flask. Elute a third time using 200 mL 50% ethyl ether in hexane (v/v) (Fraction 3). The elution patterns for the pesticides and PCBs are shown in Table 6630:IV. Concentrate fractions for 15 to 20 min as in ¶ *a* above, using hexane to prewet the column, and set water bath temperature at about 85°C. After cooling, remove Snyder column and rinse flask and its lower joint into concentrator tube with hexane. Adjust volume of each fraction to 10 mL with hexane and analyze by gas chromatography, ¶s *c*–*e* below.

2) Sulfur interference removal—Elemental sulfur usually will elute entirely in Fraction 1 of the magnesia-silica gel column cleanup. To remove sulfur interference from this fraction or the original extract, pipet 1.00 mL concentrated extract into a clean concentrator tube or TFE-sealed vial. Add 1 to 3 drops of mercury and seal.[7] Mix for 15 to 30 s. If prolonged shaking (2 h) is required, use a reciprocal shaker. Alternatively, use activated copper powder for sulfur removal.[8] Analyze by gas chromatography.

Table 6630:IV. Distribution of Chlorinated Pesticides and PCBs into Magnesia-Silica Gel Column Fractions[5]

Compound	Recovery by Fraction* %		
	1	2	3
Aldrin	100	—	—
α-BHC	100	—	—
β-BHC	97	—	—
δ-BHC	98	—	—
γ-BHC	100	—	—
Chlordane	100	—	—
4,4′-DDD	99	—	—
4,4′-DDE	98	—	—
4,4′-DDT	100	—	—
Dieldrin	0	100	—
Endosulfan I	37	64	—
Endosulfan II	0	7	91
Endosulfan sulfate	0	0	106
Endrin	4	96	—
Endrin aldehyde	0	68	26
Heptachlor	100	—	—
Heptachlor epoxide	100	—	—
Toxaphene	96	—	—
PCB-1016	97	—	—
PCB-1221	97	—	—
PCB-1232	95	4	—
PCB-1242	97	—	—
PCB-1248	103	—	—
PCB-1254	90	—	—
PCB-1260	95	—	—

* Eluent composition:
Fraction 1–6% ethyl ether in hexane,
Fraction 2–15% ethyl ether in hexane, and
Fraction 3–50% ethyl ether in hexane.

c. Gas chromatography operating conditions: Table 6630:III summarizes the recommended operating conditions for the gas chromatograph and gives retention times and MDLs that can be achieved under these conditions. Examples of separations obtained with Column 1 are shown in Figures 6630:6 –15. Other packed or capillary (open-tubular) columns,[9] chromatographic conditions, or detectors may be used if the requirements of 6630C.7 are met.

d. Calibration: Calibrate system daily by either external or internal procedure. NOTE: For quantification and identification of mixtures such as PCBs, chlordane, and toxaphene, take extra precautions.[9–11]

1) External standard calibration procedure—Prepare standards as directed in 6630C.4*g* and follow procedure of Section 6420B.5*b*3). Tabulate data and obtain calibration curve or calibration factor as directed in Section 6200B.4*c*3).

2) Internal standard calibration procedure—Prepare standards as directed in 6630C.4*g* and follow procedure of Section 6420B.5*b*3). Tabulate data and calculate response factors (RF) as directed in Section 6200B.4*c*2).

Verify working calibration curve, calibration factor, or RF on each working shift by measuring one or more calibration standards. If the response for any compound varies from the predicted response by $>\pm 15\%$, repeat test using a fresh calibration stan-

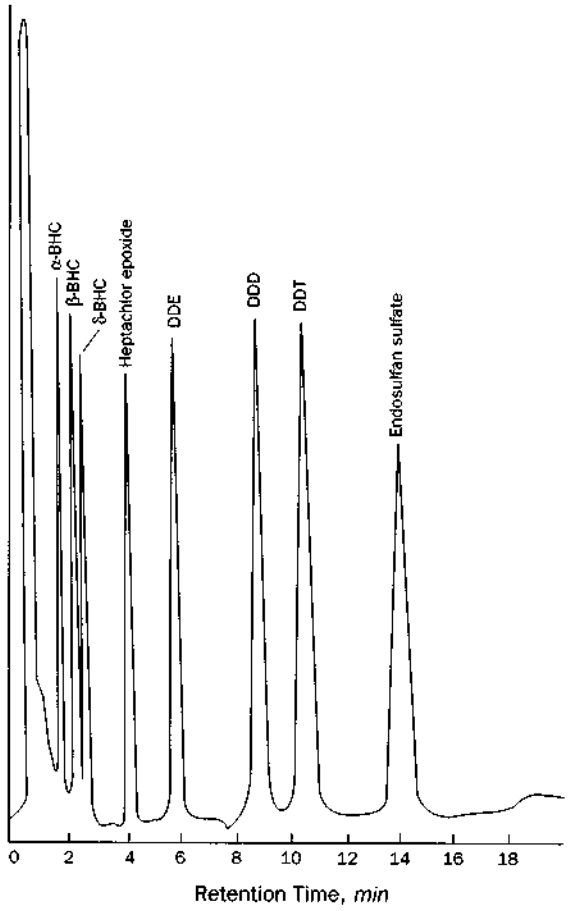


Figure 6630:6. Gas chromatogram of pesticides. Column: 1.5% SP-2250/1.95% SP-2401 on Supelcoport; temperature: 200°C; detector: electron capture.

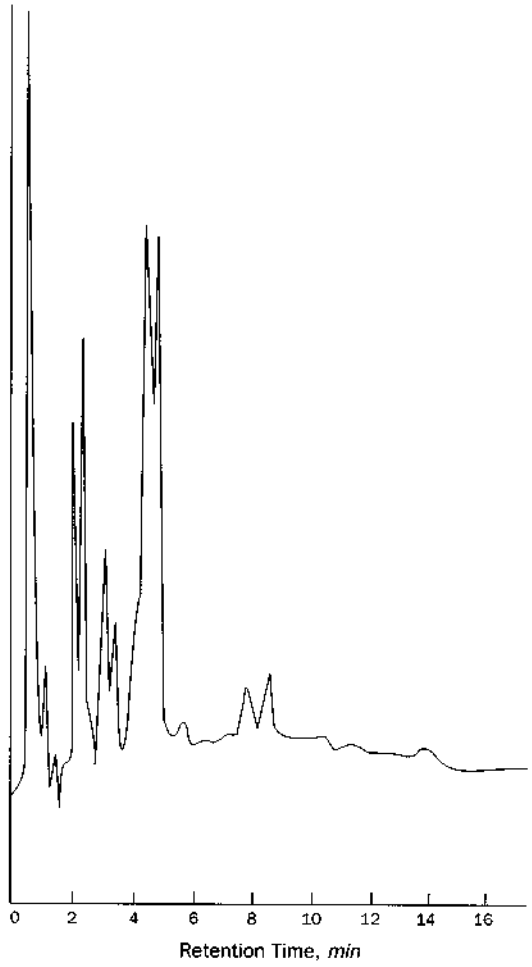


Figure 6630:7. Gas chromatogram of chlordane. Column: 1.5% SP-2250/1.95% SP-2401 on Supelcoport; temperature: 200°C; detector: electron capture.

dard. Alternatively, prepare a new calibration curve for that compound.

3) Magnesia-silica gel standardization—Gel from different batches or sources may vary in adsorptive capacity. To standardize the amount used, use the lauric acid value[12], which measures the adsorption from a hexane solution of lauric acid (mg/g gel). Determine the amount to be used for each column by dividing 110 by this ratio and multiplying the quotient by 20 g.

Before using any cleanup procedure, process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

e. Sample analysis: See Section 6420B.5*b*3). If peak response cannot be measured because of interferences, further cleanup is required.

6. Calculation

Determine concentration of individual compounds using procedures given in Section 6420B.6*a*.

If it is apparent that two or more PCB (Aroclor) mixtures are present, the Webb and McCall procedure[13] may be used to identify and quantify the Aroclors, depending on the Aroclors present. Other techniques also are available.

For multicomponent mixtures (chlordane, toxaphene, and PCBs) match peak retention times in standards with peaks in sample. Quantitate every identifiable peak unless interference with individual peaks persists after cleanup. Add peak height or peak area of each identified peak in chromatogram. Calculate as total response in sample versus total response in standard. Environmental degradation of these compounds may make identification difficult. This method is suitable only for intact mixtures such as the original Aroclor or pesticide formulation and it is not suitable for the other altered mixtures that are sometimes found in the environment. In these instances the GC peak pattern would not match the standard.

Report results in micrograms per liter without correction for recovery data. Report QC data with the sample results.

7. Quality Control

a. Quality control program: See Section 6200A.5.

b. Initial quality control: To establish the ability to generate data with acceptable precision and bias, proceed as follows: Using a pipet, prepare QC check samples at test concentrations shown in Table 6630:V by adding 1.00 mL of QC check sample

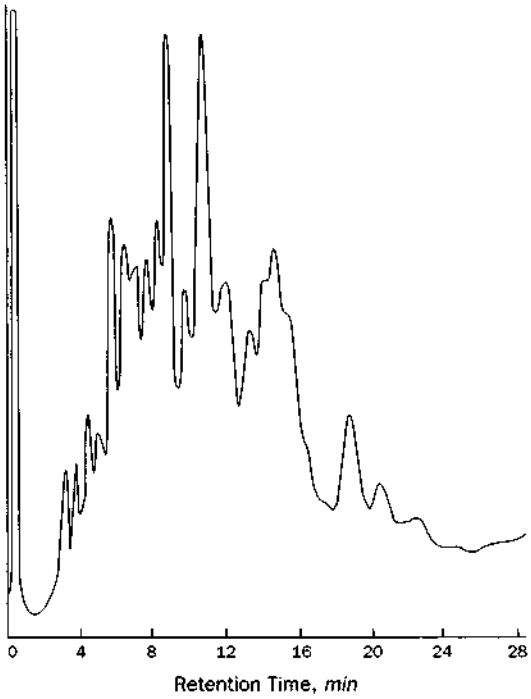


Figure 6630:8. Gas chromatogram of toxaphene. Column: 1.5% SP-2250/1.95% SP-2401 on Supelcoport; temperature: 200°C; detector: electron capture.

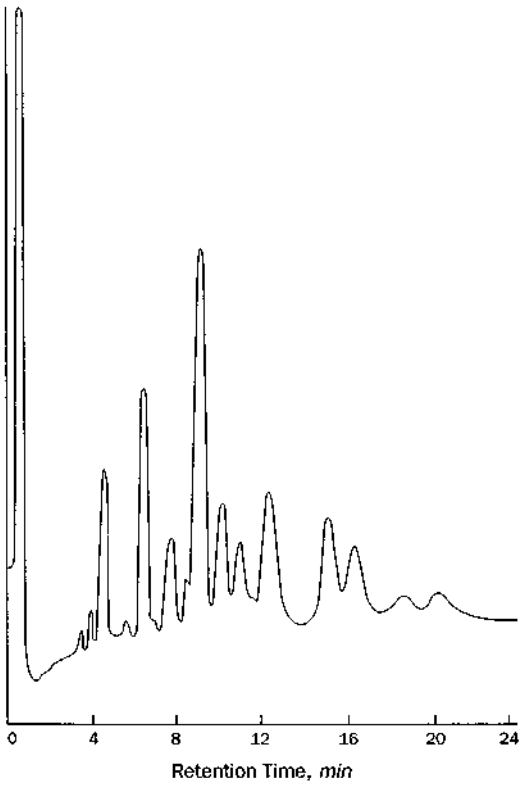


Figure 6630:9. Gas chromatogram of PCB-1016. Column: 1.5% SP-2250/1.95% SP-2401 on Supelcoport; temperature: 160°C; detector: electron capture.

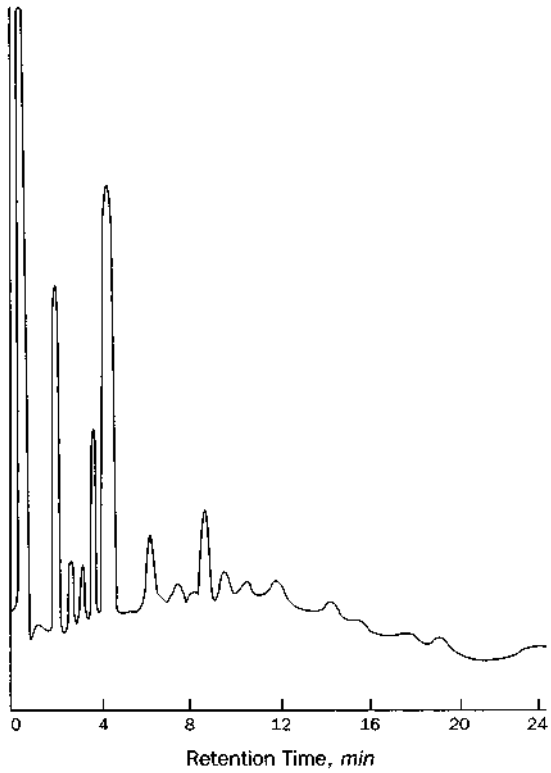


Figure 6630:10. Gas chromatogram of PCB-1221. Column: 1.5% SP-2250/1.95% SP-2401 on Supelcoport; temperature: 160°C; detector: electron capture.

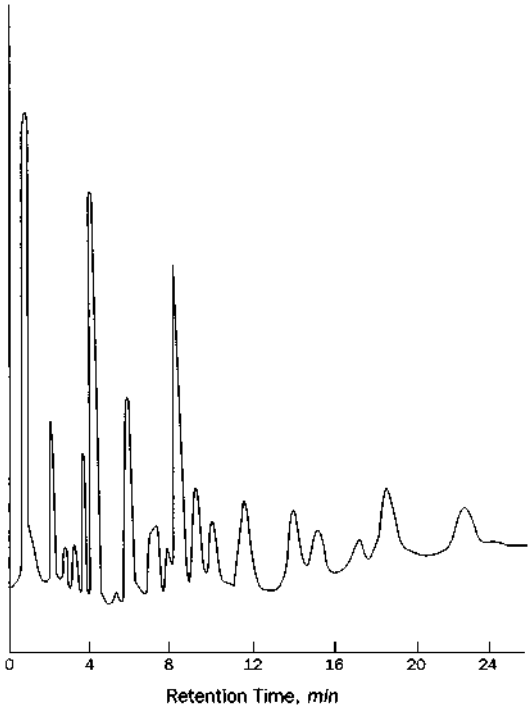


Figure 6630:11. Gas chromatogram of PCB-1232. Column: 1.5% SP-2250/1.95% SP-2401 on Supelcoport; temperature: 160°C; detector: electron capture.

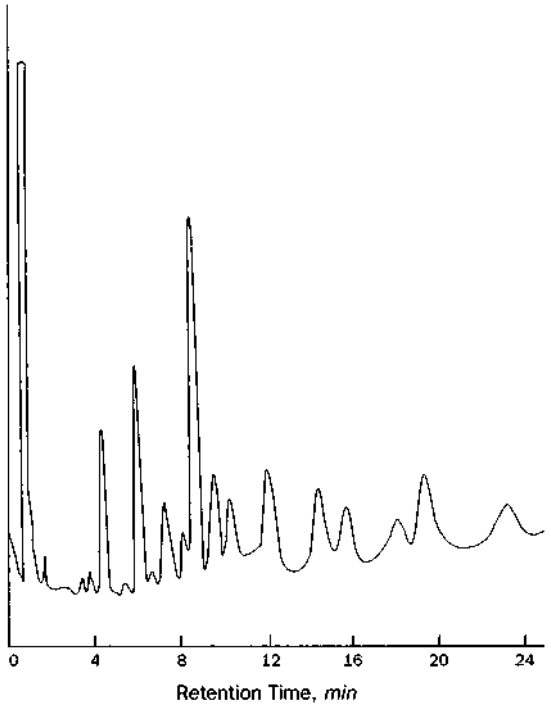


Figure 6630:12. Gas chromatogram of PCB-1242. Column: 1.5% SP-2250/1.95% SP-2401 on Supelcoport; temperature: 160°C; detector: electron capture.

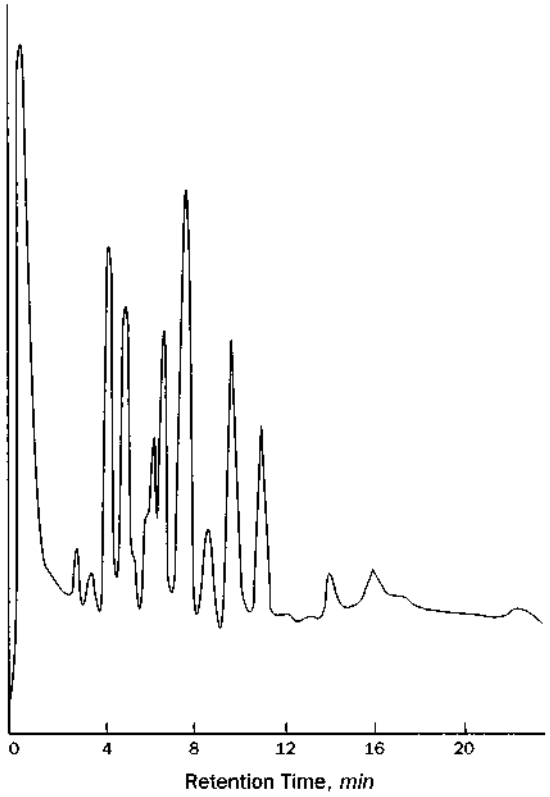


Figure 6630:14. Gas chromatogram of PCB-1254. Column: 1.5% SP-2550/1.95% SP-2401 on Supelcoport; temperature: 200°C; detector: electron capture.

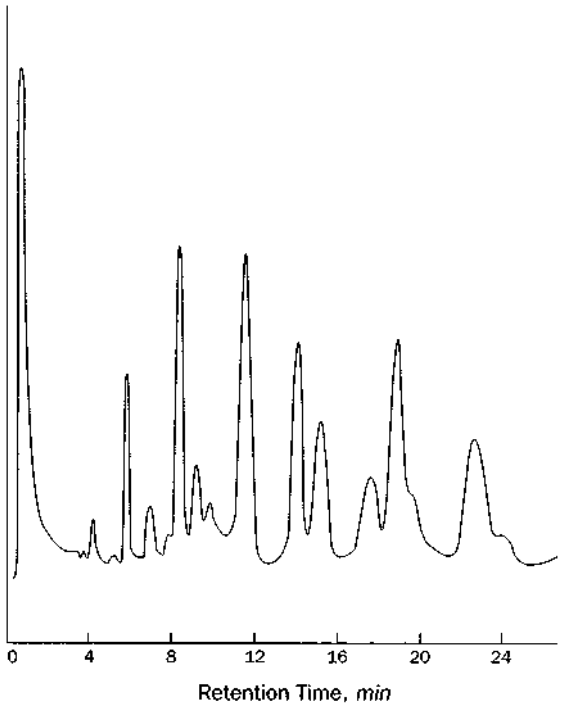


Figure 6630:13. Gas chromatogram of PCB-1248. Column: 1.5% SP-2250/1.95% SP-2401 on Supelcoport; temperature: 160°C; detector: electron capture.

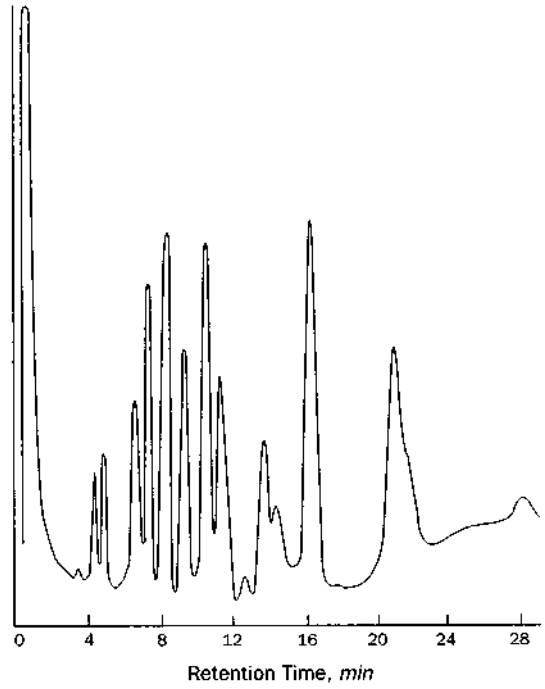


Figure 6630:15. Gas chromatogram of PCB-1260. Column: 1.5% SP-2250/1.95% SP-2401 on Supelcoport; temperature: 200°C; detector: electron capture.

TABLE 6630:V. QC ACCEPTANCE CRITERIA*

Compound	Test Conc. µg/L	Limit for s µg/L	Range for $\bar{X}$ µg/L	Range for P, P_s %
Aldrin	2.0	0.42	1.08–2.24	42–122
α-BHC	2.0	0.48	0.98–2.44	37–134
β-BHC	2.0	0.64	0.78–2.60	17–147
δ-BHC	2.0	0.72	1.01–2.37	19–140
γ-BHC	2.0	0.46	0.86–2.32	32–127
Chlordane	50	10.0	27.6–54.3	45–119
4,4′-DDD	10	2.8	4.8–12.6	31–141
4,4′-DDE	2.0	0.55	1.08–2.60	30–145
4,4′-DDT	10	3.6	4.6–13.7	25–160
Dieldrin	2.0	0.76	1.15–2.49	36–146
Endosulfan I	2.0	0.49	1.14–2.82	45–153
Endosulfan II	10	6.1	2.2–17.1	D–202
Endosulfan sulfate	10	2.7	3.8–13.2	26–144
Endrin	10	3.7	5.1–12.6	30–147
Heptachlor	2.0	0.40	0.86–2.00	34–111
Heptachlor epoxide	2.0	0.41	1.13–2.63	37–142
Toxaphene	50	12.7	27.8–55.6	41–126
PCB-1016	50	10.0	30.5–51.5	50–114
PCB-1221	50	24.4	22.1–75.2	15–178
PCB-1232	50	17.9	14.0–98.5	10–215
PCB-1242	50	12.2	24.8–69.6	39–150
PCB-1248	50	15.9	29.0–70.2	38–158
PCB-1254	50	13.8	22.2–57.9	29–131
PCB-1260	50	10.4	18.7–54.9	8–127

* s = standard deviation of four recovery measurements,
$\bar{X}$ = average recovery for four recovery measurements,
$\bar{P}$, P_s = percent recovery measured, and
D = detected; result must be >0.

NOTE: These criteria are based directly on the method performance data in Table 6630:VI. Where necessary, the limits for recovery were broadened to assure applicability of the limits to concentrations below those used to develop Table 6630:VI.

TABLE 6630:VI. METHOD PRECISION AND BIAS AS FUNCTIONS OF CONCENTRATION*

Compound	Bias, as Recovery, X' µg/L	Single-Analyst Precision, s_r µg/L	Overall Precision, S' µg/L
Aldrin	$0.81C + 0.04$	$0.16\bar{X} - 0.04$	$0.20\bar{X} - 0.01$
α-BHC	$0.84C + 0.03$	$0.13\bar{X} + 0.04$	$0.23\bar{X} - 0.00$
β-BHC	$0.81C + 0.07$	$0.22\bar{X} - 0.02$	$0.33\bar{X} - 0.05$
δ-BHC	$0.81C + 0.07$	$0.18\bar{X} + 0.09$	$0.25\bar{X} + 0.03$
γ-BHC	$0.82C - 0.05$	$0.12\bar{X} + 0.06$	$0.22\bar{X} + 0.04$
Chlordane	$0.82C - 0.04$	$0.13\bar{X} + 0.13$	$0.18\bar{X} + 0.18$
4,4′-DDD	$0.84C + 0.30$	$0.20\bar{X} - 0.18$	$0.27\bar{X} - 0.14$
4,4′-DDE	$0.85C + 0.14$	$0.13\bar{X} + 0.06$	$0.28\bar{X} - 0.09$
4,4′-DDT	$0.93C - 0.13$	$0.17\bar{X} + 0.39$	$0.31\bar{X} - 0.09$
Dieldrin	$0.90C + 0.02$	$0.12\bar{X} + 0.19$	$0.16\bar{X} + 0.16$
Endosulfan I	$0.97C + 0.04$	$0.10\bar{X} + 0.07$	$0.18\bar{X} + 0.08$
Endosulfan II	$0.93C + 0.34$	$0.41\bar{X} - 0.65$	$0.47\bar{X} - 0.20$
Endosulfan sulfate	$0.89C - 0.37$	$0.13\bar{X} + 0.33$	$0.24\bar{X} + 0.35$
Endrin	$0.89C - 0.04$	$0.20\bar{X} + 0.25$	$0.24\bar{X} + 0.25$
Heptachlor	$0.69C + 0.04$	$0.06\bar{X} + 0.13$	$0.16\bar{X} + 0.08$
Heptachlor epoxide	$0.89C + 0.10$	$0.18\bar{X} - 0.11$	$0.25\bar{X} - 0.08$
Toxaphene	$0.80C + 1.74$	$0.09\bar{X} + 3.20$	$0.20\bar{X} + 0.22$
PCB-1016	$0.81C + 0.50$	$0.13\bar{X} + 0.15$	$0.15\bar{X} + 0.45$
PCB-1221	$0.96C + 0.65$	$0.29\bar{X} - 0.76$	$0.35\bar{X} - 0.62$
PCB-1232	$0.91C + 10.79$	$0.21\bar{X} - 1.93$	$0.31\bar{X} + 3.50$
PCB-1242	$0.93C + 0.70$	$0.11\bar{X} + 1.40$	$0.21\bar{X} + 1.52$
PCB-1248	$0.97C + 1.06$	$0.17\bar{X} + 0.41$	$0.25\bar{X} - 0.37$
PCB-1254	$0.76C + 2.07$	$0.15\bar{X} + 1.66$	$0.17\bar{X} + 3.62$
PCB-1260	$0.66C + 3.76$	$0.22\bar{X} - 2.37$	$0.37\bar{X} - 4.86$

* X' = expected recovery for one or more measurements of a sample containing a concentration of C,
s_r = expected single-analyst standard deviation of measurements at an average concentration found of $\bar{X}$,
S' = expected interlaboratory standard deviation of measurements at an average concentration found of $\bar{X}$,
C = true value for the concentration, and
$\bar{X}$ = average recovery found for measurements of samples containing a concentration of C.

concentrate (6630C.4*h*) to each of four 1-L portions of reagent water. Analyze QC check samples according to the method beginning in 6630C.5*a*. Calculate average recovery and standard deviation of the recovery, compare with acceptance criteria and evaluate and correct system performance as directed in Section 6200A.5*a*1) and 2).

c. Analyses of samples with known additions: See Section 6420B.7*c*. Prepare QC check sample concentrates according to 6630C.4*h* and use Tables 6630:III and IV.

d. Quality-control check standard analysis: See Section 6420B.7*d*. Prepare QC check standard according to 6630C.4*h* and use Table 6630:V. If all compounds in Table 6630:V are to be measured in the sample in ¶ *c* above, it is probable that the analysis of a QC check will be required; therefore, routinely analyze the QC check standard with the known-addition sample.

e. Bias assessment and records: See Section 6410B.7*e*.

8. Precision and Bias

This method was tested by 20 laboratories using reagent water, drinking water, surface water, and industrial wastewaters with known additions at six concentrations over the range 0.5 to 30 μg/L for single-component pesticides and 8.5 to 400 μg/L for multicomponent samples.[14] Single-operator precision, overall precision, and method bias were found to be related directly to the compound concentration and essentially independent of sample matrix. Linear equations describing these relationships are presented in Table 6630:VI.

9. References

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2. U.S. Environmental Protection Agency. 1982. Determination of pesticides and PCBs in industrial and municipal wastewaters; EPA-600/4-82-023, National Technical Information Serv., PB82-214222. Springfield, Va.
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6630 D. Liquid–Liquid Extraction Gas Chromatographic/Mass Spectrometric Method

See Section 6410B.

6640 ACIDIC HERBICIDE COMPOUNDS*

6640 A. Introduction

1. Sources and Significance

Agricultural chemicals used for weed control can be found as contaminants in various aquatic systems.[1] Although formerly only two chlorinated phenoxy acid herbicides have been among the substances regulated by the U.S. Environmental Protection Agency in drinking water, and thus were routinely measured, several other types of carboxylic acid compounds used for their toxic effects now are under regulatory control.[2]

2. Selection of Method

The micro liquid–liquid extraction gas chromatographic method presented here is applicable to the determination of salts and esters of analyte acids. The form of each acid is not routinely distinguished by this method. Results are calculated and reported for each listed compound as the total free acid. Since only the free acid is quantified, the herbicide Lactofen will be quantified as Acifluorfen because their structures represent different esters of the same acid herbicide. Additionally, this method is unable to quantify the degradation products of Dacthal separately from the parent compound unless the optional wash step is performed. If this wash step is not performed, all forms of Dacthal (the parent compound and the mono- and di-acid degradation products) will be quantified as Dacthal.

3. Sampling and Storage

Collect grab samples in accordance with conventional sampling practices (see Section 1060B), using amber glass containers with polytetrafluoroethylene (PFTE) lined screw caps and capacities of at least 40 mL. Add sodium sulfite crystals (approximately 2 mg/40 mL sample) if residual chlorine is present in the sample. Fill sample bottles but take care not to flush out the sodium sulfite. Because the target analytes of this method are not volatile, it is not necessary to ensure that the sample bottles are completely headspace-free. Ice samples during shipment so the temperature does not exceed 10°C during the first 48 h after collection. Confirm that samples are ≤10°C when they are received at the laboratory. Store samples in the laboratory ≤6°C and protect from light until extraction. Do not freeze samples.

Because of the several pH adjustments made to the samples in the course of this method, the addition of organic or inorganic biocides, including hydrochloric acid, has been omitted. The analyst should be aware of the potential for the biological degradation of the analytes. Extract samples as soon as possible and within no >14 d.

4. References

1. U.S. Environmental Protection Agency. 1990. National Pesticide Survey: Summary Results of EPA's National Survey of Pesticides in Drinking Water Wells; EPA 57019-90-015. Cincinnati, Ohio.
2. U.S. Environmental Protection Agency. 1992. National Primary Drinking Water Regulations; Synthetic Organic Chemicals and Inorganic Chemicals; Final Rule. 40 CFR Parts 141 and 142, Part 111; *Fed. Reg.* 57, No.138.

* Approved by Standard Methods Committee, 2006. Editorial revisions, 2011. Joint Task Group: 21st Edition—Russell Chinn (chair), Wenta Liao, David J. Munch, Theresa A. Prato.

6640 B. Micro Liquid–Liquid Extraction Gas Chromatographic Method

1. General Discussion

a. Application: This is a gas chromatography (GC) method for the determination of chlorinated acids in drinking water. It can be used to measure various acidic organic compounds and their corresponding acid salts, although the form of each acid is not differentiated and the calculated amount of each is expressed as free acid. Accuracy, precision, and detection level data have been generated in reagent water and finished ground and surface waters for compounds listed in Table 6640:I. Also see 6640A.2.

b. Principle: A 40-mL sample is adjusted to pH ≥12 with 4*N* sodium hydroxide and is kept for 1 h at room temperature to hydrolyze derivatives. Note: Because the chlorphenoxy acid herbicides are formulated as a variety of esters and salts, the hydrolysis step is required and may not be skipped. After hydrolysis, an optional wash step using a 90:10 hexane:methyl *tert*-butyl ether (MtBE) mixture may be performed as a sample cleanup and to remove Dacthal. It is only necessary to perform this wash if the quantitation of the Dacthal degradation products is desired. The aqueous sample then is acidified with sulfuric acid to pH ≤1 and extracted with 4 mL of MtBE that contains the internal standard. The chlorinated acids, which have been partitioned into the MtBE, then are converted to methyl esters by derivatization with diazomethane. The target esters are separated and detected by capillary column gas chromatography using an electron capture detector (GC/ECD). Analytes are quantified using an internal-standard-based calibration curve.

c. Interferences: Clean all glassware meticulously by washing with detergent and tap water, rinsing with tap water, and rinsing with reagent water. A final rinse with solvents may be needed. In place of a solvent rinse, heat nonvolumetric glassware in a muffle furnace at 400°C for 2 h. Do not heat volumetric glass-

Table 6640:I. Single-Laboratory Method Detection Levels in Reagent Water

Compound*	Fortification Level μg/L	Primary Column MDL μg/L	Secondary Column MDL μg/L
Dalapon	0.100	0.05	0.07
3,5-Dichlorobenzoic acid	0.050	0.21	0.05
Dicamba	0.050	0.03	0.04
Dichlorprop	0.100	0.43	0.12
2,4-D	0.100	0.06	0.07
Pentachlorophenol	0.010	0.01	0.08
Silvex	0.025	0.03	0.02
2,4,5-T	0.025	0.02	0.03
2,4-DB	0.100	0.25	0.18
Chloramben	0.050	0.06	0.08
Dinoseb	0.100	0.17	0.08
Bentazon	0.100	0.07	0.19
Dacthal	0.050	0.11	0.11
Quinclorac	0.050	0.08	0.11
Picloram	0.050	0.08	0.05
Acifluorfen	0.250	0.31	0.09

* To ensure exact identification of analytes, CAS numbers are listed as follows: Acifluorfen, 50594-66-6; Bentazon, 25057-89-0; Chloramben, 133-90-4; 2,4-D, 94-75-7; Dacthal, 1861-32-1; Dalapon, 75-99-0; 2,4-DB, 94-82-6; Dicamba, 1918-00-9; 3,5-Dichlorobenzoic acid, 51-36-5; Dichloroprop, 120-36-5; Dinoseb, 88-85-7; pentachlorophenol 87-86-5; Picloram, 1918-02-1; Quinclorac, 84087-01-4; Silvex, 93-72-1; 2,4,5-T, 93-76-5.

ware in an oven above 120°C. Store glassware inverted or capped with aluminum foil. Method interferences may be caused by contaminants in solvents, reagents (including reagent water), sample bottles and caps, and other sample-processing hardware that lead to discrete artifacts and/or elevated base lines in the chromatograms. Interferences by phthalate esters can pose a major problem in pesticide analysis when an electron capture detector (ECD) is used. These compounds generally appear in the chromatogram as large peaks. Common flexible plastics contain varying amounts of phthalates that are easily extracted or leached during laboratory operations. Cross-contamination of clean glassware routinely occurs when plastics are handled during extraction steps, especially when solvent-wetted surfaces are present. Interferences from phthalates can best be minimized by avoiding the use of plastics in the laboratory. Exhaustive purification of reagents and glassware may be required to eliminate background phthalate contamination.[1,2] Routinely demonstrate that all sources of reagents are free from interferences [i.e., less than one-third the method reporting limit (MRL) for each target analyte, under the conditions of the analysis] by analyzing laboratory reagent blanks. Do not subtract blank values from sample results.

Use a grade of sodium sulfate suitable for pesticide residue analysis. If the suitability of the available sodium sulfate is in question, extract and analyze a laboratory reagent blank to test for interferences before sample processing. Matrix interferences may be caused by contaminants that are extracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the water sampled. Also, contaminants in local ground water and surface water may interfere with the analysis of Dalapon on the primary column. A slow initial temperature program can ensure separation of the interferent from Dalapon. Confirm analyte identifications with the confirmation column specified, another column that is dissimilar to the primary column, or by GC/MS if the concentrations are high enough.

d. Method detection level (MDL): Method detection levels are compound-, instrument-, and matrix-dependent. The MDL is defined as the statistically calculated minimum amount that can be measured with 99% confidence that the reported value is greater than zero.[3] Experimentally determined MDLs for the analytes are provided in Table 6640:I. The MDL differs from, and is usually lower than (but never above), the minimum reporting level (MRL). The concentration range for target analytes in this method was evaluated between 0.5 μg/L and 20 μg/L for a 40-mL sample.

e. Safety: See Section 1090. The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined. Regard each chemical compound as a potential health hazard and minimize exposure to these chemicals. Maintain in the laboratory a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of MSDSs also should be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available.[4–6]

The toxicity of the extraction solvent methyl *t*-butyl ether (MtBE) has not been well defined. Susceptible individuals may experience adverse affects upon skin contact or inhalation of vapors. Therefore, use protective clothing and gloves, and use MtBE only in a chemical fume hood or glove box. The same precaution applies to pure standard materials.

Diazomethane is a toxic carcinogen that can explode under certain conditions. Use the diazomethane generator behind a safety shield in a well-ventilated fume hood. Never allow the generator to be heated above 90°C and avoid all grinding surfaces, such as ground-glass joints, sleeve bearings, and glass stirrers. To minimize safety hazards, the diazomethane generator apparatus used in the esterification procedure produces micromolar amounts of diazomethane in solution. If the procedure is followed carefully, no possibility for explosion exists.

Although hydrogen can be safely used as a carrier gas, the potential for fire or explosion does exist if the gas system is mishandled. If unsure of the safety guidelines for using hydrogen as a carrier gas, seek advice from the instrument manufacturer.

2. Apparatus

a. Sample containers, amber glass bottles with a minimum volume of 40 mL, fitted with PTFE-lined screw caps.

b. Extraction vials, 60-mL clear glass vials with PTFE-lined screw caps.

c. Autosampler vials, 2.0-mL vials with screw or crimp cap and a PTFE-faced seal.

d. Standard solution storage containers, 10- to 20-mL amber glass vials with PTFE-lined screw caps.

e. Clear vials, 7-mL glass, disposable, with PTFE-lined screw caps for extract drying and derivatization.

f. Pasteur pipets, glass, disposable.

g. Pipets, Class A, 2.0-mL and 4.0-mL glass, or adjustable volume dispensers.

h. Volumetric flasks, Class A, suggested sizes 5 mL, 10 mL, and 100 mL.

i. Microsyringes, various sizes.

j. Balance, analytical, capable of weighing to the nearest 0.0001 g.

k. Diazomethane generator: See Figure 6251:3 for a diagram of an all-glass system custom-made for these validation studies. Micromolar generators also are available from commercial sources.* If a generator from a commercial source is used, follow manufacturer's instructions.

l. Gas chromatograph, capillary GC.† Modifications should include a high-pressure (50 lb/in.^{7}) split/splitless injector, fast temperature ramp oven (50°C/min) and a low-volume (150-μL) micro ECD detector. A data system capable of fast sampling (20 points/peak) also is required.

m. Primary GC column,‡ 180-μm ID, 40 m long, 0.2-μm film thickness, fused silica capillary with chemically bonded (14% cyanopropylphenymethylpolysiloxane), or equivalent bonded, fused silica column.

n. Confirmation GC column,§ 180-μm ID, 40 m long, 0.2-μm film thickness, fused silica capillary with chemically bonded (5% phenylmethylpolysiloxane), or equivalent bonded, fused silica column. Columns of other dimensions may be used as long as the QC criteria of the method are met.

3. Reagents

Use reagent-grade or better chemicals in all analyses. For reagents *b–f*, use high-purity HPLC-grade materials demonstrated to be free from analytes and interferences. Unless otherwise indicated, use reagents conforming to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first determined that the reagent is of sufficiently high purity to permit its use without lessening the quality of the determination.

Observe safety precautions; see 6640B.1*e*.

a. Reagent water: Use purified water that does not contain any measurable quantities of any target analytes or interfering compounds greater than 1/3 the MRL for each compound of interest.

b. Methyl tert-*butyl ether* (*MtBE*).

c. Acetone.

d. Carbitol (diethylene glycol monoethyl ether).

e. Hexane:MtBE wash solvent, 90:10 (v/v), unpreserved.

f. Hexane.

g. Sodium sulfate (Na_2SO_4), pesticide-grade, granular, anhydrous. If interferences are observed (see 6640B.1*c*), it may be necessary to heat the sodium sulfate in a shallow tray at 400°C for up to 4 h to remove phthalates and other interfering organic substances. Alternatively, extract it with methylene chloride in a Soxhlet apparatus for 48 h. Store in a capped glass bottle rather than a plastic container.

h. Acidified sodium sulfate: Acidify by slurrying 100 g of muffled sodium sulfate with enough ethyl ether to just cover the solid. Add 0.5 mL conc sulfuric acid dropwise while mixing thoroughly. Remove ether under vacuum. Mix 1 g of resulting solid with 5 mL reagent water and measure pH of the mixture. The pH must be below 4. Store in a desiccator or at 100°C to keep reagent dry.

i. Copper II sulfate pentahydrate ($CuSO_4 \cdot 5H_20$).

j. Sodium hydroxide solution (NaOH), 4*N*: Dissolve 16 g NaOH pellets in reagent water and dilute to 100 mL.

k. Potassium hydroxide solution (KOH), 37% (w/v): Dissolve 37 g KOH pellets in reagent water and dilute to 100 mL.

l. Sodium sulfite (Na_2SO_3) for use as a dechlorinating agent.

m. N-*methyl*-N-*nitroso*-p-*toluenesulfonamide*‖ *(MNTS) solution:* Prepare a solution containing 5 g MNTS in 50 mL of a 50:50 (v/v) mixture of MtBE and carbitol. This solution is stable for 1 month or longer when stored at 4°C in an amber bottle with a PTFE-lined screw cap.

n. Sulfuric acid (H_2SO_4), conc.

o. Silica gel, 35 to 60 mesh.

p. Hydrogen, 99.999% pure or better, GC carrier gas.

q. Nitrogen, 99.999% pure or better, ECD make-up gas.

r. Internal standard solutions: 4,4′-Dibromooctafluorobiphenyl (99+%) is used as an internal standard for the method. This compound has been shown to be an effective internal standard for the method analytes, but other compounds may be used if the QC requirements are met. The solutions listed in ¶s *r*1) and 2) below are stable for at least 30 d. Replace solution if ongoing QC indicates a problem.

1) *Internal standard stock solution,* 2.0 mg/mL—Prepare an internal standard stock solution by accurately weighing approximately 0.0200 g of neat 4,4′-dibromooctafluorobiphenyl. Dissolve the neat material in MtBE and dilute to volume in a 10-mL volumetric flask. Transfer solution to an amber glass vial with a PTFE-lined screw cap and store at ≤0°C. The resulting concentration of the stock internal standard solution will be approximately 2.0 mg/mL.

2) *Internal standard primary dilution standard,* 2.5 μg/mL—Prepare an internal standard fortification solution at approximately 2.5 μg/mL by adding 12.5 μL of the stock standard to 10 mL MtBE. Transfer primary dilution to an amber glass vial with a PTFE-lined screw cap and store at ≤0°C.

3) *MtBE extraction solvent with internal standard,* 2.5 μg/mL—The internal standard 4,4′-dibromooctafluorobiphenyl is added to the extraction solvent before analyte extraction to compensate for any volumetric differences encountered during sample processing. Make this solution just before extraction. The addition of 1 mL of primary dilution standard (2.5 μg/mL) to 99 mL MtBE results in a final internal standard concentration of 25 ng/mL.

s. Surrogate (SUR) analyte standard solution: 2,4-dichlorophenylacetic acid (99+%) is used as a surrogate compound to evaluate extraction and derivatization procedures. This compound is an effective surrogate for the method analytes, but other compounds may be used if QC requirements are met. The solutions listed in ¶s *s*1) and 2) below are stable for at least 30 d. Replace solution if ongoing QC indicates a problem.

1) *Surrogate stock solution,* 1.0 mg/mL—Prepare a surrogate stock standard solution of 2,4-dichlorophenylacetic acid by weighing approximately 0.0100 g of neat material. Dissolve the neat material in acetone and dilute to volume in a 10-mL volumetric flask. Transfer solution to an amber glass vial with a

* Aldrich Catalog, No. Z10, 889-8, or equivalent.
† Hewlett Packard Model 6890, or equivalent.
‡ DB-1701, or equivalent.
§ DB-5, or equivalent.
‖ Diazald®, Aldrich Chemical Co., or equivalent.

PTFE-lined screw cap and store at ≤0°C. The resulting concentration will be 1.0 mg/mL.

2) *Surrogate primary dilution standard/sample fortification solution,* 100 μg/mL—Prepare a primary dilution standard at approximately 100 μg/mL by the addition of 1 mL stock standard to 10 mL acetone. Transfer primary dilution to an amber glass vial with a PTFE-lined screw cap and store at 0°C. The addition of 10 μL of primary dilution standard to the 40-mL aqueous sample results in a surrogate concentration of 25 ng/mL.

t. Analyte standard solutions: Obtain analytes as neat or solid free acid standards or as commercially prepared ampulized solutions from a reputable standard manufacturer. Do not use pre-methylated standards for preparing analyte standards. Prepare analyte stock and primary dilution standards as described below. The solutions listed in ¶s *t*1) and 2) below are stable for at least 30 d. Replace solution if ongoing QC indicates a problem.

1) *Analyte stock standard solution*—Prepare separate stock standard solutions for each analyte of interest at a concentration of 1 to 5 mg/mL in acetone. Method analytes may be obtained as neat materials or ampulized solutions (> 99% purity) from a number of commercial suppliers. Store stock standard solutions at ≤0°C.

For analytes that are solids in their pure form, prepare stock standard solutions by accurately weighing approximately 0.01 to 0.05 g pure material in a 10-mL volumetric flask. Dilute to volume with acetone.

For analytes that are liquid in their pure form at room temperature, place approximately 9.8 mL acetone in a 10-mL volumetric flask. Let flask stand, unstoppered, for about 10 min to allow solvent film to evaporate from the inner walls of flask, and weigh to the nearest 0.1 mg. Use a 10-μL syringe and immediately add 10.0 μL standard material to the flask by keeping syringe needle just above the surface of the acetone. Be sure that the standard material falls dropwise directly into the acetone without contacting inner wall of flask. Calculate concentration in milligrams per milliliter from net gain in weight. Dilute to volume, stopper, then mix by inverting flask several times.

2) *Primary dilution standard (PDS)*—Prepare primary dilution standard solution by combining and diluting stock standard solutions with acetone. Store primary dilution standard solution at ≤0°C. As a guideline to the analyst, the analyte concentrations, in micrograms per milliliter, used in the primary dilution standard solution during method development are listed as follows.

Acifluorfen	5.0
Bentazon	10
Chloramben	5.0
2,4-D	10
Dalapon	10
2,4-DB	10
Dacthal acid degradation	5.0
Dicamba	5.0
3,5-Dichlorobenzoic acid	5.0
Dichlorprop	10
Dinoseb	10
Pentachlorophenol	1.0
Picloram	5.0
2,4,5-T	2.5
2,4,5-TP (Silvex)	2.5
Quinclorac	5.0

u. Calibration standards: Prepare a five-point calibration curve by fortifying reagent water with the primary dilution standard. Let the standard reach room temperature, then add a designated amount of each calibration standard in acetone into separate, 40-mL portions of reagent water to produce a calibration curve ranging from below or at the MRL to approximately 10 to 20 times the lowest calibration level. Treat these aqueous calibration standards like samples and therefore add all preservatives and other reagents. Extract by the procedure described below. Store calibration standard solutions in acetone at ≤0°C.

4. Procedure

a. Sample preparation: Remove samples from storage and let them equilibrate to room temperature. Place 40 mL water sample in a pre-cleaned, 60-mL glass vial with a PTFE-lined screw cap, using a graduated cylinder. Add 10 μL room temperature surrogate standard (100 μg/mL 2,4-dichlorophenylacetic acid in acetone) to the aqueous sample.

b. Hydrolysis: Because many of the herbicides included in this method are applied as a variety of esters and salts, hydrolyze them to the parent acid before extraction. Include this step in analysis of all extracted field samples, laboratory reagent blanks (LRBs), laboratory-fortified matrices (LFMs) and calibration standards. Failure to perform this step may result in data that are biased low for some targets in field samples.[7] Add 1 mL 4*N* NaOH solution to each glass vial, then cap and shake for several seconds. Check sample pH with pH paper or a pH meter. If sample does not have a pH ≥12, adjust pH by adding more 4*N* NaOH solution. Let sample remain at room temperature for 1 h, shaking contents periodically.

c. Hexane:MtBE wash (optional): Use this wash step to quantify the Dacthal degradation products from the parent Dacthal. This wash aids in sample cleanup and removes any Dacthal from the sample that would interfere with the quantitation of the Dacthal degradation products. After hydrolysis, add 5 mL 90:10 (v:v) hexane:MtBE and shake vigorously for 3 min. Let phases separate for approximately 5 min, then remove and discard the top hexane/MtBE layer. If differentiation between Dacthal and its degradation products is not required, this step may be omitted.

d. Microextraction: Adjust pH to 1 by adding approximately 1 mL conc sulfuric acid. Cap, shake, and then check pH with a pH meter or narrow-range pH paper. Add more sulfuric acid as needed to properly adjust pH.

Quickly add approximately 2 g copper II sulfate pentahydrate and shake until dissolved. This colors the aqueous phase blue and allows the analyst to better distinguish between the aqueous phase and the organic phase.

Quickly add approximately 16 g muffled sodium sulfate and shake until almost all is dissolved. Sodium sulfate addition increases the ionic strength of the aqueous phase, further driving the chlorophenoxy acids into the organic phase, and also decreases MtBE solubility in the aqueous phase, allowing greater volumetric recovery. Add this salt and the copper II sulfate pentahydrate quickly so the heat generated from addition of the acid will help dissolve the salts.

Add exactly 4.0 mL MtBE extraction solvent with internal standard to each vial and shake vigorously for 3 min. Let phases separate for approximately 5 min.

e. Preparation of diazomethane: Assemble diazomethane generator (Figure 6251:3) in a hood. See 6640B.2*k*. The collection vessel is a 10- or 15-mL glass vial equipped with a PTFE-lined screw cap and maintained at 0 to 5°C.

Add a sufficient amount of MtBE (approximately 7 mL) to first tube to cover the impinger. Add 10 mL MtBE to collection vial. Set nitrogen flow at 5 to 10 mL/min. Add 4 mL MNTS solution (6640B.3*m*) and 3 mL 37% KOH solution to the second impinger. Connect tubing as shown in Figure 6251:3 and let nitrogen flow purge diazomethane from reaction vessel into the collection vial for 30 min. Cap vial when collection is complete and maintain at 0 to 5°C. When stored at 0 to 5°C, this diazomethane solution may be used over a period of 72 h.

Using a Pasteur pipet, transfer sample extract (upper MtBE layer) to a 7-mL glass vial. Add 0.6 g acidified sodium sulfate and shake. This step dries the MtBE extract.

Using a Pasteur pipet, transfer extract to a second, 7-mL glass vial. Add 250 μL diazomethane solution to each vial. The contents of the vial should remain slightly yellow, indicating an excess of diazomethane. More diazomethane may be added if necessary. Let the esterification reaction proceed for 30 min.

Remove any unreacted diazomethane by adding 0.1 g silica gel. Effervescence (evolution of nitrogen) is an indication that excess diazomethane was present. Let extracts stand for 0.5 h.

Transfer extract to an autosampler vial. A duplicate vial may be filled with excess extract.

Analyze sample extracts as soon as possible. The sample extract may be stored up to 21 d if kept at 0°C or less. Keep extracts away from light in amber glass vials with PTFE-lined caps.

f. Gas chromatography: Use apparatus specified in 6640B.2*l, m, n,* and hydrogen carrier gas, 6640B.3*p*. **CAUTION: Observe safety guidelines when using hydrogen as a carrier gas**. If further safety information is required, seek advice from the instrument manufacturer regarding hydrogen use.

Pay strict attention to established column installation guidelines for proper cutting and placement of capillary columns within the instrument. If a loss of response is noted for an analyte, trimming approximately 1 m from the head of the column may restore response. A guard column is recommended if conditions in the laboratory necessitate frequent column trimming.

Chromatographic conditions and analyte retention times for the primary column are given in Table 6640:II A chromatogram from this column is provided in Figure 6640:1. Chromatographic conditions and analyte retention times for the secondary column are given in Table 6640:III. A chromatogram from this column is provided in Figure 6640:2.

g. Calibration: Establish GC operating parameters equivalent to the suggested specifications in Table 6640:II. Calibrate GC system using the internal standard (IS) technique. Other columns or conditions may be used if equivalent or better performance can be demonstrated. Prepare a set of at least five calibration standards as described in 6640B.3*u*. The lowest concentration of calibration standard must be at or below the MRL; preferably use a minimum of four calibration standards between the MRL and the upper quantitative range.

Use GC data system software to generate a linear regression or quadratic calibration curve using the internal standard. This curve may be generated by plotting A_x/A_{is} vs. Q_x/Q_{is}, where A_x and A_{is} are integrated peak areas of the analyte and internal standard, respectively, and Q_x and Q_{is} are quantity of analyte and internal standard injected in concentration units, respectively.

5. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 6020:I.

Quality control (QC) requirements include the initial demonstration of capability (IDC), the determination of the method detection level (MDL), and subsequent analysis in each analysis batch of a laboratory reagent blank (LRB), continuing calibration check (CCC) standards, a laboratory-fortified sample matrix (LFM), and either a laboratory-fortified sample matrix duplicate (LFMD) or a field duplicate (FD) sample. This section details the specific requirements for each QC parameter. The criteria discussed below are considered the minimum acceptable QC criteria. Laboratories are encouraged to institute additional QC practices to meet their specific needs.

Process all QC samples through all steps of the procedure (6640B.4*a–f*), including hydrolysis and methylation. Add sample preservatives as described in 6640A.3 before extracting and analyzing the QC samples.

a. Initial demonstration of capability IDC: Requirements for this demonstration are described below.

1) Initial demonstration of low system background—Before any samples are analyzed, demonstrate that a LRB is reasonably free of contamination and that the criteria in ¶ *c* below are met. Process all QC samples through all steps of the method.

2) Initial demonstration of precision—Prepare, extract, and analyze four to seven replicate LFBs fortified at 5 μg/L, or near the mid-range of the initial calibration curve. Process all QC samples through all steps of the analysis procedure. The relative standard deviation (RSD) of the results of the replicate analyses should be <20%.

3) Initial demonstration of accuracy—Using the same set of replicate data generated for ¶ *a*2) above, calculate average recovery. The average recovery of the replicate values should be within ±20% of the true value.

4) Method detection level (MDL)—Prepare, extract, and analyze at least seven replicate LFBs at a concentration estimated to be near the MDL, over a period of at least 3 d (both extraction and analysis should be conducted over at least 3 d) using the analysis procedure of 6640B.4*a–f*. Process all QC samples through all steps of the method. Select a fortification level with a signal of two to five times the noise level and at or below the laboratory's MRL. The appropriate concentration will depend on the sensitivity of the GC/ECD system. Add sample preservatives as described in 6640A.3 to these samples. Calculate the MDL as follows:

$$\mathrm{MDL} = S t_{(n-1,\ 1-\alpha=0.99)}$$

where:

S = standard deviation of replicate analyses,
$t_{(n-1,\ 1-\alpha\ =\ 0.99)}$ = Student's t value for the 99% confidence level with $n-1$ degrees of freedom, and
n = number of replicates,

TABLE 6640:II. CHROMATOGRAPHIC CONDITIONS AND AVERAGE RETENTION TIME DATA FOR PRIMARY COLUMN

Peak Number (Figure 6640:1)	Compound	Average RT* *min*	RSD %
1	Dalapon	7.06	0.017
2	3,5 Dichlorobenzoic acid	11.32	0.002
3	2,4-Dichlorophenylacetic acid (SUR)	11.80	0.002
4	Dicamba	11.91	0.002
5	Dichlorprop	12.21	0.002
6	4,4′-Dibromooctafluorobiphenyl (IS)	12.25	0.002
7	2,4-D	12.37	0.002
8	Pentachlorophenol	12.49	0.002
9	Silvex	12.70	0.002
10	2,4,5-T	12.90	0.002
11	2,4-DB	13.10	0.003
12	Chloramben	13.15	0.003
13	Dinoseb	13.44	0.003
14	Bentazon	13.60	0.003
15	Dacthal	13.68	0.002
16	Quinclorac	13.85	0.004
17	Picloram	14.02	0.003
18	Acifluorfen	15.20	0.004

* Average of seven runs.
Primary column: DB-1701, 40-m × 0.180-mm-ID, 0.20-mm film thickness, injector temperature 200°C, liner 2-mm straight Siltek® deactivated, injection volume 1 µL of 200 ng/mL (highest level component) standard, splitless injection. Hold to 1 min then purge @ 50 mL/min, detector temperature 360°C, detector make-up gas nitrogen at 20 mL/min. Temperature program: 45°C initial, program at 5°C/min to 80°C, then 50°C/min to 220°C, then 20°C/min to 280°C and hold for 3 min. Data collection via HP GC Chemstation at a rate of 20 Hz.
Carrier gas: Hydrogen (UHP).
Detector make-up gas: Nitrogen (UHP).

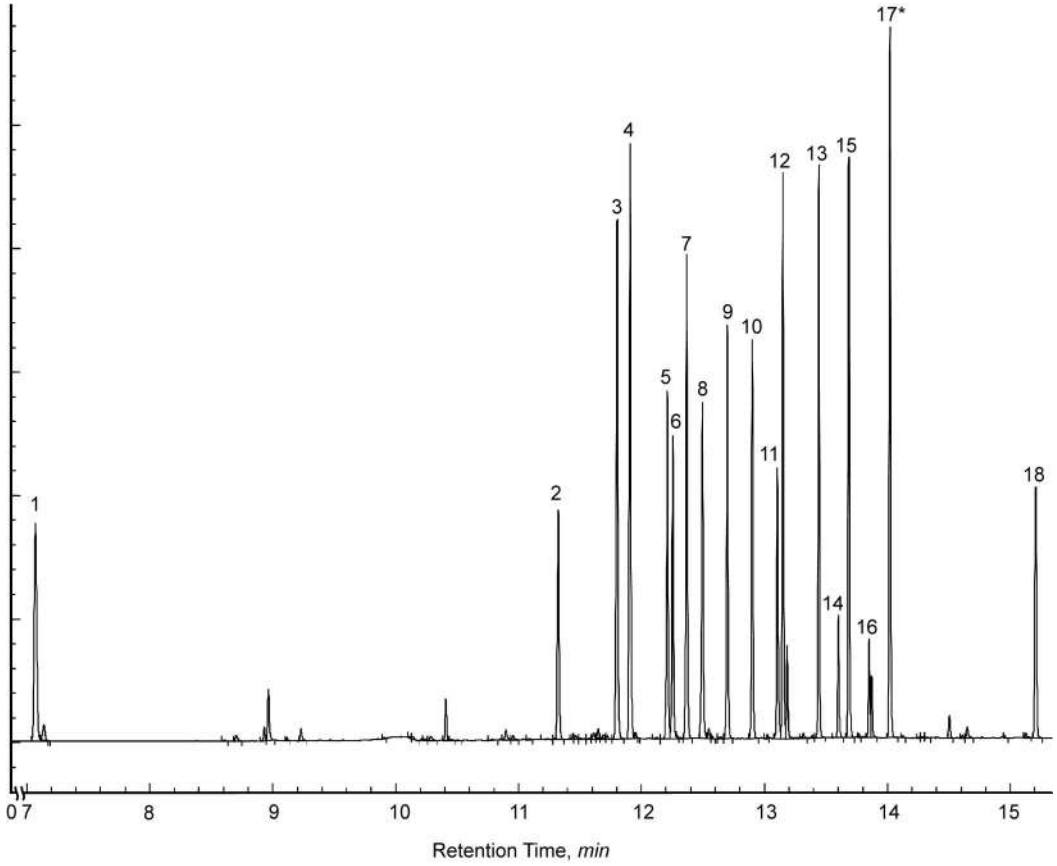


Figure 6640:1. Chromatogram of chlorphenoxy herbicides on a primary column. Peak identifications and chromatographic conditions are given in Table 6640:II. Peak has been attenuated for illustration.

TABLE 6640:III. CHROMATOGRAPHIC CONDITIONS AND AVERAGE RETENTION TIME DATA FOR CONFIRMATION COLUMN

Peak Number (Figure 6640:2)	Compound	Average RT* *min*	RSD %
1	Dalapon	5.04	0.057
2	3,5-Dichlorobenzoic acid	10.33	0.007
3	2,4-Dichlorophenylacetic acid (SUR)	11.31	0.004
4	Dicamba	11.46	0.006
5	Dichlorprop	12.19	0.006
6	4,4′-Dibromooctafluorobiphenyl (IS)	12.71	0.004
7	2,4-D	12.34	0.005
8	Pentachlorophenol	13.16	0.006
9	Silvex	13.29	0.004
10	2,4,5-T	13.48	0.005
11	2,4-DB	13.94	0.005
12	Chloramben	13.34	0.004
13	Dinoseb	14.01	0.004
14	Bentazon	14.28	0.006
15	Dacthal	15.00	0.004
16	Quinclorac	14.88	0.004
17	Picloram	14.59	0.004
18	Acifluorfen	16.38	0.003

* Average of seven runs.
Confirmation column: DB-5, 40-m × 0.180-mm-ID, 0.20-mm film thickness, injector temperature 200°C, liner 2-mm straight Siltek® deactivated, injection volume 1 µL of 100 ng/mL (highest level component) standard, splitless injection. Hold to 1 min then purge @ 50 mL/min, detector temperature 360°C, detector make-up gas nitrogen at 20 mL/min. Temperature program: 45°C initial, program at 4°C/min to 80°C, then program at 30°C/min to 280°C and hold for 2 min. Data collection via HP GC Chemstation at a rate of 20 Hz.
Carrier gas: Hydrogen (UHP).
Detector make-up gas: Nitrogen (UHP).

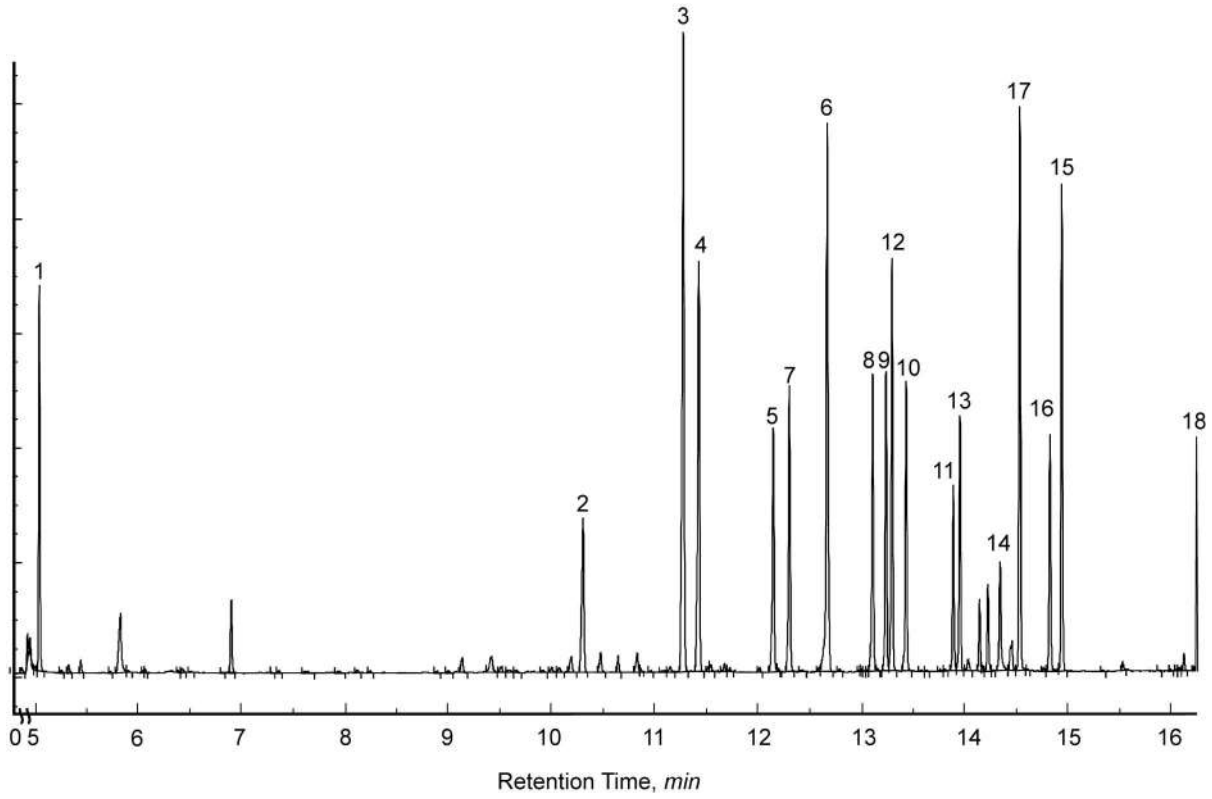


Figure 6640:2. Chromatogram of the chlorphenoxy herbicides on confirmation column. Peak identifications and chromatographic conditions are given in Table 6640:III.

NOTE: Calculated MDLs need only be less than 1/3 of the laboratory's MRL to be considered acceptable. Do not subtract blank values when performing MDL calculations. The MDL is a statistical determination of precision only.[1] If the MDL replicates are fortified at a low enough concentration, it is likely that they will not meet precision and accuracy criteria, and may result in a calculated MDL that is higher than the fortified concentration.

b. Minimum reporting level (MRL): The MRL is the threshold concentration of an analyte that a laboratory can expect to quantitate accurately in an unknown sample. The MRL should not be established at an analyte concentration that is less than either three times the MDL or a concentration yielding a signal-to-noise (S/N) ratio of less than five. Depending upon the study's data quality objectives, it may be set at a higher concentration. Although the lowest calibration standard should be at or below the MRL, the MRL cannot be established at a concentration lower than the lowest calibration standard.

c. Laboratory reagent blank (LRB): An LRB is required with each extraction batch of samples to determine the background system contamination. If the LRB produces a peak within the retention-time window of any analyte that would prevent the determination of that analyte, determine the source of contamination and eliminate the interference before processing samples. Reduce background contamination to an acceptable level before proceeding. Background from method analytes or contaminants that interfere with the measurement of method analytes should be below 1/3 of the MRL. If target analytes are detected in the LRB at concentrations equal to, or greater than, this level, then all data for the problem analyte(s) may be considered invalid for all samples in the extraction batch.

d. Continuing calibration check (CCC): A CCC is a standard, prepared in the same extraction batch as the samples of interest, that contains all compounds of interest and is extracted in the same manner as the procedural standards used to prepare the initial calibration curve. Prepare an appropriate number of CCCs with each extraction batch. The CCC verifies the initial calibration at the beginning and end of each group of analyses, and after every tenth sample. The beginning CCC for each analysis batch should be at or below the MRL in order to verify instrument sensitivity before any analyses. Subsequent CCCs should alternate between a medium and high concentration. Calculate the concentration of each analyte and surrogate in the check standard. The calculated amount for each analyte for medium and high level CCCs should be within ±30% of the true value. The calculated amount for the lowest calibration level for each analyte should be within ±50% of the true value. If these conditions do not exist, consider all data for the problem analyte invalid, and take remedial action, which may require recalibration. Reanalyze any field sample extracts analyzed since the last acceptable calibration verification after adequate calibration has been restored, with the following exception. If the continuing calibration fails because the calculated concentration is >130% (150% for the low-level CCC) for a particular target compound, and field sample extracts show no detection for that target compound, nondetects may be reported without re-analysis. Prepare calibration checks, with the samples being analyzed, at the beginning of each day that samples are analyzed, after every ten samples, and at the end of any group of sample analyses.

e. Laboratory fortified blank (LFB): Because this method uses procedural calibration standards, which are fortified reagent waters extracted with each analytical batch, there is no difference between the LFB and the continuing calibration check standard. Consequently, the analysis of an LFB is not required; however the acronym LFB is used for clarity in the IDC.

f. Internal standard (IS): Monitor the peak area of each internal standard in all injections during each analysis day. The IS response (as indicated by peak area) for any chromatographic run should not deviate by more than ±50% from the average area measured during the initial calibration for that IS. A poor injection could cause the IS area to exceed these criteria. Inject a second portion of the suspect extract to determine whether the failure is due to poor injection or instrument response drift. If the reinjected portion produces an acceptable internal standard response, report the results. If the internal standard area for the reinjected extract deviates >50% from the initial calibration average, check the CCC standards that ran before and after the sample. If the CCC fails the criteria of ¶ *d* above, recalibrate according to 6640B.4*g*. If the CCC is acceptable, repeat sample extraction provided that the sample is still within holding time.Otherwise, report results obtained from the reinjected extract, but indicate as suspect.

g. Surrogate recovery: The surrogate standard is fortified into the aqueous portion of all samples, LRBs, and LFMs and LFMDs before extraction. It is also added to the calibration curve and CCC standards. The surrogate is a means of assessing method performance from extraction to final chromatographic measurement. When surrogate recovery from a sample, blank, or CCC is <70% or >130%, check calculations to locate possible errors, standard solutions for degradation, contamination, and instrument performance. If those steps do not reveal the cause of the problem, reanalyze extract. If extract re-analysis meets the surrogate recovery criterion, report data only for re-analyzed extract. If extract reanalysis fails the 70 to 130% recovery criterion, check calibration by injecting the last calibration standard that passed. If the calibration standard fails the criteria of ¶ *d* above, recalibrate according to 6640B.4*g*. If calibration standard is acceptable, repeat sample extraction provided that the sample is still within the holding time. If sample re-extract also fails the recovery criterion, report all data for that sample as a quantitative estimate because of surrogate recovery.

h. Laboratory-fortified sample matrix and duplicate (LFM and LFMD): Include LFMs and LFMDs in each extraction batch to determine whether the sample matrix adversely affects method accuracy. If the occurrence of target analytes in the samples is infrequent, or if historical trends are unavailable, include a second LFM or LFMD from a duplicate field sample to assess method precision. Extraction batches that contain LFMDs will not require the analysis of a FD. If a variety of different sample matrices are analyzed regularly (e.g., drinking water from groundwater and surface water sources), establish method performance for each. Over time, document LFM data for all routine sample sources for the laboratory. Within each extraction batch, fortify a minimum of one field sample as an LFM for every 20 samples extracted. Prepare the LFM by adding to a sample an appropriate amount of analyte as given in 6640B.3*t*2). Select a fortifying concentration that is at least twice the matrix background concentration, if known. Use historical data and rotate through the designated concentrations when selecting a fortifying concentration.

Calculate the percent recovery *(R)* for each analyte by using the equation:

TABLE 6640:IV. METHOD PRECISION AND BIAS IN SELECTED MATRICES

		Low-Level Fortification				Mid-Level Fortification		
Matrix	Compound	Fortification Concentration µg/L	Mean Recovery %	RSD* %	S/N†	Fortification Concentration µg/L	Mean Recovery %	RSD* %
Reagent water	Dalapon	1.0	108	2.2	578	10	107	2.3
	3,5-Dichlorobenzoic acid	0.50	117	16	9	5.0	96	2.3
	Dicamba	0.50	97	3.7	35	5.0	102	1.5
	Dichlorprop	1.0	95	5.7	19	10	107	0.6
	2,4-D	1.0	98	5.2	23	10	106	1.4
	Pentachlorophenol	0.10	76	7.4	21	1.0	103	1.4
	Silvex	0.25	84	20	9	2.5	107	4.4
	2,4,5-T	0.25	96	7.3	16	2.5	105	1.3
	2,4-DB	1.0	97	8.4	6	10	93	1.6
	Chloramben	0.50	90	9.0	113	5.0	105	2.9
	Dinoseb	1.0	103	6.7	8	10	119	4.6
	Bentazon	1.0	100	2.1	41	10	98	0.7
	Dacthal	0.50	92	6.5	100	5.0	100	1.9
	Quinclorac	0.10	106	9.3	20	1.0	101	2.9
	Picloram	1.0	92	2.6	23	10	99	3.1
	Acifluorfen	0.50	77	5.9	80	5.0	107	4.5
	2,4-Dichlorophenyl-acetic acid (SUR)	25	107	8.0	0	25	112	1.3
Chlorinated surface water	Dalapon	1.0	104	2.1	—	10	100	2.5
	3,5-Dichlorobenzoic acid	0.50	110	3.0	—	5.0	104	2.6
	Dicamba	0.50	93	4.4	—	5.0	102	2.9
	Dichlorprop	1.0	93	11	—	10	101	1.2
	2,4-D	1.0	97	5.2	—	10	101	1.2
	Pentachlorophenol	0.10	86	3.2	—	1.0	100	1.0
	Silvex	0.25	103	4.3	—	2.5	101	1.0
	2,4,5-T	0.25	100	3.5	—	2.5	100	1.6
	2,4-DB	1.0	104	5.9	—	10	101	2.3
	Chloramben	0.50	93	2.0	—	5.0	102	2.6
	Dinoseb	1.0	99	1.2	—	10	101	1.6
	Bentazon	1.0	91	2.2	—	10	98	1.8
	Dacthal	0.50	92	4.6	—	5.0	99	2.0
	Quinclorac	0.10	96	15	—	1.0	105	2.5
	Picloram	1.0	102	2.4	—	10	107	3.2
	Acifluorfen	0.50	98	4.1	—	5.0	92	2.4
	2,4-Dichlorophenyl-acetic acid (SUR)	25	107	1.5	—	25	106	1.4
Chlorinated ground water	Dalapon	1.0	105	1.5	—	10	97	5.5
	3,5-Dichlorobenzoic acid	0.50	104	6.0	—	5.0	95	2.1
	Dicamba	0.50	100	2.2	—	5.0	98	1.6
	Dichlorprop	1.0	109	3.5	—	10	79	0.7
	2,4-D	1.0	108	10	—	10	99	1.6
	Pentachlorophenol	0.10	103	17	—	1.0	100	4.2
	Silvex	0.25	96	3.0	—	2.5	97	1.6
	2,4,5-T	0.25	91	3.4	—	2.5	97	1.5
	2,4-DB	1.0	94	3.1	—	10	92	1.1
	Chloramben	0.50	88	2.7	—	5.0	102	2.6
	Dinoseb	1.0	100	4.0	—	10	105	2.6
	Bentazon	1.0	101	2.8	—	10	101	1.8
	Dacthal	0.50	62‡	9.9	—	5.0	99	1.2
	Quinclorac	0.10	107	5.7	—	1.0	94	1.5
	Picloram	1.0	97	2.8	—	10	103	2.5
	Acifluorfen	0.50	92	4.2	—	5.0	84	2.5
	2,4-Dichlorophenyl acetic acid (SUR)	25	95	3.1	—	25	106	1.1

* $n = 7$

† Signal-to-noise ratios were calculated for each target compound peak by dividing peak height for each compound by peak-to-peak noise, which was determined for each component from method blank over a period of time equal to full peak width in target analyte's retention time window.

‡ Dacthal was present in the chlorinated surface water at about 1/5th fortification level. This was taken into account in the determination of the mean recovery.

Table 6640:V. Effect of Sample Holding Time on Recovery for Samples from a Chlorinated Surface Water Fortified with Method Analytes*

Compound	Recovery %			
	Day 0	Day 2	Day 7	Day 14
Dalapon	91	90	91	108
3,5-Dichlorobenzoic acid	98	97	85	91
Dicamba	92	92	96	99
Dichlorprop	89	88	91	95
2,4-D	88	85	87	92
Pentachlorophenol	92	85	86	93
Silvex	91	88	86	94
2,4,5-T	89	89	85	91
2,4-DB	87	84	86	93
Chloramben	86	86	88	100
Dinoseb	105	95	99	111
Bentazon	91	87	95	99
Dacthal	92	91	86	94
Quinclorac	90	80	84	91
Picloram	94	85	85	99
Acifluorfen	109	96	103	123

* Sample storage stability is expressed as a percent recovery value calculated as described in 6640B.5*h*.

Table 6640:VI. Effect of Extract Holding Time on Recovery for Samples from a Chlorinated Surface Water Fortified with Method Analytes*†

Compound	Recovery %	
	Initial Injection	Day 21 Reinjection
Dalapon	100	89
3,5-Dichlorobenzoic acid	104	113
Dicamba	102	104
Dichlorprop	100	101
2,4-D	101	102
Pentachlorophenol	100	102
Silvex	101	103
2,4,5-T	99	102
2,4-DB	101	102
Chloramben	102	105
Dinoseb	99	81
Bentazon	98	100
Dacthal	99	103
Quinclorac	105	127
Picloram	107	98
Acifluorfen	92	104
2,4-Dichlorophenylacetic acid	106	105

* Sample storage stability is expressed as a percent recovery value calculated as described in 6640B.5*h*.
† All samples fortified at the same level used to collect the mid-level precision and bias.

$$R = \frac{(A - B)}{C} \times 100$$

where:

A = measured concentration in fortified sample,
B = measured concentration in unfortified sample, and
C = fortification concentration.

Analyte recoveries may exhibit matrix bias. For samples fortified at or above their native concentration, recoveries should range between 70 and 130%, except for low-level fortification near or at the MRL, where 50 to 150% recoveries may be acceptable. If the accuracy of any analyte falls outside the designated range, and the laboratory performance for that analyte is shown to be in control in the LFB, the recovery may be judged to be matrix-biased. Label result for that analyte in the unfortified sample as suspect because of matrix effects.

If an LFMD is analyzed instead of a FD, calculate relative percent difference (RPD) for values in duplicate LFMs (LFM and LFMD) by using the equation:

$$RPD = \frac{LFM - LFMD}{(LFM + LFMD)/2} \times 100$$

RPDs for duplicate LFMs should fall in the range of ±30% for samples fortified at or above their native concentration. Greater variability may be observed when LFMs are spiked near the MRL. At the MRL, RPDs should fall in the range of ±50% for samples fortified at or above their native concentration. If the accuracy of any analyte falls outside the designated range and the laboratory performance for that analyte is shown to be in control in the LFB, the recovery may be judged to be matrix-biased. Label result for that analyte in the unfortified sample as suspect because of matrix effects.

i. Field duplicates (FD1 and FD2): Within each extraction batch, include a minimum of one field duplicate (FD) or LFMD (¶ *h* above). FDs check the precision associated with sample collection, preservation, storage, and laboratory procedures. If target analytes are not routinely observed in field samples, analyze a LFMD to substitute for this requirement. Extraction batches that contain LFMDs may not require the analysis of a FD. Calculate the relative percent difference (RPD) for duplicate measurements (FD1 and FD2) using the equation:

$$RPD = \frac{FD1 - FD2}{(FD1 + FD2)/2} \times 100$$

RPDs for duplicates should be in the range of ±30%. Greater variability may be observed when analyte concentrations are near the MRL. At the MRL, RPDs should fall in the range of ±50%. If the accuracy of any analyte falls outside the designated range, and the laboratory performance for that analyte is shown to be in control in the LFB, the recovery may be judged to be matrix-biased. Label result for that analyte in the unfortified sample as suspect because of matrix effects.

j. Quality control (QC) sample: Analyze a QC sample each time that new primary dilution standards, 6040B.3*t*2), are prepared. Ideally, obtain QC sample from second vendor. If a second vendor is not available, use a different lot of the standard. Although use of pre-methylated standards is prohibited for preparing analyte standard solutions, pre-methylated stan-

dards may be used to prepare the QC sample. The QC sample may be injected as a calibration standard or fortified into reagent water and analyzed as a LFB. If the QC sample is analyzed as a continuing calibration, acceptance criteria are the same as for the CCC. If the QC sample is analyzed as a LFB, acceptance criteria are the same as for a LFB. If measured analyte concentrations are not of acceptable accuracy, check entire analytical procedure to locate and correct problem source.

6. Precision and Bias

Precision and bias data for this method are presented in Table 6640:IV. The effect of sample holding time on recovery from a chlorinated surface water is shown in Table 6640:V and the effect of extract holding time in Table 6640:VI.

7. References

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2. Giam, C.S. & H.S. Chan. 1976. Control of blanks in the analysis of phthalates in air and ocean biota samples; U.S. National Bur. Standards, Special Pub. 442, p. 701.
3. Glaser, J.A., D.L. Foerst, G.D. McKee, S.A. Quave & W.L. Budde. 1981. Trace analyses for wastewaters. *Environ. Sci. Technol.* 15: 1426.
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5. U.S. Public Health Service. 1977. Carcinogens—Working with Carcinogens; Pub. No. 77-206. U.S. Dept. Health, Education, and Welfare, Public Health Serv., Center for Disease Control, National Inst. Occupational Safety & Health, Atlanta, Ga.
6. Committee on Chemical Safety. 1979. Safety in Academic Chemistry Laboratories, 3rd ed. American Chemical Soc., Washington, D.C.
7. Bassett, M.V., B.V. Pepich & D.M. Munch. 2000. The role played by basic hydrolysis in the validity of acid herbicide data — Recommendations for future preparation of herbicide performance evaluation standards. *Environ. Sci. Technol.* 34:1117.

6651 GLYPHOSATE HERBICIDE*

6651 A. Introduction

1. Sources and Significance

Glyphosate [*N*-(phosphonomethyl)glycine] is a broad-spectrum, nonselective, postemergence herbicide that has found widespread agricultural and domestic use. It is sold as a terrestrial and aquatic herbicide under the trade names Roundup® and Rodeo®. Because of low mammalian toxicity (LD_{50} = 1568 mg/kg rats; oral) there is less concern about water and food contamination than with other pesticides, but the nonselectivity of the herbicide can make nontarget phytotoxicity a problem. Glyphosate's (GLYPH) major metabolite is aminomethylphosphonic acid (AMPA). Contamination of water can occur through runoff and spray drift.

2. Selection of Method

Several methods for determination of GLYPH and AMPA in environmental samples have been developed; those using liquid chromatography are the most precise and accurate. GLYPH and AMPA are not good chromophores or fluorophores and their electrochemical or conductometric detection have not been demonstrated. Sensitive and selective detection has been achieved with the post-column reaction/fluorometric method.[1–3] The absence of a sensitive liquid chromatography technique for confirmation necessitates the use of two different stationary phases.

The liquid chromatographic method presented in 6651B is accurate and precise[3] and includes confirmation by using two columns. Gas chromatography/mass spectrometry confirmation[4] has been used when structural confirmation is required, but the method has not been tested on residues in natural waters.

3. References

1. Moye, H.A., C.J. Miles & S.J. Scherer. 1983. A simplified high-performance liquid chromatographic procedure for the determination of glyphosate herbicide and (aminomethyl)phosphonic acid in fruits and vegetables employing postcolumn fluorogenic labeling. *J. Agric. Food Chem.* 31:69.
2. Cowell, J.E., J.L. Kunstman, P.J. Nord, J.R. Steinmetz & G.R. Wilson. 1986. Validation of an analytical residue method for analysis of glyphosate and metabolite: An interlaboratory study. *J. Agric. Food Chem.* 34:955.
3. Oppenhuizen, M.E. & J.E. Cowell. 1991. Liquid chromatographic determination of glyphosate and (aminomethyl)phosphonic acid in environmental water. *J. Assoc. Offic. Anal. Chem.* 74:317.
4. Deyrup, C.L., S.M. Chang, R.A. Weintraub & H.A. Moye. 1985. Simultaneous esterification and acylation of pesticides for analysis by gas chromatography. 1. Derivatization of glyphosphate and (aminomethyl) phosphonic acid with fluorinated alcohol-perfluorinated anhydrides. *J. Agric. Food Chem.* 33:944.

4. Bibliography

Bardalaye, P.C., W.B. Wheeler & H.A. Moye. 1985. Analytical techniques of glyphosate residue analysis. *In* E. Grossbard & D. Atkinson, eds. The Herbicide Glyphosate. Butterworths, Woburn, Mass.

* Approved by Standard Methods Committee, 2005.

6651 B. Liquid Chromatographic Post-Column Fluorescence Method

1. General Discussion

a. Principle: GLYPH and AMPA are separated by anion- or cation-exchange chromatography and measured by post-column fluorescence derivatization. The post-column reactions consist of oxidation of GLYPH (a secondary amine) to glycine (a primary amine) by hypochlorite solution. Glycine then reacts with an *o*-phthalaldehyde (OPA) and mercaptoethanol (MERC) mixed reagent to form an isoindole that is measured fluorometrically. AMPA (a primary amine) reacts directly with the OPA/MERC reagent and is detected (with decreased sensitivity) in the presence of hypochlorite.

b. Interferences: No matrix interferences in water are known. GLYPH degrades in chlorinated water. GLYPH also is known to sorb strongly to minerals and glass surfaces.

c. Minimum detectable concentration: Minimum detection using this method is 25 μg/L for GLYPH and AMPA by direct injection and 0.5 μg/L with the concentration step.

2. Sampling and Storage

Collect a 500-mL representative sample in a polypropylene or amber glass container. Treatment of sample to remove residual chlorine will prevent glyphosate losses during storage. Destroy chlorine by adding 100 mg/L sodium thiosulfate. Store samples at 4°C away from light and analyze within 2 weeks.

3. Apparatus

High-performance liquid chromatograph (HPLC): An analytical system with pump, injector, detectors, and compatible strip chart recorder. Preferably use a data system for measuring peak areas and retention times. Use system capable of injecting 200-μL portions. See Figure 6651:1.

a. Analytical columns: Use either a cation exchange resin[1] or an anion-exchange resin[2] packed in a 4.6-mm × 25- to 30-cm

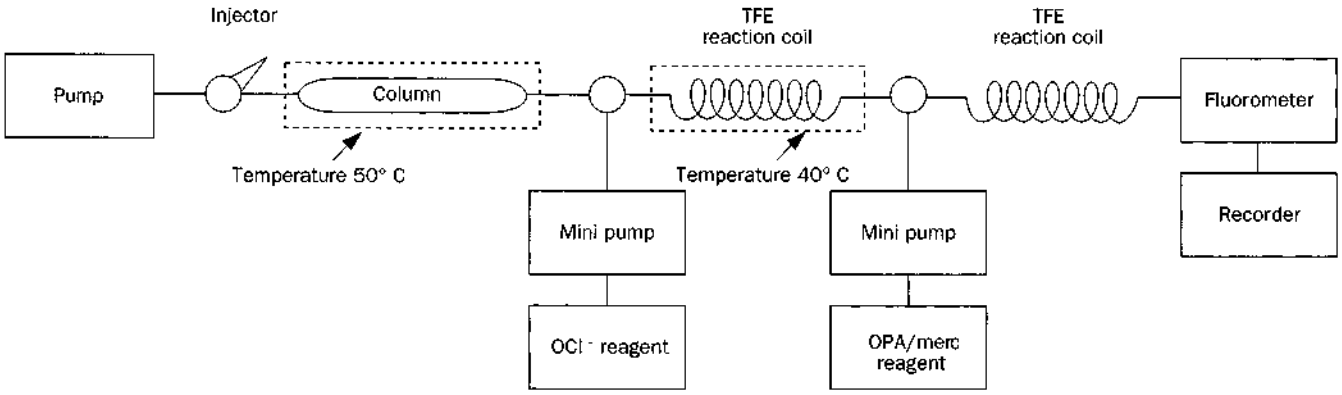


Figure 6651:1. Schematic of post-column reaction HPLC system.

column.* Heat columns to between 50 and 60°C to obtain maximum efficiency.

b. Post-column reactor: Use system consisting of two separate pumps capable of delivering reaction solutions at 0.1 to 0.5 mL/min and able to withstand pressures of up to 2000 kPa. Include two woven 1-mL TFE reaction coils[3] (0.5-mm ID × 1.4-mm OD × 5 m) with one maintained at 40°C. Turnkey post-column reactor systems are available commercially.

c. Fluorescence detector: Use filter or grating fluorimeter capable of sensitively and selectively measuring the isoindole derivative, with excitation wavelength 230 nm (deuterium), 340 nm (quartz halogen or xenon), and emission wavelength 420 to 455 nm.

4. Reagents

a. Reagent water: See Section 1080.

b. Phosphoric acid (H_3PO_4), conc.

c. Sulfuric acid (H_2SO_4), conc. Prepare anion-exchange mobile phase by adding 26 mL conc H_3PO_4 and 2.7 mL conc H_2SO_4 to 5 L water.

d. Hydrochloric acid (HCl), conc.

e. Methanol (CH_3OH) tested on HPLC and verified to give no impurity peaks.

f. Potassium dihydrogen phosphate (KH_2PO_4): Prepare cation-exchange mobile phase by dissolving 0.68 g KH_2PO_4 in 1 L methanol-water (4:96). Adjust to pH 2.1 with conc H_3PO_4. Filter through a 0.22- or 0.45-μm membrane filter and degas.

g. Disodium ethylenediamine tetraacetate dihydrate (EDTA) sodium salt solutions: Prepare a 0.001*M* solution by dissolving 0.37 g EDTA dihydrate in 1.0 L water and filter through a 0.22- or 0.45-μm filter. Prepare a 0.03*M* solution by dissolving 11.2 g EDTA dihydrate in 1.0 L water and filtering through a 0.33- or 0.45-μm filter.

h. Sodium chloride (NaCl).

i. Sodium hydroxide (NaOH).

j. Calcium hypochlorite [$Ca(OCl)_2$], 70.9% available chlorine.

k. Oxidation reagent: Dissolve 0.5 g $Ca(OCl)_2$ in 500 mL water with rapid magnetic stirring for 45 min. In a 1.0-L volumetric flask, dissolve 1.74 g KH_2PO_4, 11.6 g NaCl, 0.4 g NaOH, and 10 mL stock $Ca(OCl)_2$ solution. Dilute to volume, mix well, and filter through a 0.22- or 0.45-μm filter.

l. o-*Phthalaldehyde* [$C_6H_4(CHO)_2$] (OPA).

m. 2-mercaptoethanol ($HSCH_2CH_2OH$) (MERC).

n. Boric acid powder (H_3BO_3).

o. Potassium hydroxide (KOH).

p. Fluorogenic labeling reagent: Dissolve 100 g boric acid and 72 g KOH in about 700 mL water in a 1.0-L flask. This takes 1 to 2 h. Add 0.8 g OPA dissolved in 5 mL methanol. Add 2.0 mL MERC. Mix well.

q. Glyphosate analytical standard, N-(phosphonomethyl) glycine, 99% or greater.

r. Aminomethylphosphonic acid analytical standard, 99% or greater.

s. Glyphosate and AMPA fortification standards: Prepare a solution containing both 0.1 mg GLYPH/mL and 0.1 mg AMPA/mL in water. Make working solutions of 10.0 and 1.0 μg/mL by serial dilution of this stock solution. Store in a refrigerator, in a polypropylene or amber glass container. Prepare fresh monthly.

t. Glyphosate and AMPA HPLC calibration standards: Prepare a solution containing both 0.1 mg GLYPH/mL and 0.1 mg AMPA/mL in 0.001*M* disodium EDTA solution. Make working solutions of 1.00, 0.50, 0.10, 0.05, and 0.025 μg/mL by serial dilution. Store in a refrigerator, in a polypropylene or amber glass container. Prepare fresh monthly.

5. Procedure

a. HPLC operation: Equilibrate column at 50°C with mobile-phase flow rate of 0.5 mL/min (see Figure 6651:1). Use an approximate flow rate of 0.5 mL/min for the oxidant and 0.3 mL/min for the OPA-MERC reagent but adjust rates to obtain maximum response. While GLYPH reaches a maximum response at some flow rate of oxidative solution, the AMPA response decreases with any addition of this reagent. Thus, an oxidative reagent flow rate that gives an equal response for both GLYPH and AMPA simultaneously is considered optimum for simultaneous measurements. Reagent flow rates will differ for different mobile phases. Establish a standard curve by injecting calibration standards.

* Aminex, BioRad Labs, A-9 cation exchange and A-27 anion exchange resins, or equivalent.

Approximate retention times for GLYPH and AMPA are 13.5 and 10.0 min on the anion-exchange column and 21.5 and 30.0 min on the cation-exchange column.

b. Sample preparation: No concentration is needed for samples containing 25 μg/L or more. Fortify a 9.9-mL sample portion with 0.1 mL 0.10*M* EDTA, filter through a 0.22- or 0.45-μm filter, and inject 200 μL. Fortify portions of the same samples with known amounts of GLYPH and AMPA to determine recovery. Perform duplicate injections on at least 10% of the samples with measurable GLYPH and AMPA or 10% of fortified samples to determine precision.

To concentrate samples containing less than the detection level, transfer 250 mL to a 500-mL round-bottom flask. If suspended matter is present, filter sample through coarse filter paper.† For samples used to assess recovery, make known additions. Add 5 mL conc HCl to flask and 5 mL to sample remaining in original container. Concentrate on a rotary evaporator by slowly increasing temperature from 20 to 60°C. Before the first portion is completely evaporated, add remaining sample and two 5-mL rinses of the sample bottle. Evaporate to dryness, and if necessary, remove final traces of water with a stream of dry nitrogen. Dissolve residue in 2.9 mL of mobile phase (adjust pH to 2 if necessary) and 0.10 mL 0.03*M* EDTA solution. Filter through 0.45-μm filter to a test tube and inject into the HPLC system.

6. Calculations

Determine concentration of GLYPH and AMPA by regression analysis of the standard curve. Multiply results for samples that were concentrated by the concentration factor, 166.7 (500 mL original sample/3.0 mL), to determine the original water concentration. Report results in milligrams per liter. Report percent recovery but do not correct for recovery.

† Whatman #1, or equivalent.

7. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 6020:I.

8. Precision and Bias

For six single-operator analyses, the relative standard deviation of duplicate samples (with additions from 0.5 to 5000 μg/L) ranged from 12.1 to 20% with an average of 14.9% for glyphosate. The relative standard deviation for identical AMPA concentrations ranged from 6.5 to 28.8% with an average of 14.5%.[4]

For six single-operator analyses, recoveries of glyphosate (with additions from 0.5 to 5000 μg/L) ranged from 94.6 to 120% with an average of 104.0%. Recoveries of AMPA ranged from 86.0 to 100% with an average of 93.1%.[4]

9. References

1. Cowell, J.E., J.L. Kunstman, P.J. Nord, J.R. Steinmetz & G.R. Wilson. 1986. Validation of an analytical residue method for analysis of glyphosate and metabolite: An interlaboratory study. *J. Agric. Food Chem.* 34:955.
2. Moye, H.A., C.J. Miles & S.J. Scherer. 1983. A simplified high-performance liquid chromatographic procedure for the determination of glyphosate herbicide and (aminomethyl)phosphonic acid in fruits and vegetables employing postcolumn fluorogenic labeling. *J. Agric. Food Chem.* 31:69.
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6710 TRIBUTYL TIN*

6710 A. Introduction

1. Sources and Significance

Tributyl tin (TBT) is both a potent biocide and an endocrine disruptor.[1] Its primary uses have been as an antifouling agent in marine paint, household cleaning solutions, and cooling systems, and as a stabilizer in polyvinyl chloride (PVC) plastic. TBT has been detected in a variety of matrices, including wastewater, marine waters, shellfish tissues, and sediments.

2. Selection of Method

TBT in water or wastewater can be measured with a variety of analytical techniques, most of which involve the derivatization of TBT followed by gas chromatography as a separation technique. Detection of the derivatized TBT can be accomplished with mass spectrometry (GC/MS), flame photometric detector (GC/FPD), atomic absorption spectrometry (GC/AAS), or inductively coupled plasma (GC/ICP). Other methods include high-performance liquid chromatography (HPLC) coupled with a fluorescence detector or an ICP/MS.

This section covers the liquid/liquid extraction of TBT followed by derivatization with a Grignard reagent, hexylmagnesium bromide, and analysis by GC/MS (6710B) or GC/FPD (6710C). The detection levels for the two methods are comparable.

TBT can break down into dibutyl tin (DBT) and monobutyl tin (MBT). The extraction method presented in this section does not yield good recoveries for DBT and MBT. It is suspected that either the ionic properties of DBT and MBT lead to poor extraction recoveries or that losses of these two species occur during the extraction process. Recoveries for DBT can be improved by adding tropolone to the extraction solvent. Further study is needed to improve the recoveries of low-level MBT in an aqueous matrix.

3. Sampling and Storage

If possible, collect all samples in duplicate and one sample per sample batch in triplicate to allow for laboratory QA/QC. Suitable containers are 1-L amber glass or polycarbonate. Preserve samples with 1:1 HCl to a pH <2 and store at 4°C in the dark. TBT in water, when properly preserved and stored, is stable for at least 13 weeks.[2]

4. References

1. Takeuchi, M., K. Mizuishi & H. Toshiyuki. 2000. Determination of organotin compounds in environmental samples. *Anal. Sci.* 16:349.
2. Unger, M.A., W. G. MacIntyre, J. Greaves & R. J. Hugget. 1986. GC determination of butyl tins in natural waters by flame photometric detection of hexyl derivatives with mass spectrometric confirmation. *Chemosphere* 15:461.

5. Bibliography

Matthias, C. L., J. M. Bellama, G. J. Olson & F.E. Brinckman. 1986. Comprehensive method for determination of aquatic butyltin and butylmethyltin species at ultratrace levels using simultaneous hydridization extraction with gas chromatography-flame photometric detection. *Environ. Sci. Technol.* 20:609.

U.S. Environmental Protection Agency. 1996. DBP/ICR Analytical Methods Manual, Section 10; EPA 814-B-96-002. Off. Water, Cincinnati, Ohio.

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* Approved by Standard Methods Committee, 2004. Editorial revisions, 2011. Joint Task Group: 21st Edition Supplement—François Rodigari (chair), Philip D. Carpenter, Eric A. Crecelius, Lisa M. Ramirez, Michael A. Unger.

6710 B. Gas Chromatographic/Mass Spectrometric Method

This method is applicable to the determination of tributyl tin (TBT) in drinking water, marine water, and wastewater.

1. General Discussion

a. Principle: A measured volume of sample is extracted with methylene chloride. After a drying step followed by a solvent exchange to hexane, the sample is derivatized to tributyl hexyl tin with a Grignard reagent, hexylmagnesium bromide (HMB). Organic interferences are removed by passing the sample extract through a magnesium silica gel column. After concentration, the sample is injected into a gas chromatograph equipped with a mass spectrometer for separation and analysis. Identification and detection of TBT is performed with the mass spectrometer in selected ion monitoring mode.

b. Interferences: Most organic interferences in the samples can be removed by performing a cleanup step. Laboratory preparation of HMB is recommended because some commercially prepared solutions of HMB have been found to contain TBT as a contaminant. Preparing several blanks containing increasing amounts of HMB can assess HMB contamination.

c. Minimum detectable concentration: The method detection level (MDL)[1] for TBT in wastewater is 2 ng/L. Table 6710:I contains a single-laboratory detection level study performed in a wastewater matrix.

https://doi.org/10.2105/SMWW.2882.133

Table 6710:I. Single-Laboratory Method Detection Level in Wastewater*

Replicate No.	Concentration *ng/L*	Recovery %
1	7.8	65.2
2	8.5	70.6
3	9.6	80.3
4	9.1	75.9
5	8.0	67.0
6	9.6	80.2
7	10.0	83.4
8	9.4	78.2
Average	9.0	
Standard deviation	0.8	
Calculated MDL	2.3	

* Known addition: 5 ng TBT/L; nominal background: 7 ng/L.

Table 6710:II. Ion Abundance Criteria for Decafluorotriphenylphosphine (DFTPP)[2]

Mass *m/z*	Relative Abundance Criteria
51	10–80% of base peak
68	<2% of mass 69
70	<2% of mass 69
127	10–80% of base peak
197	<2% of mass 198
198	Base peak or >50% of 442
199	5–9% of mass 198
275	10–60% of base peak
365	<1% of base peak
441	Present and < mass 443
442	Base peak or >50% of 198
443	15–24% of mass 442

2. Apparatus

a. Gas chromatograph capable of temperature programming and equipped for splitless injection.

b. Mass spectrometer capable of scanning from 45 to 450 amu every 1 s or less, using 70 eV (nominal) in the electron impact ionization mode, producing a mass spectrum that meets all criteria in Table 6710:II when 5 ng or less of DFTPP is introduced into the chromatograph, and operating in the selected ion monitoring (SIM) mode.

c. Data system: A computer interfaced with the GC/MS with adequate software to allow continuous acquisition, storage, and processing of all mass spectra data.

d. Column: Fused silica capillary column, 30 m long with 0.25-mm ID and 0.25-μm film thickness.*

e. Balance, analytical, capable of accurately weighing to the nearest 0.1 mg.

f. Syringes, 10-, 25-, 100-, and 1000-μL volume.

g. Volumetric flasks, Class A, appropriate sizes with ground-glass stoppers.

* DB5-MS, J&W Scientific, or equivalent.

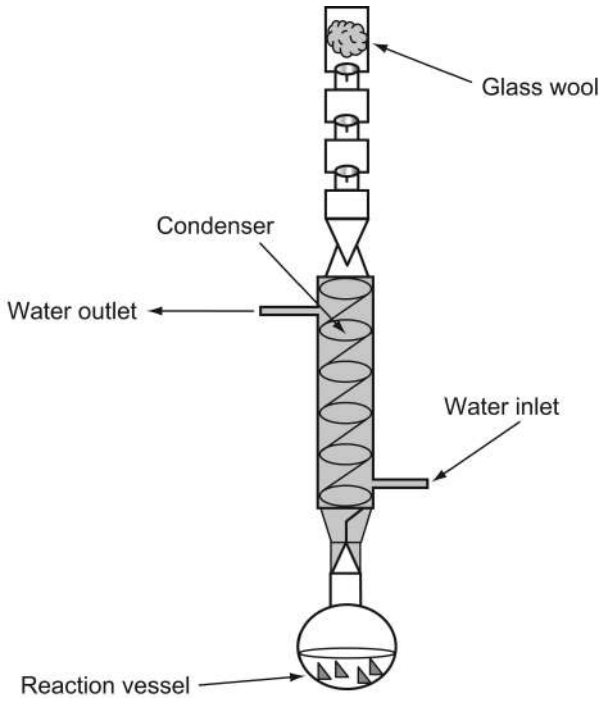


Figure 6710:1. Apparatus setup for HMB generation.

h. Hexylmagnesium bromide generation apparatus (not necessary if commercially prepared reagent is used):

1) *Boiling flask,* 50-mL.

2) *Condenser topped with a macro Snyder column* (see Figure 6710:1).

3) *Glass wool*.

4) *Ice bath,* large enough for boiling flask.

5) *Hot plate*.

i. Extraction apparatus:

1) *Continuous liquid/liquid extractors*†—Alternatively, separatory funnels may be used.

2) *Boiling flasks*—heat-resistant glass, short neck, 500-mL.‡

3) *Heating mantles*—hemispherical, for flasks with 500-mL capacity, 270 W.§

4) *Variable transformers*—0–120 V, 12 amp.‖

5) *Drying columns/cleanup columns*—250-mL reservoir, 19 cm × 300 mm.#

6) *Vials*—40-mL, precleaned.**

7) *Cooling container* (plastic or metal)—to hold ice and 40-mL extract vials.

j. Sample concentrator:†† Kuderna-Danish concentration apparatus also can be used.

† Kontes No. 584191-0000 extractor body, or equivalent.
‡ VWR No. 29113-122 (Pyrex No. 7740), or equivalent.
§ Series O, VWR No. 33787-141, or equivalent.
‖ Powerstat® VWR No. 62546-364 Model No. 3PW117C, Superior Electric, or equivalent.
Kontes Custom Glass Shop No. 34-10070, or equivalent.
** Wheaton Clean-Pak 217857, or equivalent.
†† Labconco RapidVap N_2, or equivalent.

3. Reagents

a. Extraction reagents:

1) *Reagent water*—free of observable interferences at the method detection level. Water can be obtained by using a water purification system‡‡ equipped with a UV lamp and an organic polishing cartridge. Do not use plastic pipes, especially PVC, in the plumbing of reagent water.

2) *Boiling chips* (TFE).§§

3) *Hydrochloric acid* (HCl), 1:1.‖‖‖

4) *Methylene chloride*.##

5) *Hexane*.##

6) *Tropolone*.***

7) *Sodium sulfate* (Na_2SO_4), granular †††—muffled at 400°C for 4 h.

b. Reagents for derivatization and sample cleanup: Either use reagents 1)–3) for laboratory generation of HMB, or use reagent 4).

1) *Magnesium turnings,* >99.8%.‡‡‡

2) *Diethyl ether,* anhydrous§§§, preserved with BHT.

3) *1-bromo-hexane,* >98% GC.‡‡‡

4) n-*Hexylmagnesium bromide (HMB),* 2*M*.‖‖‖‖

5) *Activated magnesium silica gel*###—60–100 mesh, baked overnight at 130°C.

6) *Sodium sulfate*—See ¶ *a*7) above.

c. Standard solutions:

1) *Stock calibration standard*—Dissolve 11.2 mg tributyl tin chloride in 1000 μL acetone. Dilute 100 μL of this solution with 1000 μL of acetone to obtain a concentration of 1.0 μg/μL. Add 2.5 μL of the 1.0-μg/μL solution to 1000 μL of hexane; final concentration is 2.5 μg tributyl tin/mL.

2) *Stock calibration check standard*—Uses a source of tributyl tin different from source of calibration stock standard. Prepare in the same manner as in ¶ *c*1) above.

3) *Surrogate standard*—Dissolve 11.4 mg tri-*n*-propyl tin chloride in 1000 μL acetone. Dilute 20 μL of this solution with 1000 μL of acetone to obtain a concentration of 200 μg/mL (stock surrogate). From the stock surrogate, prepare a working standard by diluting 50 μL of 200-μg/mL solution to 1000 μL of hexane to yield a final concentration of 10 μg tri-*n*-propyl tin/mL.

4) *Internal standard*—Use a 4-mg/mL solution of d10-phenanthrene, d10-acenaphthene, and d12-chrysene in methylene chloride. Dilute 12.5 μL of 4 mg/mL solution to 1 mL methylene chloride to prepare a concentration of 50 μg/mL.

5) *GC/MS performance check solution*—Use a solution of DFTPP, pentachlorophenol, and benzidine. Prepare a performance check solution containing 5 μg/mL DFTPP, 5 μg/mL pentachlorophenol, and 10 μg/mL benzidine by dilution of a more concentrated stock in methylene chloride.

‡‡ Millipore MQ, or equivalent.
§§ Chemware, or equivalent.
‖‖‖ EM Science Suprapur Grade, or equivalent.
EM Science Omni Solv HRGC, or equivalent.
*** Aldrich, or equivalent.
††† EM Science GR ACS Grade, or equivalent.
‡‡‡ Fluka, or equivalent.
§§§ Mallinckrodt AR Grade, or equivalent.
‖‖‖‖ TCI America, or equivalent.
Florisil, or equivalent.

TABLE 6710:III. CALIBRATION STANDARDS CONCENTRATION LEVELS AND PREPARATION METHOD

Calibration Level	Final Standard Concentration* *ng/mL*	Volume of Stock Standard Added to 10 mL Hexane *μL*	Volume of Surrogate Standard Added to 10 mL Hexane† *μL*
CC‡	100	40	20
1	10	4	20
2	50	20	20
3	100	40	20
4	200	80	20
5	500	200	20

* All calibration standards are concentrated to 1 mL after derivatization and cleanup.
† Yields a surrogate final concentration of 200 ng/mL.
‡ Calibration check.

4. Procedure

a. Standards preparation: Prepare at least five levels of calibration standards. Set lowest calibration standard concentration at three to five times the detection level. Suggested calibration levels are presented in Table 6710:III. Prepare calibration check standard (CC) from a different source and use as an independent calibration check. Prepare standards in 10 mL hexane. Add 20 μL of surrogate standard [6710B.3*c*3)] to each of the calibration standards to yield final concentration of 200 ng/mL.

Carry all prepared stock standards (10 mL each in 40-mL VOA vial) through derivatization and cleanup steps (see ¶s *d* and *e* below).

b. HMB preparation (not required if reagent is purchased): Prepare reagent no >1 d before use. Reagent will react with water. If storing reagent for more than a day, store under a nitrogen blanket or in a desiccator.

Set up reagent vessel as shown in Figure 6710:1. Acid-wash all glassware.

Weigh approximately 2.4 g magnesium turnings into a 50-mL boiling flask. Add 10 mL diethyl ether, and 20 mL 1-bromohexane. Wait for reaction to start—the solution turns cloudy and bubbles are formed. If reaction has not started within 5 min, crush magnesium with a glass stirring rod. Add 20 mL ether, then attach condenser and turn on the cooling water. Moderate reaction, using an ice bath, if flask is about to boil over. If reaction is slow, reflux on hot plate for 5 min. When bubbling stops or slows, swirl flask several times. If bubbling resumes, reaction is not complete. Repeat swirling until no bubbling is observed. Reaction is complete when there are just a few blackened metal fragments left on the bottom of the flask.

Store HMB at 4°C until use (up to 24 h).

c. Sample extraction: Mark the meniscus on side of sample container for later determination of sample volume. Adjust pH of all samples to pH <2 with 1:1 HCl. Pour entire sample into a continuous liquid/liquid extractor. Add 20 μL tripropyl tin surrogate standard [6710B.3*c*3)] to each sample, including method blank (reagent water with all reagents and preservatives added), LCS (reagent water with reagents, preservatives, and TBT), and laboratory-fortified matrix (LFM)/LFM duplicate (LFMD). Add

40 μL stock calibration check standard [6710B.3*c*2)] to the LFM/LFMD. Add 4 μL of the same solution to the LCS sample. If glass sample containers are used, add 250 mL methylene chloride to each extractor. If polycarbonate containers are used, add 350 mL methylene chloride to each extractor. If DBT and MBT are target analytes, use 0.2% (w/v) tropolone:methylene chloride as an extraction solvent. If glass containers are used, rinse sample container with 50 mL methylene chloride and add rinsate to extractor. Perform rinse twice. Do not rinse polycarbonate containers. Extract sample for 24 ± 6 h.

Determine original sample volume by refilling sample bottle to the mark and transferring the water to a 1000-mL graduated cylinder. Record sample volume to nearest 5 mL.

Prepare drying column containing 50 g muffled granular Na_2SO_4 by pre-wetting it with methylene chloride. Pass extract through column and collect in concentration vessel. Rinse extract holding flask three times with 10 mL methylene chloride and transfer each rinse to drying column. Elute drying column with 30 mL methylene chloride.

Exchange the solvent to hexane, using concentrator (6710B.2*j*). Set temperature of the concentrator at 40°C and set nitrogen gas pressure at 103 kPa (15 lb/in.^{2}). Bring extract to a final volume of approximately 2 mL. Transfer extract to 40-mL vial with a Pasteur pipet. Rinse concentrator tube three times with 2 mL hexane and transfer each rinse to the 40-mL vial. The total extract in 40-mL vial after transfer should be approximately 8 to 10 mL.

d. Derivatization: To each extract, slowly add 2.0 mL of 2.0*M* HMB and cap vial. Allow derivatization to proceed for 30 min at room temperature with constant shaking. Place vials in a rack and place rack in a cooling container with ice and water. Add 5 mL 1:1 HCl to each vial. Once vial is cool, shake for 2 min, pull off bottom aqueous layer, and discard. Add another 5 mL 1:1 HCl, shake for 2 min, and pull off and discard aqueous phase as above. Add 5 mL reagent water, shake for 2 min, and pull off and discard aqueous phase. Proceed with extraction cleanup.

TABLE 6710:IV. GAS CHROMATOGRAPH OPERATING PARAMETERS

Parameter	Setting
Initial temperature	80°C, hold for 2 min
Temperature program	8°C/min to 260°C 50°C/min to 350°C, hold for 5 min
Final temperature	350°C
Injector temperature	230°C
Transfer line	250°C
Detector temperature	200°C
Injector type	Grob-type, splitless; split on 0.7 min after injection
Sample volume	1 μL
Carrier gas	Helium at 30 cm/s (measured at 270°C)

e. Extract cleanup:[3] Activate magnesium silica gel by baking overnight at 130°C. Prepare cleanup columns by weighing 20 g activated gel into a glass cleanup column. Cover gel with 2 g of muffled granular sodium sulfate. Wash column with 70 mL hexane. Keep column wet by closing stopcock to keep 2 mm of hexane above sodium sulfate.

Transfer sample extract to a prepared column. Rinse extract vial three times with hexane, adding each rinse to cleanup column. The total hexane rinse volume is approximately 25 mL.

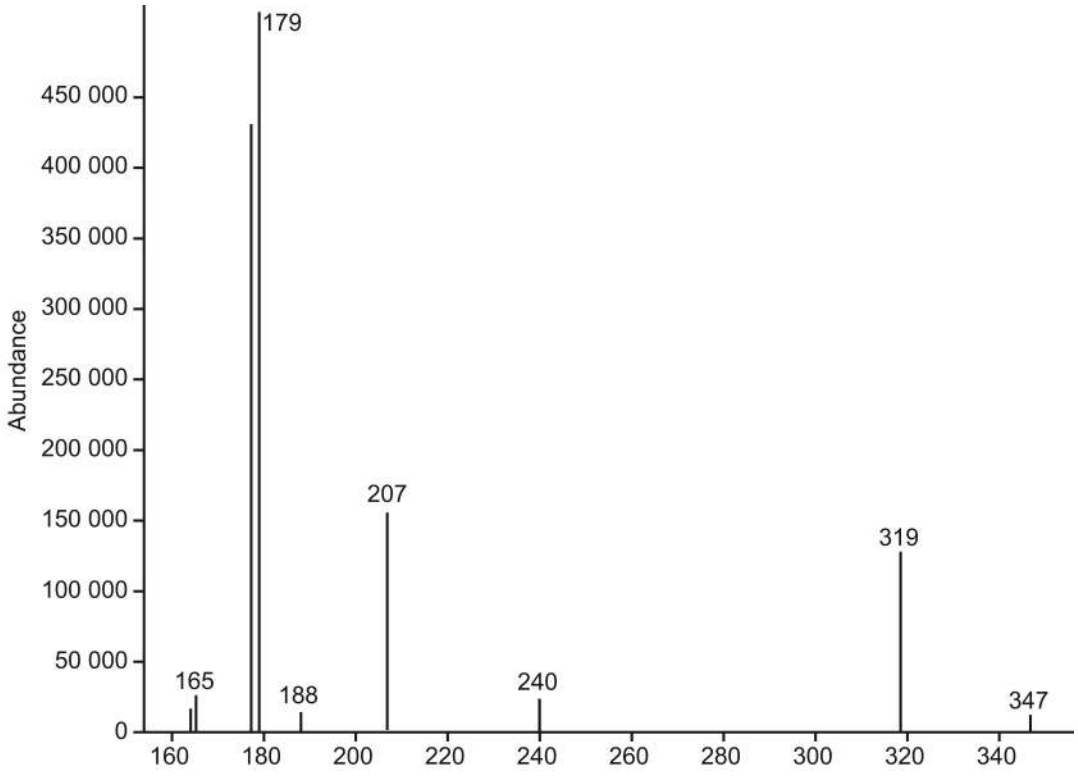


Figure 6710:2. Tributyl tin spectrum with selected ion monitoring.

Table 6710:V. Assigned Quantitation Ion and Internal Standards

Analyte/Internal Standard (IS)	Analyte/IS Quantitation Ions	Analyte/IS Qualitative Ions
Tripropyl tin/d10-acenaphthene	207/164	164, 165/165
Tributyl tin/d10-phenanthrene	179/188	177, 207, 319/ none

Table 6710:VI. Calibration Acceptance Criteria

Calibration Technique	Acceptance Criteria
Response factors (RF)	≤20% relative standard deviation (RSD)
Linear or quadratic regression	$R^2 > 0.998$

Elute column with a total of six 25-mL hexane rinses, collecting all eluent in a concentration tube.

Concentrate extract to a final volume of 1.0 mL using the concentrator. Store extracts at 4°C.

f. Instrument calibration: Set up the gas chromatograph system to operate with parameters shown in Table 6710:IV.

Perform an air/water check as recommended by the mass spectrometer manufacturer. Tune the spectrometer with perfluorotributyl amine (PFTBA) to facilitate meeting the DFTPP ion abundance criteria shown in Table 6710:II.

Inject 1 μL of GC performance check solution. The DFTPP spectrum should meet the criteria outlined in Table 6710:II. The tailing factors for benzidine and pentachlorophenol should be lower than 3 and 5, respectively. Higher tailing factors may indicate system problems: the column may need to be trimmed or baked, or the injector liner may need replacement. Resolve problems before proceeding with sample analysis.

To the calibration standards generated in ¶s *c–e* above, add 5 μL internal standard.

Inject a solvent blank followed by the calibration standards. Process calibration standards with instrument data system and prepare a calibration curve. A sample mass spectrum for tributyl tin is shown in Figure 6710:2. Suggested quantitation ions and internal standard assignments are outlined in Table 6710:V.

Acceptance criteria for the calibration curve are outlined in Table 6710:VI . If the %RSD for the calibration curve is <20%, the response can be assumed to be linear through 0 and response factors (RFs) may be used. If the %RSD is >20%, do not use RFs for quantitation purposes, but choose an appropriate regression technique.

g. Sample analysis: Add 5 μL internal standard to all samples. Inject and process all the samples in the batch, including QC samples, using the calibration curve obtained according to ¶ *f* above. Ensure that all reported concentrations are bracketed by the calibration standards. If a sample concentration exceeds the highest standard, rerun sample at the appropriate dilution to bring the concentration within the calibration range. Before rerunning the diluted sample, add required volume of internal standard to make up the loss caused by dilution. Accept or reject the sample batch on the basis of QC criteria given in 6710B.5.

5. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 6020:I.

Include a method blank, LCS, and LFM/LFMD in all analytical batches. The required QC sample types and frequencies and batch acceptance limits calculated from a single-laboratory data set are summarized in Table 6710:VII.

Develop laboratory batch acceptance limits by calculating the average percent recovery (p) and the standard deviation (s) for each of the laboratory-fortified matrix compounds after analysis of 15 to 20 laboratory-fortified matrix samples of the same matrix. Calculate average percent recovery (p) and the standard deviation (s) for each of the surrogates after analysis of 15 to 20 field samples of the same matrix, in a similar fashion. Calculate upper and lower control limit for each matrix known addition or surrogate compound:

$$\text{Upper control limit} = p + 3s$$

$$\text{Lower control limit} = p - 3s$$

Calculate warning limits as:

$$\text{Upper warning limit} = p + 2s$$

$$\text{Lower warning limit} = p - 2s$$

Table 6710:VII. Minimum QC Samples for Each Batch and Respective Acceptance Limits

QC Type	Frequency	Acceptance Range
Internal standard (IS)	Every sample	70–130% of IS response from previous CC and 50–150% of mean IS response from initial calibration
Surrogate	Every sample	29–129% recovery
Method blank	1/batch	All analytes less than MDL
LCS at 3–5 times MDL	1/batch	50–150% recovery
Calibration check (CC)	Every 10 samples; minimum 2/batch; at start and end	80–120% recovery
Laboratory-fortified matrix	10% of samples; minimum 1/batch	30–132% recovery
Laboratory-fortified matrix duplicate	10% of samples; minimum 1/batch	30–132% recovery and ≤40 relative percent difference (RPD)

Note: Ensure that laboratory meets acceptance criteria above for internal standard, method blank, LCS, and calibration check. Establish acceptance limits for recoveries of surrogate, LFM, LFM duplicate, and precision for LFM–LFMD in the laboratory.

For laboratories employing statistical software to determine these limits, the control limits approximate a 99% confidence interval around the mean recovery, while the warning limits approximate a 95% confidence interval.[4]

Ensure that laboratory meets the acceptance limits in Table 6710:VII for the internal standard, method blank, LCS, and calibration check. These QC types are used to establish that the method performance is met. Recoveries for the surrogate, LFM, LFM duplicate, and precision for the LFM-LFMD are matrix-dependent; establish these in the laboratory.

6. Precision and Bias

Single-laboratory precision and bias in a wastewater matrix are presented in Table 6710:I.

7. References

1. U.S. Environmental Protection Agency. 1984. Definition and procedure for the determination of the method detection limit. 40 CFR Part 136, Appendix B. *Fed. Reg.* 49, No. 209.
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8. Bibliography

U.S. Environmental Protection Agency. 1996. DBP/ICR Analytical Methods Manual, Section 10; EPA 814-B-96-002. Off. Water, Cincinnati, Ohio.

6710 C. Gas Chromatographic/Flame Photometric Detector Method

This method is applicable to the determination of tributyl tin (TBT) in drinking water, marine water, and wastewater. It is an adaptation of Method 6710B, modified for the analysis of tributyl tin with a flame photometric detector (FPD).

1. General Discussion

a. Principle: The principle is identical with that given in 6710B.1*a*, except that a flame photometric detector (FPD) is used for separation and analysis.

b. Interferences: See 6710B.1*b*. Because detection with GC/FPD can be subject to interferences, confirmation on a secondary column or by mass spectrometry is desirable.

c. Minimum detectable concentration: The method detection level (MDL)[1] for TBT in seawater is 1 ng/L. Table 6710:VIII contains results of a single-laboratory detection level study performed in an artificial seawater matrix.

Table 6710:VIII. Single-Laboratory Method Detection Level in Artificial Seawater*

Replicate No.	Concentration *ng/L*	Recovery %
1	1.3	120
2	1.3	120
3	0.9	82
4	0.6	55
5	0.8	73
6	1.4	79
7	0.9	82
Average	1.0	
Standard deviation	0.3	
Calculated MDL	0.96	

* Known addition: 1.1 ng TBT/L.

2. Apparatus

a. Gas chromatograph, equipped with an FPD using a >600-nm band pass filter, capable of temperature programming, and equipped for splitless injection.

b. Data system: A computer interfaced with the GC with adequate software to allow the continuous acquisition, storage, and processing of all data.

c. Other apparatus: See 6710B.2*d–j*.

3. Reagents

*a. Reagents specified in 6710B.3*a*1)–*c*3).*

b. Internal standard solution: Dissolve 44.3 mg tri-*n*-pentyl tin chloride* in 1000 μL hexane. Dilute 20 μL of this solution with 1000 μL hexane to obtain a concentration of 800 μg/mL (stock internal standard). Dilute 50 μL of this solution with 1000 μL hexane. This will yield an internal standard solution with a concentration 40 μg/mL. Add 5 μL of this solution to the calibration standards and sample extracts before derivatization and cleanup. The final concentration is 200 ng/mL of tri-*n*-pentyl tin in calibration standards and sample extracts.

* Organometallics, Inc., East Hampstead, NH, or equivalent.

TABLE 6710:IX. GAS CHROMATOGRAPH OPERATING PARAMETERS

Parameter	Setting
Initial temperature	135°C hold for 2 min
Temperature program	10°C minute to 300°C, hold 5 min
Final temperature	300°C
Injector temperature	280°C
Detector temperature	280°C
Injector type	Grob-type, splitless; split on 0.7 min after injection
Sample volume	1 μL
Carrier gas	Helium at 30 cm/s (measured at 300°C)
Make-up gas	Helium at 90 mL/min
Detector gases	Hydrogen at 152 mL/min; air1 at 97 mL/min; air2 at 188 mL/min

4. Procedure

a. Standards preparation: Prepare at least five levels of calibration standards. Set lowest calibration standard concentration at three to five times the detection level, at minimum reporting level. Suggested calibration levels are presented in Table 6710:III. Prepare calibration check standard (CC) from a different source and use as an independent calibration check. Also use CC for making known additions to samples and to prepare the laboratory control standard (LCS).

Add 5 μL of 40 μg/mL internal standard solution to all stock standards. Carry all prepared stock standards (10 mL each in 40-mL vials) through the derivatization and cleanup steps (see 6710B.4*d* and *e*).

b. HMB preparation: See 6710B.4*b*.

c. Sample extraction: Follow procedure given in 6710B.4*c*. When extraction is complete, add 5 μL of 40 ng/μL internal standard (6710C.3*b*) to every sample extract.

d. Derivatization: See 6710B.4*d*.

e. Extract cleanup: See 6710B.4*e*.

f. Instrument calibration: Set up the gas chromatograph system to operate with the parameters shown in Table 6710:IX.

Inject a solvent blank followed by the calibration standards. Process calibration standards with instrument data system and prepare a calibration curve.

Acceptance criteria for the calibration curve are outlined in Table 6710:VI. If the %RSD for the calibration curve is ≤20%, the response can be assumed to be linear through 0 and response factors may be used. If the %RSD is >20%, do not use RFs for quantitation purposes but choose an appropriate regression technique.

g. Sample analysis: Inject and process all the samples in the batch, including QC samples, using the calibration curve obtained according to ¶ *f* above. Ensure that all reported concentrations are bracketed by the calibration standards. If a sample concentration exceeds the highest standard, rerun sample at the appropriate dilution to bring the concentration within the calibration range. Accept or reject the batch on the basis of QC criteria given in 6710B.5.

5. Quality Control

See 6710B.5.

6. Precision and Bias

Single-laboratory precision and bias in an artificial seawater matrix are presented in Table 6710:VIII.

7. Reference

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1984. Definition and procedure for the determination of the method detection limit. 40CFR Part 136, Appendix B. *Fed. Reg.* 49, No. 209.

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PART 7000

RADIOACTIVITY

Part Coordinators
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Joint Task Group Chairs for the 22nd Edition

7010 Introduction .....Robert T. Shannon
7020 Quality System .....Robert T. Shannon
7030 Counting Instruments .....Robert T. Shannon
7500-Ra Radium .....Bahman Parsa

Summary of Major Changes since 2005

Quality Assurance/Quality Control (7020) was renamed Quality System and significantly revised to stay abreast of regulatory requirements and clarify the essential quality control practices for test methods in Part 7000. The gamma spectrometric method for the determination of Radium-224 (7500-Ra.E) now permits use of either Pb or Ba as a carrier.

Also, many methods now refer users to the quality control practices that are considered to be an integral part of the method.

7010 INTRODUCTION*

7010 A. General Discussion

1. Occurrence and Monitoring

Radioactivity in water and wastewater originates from both natural sources and human activities (e.g., nuclear fuel-related operations, from mining to reprocessing; medical and industrial uses of radioisotopes; worldwide fallout from atmospheric testing of nuclear devices; and enhanced concentrations of naturally occurring radionuclides). Water and wastewater monitoring programs should be designed to realistically assess the degree of radioactive contamination. In some situations (e.g., compliance monitoring for drinking water), the conditions are clearly defined.[1–3] In others, program staff may need radiation-protection professionals to determine the critical radionuclide(s), the critical pathway by which such radionuclide(s) move(s) through the environment, and the critical population group(s) exposed as a result. This approach will help narrow the list of possible radionuclides to monitor.

Monitoring-program staff can create a relevant list of the most hazardous radionuclides by examining the radioactivity concentration standards issued by the International Committee on Radiation Protection (ICRP),[4] the National Committee on Radiation Protection and Measurement (NCRP),[5–8] the U.S. Environmental Protection Agency (EPA),[1,2] and other national and international radiation protection organizations. Applicable regulatory standards and action levels may vary from state to state in the United States, as well as from country to country.

Monitoring programs should provide both assurances when environmental conditions are safe and adequate warning when they are not, so proper precautions can be taken. Therefore, baselines must be established for naturally occurring radionuclides (kinds and quantities) so changes can be measured. Decision-makers must provide laboratories with measurement quality objectives (MQOs) that unambiguously specify the type and quality of data needed to make sound judgments on the hazardous or nonhazardous nature of increased concentrations. Extensive guidance on developing MQOs is available in the Multi-Agency Radiological Laboratory Analytical Protocols (MARLAP) manual.[9]

2. Types of Measurement

Meaningful measurements depend on the careful application of good scientific techniques that meet testing objectives. Gross alpha and gross beta screening measurements are relatively inexpensive, can be completed quickly, and are useful for determining whether further analyses for specific radionuclides may be merited. However, they do not provide information about the sample's isotopic composition, cannot be used to estimate radiation dose, and are subject to significant bias if the sample contains other radionuclides or a high concentration of dissolved solids. Calibration standards that closely match the samples' characteristics and carefully applied self-absorption and cross-talk corrections may help minimize bias and optimize precision, but gross activity measurements generally cannot provide reliable, unbiased results of known precision that reflect the samples' isotopic makeup.

Specific radionuclide measurements must be made if dose estimates are needed [e.g., when gross analyses[1] results exceed established action limits (regulatory or other) or long-term trends are being monitored]. Specific measurements identify radionuclides by the type and energy of emitted radiation, chemical techniques, half-life, or some combination of these. They usually are more expensive and time-consuming than gross analyses. Gamma-emitting radionuclides can be measured rapidly with minimal sample preparation and no chemical separations via gamma spectrometry. Chemical separations can improve measurement results by increasing the quantity of sample that can be processed, thus decreasing the detection limit and minimizing interference from non-target radionuclides in samples.

Knowing the chemical and radiochemical characteristics of the measured radionuclide is critical for satisfactory results. Gross alpha and gross beta results cannot provide accurate information about radionuclides whose energies are significantly different from those of the calibration standard. When concentrating water samples via evaporation, certain radionuclides (e.g., radioiodine, polonium, tritium, and carbon-14) may be lost to volatilization. If the sample is ignited, the chance of volatilization loss is even greater.

Analysts must consider the dynamic nature of radionuclide concentrations as a result of decay and ingrowth phenomena. For example, uranium and thorium decay chain activity is rarely in secular equilibrium in water samples. So if monitoring-program staff must know the activity of an unsupported short-lived radionuclide at the time of collection, its radioactivity may need to be measured promptly following collection (before significant radionuclide ingrowth or decay occurs). If radioactive equilibria have been disturbed, samples may require multiple measurements to verify assumptions about radioactive equilibrium in the sample.

3. References

1. U.S. Environmental Protection Agency. 2000. National primary drinking water regulations; radionuclides, Clean Water Act regulations. 40 CFR Parts 9, 136, 141 & 142.
2. U.S. Environmental Protection Agency. 2002. Implementation Guidance for Radionuclides; EPA 816-F-00-002. Off. Ground Water and Drinking Water, Washington, D.C. Available at https://www.epa.gov/dwreginfo/radionuclides-rule. Accessed May 2016.
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* Approved by Standard Methods Committee, 2015.
Joint Task Group: Robert T. Shannon (chair), Catherine C. Franklin, Bahman Parsa, Shiyamalie R. Ruberu.

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5. NATIONAL COUNCIL ON RADIATION PROTECTION AND MEASUREMENTS. 2009. Ionizing Radiation Exposure of the Population of the United States; Report No. 160. Washington, D.C.
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7010 B. Sample Collection and Preservation

1. Collection

The principles of representative water and wastewater sampling apply to sampling for radioactivity testing (see Section 1060).

Because radioactive elements often are present in subnanogram quantities, a significant fraction may be lost via adsorption to the sampling container's surface. Similarly, radionuclides may be largely or wholly adsorbed to the surface of suspended particles. Such losses would compromise the integrity of the sample.

Sample container sizes vary from 0.02 to 18 L, depending on the analyses required. Use plastic (polyethylene or equivalent) or glass containers. To eliminate possible cross-contamination of subsequent samples, never reuse sample containers.

2. Preservation

For general information on sample preservation, see Section 1060. For regulatory guidance on sample handling, preservation, and holding times for measuring radionuclides in compliance with the U.S. Safe Drinking Water Act (SDWA), see Table 7010:I. Optimally, add preservative at the time of collection unless the sample will be separated into suspended and dissolved fractions. Do not delay acid addition beyond 5 d. For samples that require preservation by acidification, use concentrated nitric (HNO_3) or hydrochloric (HCl) acid to obtain a pH <2. Hold acidified samples at least 16 h, then recheck pH before analysis to verify successful acidification. For radioiodine determinations, add a reducing or oxidizing agent to ensure that iodine is present as the iodide or iodate. For radon, cool sample to slightly above freezing. For tritium (if the sample may be held long enough that biological growth is possible), the sample may be cooled or frozen.* For further details, see references.[1–3]

T1

Reagents used to preserve samples should be tested for concentrations of radioactivity that could compromise test results (e.g., by using a reagent or method blank).

3. Wastewater Samples

Wastewater typically contains more nonradioactive suspended and dissolved solids than most drinking, surface, or ground water does. Often, the sample's radioactivity is distributed between the solid and liquid phases. Using carriers in the analysis is generally ineffective unless the solid phase is dissolved; even then, the sample's high solids content may interfere with radioanalytical procedures.

Radioelements may exhibit unusual chemical characteristics due to complexing agents or the waste-production method. For example, tritium may be part of an organic compound when used in the manufacture of luminous articles. Radioiodine from hospitals may occur as complex organic compounds, while the fission products from processing spent nuclear fuels typically contain elemental and iodide forms. Uranium and thorium progeny often exist as inorganic complexes (rather than oxides) after processing in uranium mills. Strontium-90 titanate waste from radioisotope heat sources typically is more insoluble than other strontium wastes. Valuable information on the chemical composition of wastes, the behavior of radioelements, and the quantity of radioisotopes in use appears in the literature.[4–6]

* Although SDWA regulations require that samples be unpreserved, adding acid will not impair tritium analysis when using Method 7500-^{3}H.B for all other purposes.

TABLE 7010:I. SAMPLE HANDLING, PRESERVATION, AND HOLDING TIMES

CONSTITUENT	PRESERVATIVE*	CONTAINER†	MAXIMUM HOLDING TIME‡§
Gross alpha	Conc. HNO_3 to pH <2‖	P or G	6 months
Gross beta	Conc. HNO_3 to pH <2‖	P or G	6 months
Radium-226	Conc. HNO_3 or HCl to pH <2	P or G	6 months
Radium-228	Conc. HNO_3 or HCl to pH <2	P or G	6 months
Radon-222	Cool 4°C#	G with TFE-lined septum	8 d**
Uranium, natural	Conc. HNO_3 or HCl to pH <2	P or G	6 months
Radioactive strontium	Conc. HNO_3 or HCl to pH <2	P or G	6 months
Radioactive iodine	None	P or G	14 d
Tritium	None††	G††	6 months
Photon-emitters	Conc. HNO_3 or HCl to pH <2	P or G	6 months

* (All except radon-222 samples). Add preservative at time of sample collection unless suspended solids activity will be measured. If sample must be shipped to a laboratory or storage area, acidification (in original sample container) may be delayed for no more than 5 d. At least 16 h must elapse between acidification and analysis.
† P = plastic, hard or soft; G = glass, hard or soft.
‡ *Holding time* is time elapsed between sampling and analysis. In all cases, analyze samples as soon after collection as possible.
§ A 1-year holding time allows for compositing four quarterly samples.
‖ HCl may be used to acidify samples that will be analyzed for gross alpha or gross beta activities, but the chloride salts must be completely converted to nitrate salts by evaporation with nitric acid before transferring samples to planchets.
Large temperature changes will cause dissolved radon to outgas from sample. Cooling to 4°C is recommended; operationally, any temperature >0 and <6°C is acceptable.
** When analyzing short-lived radionuclides, adjust maximum holding time based on the radionuclide's half-life (e.g., 72 h for Ra-224).
†† Note that SDWA guidance specifies that glass and no preservative be used. For other programs, however, there is no technical reason that would prevent use of plastic bottles or preservation with acid for tritium sampling.

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7020 QUALITY SYSTEM*

7020 A. Quality Systems/Quality Assurance/Quality Control Program

1. Introduction

Every laboratory that analyzes environmental water and wastewater samples for radionuclides should operate under a quality system, which establishes the basis for all of the laboratory's quality assurance (QA) and quality control (QC) activities. The quality system sets forth requirements for the laboratory's organizational framework, the structure and contents of its quality manual, document controls, method validation, QC of measurement processes, internal audits, documentation, and completed-record archives. Quality-system standards commonly implemented at laboratories include The NELAC Institute Standard[1] (commonly referred to as The TNI Standard)[1] and *General Requirements for the Competence of Testing and Calibration Laboratories* (ISO 17025).[2] *The Manual for the Certification of Laboratories Analyzing Drinking Water—Criteria and Procedures, Quality Assurance*[3] also addresses QA/QC requirements established by the U.S. Environmental Protection Agency (EPA) for Safe Drinking Water Act (SDWA) compliance testing.

The laboratory's quality manual may include the laboratory's standard operating procedures (SOPs) by reference. It also addresses default QC measures applicable to instrument calibration and performance checks, background measurements, and batch QCs for precision and bias (accuracy) of analytical measurements. Also essential is a manual of analytical methods (or at least copies of approved methods).

Analysts must be trained in accordance with the quality manual and be familiar with its contents and requirements. The quality manual must also be readily accessible to them as they work in the laboratory.

2. QC Program Performance Criteria

Successful QC programs define acceptable, attainable performance criteria for precision and bias. These criteria must both reflect the laboratory's capabilities and be applicable to the programs for which analysis is performed.

At first, performance criteria can be drawn up based on either

- data derived from the laboratory's initial use of an analytical method or
- criteria set by the party requiring the water analyses (e.g., regulators).

Such criteria should be analysis- and matrix-specific and may vary by project. For example, NELAC has compiled allowable deviations for SDWA compliance-monitoring samples (see Table 7020:I). The laboratory may use published values until enough data can be compiled to set experience-based criteria that reflect actual method performance at the laboratory.

3. Minimum QC Program

Each laboratory should establish and implement QC requirements that are consistent with the data quality objectives (DQOs) and measurement quality objectives (MQOs)† of the programs they support. At minimum, the laboratory's QC program should include a number of integrated QC measures that address basic instrument performance, initial and ongoing calibrations, background measurement stability, and the precision and bias (accuracy) of each method (as measured via batch QC samples).

Typical batch QCs include

- negative controls (e.g., reagent or method blanks);
- positive controls [e.g., laboratory-fortified blanks (LFBs), laboratory control samples (LCSs), and matrix spikes (MSs)];
- reproducibility controls (e.g., sample or matrix-spike duplicates);
- sample-specific controls (e.g., acceptable range for tracer or carrier chemical yield, criteria for spectral resolution, agreement between peaks' observed energy and accepted values, etc.); and
- instrument controls (e.g., controls on background subtraction count determinations, instrument performance checks, and calibration verifications).

Such QCs can be monitored via statistical QC and tolerance charts. Certain aspects of radiochemical instrument and background QCs are instrument- or method-specific; these are dealt with in the individual methods and should be addressed in the laboratory's quality manual.

Prepare instrument QC charts[4,5,6] by plotting a reference source's results on a graph in which the abscissa is time and the ordinate is count rate, total counts, or another appropriate parameter. Determine the "true" (mean) value by averaging the results of at least 20 measurements with acceptable individual statistics. Insert lines parallel to the time axis at the mean value and the values for ± 2 and ± 3 standard deviations. These lines should include appropriate corrections for decay of the radionuclide of interest.

Sometimes the statistically determined performance may be better than the actual performance required to maintain control based on applicable DQOs and MQOs; if so, tolerance limits may be established as the control criterion. On the control chart, draw lines indicating the tolerance limits for the method's specified performance requirements, along with lines indicating its statistical performance (e.g., ± 3 standard deviations). As long as the method's statistical performance falls within the tolerance limit, the tolerance chart will supply evidence that the measurement parameter in question is under control.

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† See MARLAP[4] for definition and complete discussion of the concept and use of MQOs.

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TABLE 7020:I. LABORATORY PRECISION—ONE STANDARD DEVIATION VALUES FOR VARIOUS ANALYSES IN SAFE DRINKING WATER COMPLIANCE SAMPLES*

Analyte	Spike Level (μ†) Range	Expected Mean	Acceptable Standard Deviation (σ)
Gross Alpha	7–75 pCi/L	0.8586 μ + 1.4802	0.1610 μ + 1.1366
Gross Beta	8–75 pCi/L	0.8508 μ + 2.9725	0.0571 μ + 2.9372
Barium-133	10–100 pCi/L	0.9684 μ + −0.1424	0.0503 μ + 1.0737
Cesium-134	10–100 pCi/L	0.9369 μ + 0.0845	0.0482 μ + 0.9306
Cesium-137	20–240 pCi/L	1.0225 μ + 0.2624	0.0347 μ + 1.5185
Cobalt-60	10–120 pCi/L	1.0257 μ + 0.3051	0.0335 μ + 1.3315
Iodine-131	3–30 pCi/L	0.9711 μ + 0.8870	0.0624 μ + 0.6455
Radium-226	1–20 pCi/L	0.9253 μ + 0.3175	0.0942 μ + 0.0988
Radium-228	2–20 pCi/L	0.9243 μ + 0.2265	0.1105 μ + 0.3788
Strontium-89	10–70 pCi/L	0.9648 μ + 0.1591	0.0379 μ + 2.6203
Strontium-90	3–45 pCi/L	0.9369 μ + 0.2279	0.0902 μ + 0.5390
Tritium	1000–24000 pCi/L	0.9883 μ + −46.4776	0.0532 μ + 38.8382
Natural uranium (activity)	2–70 pCi/L	0.9568 μ + 0.0773	0.0700 μ + 0.2490
Uranium (mass)	3–104 μg/L	0.9568 μ + 0.1153	0.0700 μ + 0.3700
Zinc-65	30–360 pCi/L	1.0495 μ + 0.1245	0.0530 μ + 1.8271

* Modified from: NELAC PT for Accreditation Fields of Proficiency Testing with PTRLs, Drinking Water. Effective October 1, 2007, NELAC_DW_RAD_FOPT_Eff_2007_ 10_01-2.xls, available at: http://www.nelac-institute.org/fopt.php. Accessed December 2016.
† Acceptable Limits are $\mu \pm 3\sigma$; Warning Limits are $\mu \pm 2\sigma$.

Interpret the QC chart data objectively. When a point goes outside established limits, determine whether the excursion was random or non-statistical by running repeated measurements and applying statistical tests (e.g., a Chi-square test).

Control charts may provide evidence of trends that should trigger an investigation to determine whether a trend could affect measurements significantly enough that applicable DQOs and MQOs would not be attained. When a change is detected that will prevent MQOs from being achieved, take corrective actions (ranging from data inspection to instrument maintenance and recalibration).

Trending criteria should be as simple as possible to provide meaningful control for the related parameter. For example, multiple successive points less than one standard deviation on one side of the mean may not negatively affect DQOs/MQOs and thus may not require monitoring or corrective action.

The laboratory's quality manual should unambiguously specify all trending and QC criteria, as well as applicable corrective actions and required documentation for each QC measure implemented.

a. Instrumental QC: Count check source(s) for a predetermined time at frequencies defined in the laboratory quality manual (e.g., daily before each use for proportional, scintillation counters, or gamma spectrometers; or monthly for alpha spectrometers). Record the count rate and plot it on the specific system's control chart. Compare the value with the ± 2-σ, ± 3-σ, and/or appropriate tolerance value, and repeat the procedure if the ± 2-σ values are exceeded. Corrective action is generally required whenever repeated values fall above or below the established statistical control or tolerance limits.

For instruments that produce spectra (e.g., gamma or alpha spectrometers), analysts may track such parameters as instrument response (count rate or total counts in a given spectral area, peak channel location, or the energy of key photopeaks in the spectrum); difference in channels between two specified peaks; resolution (channel peak width at specified peak height); and certain ratios (e.g., peak-to-Compton ratio). It may or may not be necessary to track all of these routinely; see Section 7120 for specific recommendations. If the basic parameters are outside limits, it may be useful to evaluate additional parameters.

b. QC of instrument background: Perform a background subtraction count as often as the laboratory quality manual indicates (e.g., weekly or monthly). The duration of background subtraction measurements should be as long as or longer than the duration of any sample measurement from which they will be subtracted. For serial counters, such measurements are often performed with each batch of samples. Spectrometry measurements, on the other hand, often benefit from longer, less frequent counts (e.g., weekly or monthly) because these measurements produce more precise estimates of the background for specific peaks or regions of interest.

Check background-measurement results for each detection system as often as the laboratory quality manual indicates (e.g., weekly or monthly). Alternatively, background measurements may be checked with each batch of samples, or subtraction background measurements may be evaluated as background checks. At a minimum, background measurements should be frequent and sensitive enough to identify related issues that could compromise sample results.

Monitor background counts via control charts. Such counts may change slightly over time without compromising data quality. If Poisson detection statistics are used to estimate counting uncertainty and detection statistics (see 7020C), the assumption is that the background is stable between sample and background-subtraction counts, and that non-Poisson contributions to the background (e.g., electronic noise, variations in ambient background) are minimal. Comparing the background's observed variability (e.g., standard deviation) for more recent background counts to that predicted by Poisson statistics (which may be indicated via error bars for each value) may reveal excess uncertainty and could indicate significant problems with the estimates of uncertainty and detection statistics. Establish appro-

priate evaluation criteria and corrective actions to be taken if backgrounds exceed established limits.[4,5,7]

Background QC is instrument-specific. For example, for gamma spectrometry backgrounds, the QC checks and acceptance criteria needed depend on whether the detector is NaI(Tl) or high-purity germanium (see Section 7120).

c. Negative control samples (method and reagent blanks): A *method* or *reagent blank* is "a sample assumed to be essentially target-analyte-free that is carried through the radiochemical preparation, separation and measurement process in the same manner as a routine sample of a given matrix."‡ Analyze method or reagent blanks as batch QC samples frequently enough to detect deviations in measured radioactivity that represent levels of absolute bias§ that could compromise the use of associated results.

Unless otherwise required by the program, blank samples should be analyzed at the frequencies specified in the quality manual for a given analysis (generally, one per batch or 5%, whichever is more frequent). However, this guideline may be varied to fit the situation. For example, a laboratory with a heavy workload and well-established blank-analysis program may find that analyzing blanks at a 5% rate (regardless of batch size) is sufficient to determine whether the data meet established criteria. On the other hand, if a test is run less frequently or there are significant concerns about potential contamination or background control, a higher blank-analysis rate might be better.

Ideally, the blank's count rate should equal the mean background count rate, and the average of background-subtracted blank results should be zero. The calculated uncertainty of the distribution should approximate the combined standard uncertainty (CSU) for the measurement. The measured activity of approximately 19 out of 20 blanks should be less than the critical level.‖ (See 7020C.) The laboratory should define evaluation and acceptance criteria—and associated corrective actions—in its quality manual and ensure that they are consistent with the data-quality requirements of the programs they support.

d. QC for precision: The IUPAC Gold Book defines *precision* as the "closeness of agreement between independent test results obtained by applying the experimental procedure under stipulated conditions. Precision may be expressed as the standard deviation."[8] The laboratory's internal precision in performing an analytical procedure can be evaluated by analyzing duplicate aliquots of one or more samples or QC samples with each batch of samples.

Duplicate samples may be submitted to analysts on a single- or double-blind basis to effect such an evaluation. (In single-blind analysis, the analyst is aware that a duplicate sample is present. In double-blind analysis, the analyst should be unaware that any of the samples is a QC sample.) Duplicate samples may have either detectable or nondetectable amounts of radioactivity. Statistical treatment of duplicate results can be used effectively to evaluate control of analytical precision and the adequacy of uncertainty estimates both in the presence or absence of activity.

Unless otherwise required by the program, duplicate samples should be analyzed at the minimum rate specified in the quality manual (generally one duplicate sample per batch or 5%, whichever is more frequent). This guideline may be varied to fit the situation. For example, SDWA regulations stipulate more frequent duplicate analyses (e.g., 10%). Similarly, a laboratory with a heavy workload and well-established duplicate-analysis program may find that analyzing duplicates at a 5% rate is sufficient to determine whether the data meet established criteria. On the other hand, if a test is run less frequently or there are significant concerns about the reproducibility of the matrix, a higher duplicate-analysis rate would be prudent.

For results above the detection threshold where counting-related uncertainty is small (e.g., ten times the critical level activity), the absolute difference between duplicate measurements should be less than three times the acceptable standard deviation for the specific analysis (see Table 7020:I):

$$\text{Absolute Difference} = \frac{|AC_s - AC_{dup}|}{u(AC_{target})}$$

where:

AC_s = sample result (e.g., pCi/L),
AC_{dup} = duplicate sample result (e.g., pCi/L), and
$u(AC_{target})$ = the targeted combined standard uncertainty of sample result (k=1).

A more robust measure, the normalized absolute difference (NAD)# between two results, evaluates statistical agreement between two results of any magnitude based on their respective uncertainties:

$$NAD = \frac{|AC_s - AC_{dup}|}{\sqrt{u^2(AC_s) + u^2(AC_{dup})}}$$

where:

NAD = normalized absolute difference statistic,
$u^2(AC_s)$ = the square of the combined standard uncertainty of sample result (k=1), and
$u^2(AC_{dup})$ = the square of the combined standard uncertainty of duplicate sample result (k=1).

If the NAD exceeds a value of 3, the control limit, then the two results differ by more than three standard deviations** and it may be generally concluded that a problem is indicated. Examine calculations, control charts, and procedures. Take action as defined in the laboratory's quality manual, including re-analyzing samples affected by the problem unless there are indications that sample non-homogeneity would prevent reproducible laboratory

‡ See MARLAP manual glossary.
§ *Absolute bias* is a persistent deviation of the mean measured net activity of blank results from zero. See MARLAP manual for discussion.
‖ Blanks with activity exceeding the critical-level activity may be recounted once to demonstrate that the excursion is due to Poisson variability in the result. If the recount's result is less than the critical-level activity, and the control chart for blanks does not reveal problems, there is no need to recount associated samples in the batch.

MARLAP refers to NAD as Z_{rep}. It has also been called the *duplicate error ratio* (DER) and *replicate error ratio* (RER).
** Two standard deviations correspond to the 95.4% confidence level; approximately 1 in 20 results should exceed this criterion. Three standard deviations correspond to the 99.7% confidence level; approximately 3 in 1000 measurements should exceed this criterion.

subsampling (e.g., presence of hot particles in the sample). NOTE: NAD is expected to exceed a value of 2 (indicating that the two results differ by more than two standard deviations) in approximately 5% of all measurements. Repeated excursions above the warning limit (i.e., the frequency exceeds the expected rate of 5%) may indicate a problem in the analytical system and should trigger similar corrective actions to exceeding the control limit.

Relative percent difference (RPD) is also used to evaluate duplicate results. This measure does not take uncertainty into account, but it does provide useful information for programs, such as SDWA compliance testing, where only Poisson uncertainty of results is reported:

$$RPD = \frac{|AC_s - AC_{dup}|}{(AC_s + AC_{dup})/2} \times 100$$

where:

RPD = relative percent difference,
AC_s = sample result (e.g., pCi/L), and
AC_{dup} = duplicate sample result (e.g., pCi/L).

The laboratory should define all evaluation and acceptance criteria, and associated corrective actions, in its quality manual and ensure that they are consistent with the DQOs and MQOs of the programs they support. More detailed discussions of QC statistics for duplicates are given elsewhere.[3,4,5]

e. Positive controls (LFBs and matrix spikes): Analytical methods are said to be in control when they produce precise, unbiased results. Two QC measures are commonly used to assess the ongoing accuracy of analytical methods: LFBs and matrix spikes. These QC measures and their evaluation should be defined in the laboratory quality manual.

1) Laboratory-fortified blanks (also called *laboratory control samples*)—Unless otherwise required by the program, LFBs consist of demineralized water spiked with a known activity of a traceable radiostandard LFB that are analyzed at a frequency of one per batch of 20 or fewer samples. The LFB is treated and analyzed just like all the other samples in the batch. Results are compared to known values based on percent recovery:

$$\%\ \text{Recovery} = \frac{\text{Observed Result}}{\text{Known Result}} \times 100$$

where:

% Recovery = ratio of observed to known values (%),
Observed result = measured result [expressed in units used to report associated samples (e.g., pCi/L)], and
Known result = theoretical result calculated from the spike added [expressed in the same units used to report associated samples (e.g., pCi/L)].

NOTE: The result should be calculated using an aliquot size similar to those used for the associated samples.

2) Matrix spikes—The matrix spike consists of a duplicate aliquot of one batch sample that has been spiked with a known activity of a traceable radiostandard. The purpose of a matrix spike is to establish if the method or procedure is appropriate for the analysis of the particular matrix.†† Unless otherwise required by the program, and as specified in the specific method, prepare one matrix spike per batch of 20 or fewer samples for each method in which no chemical yield carrier or tracer is added to samples. The sample is processed with the rest of the batch, and results are evaluated based on percent recovery:

$$\%\ \text{MS Recovery} = \frac{AC_{MS} - AC_{Smp}}{A_{Spk}/V_{MS}} \times 100$$

where:

% MS Recovery = recovery of the matrix spike from the percent recovery of activity added to the matrix spike sample,
AC_{MS} = measured activity in matrix spike (e.g., pCi/L),
AC_{Smp} = measured activity in original sample (e.g., pCi/L),
A_{Spk} = activity added to matrix spike (e.g., pCi), and
V_{MS} = volume of aliquot taken for matrix spike (e.g., L).

3) Evaluation and control charting of LFB and matrix spike results—Laboratories should establish evaluation and acceptance criteria for LFBs and matrix spikes in their laboratory quality manuals. Plot data from these standards or known-addition samples on mean or tolerance control charts[3,5,9] and determine whether results are within established limits (or if trends, indicate problematic changes in the analytical system). If established control limits are exceeded, investigate results for potential blunders, method bias, or excess uncertainty, and take corrective action to address the root cause and prevent recurrence of the problem, up to and including selection of a method appropriate to processing the matrix in question.

f. Sample-specific QC measures: Additional QC measures are routinely used to assess the quality of radiochemical measurements on a sample-by-sample basis. While batch QCs affect the status of a batch of samples, sample-specific QC only affects the sample in question [unless it is a batch QC sample (e.g., blank, duplicate sample, LFB, or matrix spike)]. Sample-specific QC includes controls on chemical yield or spectral resolution [i.e., lower and upper limits for chemical yield and full-width-at-half-maximum (FWHM) of observed peaks in spectral results]. The laboratory should define evaluation and acceptance criteria, and associated corrective actions, in its quality manual and ensure that they are consistent with the DQOs of the programs they support.

g. External proficiency-testing programs: Laboratories should participate in external proficiency-testing programs to ensure that their measurement systems are comparable. For example, to be certified in the United States by EPA (or agreement state) to perform drinking-water compliance analyses under the SDWA, a laboratory must participate annually in a proficiency-testing (PT)

†† See MARLAP manual glossary.

program administered by a nationally accredited PT sample provider. To be accredited as a NELAC laboratory, a laboratory must participate every 6 months in a PT program administered by a nationally accredited PT sample provider. Acceptable performance for each parameter for which the laboratory is (or seeks to be) certified is demonstrated by successful analysis of at least two of the most recent three proficiency samples. Control limits have been established by NELAC. Performance is evaluated by the PT provider. (See Table 7020:I.)

Laboratories are also encouraged to participate in additional intercomparison programs [e.g., the Mixed Analyte Performance Evaluation Program (MAPEP)‡‡ and those sponsored by the International Atomic Energy Agency (IAEA)§§ and World Health Organization (WHO)].‖ ‖

h. Selection of radionuclide standards and sources: Confirm that radionuclide standard sources are traceable to the National Institute of Standards and Technology (NIST) or equivalent.

Use such standard sources, or dilutions thereof, for initial calibration. These sources may be purchased from suppliers listed in annually published buyers' guides. Before purchasing standards from commercial suppliers, it is important to specifically inquire whether the radionuclide standard of interest is traceable to a national standards body (e.g., NIST). Also, consider whether the chemical form and radionuclide purity are adequate.[4]

Calibrated radionuclide standard solutions are generally prepared for storage and shipment by the supplier in flame-sealed glass ampoules. Perform all dilutions and store radionuclides in well-sealed containers. Only use containers constructed of material that does not adsorb the radionuclides of interest.

Standard sources are radioactive sources with adequate activity and an accurately known radionuclide content and radioactive decay rate (rate of particle or photon emission). Each radionuclide standard should have a calibration certificate containing the following information:

Description:
- Principal radionuclide or parameter
- Chemical and physical form
- Solvent (where applicable)
- Carrier identity and concentration
- Mass and specific gravity or volume

Standardization:
- Activity per mass or volume
- Date and time
- Method of standardization

Accuracy:
- Combined standard uncertainty
- Coverage factor/confidence level

Purity:
- Activity of decay progeny
- Identity and concentration of other impurities and whether they are included in principal activity

Assumptions:
- Decay scheme
- Half-life and uncertainty
- Equilibrium ratios

Production:
- Production method
- Date of separation

Usable lifetime/expiration date

After preparation and before use, verify the activity concentration of liquid radioactivity standards and tracers by comparing them to a standard obtained from a second, independent source (where possible). Also, verify that levels of impurities will meet the requirements of the method in question. Verification procedures and acceptance criteria should be defined in the laboratory's quality manual.

Use instrument check sources to demonstrate the instrument's continuing stability and, by inference, existing calibrations by evaluating key operating parameters (e.g., checks of detector response and/or energy calibration). The sources should be of sufficient radiochemical purity, the radionuclides should ideally be long-lived enough to permit use over the period of their intended use, and the sources should have enough activity to minimize counting uncertainty. Check sources, however, need not have known disintegration rates (i.e., need not be a traceable standard source).

Standard reference materials are radioactive materials with accurately known radionuclide content or radioactive decay rate (rate of particle decay or radiation emission). They may be used as internal LCSs, internal tracers, or matrix and blind known additions. They should provide traceability to national standards, such as those provided by NIST.

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7020 B. Quality Control for Wastewater Samples

Generally, it is difficult to perform collaborative (interlaboratory) analyses of real-matrix wastewater samples because the composition of elements and solids varies from one facility to the next. The methods included herein have been evaluated via homogeneous samples. They presume that nonhomogeneous samples have been homogenized to ensure that any subsamples taken are representative of the sample delivered to the laboratory. Although reference samples used for collaborative testing may not contain radionuclides exhibiting interferences (e.g., short-lived radionuclides would decay during preparation and shipment), analytical methods should include critical steps to eliminate interferences that may be present in samples for which the method is applicable.

7020 C. Statistics

Section 1010B, which discusses statistics applicable to chemical analyses, also applies to radioactivity examinations. However, certain statistical concepts particular to radioactivity measurements are discussed below.

1. Propagation of Uncertainty

It is generally necessary to calculate the uncertainty of a value that is not the result of a single, direct measurement but rather is derived from a number of measurements via a mathematical formula. The uncertainty of each direct measurement is either known or can be computed or otherwise estimated. The combined uncertainty of each calculated result may then be derived mathematically from them. Statistically, this is known as *propagation of uncertainty*.

The propagation of uncertainty typically involves combining the sources of uncertainty associated with determining the concentration of radionuclides in environmental samples (e.g., soil, air, milk, or water). These should include uncertainty for each measurement performed at the laboratory (e.g., sample preparation and subsampling, chemical separations and preparation of the sample test source, and analysis) but should not reflect uncertainty associated with sampling before receipt at the laboratory. Any variable that contributes uncertainty to the final measurement must be considered when evaluating the overall uncertainty of the measurement.

Hereafter, the following notation will be used in formulas to express uncertainties. The symbol $u(x)$ denotes the standard uncertainty of variable x, whereas $u^2(x)$ denotes the square of the standard uncertainty of x. Similarly, $u_{cC}(x)$ denotes the standard uncertainty of x due only to counting (i.e., count uncertainty) while $u_c(x)$ denotes the combined standard uncertainty of x, which reflects other sources of uncertainty beyond just count uncertainty.

The law of propagation of uncertainty is applicable because variance is an additive property. According to the propagation of uncertainty formula, when noncorrelated quantities are added or subtracted, the total variance equals the sum of the variances of all components that contribute to the measurement's overall uncertainty:

$$u_c^2(A) = u_1^2(A) + u_2^2(A) + u_3^2(A) + \ \ldots \ + u_n^2(A)$$

Thus, the combined standard* uncertainty (CSU) for a measurement, $u_c(\mathrm{A})$, is simply the square root of the variance:

$$u_C(A) = \sqrt{u_1^2(A) + u_2^2(A) + u_3^2(A) + \ \ldots \ + u_n^2(A)}$$

A number of propagation-of-uncertainty formulas can be used to determine uncertainties for radionuclide concentrations in water (see Table 7020:II). The one most widely used in nuclear-counting statistics is the first formula, where X = the total number of counts observed when measuring the sample and Y = the background counts. The propagation and reporting of uncertainty are dealt with in great detail elsewhere.[1,2,3]

Table 7020:II. Propagation-of-Uncertainty Formulas

Model Equation	Uncertainty Expression
$Q = X + Y$ or $Q = X - Y$	$u(Q) = \sqrt{\sigma_X^2 + \sigma_Y^2}$
$Q = aX + bY$ or $Q = aX - bY$	$u(Q) = \sqrt{a^2\sigma_X^2 + b^2\sigma_Y^2}$
$Q = XY$	$u(Q) = XY\sqrt{\frac{\sigma_X^2}{X^2} + \frac{\sigma_Y^2}{Y^2}}$
$Q = \frac{X}{Y}$	$u(Q) = \frac{X}{Y}\sqrt{\frac{\sigma_X^2}{X^2} + \frac{\sigma_Y^2}{Y^2}}$

* See MARLAP Manual, Chapter 18, and the BIPM Guide to Uncertainty in Measurements.[1,2]

2. Standard Deviation and Counting Uncertainty

A measurement's variability is described by the standard deviation, which can most accurately be estimated by replicate measurements of the quantity in question. Because replicate measurements of each quantity are impractical for most routine sample measurements, the standard uncertainty associated with measurements is often estimated based on knowledge of the characteristics of the quantity being measured. For example, a lower boundary on the most significant source of uncertainty for very low-activity measurements, often called *counting uncertainty,* can be estimated based on the radioactivity measurements' inherent variability due to the random nature of radioactive decay, which is described by the Poisson distribution. In the ideal case, the standard deviation (uncertainty) of this distribution for a large number of events (N) equals its square root, or

$$u(N) = \sqrt{N}$$

When the number of counts in a measurement (N) is more than about 20, the Poisson distribution increasingly approximates the normal (Gaussian) distribution. For the measurement of radioactivities very close to background, when there are no other significant non-Poisson contributions to the count uncertainty, this approximation can be used to simplify the computation of standard counting uncertainties and thus may facilitate reasonably accurate estimates of the uncertainty of results without needing to perform replicate counts.†

More often, the variable of concern is the standard deviation of the count rate for a single measurement, R, due to uncertainty (counts per unit time):

$$R = \frac{N}{t}$$

where:

t = the duration of the count.

The standard deviation of the count rate, when the appropriate substitutions are made, can be expressed as follows:

$$u(R) = \frac{\sqrt{N}}{t} = \frac{\sqrt{Rt}}{t} = \frac{\sqrt{R}}{t}$$

In practice, all counting instruments have a background counting rate (R_B) when no sample is present. With a sample of a count rate R_s, the net count rate (R_n) due to the sample is:

$$R_n = R_S - R_B$$

† This condition should be ensured by evaluating checks of the background stability, as described in 7020B.3*b*. If other sources of uncertainty are present, their contribution to the overall uncertainty should be estimated and accounted for when estimating measurement uncertainties. See the MARLAP Manual, Chapter 20.

Via uncertainty propagation methods, the standard deviation of R_n (the sample's net count rate) is calculated as follows:

$$u(R_n) = \sqrt{\frac{R_S}{t_S} + \frac{R_B}{t_B}}$$

where:

R_S = the count rate of the sample,
R_B = the background subtraction count rate,
t_S = elapsed duration of the sample count, and
t_B = elapsed duration of the background subtraction count.

The duration of the count for a given set of conditions depends on the detection limit required (see below). The count time can be divided into equal periods to check the constancy of the observed count rate. For low-level counting, where the net count rate is of the same order of magnitude as the background, the background subtraction count should be counted as long as, or longer than, the sample count to which it will be applied. The uncertainty thus calculated, however, reflects a minimum value for uncertainty associated with the radioactive disintegration process' inherent variability. In most cases, it is smaller than the standard deviation of the total analysis. At or near background, the counting uncertainty is the major contributor to the total or combined uncertainty. As concentration levels increase, the relative counting uncertainty decreases and other sources of uncertainty become major contributors to the measurement's combined uncertainty.

To calculate the expanded uncertainty, multiply the standard uncertainty (1σ) by a coverage factor (k) to report expanded uncertainty, *U(AC)*, at different confidence levels.

$$U(AC) = k \times u(AC)$$

where:

U(AC) = expanded combined uncertainty of the activity concentration,
k = coverage factor (e.g., 1.96 for 95% confidence), and
u(AC) = standard uncertainty of the activity concentration.

All radioactivity measurements should be reported with their associated CSU (X.XX ± k × CSU) in appropriate activity-concentration units. The respective value for the coverage factor for the uncertainty (k) or the confidence interval should always be clearly reported with the reported uncertainty.

3. Detection Limits

a. Detection decisions: In analyte detection, the *critical value* is defined in the MARLAP Manual[1] as:

> "The minimum measured value (e.g., of the instrument signal or the analyte concentration) required to give confidence that a positive (nonzero) amount of analyte is present in the material analyzed. The critical value is sometimes called the *critical level* or *decision level*."[4]

Various conventions have been used to estimate the critical value. The procedure recommended here is based on hypothesis

testing.[1,5] If there are no interferences and the observed uncertainty for a representative blank approximates the Poisson uncertainty, then the critical value may be estimated as follows:

$$L_c = K_\alpha \sqrt{\frac{N_B}{t_B \times t_s} \times \left(1 + \frac{t_s}{t_B}\right)} = K_\alpha \sqrt{\frac{R_B}{t_s} \times \left(1 + \frac{t_s}{t_B}\right)}$$

where:

K_α = value for upper percentile of standard normal distribution corresponding to a preselected risk of concluding falsely that activity is present when it is not (α),
N_B = total counts during the counting period of a representative blank or background (cpm),
t_B = duration of the count of the representative blank or background (min),
t_s = duration of the count of a sample (min), and
R_B = count rate of a representative blank or background (cpm).

Generally for the 95% confidence level ($\alpha = 0.05$ and $K_\alpha = 1.645$), the amount of radioactivity needed to provide confidence that a positive (nonzero) amount of analyte detected is actually present in the analyzed material is

$$L_c = 1.645 \sqrt{\frac{R_B}{t_s} \times \left(1 + \frac{t_s}{t_B}\right)}$$

When the sample and background count duration are equal, the above equation simplifies to

$$L_c = 2.33 \sqrt{\frac{R_B}{t_s}}$$

This formulation applies only to situations in which a large number of background counts are observed during the counting period (e.g., >100 counts). For formulations that address, for example, situations commonly encountered during alpha counting when <100 background counts are observed during the counting period, see Chapter 20 of the MARLAP Manual. The manual also addresses how to accommodate uncertainty in excess of that predicted by Poisson counting uncertainty.

In practice, the critical level is reported as a concentration in the same units as the sample result. To convert L_C to the critical level concentration, L_C *(pCi/L* or *pCi/g)*, the L_C is divided by the same factors used to convert the net count rate to activity concentration. Thus, the critical level concentration could be calculated as:

$$L_c(pCi/L \cdots \text{or} \cdots pCi/g) = \frac{L_c}{\varepsilon \times A \times Y \times I \times D \times V \times F}$$

where:

L_c*(pCi/L or pCi/g)* = critical level concentration (pCi/L or pCi/g)
L_c = critical level concentration in counts per minute
ε = counting efficiency (cpm/decay particle),
A = abundance (decay particles per disintegration),
Y = chemical yield (fractional),
I = correction factor for ingrowth (fractional),
D = correction for decay (fractional),
V = volume or mass of sample aliquot (e.g., L or g), and
F = factor to convert dpm to desired reporting units (e.g., 2.22 dpm/pCi).

This is a generalized formula. Please refer to equations defined in the specific methods.

b. Minimum detectable activity: The *minimum detectable activity* (MDA) is the smallest amount of radioactivity in a sample that will yield a net count for which there is a predetermined level of confidence that radioactivity will be measured. Two types of errors may occur when making detection decisions: Type I (false detection) and Type II (false nondetection).

Various conventions have been used to estimate the MDA. Concepts such as the lower limit of detection (LLD) are based on equivalent concepts, although the specific derivation may vary. This discussion will focus on the MDA and the minimum detectable concentration (MDC).

The recommended procedure for estimating MDA is based on hypothesis testing.[1,5] If there are no interferences, and the observed uncertainty for a representative blank approximates the Poisson uncertainty, then the MDA may be approximated (in counts) as follows:

$$\text{MDA} = K_\beta^2 + 2L_c$$

where:

K_β = corresponding value for predetermined degree of confidence for detecting presence of activity (1-β).

If count duration for the sample and representative blank or background are unequal, and both Type I and II errors are set to 5% ($\alpha = \beta = 0.05$ and $K_\alpha = K_\beta = 1.645$), then MDA would be:

$$\text{MDA} = 2.71/t_s + 3.29 \sqrt{\frac{N_B}{t_B \times t_s} \times \left(1 + \frac{t_s}{t_B}\right)}$$

$$= 2.71/t_s + 3.29 \sqrt{\frac{R_B}{s} \times \left(1 + \frac{t_s}{t_B}\right)}$$

When the durations of both sample and representative blank or background counts are equal, this simplifies to:

$$\text{MDA} = \frac{2.71}{t_s} + 4.65 \times \sqrt{\frac{R_B}{t_s}}$$

This formulation applies to situations in which more than approximately 100 background counts will be observed during the counting period. For situations in which fewer background counts will occur (e.g., alpha counting or very short counting

intervals) and for recommendations on accommodating uncertainty in excess of that predicted by the Poisson counting uncertainty, see Chapter 20 of the MARLAP Manual.

In practice, MDA is reported in terms of concentration in the same units as the sample result. To convert MDA to MDC, divide MDA by the same factors used to convert the net count rate to activity concentration. Thus, MDC could be calculated as:

$$MDC = \frac{MDA}{\varepsilon \times A \times Y \times I \times D \times V \times F}$$

where:

MDC = minimum detectable concentration (pCi/L or pCi/g)
MDA = critical level concentration in net counts per minute (cpm)
ε = counting efficiency (cpm/decay particle),
A = abundance (decay particles per disintegration),
Y = chemical yield (fractional),
I = correction factor for ingrowth (fractional),
D = correction for decay (fractional),
V = volume or mass of sample aliquot (e.g., L or g), and
F = factor to convert dpm to desired reporting units (e.g., 2.22 dpm/pCi).

This is a generalized formula. Please refer to equations defined in the specific methods.

c. SDWA detection limit: When radioactivity concentrations are monitored to determine compliance with SDWA, the required detection capability is defined in terms of the *detection limit* ["that concentration which can be counted with a precision of plus or minus 100 percent at the 95 percent confidence level (1.96σ, where σ is the standard deviation of the net counting rate of the sample)"].[6] The detection limit is calculated as[4]:

$$DL_{DW} = \frac{\frac{1.96^2}{2t_S} \times \left[1 + \sqrt{1 + \frac{4t_S^2}{1.96^2} \times R_B \times \left(\frac{1}{t_S} + \frac{1}{t_B}\right)}\right]}{\varepsilon \times A \times Y \times I \times V \times D \times F}$$

where:

DL_{DW} = SDWA detection limit, in units or activity/mass or volume,
R_B = count rate for background subtraction count (cpm),
ε = counting efficiency (cpm/decay particle),
A = abundance (decay particles per disintegation),
Y = chemical yield (fractional),
I = correction factor for ingrowth (fractional),
V = volume or mass of sample aliquot (e.g., L or g),
D = correction for decay (fractional), and
F = factor for converting from dpm to desired reporting units (e.g., 2.22 dpm/pCi).

This is a generalized formula. Please refer to equations defined in the specific methods.

4. References

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7020 D. Calculation and Expression of Results

The results of radioactivity analyses usually are reported in terms of "activity" per unit volume or mass. The SI*-recognized unit for activity is the Becquerel (Bq), which is equal to 1 disintegration per second. In the United States, a commonly used unit for reporting environmental concentrations is the picocurie (pCi), which is equal to 0.037 Bq, 2.22 disintegrations per minute (dpm), or 1×10^{-6} microcuries (μCi).

Specific formulas for calculating activity per volume or mass are presented in the individual methods; while each is specific to the method, they are based on the following general formula:

$$AC = \frac{R_S - R_B}{\varepsilon \times A \times Y \times I \times V \times D \times F}$$

where:

* International System of Units.

AC = activity per unit volume, in units or activity/mass or volume,
R_S = count rate for the sample count (cpm),
R_B = count rate for background subtraction count (cpm),
ε = counting efficiency (cpm/decay particle),
A = abundance (decay particles per disintegration),
Y = chemical yield (fractional),
I = correction factor for ingrowth (fractional),
V = volume or mass of sample aliquot (e.g., L or g),
D = correction for decay (fractional), and
F = factor for converting from dpm to desired reporting units (e.g., 2.22 dpm/pCi).

Every result must be reported with an estimate of its measurement uncertainty [i.e., the combined standard uncertainty (CSU)], generally in terms of absolute activity concentration (pCi/L or Bq/kg). Because it is the CSU that determines a result's uncertainty, each result should be rounded based on its uncertainty. Generally, uncertainty is reported to one or two significant figures, and the magnitude of the measured activity is adjusted to match the final significant digit of the uncertainty. If the uncertainty is reported to two significant figures, a measured result of 0.12345 ± 0.06789 would be reported as 0.123 ± 0.068. Similarly, a result of 12 345 ± 6789 would be reported as 12 300 ± 6800.

In general, results should never be censored via comparison to an MDC, LLD, or critical value; positive, zero, and negative results should be reported as calculated (i.e., do not report "not detected," "ND," or "less than" values).

Program requirements may also dictate the data-reporting format. For example, SDWA compliance-testing regulations require that only the counting uncertainty be reported. Radiochemical data for other programs, however, should be reported with an estimate of the result's CSU, as described above.

The following formula illustrates calculation of (expanded) counting uncertainty at the 95% confidence level, $U_{cC}(AC)$:

$$U_{cC}(AC) = \frac{1.96 \times \sqrt{\frac{R_s}{t_s} + \frac{R_B}{t_B}}}{\varepsilon \times A \times Y \times I \times V \times D \times F}$$

where:

t_S = count duration for the sample (min),
t_B = count duration for the background subtraction count (min), and
R_S,R_B,ε, AC, A, Y, I, V, D, and F are as previously defined.

7030 COUNTING INSTRUMENTS*

7030 A. Introduction

Radiochemical analytical instruments operate on the principle that the energy expended by a radiation event is detected and recorded by an instrument suitable for the type of radiation emitted. The counting instruments described below are commonly found in radioanalytical laboratories. Other less common instruments may also be used for analysis. Instrument background and fractional counting efficiency must be measured and integrated into the sample calculations. These characteristics can be compared with historical data and used to evaluate instrument stability.

* Approved by Standard Methods Committee, 2011.
Joint Task Group: 22nd Edition—Robert T. Shannon.

7030 B. Description and Operation of Instruments

1. Gas-Flow Proportional Counters

Gas-flow proportional counters (GPCs) detect and quantify alpha- and beta-emitting radionuclides. If the sample contains multiple radionuclides, the instrument will detect and count all emissions regardless of their radionuclide source or energy. The analysis may be made radionuclide-specific by combining this detection method with a radiochemical separation to segregate the desired radionuclide. The radionuclide (^{226}Ra, Th, U, etc.) is usually precipitated and mounted as a thin layer (generally less than 5 mg/cm^2) on planchets.

a. Types of GPCs commonly used for environmental analyses: GPCs operate on the principle that radioactive decay particles, such as alpha and beta particles, cause ionization in a gas-filled detector. The liberated electrons are accelerated in an electrical field placed across the detector cell, thereby amplifying the signal. The signal is collected at the detector's anode, creating a pulse of electricity that then can be processed. The combined magnitude of all pulses collected from one decay particle is proportional to the energy deposited in the detector by that particle—hence the term *proportional count*.

Select the detector fill gas to optimize amplification. Although other gases have been used successfully, the most frequently used mixture in low-background GPCs is called P-10; it consists of 90% argon and 10% methane.

Alpha particles may be determined by operating the detector at the alpha plateau, where the voltage is low enough that the detector does not respond to beta particles. Detectors are frequently operated at the higher-voltage beta plateau, where the detector responds to both alpha and beta particles. Because each pulse produced is proportional to the energy deposited in the active volume of the detector by the decay particle, pulse-height analysis (PHA) can be used to discriminate between the larger pulses associated with alpha particles and the smaller pulses associated with beta particles.

b. Internal and thin-windowed GPCs: Internal and thin-windowed GPCs are commonly used for low-level environmental measurements. With internal GPCs, the test source is introduced directly into the counting chamber, which is then filled with the counting gas. With thin-window GPCs, the test source is positioned outside the active volume of the detector, next to an entrance window generally constructed of a very thin Mylar™ film. The entrance window allows decay particles emitted from the source to enter the active volume of the detector.

Thin-windowed GPCs are marginally less sensitive to low-energy decay particles than internal GPCs due to the less favorable counting geometry associated with positioning the source outside the active volume of the detector. Additionally, there are losses due to the decay particles' interaction with air and the window between the test source and the active volume. In contrast, windowed GPCs are much less subject to counting-chamber contamination and damage due to loose residues, losses due to residual moisture, and corrosion due to vapors commonly associated with test sources. Prepare all GPC test sources in a manner that minimizes friable particles and vapors from moisture or solvents because these can contaminate or damage the detector chamber (internal GPCs) or detector entrance window (thin-windowed GPCs).

c. Detection system components: The instrument consists of a counting chamber, pre-amplifier, amplifier, scaler, high-voltage power supply, timer, pulse-shape discriminator, and register. Use the specified counting gas and accessories, make adjustments for sensitivity, and operate in accordance with both the method and the manufacturer's instructions.

d. Background-reduction measures: Low backgrounds are important for environmental analysis. One technique for minimizing background activity is constructing the detection system with low-background materials. Two other techniques used for background reduction—passive and active shielding—are designed to minimize the effect of external radiation (e.g., cosmic radiation as well as gamma and X-ray radiation from terrestrial sources, building materials, and radioactive sources at the laboratory). A passive shield typically consists of a 5- to 10-cm-thick layer of lead surrounding the detector. An active shield involves a "guard" detector placed next to the primary detector so any radiation that impinges on the primary detector must pass through the guard. The primary detector is operated in anti-coincidence mode (i.e., any counts registered concurrently in the primary and guard detectors are not registered as valid sample counts). With these measures, modern low-background GPCs

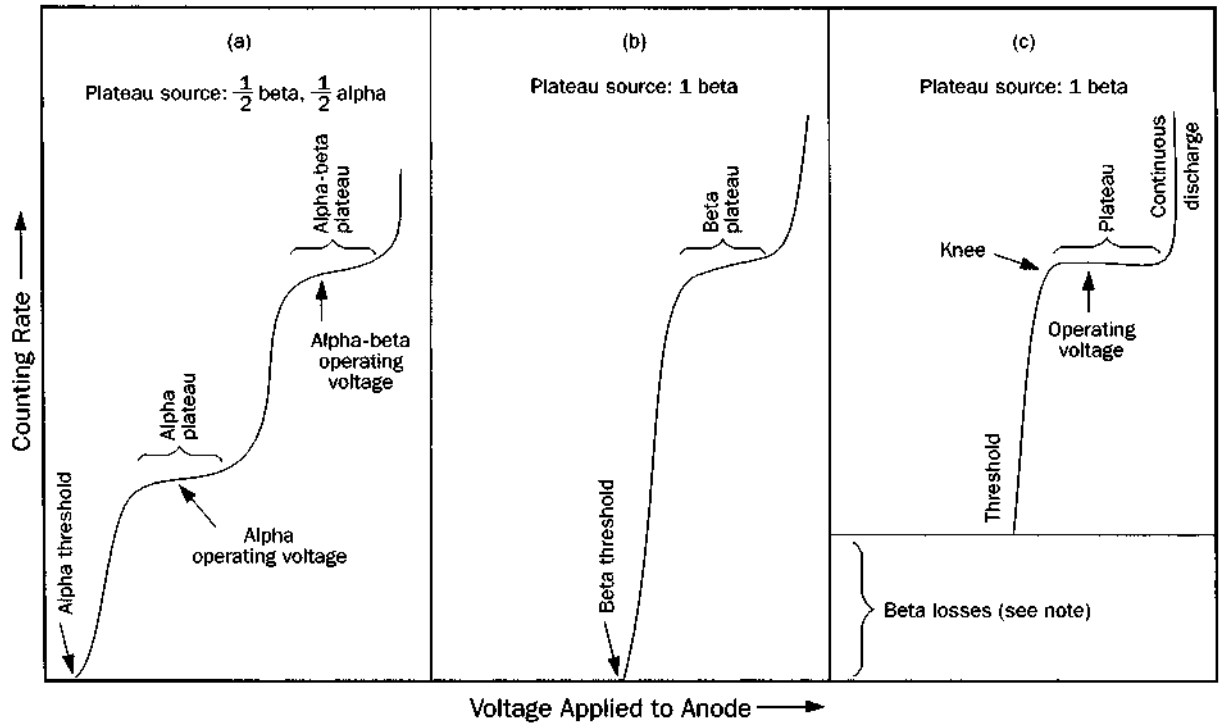


Figure 7030:1. Shape of counting rate-anode voltage curves. Key: (a) and (b) are for internal proportional counter with P-10 gas; (c) is for end-window Geiger-Mueller counter with Geiger gas (NOTE: Beta losses depend on radiation energy and thickness of window and air path.)

can routinely achieve background count rates on the order of 0.05 to 0.2 alpha counts per minute (cpm) and 0.5 to 2 beta cpm.

e. Availability: GPC detection systems are commercially available in a number of assembled configurations. Detectors may be configured alone or in arrays (e.g., of 4, 8, and 10 detectors), and may incorporate automatic sample changers. The most commonly encountered detector diameter is approximately 5 cm. Ten-centimeter detectors are also available; they are most frequently used to count larger sources (e.g., air filters).

f. Instrument setup:

1) Radioactive sources—See Section 7020A.3*h*.

2) Voltage plateau (alpha or beta)—A *voltage plateau* is a curve used to determine an operating voltage that will provide reproducible count rates. It is obtained by placing an appropriate alpha or beta source in each detector and varying the detector voltage until the change in the count rate reaches a minimum. Further increases in voltage will produce little change in overall detector response until the plateau region is exceeded. **CAUTION: Continuous discharge at too high a voltage will damage the instrument.**

The alpha plateau is generally a region in the 300 to 800 V range (depending on the detector) where the instrument responds to an alpha source but not a beta source. The beta plateau is generally a region in the 1000 to 1700 V range where the instrument responds to both alpha and beta radiation. f1

The relative counting rate (ordinate) is plotted against the voltage (abscissa). The operating point is generally established on the plateau curve at a point marginally beyond the knee of the curve (see Figure 7030:1), where instrument response to a point source varies by less than 5% per 100-V change in voltage. Distributed planar test sources are the most common test source counted in most laboratories. When plateaus are obtained using distributed sources, the slope of the curve at the operating voltage may nominally exceed a target of 5%/100 V. Check the plateau to ensure that the optimal operating voltage has not changed by performing instrument response and background checks after each change of counting gas.

Most modern GPC software contains utilities for performing plateau curve acquisition and establishing operating voltage setpoints. Consult the instrument manual for setup details.

3) Pulse-height discriminator setup—GPCs use pulse-height analysis (PHA) to discriminate between pulses originating from alpha or beta particles based on the amplitude of the pulses they produce. Due to the physics of the measurement, alpha pulses are generally much larger than beta pulses. *Crosstalk* refers to the unavoidable misclassification of pulses during simultaneous counting for alpha and beta. For example, pulses resulting from alpha decays may be misidentified as beta pulses (alpha-to-beta crosstalk), or pulses resulting from beta decays may be misidentified as alpha pulses (beta-to-alpha crosstalk). Alpha particles are much more strongly attenuated than beta particles, so they often result in smaller pulses that may be misclassified as beta pulses. In other words, alpha-to-beta crosstalk will be much larger than beta-to-alpha crosstalk.

Setting the pulse-height discriminator establishes the instrument's crosstalk response. Some instruments require that discriminators be set up after the operating voltage is established, while others are configured at the factory. Consult the instrument manual for setup details. As an overall measurement strategy, setting the discriminators to minimize beta-to-alpha crosstalk (e.g., to ~0.1%) will decrease the magnitude of crosstalk cor-

rections and improve the quality of simultaneous alpha and beta measurements.

Once the operating voltage and discriminator settings have been established, they must be documented and locked down. Changing either parameter invalidates all previously established performance check data and instrument calibrations, and the instrument will have to be recalibrated.

4) Background (alpha or beta)—Most detectors have a background count rate that is due to several factors: cosmic radiation; radionuclides contained in instrument materials; the counting room's construction material; electrical/electronic noise; and/or the proximity of radioactive sources. The background is roughly proportional to the size or mass of the counting chamber or detector but can be reduced by shielding or anti-coincidence guard circuitry (see ¶ *d* above).

a) Determination of background count rate—The background count rate is subtracted from each measurement to determine the "net" activity of the sample test source. Determine the background count rate by counting an empty planchet in the counting chamber in the same configuration as the sample test source. The background count duration should be as long as or longer than the counts from which it will be subtracted.

b) Control of backgrounds—Verify the continuing stability of the background count rate during the count by tracking background performance via control charts. Also, evaluate method blank control charts for persistent high or low bias in results (i.e., absolute bias), then determine the cause and correct it. If the cause is intrinsic to the method, a correction factor (independent of QC samples) may be developed and applied to eliminate absolute bias as long as the additional correction's uncertainty is reflected in the combined standard uncertainty reported for the sample result.

5) Initial calibration (alpha, beta, or radionuclide specific)—The purpose of initial calibration is to empirically derive counting efficiency factors for a geometry that matches those of the test samples, thus ensuring that the correction for efficiency is accurate. Parameters that must match include radionuclide, matrix composition, and density/thickness (which causes self-absorption of alpha or beta particles in the sample matrix). Each detector's detection efficiency must be determined separately. Initial calibration is procedure-dependent; see individual procedures for specific instructions. Absorption curve values do not need to be re-established after initial calibration as long as continuing calibration (source check) response is monitored regularly and indicates that the instrument has remained stable between calibration and sample measurements.

6) Continuing calibration (source check)—Verify instrument stability at the operating voltage by counting separate check sources for alpha and beta (where applicable) on each detector. Monitor the major and minor channel count rates (i.e., for an alpha source, alpha is the major channel and beta is the minor channel) for each source counted and plot the results on a control/tolerance chart to demonstrate the continuing stability of the detector efficiency and crosstalk (see Section 7020A). The tests and acceptance criteria used should be addressed in the laboratory quality manual. If the checks do not provide evidence of continuing instrument stability, the detector should be taken offline until the problem is fixed and the instrument is recalibrated, or until continuing checks demonstrate that maintenance has not changed instrument response.

7) Sample counting—Place the prepared sample test source in the detector in a geometry consistent with initial calibration. For internal GPCs, ensure that there is electrical contact between planchet and chamber, and flush the chamber with counting gas. Count for a preset duration or a preset count to give the desired counting uncertainty (see Section 7020D).

2. Alpha Scintillation Counter

Alpha scintillation counters detect and quantify alpha-emitting radionuclides. If the sample contains multiple radionuclides, the instrument will detect and count all alpha emissions regardless of their radionuclide source or energy. The analysis may be made more radionuclide-specific by using a radiochemical separation to isolate the desired radionuclide. This radionuclide (^{226}Ra, Th, U, etc.) is usually precipitated and mounted as a thin layer ($\leq$5 mg/cm^2) on planchets. Radon-222 (radium-226 by radon emanation) also can be counted in alpha scintillation cells in a modified sample chamber (see Section 7500-Ra.C).

a. Principle and uses: An alpha particle interacts with zinc sulfide phosphor (which contains a silver activator), exciting the scintillator's atoms. When the atoms return to the ground state, they emit the energy as visible light. This process is called *scintillation*. The light is further transformed into an electrical current via an attached PMT, which amplifies the electrical current into a measurable pulse. The pulses trigger a scaler, which registers each pulse as a "count." Depending on the amount of radionuclide present and statistics required, count a sample long enough to obtain required sensitivity. The counter is calibrated with a thin-layered precipitate of a radionuclide or an electrodeposited radionuclide.

b. Components: The alpha scintillation counter consists of a light-tight sample chamber with a phosphor detector coupled to a photomultiplier tube (PMT) and sample holder, a high-voltage supply, an amplifier-discriminator, a scaler, and readout capability. Generally, the PMT's window has a larger diameter than that of the samples. Put the phosphor between the sample and the PMT. The distance between the sample surface and PMT face is usually about 3 to 5 mm. Arrange the phosphor so the PMT window is in optical contact with the scintillator. Under these conditions, the counting efficiency can be 35 to 40%. For details on operating and calibrating an alpha scintillation counter, see manufacturer's instructions.

c. Performance verification:

1) Radioactive source—See Section 7020A.3*h*.

2) Plateau—In accordance with manufacturer recommendations, use a radioactive source to find the operating voltage where the count rate is consistent over some specified voltage range.

3) Background—Two backgrounds are evaluated with alpha scintillation counters: electronic instrument background and chamber background. The electronic instrument background characteristically measures the PMT's electronic noise. It is determined periodically (e.g., annually) by counting with an empty chamber and should vary from 0 to 1 cpm.

The chamber background is counted with the zinc sulfide phosphor or scintillation cell in place. This background is caused by contaminated instrument parts, counting-room construction materials, and/or nearby sources of radioactivity. When measuring chamber background, use a duration as long or longer than

the longest sample counting duration; this background is subtracted from gross sample results.

4) Initial calibration—The purpose of initial calibration is to determine the scintillation counting efficiency based on sample thickness (which causes absorption of some alpha or beta particles in the sample matrix) or cell geometry (in the case of scintillation cells). Initial calibration is method-dependent; see individual methods for specific instructions.

Absorption curve values do not need to be re-established after initial calibration as long as the check source response is monitored regularly and control charts indicate instrument stability. Recalibrate scintillation cells periodically.

5) Continuing calibration (source check)—See 7030B.1*f* 6).

6) Sample counting—Place prepared sample in the counting chamber according to manufacturer's instructions. Take the following precautions:

- ensure that counting chamber is light-tight;
- ensure that PMT is not exposed to direct light while high voltage is applied;
- let sample chamber adapt to the dark before starting count;
- count for a preset duration or count, to give the desired counting precision;
- ensure that sample is in contact with the phosphor; and
- count samples in a geometry consistent with initial calibration.

3. Liquid Scintillation Counters

A liquid scintillation spectrometer system uses one or more PMTs to count the number of scintillations (photons of light) emitted from a sample vial. The number of photons produced in the scintillator is proportional to the particle's initial energy. This information helps determine the specific radionuclide present.

a. Principle and uses: A sample containing beta- (or alpha-) emitting radionuclides is mixed in a liquid scintillator. Each beta (or alpha) particle transfers kinetic energy to solvent and phosphor molecules in the liquid scintillation cocktail via a large number of collisions. As the excited molecules return to ground state, they emit photons of light that are detected by PMTs in the instrument. Although the number of photons produced for any given decay event is proportional to the energy of the decay particle, it is notable that alpha particles transfer energy at roughly one-tenth the rate of beta particles and thus result in lower energy pulses. The PMTs amplify the incoming light into an electronic pulse. In most instruments currently in use at radiochemistry laboratories, background is significantly reduced by operating two PMTs in coincidence. This means that signal must be received from both PMTs within the same time interval (e.g., within 20 ns of each other) to yield a valid pulse. Coincident signals are summed, digitized according to their pulse-height and then stored in a multichannel analyzer in a channel corresponding to the energy of the original radiation.

Although the instrument outputs spectrometric data, the continuous nature of beta spectra combined with the instrument's limited resolution limits the instrument's use for spectrometric determinations. Most frequently, LS counting is combined with chemical separations to perform sensitive, radionuclide-specific determinations of alpha and beta emitters. Liquid scintillation is commonly used to determine the activities of some alpha (e.g., ^{222}Rn and daughters) and most low-energy beta emitters (e.g., ^{3}H and ^{14}C). Counting efficiencies approaching 100% can be obtained from alpha- and higher-energy beta-emitting radionuclides.

b. Components: The system consists of a liquid scintillator [organic scintillator(s) diluted with an appropriate solvent], a polyethylene or glass vial, a liquid scintillation counter (with one or more PMTs coupled to a single- or multichannel analyzer), and a readout device.

c. Performance verification:

1) Radioactive sources—See Section 7020A.3*h*.

2) Background—Consider three backgrounds when dealing with liquid scintillation spectrometers: electronic or instrument background, chamber background, and background for subtraction.

a) Electronic or instrument background—Periodically determine background using an empty counting chamber and a dark vial. Some manufacturers supply a dark or "black" vial [a counting vial filled with black material (e.g., graphite)] for this purpose.

b) Chamber background—The "unquenched background" is determined using a vial supplied by the manufacturer. This background is used only to assess daily instrument performance and should never be subtracted from sample results.

c) Background for subtraction—A method-specific background is determined and subtracted from each sample test source result using vials identical to those used for samples, except that they are free of activity. The background subtraction sample is not a QC sample; it is part of the analytical process and should be prepared independently of QC reagent or method blanks. When analyzing tritium, for example, mix the same type of vial with cocktail from the same production lot, using the same sample-to-cocktail ratio with an aliquant of purified "dead" water (e.g., a fossil source of water from which tritium has already decayed).

3) Initial calibration—Before sample analysis, the counting conditions may be optimized for measurements with constant quench. For variable-quench measurements, counting regions may be optimized if they take into account variation in the region of interest as a function of quench. Some analysts maximize the figure of merit (FOM):

$$E^2/B$$

where:

E = efficiency and
B = background count rate.

Optimum counting conditions should not be re-established after initial calibration because changing the analysis window will affect the efficiency that is determined. Once optimum conditions have been selected (via a pure source), determine sample counting efficiency by one of several methods (see Sections 7500-^{3}H and 7500-Rn): standard additions, quench curve, or prepared laboratory standard.

4) Continuing calibration (source check)—See Section 7500-Rn for specific calibration procedures for radon-222 by liquid scintillation counting.

5) Sample counting—Place prepared samples in the counting chamber, let samples adapt to the dark, and count for the preset count or duration needed to obtain the desired sensitivity.

As with other counting techniques, deal with any interferences to the method. Such interferences include quenching (chemical, color, or particle quenching), chemiluminescence/photoluminescence, static electricity, scintillation volume variations, sample homogeneity, background, multiple radionuclides, and phase separation.

4. Alpha Spectrometers

a. Principle and uses: An alpha spectrometer detects, identifies, and quantifies specific alpha-emitting radionuclides. The radionuclides should be chemically separated from samples and deposited as a thin layer on filter papers or electrodeposited on metal disks. When a sample's alpha emissions cause ionization in a solid-state detector, a pulse of current is produced that has an amplitude proportional to the alpha particle energy.

The current is collected, amplified, sorted according to deposited alpha energy, and displayed on a multichannel analyzer. Chemical yield tracers should be added to each sample to ensure accurate quantitation.

b. Components: An alpha spectrometer consists of a sample chamber (with detector, detector/sample holder, and vacuum chamber); a mechanical vacuum pump; detector bias voltage supply; preamplifier; amplifier; multichannel analyzer (with ADC and memory storage); and data readout capability. For details on calibration and operations, see the operations manual provided by the manufacturer.

c. Performance verification:

1) Radioactive source—See Section 7020A.3*h*.

2) Detector voltage—Set detector operating voltage in accordance with the manufacturer's recommendations.

3) Background—Determine the chamber background count using a clean filter or metal disk identical to those on which samples are mounted. The counting duration should be at least as long as the longest sample counting duration. Monitor the background count rate (which will be subtracted from the sample count rate) via a control chart to demonstrate its stability over the most recent 3 to 10 counts and to identify potential long-term trends that could prevent the detector from providing results that meet detection limit requirements.

4) Initial calibration—The purpose of initial calibration is to establish a detector's energy calibration and counting efficiency. Use a standard source, as described in Section 7020A.3*h*. Before beginning calibration, ensure that the counting vacuum chamber is well sealed and has an adequate, consistent vacuum source. Inadequate vacuum will result in a distorted alpha spectrum. Inconsistent vacuum can cause significant drift of the alpha spectra.

5) Continuing calibration (source check)—Verify detector efficiency and energy calibration stability. A mixed alpha-emitting isotopic source containing at least two distinct peaks (see Section 7020A.3*h*) allows the energy calibration to be confirmed by permitting identification of specific alpha-emitting radionuclides. For criteria, see 7030B.1*f* 6).

6) Sample counting—Place prepared sample in the counting chamber according to manufacturer's instructions. Take the following precautions:

- ensure that the air in the counting chamber is slowly evacuated to the level of vacuum defined in the laboratory's SOPs;
- count for a preset duration or preset count, to give the desired counting precision;
- ensure that the sample is properly positioned on the sample holder; and
- after counting, release vacuum *slowly* to minimize the risk of contaminating chamber and detector.

Table 7030:I. Energy Resolution for Various Detector Types

Detector	Resolution at Given Energy		
	5.9 keV	122 keV	1332 keV
Proportional counter	1.2	–	–
X-ray NaI(Tl)	3.0	12.0	–
3 × 3 NaI(Tl)	–	12.0	60
Si(Li)	0.16	–	–
Planar Ge	0.18	0.5	–
Coaxial Ge	–	0.8	1.8

5. Gamma Spectrometers

Gamma spectrometry identifies and quantifies specific energy photons (gamma rays), thereby quantitating specific radionuclides.

a. Principle and uses: Gamma rays from a sample enter the sensitive volume of the detector and cause ionization. The liberated charge is converted into a voltage pulse whose amplitude is proportional to the photon energy. Pulses are stored in sequence in finite energy-equivalent increments over the desired spectrum. After sample counting, the accumulated pulses over a certain area result in photopeaks that can be identified and attributed to specific radionuclides based on their characteristic energies.

b. Components: A gamma spectrometer consists of a detector, pre-amplifier and detector bias supply, pulse-height analyzer system, data readout capability, and shielded sample enclosure. The pulse-height analyzer system consists of a linear amplifier, an analog-to-digital converter (ADC), memory storage, and a logic control mechanism. The logic-control capabilities allow data storage in various modes and data display or recall. For details on operating and calibrating a gamma spectrum analyzer, see manufacturer's instructions.

Commonly used gamma detectors consist of intrinsic or high-purity germanium (HPGe). Lithium drifted germanium [Ge(Li)] detectors are less robust, and many have been replaced by HPGe detectors. Sodium iodide [NaI(Tl)] and silicon [Si(Li)] detectors are sometimes encountered, but have limited application (primarily to screen samples or analyze single gamma-emitting radionuclides). Germanium detectors offer excellent resolution and reasonable to good efficiency (depending on the application and cost). NaI(Tl) detectors have poor resolution and reasonable to good efficiency at reasonable cost. Si(Li) detectors have good efficiency and reasonable resolution for low-energy X-rays (10 to 200 KeV). Other detectors are available. See Table 7030:1 for typical resolution for several detector types. T1

c. Performance verification:

1) Radioactive sources—See Section 7020A.3*h*.

2) Detector voltage—Set detector operating voltage according to manufacturer's recommendations.

3) Background—Background counts are the result of a contaminated sample chamber and cosmic, natural, and worldwide fallout in the detector shielding. Determine the detector background count using an appropriate volume of reagent-grade water in the desired geometry. The background counting duration should be at least as long as the longest sample counting duration.

4) Initial calibration—Establish a detector's energy calibration, shape, and counting efficiency per sample geometry. Use a standard source, as described in Section 7020A.3*h*.

5) Continuing calibration (source check)—Verify the detector's efficiency and energy calibration stability (see Section 7020A.3*a*). Use a standard source, as described in Section 7020A.3*h*.

6) Sample counting—Place prepared sample in the counting chamber and proceed according to the instrument manufacturer's instructions.

d. Gamma scintillation: A common gamma spectroscopy system is the sodium iodide, thallium-activated [NaI(Tl)] crystal system using scintillation phenomenon. The high atomic number of iodine in NaI gives good counting efficiency. A small amount of Tl is added to activate the crystal. The best achievable resolution is about 7% with the ^{137}Cs gamma ray at 662 KeV gamma ray [in a 3-in.-diam × 3-in.-long (7.6-cm-diam × 7.6-cm-long) crystal] and slightly worse for smaller or larger detectors.

The light decay time constant in NaI is about 0.25 μs. Typical charge-sensitive pre-amplifiers translate this into an output pulse rise time of about 0.5 μs. Fast coincidence measurements cannot achieve the brief resolving times that are possible with plastic scintillators, especially at low gamma-ray energies. Many configurations of NaI detectors are available commercially, ranging from very thin crystals for X-ray measurements to large crystals with multiple phototubes. Crystals built with a well to allow nearly spherical (4π) counting of weak samples also are a standard configuration. This type of detector is used primarily for screening measurements, or when a sample is known to contain only one radionuclide and its limited resolution permits accurate measurements.

e. Solid state detectors: A common high-resolution germanium detector consists of a sensitive-volume cylinder containing a diode with more than 30 cm^3 of sensitive volume and a dipstick immersed in liquid nitrogen in a large cryostat; a pre-amplifier; and a detector bias voltage supply. The detector is cooled with liquid nitrogen to protect the diode and reduce electronic noise generation. Keep intrinsic germanium detectors at the temperature of liquid nitrogen. Use a linear amplifier that will maintain the detector's pulse resolution.

In Ge diode detector systems, gamma photons interacting with the detector cause detector atoms to ionize. A bias voltage to the detector allows a charge proportional to deposited photon energy to collect, with resolving times of 10^{-9} to 10^{-13} s. Such systems have exceptionally high resolution.

f. Data system: The data systems contain one or more of the following: a visual display for the gamma spectrometer readout indicator, a digital printer, and a computer terminal with associated capabilities. An oscilloscope is helpful in aligning the instrument with standards (e.g., ^{60}Co, ^{137}Cs, ^{207}Bi, and ^{154}Eu). Computer capability is essential in both data reduction and complex sample analysis.

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7040 FACILITIES*

7040 A. Counting Room

Because the quality of low-level radioactivity measurements improves when background radiation levels are low and stable, facility designers should carefully evaluate the counting room's location and design with facility-specific contributors to background radiation in mind. As a result, the counting room's location, design, and construction may vary significantly from one laboratory to the next. For example, locating a counting room in a lower story of a facility may provide more shielding from cosmic radiation, while locating it in a higher story may better limit terrestrial radiation. Avoid transporting, handling, or storing materials containing even moderate levels of radioactivity (e.g., samples, sample residuals, radioactive standards, or wastes) in or near the instrument area. Select building materials for walls, floor, and ceiling that minimize background radiation from such radionuclides as postassium-40 (^{40}K) and uranium and thorium chain radionuclides. Also, construct exposed surfaces (floors, walls, ceilings, countertops, etc.) from materials that are easy to clean if contamination occurs. Provide enough room in the instrument area to allow sufficient access for required periodic maintenance.

An appropriate heating, ventilation, and air-conditioning (HVAC) system should be provided to maximize instrument stability. In many cases, a humidity-control system will also prove valuable for instrument stability. As with all electronic equipment, follow the instrument manufacturer's recommendations for operating temperature and humidity as closely as possible.

Consider that not all counting instruments have voltage-regulating or power-conditioning circuitry suitable for controlling minor fluctuations in line voltage. Additionally, most power lines in laboratories (even new construction) are not designed to control power fluctuations for the building. Voltage fluctuations can cause changes in background and in the instrument's resolution and energy calibration. The effect of unusual fluctuations can be minimized using an auxiliary voltage regulator/transformer. The risk of losing analytical data due to power interruptions can be minimized by using uninterruptable power supplies (UPS).

Although a "clean room" is not required to control contamination, the counting room should be adequately segregated from the area(s) where samples are physically and chemically processed to minimize exposure to dust and fumes, thereby minimizing contamination and protecting the instrumentation's electrical stability. Minimize the migration of radioactive contamination by locating the counting room in an area with minimal traffic flow and regulate the HVAC system so air flows from areas of least to greatest potential contamination (i.e., from counting areas to chemical-separation areas and then to sample-preparation areas). Implementing measures to minimize fluctuation in radon levels will significantly improve the quality of low-level measurements that the facility can perform.

* Approved by Standard Methods Committee, 2013.
Joint Task Group: Robert T. Shannon (chair), Catherine C. Franklin, Bahman Parsa, Shiyamalie Ruberu.

7040 B. Radiochemistry Laboratory

When designing a radiochemistry laboratory, one of the prime considerations is contamination control. In general, environmental radioactivity measurements are so sensitive that the quality of low-level measurements can be compromised by radioactivity levels that are often much lower than those that pose concern to human health. This should not be surprising because environmental radioactivity measurements are designed, wherever possible, to provide early warning of potential health concerns.

The radioactivity levels found in most environmental samples normally will not produce cross-contamination problems when good laboratory practices are followed. Samples that contain non-environmental (or unknown) levels of radioactivity should be segregated from samples containing environmental levels of radioactivity. In work areas where elevated-activity-level samples or radioactive standards are being handled, take care to minimize contamination of surfaces, other samples, and personnel. Work surfaces should be composed of acid-resistant, nonporous material and covered with absorbent paper.

1. Chemical Reagents and Reagent-Grade Water

Although it is generally appropriate to use high-purity reagents, such as Reagent A.C.S. chemicals or the equivalent, Reagent A.C.S. specifications generally do not address radioactive contaminants. Many reagents contain some radioactivity and other impurities that could bias measurements if not accounted for, so the reagents should undergo acceptance testing to characterize them appropriately before use in radioactivity analysis. Quantify reagents' radioactive contributions by analyzing reagent blanks. Also include reagent blanks with every analysis batch, and ensure that the evaluation of the blank will detect bias that could compromise use of the results. For example, if barium is to be used as a carrier for radium, analysts should initially determine the isotopic

radium content of the barium salt used for the analysis. Rare-earth carriers, such as yttrium or cerium, may be contaminated with thorium from the original rare-earth ore, which would compromise the use of these carriers to prepare microprecipitated test sources for alpha spectrometry measurements. If reagents have radioactivity levels high enough to produce detectable bias in sample measurements, then find a less-contaminated source for the reagent or else purify it to remove the unacceptable radioactivity levels before use. If such measures are unsuccessful, correct results and account for the additional uncertainty associated with this correction when reporting sample results.

Laboratory water (e.g., distilled or deionized/demineralized water) generally is used in analytical laboratories for dilution, reagent preparation, and final glassware rinses. In certain cases, ordinary laboratory water may not be pure enough for certain applications in the environmental radiochemistry laboratory (e.g., background subtraction samples for gamma spectroscopy and liquid scintillation counting). Where radon and its progeny are analytes of concern in gamma spectrometry measurements, remove radon by purging the laboratory water with aged (30-d) air or an inert gas (e.g., nitrogen), or by aging the distilled water for 30 d to allow the radon and progeny to decay. Tritium is an integral constituent of water (i.e., hydrogen) and cannot be removed by purifying the water. Tritium is produced cosmogenically in the atmosphere and is found in surface waters. If water containing tritium is used for liquid scintillation measurements, positive and negative bias can result. Avoid this by using "fossil" or "dead" water that has been isolated from surface waters for a number of tritium half-lives (e.g., >125 years). While this is often obtained from deep wells, there is no guarantee that water from a deep well has been isolated from sources of surface water long enough to contain acceptably low levels of tritium.

2. Apparatus and Glassware

Generally, glassware in radiochemistry laboratories should be treated as it is in any analytical laboratory (an excellent discussion of the kinds of labware available, the use of volumetric ware, and various cleaning protocols is available[1]). That said, certain aspects of glassware use are peculiar to radiochemistry due to the extremely low concentrations of radioactive constituents. Use disposable labware wherever possible. Treat reusable glassware and apparatus used to prepare standards or handle elevated-activity samples as if they were contaminated; discard or segregate them for further use with samples of comparable activity. [As a general rule, do not analyze samples side-by-side if the samples' activity levels vary by three orders of magnitude (e.g., fCi vs. pCi, or pCi vs. nCi).] Incorporate acid soaking and rinsing, and use detergents that contain complexing or chelating agents in labware-washing protocols. Replace washwater frequently and periodically screen washwater for radioactivity to ensure that it does not become a source of contamination. Do not reuse planchets.

3. Reference

1. U.S. Environmental Protection Agency, U.S. Department of Defense, U.S. Department of Energy, U.S. Department of Homeland Security, U.S. Nuclear Regulatory Commission, U.S. Food and Drug Administration, U.S. Geological Survey, National Institute of Standards and Technology. 2004. Contamination from Sources in the Laboratory. Section 12.2.2 *in* Multi-Agency Radiological Laboratory Analytical Protocols Manual (MARLAP); NUREG-1576; EPA 402-B-04-001A; NTIS PB2004-105421. Washington, D.C.

7040 C. Laboratory Safety

Each laboratory should have a safety plan as part of its standard operating procedures (SOPs). While specific safety criteria are beyond the scope of this discussion, apply general and customary safety practices as a part of good laboratory procedures. Use fume hoods where appropriate, wear safety glasses or a shield for eye protection, and wear protective clothing at all times. When an approved method includes safety practices, follow them strictly.

Treat each chemical as a potential health hazard and keep exposure as low as reasonably achievable. Maintain a file of current, applicable regulations about safely handling the chemicals specified in radiochemical analysis methods. Make available a reference file of materials-handling information [e.g., safety data sheet (SDS)] to all personnel who may work with such items.

7040 D. Pollution Prevention

1. Management Techniques

The U.S. Environmental Protection Agency has established a preferred hierarchy of environmental management techniques; pollution prevention is its first choice. *Pollution prevention* includes any technique that reduces or eliminates the quantity or toxicity of waste generated. Numerous pollution-prevention opportunities exist; use them whenever feasible. When pollution prevention is infeasible, the next recommended option is recycling.

Further lab-related information about pollution prevention is available.[1]

2. Reference

1. American Chemical Society. 1985. Less is Better: Laboratory Chemical Management for Waste Reduction. Washington, D.C.

7040 E. Waste Management

1. Management Techniques

Establish laboratory waste-management practices consistent with all applicable rules and regulations for controlling radioactive and hazardous materials. Protect air, water, and land by

- minimizing and controlling all releases from hoods and bench operations;
- complying with both the letter and spirit of any sewer-discharge permits and regulations; and
- complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land-disposal restrictions.

Further information on waste management is available.[1]

2. Reference

1. American Chemical Society. 1990. The Waste Management Manual for Laboratory Personnel. Washington, D.C.

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7110 GROSS ALPHA AND GROSS BETA RADIOACTIVITY (TOTAL, SUSPENDED, AND DISSOLVED)*

7110 A. Introduction

1. Occurrence

a. Natural radioactivity: Uranium, thorium, and radium are naturally occurring radioactive elements that have a long series of radioactive daughters that emit alpha, beta, and/or gamma radiations until a stable end-element is produced. These naturally occurring elements, through their radioactive daughter gases, radon and thoron, cause airborne activity and contribute to the radioactivity of rain and groundwaters. Additional naturally radioactive elements include potassium-40, rubidium-87, samarium-147, lutetium-176, and rhenium-187.

b. Artificial radioactivity: With the development and operation of nuclear reactors and radionuclide-generating devices, large quantities of radioactive elements are being produced. These include almost all the elements in the periodic table.

2. Significance

Regular measurements of gross alpha and gross beta activity in water may be invaluable for early detection of radioactive contamination and indicate the need for supplemental data on concentrations of more hazardous radionuclides.

With the simpler techniques for routine measurement of gross alpha and beta activity, the presence of contamination may be determined in a matter of hours, whereas days may be required to make the radiochemical analyses necessary to identify radionuclides present.

3. Bibliography

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1976. Drinking Water Regulations. Radionuclides. *Fed. Reg.* 41:28402.

* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011. Joint Task Group: James W. Mullins (chair).

7110 B. Evaporation Method for Gross Alpha-Beta

1. General Discussion

a. Selection of counting instrument: The thin-window, heavily shielded, gas-flow, anticoincidence-circuitry proportional counter is the recommended instrument for counting gross alpha and beta radioactivity because of its superior operating characteristics. These include a very low background and a high sensitivity to detect and count an alpha and beta radiation range that is reasonable but not so wide as that of internal proportional counters. Calibrate the instrument by adding standard nuclide portions to media comparable to the samples and preparing, mounting, and counting the standards exactly as the samples.

An internal proportional or Geiger counter also may be used; however, the internal proportional counter has a higher background for beta counting than the thin-window counter and alpha activity cannot be determined separately with a Geiger counter. Alpha activity can be measured with either a thin-window or internal proportional counter; counting efficiency is higher for the internal counter.

b. Calibration standard: When gross beta activity is assayed in samples containing mixtures of naturally radioactive elements and fission products, the choice of a calibration standard may influence the beta results significantly because self-absorption factors and counting chamber characteristics are beta-energy-dependent.

A standard solution of cesium-137, certified by the National Institute of Standards and Technology (NIST) or traceable to a certified source, is recommended for calibration of counter efficiency and self-absorption for gross beta determinations. The half-life of cesium-137 is about 30 years. The daughter products after beta decay of cesium-137 are stable barium-137 and metastable barium-137, which in turn disintegrates by gamma emission. For this reason, the standardization of cesium-137 solutions may be stated in terms of the gamma emission rate per milliliter or per gram. However, one has to consider the conversion electrons emitted by metastable barium-137 when using cesium-137 as a gross beta counting standard. It is common practice to calibrate cesium-137 on the basis of the gamma rays emitted by metastable barium-137 (85% of Cs-137 disintegrations), but a calculation of total electrons emitted must include the conversion electrons from barium-137m (9.5% of Cs-137 emissions). To convert gamma rate to equivalent beta disintegration rate, multiply calibrated gamma emission rate by 1.095/0.85 = 1.29.

Strontium-90 in equilibrium with its daughter yttrium-90 also is a suitable gross beta standard; its use is recommended by EPA.[1] For gross alpha activity, the recommended standards are natural uranium and thorium-230. Plutonium-239 and americium-241 also are widely used.

Note that gross alpha and beta results are meaningless unless the calibration standard also is reported.

c. Radiation lost by self-absorption: The radiation from alpha-emitters having an energy of 8 MeV and from beta-emitters having an energy of 60 keV will not escape from the sample if

the emitters are covered by a sample thickness of 5.5 mg/cm^2. The radiation from a weak alpha-emitter will be stopped if covered by only 4 mg/cm^2 of sample solids. Consequently, for low-level counting it is imperative to evaporate all moisture and preferable to destroy organic matter before depositing a thin film of sample solids from which radiation may enter the counter. In counting water samples for gross beta radioactivity, a solids thickness of 10 mg/cm^2 or less on the bottom area of the counting pan is recommended. For the most accurate results, determine the self-absorption factor as outlined below.

d. Calibration of overall counter efficiency: Correct observed counting rate for geometry, back-scatter, and self-absorption (sample absorption).

Although it is useful to know the variation in these individual factors, determine overall efficiency by preparing standard sample sources and unknowns.

1) For measuring mixed fission products or beta radioactivity of unknown composition, use a standard solution of cesium-137 or strontium-90 in equilibrium with its daughter yttrium-90.

Prepare a standard (known disintegration rate) in an aqueous solution of sample solids similar in composition to that present in samples. Dispense increments of solution in tared pans and evaporate. Make a series of samples having a solids thickness of 1 to 10 mg/cm^2 of bottom area in the counting pan. Evaporate carefully to obtain uniform solids deposition. Dry (103 to 105°C), weigh, and count. Calculate the ratio of counts per minute to disintegrations per minute (efficiency) for different weights of sample solids. Plot efficiency as a function of sample thickness and use the resulting calibration curve to convert counts per minute (cpm) to disintegrations per minute (dpm).

2) If other radionuclides are to be tested, repeat the above procedure, using certified solutions of each radionuclide. Avoid unequal distribution of sample solids, particularly in the 0- to 3-mg/cm^2 range, in both calibration and sample preparation.

3) For alpha calibration, proceed as above, using a standard solution of natural uranium salt in secular equilibrium (not depleted uranium), thorium-230, plutonium-239, or americium-241. Recount alpha standard at the beta operating voltage and determine alpha amplification factor (7110B.5*b*). Report calibration standard used with results.

e. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 7020.

2. Apparatus

a. Counting pans, of metal resistant to corrosion from sample solids or reagents, about 50 mm diam, 6 to 10 mm in height, and thick enough to be serviceable for one-time use. Stainless steel planchets are recommended for acidified samples.

b. Thin end-window proportional counter, capable of accommodating a counting pan.

c. Alternate counters: Other beta counters are internal proportional and Geiger counters.

*d. Membrane filter,** 0.45-μm pore diam.

e. Gooch crucibles.

f. Counting gas, as recommended by the instrument manufacturer.

3. Reagents

a. Methyl orange indicator solution.

b. Nitric acid, HNO_3, 1*N*.

c. Clear acrylic solution: Dissolve 50 mg clear acrylic† in 100 mL acetone.

d. Ethyl alcohol, 95%.

e. Conducting fluid:‡ Prepare according to manufacturer's directions (for internal counters).

f. Standard certified thorium-230, cesium-137, or strontium-90–yttrium-90 solution.

g. Standard certified americium-241, plutonium-239, or natural uranium solution. For natural uranium, use material in secular equilibrium.

h. Reagents for wet-combustion procedure:

1) *Nitric acid,* HNO_3, 6*N*.

2) *Hydrogen peroxide solution:* Dilute 30% H_2O_2 with an equal volume of water.

4. Procedure

a. Total sample activity:

1) For each 20 cm^2 of counting pan area, take a volume of sample containing not more than 200 mg residue for beta examination and not more than 100 mg residue for alpha examination. The specific conductance test on a nonpreserved sample helps to select the appropriate sample volume.

2) Evaporate by either of the following techniques:

a) Add sample directly to a tared counting pan in small increments, with evaporation at just below boiling temperature. This procedure is not recommended for large samples.

b) Place sample in a borosilicate glass beaker or evaporating dish, add a few drops of methyl orange indicator solution, add 1*N* HNO_3 dropwise to pH 4 to 6, and evaporate on a hot plate or steam bath to near dryness. Avoid baking solids on evaporation vessel. Transfer to a tared counting pan with the aid of a rubber policeman and distilled water from a wash bottle. Using a rubber policeman, thoroughly wet walls of evaporating vessel with a few drops of acid and transfer washings to counting pan. (Excess alkalinity or mineral acidity is corrosive to aluminum counting pans.)

3) Complete drying in an oven at 103 to 105°C, cool in a desiccator, weigh, and keep dry until counted.

4) Treat sample residues having particles that tend to be airborne with a few drops of clear acrylic solution, then air- and oven-dry and weigh.

5) With a thin end-window counter count alpha and/or beta activity.

6) Store sample in a desiccator and count for decay if necessary. Radionuclides that are volatile under the sample preparation conditions of this method will not be measured. In some geographic areas nitrated water solids (sample evaporated with nitric acid present) will not remain at a constant weight after being dried at 105°C for 2 h and then exposed to the atmosphere before and during counting. Other radioactive substances (such as some chemical forms of radioiodine) also may be lost during

* Type HA, Millipore Filter Corp., Bedford, MA, or equivalent.

† Lucite, or equivalent.

‡ Anstac 2M, Chemical Development Corporation, Danvers, MA, or equivalent.

sample evaporation and drying. Heat such samples to a dull red heat for a few minutes to convert the salts to oxides. Sample weights then usually are sufficiently stable to give consistent counting rates and a correct counting efficiency can be assigned. Radioisotopes such as those of cesium may be lost when samples are heated to dull red color. Such losses are limitations of the test method.

b. Activity of dissolved matter: Proceed as in ¶ *a*1) above, using a sample filtered through a 0.45-μm membrane filter.

c. Activity of suspended matter:

1) For each 10 cm^2 of membrane filter area, take a volume of sample not to exceed 50 mg suspended matter for alpha assay and not to exceed 100 mg for beta assay.

2) Filter sample through membrane filter with suction; then wash sides of filter funnel with a few milliliters of distilled water.

3) Transfer filter to a tared counting pan and oven-dry.

4) If sample is to be counted in an internal counter, saturate membrane with alcohol and ignite. (When beta or alpha activity is counted with another type of counter, ignition is not necessary provided that the sample is dry and flat.) When burning has stopped, direct flame of a Meker burner down on the partially ignited sample to fix sample to pan.

5) Cool, weigh, and count alpha and beta activities.

6) If sample particles tend to be airborne, treat sample with a few drops of clear acrylic solution, air-dry, and count.

7) Alternatively, prepare membrane filters for counting in internal counters by wetting filters with conducting fluid, drying, weighing, and counting. (Include weight of membrane filter in the tare.)

d. Activity of suspended matter (alternate): If it is impossible to filter sewage, highly polluted waters, or industrial wastes through membrane filters in a reasonable time, proceed as follows:

1) Determine total and dissolved activity by the procedures given in 7110B.4*a* and *b* and estimate suspended activity by difference.

2) Filter sample through an ashless mat or filter paper of stated porosity. Dry, ignite, and weigh suspended fixed residue. Transfer and fix a thin uniform layer of sample residue to a tared counting pan with a few drops of clear acrylic solution. Dry, weigh, and count in a thin end-window counter for alpha and beta activity.

e. Activity of nonfatty semisolid samples: Use the following procedure for samples of sludge, vegetation, soil, etc.:

1) Determine total and fixed solids of representative samples according to Section 2540.

2) Reduce fixed solids of a granular nature to a fine powder with pestle and mortar.

3) Transfer a maximum of 100 mg fixed solids for alpha assay and 200 mg fixed solids for beta assay for each 20 cm^2 of counting pan area (see NOTE below).

4) Distribute solids to uniform thickness in a tared counting pan by (*a*) spreading a thick aqueous cream of solids that is weighed after oven-drying, or (*b*) dispensing dry solids of known weight and spreading with acetone and a few drops of clear acrylic solution.

5) Oven-dry at 103 to 105°C, weigh, and count.

NOTE: The fixed residue of vegetation and similar samples usually is corrosive to aluminum counting pans. To avoid difficulty, use stainless steel pans or treat a weighed amount of fixed residue with HCl or HNO_3 in the presence of methyl orange indicator to pH 4 to 6, transfer to an aluminum counting pan, dry at 103 to 105°C, reweigh, and count.

f. Alternate wet-combustion procedure for biological samples: Some samples, such as fatty animal tissues, are difficult to process according to ¶ *e* above. An alternate procedure consists of acid digestion. Because a highly acid and oxidizing state is created, volatile radionuclides may be lost under these conditions.

1) To a 2- to 10-g sample in a tared silica dish or equivalent, add 20 to 50 mL 6*N* HNO_3 and 1 mL 15% H_2O_2 and digest at room temperature for a few hours or overnight. Heat gently and, when frothing subsides, heat more vigorously but without spattering, until nearly dry. Add two more 6*N* HNO_3 portions of 10 to 20 mL each, heat to near boiling, and continue gentle treatment until dry.

2) Ignite in a muffle furnace for 30 min at 600°C, cool in a desiccator, and weigh.

3) Continue the test as described in 7110B.4*e*3)–5).

5. Calculation and Reporting

a. Alpha activity: Calculate alpha activity, in picocuries per liter, by the equation

$$\text{Alpha} = \frac{\text{net cpm} \times 1000}{2.22e\ \nu}$$

where:

e = calibrated overall counter efficiency (see 7110B.1*d*), and
ν = volume of sample counted, mL.

Express the counting error as described in ¶ *c* below. Similarly, calculate and report alpha activity in picocuries per kilogram of moist biological material or per kilogram of moist and per kilogram of dry silt.

b. Beta activity: Calculate and report gross beta activity and counting error in picocuries per liter of fluid, per kilogram of moist (live weight) biological material, or per kilogram of moist and per kilogram of dry silt, according to ¶s *a*, above, and *c*, below.

To calculate picocuries of beta activity per liter, determine the value of *e* in the above equation as described in 7110B.1*d*.

When beta activity is counted in the presence of alpha activity by gas-flow proportional counting systems (at the beta plateau) alpha particles also are counted. Because alpha particles are more readily absorbed by increased sample thickness than beta particles, alpha/beta count ratios vary. Therefore, prepare a calibration curve by counting standards (americium-241, thorium-230, or plutonium-239) with increasing solids thickness, first on the alpha plateau, then on the beta plateau. Plot the ratios of the two counts against mg/cm^2 thickness, determine the alpha amplification factor (*M*), and correct the amplified alpha count on the beta plateau for the sample.

$$M = \frac{\text{net cpm on beta plateau}}{\text{net cpm on alpha plateau}}$$

If significant alpha activity is indicated by the sample alpha plateau count, determine beta activity by counting the sample at the beta plateau and calculating:

$$\text{Beta, pCi/L} = \frac{B - AM}{2.22 \times D \times V}$$

where:

B = net beta counts at the beta plateau,
A = net alpha counts at the alpha plateau,
M = alpha amplification factor (from ratio plot),
2.22 = dpm/pCi,
D = beta counting efficiency, cpm/dpm, and
V = sample volume, L.

Some gas-flow proportional counters have electronic discrimination to eliminate alpha counts at the beta operating voltage. For these instruments the alpha amplification factor will be less than 1.

Where greater precision is desired, for example, when the count of alpha activity at the beta plateau is a substantial fraction of the net counts per minute of gross beta activity, the beta counting error equals $(E_a^2 + E_b^2)^{1/2}$, where E_a is the alpha counting error and E_b the gross beta counting error.

c. Counting error: Determine the counting error, E (in picocuries per sample), at the 95% confidence level from:

$$E = \frac{1.96\mu(R_n)}{2.22e}$$

where $\mu(R)$ is calculated as shown in Section 7020C.2, using $t_1 = t_2$ (in minutes); and e, the counter efficiency, is defined and calculated as in 7110B.1*d*.

d. Miscellaneous information to be reported: In reporting radioactivity data, identify adequately the sample, sampling station, date of collection, volume of sample, type of test, type of activity, type of counting equipment, standard calibration solutions used (particularly when counting standards other than those recommended in 7110B.1*d* are used), time of counting (particularly if short-lived isotopes are involved), weight of sample solids, and kind and amount of radioactivity. So far as possible, tabulate the data for ease of interpretation and incorporate repetitious items in the table heading or in footnotes. Unless especially inconvenient, do not change quantity units within a given table. Always report the counting error to assist in interpretation of results.

6. Precision and Bias

In a collaborative study of two sets of paired water samples containing known additions of radionuclides, 15 laboratories determined the gross alpha activity and 16 analyzed gross beta activity. The samples contained simulated water minerals of approximately 350 mg fixed solids/L. The alpha results of one laboratory were rejected as outliers.

The average recoveries of added gross alpha activity were 86, 87, 84, and 82%. The precision (random error) at the 95% confidence level was 20 and 24% for the two sets of paired samples. The method was biased low, but not seriously.

The average recoveries of added gross beta activity were 99, 100, 100, and 100%. The precision (random error) at the 95% confidence level was 12 and 18% for the two sets of paired samples. The method showed no bias.

7. Reference

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7110 C. Coprecipitation Method for Gross Alpha Radioactivity in Drinking Water

1. General Discussion

The Evaporation Method for Gross Alpha-Beta (7110B) does not separate alpha-(or beta-) emitting radionuclides from the sample's dissolved solids. For drinking water samples with high dissolved solids content, e.g., 500 mg/L or higher, Method 7110B is severely limited because of the small sample size possible and the very long counting times necessary to meet required sensitivity (3 pCi/L). The coprecipitation procedure eliminates the problem of high dissolved solids and gives increased sensitivity.

a. Principle: All alpha-emitting radionuclides of interest (mainly radium, uranium, and thorium isotopes) are coprecipitated with barium sulfate and iron hydroxide as carriers, thereby separating alpha-emitting radionuclides from other sample dissolved solids. The combined precipitates are filtered and counted

for alpha activity. Relatively large samples can be analyzed so that sensitivity is improved and counting time is minimized.

b. Interferences: Allow at least 3 h for decay of radon progeny before beginning the alpha count.

Soluble ions that coprecipitate and add to the mixed barium sulfate and iron hydroxide precipitate weights result in counting efficiencies that are biased low.

Iron hydroxide precipitates collected on membrane filters without a holding agent flake when dried and are easily lost from the filter. Add 5 mg paper pulp fiber to the sample to help secure the iron hydroxide to the filter. Preferably use glass fiber filters because the surface glass fibers help to secure the precipitate.

c. Calibration: Add at least 100 pCi standard alpha-emitter activity to 500-mL portions of tap water in separate beakers. Add 2.5 mL conc HNO_3 to each beaker. Determine counting efficiency (cpm/pCi) for the alpha-emitter by taking these known additions through the procedure. Make at least six replicate determinations to determine counting efficiency.[1]

Use 500-mL portions with no addition for blank corrections of alpha activity in the tap water and reagents.

$$\text{Efficiency, cpm/pCi} = \frac{C_a - C_b}{\text{pCi}}$$

where:

C_a = sample with added activity, mean cpm,
C_b = mean blank cpm, and
pCi = activity added.

Preferably use thorium 230 (a pure alpha-emitter) for gross alpha efficiency calibration. As noted in 7110B.1*b*, other alpha-counting standards may be used.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 7020.

2. Apparatus

a. Hot plate/magnetic stirrer and stirring bars.

b. Filter membranes, 47-mm diam, 0.45-μm pore size, or glass fiber filters.*

c. Drying lamp.

d. Planchets, stainless steel, 2-in. diam.

e. Alpha scintillation counter or low-background proportional counter.

3. Reagents

a. Ammonium hydroxide, NH_4OH, 6*N*.

b. Barium carrier, 5 mg Ba^{2+}/mL: Dissolve 4.4 g $BaCl_2 \cdot 2H_2O$ in 500 mL distilled water.

c. Bromocresol purple, 0.1%: Dissolve 100 mg water-soluble reagent in 100 mL distilled water.

d. Iron carrier, 5 mg Fe^{3+}/mL: Dissolve 17.5 g $Fe(NO_3)_3 \cdot 9H_2O$ in 200 mL distilled water containing 2 mL 16*N* HNO_3. Dilute to 500 mL.

e. Sulfuric acid, H_2SO_4, 2*N:* Dilute 55 mL conc H_2SO_4 to 1 L with distilled water.

f. Paper pulp/water mixture: Add a 0.5-g paper pulp pellet to 500 mL distilled water in a plastic bottle. Add 5 drops diluted (1 + 4) detergent. Cap bottle and stir vigorously for 3 h before use. Stir mixture whenever a portion is taken.

g. Detergent: 1 part detergent + 4 parts distilled water.†

4. Procedure

To a sample of 500 mL to 1 L, or sample diluted to 500 mL, add 5 drops of diluted detergent. Place sample on magnetic stirrer/hot plate and, while stirring, gently add 20 mL 2*N* H_2SO_4. Boil for 10 min to flush CO_2 (from carbonates and bicarbonates) from sample. Radon also is flushed. Reduce temperature to below boiling, continue stirring, and add 1 mL barium carrier solution. Continue stirring for 30 min. Add 1 mL bromocresol purple indicator solution, 1 mL iron carrier solution, and 5 mL stirred paper pulp/water reagent.

Continue stirring and add 6*N* NH_4OH dropwise until there is a distinct color change (yellow to purple). Continue warming and stirring for 30 min. Filter sample through a glass fiber filter (or membrane filter if further analysis is to be done). Quantitatively transfer all precipitate to the filter. Wash precipitate with 25 mL distilled water. Hold filter for 3 h for collected radon progeny to decay. Dry filter at 105°C or under a mild heat lamp.

Count filter for gross alpha activity.

Prepare a reagent blank precipitate to determine reagent alpha activity background.

5. Calculations

$$\text{Gross alpha activity, pCi/L} = \frac{C_a - C_b}{EV}$$

where:

E = counter efficiency, cpm/pCi,
V = volume analyzed, L,
C_a = sample counts per minute, cpm, and
C_b = reagent blank, cpm.

6. Precision and Bias

In collaborative test with 18 laboratories participating,[2] gross alpha activities for four different samples were calculated with four different alpha-emitting radionuclide standard counting efficiencies. Thorium-230, a pure alpha-emitter, appeared to be the best standard for gross alpha counting efficiency.

Water samples A, B, C, and D contained gross alpha concentrations of 74.0, 52.6, 4.8, and 10.0 pCi/L, respectively, at 3 h after separation of alpha-emitting radionuclides by coprecipitation with iron hydroxide and barium sulfate. Test results using the thorium-230 counting efficiency showed coefficients of variation for repeatability (within laboratory precision) of 7.9, 7.8, 8.7, and 8.8%, respectively, for an average of 8.3%. Coefficients of variation for reproducibility (combined within and between

* Gelman Type A/E, Millipore Type AP, or equivalent.

† Rohm and Haas Triton N101 or Triton X100, or equivalent.

laboratory precision) of 20.4, 16.8, 18.7, and 18.5%, respectively, were obtained for an average of 18.6%.

A comparison of the 18 laboratory grand average results (calculated with the ^{230}Th counting efficiency) and known gross alpha particle concentrations showed accuracy indexes of 91.9, 99.4, 122, and 94.5%, respectively, for an average accuracy index of 102%. The *t*-test for bias showed a significant positive bias for Sample C but no significant bias for the other three samples.

7. References

1. U.S. Environmental Protection Agency. 1984. EERF Radiochemistry Procedures Manual. 00-02 Radiochemical Determination of Gross Alpha Activity in Drinking Water by Coprecipitation. EPA-520/5-84-006, USEPA ORP-EERF, Montgomery, Ala.
2. Whittaker, E.L. 1986. Test Procedure for Gross Alpha Particle Activity in Drinking Water, Interlaboratory Collaborative Study. EPA-600/4-86/027 July 1986, Pre-issue copy. USEPA EMSL-Las Vegas, Las Vegas, Nev.

7120 GAMMA-EMITTING RADIONUCLIDES*

7120 A. Introduction

For information about occurrence of natural and artificial radioactivity, see Section 7110A.1.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2011.
Joint Task Group: 20th Edition—James W. Mullins.

7120 B. Gamma Spectroscopic Method

1. General Discussion

a. Application: This method describes the use of gamma spectroscopy, using either germanium (Ge) diodes or thallium-activated sodium iodide [NaI(Tl)] crystals, for the measurement of gamma photons emitted from radionuclides present in water. The method is applicable to samples that contain radionuclides emitting gamma photons with energies ranging from about 60 to 2000 keV.

The method can be used for qualitative and quantitative determinations with Ge detectors or for screening and semi-quantitative and semi-qualitative determinations with [NaI(Tl)] detectors. Exact quantitation using NaI is possible for single nuclides or when the gamma emissions are limited to a few well-separated energies. Detection limits for typical counting systems range from a few picocuries (pCi) of gamma activity for a 100-min count to approximately 100 pCi for a 5-min count, depending on counting geometry and gamma ray energy and abundance.

Determine energy and efficiency calibrations for each detector at several energies between 50 and 2000 keV for the geometries of interest. Gamma ray libraries[1,2] for Ge spectrometry should contain the nuclides and gamma ray lines most likely to be found in water samples. Have computer software available to list the contents of the library and to add more nuclides and gamma ray lines to the library for peak search routines.

b. Principle: Because gamma spectroscopy is nondestructive, it is possible to analyze for gamma-emitting radionuclides without separating them from the sample matrix. This technique makes it possible to identify and quantitate gamma-emitting radionuclides when the gross beta screen has been exceeded or it is otherwise necessary to define the contribution of gamma-emitters to the total radioactivity present.

A homogeneous water sample is put into a standard geometry for gamma counting. The counting efficiency for this geometry must have been determined with a mixed energy gamma standard containing known radionuclide activities. Sample portions are counted long enough to meet the required sensitivity of measurement.

The gamma spectrum is printed out and/or stored in the appropriate computer-compatible device for data processing (calculation of sample radionuclide concentrations).

Consult a good text on gamma ray spectrometry[3] for a more detailed discussion.

c. Sampling and storage: See Table 7010:I.

d. Interferences: Significant interference occurs when a sample is counted with a NaI(Tl) detector and the sample radionuclides emit gamma photons of nearly identical energies. Such interference is greatly reduced by counting the sample with a Ge detector. Higher-energy gammas that predominate may completely mask minor, less energetic photopeaks for both Ge and NaI detectors by increasing the baseline or Compton continuum.

Interferences can occur with Ge detectors from cascade peak summing, which results when two or more gamma rays are emitted in one disintegration (e.g., with cobalt-60) where 1172 and 1333 keV gamma rays are emitted in cascade. These can be detected together to produce a sum peak at 2505 keV or a count in the continuum between the individual peaks and the sum peak, thus causing the loss of counts from one or both of the other two peaks. Cascade summing (as distinct from random summing) is geometry/counting efficiency dependent (but not count rate dependent) with the effect, and hence error, increasing as tighter geometries and more efficient detectors are used. This problem has become more commonplace with the availability of larger, more affordable, and more efficient Ge detectors.

Sample homogeneity is important to gamma count reproducibility and counting efficiency validity. When sample radionuclides are adsorbed on the walls of the counting container, the sample is no longer homogeneous. This problem can be lessened by adding 15 mL 1*N*HNO_3/L sample at collection.

Sample density and composition can affect data quality. Prepare efficiency calibration standards in the same geometry and density as the samples. Ensure reproducible sample geometry to limit bias.[4] Plexiglass spacers may be useful in producing consistent sample positions. Random noise produced by vibration or by improper grounding can increase peak width and introduce additional uncertainty.

e. Safety: No unusual hazards are associated with the reagents used in this procedure. Follow routine safety precautions (i.e., wear laboratory coat, plastic gloves, and safety glasses and use a hood) when transferring samples and standards and preparing standards when solutions of gamma-emitting radionuclides are used. Cool germanium diodes with liquid nitrogen when they are being used to count samples. Take care when transferring liquid nitrogen from the storage dewar to the dewar used to supply coolant for the germanium diode. Use cryogenic gloves, protective clothing, and eye protection.

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2. Apparatus

a. Detector, large-volume (>50 cm^3) germanium-diode detector or 10.2-cm × 10.2-cm (4-in. × 4-in.) thallium-activated sodium iodide crystal [NaI(Tl)] detector. Smaller detectors may be acceptable if inherent limitations, such as reduced counting efficiency, are taken into account. Be sure that large detectors can accommodate re-entrant (Marinelli) beakers. Germanium (Ge) detectors are preferred because of better photon energy resolution. Despite the possibly higher counting efficiencies of NaI(Tl) detectors, the considerably narrower peak shape from a Ge detector leads to fewer baseline counts, thereby improving peak sensitivity. Preferably do not use NaI(Tl) detectors to make both qualitative and quantitative analyses for samples containing multiple gamma-emitting radioisotopes; however, they are preferred if a single nuclide is being quantitated. Ge detectors may be of either intrinsic (pure) germanium type or lithium-drifted germanium [Ge(Li)] type. Both require use of liquid nitrogen for cooling when bias voltage is applied to the detector. Intrinsic Ge detectors can be stored or shipped at ambient temperatures; Ge(Li) detectors must be cooled with liquid nitrogen at all times to avoid damage to detector.

b. Gamma-ray spectrometer plus analyzer with at least 2048 channels for Ge or 256 to 512 for NaI(Tl). See Section 7030B.5.

c. Counting container, standard geometry for either detector [e.g., 0.5-L cylindrical container, 0.45-L or 4-L re-entrant (Marinelli)[4] polyethylene beaker]. Counting containers of other sizes often are used.

d. Computer: Use a data acquisition system including a computer (PC, networked-PCs, or larger) supplied with software to automate the processing of raw spectral data as outlined in 7120B.4 and 5, below. Software should contain algorithms to:

- perform energy and efficiency calibrations;
- locate peaks [deconvoluting multiplets as needed; that may require a separate routine(s) for low-resolution NaI counting data];
- perform peak integrations;
- perform nuclide searches;
- do activity calculations (result, uncertainty, and MDA); and
- manage the library reference information needed to support these actions.

e. High-voltage power supply.

f. Amplifier, suitable for spectroscopy with gain and shaping time adjustments and baseline restoration.

g. Analog-to-digital converter and spectrum storage device.

3. Reagents

a. Distilled or deionized water, radon-free, for standard preparation and sample dilution.

b. Nitric acid (HNO_3), 1*N*.

4. Procedure

a. Energy calibration: Use NIST or NIST-traceable, or equivalent standards. For a Ge system, adjust analyzer amplifier gain and analog-to-digital converter zero offset to locate each photopeak in its appropriate channel. A 0.5- or 1.0-keV per channel calibration is recommended. If the system is calibrated for 1 keV per channel with channel zero representing 0 keV, the energy will be equal to the channel number. Check and adjust the pole zero cancellation of the amplifier output if required.[5]

Use a standard containing a mixture of gamma energies from about 100 to 2000 keV for energy calibration. Multiline gamma standards can be obtained commercially or can be prepared by the user. Some laboratories use radium-226 and daughters in equilibrium or europium-152 for this purpose. NIST SRM 4275 is a solid source that is useful for energy calibration and routine monitoring of instrument performance. Solid sources prepared on plastic mounts are stable and are recommended. Count energy calibration standards long enough to minimize uncertainty due to counting statistics; as a rule of thumb accumulate 10 000 counts in each photopeak area resulting in a counting error of 1%.

For a NaI(Tl) system, a 10- or 20-keV per channel calibration is adequate. A solid multipeak standard source (e.g., bismuth-207) is satisfactory for energy calibration.

b. Efficiency calibration: Use NIST, NIST-traceable, or equivalent standards with minimal cascade summing concerns. Use a known amount of a multipeak standard or various radionuclides that emit gamma photons with energies well spaced and distributed over the normal range of analysis; put these into each container geometry and gamma count for a photopeak spectrum accumulation. Count efficiency calibration standards long enough to minimize uncertainty due to counting statistics; as a rule of thumb accumulate 10 000 counts in each photopeak area, resulting in a counting error of 1%.

Determine counting efficiencies for the various gamma energies (photopeaks) from the activity counts of the known-value samples as follows:

$$E = \frac{C}{A \times B}$$

where:

E = efficiency (expressed as counts per minute/gamma rays emitted per minute),
C = net count rate, cpm (integrated counts in the photopeak above the baseline continuum divided by the counting duration),
A = activity of radionuclide added to the given geometry container, dpm (corrected for decay, if necessary), and
B = gamma-ray abundance of the radionuclide being measured, gammas/disintegration.

Plot counting efficiency against gamma energy for each container geometry and for each detector that is to be used. For NaI systems, prepare a library of radionuclide spectra from counts of known radionuclide-water sample concentrations at standard sample geometries.

c. Sample measurement: Measure sample portion in a standard-geometry container calibrated as directed in ¶s *a* and *b* above. Place container and sample on a shielded Ge or NaI(Tl) detector and gamma count for a period of time that will meet the required sensitivity. Print gamma spectrum and/or store the spectrum on the appropriate computer-compatible device.

5. Calculations

The equations (7120B.4 and 5) describe the fundamental relationships between the defined variables and could be used if the calculations were to be performed manually. NaI spectral data, with their high probability for peak overlap due to low resolution, often

require complex peak unfolding routines. Modern gamma spectroscopy systems rely on vendor-supplied computer software to process the 256 to 500-plus (for NaI systems) and up to 8000 (for Ge systems) data points. The supplied software should be accompanied by documentation describing the algorithms, which must incorporate the fundamental relationships presented here.

Determine isotopes indicated by the gamma spectrum as follows: Identify all photopeak energies, integrate photopeak regions of the spectrum and subtract the area under the baseline continuum to determine the true photopeak area, and identify isotopes by their appropriate photopeaks, and ratios to each other when more than one gamma photon is emitted by an isotope.

Calculate the sample radionuclide concentrations as follows:

$$A' = \frac{C}{2.22 \times B \times E \times V \times e^{-\lambda t}}$$

where:

A' = sample radionuclide concentration, pCi/L,
V = sample volume, L,
$e^{-\lambda t}$ = decay factor (corrected to sample collection time), with λ = decay constant for the gamma-emitting radionuclide being analyzed, and t = duration of time from sample collection to counting,
2.22 = conversion factor from dpm to pCi,

and B, C, and E are as defined in 7120B.4*b*. Calculate the 2σ counting error term for gamma-emitters as follows:

$$2\sigma, \text{pCi/L} = \frac{2\sqrt{\frac{C + 2G}{t_c}}}{2.22 \times B \times E \times V \times e^{-\lambda t}}$$

where:

G = photopeak area below continuum, cpm,
t_c = counting duration, min,
and other terms are as defined above.

Report the result and counting error together in the form:

$$X \pm 2\sigma, \text{pCi/L}$$

Vendor-supplied software usually can calculate a Total Propagated Uncertainty (TPU). If so, the 2σ value reflects the total uncertainty and not just the counting error. The vendor-supplied software also should calculate a minimum detectable concentration (MDC) (see Section 7020C.3*b* for a general discussion). Report concentration, uncertainty, and MDC for each sample.

6. Quality Control

The quality control practices considered to be an integral part of each method can be found in Section 7020.

a. Duplicates: Make duplicate analyses for one out of every ten samples. (See Section 7020A.3*d*.) If it is known or strongly suspected that the sample(s) contain no detectable gamma-emitting radionuclides, do not use the sample(s) for duplicate analysis. In such a case, rely on the results of known-addition samples as described below. If desired, analyze known-addition samples in duplicate.

b. Known-addition sample: Analyze a known-addition sample for one out of every ten samples.

c. Background: See Sections 7030B.5*c*3) and 7020A.3*b*. Find and identify any background lines that may be present. If present, subtract the background counts line by line from the sample photopeaks. Even if background does not significantly affect results, monitor it to ensure system integrity. Weekly background counts for durations longer than normal sample counting durations may be needed to quantify low-level activity from nuclides such as cobalt-60.

d. Energy calibration: Check the energy calibration daily or before each use with a multi-line source as in 7120B.4*a*.

e. Efficiency check: Check detector efficiency daily or before each use with a stable multiline source in a reproducible geometry.

f. Records: Collect and maintain results from duplicate pairs and check standards. Include date, results, analyst's name, and any comments relevant to the evaluation of these data.

7. Precision and Bias

The precision of an individual measurement by gamma spectrometry can be improved by increasing sample counting duration. It may be necessary to gamma count for as much as 1000 min to reach desired precision. Other ways of increasing precision of an individual measurement are to increase sample volume, use a more efficient detector, or concentrate the sample. To obtain accurate results, calibrate carefully and use standardized radionuclides at the proper activity and purity levels.

Collaborative test data for a closely defined technique or procedure were not available for gamma-emitters in water. However, data from EPA's Environmental Radioactivity Performance Evaluation Studies Program are presented here. Table 7120:I is a summary of the recovery, within-laboratory variance, S_r, and total-error variance, S_R, regression line equations for each gamma-emitter studied. These data are from the analysis of gamma-emitting radionuclides in standard samples by participants in the program from 1981 to 1998. It is not possible to say how many investigators used NaI(Tl) detectors in the early years of the data collection period. It is believed that most, if not all, are now using germanium detectors, and that the data are comparable among all participating laboratories. The gamma spectral data from the Gamma Performance Evaluation and Blind Samples from February 1981 through November 1998 (April 1981 through September 1998 for iodine-131) were summarized by study and the data were arrayed for analyses. See Table 7120:II. T1 T2

Regression equations were generated for: recovery, grand average of each study; estimate of precision, standard deviation (1 σ) of the mean value of each study, $S_{\bar{x}}$; within-laboratory standard deviation (1 σ), also known as the repeatability or random error, S_r; between-laboratory standard deviation (1 σ), also known as the systematic error, S_L; and total error from within and between labs, also known as reproducibility, S_R. S_R equals the square root of the sum of the variance of the within-laboratory error and the between-laboratory error (i.e., the reproducibility variance is equal to the sum of the random variance and the systematic variance).

$$S_R^2 = S_r^2 + S_L^2$$

$$S_R = \sqrt{S_r^2 + S_L^2}$$

TABLE 7120:I. GAMMA-EMITTERS RECOVERY AND PRECISION ESTIMATE REGRESSION LINE EQUATIONS

Nuclide	Recovery	S_r *pCi/L*	S_R *pCi/L*
Iodine-131*	$y = 1.010x - 0.31$	$y = 0.033x + 1.68$	$y = 0.071x + 2.41$
Cesium-137	$y = 1.012x + 0.62$	$y = 0.018x + 1.49$	$y = 0.043x + 1.97$
Cesium-134	$y = 0.919x + 0.56$	$y = 0.023x + 1.30$	$y = 0.053x + 1.70$
Barium-133	$y = 0.966x + 0.18$	$y = 0.021x + 1.70$	$y = 0.054x + 2.35$
Ruthenium-106	$y = 0.928x + 2.33$	$y = 0.051x + 3.68$	$y = 0.072x + 7.28$
Zinc-65	$y = 1.023x + 0.11$	$y = 0.026x + 2.81$	$y = 0.052x + 3.74$
Cobalt-60	$y = 0.985x + 0.81$	$y = 0.021x + 1.40$	$y = 0.043x + 1.91$
Chromium-51	$y = 0.997x + 0.30$	$y = 0.058x + 3.99$	$y = 0.081x + 8.67$

* Analyzed singly as a separate study from the other gamma-emitters.

TABLE 7120:II. GAMMA-EMITTERS STUDY: SUMMARY OF PARTICIPANTS

Nuclide	No. of Studies*	Concentration Range *pCi/L*	No. of Participants†	No. of Acceptables‡	No. where $\sigma \neq 0$§
Iodine-131	37	6.1–148	42–119	39–115	34–105
Cesium-137	84	4–197	45–197	43–180	30–158
Cesium-134	84	2–105	17–196	15–183	8–165
Barium-133	23	25–745	118–192	107–181	94–156
Ruthenium-106	35	15–252	20–193	17–179	11–135
Zinc-65	47	10–300	61–195	55–182	50–163
Cobalt-60	74	8–99	53–196	52–183	48–165
Chromium-51	19	21–302	34–124	32–121	16–112

* Samples with concentrations equal to 0 pCi/L were not included.
† Total number of participants in study, even though all of the data may not have been used.
‡ Number of participants used in calculating grand average and standard deviation of grand average.
§ Participants reporting within laboratory variance equal to zero were not used in calculating the study within-laboratory, between-laboratory, and total error variance.

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7500-Cs RADIOACTIVE CESIUM*

7500-Cs A. Introduction

Radioactive cesium has been considered one of the more hazardous radioactive nuclides produced in nuclear fission. Upon ingestion, like potassium, cesium distributes itself throughout the soft tissue and has a relatively short residence time in the body. Half-lives of ^{134}Cs and ^{137}Cs are 2 and 30 years, respectively, both being beta- and gamma-emitters.

* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011.

7500-Cs B. Precipitation Method

1. General Discussion

Principle: If the activity of cesium is high, radioactive cesium can be determined directly by gamma-counting a large liquid sample (4 L) or the sample can be evaporated to dryness and counted. For lower-level environmental samples, add cesium carrier to an acidified sample and collect the cesium as phosphomolybdate. This is purified and precipitated as Cs_2PtCl_6 for counting. If total radiocesium determined by beta-counting exceeds 30 pCi/L, determine ^{134}Cs and ^{137}Cs by gamma spectrometry.

The quality control practices considered to be an integral part of each method can be found in Section 7020.

2. Apparatus

a. Magnetic stirrer with TFE-coated magnet bar.
b. Centrifuge, bench-size clinical, and centrifuge tubes.
c. Filter papers and glass fiber filter*, 2.4 cm diam.
d. pH paper, wide range, 1 to 11 pH.
e. Filtering apparatus: See Section 7500-Sr.B.2*c*.
f. Counting instruments: Use either a low-background beta counter (see Section 7030B.1) or a gamma spectrometer (see Section 7030B.5).

3. Reagents

a. Ammonium phosphomolybdate reagent ($H_{12}Mo_{12}N_3O_{40}P$): Dissolve 100 g molybdic acid (85% MoO_3) in a mixture of 240 mL distilled water and 140 mL conc ammonium hydroxide (NH_4OH). When solution is complete, filter and add 60 mL conc nitric acid (HNO_3). Separately mix 400 mL conc HNO_3 and 960 mL distilled water. After both solutions cool to room temperature, add, with constant stirring, the $(NH_4)_6Mo_7O_{24}$ solution to the HNO_3 solution. Let stand for 24 h. Filter† and discard insoluble material.

Collect filtrate in a 3-L beaker and heat to 50 to 55°C (never above 55°C). Remove from heating unit. Add 25 g sodium dihydrogen phosphate (NaH_2PO_4) dissolved in 100 mL distilled water, stir occasionally for 15 min, and let settle (approximately 30 min). Filter and wash precipitate with 1% potassium nitrate (KNO_3) and finally with distilled water. Dry precipitate and paper at 100°C for 3 to 4 h. Transfer solid $(NH_4)_3PMo_{12}O_{40}$ to a weighing bottle and store in a desiccator.

b. Chloroplatinic acid, 0.1*M:* Dissolve 51.8 g $H_2PtCl_6 \cdot 6H_2O$ in distilled water and dilute to 1000 mL.

c. Cesium carrier: Dissolve 1.267 g cesium chloride (CsCl) in distilled water and dilute to 100 mL; 1 mL = 10 mg Cs.

d. Calcium chloride, 3*M:* Dissolve 330 g $CaCl_2$ in distilled water and dilute to 1000 mL.

e. Ethanol, 95%.

f. Hydrochloric acid (HCl), conc, 6*N*, 1*N*.

g. Sodium hydroxide (NaOH), 6*N*.

4. Procedure

a. To a 1-L sample, add 1.0 mL cesium carrier and enough conc HCl to make the solution about 0.1*N* HCl (about 8.6 mL). Slowly add 1 g $(NH_4)_3PMo_{12}O_{40}$ and stir for 30 min using a magnetic stirrer at 800 rpm. Let precipitate settle for at least 4 h and discard supernatant by decanting or using suction (provided by an inverted glass funnel connected to a vacuum source). Using a stream of 1*N* HCl, quantitatively transfer precipitate to a centrifuge tube. Centrifuge and discard supernatant. Wash precipitate with 20 mL 1*N* HCl and discard wash solution.

b. Dissolve precipitate by dropwise addition of 3 to 5 mL 6*N* NaOH. Heat over a flame for several minutes to remove ammonium ions. (Moist pH paper turns green as long as NH_3 vapors are evolved.) Dilute to 20 mL with distilled water. Add 10 mL 3*M* $CaCl_2$ and adjust to pH 7 with 6*N* HCl to precipitate $CaMoO_4$. Stir, centrifuge, and filter‡ supernatant into a 50-mL centrifuge tube. Wash precipitate remaining in the original centrifuge tube with 10 mL distilled water, filter through the same filter paper, and combine the wash with filtrate. Discard precipitate and filter paper.

c. Add 2 mL 0.1*M* H_2PtCl_6 and 5 mL ethanol. Cool and stir in ice bath for 10 min. Using distilled water transfer to a tared glass-fiber filter. Wash with successive portions of distilled water, 1*N* HCl, and ethanol.

* Whatman No. 41, 9 cm diam; Whatman No. 42, 2.4 cm diam; or equivalent.
† Whatman No. 42 filter paper or equivalent.
‡ Whatman No. 41 filter paper or equivalent.

d. Dry at 110°C for 30 min, cool, weigh, mount on a nylon disk and ring with polyester plastic§ cover, and beta-count or gamma-scan for ^{134}Cs and ^{137}Cs.

5. Calculation

Calculate the concentration of radiocesium as follows:

$$\text{Cs, pCi/L} = \frac{C}{2.22 \times E\ V\ R}$$

where:

C = net count rate, cpm,
E = counter efficiency,
V = volume of sample, L, and
R = fractional chemical yield
$= \dfrac{\text{recovered } Cs_2PtCl_6\text{, mg} \times 0.3945}{\text{added Cs carrier, mg}}$

§ Mylar or equivalent.

6. Bibliography

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7500-^{3}H TRITIUM*

7500-^{3}H A. Introduction

Tritium exists fairly uniformly in the environment as a result of natural production by cosmic radiation and residual fallout from nuclear weapons tests. This background level gradually is being increased by the use of nuclear reactors to generate electricity, although tritium from this source is only a small proportion of environmental tritium. Nuclear reactors and fuel-processing plants are localized sources of tritium because of discharges during normal operation. This industry is expected to become the major source of environmental

* Approved by Standard Methods Committee, 2000.

tritium contamination in the future. Tritium is produced in light-water nuclear reactors by ternary fission, neutron capture in coolant additives, control rods and plates, and activation of deuterium. About 1% of the tritium in the primary coolant is released in gaseous form to the atmosphere; the remainder eventually is released in liquid waste discharges. Most tritium produced in reactors remains in the fuel and is released when fuel is reprocessed.

Naturally occurring tritium is most abundant in precipitation and lowest in aged water because of its physical decay by beta emission to helium. The maximum beta energy of tritium is 0.018 MeV and its half-life is 12.26 years.

7500-^{3}H B. Liquid Scintillation Spectrometric Method

1. General Discussion

a. Principle: A sample is treated by alkaline permanganate distillation to hold back most quenching materials, as well as radioiodine and radiocarbon. Complete transfer of tritiated water is assured by distillation to near dryness. A subsample of distillate is mixed with scintillation solution and the beta activity is counted on a coincidence-type liquid scintillation spectrometer. The scintillation solution consists of 1,4-dioxane, naphthalene, POPOP, and PPO.* The spectrometer is calibrated with standard solutions of tritiated water; then background and unknown samples are prepared and counted alternately, thus nullifying errors that could result from instrument drift or from aging of the scintillation solution.

b. Interferences: Sample distillation effectively removes nonvolatile radioactivity and the usual quenching materials. For waters containing volatile organic or radioactive materials, use wet oxidation (Section 4500-N_{org}) to remove interference from quenching due to volatile organic material. Distillation at about pH 8.5 holds back volatile radionuclides such as iodides and bicarbonates. Double distillation with an appropriate delay (10 half-lives) between distillations may be required to eliminate interference from volatile daughters of radium isotopes. Some clear-water samples collected near nuclear facilities may be monitored satisfactorily without distillation, especially when the monitoring instrument is capable of discriminating against beta radiation energies higher than those in the tritium range.

2. Apparatus

a. Liquid scintillation spectrometer, coincidence-type.

b. Liquid scintillation vial: 20-mL; polyethylene, low-K glass, or equivalent bottles.

c. Distillation apparatus: 250-mL round-bottom distillation flask, connecting side-arm adapter, condenser, and heating mantle.

3. Reagents

a. Scintillation solution: Thoroughly mix 4 g PPO, 0.05 g POPOP, and 120 g solid naphthalene in 1 L spectroquality 1,4-dioxane. Store in dark bottle. Solution is stable for 2 months. Alternatively, use a commercially prepared scintillation solution available from suppliers of liquid scintillation materials.

b. Low-background water: Use water with no detectable tritium activity (most deep well waters are low in tritium).

c. Standard tritium solution: Dilute available tritium standard solution to approximately 1000 dpm/mL with low-background water.

d. Sodium hydroxide, NaOH, pellets.

e. Potassium permanganate, $KMnO_4$.

4. Procedure

Add three pellets NaOH and 0.1 g $KMnO_4$ to 100 mL sample in 250-mL distillation flask. Distill at 100 to 105°C, discard first 10 mL distillate, and collect next 50 mL. Thoroughly mix 4 mL distillate with 16 mL scintillation solution in tightly capped vial.

Prepare low-background water and standard tritium solution in same manner as samples.

Hold samples, background, and standards in the dark for 3 h. Count samples containing less than 200 pCi/mL for 100 min and samples containing more than 200 pCi/mL for 50 min.

5. Calculations and Reporting

a. Calculate and report tritium, ^{3}H, in picocuries per milliliter (pCi/mL) or its equivalent, nanocuries per liter (nCi/L) as follows:

$$^3\mathrm{H} = \frac{(C - B)}{(E \times 4 \times 2.22)}$$

where:

C = gross counting rate for sample, cpm,
B = background counting rate, cpm,
E = counting efficiency, $(S - B)/D$,
S = gross counting rate for standard solution, cpm, and
D = tritium activity in standard sample, dpm, corrected for decay to time of counting.

b. Calculate the counting error at the 95% confidence level based on the equation for $\sigma(R)$ given in Section 7020C. A total count of 40 000 within 1 h for a background count rate of about 50 cpm gives a counting error slightly in excess of 1% at the 95% confidence level.

6. Precision and Bias

Samples with tritium activity above 200 pCi/mL can be analyzed with precision of less than ±6% at the 95% confidence

* POPOP = 1,4-di-2-(5-phenyloxazolyl) benzene; PPO = (2,5- diphenyloxazole).

level and those with 1 pCi/mL can be analyzed with a precision of less than ±10%.

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7500-I RADIOACTIVE IODINE*

7500-I A. Introduction

1. Occurrence and Significance

Radioiodine that results from testing nuclear devices or is released during use and processing of reactor fuels is a major concern in radioactivity monitoring. Fission products may contain iodine-129 through iodine-135. Iodine-129 has a half-life of 1.6×10^7 years but a relatively low specific activity (1.73×10^{-4} Ci/g for ^{129}I as compared to 1.24×10^5 Ci/g for ^{131}I). The half-life of ^{131}I is 8 d while for the other isotopes it is shorter (35 min to 21 h). At present, only ^{131}I is likely to be found in water. When ingested or inhaled, it concentrates in the thyroid gland and may cause thyroid cancer.

2. Selection of Method

Of the three methods, the precipitation method (7500-I.B) is preferred because it is simple and involves the least time. Method 7500-I.C, in which iodide is concentrated by absorption on an anion resin, purified, and counted in a beta-gamma coincidence system, is sensitive and accurate. Method 7500-I.D uses distillation. With each method it is possible to reach the EPA recommended detection limit of 1 pCi ^{131}I/L.

3. Bibliography

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* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011.

7500-I B. Precipitation Method

1. General Discussion

a. Principle: Iodate carrier is added to an acidified sample and, after reduction with Na_2SO_3 to iodide, the ^{131}I is precipitated with $AgNO_3$. The precipitate is dissolved and purified with zinc powder and H_2SO_4 and the solution is reprecipitated as PdI_2 for counting.

b. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 7020.

2. Apparatus

a. Counting instrument: Low-background beta counter (Section 7030B.1) or gamma spectrometer (Section 7030B.5).

b. Fine-fritted glass funnel.

c. Filter apparatus: Two-piece filter funnel with filtering equipment.*

d. Filter materials: Filter paper;† glass-fiber filter, 2.4 cm diam; or 0.8-μm pore-diam membrane filter, 4.7 cm diam.

3. Reagents

a. Ammonium hydroxide (NH_4OH), 6*N*.

b. Ethanol, 95%.

c. Hydrochloric acid (HCl), 6*N*.

d. Iodate carrier: Dissolve 1.685 g KIO_3 in distilled water and dilute to 100 mL. Store in dark flask; 1 mL = 10 mg I.

e. Nitric acid (HNO_3), conc.

f. Palladium chloride ($PdCl_2$): Dissolve 3.3 g $PdCl_2$ in 100 mL 6*N* HCl; 1 mL = 20 mg Pd.

g. Silver nitrate ($AgNO_3$), 0.1*M:* Dissolve 17 g $AgNO_3$ in distilled water and dilute to 1000 mL. Store in dark flask.

h. Sodium sulfite (Na_2SO_3), 1*M* (freshly prepared): Dissolve 6.3 g Na_2SO_3 in distilled water and dilute to 50 mL.

i. Sulfuric acid (H_2SO_4), 2*N*.

j. Zinc, powder, reagent grade.

4. Procedure

a. To a 2000-mL sample, add 15 mL conc HNO_3 and 1.0 mL iodate carrier. Mix well. Add 4 mL freshly prepared 1*M* Na_2SO_3 and stir for 30 min. Add 20 mL 0.1*M* $AgNO_3$, stir for 1 h, and let settle for 1 h. Decant and discard as much of the supernatant as possible. Filter remainder through a glass-fiber filter and discard filtrate.

b. Transfer filter to a centrifuge tube and slurry with 10 mL distilled water. Add 1 g zinc powder and 2 mL 2*N* H_2SO_4 and stir frequently for at least 30 min. Filter, with vacuum, through a fine-fritted glass funnel and collect filtrate in an Erlenmeyer flask. Wash both residue and filter with a minimum quantity of distilled water and add wash water to filtrate. Discard residue.

c. Add 2 mL 6*N* HCl and heat in water bath at 80°C for 10 min. Add 1 mL 0.2*M* $PdCl_2$ and digest for at least 5 min. Centrifuge and discard supernatant.

* Fisher Filtrator or equivalent.
† Whatman No. 42, or equivalent.

d. Dissolve precipitate in 5 mL 6*N* NH_4OH and heat in boiling water bath for 5 min. Filter through a glass-fiber filter and collect filtrate in a centrifuge tube. Discard filter and residue.

e. Neutralize filtrate with 6*N* HCl, add 2 mL in excess, and heat in a water bath. Add 1 mL 0.2*M* $PdCl_2$ to reprecipitate PdI_2 and digest for 10 min. Cool slightly and transfer to a tared filter with distilled water. Wash successively with 5-mL portions of distilled water and 95% ethanol. Dry in a vacuum oven at 60°C for 1 h, weigh precipitate, mount, and beta-count.

f. If final PdI_2 precipitate on a glass-fiber filter is counted in a low-background beta counter, the background counting rate is relatively high (about 1.3 cpm). If precipitate is collected on a 0.8-μm membrane filter and dried for 30 min at 70°C it may be counted in a beta-gamma coincidence scintillation system with a background rate of less than 0.1 cpm.

If a low-background counter is used, confirm identity of ^{131}I by recounting precipitate after about 1 week to check the half-life.

5. Calculation

Calculate concentration of radioiodine as follows:

$$^{131}I,\ pCi/L = \frac{C}{2.22 \times EVR \times A}$$

where:

C = net count rate, cpm,
E = counting efficiency of ^{131}I as function of mass of PdI_2 precipitate,
V = volume of sample, L,
R = fractional chemical yield $= \frac{\text{recovered } PdI_2 \times 0.0704}{\text{added iodine carrier}}$, and
A = ^{131}I decay factor for the time interval between sample collection and measurement.

6. Bibliography

U.S. Environmental Protection Agency. 1980. Prescribed Procedures for Measurement of Radioactivity in Drinking Water; EPA-600/4-

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7500-I C. Ion-Exchange Method

1. General Discussion

a. Principle: A known amount of inactive iodine in the form of KI is added as a carrier and the sample is taken through an oxidation-reduction step using hydroxylamine and sodium bisulfite to convert all iodine to iodide. Iodine, as the iodide, is concentrated by absorption on an anion-exchange column. Following an NaCl wash, iodine is eluted with sodium hypochlorite. Iodine in the iodate form is reduced to I_2, extracted into CCl_4, and back-extracted as iodide into water. The iodine finally is precipitated as PdI_2.

b. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 7020.

2. Apparatus

a. Counting instrument: Low-background beta counter (Section 7030B.1) or gamma spectrometer (Section 7030B.5).
b. Chromatographic column, 2 cm × 15 cm.
c. Vacuum filter holder, 2.5 cm^2 filter area.
*d. Filter paper,** 2.4 cm diam.
e. Vacuum oven.

3. Reagents

a. Iodine carrier: Weigh approximately 13 g dried KI to the nearest 0.1 mg. Dissolve in a 1-L volumetric flask containing 100 mL distilled water. Add 10 mL 1*M* $NaHSO_3$ and dilute to mark with distilled water. Concentration of carrier I, mg/L = g KI × 0.7644.

b. Ethanol, absolute.

c. Hydroxylamine hydrochloride, 1*M:* Dissolve 6.95 g $NH_2OH \cdot HCl$ in distilled water and dilute to 100 mL.

d. Nitric acid (HNO_3), conc, 8*N*, 1.6*N*.

e. Sodium bisulfite, 1*M:* Dissolve 1.04 g $NaHSO_3$ in distilled water and dilute to 10 mL.

f. Sodium hydroxide, 12*N:* Dissolve 480 g NaOH in distilled water and dilute to 1 L.

g. Sodium hypochlorite (NaOCl), 5%: Use available household bleach.

h. Anion-exchange resin.†

i. Carbon tetrachloride (CCl_4), reagent grade.

j. Hydrochloric acid (HCl), 3*N*, 1*N*.

k. Palladium chloride: Dissolve 3.3 g $PdCl_2$ in 100 mL 6*N* HCl; 1 mL = 20 mg Pd.

l. Sodium chloride (NaCl), 2*M:* Dissolve 117 g NaCl in distilled water and dilute to 1 L.

m. Hydroxylamine hydrochloride wash solution: Add 20 mL conc HNO_3 and 20 mL 1*M* $NH_2OH \cdot HCl$ to 100 mL distilled water.

4. Procedure

a. To 1 L sample in a beaker add, while stirring, 2.0 mL iodine carrier and 5 mL 5% NaOCl, and heat for 2 to 3 min to complete oxidation. After the interchange reaction (2 to 3 min), slowly add 5 mL conc HNO_3. Add 25 mL 1*M* $NH_2OH \cdot HCl$ and stir. Let

* Whatman No. 42, or equivalent.

† Dowex 1 × 8, 50-100 mesh, chloride form, or equivalent.

reaction go on for a few seconds, add 10 mL 1*M* $NaHSO_3$, and adjust pH to 6.5 with 12*N* NaOH or 1.6*N* HNO_3. Stir thoroughly for a few minutes. (Stir samples containing a large amount of organic material, such as muddy water, for 45 min.) Filter through a glass-fiber filter to remove suspended matter. Discard residue.

b. Pour 20 mL anion-exchange resin into a column and wash sides down with distilled water. Pass sample through ionexchange column at a flow rate of 20 mL/min. Discard effluent. Wash column with 200 mL distilled water and then with 100 mL 2*M* NaCl at a flow rate of 4 mL/min. Discard wash solutions.

c. Add 50 mL 5% NaOCl in 10- to 20-mL increments, stirring the resin as needed to eliminate gas bubbles, and maintain a flow rate of 2 mL/min. To the eluted volume of 50 to 60 mL, collected in a beaker, carefully add 10 mL conc HNO_3 to make sample 2 to 3*N* in HNO_3 and transfer to a separatory funnel. (Add acid slowly with stirring until vigorous reaction subsides.)

d. Add 50 mL CCl_4 and 10 mL 1*M* $NH_2OH \cdot HCl$. Extract iodine into organic phase by shaking for about 2 min. Let phases separate and transfer organic phase to another separatory funnel. Add 25 mL CCl_4 and 5 mL 1*M* $NH_2OH \cdot HCl$ to the first separatory funnel and shake for 2 min. Combine organic phase with the one obtained from the first extraction. Discard aqueous phase. Add 20 mL $NH_2OH \cdot HCl$ wash solution to the organic phase and shake for 2 min. Let phases separate and transfer organic phase to a clean separatory funnel. Discard wash solution.

e. Add 25 mL distilled water and 10 drops 1*M* $NaHSO_3$ to organic phase. Shake for 2 min, let phases separate, and discard organic phase. Transfer aqueous phase to a beaker. Add 10 mL 3*N* HCl. Using a stirrer-hot plate, boil and stir the sample until it evaporates to 10 to 15 mL or begins to turn yellow.

f. Add 1.0 mL $PdCl_2$ solution dropwise. Rinse sides of beaker with 1*N* HCl and add sufficient 1*N* HCl to make a volume of 30 mL. Continue stirring until cool. Place beaker in a stainless steel tray and store at about 4°C overnight.

g. Filter through a tared filter mounted in a filter holder. Wash residue with 1*N* HCl and then with absolute alcohol. Dry in a vacuum oven at 60°C for 1 h. Cool in a desiccator, weigh precipitate, then seal it between polyester tape and polyester plastic film,‡ with the film over the precipitate. Count with a beta-gamma coincidence system.

5. Calculation

Calculate ^{131}I, pCi/L, as in 7500-I.B.5.

6. Bibliography

American Society for Testing & Materials. 1972 Book of ASTM Standards. Part 23; D 2334-68. Philadelphia, Pa.

Gabay, J.J., C.J. Paperiello, S. Goodyear, J.C. Daly & J.M. Matuszek. 1974. A method of determining ^{129}I in milk and water. *Health Phys.* 26:89.

‡ Mylar, or equivalent.

7500-I D. Distillation Method

1. General Discussion

a. Principle: Iodine carrier is added to an acidified sample and iodine is distilled into a caustic solution. The distillate is acidified and the iodine is extracted into CCl_4. After back-extraction as iodide, the iodine is purified as PdI_2 for counting.

b. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 7020.

2. Apparatus

a. Distillation apparatus and 3-L round-bottom flask.

b. Separatory funnel, 60 mL.

c. Filter apparatus: Two-piece filter funnel with filtering equipment.*

d. Filter paper: See 7500-I.B.2*d*.

3. Reagents

a. Ammonium hydroxide (NH_4OH), conc.

b. Carbon tetrachloride (CCl_4).

c. Ethanol, 95%.

d. Hydrochloric acid (HCl), 6*N*, 1*N*.

e. Iodide carrier: Dissolve 2.616 g KI in distilled water, add 2 drops $NaHSO_3$, and dilute to 100 mL. Store in dark flask. 1 mL = 20 mg I.

f. Nitric acid (HNO_3), conc.

g. Palladium chloride: Dissolve 3.3 g $PdCl_2$ in 100 mL 6*N* HCl; 1 mL = 20 mg Pd.

h. Sodium bisulfite ($NaHSO_3$), 1*M:* Dissolve 5.2 g $NaHSO_3$ in distilled water and dilute to 50 mL. Prepare only in small quantities.

i. Sodium hydroxide (NaOH), 0.5*N*.

j. Sodium nitrite ($NaNO_2$), 1*M:* Dissolve 69 g $NaNO_2$ in distilled water and dilute to 1 L.

k. Sulfuric acid (H_2SO_4), 12*N*.

l. Tartaric acid ($C_4H_6O_6$), 50%: Dissolve 50 g $C_4H_6O_6$ in distilled water and dilute to 100 mL.

4. Procedure

a. To a 2000-mL sample in a 3-L round-bottom flask, add 15 mL 50% $C_4H_6O_6$ and 1.0 mL iodide carrier. Mix well, cautiously add 25 mL cold conc HNO_3, and close distillation apparatus (Figure 7500-I:1). **F1**

* Fisher Filtrator, or equivalent.

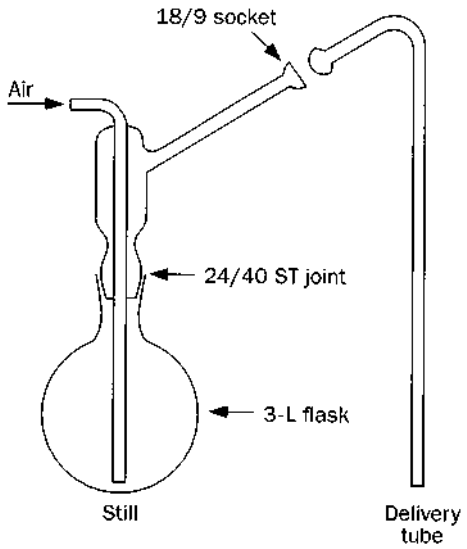


Figure 7500-I:1. Distillation apparatus for iodine analysis (not to scale).

b. Connect an air line to still inlet, adjust flow rate to about 2 bubbles/s, and distill for at least 15 min into 15 mL 0.5*N* NaOH. Cool and transfer NaOH solution to a 60-mL separatory funnel. Discard still residue.

c. Adjust distillate to slightly acid with 1 mL 12*N* H_2SO_4 and oxidize with 1 mL 1*M* $NaNO_2$. Add 10 mL CCl_4 and shake for 1 to 2 min. Transfer organic layer to a clean 60-mL separatory funnel containing 2 mL 1*M* $NaHSO_3$.

d. Add 5 mL CCl_4 and 1 mL 1*M* $NaNO_2$ to original separatory funnel containing the aqueous layer and shake for 2 min. Combine organic fractions. Repeat and discard aqueous layer.

e. Shake separatory funnel thoroughly until CCl_4 layer is decolorized; let phases separate and transfer aqueous layer to a centrifuge tube. Add 2 mL 1*M* $NaHSO_3$ to the separatory funnel containing CCl_4 and shake for several minutes. When phases separate, add aqueous layer to centrifuge tube. Add 1 mL distilled water to separatory funnel and shake for several minutes. When the phases separate, add aqueous layer to centrifuge tube. Discard organic layer.

f. To combined aqueous fractions, add 2 mL 6*N* HCl and heat in water bath at 80°C for 10 min. Add 1.0 mL $PdCl_2$ solution dropwise, with stirring, and digest for 15 min.

g. Cool, stir precipitate, and transfer to a tared filter mounted in a two-piece funnel. Let precipitate settle by gravity for uniform deposition, then apply suction. Wash residue with 10 mL 1*N* HCl, 10 mL distilled water, and then with 10 mL 95% ethanol. Dry in a vacuum oven at 60°C for 1 h. Cool in desiccator, weigh, mount, and make beta count.

5. Calculation

Calculate the concentration of radioiodine as given in 7500-I.B.5.

6. Bibliography

AMERICAN SOCIETY FOR TESTING & MATERIALS. 1972 Book of ASTM Standards. Part 23; D 2334-68. Philadelphia, Pa.

7500-Ra RADIUM*

7500-Ra A. Introduction

1. Occurrence

Radium is a radioactive member of the alkaline earth family and is widely disseminated throughout the earth's crust. It has four naturally occurring isotopes: 11.43-d ^{223}Ra, 3.66-d ^{224}Ra, 1600-year ^{226}Ra, and 5.75-year ^{228}Ra. Radium-223 is a member of the ^{235}U series, ^{224}Ra and ^{228}Ra are members of the thorium series, and ^{226}Ra is a member of the ^{238}U series. The contribution of ^{228}Ra (a beta-emitter) to the total radium alpha activity is negligible because of the 1.9-year half-life of its first alpha-emitting daughter product, ^{228}Th. The other three radium isotopes are alpha-emitters; each gives rise to a series of relatively short-lived daughter products, including three more alpha-emitters.

Because of their longer half-lives and health significance, ^{226}Ra and ^{228}Ra are the most important radium isotopes found in water. Even though it has a short half-life, ^{224}Ra in groundwater is important in certain geographical areas because of their geochemistry and the short delivery time of well water to consumers. Radium is a bone-seeker, and high concentrations in bone can lead to malignancies.

* Approved by Standard Methods Committee, B, C, D, 2001; A, E, 2007. Editorial revisions, 2011.
Joint Task Group: 21st Edition—Bahman Parsa (chair), Edmond J. Baratta, Loren A. Berge, John G. Griggs, Nancy E. Kinner, David E. McCurdy, James W. Mullins, Stephen H. Pia. 7500-Ra.A, E: Bahman Parsa (chair), Edmond J. Baratta, Loren A. Berge, James W. Mullins, Stephen H. Pia, Robert Rosson, Shiyamalie R. Ruberu, Carolyn T. Wong.

2. Selection of Method

Four common methods for measuring radium are: alpha counting of barium–radium sulfate precipitate that has been purified; measurement of ^{222}Rn produced from ^{226}Ra in a sample or in a soluble concentrate isolated from the sample; measurement of ^{228}Ac from ^{228}Ra by beta-counting; and measurement of ^{224}Ra, ^{226}Ra, and ^{228}Ra by gamma-counting their respective ^{212}Pb, ^{214}Pb (and/or ^{214}Bi), and ^{228}Ac decay products.

The determination of radium by precipitation (7500-Ra.B) includes all alpha-emitting radium isotopes; it is a screening technique particularly applicable to drinking water. As long as the concentration of radium is less than the ^{226}Ra plus ^{228}Ra drinking water standard, examination by a more specific method is seldom needed. This method also is applicable to sewage and industrial wastes, provided that steps are taken to destroy organic matter and eliminate other interfering ions (see Section 7110). However, avoid igniting sample ash.

The emanation technique (7500-Ra.C), based on measurement of ^{222}Rn, is nearly, but not absolutely, specific for ^{226}Ra. Procedures for soluble, suspended, and total ^{226}Ra are given.

The sequential precipitation method (7500-Ra.D) can be used to measure either ^{228}Ra alone or ^{228}Ra and ^{226}Ra.

The gamma-counting method (7500-Ra.E) is used to measure ^{224}Ra, ^{226}Ra, and ^{228}Ra either concurrently or sequentially.

7500-Ra B. Precipitation Method

1. General Discussion

a. Application: This method is suitable for determination of the alpha-emitting isotopes of radium.

b. Principle: Because of the difference in half-lives of the nuclides in the series, including the alpha-emitting Ra isotopes, these isotopes can be identified by the rate of ingrowth and decay of their daughters in a barium sulfate precipitate.[1–3] The ingrowth of alpha activity from ^{226}Ra increases at a rate governed primarily by the 3.8-d half-life of ^{222}Rn. The ingrowth of alpha activity in ^{223}Ra is complete by the time a radium–barium precipitate can be prepared for counting. The ingrowth of the first two alpha-emitting daughters of ^{224}Ra is complete within a few minutes and the third alpha daughter activity increases at a rate governed by the 10.6-h half-life of ^{212}Pb. The activity of the ^{224}Ra itself, with a 3.6-d half-life, also is decreasing, leading to a rather complicated ingrowth and decay curve.

Lead and barium carriers are added to the sample containing alkaline citrate, then sulfuric acid (H_2SO_4) is added to precipitate radium, barium, and lead as sulfates. The precipitate is purified by washing with nitric acid (HNO_3), dissolving in alkaline EDTA, and reprecipitating as radium–barium sulfate after pH adjustment to 4.5. This slightly acidic EDTA keeps other naturally occurring alpha-emitters and the lead carrier in solution.

c. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 7020.

2. Apparatus

a. Counting instruments: One of the following is required:

1) *Internal proportional counter,* gas-flow, with scaler and register;

2) *Alpha scintillation counter,* silver-activated zinc sulfide phosphor deposited on thin polyester plastic, with photomultiplier tube, scaler, timer, and register; or

3) *Proportional counter,* thin end-window, gas-flow, with scaler and register.

b. Membrane filter holder, or stainless steel or TFE filter funnels, with vacuum source.*

c. Membrane filters† or *glass fiber filters.*‡

* Fisher Filtrator or equivalent.
† Millipore Type HAWP or equivalent.
‡ No. 934-AH, diameter 2.4 cm, H. Reeve Angel and Co., or equivalent.

3. Reagents

a. Citric acid, 1*M:* Dissolve 210 g $H_3C_6H_5O_7 \cdot H_2O$ in distilled water and dilute to 1 L.

b. Ammonium hydroxide, conc and 5*N:* Verify strength of old 5*N* NH_4OH solution before use.

c. Lead nitrate carrier: Dissolve 160 g $Pb(NO_3)_2$ in distilled water and dilute to 1 L; 1 mL = 100 mg Pb.

d. Stock barium chloride solution: Dissolve 17.79 g $BaCl_2 \cdot 2H_2O$ in distilled water and dilute to 1 L in a volumetric flask; 1 mL = 10 mg Ba.

e. Barium chloride carrier: To a 100-mL volumetric flask, add 20.00 mL stock $BaCl_2$ solution using a transfer pipet, dilute to 100 mL with distilled water, and mix; 1 mL = 2.00 mg Ba.

f. Methyl orange indicator solution.

g. Phenolphthalein indicator solution.

h. Bromcresol green indicator solution: Dissolve 0.1 g bromcresol green sodium salt in 100 mL distilled water.

i. Sulfuric acid (H_2SO_4), 18*N*.

j. Nitric acid (HNO_3), conc.

k. EDTA reagent, 0.25*M:* Add 93 g disodium ethylenediaminetetraacetate dihydrate to distilled water, dilute to 1 L, and mix.

l. Acetic acid, conc.

m. Ethyl alcohol, 95%.

n. Acetone.

o. Clear acrylic solution:§ Dissolve 50 mg clear acrylic in 100 mL acetone.

p. Standard ^{226}Ra solution: Prepare as directed in 7500-Ra.C.3*d–f*, except that in 7500-Ra.C.3*f* (standard ^{226}Ra solution), add 0.50 mL $BaCl_2$ stock solution (7500-Ra.C.3*a*) before adding the ^{226}Ra solution; 1 mL final standard radium solution so prepared contains 2.00 mg Ba/mL and approximately 3 pCi ^{226}Ra/mL after the necessary correcting factors are applied.

4. Procedure for Radium in Drinking Water and for Dissolved Radium

a. To 1 L sample in a 1500-mL beaker, add 5 mL 1*M* citric acid, 2.5 mL conc NH_4OH, 2 mL $Pb(NO_3)_2$ carrier, and 3.00 mL $BaCl_2$ carrier. In each batch of samples include a distilled water blank.

b. Heat to boiling and add 10 drops methyl orange indicator.

c. While stirring, slowly add 18*N* H_2SO_4 to obtain a permanent pink color; then add 0.25 mL acid in excess.

d. Boil gently 5 to 10 min.

e. Set beaker aside and let stand until precipitate has settled (3 to 5 h or more).‖

f. Decant and discard clear supernate. Transfer precipitate to a 40-mL or larger centrifuge tube, centrifuge, decant, and discard supernate.

g. Rinse wall of centrifuge tube with a 10-mL portion of conc HNO_3, stir precipitate with a glass rod, centrifuge, and discard supernate. Repeat rinsing and washing two more times.

h. To precipitate, add 10 mL distilled water and 1 to 2 drops phenolphthalein indicator solution. Stir and loosen precipitate from bottom of tube (using a glass rod if necessary) and add 5*N* NH_4OH, dropwise, until solution is definitely alkaline (red). Add 10 mL EDTA reagent and 3 mL 5*N* NH_4OH. Stir occasionally for 2 min. Most of the precipitate should dissolve, but a slight turbidity may remain.

i. Warm in a steam bath to clear solution (about 10 min), but do not heat for an unnecessarily long period.# Add conc acetic acid dropwise until red color disappears; add 2 or 3 drops bromcresol green indicator solution and continue to add conc acetic acid dropwise, while stirring with a glass rod, until indicator turns green (aqua).** $BaSO_4$ will precipitate. Note date and time of precipitation as zero time for ingrowth of alpha activity. Digest in a steam bath for 5 to 10 min, cool, and centrifuge. Discard supernate. The final pH should be about 4.5, which is sufficiently low to destroy the Ba-EDTA complex, but not Pb-EDTA. A pH much below 4.5 will precipitate $PbSO_4$.

j. Wash Ba-Ra sulfate precipitate with distilled water and mount in a manner suitable for counting as given in ¶s *k*, *l*, or *m* below.

k. Transfer Ba-Ra sulfate precipitate to a tared stainless steel planchet with a minimum of 95% ethyl alcohol and evaporate under an infrared lamp. Add 2 mL acetone and 2 drops clear acrylic solution, disperse precipitate evenly, and evaporate under an infrared lamp. Dry in oven at 110°C, weigh, and determine alpha activity, preferably with an internal proportional counter. Calculate net counts per minute and weight of precipitate.

l. Weigh a membrane filter, a counting dish, and a weight (glass ring) as a unit. Transfer precipitate to tared membrane filter in a holder and wash with 15 to 25 mL distilled water. Place membrane filter in dish, add glass ring, and dry at 110°C. Weigh and count in one of the counters mentioned under 7500-Ra.B.2*a*. Calculate net counts per minute and weight of precipitate.

m. Add 20 mL distilled water to the Ba-Ra sulfate precipitate, let settle in a steam bath, cool, and filter through a special funnel with a tared glass fiber filter. Dry precipitate at 110°C to constant weight, cool, and weigh. Mount precipitate on a nylon disk and ring with an alpha phosphor on polyester plastic film,[4] and count in an alpha scintillation counter. Calculate net counts per minute and weight of precipitate.

n. If the isotopic composition of the precipitate is to be estimated, perform additional counting as mentioned in the calculation below.

o. Determination of combined efficiency and self-absorption factor: Prepare standards from 1 L distilled water and the standard ^{226}Ra solution (7500-Ra.B.3*p*). Include at least one blank. The barium content will impose an upper limit of 3.0 mL on the volume of the standard ^{226}Ra solution that can be used. If x is volume of standard ^{226}Ra solution added, then add $(3.00 - x)$ mL $BaCl_2$ carrier (7500-Ra.B.3*e*). Analyze standards as samples, beginning with 7500-Ra.B.4*a*, but omit 3.00-mL $BaCl_2$ carrier.

From the observed net count rate, calculate the combined factor, *bc*, from the formula:

§ Lucite or equivalent.

‖ If original concentrations of isotopes of radium other than ^{226}Ra are of interest, note date and time of this original precipitation as the separation of the isotopes from their parents; use a minimal settling time and complete procedure through 7500-Ra.B.4*j* without delay. Assuming the presence of and separation of parents, decay of ^{223}Ra and ^{224}Ra begins at the time of the first precipitation, but ingrowth of decay products is timed from the second precipitation (7500-Ra.B.4*i*). The time of the first

If solution does not clear in 10 min, cool, add another mL 5*N* NH_4OH, let stand 2 min, and heat for another 10-min period.

** The endpoint is most easily determined by comparison with a solution of similar composition that has been adjusted to pH 4.5 using a pH meter.

$$bc = \frac{\text{net cpm}}{ad \times 2.22 \times \text{pCi } ^{226}\text{Ra}}\text{††}$$

where:

ad = ingrowth factor (see 7500-Ra.B.5) multiplied by chemical yield.

If all chemical yields on samples and standards are not essentially equal, the factor bc will not be a constant. In this event, construct a curve relating the factor bc to varying weights of recovered $BaSO_4$.

5. Calculation

$$\text{Radium, pCi/L} = \frac{\text{net cpm}}{a \times b \times c \times d \times e \times 2.22}$$

where:

a = ingrowth factor (as shown in the following tabulation):

Ingrowth h	Alpha Activity from ^{226}Ra
0	1.000
1	1.016
2	1.036
3	1.058
4	1.080
5	1.102
6	1.124
24	1.489
48	1.905
72	2.253

b = efficiency factor for alpha counting,
c = self-absorption factor,
d = chemical yield, and
e = sample volume, L.

The calculations are based on the assumption that the radium is ^{226}Ra. If the observed concentration approaches 3 pCi/L, it may be desirable to follow the rate of ingrowth and estimate the isotopic content[2,3] or, preferably, to determine ^{226}Ra by ^{222}Rn.

The optimum ingrowth periods can be selected only if the ratios and identities of the radium isotopes are known. The number of observed count rates at different ages must be equal to or greater than the number of radium isotopes present in a mixture. In the general case, suitable ages for counting are 3 to 18 h for the first count; for isotopic analysis, additional counting at 7, 14, or 28 d is suggested, depending on the number of isotopes in mixture. The amounts of the various radium isotopes can be determined by solving a set of simultaneous equations.[3] This approach is most satisfactory when ^{226}Ra is the predominant isotope; in other situations, the approach suffers from statistical counting errors.

6. Precision and Bias

In a collaborative study, 20 laboratories analyzed four water samples for total (dissolved) radium. The radionuclide composition of these reference samples is shown in Table 7500-Ra:I. Note that Samples C and D had a ^{224}Ra concentration equal to that of ^{226}Ra.

Table 7500-Ra:I. Chemical and Radiochemical Composition of Samples Used to Determine Bias and Precision of Radium-226 Method

Radionuclide Composition	Samples			
	Pair 1		Pair 2	
	A	B	C	D
^{226}Ra,* pCi/L	12.12	8.96	25.53	18.84
^{228}Th,* pCi/L	none	none	25.90	19.12
Uranium, natural, pCi/L	105	77.9	27.7	20.5
^{210}Pb,* pCi/L	11.5	8.5	23.7	17.5
^{90}Sr,* pCi/L	49.1	36.3	13.9	10.2
^{137}Cs, pCi/L	50.3	37.2	12.7	9.5
NaCl, mg/L	60	60	300	300
$CaSO_4$, mg/L	30	30	150	150
$MgCl_2 \cdot 6H_2O$, mg/L	30	30	150	150
KCl, mg/L	5	5	10	10

* Daughter products were in substantial secular equilibrium.

The four results from each of two laboratories and two results from a third laboratory were rejected as outliers. The average recoveries of ^{226}Ra from the remaining A, B, C, and D samples were 97.5, 98.7, 94.9, and 99.4%, respectively. At the 95% confidence level, the precision (random error) was 28% and 30% for the two sets of paired samples. The method is biased low for ^{226}Ra, but not seriously. The method appears satisfactory for ^{226}Ra alone or in the presence of an equal activity of ^{224}Ra when correction for ^{224}Ra interference is made from a second count.

For the determination of ^{224}Ra in Samples C and D, the results of two laboratories were excluded. Hence the average recoveries were 51 and 45% for Samples C and D, respectively. At the 95% confidence level, the precision was 46% for this pair of samples. The results indicated that the method for ^{224}Ra is seriously biased low. When the recoveries for ^{224}Ra did not agree with those for ^{226}Ra, this may have been due, in part, to incomplete instructions given in the method to account for the transitory nature of ^{224}Ra activity. The method as given here contains footnotes calling attention to the importance of the time of counting. Still uncertain is the degree of separation of ^{224}Ra from its parent, ^{228}Th, in 7500-Ra.B.4*a–g*.

Radium-223 and ^{224}Ra analysis by this method may be satisfactory, but special refinements and further investigations are required.

7. References

1. Kirby, H.W. 1954. Decay and growth tables for naturally occurring radioactive series. *Anal. Chem.* 26:1063.
2. Sill, C. 1960. Determination of radium-226, thorium-230, and thorium-232; Rep. No. TID 7616 (Oct.). U.S. Atomic Energy Comm., Washington, D.C.
3. Goldin, A.S. 1961. Determination of dissolved radium. *Anal. Chem.* 33:406.
4. Hallden, N.A. & J.H. Harley. 1960. An improved alpha-counting technique. *Anal. Chem.* 32:1961.

†† See calculation that follows.

7500-Ra C. Emanation Method

1. General Discussion

a. Application: This method is suitable for the determination of soluble, suspended, and total ^{226}Ra in water. In this method, *total* ^{226}Ra means the sum of suspended and dissolved ^{226}Ra. Radon means ^{222}Rn unless otherwise specified.

b. Principle: Radium in water is concentrated and separated from sample solids by coprecipitation with a relatively large amount of barium as the sulfate. The precipitate is treated to remove silicates, if present, and to decompose insoluble radium compounds, fumed with phosphoric acid to remove sulfite (SO_3^{2-}), and dissolved in hydrochloric acid (HCl). The completely dissolved radium is placed in a bubbler, which is then closed and stored for a period of several days to 4 weeks for ingrowth of radon. The bubbler is connected to an evacuated system and the radon gas is removed from the liquid by aeration, dried with a desiccant, and collected in a counting chamber. The counting chamber consists of a dome-topped scintillation cell coated inside with silver-activated zinc sulfide phosphor; a transparent window forms the bottom (Figure 7500-Ra:1). The chamber rests on a photomultiplier tube during counting. About 4 h after radon collection, the alpha-counting rate of radon and decay products is at equilibrium, and a count is obtained and related to ^{226}Ra standards similarly treated.

The counting gas used to purge radon from the liquid to the counting chamber may be helium, nitrogen, or aged air.

Some radon (emanation) techniques employ a minimum of chemistry but require high dilution of the sample and large chambers for counting the ^{222}Rn.[1] Others involve more chemical separation, concentration, and purification of ^{226}Ra before de-emanation into counting cells of either the ionization or alpha scintillation types. The method[2] given here requires a moderate amount of chemistry coupled with a sensitive alpha scintillation count of ^{222}Rn plus daughter products in a small chamber.[3]

c. Concentration techniques: The chemical properties of barium and radium are similar; therefore, because barium does not interfere with de-emanation, as much as 100 mg may be used to aid in coprecipitating radium from a sample to be placed in a single radon bubbler. However, because some ^{226}Ra is present in barium salts, reagent tests are necessary to account for ^{226}Ra introduced in this way.

d. Interferences: Only the gaseous alpha-emitting radionuclides, ^{219}Rn (actinon) and ^{220}Rn (thoron), can interfere. Interference from these radionuclides would be expected to be very rare in water not contaminated by such industrial wastes as uranium mill elements.[2] The half-lives of these nuclides are only 3.92 and 54.5 s, respectively, so only their alpha-emitting decay products interfere.

Interference from stable chemicals is limited. Small amounts of lead, calcium, and strontium, collected by the barium sulfate, do not interfere. However, lead may cause deterioration of platinum ware. Calcium at a concentration of 300 mg/L and other dissolved solids (in brines) at 269 000 mg/L cause no difficulty.[4]

The formation of precipitates in excess of a few milligrams during the ^{222}Rn ingrowth period is a warning that modifications[2] may be necessary because ^{222}Rn recovery may be impaired.

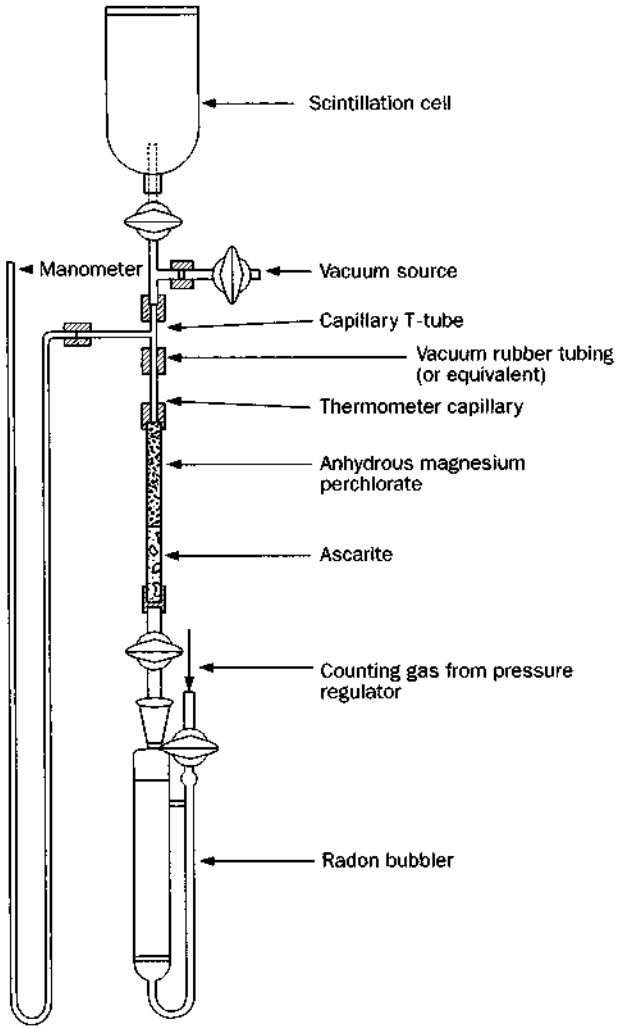


Figure 7500-Ra:1. De-emanation assembly.

e. Minimum detectable concentration: The minimum detectable concentration depends on counter characteristics, background-counting rate of scintillation cell, length of counting period, and contamination of apparatus and environment by ^{226}Ra. Without reagent purification, the overall reagent blank (excluding background) should be between 0.03 and 0.05 pCi ^{226}Ra, which may be considered the minimum detectable amount under routine conditions.

f. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 7020.

2. Apparatus

The de-emanation assembly is shown in Figure 7500-Ra:1, and its major components are described in ¶s *b–e*, below.

a. Scintillation counter assembly with a photomultiplier (PM) tube 5 cm or more in diameter, normally mounted, face up, in a light-tight housing. The photomultiplier tube, preamplifier, high-voltage supply, and scaler may be contained in one chassis; or

the PM tube and preamplifier may be used as an accessory with a proportional counter or a separate scaler. A high-voltage safety switch should open automatically when the light cover is removed, to avoid damage to the photomultiplier tube.

Use a preamplifier with a variable gain adjustment. Equip counter with a flexible ground wire attached to the chassis and to the neck of the scintillation cell by an alligator clip or similar device. Ascertain operating voltage by determining a plateau using ^{222}Rn in the scintillation cell as the alpha source; the slope should not exceed 2%/100 V. Calibrate and use counter and scintillation cell as a unit when more than one counter is available. The background-counting rate for the counter assembly without the scintillation cell in place should be 0.00 to 0.03 cpm.

b. Scintillation cells,[2,3] Lucas-type, preferably having a volume of 95 to 140 mL, made in the laboratory, or commercially available.*

c. Radon bubblers, capacity 18 to 25 mL.† Use gas-tight glass stopcocks and a fritted glass disk of medium porosity.‡ Use one bubbler for a standard ^{226}Ra solution and one for each sample and blank in a batch.[2]

d. Manometer, open-end capillary tube or vacuum gauge having volume that is small compared to volume of scintillation cell, 0 to 760 mm Hg.

e. Gas purification tube, 7 to 8 mm OD standard-wall glass tubing, 100 to 120 mm long, constricted at lower end to hold glass wool plug; thermometer capillary tubing.

f. Sample bottles, polyethylene, 2- to 4-L capacity.

g. Membrane filters.§

h. Gas supply: Helium, nitrogen, or air aged in high-pressure cylinder with two-stage pressure regulator and needle valve. Helium is preferred.

i. Silicone grease, high-vacuum.

j. Sealing wax, low-melting.‖

k. Laboratory glassware: Excepting bubblers, decontaminate all glassware before and between uses by heating for 1 h in EDTA decontaminating solution at 90 to 100°C, then rinse in water, 1*N* HCl, and again in distilled water to dissolve barium (radium) sulfate, $Ba(Ra)SO_4$.

Removal of previous samples from bubblers and rinsing is described in 7500-Ra.C.5*a*17). More extensive cleaning of bubblers requires removal of wax from joints, silicone grease from stopcocks, and the last traces of barium-radium compounds.

l. Platinum ware: Crucibles (20 to 30 mL) or dishes (50 to 75 mL), large dish (for flux preparation), and platinum-tipped tongs (preferably Blair type). Clean platinum ware by immersion and rotation in a molten bath of potassium pyrosulfate, remove, cool, rinse in hot tap water, digest in hot 6*N* HCl, rinse in distilled water, and finally flame over a burner.

3. Reagents

a. Stock barium chloride solution: Dissolve 17.79 g $BaCl_2 \cdot 2H_2O$ in distilled water and dilute to 1 L; 1 mL = 10 mg Ba.

b. Dilute barium chloride solution: Dilute 200.0 mL stock barium chloride solution to 1000 mL as needed; 1 mL = 2.00 mg Ba. Let stand 24 h and filter through a membrane filter.

Optionally, add approximately 40 000 dpm of ^{133}Ba to this solution before dilution. Take account of the stable barium carrier added with the ^{133}Ba and with the diluting solution, so the final barium concentration is near 2 mg/L. The use of ^{133}Ba provides a convenient means of checking on the recovery of ^{226}Ra from the sample; see 7500-Ra.C.7. Use the $BaCl_2$ solution containing ^{133}Ba in steps described in 7500-Ra.C.5*a*3), *b*8), and *c*3). Do *not* use in *d* below; instead, use a separate dilution of stock $BaCl_2$ solution for preparing ^{226}Ra standard solutions.

c. Acid barium chloride solution: To 20 mL conc HCl in a 1-L volumetric flask, add dilute $BaCl_2$ solution to the mark and mix.

d. Stock radium-226 solution: Take every precaution to avoid unnecessary contamination of working area, equipment, and glassware, preferably by preparing ^{226}Ra standards in a separate area or room reserved for this purpose. Obtain a National Institute of Standards and Technology (NIST) gamma ray standard containing 0.1 μg ^{226}Ra as of date of standardization. Using a heavy glass rod, cautiously break neck of ampule, which is submerged in 300 mL acid $BaCl_2$ solution in a 600-mL beaker. Chip ampule unit until it is thoroughly broken or until hole is large enough to give complete mixing. Transfer solution to a 1-L volumetric flask, rinse beaker with acid $BaCl_2$ solution, dilute to mark with same solution, and mix; 1 mL = approximately 100 pg ^{226}Ra.

Determine the time in years, *t*, since the NIST standardization of the original ^{226}Ra solution. Calculate pCi ^{226}Ra/mL as:

$$\text{pCi } ^{226}\text{Ra} = [1 - (4.3 \times 10^{-4})(t)]\ [100]\ [0.990]$$

e. Intermediate radium-226 solution: Dilute 100 mL stock ^{226}Ra solution to 1000 mL with acid $BaCl_2$ solution; 1 mL = approximately 10 pCi ^{226}Ra.

f. Standard radium-226 solution: Add 30.0 mL intermediate ^{226}Ra solution to a 100-mL volumetric flask and dilute to mark with acid $BaCl_2$ solution; 1 mL = approximately 3 pCi ^{226}Ra and contains about 2 mg Ba. See ¶ *d et seq.* above for correction factors.

g. Hydrochloric acid (HCl), conc, 6*N*, 1*N*, and 0.1*N*.

h. Sulfuric acid (H_2SO_4), conc and 0.1*N*.

i. Hydrofluoric acid (HF), 48%, in a plastic dropping bottle. **(CAUTION.)**

j. Ammonium sulfate solution: Dissolve 10 g $(NH_4)_2SO_4$ in distilled water and dilute to 100 mL in a graduated cylinder.

k. Phosphoric acid (H_3PO_4), 85%.

l. Ascarite, 8 to 20 mesh.

m. Magnesium perchlorate, anhydrous desiccant.

n. EDTA decontaminating solution: Dissolve 10 g disodium ethylenediaminetetraacetate dihydrate and 10 g Na_2CO_3 in distilled water and dilute to 1 L in a graduated cylinder.

o. Special reagents for total and suspended radium:

1) *Flux:* Add 30 mg $BaSO_4$, 65.8 g K_2CO_3, 50.5 g Na_2CO_3, and 33.7 g $Na_2B_4O_7 \cdot 10H_2O$, to a 500-mL platinum dish. Mix thoroughly and heat cautiously to expel water, then fuse and mix thoroughly by swirling. Cool flux, grind in a porcelain mortar to pass a 10- to 12-mesh (or finer) screen, and store in an airtight bottle.

* William H. Johnston Laboratories, 3617 Woodland Ave., Baltimore, MD 21215.
† Available from Corning Glass Works, Special Sales Section, Corning, NY 14830.
‡ Corning or equivalent.
§ Type HAWP, Millipore Filter Corp., Bedford, MA, or equivalent.
‖ Pyseal, Fisher Scientific Co., Pittsburgh, PA, or equivalent.

2) *Dilute hydrogen peroxide solution:* Dilute 10 mL 30% H_2O_2 to 100 mL in a graduated cylinder. Prepare daily.

4. Calibration of Scintillation Counter Assembly

a. Test bubblers by adding about 10 mL distilled water and passing air through them at the rate of 3 to 5 mL (free volume)/min. Air should form many fine bubbles rather than a few large ones; the latter condition indicates nonuniform pores. Do not use bubblers requiring excessive pressure to initiate bubbling. Fritted-glass disks of medium porosity (7500-Ra.C.2*c*) usually are satisfactory.

b. Apply silicone grease to stopcocks of a bubbler and, with gas inlet stopcock closed, add 1 mL stock $BaCl_2$ solution and 10 mL (30 pCi) standard ^{226}Ra solution, and fill bubbler two-thirds to three-fourths full with additional acid $BaCl_2$ solution.

c. With bubbler in a clamp or rack, dry joint with lint-free paper or cloth, warm separate parts of the joint, apply sealing wax sparingly to the male part, and make the connection with a twisting motion to spread the wax uniformly in the ground joint. Let cool. Establish zero ingrowth time by purging liquid with counting gas for 15 to 20 min according to ¶ *j* below and adjust inlet pressure to produce a froth a few millimeters thick. Close stopcocks, record date and time, and store bubbler, preferably for 3 weeks or more (with most samples) before collecting and counting ^{222}Rn. A much shorter ingrowth period of 16 to 24 h is convenient for a standard bubbler. Obtain an estimate of ^{222}Rn present at any time from the B columns in Table 7500-Ra:II.

d. Attach scintillation cell as shown in Figure 7500-Ra:1;# substitute a glass tube with a stopcock for bubbler so the compressed gas can be turned on or off conveniently. Open stopcock on scintillation cell, close stopcock to gas, and gradually open stopcock to vacuum source to evacuate cell. Close stopcock to vacuum source and check manometer reading for 2 min to test system, especially the scintillation cell, for leaks.

e. Open stopcock to counting gas and cautiously admit gas to scintillation cell until atmospheric pressure is reached.

f. Center scintillation cell on photomultiplier tube, cover with light-tight hood and, after 10 min, obtain a background counting rate (preferably over a 100- to 1000-min period, depending on concentration of ^{226}Ra in samples). *Do not expose phototube to external light with the high voltage applied.*

g. Repeat ¶s *d–f* above for each scintillation cell.

h. If the leakage test and background are satisfactory, continue calibration.

i. With scintillation cell and standard bubbler (¶ *c* above) on vacuum train, open stopcock on scintillation cell and evacuate scintillation cell and purification system (Figure 7500-Ra:1) by opening stopcock to vacuum source. Close stopcock to vacuum source. Check system for leaks as in ¶ *d* above.

j. Adjust gas regulator (diaphragm) valve so a very slow stream of gas will flow with the needle valve open. Attach gas supply to inlet of bubbler.

k. Note time as beginning of an approximately 20-min de-emanation period. Very cautiously open bubbler outlet stopcock to equalize pressure and transfer all or most of the fluid in the inlet side arm to bubbler chamber.

l. Close outlet stopcock and very cautiously open inlet stopcock to flush remaining fluid from side arm and fritted disk. Close inlet stopcock.

m. Repeat ¶s *h* and *l* above, four or five times, to obtain more nearly equal pressures on the two sides of bubbler.

n. With outlet stopcock fully open, cautiously open inlet stopcock so gas flow produces a froth a few millimeters thick at surface of bubbler solution. Maintain flow rate by gradually increasing pressure with regulator valve and continue de-emanation until pressure in cell reaches atmospheric pressure. Total elapsed time for the de-emanation should be 15 to 25 min.

o. Close stopcocks to scintillation cell, close bubbler inlet and outlet, shut off and disconnect gas supply, and record date and time as the ends of the ^{222}Rn ingrowth and de-emanation periods and as the beginnings of decay of ^{222}Rn and ingrowth of decay products.

p. Store bubbler for another ^{222}Rn ingrowth in the event a subsequent de-emanation is desired (Table 7500-Ra:II). The standard bubbler may be kept indefinitely.

q. Four hours after de-emanation, when daughter products are in virtual transient equilibrium with ^{222}Rn, place scintillation cell on photomultiplier tube, cover with light-tight hood, let stand for at least 10 min, then begin counting. Record date and time counting was started and finished.

r. Correct net counting rate for ^{222}Rn decay (Table 7500-Ra:II) and relate it to picocuries ^{226}Ra in standard bubbler (see 7500-Ra.C.6*a*). Unless the scintillation cell is physically damaged, the calibration will remain essentially unchanged for years. Occasional calibration is recommended.

s. Repeat ¶s *h–r* above on each scintillation cell.

t. To remove ^{222}Rn and prepare scintillation cell for reuse, evacuate and cautiously refill with counting gas. Routinely, repeat evacuation and refilling twice, and repeat process more times if the cells have contained a high ^{222}Rn activity. (Decay products with a half-life of approximately 30 min will remain in the cell. Do not check background on cells until activity of decay products has had time to decay to insignificance.)

5. Procedure

a. Soluble radium-226:

1) Using a membrane filter, filter at least 1 L sample or a volume containing up to 30 pCi ^{226}Ra and transfer to a polyethylene bottle as soon after sampling as possible. Save the suspended matter for determination by the procedure described in 5*b*, below. Record sample volume filtered if suspended solids are to be analyzed as in the procedure for ^{226}Ra in suspended matter.

2) Add 20 mL conc HCl/L of filtrate and continue analysis when convenient.

3) Add 50 mL dilute $BaCl_2$ solution, with vigorous stirring, to 1020 mL acidified filtrate [¶ *a*2) above] in a 1.5-L beaker. In each batch of samples include a reagent blank consisting of distilled water plus 20 mL conc HCl.

4) Cautiously, with vigorous stirring, add 20 mL conc H_2SO_4. Cover beaker and let precipitate overnight.

The system as described and shown in Figure 7500-Ra:1 is considered minimal. In routine work, use manifold systems and additional, more precise needle valves. An occasional drop of solution will escape from the bubbler; provide enough free space beyond the outlet stopcock to accommodate this liquid, preventing its entrance into the gas-purifying train.

TABLE 7500-Ra:II. FACTORS FOR DECAY OF RADON-222, GROWTH OF RADON-222 FROM RADIUM-226, AND CORRECTION OF RADON-222 ACTIVITY FOR DECAY DURING COUNTING

	Factor for Decay of Radon-222		Factor for Growth of Radon-222 from Radium-226		Factor for Correction of Radon-222 Activity for Decay during Counting		Factor for Decay of Radon-222		Factor for Growth of Radon-222 from Radium-226		Factor for Correction of Radon-222 Activity for Decay during Counting
	$A = e^{-\lambda t}$		$B = 1 - e^{-\lambda t}$		$C = \lambda t/(1 - e^{-\lambda t})$		$A = e^{-\lambda t}$		$B = 1 - e^{-\lambda t}$		$C = \lambda t/(1 - e^{-\lambda t})$
Time	*Hours*	*Days*	*Hours*	*Days*	*Hours*	Time	*Hours*	*Days*	*Hours*	*Days*	*Hours*
0.0	1.0000		0.000 00		1.000	29	0.8034	0.0052	0.1966	0.9948	1.113
0.2	0.9985		0.001 51		1.001	30	0.7973	0.0044	0.2027	0.9956	1.118
0.4	0.9970		0.003 01		1.001						
0.6	0.9955		0.004 52		1.002	31	0.7913	0.0036	0.2087	0.9964	1.122
0.8	0.9940		0.006 02		1.003	32	0.7854	0.0030	0.2146	0.9970	1.126
						33	0.7795	0.0025	0.2205	0.9975	1.130
1	0.9925	0.8343	0.007 52	0.1657	1.004	34	0.7736	0.0021	0.2264	0.9979	1.134
2	0.9850	0.6960	0.014 99	0.3040	1.008	35	0.7678	0.0018	0.2322	0.9982	1.138
3	0.9776	0.5807	0.022 40	0.4193	1.011						
4	0.9703	0.4844	0.029 75	0.5156	1.015	36	0.7620	0.0015	0.2380	0.9985	1.142
5	0.9630	0.4041	0.037 05	0.5959	1.019	37	0.7563	0.0012	0.2437	0.9988	1.146
						38	0.7506	0.0010	0.2494	0.9990	1.150
6	0.9557	0.3372	0.044 29	0.6628	1.023	39	0.7449	0.0009	0.2551	0.9991	1.154
7	0.9485	0.2813	0.051 48	0.7187	1.027	40	0.7393	0.0007	0.2607	0.9993	1.159
8	0.9414	0.2347	0.058 61	0.7653	1.031						
9	0.9343	0.1958	0.065 69	0.8042	1.034	41	0.7338	0.0006	0.2662	0.9994	1.163
10	0.9273	0.1633	0.072 72	0.8367	1.038	42	0.7283	0.0005	0.2717	0.9995	1.167
						43	0.7228	0.0004	0.2772	0.9996	1.171
11	0.9203	0.1363	0.079 69	0.8637	1.042	44	0.7173	0.0003	0.2827	0.9997	1.175
12	0.9134	0.1137	0.086 62	0.8863	1.046	45	0.7120	0.0003	0.2880	0.9997	1.179
13	0.9065	0.0948	0.093 49	0.9052	1.050						
14	0.8997	0.0791	0.100 31	0.9209	1.054						
15	0.8929	0.0660	0.107 07	0.9340	1.058	46	0.7066	0.0002	0.2934	0.9998	1.184
						47	0.7013	0.0002	0.2987	0.9998	1.188
16	0.8862	0.0551	0.1138	0.9449	1.062	48	0.6960	0.0002	0.3040	0.9998	1.192
17	0.8795	0.0459	0.1205	0.9541	1.066	49	0.6908	0.0001	0.3092	0.9999	1.196
18	0.8729	0.0383	0.1271	0.9617	1.069	50	0.6856	0.0001	0.3144	0.9999	1.201
19	0.8664	0.0320	0.1336	0.9680	1.073						
20	0.8598	0.0267	0.1402	0.9733	1.077	51	0.6804	0.0001	0.3196	0.9999	1.205
						52	0.6753	0.0001	0.3247	0.9999	1.209
21	0.8534	0.0223	0.1466	0.9777	1.081	53	0.6702	0.0001	0.3298	0.9999	1.213
22	0.8470	0.0186	0.1530	0.9814	1.085	54	0.6652	0.0001	0.3348	0.9999	1.218
23	0.8406	0.0155	0.1594	0.9845	1.089	55	0.6602	0.0000	0.3398	1.0000	1.222
24	0.8343	0.0129	0.1657	0.9871	1.093						
25	0.8280	0.0108	0.1720	0.9892	1.097	56	0.6552	0.0000	0.3448	1.0000	1.226
						57	0.6503	0.0000	0.3497	1.0000	1.231
26	0.8218	0.0090	0.1782	0.9910	1.101	58	0.6454	0.0000	0.3546	1.0000	1.235
27	0.8156	0.0075	0.1844	0.9925	1.105	59	0.6405	0.0000	0.3595	1.0000	1.239
28	0.8095	0.0063	0.1905	0.9937	1.109	60	0.6357	0.0000	0.3643	1.0000	1.244

5) Filter supernate through a membrane filter, using 0.1*N* H_2SO_4 to transfer Ba-Ra precipitate to filter, and wash precipitate twice with 0.1*N* H_2SO_4.

6) Place filter in a platinum crucible or dish, add 0.5 mL HF and 3 drops (0.15 mL) $(NH_4)_2SO_4$ solution, and evaporate to dryness.

7) Carefully ignite over a small flame until carbon is burned off; cool. (After filter is charred, a Meker burner may be used.)

8) Add 1 mL H_3PO_4 with a calibrated dropper and heat on hot plate at about 200°C. Gradually raise temperature and maintain at about 300 to 400°C for 30 min.

9) Swirl vessel over a low Bunsen flame, adjusted to avoid spattering, while covering the walls with hot H_3PO_4. Continue to heat for a minute after precipitate fuses into a clear melt (just below redness) to ensure complete removal of SO_3.

10) Fill cooled vessel one-half full with 6*N* HCl, heat on steam bath, then gradually add distilled water to within 2 mm of top of vessel.

11) Evaporate on boiling steam bath until there are no more vapors of HCl.

12) Add 6 mL 1*N* HCl, swirl, and warm to dissolve $BaCl_2$ crystals.

13) Close gas inlet stopcock, add a drop of water to the fritted disk of the fully greased and tested radon bubbler, and transfer sample from platinum vessel to bubbler with a medicine dropper. Use dropper to rinse vessel with at least three 2-mL portions of distilled water. Add distilled water until bubbler is two-thirds to three-fourths full.

14) Dry, wax if necessary, and seal joint. Establish zero ingrowth time as instructed in 7500-Ra.C.4*c*.

15) Close stopcocks, record date and time, and store bubbler for ^{222}Rn ingrowth, preferably for 3 weeks for low concentrations of ^{226}Ra.

16) De-emanate and count ^{222}Rn as instructed for calibrations in 7500-Ra.C.4*i–r*, with sample replacing standard bubbler.

17) The sample in the bubbler may be stored for a second ingrowth, or it may be discarded and the bubbler cleaned for reuse. (A bubbler is readily cleaned while in an inverted position by attaching a tube from a beaker containing 100 mL 0.1*N* HCl to the inlet and attaching another tube from outlet to a suction flask. Alternately open and close outlet and inlet stopcocks to pass the acid rinse water sequentially through the fritted disk, accumulate in the bubbler, and flush into the suction flask. Drain bubbler with the aid of vacuum, heat ground joint gently to melt wax, and separate joint. More extensive cleaning, as indicated in 7500-Ra.C.2*k*, may be necessary if the bubbler contained more than 10 pCi ^{226}Ra.)

b. Radium-226 in suspended matter:

1) Suspended matter in water usually contains siliceous materials that require fusion with an alkaline flux to ensure recovery of radium. Dry suspended matter (up to 1000 mg inorganic material) retained on the membrane filter specified in ¶ *a*1) above in a tared platinum crucible and ignite as in ¶ *a*7) above.

2) Weigh crucible to estimate residue.

3) Add 8 g flux/g residue, but not less than 2 g flux, and mix with a glass rod.

4) Heat over a Meker burner until melting begins, being careful to prevent spattering. Continue heating for 20 min after bubbling stops, with an occasional swirl of the crucible to mix contents and achieve a uniform melt. A clear melt usually is obtained only when the suspended solids are present in small amount or have a high silica content.

5) Remove crucible from burner and rotate as melt cools to distribute it in a thin layer on crucible wall.

6) When cool, place crucible in a covered beaker containing 120 mL distilled water, 20 mL conc H_2SO_4, and 5 mL dilute H_2O_2 solution for each 8 g flux. (Reduce acid and H_2O_2 in proportion to flux used.) Rotate crucible to dissolve melt if necessary.

7) When melt is dissolved, remove and rinse crucible into beaker. Save crucible for ¶ *b*10) below.

8) Heat solution and slowly add 50 mL dilute $BaCl_2$ solution with vigorous stirring. Cover beaker and let stand overnight for precipitation. (Precipitation with cool sample solution also is satisfactory.)

9) Add about 1 mL dilute H_2O_2 and, if yellow color (from titanium) deepens, add more H_2O_2 until there is no further color change.

10) Continue analysis according to ¶s *a*5)–16).

11) Calculate result as directed in 7500-Ra.C.6*a* and *b*, taking into account that the suspended solids possibly were contained in a sample volume other than 1 L [see ¶ *a*1) above].

c. Total radium-226:

1) Total ^{226}Ra in water is the sum of soluble and suspended ^{226}Ra as determined in 7500-Ra.C.5*a* and *b*, or it may be determined directly by examining the original water sample that has been acidified with 20 mL conc HCl/L sample and stored in a polyethylene bottle.

2) Thoroughly mix acidified sample and take 1020 mL or a measured volume containing not more than 1000 mg inorganic suspended solids.

3) Add 50 mL dilute $BaCl_2$ solution and slowly, with vigorous stirring, add 20 mL conc H_2SO_4/L sample. Cover and let precipitate overnight.

4) Filter supernate through membrane filter and transfer solids to filter as in ¶ *a*5) above.

5) Place filter and precipitate in tared platinum crucible and proceed as in ¶s *b*2)–10) above but with the following changes in the procedure given in ¶ *b*8): Omit add-ing dilute $BaCl_2$ solution, digest for 1 h on a steam bath, and filter immediately after digestion without stirring up $BaSO_4$. (If these changes are not made, filtration will be very slow.)

6) Calculate total ^{226}Ra concentration as directed in 7500-Ra.C.6*a* and *b*.

6. Calculations

a. Calculate the ^{226}Ra in a bubbler, including reagent blank, as follows:

$$^{226}\text{Ra, pCi} = \frac{R_s - R_b}{R_c} \times \frac{1}{1 - e^{-\lambda t_1}} \times \frac{1}{e^{-\lambda t_2}} \times \frac{\lambda t_3}{1 - e^{-\lambda t_3}}$$

where:

λ = decay constant for ^{222}Rn, 0.007 55/h,
t_1 = time interval allowed for ingrowth of ^{222}Rn, h,
t_2 = time interval between de-emanation and counting, h,
t_3 = time interval of counting, h,
R_s = observed counting rate of sample in scintillation cell, cph,
R_b = (previously) observed background counting rate of scintillation cell with counting gas, cph,
R_c = calibration constant for scintillation cell [i.e., observed net counts per hour, corrected by use of ingrowth and decay factors (*C*/*AB* below) per picocurie of Ra in standard],

or:

$$^{226}\text{Ra, pCi} = \frac{(R_s - R_b)}{R_c} \times \frac{C}{AB}$$

where:

A = factor for decay of ^{222}Rn (see Table 7500-Ra:II),
B = factor for growth of ^{222}Rn from ^{226}Ra (see Table 7500-Ra:II), and
C = factor for correction of ^{222}Rn activity for decay during counting (see Table 7500-Ra:II).

For nontabulated times, obtain decay factors for ^{222}Rn by multiplying together the appropriate tabulated "day" and "hour" decay factors, interpolating for less than 0.2 h if indicated by the precision desired. Obtain ^{222}Rn growth factors for nontabulated times most accurately, especially for short periods (e.g., in calibrations), by calculation from ^{222}Rn decay factors given in

Column A and using formula given in heading for Column B (of Table 7500-Ra:II). Linear interpolations are satisfactory for routine samples. Obtain the decay-during-counting factors by linear interpolation for all nontabulated times.

In calculating cell calibration constants, use the same equation, but picocuries of ^{226}Ra is known and R_c is unknown.

b. Convert the activity into picocuries per liter of soluble, suspended, or total ^{226}Ra by the following equation:

$$^{226}\text{Ra, pCi/L} = \frac{(D - E) \times 1000}{\text{mL sample}}$$

where:

D = pCi ^{226}Ra found in sample, and
E = pCi ^{226}Ra found in reagent blank.

7. Recovery of Barium (Radium-226) (Optional)

If ^{133}Ba was added in reagent *b*, check recovery of Ba by removing sample from bubbler, adjusting its volume appropriately, gamma-counting it under standardized conditions, and comparing the result with the count obtained from a 50-mL portion (evaporated if necessary to reduce volume) of dilute barium solution also counted under standardized conditions; add 1 mL H_3PO_4 to the latter portion before counting. The assumption that the Ba and ^{226}Ra are recovered to the same extent is valid in the method described.

NOTE: ^{226}Ra and its decay products interfere slightly even if a gamma spectrometer is used. The technique works best when the ratio of ^{133}Ba to ^{226}Ra is high.

Determinations of recovery are particularly helpful with irreplaceable samples, both in gaining experience with the method and in applying the general method to unfamiliar media.

8. Precision and Bias

In a collaborative study, seven laboratories analyzed four water samples for dissolved ^{226}Ra by this method. No result was rejected as an outlier. The average recoveries of added ^{226}Ra from Samples A, B, C, and D (below) were 97.1, 97.3, 97.6, and 98.0%, respectively. At the 95% confidence level, the precision (random error) was 6% and 8% for the two sets of paired samples. Because of the small number of participating laboratories and the low values for random and total errors, there was no evidence of laboratory systematic errors. Neither ^{224}Ra at an activity equal to that of the ^{226}Ra nor dissolved solids up to 610 mg/L produced a detectable error in the results.

Test samples consisted of two pairs of simulated moderately hard and hard water samples containing known amounts of added the ^{226}Ra and other radionuclides. The composition of the samples with respect to nonradioactive substances was the same for a pair of samples but varied for the two pairs. The radiochemical composition of the samples is given in Table 7500-Ra:I.

9. References

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7500-Ra D. Sequential Precipitation Method

1. General Discussion

a. Application: This method can be used to determine soluble ^{228}Ra alone or soluble ^{228}Ra plus ^{226}Ra.

b. Principle: Radium-228 and ^{226}Ra in water are concentrated and separated by coprecipitation with barium and lead as sulfates and purified by EDTA chelation. After 36-h ingrowth of ^{228}Ac from ^{228}Ra, ^{228}Ac is carried on yttrium oxalate, purified, and beta-counted. Radium-226 in the supernatant is precipitated as the sulfate, purified, and alpha-counted (7500-Ra.B) or it is transferred to a radon bubbler and determined by the emanation procedure (7500-Ra.C), which is the preferred method.

If analysis of ^{226}Ra is not required, the procedure for ^{228}Ra may be terminated by beta-counting the yttrium oxalate precipitate with a follow-up precipitation of barium sulfate for yield determination. If it is determined that ^{228}Ra is absent, the ^{226}Ra fraction may be alpha-counted directly. If ^{228}Ra is present, ^{226}Ra must be determined by radon emanation.

c. Sampling and storage: To drinking water or a filtered sample of turbid water, add 2 mL conc nitric acid (HNO_3)/L sample at the time of collection or immediately after filtration.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 7020.

2. Apparatus

a. Counting instruments: One of the following is required:

1) *Internal proportional counter,* gas flow, with scaler, timer, and register; or a thin end-window (polyester plastic)* proportional counting chamber with scaler, timer, register amplifier, and preferably having an anticoincident system (low background).

2) *Scintillation counter assembly:* See 7500-Ra.C.2*a*. This equipment is necessary only if ^{226}Ra is determined sequentially with ^{228}Ra and is analyzed by emanation of radon.

b. Centrifuge, bench-size clinical, with polypropylene tubes.

* Mylar, or equivalent.

c. Filter funnels, for 2.4-cm filter paper.
d. Stainless steel pans, 5.1 cm.
e. Infrared drying lamp assembly.
f. Magnetic stirrer hot plate.
g. Membrane filters, 47-mm diam, 0.45-μm pore diam.†

3. Reagents

a. Acetic acid, conc.
b. Acetone, anhydrous.
c. Ammonium hydroxide (NH_4OH), conc.
d. Ammonium oxalate solution: Dissolve 25 g $(NH_4)_2C_2O_4$ in distilled water and dilute to 500 mL.
e. Ammonium sulfate solution: Dissolve 20 g $(NH_4)_2SO_4$ in a minimum of distilled water and dilute to 100 mL.
f. Ammonium sulfide solution: Dilute 10 mL $(NH_4)_2S$ (20 to 24%) to 100 mL with distilled water.
g. Barium carrier standardized: Dissolve 2.846 g $BaCl_2 \cdot 2H_2O$ in distilled water, add 0.5 mL conc HNO_3, and dilute to 100 mL; 1 mL = 16 mg Ba.
h. Citric acid, 1*M:* See 7500-Ra.B.3*a*.
i. EDTA reagent, 0.25*M:* See 7500-Ra.B.3*k*.
j. Ethanol, 95%.
k. Lead carrier:
1) *Solution A*—Dissolve 2.397 g $Pb(NO_3)_2$ in distilled water, add 0.5 mL conc HNO_3, and dilute to 100 mL; 1 mL = 15 mg Pb.
2) *Solution B*—Dilute 10 mL Solution A to 100 mL with distilled water; 1 mL = 1.5 mg Pb.
l. Methyl orange indicator solution: Dissolve 0.1 g methyl orange powder in 100 mL distilled water.
m. Nitric acid (HNO_3), conc, 6*N*, and 1*N*.
n. Sodium hydroxide, 18*N:* Dissolve 720 g NaOH in 500 mL distilled water and dilute to 1 L.
o. Sodium hydroxide, 10*N:* Dissolve 400 g NaOH in 500 mL distilled water and dilute to 1 L.
p. Sodium hydroxide (NaOH), 1*N*.
q. Strontium-yttrium mixed carrier:
1) *Solution A*—Dilute 10.0 mL yttrium carrier to 100 mL.
2) *Solution B*—Dissolve 0.4348 g $Sr(NO_3)_2$ in distilled water and dilute to 100 mL. Combine equal volumes of Solutions A and B; 1 mL = 0.9 mg Sr and 0.9 mg Y.
r. Sulfuric acid (H_2SO_4), 18*N*.
s. Yttrium carrier: Add 12.7 g Y_2O_3 (Section 7500-Sr.B.3*d*) to an Erlenmeyer flask containing 20 mL distilled water. Heat to boiling and, while stirring with a magnetic stirring hot plate, add small portions of conc HNO_3. (About 30 mL is necessary to dissolve the Y_2O_3. Small additions of distilled water also may be needed to replace water lost by evaporation.) After total dissolution, add 70 mL conc HNO_3 and dilute to 1 L with distilled water; 1 mL = 10 mg Y.

4. Procedure

a. Radium-228:
1) For 1 L sample add 5 mL 1*M* citric acid and a few drops methyl orange indicator. The solution should be red. Add 10 mL lead carrier (Solution A), 2.0 mL barium carrier, and 2 mL yttrium carrier; stir well. Heat to incipient boiling and maintain at this temperature for 30 min.
2) Add conc NH_4OH until a definite yellow color is obtained; add a few drops excess. Precipitate lead and barium sulfates by adding 18*N* H_2SO_4 until the red color reappears; add 0.25 mL excess. Add 5 mL $(NH_4)_2SO_4$ solution/L sample. Stir frequently and hold at about 90°C for 30 min.
3) Cool and filter with suction through a membrane filter. Quantitatively transfer precipitate to filter. Carefully place filter in a 250-mL beaker. Add about 10 mL conc HNO_3 and heat gently until the filter dissolves completely. Using conc HNO_3 transfer precipitate to a centrifuge tube. Centrifuge and discard supernatant.
4) Wash precipitate with 15 mL conc HNO_3, centrifuge, and discard supernatant. Repeat wash and centrifuge again. Add 25 mL EDTA reagent, heat in a hot water bath, and stir well. Add a few drops 10*N* NaOH if the precipitate does not dissolve readily.
5) Add 1 mL strontium-yttrium mixed carrier and stir thoroughly. Add a few drops 10*N* NaOH if any precipitate forms. Add 1 mL $(NH_4)_2SO_4$ solution and stir thoroughly. Add conc acetic acid until $BaSO_4$ precipitates; add 2 mL excess. The pH should be about 4.5. Digest in a hot water bath (80°C) until precipitate settles. Centrifuge and discard supernatant.
6) Add 20 mL EDTA reagent, heat in a hot water bath, and stir until precipitate dissolves. Repeat ¶ *a*5) above. Note time of last $BaSO_4$ precipitation as zero time for ingrowth of ^{228}Ac. Dissolve precipitate in 20 mL EDTA reagent, add 0.5 mL yttrium carrier and 1 mL lead carrier (Solution B). If any precipitate forms, dissolve by adding a few drops 10*N* NaOH. Mix well, cap tube, and age at least 36 h.
7) Add 0.3 mL $(NH_4)_2S$ solution and mix well. Add 10*N* NaOH dropwise with vigorous stirring until PbS precipitates; add 10 drops excess. Stir intermittently for about 10 min. Centrifuge and decant supernatant into a clean tube.
8) Add 1 mL lead carrier (Solution B), 0.1 mL $(NH_4)_2S$ solution, and a few drops 10*N* NaOH. Repeat precipitation of PbS. Centrifuge and filter supernatant through filter paper‡ into a clean tube. Wash filter with a few milliliters of distilled water. Discard residue.
9) Add 5 mL 18*N* NaOH (make at least 2*N* in OH^-). Because of the short half-life of ^{228}Ac (6.13 h) complete the following procedure without delay. Mix well and digest in a hot water bath until $Y(OH)_3$ coagulates. Centrifuge and decant supernatant into a beaker. Cover beaker and save supernatant for ^{226}Ra analysis (¶s *b* or *c* below). Note time of $Y(OH)_3$ precipitation; this is the end of ^{228}Ac ingrowth and beginning of ^{228}Ac decay. [t_3 = time in minutes between last $BaSO_4$ and first $Y(OH)_3$ precipitations.] Dissolve precipitate in 2 mL 6*N* HNO_3. Heat and stir in a hot water bath about 5 min. Add 5 mL distilled water and reprecipitate $Y(OH)_3$ with 3 mL 10*N* NaOH. Heat and stir in a hot water bath until precipitate coagulates. Centrifuge and discard supernatant.
10) Dissolve precipitate with 1 mL 1*N* HNO_3 and heat in hot water bath for several minutes. Dilute to 5 mL with distilled water and add 2 mL ammonium oxalate solution. Heat to coagulate, centrifuge, and discard supernatant. Add

† Gelman Ga-6, or equivalent.
‡ Whatman No. 42, or equivalent.

10 mL distilled water, 6 drops 1*N* HNO_3, and 6 drops ammonium oxalate solution. Heat and stir in a hot water bath for several minutes. Centrifuge and discard supernatant. Transfer quantitatively to a tared stainless-steel planchet using a minimum quantity of distilled water. Dry under an infrared lamp to constant weight and count in a low-background beta counter. [t_1 = time in minutes between first $Y(OH)_3$ precipitation and counting.]

If analysis of ^{226}Ra is not required, complete ¶s *b*1) and 3) below to obtain the fractional barium yield to be used in calculating ^{228}Ra activity.

b. Radium by precipitation:

1) To the supernatant saved in ¶ *a*9) above add 4 mL conc HNO_3 and 2 mL $(NH_4)_2SO_4$ solution, mixing well after each addition. Add conc acetic acid until $BaSO_4$ precipitates; add 2 mL excess. Digest on a hot plate until precipitate settles. Centrifuge and discard supernatant.

2) Add 20 mL EDTA reagent, heat in a hot water bath, and stir until precipitate dissolves. Add a few drops 10*N* NaOH if precipitate does not dissolve readily. Add 1 mL strontium-yttrium mixed carrier and 1 mL lead carrier (Solution B), and stir thoroughly. Add a few drops 10*N* NaOH if any precipitate forms. Add 1 mL $(NH_4)_2SO_4$ solution and stir thoroughly. Add conc acetic acid until $BaSO_4$ precipitates; add 2 mL excess. Digest in a hot water bath until precipitate settles. Centrifuge, discard supernatant, and note time.

3) Wash precipitate with 10 mL distilled water. Centrifuge and discard supernatant. Transfer quantitatively to a tared stainless-steel planchet using a minimum quantity distilled water. Dry under an infrared lamp to constant weight. If after sufficient beta decay of the actinium fraction ^{228}Ra is found to be absent, make a direct alpha count for ^{226}Ra. If ^{228}Ra is present, determine ^{226}Ra by radon emanation, ¶ *c* below.

4) Count immediately in an alpha proportional counter.

c. Radium-226 by radon: Transfer the final precipitate obtained in *b* above to a small beaker using a rubber policeman and 14 mL EDTA reagent. Add a few drops 10*N* NaOH and heat on a hot plate to dissolve. Cool and transfer to a radon bubbler (Figure 7500-Ra:1) rinsing beaker with 1 mL EDTA reagent. Proceed as in 7500-Ra.C beginning with 7500-Ra.C.5*a*14).

5. Calculation

a. Calculation of ^{228}Ra concentration:

$$^{228}\text{Ra, pCi/L} = \frac{C}{2.22 \times EVR} \times \frac{\lambda t_2}{(1 - e^{-\lambda t_2})} \times \frac{1}{(1 - e^{-\lambda t_3})} \times \frac{1}{e^{-\lambda t_1}}$$

where:

C = average net count rate, cpm,
E = counter efficiency for ^{228}Ac,
V = sample volume L,
R = fractional chemical yield of yttrium carrier, 7500-Ra.D.4*a*10), multiplied by fractional chemical yield of barium carrier, 7500-Ra.D.4*b*3),
λ = decay constant of ^{228}Ac, 0.001 884/min,
t_1 = time between first $Y(OH)_3$ precipitation and start of counting, min,
t_2 = counting time, min, and
t_3 = ingrowth time of ^{228}Ac between last $BaSO_4$ precipitation and first $Y(OH)_3$ precipitation, min.

The factor $\lambda t_2/(1 - e^{-\lambda t_2})$ corrects average count rate to count rate at beginning of counting time.

b. Calculation of ^{226}Ra (plus any ^{224}Ra and ^{223}Ra) concentration: See 7500-Ra.B.5.

c. Calculation of ^{226}Ra (emanation) concentration: See 7500-Ra.C.6.

6. Bibliography

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7500-Ra E. Gamma Spectroscopy Method

1. General Discussion

a. Application: The gamma spectroscopy method for measuring ^{224}Ra, ^{226}Ra, and ^{228}Ra in ground and surface water may be used for drinking water. The radium isotopes can be assayed concurrently or individually in one sample-preparation procedure.

Ensure that the analyst is experienced with gamma-ray measurements and the mathematics and implications of transient equilibrium, because commercial gamma software does not have the capability to calculate sample concentration for transient equilibrium.

Preferably, have the analyst prepare at least three samples with known additions by the method before it is used for samples. This method also can be the basis for standard operating procedures (SOPs) that may differ with respect to the intrinsic efficiency of the detector, the method of precipitating radium, the method of yield determination, and the sample volume used with the chemistry.

b. Principle: Radium in water is separated from a 1- to 3-L sample by a fast lead sulfate ($PbSO_4$) or barium sulfate ($BaSO_4$) coprecipitation technique. The radium isotopes ^{224}Ra, ^{226}Ra, and ^{228}Ra are measured through their respective gamma-ray-emitting decay products, ^{212}Pb, ^{214}Pb (and/or ^{214}Bi), and ^{228}Ac, by means of a high-resolution intrinsic Ge detector. If ^{224}Ra is to be measured, prepare and count the water sample during the 2- to 4-d window after sample

collection because of this isotope's short half-life and radioactive growth/decay considerations (7500-Ra.E.5). If the presence of unsupported ^{212}Pb (^{212}Pb not due to ingrowth from ^{224}Ra parent) is suspected, at least two consecutive countings are required to assess this contribution. Repeat the counting after about 21 d to ensure that ^{226}Ra progeny are in equilibrium with their parent. At this point, the ^{228}Ac equilibration with its ^{228}Ra parent is already established. NOTE: Secular equilibrium of ^{228}Ac with ^{228}Ra is achieved about 36 to 60 h after precipitation (about 6 to 10 half-lives of ^{228}Ac).

c. Interferences: Ensure, by running laboratory blanks, that all materials used in the analysis are free from interferences under the conditions of analysis.

Lead-214, a ^{226}Ra progeny, has a 241.9-keV gamma ray (7.5% abundance). Similarly, ^{224}Ra has a 240.8-keV gamma ray (3.9% abundance). Resolve the photopeaks due to these two gamma rays correctly in the gamma-ray spectrum to minimize interference with the ^{212}Pb 238.6-keV peak, thus avoiding high bias for ^{224}Ra determination.

The carryover of ^{228}Th into the Pb(Ra)SO_4 precipitate has been measured to be about 5%. This is insignificant for drinking water samples because the typical range of activities for thorium radionuclides is below 0.1 pCi/L. Consequently, the ^{224}Ra activity due to ingrowth, during the time span between sample preparation and counting, from the carried-over ^{228}Th also is negligible and can be disregarded.

2. Apparatus

a. Analytical balance, 0.1 mg sensitivity.
b. Beakers, 4-L.
c. Watch glasses.
d. Drying lamp.
e. Gamma-ray spectroscopy system with high-resolution intrinsic Ge detector (or lithium drifted Ge detector).
f. Magnetic stirrers/hot plates and stirring bars.
g. Membrane filters, 47-mm diam, 0.45-μm porosity.
h. Petri dishes, approximately 3-in. diam.
i. Planchets, stainless steel, 5-cm diam, either shallow or deep type.
j. Polyethylene squeeze-bottles, 250- or 500-mL capacity.
k. Retaining rings, O-rings, or adhesives.
l. Snap-ring pliers to insert retaining rings.
m. Stirring-bar retriever.
n. Suction filter apparatus.
o. Volumetric flasks, 100-, 500-, 1000-, and 2000-mL.
p. Volumetric pipets, 1.0-, 5-, and 20-mL.

3. Reagents

a. For procedure using lead as the carrier in the coprecipitation step:

1) *Lead carrier*, 100 mg Pb^{2+}/mL—Dissolve 160 g reagent-grade $Pb(NO_3)_2$ in 800 mL distilled water and dilute to 1 L.

2) *Lead sulfate wash solution*—Dissolve 25 g anhydrous sodium sulfate in 1 L 1% sulfuric acid.

3) *Mixed-nuclide gamma-ray standard solution,* traceable to NIST—Prepare the source in a similar geometry as the precipitated sample for detector efficiency calibration. Alternately, purchase a similar source from a commercial supplier.

4) *Radium-226 and ^{228}Ra standards*, for preparation of known-addition QC samples and calibration of instrumentation (see Section 7020A.3*c, d,* and *e*).

5) *Reagent water,* distilled or deionized.

6) *Sodium sulfate* (Na_2SO_4), anhydrous.

7) *Sulfuric acid* (H_2SO_4), 18*M*.

b. For procedure using barium as the carrier in the coprecipitation step:

1) *Barium carrier,* 9 mg Ba^{2+}/mL—Dissolve 16.01 g reagent-grade $BaCl_2 \cdot 2H_2O$ in reagent water, add 5 mL 16*N* HNO_3, and dilute to 1 L with reagent water.

2) *Diethyl ether.*

3) *Ethanol.*

4) *Hydrochloric acid* (HCl), 12*N*.

5) *Mixed-nuclide gamma-ray standard solution*—See ¶ *a*3) above.

6) *Nitric acid* (HNO_3), 16*N*.

7) *Radium-226 and ^{228}Ra,* for preparation of known-addition QC samples and calibration of instrumentation (see Section 7020A.3*c, d,* and *e*).

8) *Reagent water,* distilled or deionized.

9) *Sulfuric acid* (H_2SO_4), 18*N*.

4. Procedure

a. Sample collection and preservation: Collect water samples in plastic bottles and acidify in the field, immediately after collection, with HNO_3 or HCl to a pH of 2.0 or lower. Record time and date of sample collection. If sample is not preserved in the field, acidify it in the laboratory and let it stand for at least 16 h before chemical separation.

b. Chemical separation: Use procedure 1) or 2) below.

1) Separation with lead as the carrier in coprecipitation—Measure 3 L sample by weight and transfer to a 4-L beaker. NOTE: For other sample portions, vary the concentrations of chemicals used in the subsequent steps proportionally, except for the lead carrier solution.

To the 3-L portion, add 24 g anhydrous sodium sulfate and 18 mL conc H_2SO_4. Place a magnetic stirring bar in the beaker, cover with a watch glass, and bring sample to a rolling boil on magnetic stirrer/hot plate. While stirring and boiling, add 1.0 mL lead carrier solution (100 mg/mL) dropwise to each beaker and continue boiling for 2 min. Then, add another 1.0 mL lead carrier solution similarly, and boil an additional 15 min. NOTE: In most cases, the rolling boiling water is sufficient to facilitate rapid and nearly homogeneous $PbSO_4$ precipitation. For samples with high total dissolved solids (TDS), constant stirring of water sample is recommended to avoid bumping.

Remove stirring bar and place beaker in an ice bath to cool. (Cooling without ice bath takes several hours.)

Pre-weigh a membrane filter. Isolate the lead sulfate precipitate by carefully pouring contents of beaker through filtration system fitted with pre-weighed filter. Use lead sulfate wash solution to complete the transfer.

Transfer filter to a planchet. Insert a retaining ring using snap-ring pliers, and place planchet in a Petri dish. Alternate means of securing the filter paper in a flat position, such as an

O-ring may be used. Retain filter after gamma-ray measurement for chemical yield determination.

2) Separation with barium as the carrier in coprecipitation—Measure 3 L sample by weight and transfer to a 4-L beaker. NOTE: For other sample portions, vary the concentrations of chemicals used in the subsequent steps proportionally except for the barium carrier solution. Add 30 mL of 12*N* HCl and stir. Using a volumetric pipet, add 15.0 mL barium carrier. Stir and heat to boiling. Precipitate barium sulfate by adding 30 mL of 18*N* H_2SO_4, stirring frequently. Boil for 30 min. Store overnight to let precipitate settle, or for fast settling, cool for 30 min in ice bath.

Pre-weigh a membrane filter. Isolate the barium sulfate precipitate by carefully pouring contents of beaker through the filtration system fitted with pre-weighed filter. Quantitatively transfer precipitate to filter by rinsing remaining particles from beaker with a jet of water.

Dry the precipitate on the filter with 10 mL ethanol, followed by 10 mL diethyl ether. Transfer filter to a planchet and secure filter paper in a flat position via double-sided transparent tape or a ring. Place planchet in a Petri dish. Retain filter for chemical yield determination.

c. Instrumental analysis:

1) Sample counting procedure—Reproducibly position the Petri dish containing coprecipitated sample source planchet on an intrinsic Ge detector. When performing gamma-ray measurements, observe the following scheme in accordance with the particular radium isotope of interest:

a) Radium-224 assay—This assay consists of one 1000-min overnight counting [or an appropriate count time to reach the desired minimum detectable concentration (MDC)] of the prepared sample 2 to 4 d after sample collection date. Use the 238.6-keV ^{212}Pb photopeak to determine the ^{212}Pb activity. Apply the standard decay and ingrowth equations to calculate the parent ^{224}Ra activity.

b) Radium-226 and ^{228}Ra assay—This assay consists of one 1000-min overnight counting (or an appropriate count time to reach the desired MDC) of the sample about 21 d after preparation. The gamma rays of ^{226}Ra progeny ^{214}Pb/^{214}Bi and of ^{228}Ra progeny ^{228}Ac are used for determinations. Allow adequate time between Ra separation and counting to establish equilibrium (ingrowth periods of 2 d and 21 d for ^{228}Ra and ^{226}Ra, respectively).

2) Chemical yield determinations—After gamma-ray spectroscopy measurements are completed, weigh filter paper (to nearest 0.1 mg) containing $PbSO_4$ or $BaSO_4$ precipitate for chemical yield determination.

3) Detector efficiency calibration—Distribute a portion of the mixed-gamma standard solution [7500-Ra.E.3*a*3)] uniformly as very fine droplets over a membrane filter (7500-Ra.E.2*g*), or use a similar source from a commercial supplier. Count prepared calibration standard on the Ge detector. Select count time to achieve a relative counting uncertainty of 1% (about 10,000 net counts in each photopeak used for the calibration. Alternately, the detector can be calibrated specifically for the gamma rays associated with Ra progeny detection using a calibration source prepared by using deionized water fortified with ^{224}Ra, ^{226}Ra, and ^{228}Ra standards, and carried through the entire sample preparation process. This calibration accounts for any summing effects as with the ^{214}Bi 609-keV gamma rays.

4) Background determination—Prepare a background sample, consisting of a membrane filter on a planchet without application of standard solution, and count for 1000 min or longer. The spectrum obtained from the analysis of background sample detector is used to create distinct background files. For each sample analysis, subtract the contributions of background peaks (as determined from the background file) from the sample peaks.

5. Calculation

a. Radium-224 measurement:

1) Lead-212 activity—Calculate ^{212}Pb activity with the equation:

$$A_{212} = \frac{C_s - C_b}{E \times A \times V \times 2.22 \times Y}\left(\frac{\lambda_{224} t_s}{1 - e^{-\lambda_{224} t_s}}\right)$$

where:

A_{212} = ^{212}Pb activity at start of count time, pCi/L,
C_s = sample net count rate under 238.6-keV photopeak region, cpm,
C_b = background net count rate for 238.6-keV photopeak region, cpm,
E = detection efficiency for 238.6-keV gamma ray, counts/gamma,
A = fractional gamma-ray abundance for 238.6-keV ^{212}Pb, 0.436 gamma/disintegration,
V = volume of sample, L,
2.22 = conversion factor from dpm to pCi,
Y = chemical recovery,
λ_{224} = ^{224}Ra decay constant = 1.31×10^{-4}/min, and
t_s = sample count time, min.

The multiplicative factor inside the parentheses in this equation represents the correction of ^{212}Pb activity for decay during the counting period.

2) Radium-224 activity—The correlation between ^{224}Ra parent and ^{212}Pb decay products is expressed as:

$$A_{212} = \frac{\lambda_{212}}{\lambda_{212} - \lambda_{224}} A^0{}_{224}(e^{-\lambda_{224} t} - e^{-\lambda_{212} t})$$

$$= 1.14 A^0{}_{224}(e^{-\lambda_{224} t} - e^{-\lambda_{212} t})$$

where:

λ_{212} = ^{212}Pb decay constant (1.086×10^{-3}/min),
$A^0{}_{224}$ = ^{224}Ra activity at sample-collection time, and
t = time from sample collection to the start of counting.

When the two equations above are combined,

$$A^0{}_{224} = \frac{C_s - C_b}{1.14 \times E \times A \times V \times 2.22 \times Y}\left(\frac{1}{e^{-\lambda_{224} t} - e^{-\lambda_{212} t}}\right)\left(\frac{\lambda_{224} t_s}{1 - e^{-\lambda_{224} t_s}}\right)$$

3) Minimum detectable count rate (MDR)—The MDR is the mean expected count rate of samples having a 5% probability of not being detected where activity is present. The MDR may be calculated from the formula:

$$MDR = \frac{2.71}{t_b} + \left[3.29 \times \sqrt{\frac{(C_c + C_b)}{t_b} \times \left(1 + \frac{t_b}{t_s}\right)}\right]$$

where:

t_b = background count time, and
C_c = Compton continuum background count rate under 238.6-keV photopeak region.

In the more specific case where sample count time is equal to background count time, $t_b = t_s$, this is simplified to

$$MDR = \frac{2.71}{t_s} + 4.65\sqrt{(C_c + C_b)/t_s}$$

4) Minimum detectable concentration (MDC)—The MDC for ^{212}Pb is calculated from the MDR as follows:

$$MDC_{212} = \frac{MDR}{E \times A \times V \times 2.22 \times Y}\left(\frac{\lambda_{224} t_s}{1 - e^{-\lambda_{224} t_s}}\right)$$

The MDC for ^{224}Ra at sample collection time, MDC^0_{224}, can be calculated using the first equation in ¶ 2) above and expressed as:

$$MDC^0_{224} = \frac{MDC_{212}}{1.14(e^{-\lambda_{224} t} - e^{-\lambda_{212} t})}$$

5) Uncertainties—If C_g represents the gross count rate in the 238.6−keV region, then:

$$C_s = C_g - C_c$$

The standard deviation associated with C_s is equal to $\sqrt{(C_g + C_c)/t_s}$ and the standard deviation associated with the background subtracted sample count rate under the 238.6-keV photopeak region (i.e., $C_s - C_b$) is given by

$$\sqrt{[(C_g + C_c)/t_s] + [C_b/t_b]}$$

and the relative count uncertainty (in percent) at the 95% confidence level is:

$$\%\ 1.96\ \text{SD} = \frac{1.96\sqrt{[(C_g + C_c)/t_s] + [C_b/t_b]} \times 100}{C_s - C_b}$$

b. Radium-226 and ^{228}Ra determinations: If commercial gamma software is available, ^{226}Ra and ^{228}Ra activities are measured through their respective progeny, ^{214}Pb (or ^{214}Bi) and ^{228}Ac, and are reported in the appropriate result documentation. Otherwise, the activity equation for both isotopes can be written as:

$$A_s = \frac{1}{2.22 \times n \times V \times Y \times D}\sum_{i=1}^{n}\frac{C_i}{E_i A_i}$$

where:

A_s = sample activity concentration, pCi/L,
n = the number of photopeaks used (ordinarily n=2),
V = volume of sample, L,
Y = chemical yield,
D = correction factor for decay/ingrowth,
C_i = net count rate in counts per minute for the ith peak, corrected for baseline, background, and interferences,
i = peak number (i=1, 2, . . . , n)
E_i = detection efficiency for peak i, and
A_i = branching fraction for peak i.

Other unit conversions can be handled by including a constant factor in the denominator of the expression for A_S.

The equation for the combined standard uncertainty (u) of A_S is shown below.

$$u_c(A_s) = \frac{1}{n \times V \times Y \times D}\sum_{i=1}^{n}\sqrt{\frac{u^2(C_i)}{E_i^2 A_i^2}}$$

The MDC for each photopeak i used in the analysis is calculated as:

$$MDC_i = \frac{MDR_i}{2.22 \times E_i \times A_i \times Y \times D_s}$$

where:

MDR_i is defined as in 7500-Ra.E.5*a*3).

TABLE 7500-Ra:III. RESULTS OF ^{224}Ra COLLABORATIVE STUDY

Portion Size *L*	Sample Concentration *pCi/L**	No. of Results	SD† *pCi/L*	Repeatability (Within-Laboratory SD) *pCi/L*	Reproducibility (Between-Laboratory Error) *pCi/L*	Total Uncertainty *pCi/L*
1	3.6	2	1.6	1.4	0.7	1.6
	5.8	4	1.3	1.7	0.6	1.8
	12.2	5	1.6	1.2	1.4	1.9
3	2.7	4	0.3	0.1	0.3	0.3
	5.8	8	1.0	1.2	0.9	1.5
	12.0	10	0.6	0.4	0.6	0.7
6	3.4	2	0.1	—	—	—
	6.0	4	0.2	—	—	—
	14.3	5	0.3	—	—	—

* Mean of individual laboratory means.
† Standard deviation.

Table 7500-Ra:IV. ^{226}Ra and ^{228}Ra Collaborative Study: Interlaboratory Results for Accuracy and Precision

	^{226}Ra		^{228}Ra	
	% Recovery	% RPD*	% Recovery	% RPD*
Average	100	7	101	6
SD	7	6	8	6
RPD Limit	—	17	—	18
Recovery lower limit	87	—	84	—
Recovery upper limit	115	—	117	—

* RPD = relative percent difference. Note: $N = 20$.
Based on data in DeHart, J.M. 2003 (corrected 2007). Radiochemistry. Alternate Test Procedure (ATP) Validation Study Report. EPA Sample Control Center (operated by CSC), Alexandria, Va.

When n photopeaks are used for each determination, the total MDC is:

$$\text{Total } MDC = \sum_{i=1}^{n} (MDC_i^2)^{1/2}/n$$

6. Quality Control

The quality control practices considered to be an integral part of each method can be found in Section 7020.

7. Precision and Bias

a. Radium-224 determination: A collaborative study, consisting of three laboratories analyzing three New Jersey groundwater samples at three concentration levels, was conducted to determine precision. The results are summarized in Table 7500-Ra:III. The true activity concentrations were not known, but it was known that the samples were at three distinct concentration levels. The samples were analyzed by gamma spectroscopy of the original water and radiochemical analysis of 1-L, 3-L, and 6-L portions of the water. Two laboratories analyzed the 1-L and 3-L portions, while the third analyzed the 6-L portions. The results of an analysis of variance (calculated via standard statistical methods) of the 3-L and 1-L portion data are given in the table. The precipitates from these portions were gamma-counted for various count times, but the vast majority were counted for 1000 min. The data for the 6-L portions are presented with the standard deviations for the three samples.

The chemical recoveries of lead carrier were determined by single-laboratory testing and multilab testing as described above. The chemical yields are 90% or greater and can be determined gravimetrically.

The radiochemical bias of the method was determined by single-laboratory analysis of water samples fortified at two levels with ^{228}Ra standard solution, in equilibrium with its progeny, including ^{224}Ra. For ^{224}Ra sample concentration of 1350 pCi/L, recovery was 98.5% and bias −1.5%; for concentration of 53.4 pCi/L, recovery was 108.9% and bias 8.9%.

Two approaches to gamma spectroscopic determination of ^{224}Ra, comparing the use of Pb versus Ba carriers were evaluated by comparing results of analyses of 20 randomly selected groundwater split samples between the New Jersey Department of Health and Senior Services (NJDHSS) Radiation Laboratory and U.S. Geological Survey Laboratory in Reston, Va. Aside from subtle differences, the two methods use similar approaches to the isolation of radium and its quantitation via gamma ray spectroscopy. Concentrations in the samples analyzed ranged from less than 0.5 to 12.6 pCi/L, with a median of 2.7 pCi/L. Results of the two-tailed paired t test indicated that the differences between concentrations in sample pairs were not different from zero at the 95% confidence level.

b. Radium-226 and radium-228 determinations: A data validation study was performed with the participation of the Georgia Institute of Technology Environmental Resource Center and the New Jersey Department of Health and Senior Services (NJDHSS) Radiation Laboratory. The study was based on an Alternate Test Procedure protocol developed by the U.S. Environmental Protection Agency. The process included background characterization, method detection limit, and method ruggedness studies. As a part of the study, samples from three public water supplies in different physiographic regions of Georgia were analyzed. The participating laboratories followed the sample preparation using Ba as the

Table 7500-Ra:V. ^{226}Ra and ^{228}Ra Collaborative Study: Lead Carrier Equivalency Study, LFM, LFMD Sample Results*

Sample ID	^{226}Ra Addition *pCi/L*	^{226}Ra Measured Value *pCi/L*	^{226}Ra Percent Recovery	^{226}Ra Measured Value Percent Difference	^{228}Ra Addition *pCi/L*	^{228}Ra Measured Value *pCi/L*	^{228}Ra Percent Recovery	^{228}Ra Measured Value Percent Difference
304328	0	2.2	—	—	0	5.2	—	—
304328 spk-1	21.3	23.2	98.6	5.0	24.6	28.1	93.1	4.9
304328 spk-2	21.3	24.4	104		24.6	29.5	98.8	
304329	0	3.3	—	—	0	0.1	—	—
304329 spk-1	21.4	24.3	98.1	0.82	24.7	23.1	93.1	8.3
304329 spk-2	21.5	24.5	98.6		24.7	25.1	101	
304327	0	3.3	—	—	0	0.5	—	—
304327 spk-1	21.3	23.9	96.7	1.2	24.7	23.5	93.1	4.6
304327 spk-2	21.3	24.2	98.1		24.7	24.6	97.6	

* Based on data in DeHart, J.M. 2003. Radiochemistry, Alternate Test Procedure (ATP) Validation Study Report, EPA Sample Control Center (operated by CSC), Alexandria, Va. (percent recovery and RPD values recalculated/rerounded). Percent Recovery for each LFM and LFMD calculated following subtraction of result for the associated unfortified sample. Relative percent difference calculated based on percent recovery for laboratory-fortified matrix (LFM) and laboratory-fortified matrix duplicate (LFMD) pairs.

carrier for the coprecipitation step. In addition, to demonstrate the equivalency between the two carriers, the NJDHSS Radiation Laboratory performed the same analyses, using Pb as the carrier in the coprecipitation step. Results are presented in Tables 7500-Ra:IV and V.

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7500-Rn RADON*

7500-Rn A. Introduction

1. Occurrence and Significance

Radon-222 is a gaseous decay product of naturally occurring radium-226. It is an alpha-emitter with a 3.82-d half-life, and normally is of concern only in groundwater. It is considered to be carcinogenic, as are its short-lived daughters. In household air, radon may originate from radium in building materials and the surrounding soil. Where radon concentration in the water supply is high, the water also can be a major source of radon in household air. While radon dissolves readily in water and other solvents, it is easily displaced from water by air; thus, aeration of radon-bearing water in normal household uses can release a significant fraction of the dissolved radon to the air.[1–3]

The average ^{222}Rn concentration in community groundwater systems in the U.S. is estimated to range from 200 to 600 pCi/L,[2–6] with some individual wells having much higher concentrations.

* Approved by Standard Methods Committee, 2006. Editorial revisions, 2011. Joint Task Group: 20th Edition—Stephen H. Pia (chair), Loren A. Berge, C. Richard Cothern, Paul B. Hahn, Vernon F. Hodge, Nancy E. Kinner, S. Kusum Perera, George S. Uyesugi.

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7500-Rn B. Liquid Scintillation Method

1. General Discussion

a. Principle: This method is specific for radon-222 (radon) in drinking water. Radon is partitioned selectively into a mineral-oil scintillation cocktail immiscible with the water sample. The sample is dark-adapted and equilibrated, and then counted in a liquid scintillation counter using a region or window of the energy spectrum optimal for radon alpha particles. Results are reported as pCi/L.

The procedure has been developed for the analysis of radon in drinking water supplies from groundwater and surface-water sources. Applications of this analytical procedure to matrices other than drinking water have not been studied; use caution in analyzing any such samples.

b. Interferences: There are no known chemical interferences from species found in drinking water nor from the dilute concentration of acid that may be present in the calibration standards. Uranium, radium, or other radioactive elements would cause a positive bias, if present in quantities significantly greater than the radon.

Diffusion of radon is affected by temperature and pressure. Let samples equilibrate to room temperature before processing.

Precision and accuracy of the method are affected by the background in the energy window used for analysis. A procedure is provided for selection of the analytical window to minimize the background contribution to the measurement.

Some cocktails will become progressively quenched by atmospheric oxygen after opening. This problem has not been noted for the mineral-oil-based cocktail. For other than mineral-oil-based cocktails, check weekly for quenching.

Radon has an affinity for some plastics used in sample containers. Use only glass sample containers or glass scintillation vials with TFE or foil-lined caps.

c. Sample preservation, storage, and holding time: Collect samples from a nonaerated faucet that has been allowed to flow for sufficient time so that the sample is representative of the water in the distribution system or well. The following procedure will minimize the loss of radon from the sample during collection:

Place a glass sample vial in a 300- to 600-mL beaker or other suitable container; attach delivery tube to faucet, and start the flow. Make sure that delivery tube does not let bubbles enter the sample. Fill vial to prevent its floating, then fill beaker until vial is submerged. Place tip of delivery tube about two thirds of the way into vial and fill until approximately two or more vial volumes (50 to 100 mL) have been displaced. Carefully remove vial by hand or with a pair of 25-cm (10 in.) tweezers and cap vial with a TFE or foil-lined cap. Cap sample vials underwater,

if possible. Invert sample and check for air bubbles. If any bubbles are present, discard sample and repeat sampling procedure.

Alternatively, collect samples in containers other than scintillation vials, with similar precautions.

Record date and time of sample collection and store sample in a cooler. Transport samples to laboratory in a cooler or other suitable insulated package to avoid large temperature changes and outgassing of radon. Begin counting within 4 d or applicable regulatory specified holding time.

d. Minimum detectable concentration: 18 pCi/L for a 50-min count time, 6 cpm background, 6 cpm/pCi calibration factor, and energy region optimized by the procedure in 7500-Rn.B.4*b*.

2. Apparatus

a. Pipet: 5-mL mechanical pipet or syringe.

b. Scintillation cocktail dispenser adjustable to deliver 5 mL.

c. Liquid scintillation counter: Preferably use a system permitting automatic spectral analysis.

d. Faucet connector or universal faucet adapter.

e. Plastic tubing for connector or adapter.

f. Scintillation vials: 23-mL glass vials with caps, TFE or foil-lined.

g. Volumetric glassware.

h. Sample storage and shipping containers, insulated.

3. Reagents

a. Scintillation cocktail: Water-immiscible high-efficiency mineral oil cocktail or other commercial equivalent.

b. Hydrochloric acid (HCl), conc.

c. Water, radon-free demineralized or equivalent.

d. Radium solution: Use two dilutions for calibration and check standards. Use NIST-traceable (explicit or implicit) radium-226 standard solution.

4. Procedure

a. Calibration: Prepare 100 mL radium-226 in water standard such that the final activity will be approximately 8000 pCi/L by the procedure suggested below. Transfer standard to a scintillation vial or other suitable container, seal, and record initial mass to nearest 0.0001 g. To a 100-mL volumetric flask add 20 mL water and 0.5 mL conc HCl; stopper. Transfer with a pipet, or suitable dropper, the required mass of radium solution into flask; re-weigh vial. Obtain actual mass of radium solution added by difference of final and initial weights. Fill to mark and mix.

Transfer 15 mL diluted standard into scintillation vial, to which has been added 5 mL mineral oil cocktail. Prepare at least three standards and three backgrounds using distilled or deionized water. The relative percent deviation spread of the resultant calibration factors should be $<5\%$.

Set standards and background samples aside for at least 25 d (99% ingrowth) to allow radon progeny to attain secular equilibrium with radium-226. Determine optimal analytical window as outlined in ¶ *b* below. After ingrowth period, let sample dark-adapt for 3 h if necessary and count for 50 min. Repeat counting two additional times. From pooled results calculate a system calibration factor by the following expression:

$$CF = \frac{S - B}{C \times V}$$

where:

CF = calibration factor, cpm/pCi,
S = standard counting rate, cpm,
B = background counting rate, cpm,
C = concentration of radium-226 standard, pCi/L, and
V = volume of standard used, 0.015 L.

b. Selecting optimal window: Count a radon standard for 5 min or sufficient time to acquire several thousand counts or more in the alpha region and generate a sample spectrum. For greater clarity use a log scale for the channel number or energy axis if possible.

The alpha activity region of interest will be obvious as one or two large peaks at the higher end of the energy spectrum. The lower peak is the doublet of radon-222 and polonium-218 and the higher peak is that of polonium-214. The optimal window is formed by extending the region by approximately 10 channels on each side of the alpha peaks. Use this window for subsequent calibration and analysis. Calibration factor should be at least 6 cpm/pCi with the background not exceeding 6 cpm.

For counters not having a spectrum display, set window initially wide-open and count for sufficient time to obtain several thousand counts. Adjust energy window to a width of 5% of full scale at upper end of scale (95 to 100%) and determine count rate in the region. Repeat counts at successively lower regions using the same 5% interval (90 to 95, 85 to 90, 80 to 85, etc.). Plot count rate versus midpoint of interval and choose region of interest, which will be evident by one or two prominent peaks in the upper half of the energy scale. Calibration factor should be at least 6 cpm/pCi with the background not exceeding 6 cpm.

c. Analysis of samples: Carefully remove by pipet 8.5 mL sample from the scintillation vial used for collection and add 5 mL water-immiscible scintillation cocktail. Alternatively, pipet, without turbulence, a 15-mL portion to a scintillation vial containing 5 mL cocktail if sample was collected in a different container.

Cap and shake sample for 30 s and set aside in the dark for a minimum of 3 h to equilibrate radon progeny and dark-adapt sample. Count all samples within the regulation specified holding time. The time of sample collection is the initial time for decay correction.

Count a standard for 5 min or longer if required and either examine spectrum or compare results to previous standards to determine if there has been any shift or quench due to changes in the cocktail or instrument drift. Count samples for 50 min or to a percent 2σ counting error of 10% or for a period of time to achieve an uncertainty in the net counting rate corresponding to program data quality objectives using optimized window settings for alpha counting. Make sure the expression

used includes the background in the uncertainty computation. This may have to be done manually because most instruments calculate the uncertainty only for the gross counting rate.

5. Calculations

Calculate concentration of radon-222 in pCi/L from the following equation:

$$\text{Rn, pCi/L} = \frac{G - B}{CF \times D \times V}$$

where:

G = gross counting rate of sample, cpm,
B = background counting rate, cpm,
CF = calibration factor (see 7500-Rn.B.4*a*),
V = volume of sample (~0.015 L), and
D = decay factor for Rn-222 between time of collection and midpoint of counting period for that sample.

Calculate 2σ (95% confidence level) counting uncertainty, as:

$$2\sigma\text{, pCi/L} = \frac{2 \times \sqrt{\frac{G}{T_G} + \frac{B}{T_B}}}{CF \times D \times V}$$

where:

T_G = duration of sample count, and
T_B = duration of background count.

Report 2σ uncertainty with each drinking water radioactivity concentration result. This term represents the uncertainty due to the random nature of radioactive decay; it is related to count time and can be used to determine whether sample was counted long enough to satisfy any required precision criteria. If percent counting error (2σ counting error divided by activity concentration) exceeds precision requirements, count sample longer or reduce the holding time on re-collected samples.

Report result and counting error together in the form:

$$X \pm 2\sigma \text{ pCi/L, } 2\sigma \text{ counting error}$$

For example, for a water sample with calculated radon-222 concentration of 285 pCi/L and 2σ counting error of 27 pCi/L, report result as:

$$^{222}\text{Rn: } 285 \pm 27 \text{ pCi/L, } 2\sigma \text{ counting error}$$

6. Quality Control

The quality control practices considered to be an integral part of each method can be found in Section 7020.

a. Background samples: Include a minimum of two background samples with each batch of 20 samples. Place backgrounds as first and next-to-last samples of batch. Use average of these backgrounds to calculate results for batch. The background should be ≤6 cpm.

For a suitable background sample, use laboratory deionized water, or prepare by boiling 2 L laboratory radium- and uranium-free tap water to remove residual radon if present. Store the cooled tap water in a capped 2-L bottle.

b. Duplicate samples: Collect duplicate field samples for one out of every ten samples. Preferably collect all samples in duplicate if the number of samples from an individual client represents a single source. Ensure that at least 10% of the samples analyzed daily are duplicates, and that duplicate analyses have a relative percent difference (RPD) less than or equal to the percent 2σ counting error or 10% of the decay-corrected radon concentration, whichever is greater. Relative percent difference is calculated by the following expression:

$$RPD = \frac{|\text{Analysis 1} - \text{Analysis 2}| \times 200}{\text{Analysis 1} + \text{Analysis 2}}$$

Record the RPD and note acceptability of the duplicate analysis. If RPD exceeds the limits, recount duplicates. If results still exceed limits but RPD for the quality control check standard is acceptable, a problem with the sampling procedure may exist. Resolve problem before collecting and analyzing additional samples.

c. Quality control check standard (QCCS): QCCSs are prepared from a dilution of radium different from that used to prepare standards and should have a nominal activity of ~8000 pCi/L. Place first QCCS immediately after first background and before first sample. Place additional QCCS after every tenth sample in batch, and final QCCS as last sample of the batch.

The relative percent difference (RPD) between sequential pairs of QCCS samples must be less than or equal to the 2σ counting error or 10% of the known value of the QCCS sample, whichever is greater. If RPD exceeds this value, recount the pair of QCCS samples. If RPD is still unacceptable, standards and/or instrument are suspect. Resolve problem and rerun samples between suspect QCCS.

d. Records: Collect and maintain results from backgrounds, duplicate pairs, and QCCS standards in a bound notebook; include date, results, name of analyst, and comments relevant to data evaluation.

Plot averages of backgrounds and QCCS standards on a control chart for the counter.

7. Precision and Bias

A collaborative study of this method composed of 36 participants[1] produced the results shown below:

Sample Conc. *pCi/L*	Accuracy %	Repeatability *pCi/L*	Reproducibility *pCi/L*	Bias %
111	101–102	9	12	0.7–2.3
153	102–103	10	16–18	2.3–3.4

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7500-Sr TOTAL RADIOACTIVE STRONTIUM AND STRONTIUM-90*

7500-Sr A. Introduction

The important radioactive nuclides of strontium produced in nuclear fission are ^{89}Sr and ^{90}Sr. Strontium-90 is one of the most hazardous of all fission products. It decays slowly, with a half-life of 28 years. Upon ingestion, strontium is concentrated in the bone.

The method presented in this section is designed to measure total radioactive strontium (^{89}Sr and ^{90}Sr) or ^{90}Sr alone in drinking water or in filtered raw water. It is applicable to sewage and industrial wastes provided that steps are taken to destroy organic matter and eliminate other interfering ions.

* Approved by Standard Methods Committee, 2001. Editorial revisions, 2011.

7500-Sr B. Precipitation Method

1. General Discussion

a. Principle: A known amount of inactive strontium ions, in the form of strontium nitrate, $Sr(NO_3)_2$, is added as a "carrier." The carrier, alkaline earths, and rare earths are precipitated as the carbonate to concentrate the radiostrontium. The carrier, along with the radionuclides of strontium, is separated from other radioactive elements and inactive sample solids by precipitation as $Sr(NO_3)_2$ from fuming nitric acid solution. The strontium carrier, together with the radionuclides of strontium, finally is precipitated as strontium carbonate, $SrCO_3$, which is dried, weighed to determine recovery of carrier, and measured for radioactivity. The activity in the final precipitate is due to radioactive strontium only, because all other radioactive elements have been removed. A correction is applied to compensate for losses of carrier and activity during the various purification steps. A delay in the count will give an increased counting rate due to the ingrowth of ^{90}Y.

b. Concentration techniques: Because of the very low amount of radioactivity, a large sample must be taken and the activity concentrated by precipitation. $Sr(NO_3)_2$ and barium nitrate, $Ba(NO_3)_2$, carriers are added to the sample. Sodium carbonate is then added to concentrate radiostrontium by precipitation of alkaline earth carbonates along with other radioactive elements. The supernate is discarded. The precipitate is dissolved and reprecipitated to remove interfering radionuclides.

c. Interference: Radioactive barium (^{140}Ba, ^{140}La) interferes in the determination of radioactive strontium inasmuch as it precipitates with the radioactive strontium. Eliminate this interference by adding inactive $Ba(NO_3)_2$ carrier and separating this from the strontium by precipitating barium chromate in acetate buffer solution. Radium isotopes also are eliminated by this treatment.

In hard water, some calcium nitrate may be coprecipitated with $Sr(NO_3)_2$ and can cause errors in recovery of the final precipitate and in measuring its activity. Eliminate this interference by repeated precipitations of strontium as the nitrate followed by leaching the $Sr(NO_3)_2$ with acetone **(CAUTION)**.

For total radiostrontium, count the precipitate within 3 to 4 h after the final separation and before ingrowth of ^{90}Y.

d. Determination of ^{90}Sr: Because it is impossible to separate the isotopes ^{89}Sr and ^{90}Sr by any chemical procedure, the amount of ^{90}Sr is determined by separating and measuring the activity of ^{90}Y, its daughter. After equilibrium is reached, the activity of ^{90}Y is exactly equal to the activity of ^{90}Sr. Two alternate procedures are given for the separation of ^{90}Y. In the first method, ^{90}Y is separated by extraction into tributyl phosphate from concentrated nitric acid (HNO_3) solution. It is back-extracted into dilute HNO_3 and evaporated to dryness for beta counting. The second method consists of adding yttrium carrier, separating by precipitation as yttrium hydroxide, $Y(OH)_3$, and finally precipitating yttrium oxalate for counting.

e. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 7020.

2. Apparatus

a. Counting instruments: Use either an internal proportional counter, gas-flow, with scaler, timer, and register; or a thin end-window (polyester plastic film*) proportional or G-M counting chamber with scaler, timer, register amplifier, and preferably having an anticoincident system (low background).

b. Filter paper,† 2.4-cm diam; or glass fiber filters, 2.4-cm diam.

c. Two-piece filtering apparatus for 2.4-cm filters such as TFE filter holder,‡ stainless steel filter holder, or equivalent.

d. Stainless steel pans, about 50 mm diam and 7 mm deep, for counting solids deposited on pan bottom. For counting precipitates on 2.4-cm filters, use nylon disk with ring§ on which the filter samples are mounted and covered by 0.25 mil film.

3. Reagents

a. Strontium carrier, 10 mg Sr^{2+}/mL, standardized: Carefully add 24.16 g $Sr(NO_3)_2$ to a 1-L volumetric flask and dilute with distilled water to the mark. For standardization, pipet three

* Mylar, E. I. du Pont de Nemours, Wilmington, DE, or equivalent.
† Whatman No. 42, or equivalent.
‡ Flurolon Laboratory, Box 305, Caldwell, NJ.
§ Control Molding Corp., Staten Island, NY, or equivalent.

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10.0-mL portions of strontium carrier solution into 40-mL centrifuge tubes and add 15 mL 2*N* Na_2CO_3 solution. Stir, heat in a boiling water bath for 15 min, and cool. Filter $SrCO_3$ precipitate through a tared fine-porosity sintered-glass crucible of 15-mL size. Wash precipitate with three 5-mL portions of water and then with three 5-mL portions of absolute ethanol (or acetone). Wipe crucible with absorbent tissue and dry to constant weight in an oven at 110°C (20 min). Cool in a desiccator and weigh.

$$\text{Sr, mg/mL} = \frac{(\text{mg } SrCO_3)\ (0.5935)}{10}$$

b. Barium carrier, 10 mg Ba^{2+}/mL: Dissolve 19.0 g $Ba(NO_3)_2$ in distilled water and dilute to 1 L.

c. Rare earth carrier, mixed: Dissolve 12.8 g cerous nitrate hexahydrate, $Ce(NO_3)_3 \cdot 6H_2O$, 14 g zirconyl chloride octahydrate, $ZrOCl_2 \cdot 8H_2O$, and 25 g ferric chloride hexahydrate, $FeCl_3 \cdot 6H_2O$, in 600 mL distilled water containing 10 mL conc HCl, and dilute to 1 L.

d. Yttrium carrier: Dissolve 12.7 g yttrium oxide,‖ Y_2O_3, in 30 mL conc HNO_3 by stirring and warming. Add an additional 20 mL conc HNO_3 and dilute to 1 L with distilled water; 1 mL is equivalent to 10 mg Y, or approximately 34 mg $Y_2(C_2O_4)_3 \cdot 9H_2O$. Determine exact equivalence by precipitating yttrium carrier in acid solution according to 7500-Sr.B.4*c*2)–8), or by extracting yttrium carrier in acid solution according to 7500-Sr.B.4*b*3)–11).

e. Acetate buffer solution: Dissolve 154 g $NH_4C_2H_3O_2$ in 700 mL distilled water, add 57 mL conc acetic acid, adjust pH to 5.5 by dropwise addition of conc acetic acid or 6*N* NH_4OH as necessary, and dilute to 1 L.

f. Acetic acid, 6*N*.

g. Acetone, anhydrous.

h. Ammonium hydroxide (NH_4OH), 6*N*.

i. Hydrochloric acid (HCl), 6*N*.

j. Methyl red indicator, 0.1%: Dissolve 0.1 g methyl red in 100 mL distilled water.

k. Nitric acid (HNO_3), fuming (90%), conc, 14*N*, 6*N*, and 0.1*N*.

l. Oxalic acid, saturated solution: Dissolve approximately 11 g $H_2C_2O_4 \cdot 2H_2O$ in 100 mL distilled water.

m. Sodium carbonate solution, 1*M:* Dissolve 124 g $Na_2CO_3 \cdot H_2O$ in distilled water and dilute to 1 L.

n. Sodium chromate solution, 0.5*M:* Dissolve 117 g $Na_2CrO_4 \cdot 4H_2O$ in distilled water and dilute to 1 L.

o. Sodium hydroxide, 6*N:* Dissolve 240 g NaOH in distilled water and dilute to 1 L.

p. Tributyl phosphate, reagent grade: Shake with an equal volume of 14*N* HNO_3 to equilibrate. Separate and discard the HNO_3 washings.

4. Procedure

a. Total radiostrontium:

1) To 1 L of drinking water, or a filtered sample of raw water in a beaker, add 2.0 mL conc HNO_3 and mix. Add 2.0 mL each of strontium and barium carriers and mix well. (A precipitate of $BaSO_4$ may form if the water is high in sulfate ion, but this will cause no difficulties.) A smaller sample may be used if it contains at least 25 pCi strontium. The suspended matter that has been filtered off may be digested [Section 7110B.4*f*1)], diluted, and analyzed separately.

2) Heat to boiling, then add 20 mL 6*N* NaOH and 20 mL 1*M* Na_2CO_3. Stir and let simmer at 90 to 95°C for about 1 h.

3) Set beaker aside until precipitate has settled (about 1 to 3 h).

4) Decant and discard clear supernate. Transfer precipitate to a 40-mL centrifuge tube and centrifuge. Discard supernate.

5) Add, dropwise (**Caution: Effervescence**), 4 mL conc HNO_3. Heat to boiling, stir, then cool under running water.

6) Add 20 mL fuming HNO_3, cool 5 to 10 min in ice bath, stir, and centrifuge. Discard supernate.

7) Add 4 mL distilled water, stir, and heat to boiling to dissolve the strontium. Centrifuge while hot to remove remaining insolubles and decant supernate to a clean centrifuge tube. Add 2 mL 6*N* HNO_3, heat to boiling, centrifuge while hot, and combine supernate with aqueous supernate. Discard insoluble residue of SiO_2, $BaSO_4$, etc.

8) Cool combined supernates, then add 20 mL fuming HNO_3, cool 5 to 10 min in ice bath, stir, centrifuge, and discard supernate.

9) Add 4 mL distilled water and dissolve by heating. Repeat ¶ *a*8) above.

10) Repeat ¶ *a*9) above if more than 200 mg Ca were present in the sample.

11) After last HNO_3 precipitation, invert tube in a beaker for about 10 min to drain off most excess HNO_3. Add 20 mL anhydrous acetone, stir thoroughly, cool, and centrifuge. Discard supernate (**Caution**).

12) Dissolve precipitate of $Sr(NO_3)_2$+ $Ba(NO_3)_2$ in 10 mL distilled water and boil for 30 s to remove any remaining acetone.

13) Add 0.25 mL (5 drops) mixed rare earth carrier and precipitate rare earth hydroxides by making solution basic with 6*N* NH_4OH. Digest in a boiling water bath for 10 min. Cool, centrifuge, and decant supernate to a clean tube. Discard precipitate.

14) Repeat ¶ *a*13) above.

Note the time of rare earth precipitation, which marks the beginning of the ^{90}Y ingrowth period. Do not delay procedure more than a few hours after the separation; otherwise, false results will be obtained because of ingrowth of ^{90}Y.

15) Add 2 drops methyl red indicator and then add 6*N* acetic acid dropwise with stirring until indicator changes from yellow to red.

16) Add 5 mL acetate buffer solution, heat to boiling, and add dropwise, with stirring, 2 mL Na_2CrO_4 solution. Digest in a boiling water bath for 5 min. Cool, centrifuge, and decant supernate to a clean tube. Discard residue.

17) Add 2 mL 6*N* NaOH, add 5 mL 1*M* Na_2CO_3 solution, and heat to boiling. Cool in an ice bath (about 5 min) and centrifuge. Discard supernate.

18) Add 15 mL distilled water, stir, centrifuge, and discard wash water.

19) Repeat ¶ *a*18) above, and proceed either as in ¶ *a*20)a) or *a*20)b) below. *Save this precipitate if a determination of ^{90}Sr is required.*

20) Either:

‖ Yttrium oxide, Code 1118, American Potash and Chemical Corp., West Chicago, IL, or equivalent. Yttrium oxide of purity less than Code 1118 may require purification because of radioactivity contamination.

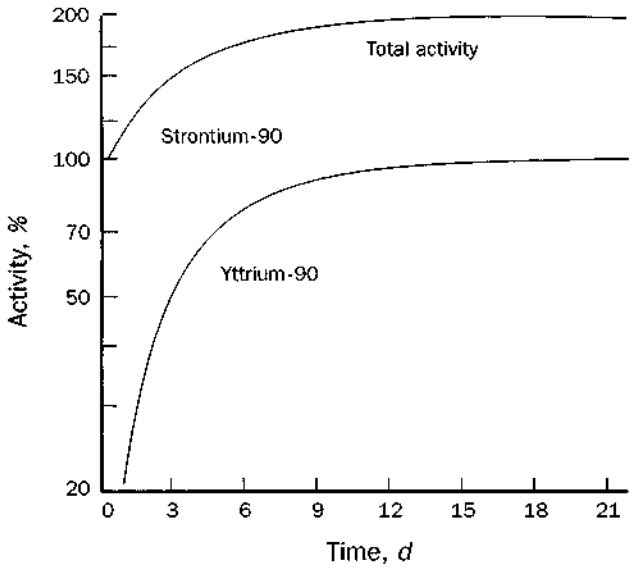


Figure 7500-Sr:1. Yttrium-90 vs. strontium-90 activity as a function of time.

a) Slurry precipitate with a small volume of distilled water and transfer to a tared stainless steel pan; dry under an infrared lamp, cool, weigh, and count# the precipitate of $SrCO_3$;** or

b) Transfer precipitate to a tared paper or glass filter mounted in a two-piece funnel. Allow gravity settling for uniform deposition and then apply suction. Wash precipitate with three 5-mL portions of water, three 5-mL portions of 95% alcohol, and three 5-mL portions of ethyl ether or acetone. Dry in an oven at 110 to 125°C for 15 to 30 min, cool, weigh,** mount on a nylon disk and ring with polyester plastic film cover, and count.

21) Calculation

$$\text{Total Sr activity, pCi/L} = \frac{b}{adf \times 2.22}$$

where:

a = beta counter efficiency [see ¶ *a*22) below],

$d = \dfrac{\text{mg final } SrCO_3 \text{ precipitate}}{\text{mg } SrCO_3 \text{ in 2 mL of carrier}}$
= correction for carrier recovery [see ¶ *a*23) below],

f = sample volume, L,

b = beta activity, net cpm = $(i/t)-k$,

i = total counts accumulated,

t = time of counting, min, and

k = background, cpm.

22) Counting efficiency—As a first estimate, when mounting sample according to ¶ *a*20)a) above, convert counts per minute to disintegrations per minute, based on the beta activity of cesium-137 standard solutions having a sample thickness equivalent to that of the $SrCO_3$ precipitate. More precise measurements may follow a second count after substantial ingrowth of ^{90}Y from ^{90}Sr, but this precision is not warranted for the usual total radiostrontium determination. When mounting samples according to ¶ *a*20)b) above, determine self-absorption curves by separately precipitating standard solutions of ^{89}Sr and ^{90}Sr as the carbonate (see gross beta in Section 7110).

23) Correction for carrier recovery—20 mg Sr are equivalent to 33.7 mg $SrCO_3$. Should more than traces of stable strontium be present in the sample, it would act as carrier; hence its determination by flame photometric or atomic absorption spectrometric method would be required.

b. Strontium-90 by extraction of yttrium-90:†† Store $SrCO_3$ precipitate, as in ¶ *a*20) above, for at least 2 weeks to allow ingrowth of ^{90}Y and then proceed as directed here or in an alternate procedure in ¶ *c* below.

1) Transfer of precipitate to separatory funnel—Either

a) Place a small funnel upright into mouth of a 60-mL separatory funnel; then place pan with precipitate, as in ¶ *a*20)a) above, in funnel and add, dropwise, 1 mL 6*N* HNO_3 (**CAUTION: effervescence**); tilt pan to empty into funnel and rinse pan twice with 2-mL portions of 6*N* HNO_3; or ¶ *a*20)b) above,

b) Uncover precipitate from filter, as in 7500-Sr.B.4*a*20)b), and transfer filter with forceps to upright funnel in mouth of 60-mL separatory funnel as in ¶ *b*1)a) above. Dislodge bulk of precipitate into funnel stem. Dropwise, add with caution 1 mL 6*N* HNO_3 to filter, removing residual precipitate and dissolving bulk precipitate. Rinse filter and funnel twice with 2-mL portions 6*N* HNO_3.

2) Remove filter or pan and add 10 mL fuming HNO_3 to separatory funnel through upright funnel.

3) Remove upright funnel and add 1 mL yttrium carrier in a separatory funnel.

4) Add 5.0 mL tributyl phosphate reagent, shake thoroughly for 3 to 5 min, allow phases to separate, and transfer aqueous layer to a second 60-mL separatory funnel.

5) Add 5.0 mL tributyl phosphate reagent, shake 5 min, allow phases to separate, and transfer aqueous layer to a third 60-mL separatory funnel.

6) Combine organic extractants in the first and second funnels into one funnel and wash organic phase twice with 5-mL portions 14*N* HNO_3. Record time as the beginning of ^{90}Y decay (combine acid washings with aqueous phase in third funnel if a second ingrowth of ^{90}Y is desired).

7) Back-extract ^{90}Y from combined organic phases with 10 mL 0.1*N* HNO_3 for 5 min.

8) Continue as in ¶s *c*6)–8) below or transfer aqueous phase from ¶ *b*7) above into a 50-mL beaker and evaporate on a hot plate to 5 to 10 mL.

9) Repeat ¶ *b*7) above and transfer aqueous phase to beaker in ¶ *b*8) above; evaporate to 5 to 10 mL.

10) Transfer residual solution in beaker to a tared stainless steel counting pan and evaporate.

Strontium-90 in thick samples is counted with low efficiency; hence, a first count within hours favors ^{89}Sr counting, and a recount after 3 to 6 d that exceeds the first count provides a rough estimate of the ^{90}Y ingrowth—see Figure 7500-Sr:1 and R.J. Velten (1966).

** When a determination of total strontium is not required, weigh precipitate [¶ *a*20)a) or *a*20)b)] for carrier recovery but do not count. Then proceed with ^{90}Sr determination according to 7500-Sr.B.4*b*.

†† See footnote to 7500-Sr.B.4*a*20)a) when a determination for only ^{90}Sr is required.

11) Rinse beaker twice with 2-mL portions of 0.1*N* HNO_3; add rinsings to counting pan, evaporate to dryness, and weigh.

12) Count in an internal proportional or end-window counter and calculate ^{90}Sr as given in ¶ *c*9) below.

c. Strontium-90 by oxalate precipitation of yttrium-90:††

1) Quantitatively transfer $SrCO_3$ precipitate to a 40-mL centrifuge tube with 2 mL 6*N* HNO_3. Add acid dropwise during dissolution (**Caution—effervescence**). Use 0.1*N* HNO_3 for rinsing.

2) Add 1 mL yttrium carrier, 2 drops methyl red indicator and, *dropwise,* add conc NH_4OH to the methyl red endpoint.

3) Add 5 mL more conc NH_4OH and *record the time,* which is the end of ^{90}Y ingrowth and the beginning of decay; centrifuge and decant supernate to a beaker (save supernate and washings for a second ingrowth if desired).

4) Wash precipitate twice with 20-mL portions hot distilled water.

5) Add 5 to 10 drops of 6*N* HNO_3, stir to dissolve precipitate, add 25 mL distilled water, and heat in a water bath at 90°C.

6) Gradually add 15 to 20 drops saturated oxalic acid reagent with stirring and adjust to pH 1.5 to 2.0 (pH meter or indicator paper) by adding conc NH_4OH dropwise. Digest precipitate for 5 min and cool in an ice bath with occasional stirring.

7) Transfer precipitate to a tared glass fiber filter in a two-piece funnel. Let precipitate settle by gravity (for uniform deposition) and apply suction. Wash precipitate in sequence with 10 to 15 mL hot distilled water and then three times with 95% ethyl alcohol and three times with diethyl ether.

8) Air-dry precipitate with suction for 2 min, weigh, mount on a nylon disk and ring with polyester plastic film cover, count, and calculate ^{90}Sr as follows.

9) Calculation

$$^{90}\text{Sr, pCi/L} = \frac{\text{net cpm}}{a\ b\ c\ d\ f\ g \times 2.22}$$

where:

a = counting efficiency for ^{90}Y,
b = chemical yield of extracting or precipitating ^{90}Y,
c = ingrowth correction factor if not in secular equilibrium,
d = chemical yield of strontium determined gravimetrically or by flame photometry,
f = volume of original sample, L,
g = ^{90}Y decay factor, $e^{-\lambda t}$, and
e = base of natural logarithms,
λ = $0.693/T_{1/2}$, where $T_{1/2}$ for ^{90}Y is 64.2 h, and
t = time between separation and counting, h.

5. Precision and Bias

In a collaborative study of two sets of paired, moderately hard water samples containing known additions of radionuclides, 12 laboratories determined the total radiostrontium and 10 laboratories determined ^{90}Sr. The results of one sample from one laboratory were rejected as outliers.

The average recoveries of added total radiostrontium from the four samples were 99, 99, 96, and 93%. The precision (random error) at the 95% confidence level was 10 and 12% for the two sets of paired samples. The method was slightly biased on the low side.

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7500-^{3}H TRITIUM*

7500-^{3}H A. Introduction

Tritium exists fairly uniformly in the environment as a result of natural production by cosmic radiation and residual fallout from nuclear weapons tests. This background level gradually is being increased by the use of nuclear reactors to generate electricity, although tritium from this source is only a small proportion of environmental tritium. Nuclear reactors and fuel-processing plants are localized sources of tritium because of discharges during normal operation. This industry is expected to become the major source of environmental tritium contamination in the future. Tritium is produced in light-water nuclear reactors by ternary fission, neutron capture in coolant additives, control rods and plates, and activation of deuterium. About 1% of the tritium in the primary coolant is released in gaseous form to the atmosphere; the remainder eventually is released in liquid waste discharges. Most tritium produced in reactors remains in the fuel and is released when fuel is reprocessed.

Naturally occurring tritium is most abundant in precipitation and lowest in aged water because of its physical decay by beta emission to helium. The maximum beta energy of tritium is 0.018 MeV and its half-life is 12.26 years.

* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011.

7500-^{3}H B. Liquid Scintillation Spectrometric Method

1. General Discussion

a. Principle: A sample is treated by alkaline permanganate distillation to hold back most quenching materials, as well as radioiodine and radiocarbon. Complete transfer of tritiated water is assured by distillation to near dryness. A subsample of distillate is mixed with scintillation solution and the beta activity is counted on a coincidence-type liquid scintillation spectrometer. The scintillation solution consists of 1,4-dioxane, naphthalene, POPOP, and PPO.* The spectrometer is calibrated with standard solutions of tritiated water; then background and unknown samples are prepared and counted alternately, thus nullifying errors that could result from instrument drift or from aging of the scintillation solution.

b. Interferences: Sample distillation effectively removes nonvolatile radioactivity and the usual quenching materials. For waters containing volatile organic or radioactive materials, use wet oxidation (Section 4500-N_{org}) to remove interference from quenching due to volatile organic material. Distillation at about pH 8.5 holds back volatile radionuclides such as iodides and bicarbonates. Double distillation with an appropriate delay (10 half-lives) between distillations may be required to eliminate interference from volatile daughters of radium isotopes. Some clear-water samples collected near nuclear facilities may be monitored satisfactorily without distillation, especially when the monitoring instrument is capable of discriminating against beta radiation energies higher than those in the tritium range.

c. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 7020.

* POPOP = 1,4-di-2-(5-phenyloxazolyl) benzene; PPO = (2,5- diphenyloxazole).

2. Apparatus

a. Liquid scintillation spectrometer, coincidence-type.

b. Liquid scintillation vial: 20-mL; polyethylene, low-K glass, or equivalent bottles.

c. Distillation apparatus: 250-mL round-bottom distillation flask, connecting side-arm adapter, condenser, and heating mantle.

3. Reagents

a. Scintillation solution: Thoroughly mix 4 g PPO, 0.05 g POPOP, and 120 g solid naphthalene in 1 L spectroquality 1,4-dioxane. Store in dark bottle. Solution is stable for 2 months. Alternatively, use a commercially prepared scintillation solution available from suppliers of liquid scintillation materials.

b. Low-background water: Use water with no detectable tritium activity (most deep well waters are low in tritium).

c. Standard tritium solution: Dilute available tritium standard solution to approximately 1000 dpm/mL with low-background water.

d. Sodium hydroxide (NaOH) pellets.

e. Potassium permanganate ($KMnO_4$).

4. Procedure

Add three pellets NaOH and 0.1 g $KMnO_4$ to 100 mL sample in 250-mL distillation flask. Distill at 100 to 105°C, discard first 10 mL distillate, and collect next 50 mL. Thoroughly mix 4 mL distillate with 16 mL scintillation solution in tightly capped vial.

Prepare low-background water and standard tritium solution in same manner as samples.

Hold samples, background, and standards in the dark for 3 h. Count samples containing less than 200 pCi/mL for 100 min and samples containing more than 200 pCi/mL for 50 min.

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5. Calculations and Reporting

a. Calculate and report tritium, ^{3}H, in picocuries per milliliter (pCi/mL) or its equivalent, nanocuries per liter (nCi/L) as follows:

$$^3\mathrm{H} = \frac{(C - B)}{(E \times 4 \times 2.22)}$$

where:

C = gross counting rate for sample, cpm,
B = background counting rate, cpm,
E = counting efficiency, $(S - B)/D$,
S = gross counting rate for standard solution, cpm, and
D = tritium activity in standard sample, dpm, corrected for decay to time of counting.

b. Calculate the counting error at the 95% confidence level based on the equation for $u(R_n)$ given in Section 7020C.2. A total count of 40 000 within 1 h for a background count rate of about 50 cpm gives a counting error slightly in excess of 1% at the 95% confidence level.

6. Precision and Bias

Samples with tritium activity above 200 pCi/mL can be analyzed with precision of less than ±6% at the 95% confidence level and those with 1 pCi/mL can be analyzed with a precision of less than ±10%.

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7500-U URANIUM*

7500-U A. Introduction

1. Occurrence

Uranium, the heaviest naturally occurring element, is a mixture of three radioactive isotopes: uranium-238 (99.275%), uranium-235 (0.72%), and uranium-234 (0.005%). Most drinking-water sources, especially ground waters, contain soluble carbonates and bicarbonates that complex and keep uranium in solution.

2. Selection of Method

Method 7500-U.B, a radiochemical procedure, determines total uranium alpha activity without making an isotopic uranium analysis. Method 7500-U.C is a radiochemical procedure that determines the isotopic content of the uranium alpha activity; it is consistent with determining the differences among naturally occurring, depleted, and enriched uranium.

* Approved by Standard Methods Committee, 2000. Editorial revisions, 2011. Joint Task Group: 20th Edition—Edmond J. Baratta (chair), Paul B. Hahn.

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7500-U B. Radiochemical Method

1. General Discussion

a. Principle: The sample is acidified with hydrochloric or nitric acid and boiled to eliminate carbonate and bicarbonate ions. Uranium is coprecipitated with ferric hydroxide and subsequently separated. The ferric hydroxide is dissolved, passed through an anion-exchange column, and washed with acid, and the uranium is eluted with dilute hydrochloric acid. The acid eluate is evaporated to near dryness, the residual salt is converted to nitrate, and the alpha activity is counted.

b. Interference: The only alpha-emitting radionuclide that may be carried through this procedure is protactinium-231. However, this isotope, which is a decay product of uranium-235, causes very little interference. Check reagents for uranium contamination by analyzing a complete reagent blank.

c. Sampling: Preserve sample by adjusting its pH to <2 with HCl or HNO_3 at time of collection.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 7020.

2. Apparatus

a. Counting instrument, gas-flow proportional or alpha scintillation counting system.

b. Ion-exchange column, approximately 13 mm ID × 150 mm long with 100-mL reservoir.

c. Membrane filter apparatus, 47-mm diam.

3. Reagents

a. Ammonium hydroxide (NH_4OH), 5*N*, 1%.

b. Anion-exchange resin.*

c. Ferric chloride carrier: Dissolve 9.6 g $FeCl_3 \cdot 6H_2O$ in 100 mL 0.5*N* HCl; 1 mL = 20 mg Fe^{3+}.

d. Hydriodic acid (HI), 47%.

e. Hydrochloric acid (HCl), conc, 8*N*, 6*N*, 0.1*N*.

f. Iodic acid, 1 mg/mL: Dissolve 100 mg HIO_3 in 100 mL 4*N* HNO_3.

g. Nitric acid (HNO_3), conc, 4*N*.

h. Sodium hydrogen sulfite, 1%: Dissolve 1 g $NaHSO_3$ in 100 mL 6*N* HCl.

i. Uranium standard solution:† Dissolve 177.3 mg natural undepleted uranyl acetate, $UO_2(C_2H_3O_2)_2 \cdot 2H_2O$, in 1000 mL 0.2*N* HNO_3; 1 mL = 100 μg U = 150 dpm U = 67.6 pCi U. NOTE: Commonly available uranyl salts may be formed from depleted uranium; verify isotopic composition before use.

4. Calibration

Determine counting efficiency, *E*, for a known amount of uranium standard solution (about 750 dpm) evaporated from 6 to

* Dowex 1×4, 100-200 mesh, chloride form, or equivalent.

† A uranium oxide assay standard, CRM 129, is available for purchase from U.S. Department of Energy, Chicago Operations Office, New Brunswick Laboratory, D-350, 9800 South Cass Avenue, Argonne, IL 60439.

8 mL of 1 mg/mL HIO_3 solution in a 50-mm-diam stainless steel planchet. After flaming planchet, count for at least 50 min. Run a reagent blank with the standard portions and count.

$$\text{Counting efficiency, } E = \frac{C - B}{D}$$

where:

C = gross alpha count rate of standard, cpm,
B = alpha background count rate, cpm, and
D = disintegration rate of uranium standard, dpm.

Determine uranium recovery factor by adding a measured amount of uranium standard to the same volume of sample and taking it through the entire procedure. Alpha count the separated, evaporated, and flamed uranium planchet. Determine the recovery factor on at least 10% of all drinking water samples. For non-drinking water samples, it may be necessary to determine the recovery factor in every sample.

$$\text{Recovery factor, } R = \frac{C' - B'}{DE}$$

where:

C' = gross count rate of sample with added uranium, cpm,
B' = count of reagent blank, cpm,
D = disintegration rate of uranium standard, dpm, and
E = counting efficiency.

5. Procedure

a. If the sample has not been acidified, add 5 mL conc HCl or HNO_3 to 1 L sample in a 1500-mL beaker. Add 1 mL $FeCl_3$ carrier. In each batch of samples include a distilled-water blank. Cover with watch glass and heat to boiling for 20 min. If pH is greater than 1, add conc HCl or HNO_3 dropwise to bring pH to 1. While sample is boiling, gently add 5*N* NH_4OH from a polyethylene squeeze bottle with the delivery tube inserted between the watch glass and the beaker lip. Add 5*N* NH_4OH until turbidity persists while boiling continues; then add 10 mL more. Continue boiling for 10 min more, then set aside for 30 min to cool and settle. After sufficient settling, decant and filter supernate through a 47-mm, 0.45-μm membrane filter using a large filtering apparatus. Slurry the remaining precipitate, transfer to the filtering apparatus, and filter with suction. Complete transfer using 1% solution of NH_4OH delivered from a polyethylene squeeze bottle. Place filtering apparatus over a clean 250-mL filtering flask, add 25 mL 8*N* HCl to dissolve precipitate, and filter. Wash filter with an additional 25 mL 8*N* HCl. (Alternatively, use centrifugation in place of filtration as in 7500-U.C.4*a*.)

b. Prepare an ion-exchange column by slurrying the anion-exchange resin with 8*N* HCl and pouring it into a 13-mm-ID column to give a resin bed height of about 80 mm. Transfer solution to the 100-mL reservoir of the ion-exchange column. Rinse side-arm filtering flask twice with 25-mL portions of 8*N* HCl. Combine in the ion-exchange reservoir. Pass sample solution through the anion-exchange column at a flow rate of not more than 5 mL/min. After sample has passed through column, elute the iron (and plutonium if present) with six column volumes of freshly prepared 8*N* HCl containing 1 mL 47% HI /9 mL 8*N* HCl. Wash column with two additional column volumes of 8*N* HCl. Discard all washes. Elute uranium into a 100-mL beaker with six column volumes of 0.1*N* HCl. Evaporate acid eluate to near dryness and convert residue to the nitrate form by three successive treatments with 5-mL portions of conc HNO_3, evaporating to near dryness each time. *Do not bake.* Dissolve residue (of which there may be very little visible) in 2 mL 4*N* HNO_3. Using a transfer pipet, transfer to a marked planchet. Complete transfer by rinsing beaker three times with 2-mL portions of 4*N* HNO_3. Evaporate planchet contents to dryness under a heat lamp, flame to remove traces of HIO_3, cool, and count for alpha activity.

c. To regenerate anion-exchange resin column, pass three column volumes of 1% $NaHSO_3$ in 6*N* HCl through the column, follow with six column volumes of 6*N* HCl, and then three column volumes of distilled water. Do not let resin become dry. When ready for the next set of samples, equilibrate by passing six column volumes of 8*N* HCl through the column.

6. Calculations

$$\text{Uranium alpha activity, pCi/L} = \frac{C'' - B'}{2.22 \times ERV}$$

where:

C'' = gross count rate of sample, cpm,
V = volume of sample, L, and
other factors are as defined above.

7. Precision and Bias

In a collaborative study, three sets of triplicate samples with known additions of uranium were analyzed by 18 laboratories. The average recovery was 91.5%. The estimated average 95% repeatability interval was 29.3% of the uranium concentration over the range of 8 to 75 pCi/L. The estimated average 95% reproducibility interval was 37.2% over the same range.

7500-U C. Isotopic Method

1. General Discussion

a. Principle: The sample is acidified with hydrochloric or nitric acid and uranium-232 is added as an isotopic tracer. Uranium is separated as in the radiochemical method (see 7500-U.B) and is electrodeposited onto a stainless steel disk for counting by alpha pulse height analysis using a silicon surface barrier detector.

b. Interferences: The only alpha-emitting radionuclide that may be carried through the procedure is protactinium-231. The presence of this radionuclide can be determined from the alpha spectrum and the interference subtracted. Check reagents for uranium contamination by analyzing a complete reagent blank.

c. Sampling: Preserve sample by adjusting its pH to <2 with HCl or HNO_3 at the time of collection.

d. Quality control (QC): The QC practices considered to be an integral part of each method can be found in Section 7020.

2. Apparatus

a. Counting instrument, alpha spectrometer (see Section 7030B.4), giving a resolution of 50 keV (FWHM) or better and having a counting efficiency greater than 15%.

b. Ion-exchange column, 13 mm ID × 150 mm long with 100-mL reservoir.

c. Electrodeposition apparatus as shown in Figure 7500-U:1. Although the electrodeposition cell is surrounded by water the water is not circulated because cooling is unnecessary. The cathode slide has mirror finish, is 0.05 cm thick, and has an exposed electrodeposition area of 2 cm^2. The anode is a 1-mm-diam platinum wire with an 8-mm-diam loop at the end above the cathode.

d. DC power supply, 0 to 12 V at 0 to 2 amp, for electrodeposition.

e. Centrifuge, capable of handling 100-mL or larger centrifuge bottles.

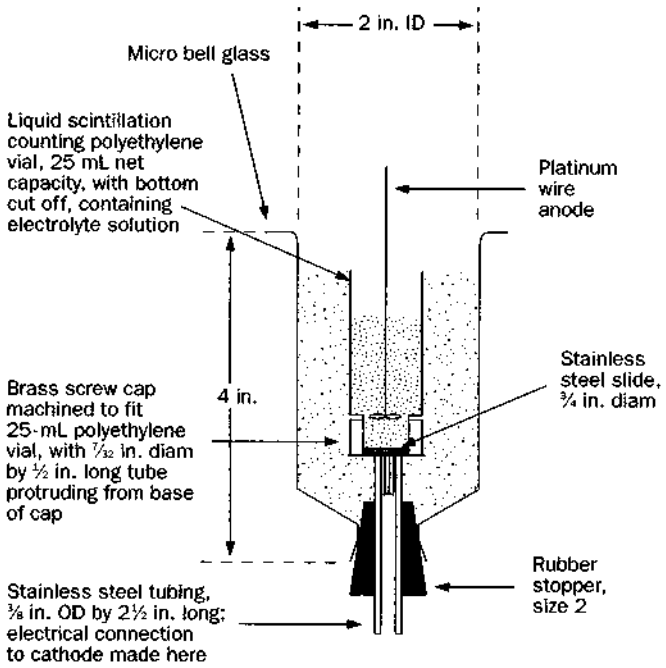


Figure 7500-U:1. Electrodeposition apparatus. To obtain dimensions in centimeters, multiply dimensions in inches by 2.54.

3. Reagents

In addition to reagents in 7500-U.B.3*d–g*, the following are needed:

a. Ammonium hydroxide (NH_4OH), 5*N*, 1.5*N*, and 0.15*N*.

*b. Anion-exchange resin.**

c. Ethyl alcohol, made slightly basic with a few drops of conc NH_4OH/100 mL.

d. Pre-adjusted electrolyte [$(NH_4)_2SO_4$], 1*M*, adjusted to pH 3.5 with conc NH_4OH and conc H_2SO_4.

e. Sulfuric acid (H_2SO_4), conc, 3.6*N*.

f. Sodium hydrogen sulfate, about 5% in 18*N* H_2SO_4. Dissolve 10 g $NaHSO_4 \cdot H_2O$ in 100 mL water and carefully add 100 mL conc H_2SO_4.

g. Thymol blue indicator, sodium salt, 0.04% solution.

h. Uranium-232 tracer solution, 10 dpm/mL in 1*N* HNO_3: If possible use a ^{232}U standard solution from, or traceable to, the National Institute for Standards and Technology (NIST). Standardize a freshly purified solution of ^{232}U by thoroughly mixing a known amount with a known amount of another uranium standard such as ^{236}U or natural uranium, and electroplating the mixture. Determine specific activity of the ^{232}U solution from an alpha pulse height analysis of the electroplated mixture. Alternatively, evaporate weighed portions of a freshly purified ^{232}U solution (free of HCl) on stainless steel slides and count with a 2π proportional counter. Determine efficiency of the 2π counter accurately with a NIST alpha-particle standard. When using this standard, correct for resolving time and backscattering if necessary.

* Bio Rad AGl-X4, 100-200 mesh, chloride form, or equivalent.

4. Procedure

a. If the sample has not been acidified, add 5 mL conc HCl or conc HNO_3 to 1 L sample in a 1500-mL beaker. Mix and check pH. If pH is greater than 1, add conc HCl or HNO_3 dropwise to bring the pH to 1. Add 1.0 mL uranium-232 tracer solution and 1 mL $FeCl_3$ carrier. Cover, boil, add NH_4OH, cool, and let settle as directed in 7500-U.B.5*a*. Decant supernate, being careful not to remove any precipitate. Slurry precipitate and supernate and transfer to a centrifuge bottle. Centrifuge and pour off remaining supernate. Dissolve precipitate with 8*N* HCl. Dilute to approximately 50 mL with 8*N* HCl.

b. Prepare ion-exchange column and transfer sample solution to reservoir as directed in 7500-U.B.5*b*. Rinse centrifuge bottle twice with 25-mL portions of 8*N* HCl, and add rinse to the reservoir. Follow anion-exchange and uranium-elution proce-

dures of 7500-U.B.5*b*. Evaporate sample to about 20 mL and add 5 mL conc HNO_3. Evaporate sample to near dryness.

c. Add 2 mL 5% $NaHSO_4$ solution. Add 5 mL conc HNO_3, mix well, and evaporate to dryness but do not bake. Warm and dissolve in 5 mL preadjusted electrolyte. Transfer to electrodeposition cell using an additional 5 to 10 mL electrolyte in small increments to rinse the sample beaker. Add 3 or 4 drops thymol blue indicator solution. If the color is not salmon pink, add 3.6*N* H_2SO_4 (or conc NH_4OH) until this color is obtained. Place platinum anode in solution so that it is about 1 cm above the stainless steel slide that serves as the cathode. Connect electrodes to power supply and adjust to give a current of 1.2 amp (constant current power supplies will not require further adjustments during electrodeposition). Continue electrodeposition for 1 h. When electrodeposition is to be ended, add 1 mL conc NH_4OH and continue for 1 min. Remove anode from cell and then turn off power. Discard solution in cell and rinse two or three times with 0.15*N* NH_4OH. Disassemble cell and wash slide with ethyl alcohol that has been made basic with NH_4OH. Dry slide over a hot plate. Measure activity of the uranium isotopes using an alpha spectrometer (see Section 7030B.4) within a week of preparation.†

5. Calculations

a. Determine total counts for each uranium isotope by summing the counts in the peak at the energy corresponding to the isotope. If two isotopes are close in energy, complete resolution may not be possible. Subtract background from each peak. Make a blank correction for each peak, if necessary.

b. Calculate concentration of each uranium isotope as follows:

$$U_i, \text{pCi/L} = \frac{C_i \times A_t}{2.22 \times C_t V}$$

where:

U_i = concentration of uranium isotope being determined,
C_i = net sample counts in the energy region corresponding to uranium isotope being measured,
A_t = activity of added uranium-232 tracer, dpm,
C_t = net sample counts in the energy region corresponding to uranium-232 tracer, and
V = sample volume, L.

6. Calibration

To calculate uranium recovery, determine absolute counting efficiency (E) of the alpha spectrometer. To determine efficiency count a standard source of a known alpha activity having the same active area as the samples.

$$E = \frac{C_s - B}{D}$$

where:

C_s = gross count rate in the energy region corresponding to the energy of the standard, cpm,
B = background count rate in the energy region corresponding to the energy of the standard, cpm, and
D = disintegration rate of standard, dpm.

$$\text{Recovery factor, } R = \frac{C_t}{t \times A_t E}$$

where:

t = sample counting time, min.

7. Precision and Bias

In a collaborative study, four sets of duplicate samples with known additions of uranium isotopes were analyzed by eight laboratories. Results agreed within 5% of the reference values, except for very low concentrations of uranium (concentrations approaching MDL due to background). Levels less than 0.1 pCi/L can be detected by this method.

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† Electrodeposition was the recommended technique for alpha spectroscopy source preparation in the collaborative test of this method. A number of laboratories are currently using a rare earth fluoride co-precipitation technique for alpha spectroscopy source preparation. If adequate resolution can be obtained, the rare earth fluoride source preparation should be adequate as an alternative to electrodeposition.

PART 8000

TOXICITY

Part Coordinator
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8050 Bacterial Bioluminescence ..... Ruth M. Sofield
8113 Macroalgae ..... Lan C. Wiborg
8310 Protozoa ..... James R. Pratt
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8712 *Ceriodaphnia* ..... Sandra Valeria Buratini
8750 Aquatic Insects ..... Paul K. Sibley
8910 Fish ..... Gabriela Rodríguez-Fuentes and Xin Deng
8921 Fathead Minnow ..... Mary Ann Rempel-Hester

Summary of Major Changes since 2012

The Introduction (8010) includes a corrected growth-rate equation. Quality Assurance/Quality Control (8020) adds residual chlorine to the list of analytes to be measured upon receipt for National Pollutant Discharge Elimination System (NPDES) effluent samples. It also includes new factors to consider when deciding to sieve sediment samples, as well as information to request from animal suppliers.

Editorial revisions were made to Bacterial Bioluminescence (8050), Protozoa (8310), and Rotifers (8420). New references were added and others were updated.

Annelids (8510) now includes a *Neanthes arenaceodentata* 28-d early-life-stage toxicity test procedure and an updated *N. arenaceodentata* 20-d procedure.

Test procedures were updated in Macroalgae (8113), Mollusks (8610), *Daphnia* (8711), *Ceriodaphnia* (8712), Aquatic Insects (8750), Fish (8910), and Fathead Minnow (8921).

Minor clarifications have been made in all sections, and references and bibliographies have been updated.

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8010 INTRODUCTION*

8010 A. General Discussion

1. Uses of Toxicity Tests

Toxicity tests are desirable in water-quality evaluations because chemical and physical tests alone are not sufficient to assess potential effects on aquatic biota.[1–3] For example, the effects of chemical interactions and the influence of complex matrices on toxicity cannot be determined from chemical tests alone. Different species of aquatic organisms are not equally susceptible to the same toxic substances, nor are organisms equally susceptible throughout the life cycle. Even previous exposure to toxicants can alter susceptibility. In addition, organisms of the same species can respond differently to the same level of a toxicant from time to time, even when all other variables are held constant.

Toxicity tests are useful for a variety of purposes, which include determining:

- suitability of environmental conditions for aquatic life;
- favorable and unfavorable environmental factors, such as dissolved oxygen (DO), pH, temperature, salinity, or turbidity;
- effect of environmental factors on waste toxicity;
- toxicity of wastes to a test species;
- relative sensitivity of aquatic organisms to an effluent or toxicant;
- amount and type of waste treatment needed to meet water pollution control requirements;
- effectiveness of waste treatment methods,
- permissible effluent discharge rates; and
- compliance with water-quality standards, effluent requirements, and discharge permits.

In such regulatory assessments, use toxicity test data with receiving-water and site-specific discharge data on volumes, dilution rates, and exposure times and concentrations.

2. Test Procedures

Use correct terminology (see 8010B) and environmentally relevant test procedures to meet regulatory, legal, and research objectives.[3–8]

The procedures given below allow measurement of biological responses to known and unknown concentrations of materials in both fresh and saline waters. These toxicity tests are applicable to both routine monitoring requirements and research needs. Refer to Part 9000 for microbiological methods and Part 10000 for field and other types of biological laboratory methods for water-quality evaluations. Refer to Section 10900 for identification aids for aquatic organisms.

Reasonable uniformity of procedures and of data presentation is essential. The use of standardized methods described below will ensure adequate uniformity, reproducibility, and general usefulness of results without interfering unduly with the tests' adaptability to local circumstances.

Quality assurance (QA) practices for toxicity test methods include all aspects of the test that affect data quality. These include sampling and handling, source and condition of test organisms, performance of reference toxicant tests, and the test procedures themselves. Quality assurance/quality control (QA/QC) guidelines are available for single compound testing and general laboratory practices[9] and for effluent evaluations in technical guidance manuals for conducting acute and short-term chronic toxicity tests with effluents.[10–12]

Other publications covering aspects of toxicity test procedures are available.[8,13–15] Also, see 8010I.

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* Approved by Standard Methods Committee, 2015.
Joint Task Group: Wayne L. McCulloch (chair), Michael K. Chanov, William L. Goodfellow, William J. Rue.

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8010 B. Terminology

An aquatic toxicity test is a procedure in which the responses of aquatic organisms are used to detect or measure the presence or effect of one or more substances, wastes, or environmental factors, alone or in combination.

1. General Terms

Acclimate—to accustom test organisms to different environmental conditions, such as temperature, light, and water quality.
Control—treatment in a toxicity test that duplicates all the conditions of the exposure treatment but contains no test material.
Definitive test—toxicity test designed to establish the concentration at which a particular endpoint occurs. Exposures for these tests are longer than for screening or range-finding tests, incorporating multiple concentrations at closer intervals and multiple replicates.
Range-finding test—preliminary test designed to establish a solution's approximate toxicity. Test design incorporates multiple, widely spaced concentrations with single replicates; exposure is usually 8 to 24 h.
Response—the measured biological effect of the variable tested. In acute toxicity tests, the response usually is death or immobilization. In chronic tests, the response can include reduced growth, abnormal development, or reduced reproduction. In plant toxicity tests, the response can be death, growth inhibition, or reproductive inhibition. In biostimulation tests, the response is biomass increase.
Screening test—toxicity test to determine if an impact is likely to be observed; test design incorporates one concentration, multiple replicates, exposure 24 to 96 h for aqueous tests and up to 10 days for sediment tests.

2. Toxicity Terms

Acute toxicity—relatively short-term lethal or other effect, usually defined as occurring within 4 d for fish and macroinvertebrates, and within 2 d for organisms with shorter life spans.
Asymptotic LC_{50}—toxicant concentration at which the median lethal concentration (LC_{50}) approaches a constant for a prolonged exposure time.
Chronic toxicity—toxicity involving a stimulus that lingers or continues for a relatively long time, often one-tenth of the life span or more. *Chronic* should be considered a relative term, depending on an organism's life span. A chronic toxic effect can be measured in terms of reduced growth, reduced reproduction, etc., in addition to lethality.
Dose—amount of toxicant that enters the organism. Dose and concentration are not interchangeable.
Effective concentration (EC_P)—toxicant concentration estimated to cause a specified effect in a designated proportion (P) of test organisms. The effect is usually sublethal (e.g., a change in respiration rate or loss of equilibrium). The exposure time also is specified; for example, the 96-h EC_{50} for loss of equilibrium is the effective concentration for 50% of test organisms to exhibit this effect within 96 h.
Exposure time—time test organism is exposed to test solution.
Inhibition concentration (IC_P)—toxicant concentration estimated to cause a specified percentage (P) of inhibition or impairment in a qualitative biological function. For example, an IC_{25} could be the concentration estimated to reduce larval fish growth by 25%, relative to the control. Use this term with any toxicological test that measures a change in rate (respiration, number of progeny, decrease in number of algal cells, etc).
Lethal concentration (LC_P)—toxicant concentration estimated to produce death in a specified proportion (P) of test organisms. Usually defined as median (50%) lethal concentration (LC_{50})—the concentration killing 50% of exposed organisms within a specific exposure period (e.g., 96-h LC_{50}).
Lowest-observed-effect concentration (LOEC)—in a full- or partial-life-cycle test, the lowest toxicant concentration in which the values for the measured response are statistically significantly different from those in the control.
Median tolerance limit (TL_m)—test material concentration at which 50% of test organisms survive for a specified exposure time. This term has been superseded by LC_{50} and EC_{50}.
No-observed-effect concentration (NOEC)—in a full- or partial-life-cycle test, the highest toxicant concentration in which the values for the measured response are not statistically significantly different from those in the control.
Toxicity—potential or capacity of a test material (generally a poison or mixture of poisons) to adversely affect living organisms. Toxicity is a result of dose or exposure concentration and exposure time, modified by such variables as temperature, chemical form, and availability.

3. Biostimulation Terms

Limiting nutrient—among the required nutrients, the one whose quantity is inadequate for growth (while others remain sufficient).
Maximum standing crop—maximum weight of organisms during a test, specified as wet or dry weight.
Nutrient—specific substance required for organism growth.

4. Solution Renewal Terms

Flow-through test—test in which solution is replaced continuously in test chambers throughout the test duration.
Renewal test—tests in which organisms are exposed to solutions of the same composition that are renewed periodically (usually 24-h intervals) during the test period. This is accomplished by transferring test organisms or replacing test solution.
Static test—test in which solutions and test organisms are placed in test chambers and kept there for the duration of the test.

5. Evaluation of Results Terms

Acute-to-chronic ratio—numerical relationship between acute and chronic toxicity that is applied to acute toxicity test values to estimate toxicant concentration that is safe for chronic or long-term exposure of a test organism.
Chronic value (ChV)—geometric mean of the NOEC and LOEC from partial- and full-life-cycle tests and early-life-stage tests.
Maximum allowable toxicant concentration (MATC)—toxicant concentration that may be present in a receiving water without causing significant harm to productivity or other uses. MATC is determined by long-term tests of either partial life cycle with sensitive life stages or a full life cycle of the test organism.

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8010 C. Basic Requirements for Toxicity Tests

1. General Requirements

The basic requirements and desirable conditions for toxicity tests are:

- an abundant supply of water of desired quality (see 8010E.4*b*);
- an adequate and effective flowing water system constructed of nonpolluting or absorbing materials (see 8010F.1*a*);
- adequate space and well-planned holding, culturing, and testing equipment and facilities (see 8010E.3);
- an adequate source of healthy experimental organisms (see 8010E.4); and
- appropriate lighting facilities for plant toxicity tests.

Much valuable information and advice regarding general requirements and desirable conditions for toxicity testing are available.[1–9]

2. Requirements for Specific Test Purposes

The facilities, equipment, and water supplies needed for effective tests depend on the type of tests and their objectives.[6] For effluent and monitoring compliance tests requiring receiving water as the dilution water, use water immediately upstream and outside the waste's zone of influence. When studies require the use of laboratory-grade water, use a pollution-free water supply that provides for acceptable survival, growth, and reproduction of the aquatic test organisms to be studied. The most important requirements for designing a toxicity testing program are defining study objectives and establishing QC practices to ensure that the data are of sufficient quality to address the objectives and ensure credibility.

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8010 D. Conducting Toxicity Tests

1. Types of Toxicity Tests: Their Uses, Advantages, and Disadvantages

Toxicity tests are classified according to

- duration—short-, intermediate-, and/or long-term;
- method of adding test solutions—static, renewal, or flow-through; and
- purpose—effluent quality monitoring, single compound testing, relative toxicity, relative sensitivity, taste or odor, or growth rate, etc.

Short-term toxicity tests are used for routine monitoring suitable for effluent-discharge permit requirements and for exploratory tests. They may use endpoints other than mortality. Acute definitive tests typically use mortality as an endpoint or other discrete observations to determine effects due to the toxicant (i.e., LC_{50} or EC_{50} values). These tests also may be used to indicate a suitable range of toxicant concentrations for intermediate- and long-term tests. Short-term tests are preferred for obtaining toxicity data as rapidly and inexpensively as possible (when that is the goal; otherwise, longer tests that provide more details for a given purpose may be preferred). They are valuable for estimating overall toxicity, screening test solutions or materials for which toxicity data do not exist, assessing relative toxicity of different toxicants or wastes to selected test organisms, or determining the relative sensitivity of different organisms to different conditions of such variables as temperature and pH. The results of these tests can be used to calculate acceptable concentrations for rapid exposures (e.g., when organisms pass through an effluent zone of initial dilution or a mixing zone).

Intermediate-term toxicity tests typically are used when more time is needed to determine the toxicant's effect on various life stages of long-life-cycle organisms, and to indicate toxicant concentrations for life-cycle tests.

Long-term toxicity tests are generally used for estimating chronic toxicity. Long-term testing may include early-life-stage, partial-life-cycle, or full-life-cycle testing. Exposures may be as short as 7 d (to expose specific portions of an organism's life cycle) or as long as 21–28 d to several months or longer for traditional partial-life-cycle and full-life-cycle tests with fish.

To establish a successful testing program, consider the following: Be cautious when using static tests to evaluate solutions containing high levels of biochemical oxygen demand (BOD), chemical oxygen demand (COD), and/or bacteria. Such tests can be conducted successfully if rigorous DO monitoring and acceptable aeration are incorporated to provide adequate oxygen. During testing, concentrations of volatile or unstable toxicants may drop, causing discrepancies between assumed and actual exposure. On the other hand, ammonia and other metabolic products may rise to undesirably high levels, stressing or killing test organisms and causing analysts to overestimate toxic response. Also, toxicant levels may drop due to toxicant adsorption to sediments, test chamber walls, or the test organisms' food; combination with the organisms' mucus or metabolic products; or intake into their bodies. These problems can be mitigated by frequently renewing test solution or using flow-through test methods, albeit at the expense of greater organism manipulation and use of more complicated test equipment.

Flow-through toxicity tests are desirable for samples containing high levels of BOD or COD, or unstable or volatile substances. Organisms with high metabolic rates are difficult to maintain under static exposure conditions; flow-through tests keep the environment well oxygenated and continuously remove metabolic wastes. Use flow-through toxicity tests whenever the test solution is known or expected to degrade rapidly—this often can be clarified by comparing test-organism viability or survival time in fresh solution and a corresponding 2-d-old solution (provided that DO is adequate throughout both tests). Flow-through toxicity tests are also desirable for industrial effluents and chemicals that are removed appreciably from solution via precipitation, test organisms, or other means.

LC_{50} values may be useful measures of acute toxicity but are not concentrations that are safe or harmless in aquatic habitats. Waste concentrations that are not demonstrably toxic in 96 h may be toxic at longer exposure periods in a receiving water. Thus, the 96-h LC_{50} may represent only a fraction of long-term toxicity. When estimating safe discharge rates or dilution ratios for effluents or other pollutants based on acute toxicity evaluations, use acute-to-chronic ratios determined primarily from life-cycle tests; however, NOEC values determined from shorter-duration chronic toxicity tests can be used. Even the provision of an apparently ample margin of safety can fail to accomplish its purpose if the cumulative toxicity cannot be predicted from acute toxicity results.

No single, simple acute-to-chronic ratio is valid for all wastes or toxicants; however, acute-to-chronic ratios of 10 for whole effluents, 20 for nonpersistent chemicals (half life of <8 weeks), and 100 for persistent chemicals (half life >8 weeks) are often used by regulators when data are insufficient to calculate a specific acute-to-chronic ratio.[1,2] Likewise, the constituents of a complex waste responsible for acute toxicity may or may not be those responsible for chronic or cumulative toxicity observed in diluted waste that is no longer acutely toxic. The chronic toxicity

may be lethal after a long exposure period or may only impair biological function. Knowledge of a waste's acute toxicity often can be helpful in predicting and preventing acute damage to aquatic life in receiving waters, as well as in regulating toxic waste discharges.

2. Short-Term Toxicity Tests

a. Range-finding toxicity tests: For effluents or materials of unknown toxicity, conduct short-term (usually 24- or 48-h) small-scale range-finding or exploratory tests to determine approximate concentration range to be included in definitive short-term tests. For effluents with low or slow-acting toxicity, 48- or 96-h tests may be necessary. Expose test organisms to a wide range of concentrations of the test substance, usually in a logarithmic ratio (e.g., 0.01, 0.1, 1, 10, and 100% of sample). Attempt to include concentrations that will kill all organisms and others that will kill few or no organisms. For short-term definitive tests, select a geometrically spaced series of concentrations between the highest concentration that killed no or few test organisms and the lowest concentration that killed most or all test organisms.

Prepare test concentrations as described in 8010F.2*b*.

b. Short-term definitive tests: Because death is an important, easily detected adverse effect, the most commonly used tests are for acute lethality. These tests are most appropriate for routine monitoring and checking conformity with National Pollutant Discharge Elimination System (NPDES) requirements.[3] If it is not possible to perform a range-finding toxicity test before a definitive acute toxicity test, using a concentration series with a 0.5 (100, 50, 25, 12.5, 6.25%) or 0.3 (100, 30, 10, 3, 1%) dilution factor may be appropriate. Short-term tests may be static, renewal, or flow-through. Exposure periods for these tests usually are 48 or 96 h. Static or renewal tests often are used when the test organisms are phyto- or zooplankton because these organisms are easily washed out in flow-through tests. Static and renewal tests are considerably less expensive to perform than flow-through tests. Overnight express-mail shipments of samples often make static and renewal tests the method of choice for regulatory compliance testing.

Test solutions may be renewed daily if required due to oxygen demand, an unstable or volatile toxicant, or (in the case of whole effluents) daily variation in effluent composition. Renewals also may be less frequent. If the test material has high BOD and/or COD level(s) or is relatively unstable, use test vessels with maximum surface area-to-volume ratio, or use the renewal or flow-through technique.

Test duration is determined by toxicant and test objectives, and usually is the same for different groups of organisms. For short-life-cycle organisms (e.g., phytoplankton), the usual exposure time can cover many generations. Determine test duration, in part, by the length of life cycle. Generally, expose fish and large invertebrates in static and static renewal tests for 96 h and in flow-through tests for an equal period unless toxicant composition varies. If so, longer exposure may be useful to assess the effects of toxicant variability. Expose *Daphnia* and *Ceriodaphnia* for 48 h. Short-term tests have been limited arbitrarily to 96 h, but longer tests sometimes are desirable because death does not always occur within the 48- or 96-h period. When some test animals, though still alive, are dying or evidently affected after 96-h exposure, prolong the test or express test results as a 48- or 96-h EC_{50}, defining the observed effect.

If tests are continued for longer periods, the test organisms may need to be fed. Feed test organisms as directed in specific sections of Part 8000. Record feeding and ensure that it is equivalent in each container.

Special tests may be conducted on altered or treated effluent samples to obtain more toxicity information. For example, effluent–dilution water mixtures may be aged 24 to 48 h before adding test organisms in order to determine changes in toxicity. When special tests are conducted, describe methods in detail.

3. Intermediate-Term Toxicity Tests

There are no sharp time distinctions between short- and intermediate-term or between intermediate- and long-term tests. Usually, tests lasting 10 d or less are considered short-term, while intermediate tests may last from 11 to 90 d. The length of the test organism's life cycle helps determine the duration of short-, intermediate-, or long-term tests for that species. Intermediate-term tests may be static, renewal, or flow-through, but flow-through tests are recommended for most situations. For test conduct, see 8010F.3*a*.

4. Long-Term, Partial-, or Complete-Life-Cycle Toxicity Tests

With few exceptions, use flow-through tests with exposure extending over as much of the life cycle as possible. Continue tests from egg to egg or beyond, or for several life cycles for smaller forms. Determine the maximum concentrations of toxicant that do not produce harmful effects with continuous exposure. The overall objective of this type of test is to determine NOECs or ChV of effluents, toxicants, or wastes. Use life-cycle tests whenever possible to determine acute-to-chronic ratios and the effects on growth, reproduction, development of sex products, maturation, spawning, success of spawning and hatching, survival of larvae or fry, growth and survival of different life stages, deformities, behavior, and bioaccumulation—although bioaccumulation (or bioconcentration) often is determined with more mature animals in specially designed tests.[4]

In life-cycle or partial-life-cycle tests, ensure that water-quality factors (e.g., temperature, pH, salinity, and DO) follow the natural seasonal cycle unless the test objective is to study one of these factors. It may be essential that the natural annual cycle be duplicated if the development of sex products, spawning, and development of eggs and larvae are to be normal. Whenever possible, do not let toxicant concentrations vary by more than $\pm 15\%$ from the selected concentration because of uptake by test organisms, absorption, precipitation, or other factors.

In these tests, select five or more concentrations on the basis of short- or intermediate-term tests and set up the exposure chambers at least in duplicate. Vary exposure chambers, spawning chambers, and other equipment to meet the different organisms' needs. (See Sections 8111–8910.) Other apparatus, water supplies, and analytical determinations are listed in 8010E.

5. Short-Term Tests for Estimating Chronic Toxicity

Tests are available to estimate long-term effects of a toxicant or effluent after a relatively short (7 d) exposure. Endpoints for

these tests, called *chronic estimator* or *rapid bioassessment* tests, include lethality, reproductive potential, and growth. Tests estimating chronic toxicity frequently are being included as biomonitoring requirements in discharge permits. The long duration of life-cycle or early-life-stage chronic tests increases the cost and reduces laboratories' ability to conduct long-term tests successfully as the demand for testing increases. The U.S. Environmental Protection Agency (EPA) has published a number of short-term chronic estimation toxicity test methods for fresh- and saltwater invertebrates and fishes.[5,6] These tests were designed to evaluate effluent toxicity and may not be appropriate for other testing requirements (pre-manufacturing testing, development of water quality criteria, etc.).

6. Special-Purpose Toxicity Tests

a. Relative sensitivity to a toxicant: To rank the sensitivity of different species to a toxicant, use a standard water and standard exposure conditions. Select exposure conditions [e.g., temperature, DO, pH, carbon dioxide (CO_2), light, and salinity] in a favorable range for the test species and keep conditions constant throughout the test.

b. Relative sensitivity of various toxicants to selected species: These tests resemble sensitivity tests because the selected test conditions, dilution waters, and test species are kept constant and standard. Prevent any change in sensitivity of test organisms during the tests. If possible, select species from several different groups: an alga, microcrustacean, macrocrustacean, insect, mollusk, or fish.

c. Toxicity reduction evaluation: Use acute and chronic toxicity tests to determine the toxicant in the effluent. A toxicity reduction evaluation (TRE) is a phased approach that

- characterizes the acute or chronic toxicity of an effluent;
- identifies the toxicant(s) of concern [this phase often is termed *toxicity identification evaluation* (TIE)]; and
- confirms toxicity.

This approach is then used to evaluate the removal of toxicant(s) via pretreatment or changes at the wastewater treatment plant. EPA guidance manuals for performance of TIEs[7–11] and generalized TRE protocols for municipal[12] and industrial[13] facilities are available. Other TRE protocols are available.[14–19]

d. Flesh tainting tests: **CAUTION: Perform such tests only when there is assurance that the intake of potentially tainting substances via organism consumption is safe. If sufficient information on the substances is not available, replace consumption with smell.** Use these tests to determine the maximum concentrations of wastes and materials that do not taint the flesh of edible aquatic organisms. Expose organisms that are large enough to supply portions for a taste panel. Set up exposure tanks as for other flow-through tests. Perform range-finding tests over a wide concentration range to determine the concentrations for a more definitive series of tests.

After exposure, prepare test organisms for taste testing. Clean, prepare for cooking (without seasoning), wrap in aluminum foil, and bake in an oven. When organisms are cooked, divide them into portions, wrap in aluminum foil, assign a code number, and distribute to a taste panel while still warm, along with samples of unexposed organisms similarly cooked, wrapped, and coded. Record the panel's observations on a prepared form and determine the highest concentration of test material that does not cause detectable tainting based on either taste or smell. Several tests may be necessary.

e. Growth-rate determinations: Growth rate is an important response of both algae and fish to toxicants and environmental factors. This section focuses on fish; for a discussion related to algae, see Section 8111G.3*c*. Always report details of the fish-feeding method in growth studies. Three techniques are available:

1) Unrestricted food supply—Provide attractive and palatable food (usually live food, such as *Daphnia*, tubificid worms, or brine shrimp) continuously in greater quantities than fish can consume. It is desirable to make a mass balance of food consumption by weighing food introduced and uneaten food removed.

2) Intermittent satiated food supply—Provide all the attractive food that fish can consume at feeding time once or twice daily. After fish cease to feed, remove all uneaten food.

3) Uniformly restricted food supply—Once or twice per day, provide all fish with an amount of food that they will consume completely without exception. Ideally, hold fish separately in individual aquariums or compartments. For fish held together, feed so all fish have an equal opportunity to consume food. Uniformity of temperature and DO helps to ensure equal feeding of a group of fish.

While growth studies usually have been conducted with unrestricted and intermittent satiated feeding techniques, it is recommended that each study include at least one test series using uniformly restricted food supply. Only this technique can reveal whether growth rate differences are not the result of the toxicant's effect on appetite or food-consumption rate. The presence of an abundant food supply can obscure toxic effects. For example, fish exposed to toxicants, such as cyanide or pentachlorophenol, increase food-consumption rate to compensate partially for loss of food-utilization efficiency caused by the toxicant. This may not be possible in natural conditions, where food supply may be limited.

Ideally, include a series of tests with different, uniformly restricted food rations in which the lowest ration is near that which results in no growth (or weight) loss in the control. This is the maintenance level. Determine the effect of the studied variable at any level of food availability and consumption by relating observed growth rates to, for example, toxicant concentration, at each feeding level.

Juvenile fish may gain enough weight in 1 to 3 weeks to determine growth rate satisfactorily. Longer exposures are needed to determine long-term effects (e.g., acclimation or accumulative toxicity); in these tests, weigh fish approximately every 10 d.

Report results as specific growth rates, computed as follows:

$$\text{Growth rate} = \frac{\text{Weight gain, g}}{\text{time interval, d}} \times \frac{1}{\text{mean weight, g}}$$

where:

$$\text{mean weight} = [\text{weight at start of time interval (g)} + \text{weight at end of time interval (g)}] \div 2$$

Determine dry weight, wet weight, and fat (lipid) content of fish at the beginning and end of a test. Weight gain due to

increased fat content is not universally considered true growth; some investigators consider that true growth occurs only when protein content increases. However, fat storage is important ecologically and bioenergetically because fat can be used as an energy source during periods of malnutrition, reproduction, and overwintering survival. Fat content also is important in the dynamics of toxicant uptake, storage, and depuration. Fat and water content should typify that of the target species.

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8010 E. Preparing Organisms for Toxicity Tests

1. Selecting Test Organisms

The prime considerations in selecting test organisms are:

- their sensitivity to the factors under consideration;
- their geographical distribution, abundance, and availability within a practical size range throughout the year;
- their recreational, economic, and ecological importance and relevance to the purpose of the study;
- their abiotic requirements and whether these requirements approach the conditions normally found at the study site;

- the availability of culture methods for rearing them in the laboratory and a knowledge of their physiological and nutritional requirements; and
- their general physical condition and freedom from parasites and disease.

To select a best species, consider available information on sensitivity, consult with local authorities in pollution control or fish and wildlife agencies, or determine sensitivity with short-term tests. Select the test species based on the considerations listed above, as well as organism size and life-cycle length. Sections 8110–8930 list plant, invertebrate, and fish species that are commonly used in aquatic toxicity testing. When testing early life stages of organisms, species with a short life cycle are most cost-effective, but some tests require larger organisms with long life cycles (e.g., bioaccumulation or *in situ* biomonitoring studies).

For studies to determine effluent effects, select representative species in the affected area. In most cases, it is better to use laboratory-cultured species than those collected in the field. Laboratory-cultured organisms—either from in-house cultures or purchased from commercial bioassay organism suppliers—are of known age and quality, while those taken from the field may already have been selected or biased for the more resistant members of the population. This allows for use of the most sensitive life stages throughout the year. Their use also may be more cost-effective and allows for better QA/QC. When field-collected or obtained from commercial suppliers, species must be confirmed by a qualified taxonomist. For each series of tests, use organisms

- from a single source,
- that are nearly uniform in size (for fish, the largest individual is ≤50% longer than the shortest), and
- of the same age group or life stage.

Optimally, conduct reference toxicant tests on cultured stocks and on lots of acquired or collected organisms.[1] Report time, place, source, and culture history for cultured organisms and method of collection, transportation, handling, and acclimation of acquired or collected organisms, along with their response to reference toxicants.

Knowledge of their environmental requirements and food habits is important in selecting test organisms. Methods for laboratory holding and culturing are well described for a number of standard test species. When the purpose of the testing is site-specific, it may be necessary to collect certain life stages of selected organisms from the field for testing.

2. Collecting Test Organisms

Preferably, use standard laboratory-reared organisms. In special instances (e.g., when it is important to incorporate genetic variability from wild populations), use species indigenous to the receiving water. This is particularly important for organisms with recreational, commercial, or ecological significance.

When designing a test, consider any unusual past conditions to which the organisms may have been exposed (pesticides, various industrial effluents, waste treatment plants, return flows, etc.). The interactions between a new toxicant and those already being discharged to the receiving water may be important. Do not collect test organisms from polluted areas where they are in poor condition, diseased, or deformed; are infested with parasites; or have unusually high body burdens of chemicals. Avoid testing organisms with questionable histories.

Many smaller invertebrates and fish can be collected along the shore in dip nets, in coarse plankton nets, or by hand. Use seines to catch larger species near shore. Traps, fyke nets, and trawls are valuable collection tools but may be selective for some species. Otter trawls collect benthic species effectively, and midwater trawls are effective for pelagic species. Various dredges are available to collect benthic species from different types of bottoms or to collect different sizes of organisms. Commercially important species (e.g., lobster, blue crab, and dungeness crab) may be trapped by the researcher or purchased from commercial suppliers if proper care is taken before purchase. Species that colonize surfaces (e.g., barnacles) may be harvested from hard surfaces submerged in water. Ensure that organisms are not damaged during collection, transfer, and transport.

When seining or using trawls, make short hauls. Avoid collecting significant amounts of plant materials, debris, mud, sand, or gravel in net or in bag of seine because these will injure the animals. Always leave seine bag in the water at end of haul, stretch out wings of seine, open bag entrance, dip out organisms with a bucket or hand net, and transfer directly to prepared holding tanks. Do not expose delicate, easily damaged species to air. Remove larger, more hardy species with soft-mesh dip nets. Do not collect too many animals at once. After raising a trawl up to the boat, bring it over the side without delay and avoid letting the catch hit the boat. Immerse the portion of net containing specimens in a tank of water. Open trawl and remove desired animals by dipping with a bucket or soft-mesh hand net. Fill tanks with adequate quantities of clean water before beginning a haul. Transfer organisms to tanks as rapidly and carefully as possible.

If organisms will be transported any distance by boat, hold in aerated live boxes. If transported by truck, put them in large baffled and insulated tanks filled with water from area in which they were collected. Aerate the water and maintain at collection temperature. Determine water temperature, salinity, DO, and pH at the collection site. Do not handle organisms more than necessary. Make transfers with suitable containers or hand nets, or for small organisms, via large-bore pipets. Use hand nets made of soft material with several layers around the net rim and no sharp points or projections. Clean and sterilize all equipment before use. Avoid overcrowding organisms during transport. Aeration, oxygenation, water exchange, and cooling may reduce distress. Avoid cold shock as much as overheating.

Observe collected animals for possible injury resulting from transport to the laboratory. Examine smaller forms under a dissecting microscope. Criteria for assessing injury depend on the species and are more difficult for sluggish ones. Useful criteria include lost or damaged appendages, inability to maintain a normal body posture (e.g., dorsal side uppermost), abnormal locomotion, refusal to feed, discoloration, or uncoordinated movements of the mouth or other body parts.

For more information on collecting aquatic organisms, see Part 10000.

3. Handling, Holding, and Conditioning Test Organisms

During transport to the laboratory, organisms sometimes are crowded, bruised, and otherwise stressed, thereby increasing their susceptibility to disease. To avoid outbreaks of disease in stock tanks, treat organisms during transit or on arrival in accordance with procedures in 8010E.5 and as suggested for each group (Sections 8211–8910). Hold field-collected fish in quarantine for at least 7 d to observe for parasites and disease, and to recover from collection and transport stress; observe invertebrates for at least 2 d. Do not use the animals if more than 10% of them die after the second day or if they are parasitized or diseased beyond control. Clean and sterilize all contacted containers and equipment, and collect another supply from a different area if possible.

Because it is not always possible to collect from unpolluted areas and the collector cannot always be sure that a particular organism has not been exposed to a toxicant, for certain types of tests it may be necessary to sample collected individuals to determine whether they already have accumulated the pesticides, heavy metals, and toxic materials to be studied. Check the animals or materials collected as food for test organisms for both disease and concentrations of the pesticides, heavy metals, and toxic materials to be studied. Feed test organisms daily during quarantine.

After quarantine period, transfer disease-free animals to regular stock tanks. Discard organisms that touch dry surfaces, are dropped, or are injured during handling. To avoid unnecessary stress, do not subject organisms to rapid temperature or water-quality changes. In general, change water temperature less than 3°C in any 24-h period. Use an even smaller rate of temperature change for stenothermal, deep-water species. Preferably keep DO concentrations at or near saturation, but never $<60\%$ or $>100\%$ of saturation. After transfer to stock-holding tanks, begin a slow acclimation to laboratory conditions (e.g., temperature, salinity, and hardness). The acclimation period will be governed by type of organism and extent of changes in water quality. For forms with a life cycle of several months or more, use an acclimation period of at least 2 to 3 weeks.

Inspect organisms closely and frequently for stress, unusual behavior, parasites or disease, changes in color, or failure to eat. Avoid crowding. Provide adequate flow-through water so such characteristics as DO, pH, CO_2, salinity, hardness, and ammonia (NH_3) are favorable. Check temperature and DO frequently. Do not let metabolic products accumulate. Generally, use a flow-through rate of 6 to 10 tank volumes/d. Usually, greater amounts of flow-through water are required for smaller organisms on a weight–volume basis. For small organisms, use a water flow of at least 3 L/d/g. When brood stock are being held, periodic or continuous treatment for parasite and disease control may be required.[2–10]

Clean tanks and equipment thoroughly and often, removing or flushing out all growths and wastes, preferably daily but at least twice per week. Remove all uneaten food within 24 h. Use different sets of nets and other equipment for different groups of organisms, and clean and sterilize them between uses. When handling is necessary, clean hands and nets before touching organisms. Cover tanks and containers to prevent organisms from jumping out. Shield tanks via curtains or some other means to protect organisms from unnecessary disturbances and noise. Provide photoperiods and light intensities favorable to the organisms (see 8010F.3*f*). Begin acclimation to test conditions at a suitable interval in advance of testing.

It is of utmost importance that animals be kept in excellent condition before the tests. Make no abrupt changes in environmental conditions; preferably follow natural seasonal variations in environmental conditions (e.g., temperature and daylight patterns). Many water supplies are supersaturated with gases, especially in winter when very cold water is brought into the laboratory and warmed. Because there is a danger of gas-bubble disease, keep incoming water in an open system and let it cascade over baffles or otherwise aerate it to bring dissolved gases into equilibrium with the air, or strip supersaturated nitrogen with pure oxygen.[11]

Acclimate freshwater arthropods by rearing them in dilution water at test temperatures, unless temperature is one of the factors being studied. Acclimate other organisms to the dilution water and test temperatures by gradually changing the water from 100% holding water to 100% dilution water over several days. Keep all organisms in 100% dilution water for at least 2 d before use. Do not use a group of organisms if more than 10% die during the 48 h immediately before the test begins.[1] If a group fails to meet these criteria, discard it or re-treat, hold, and reacclimate if necessary.

Make necessary provisions for organisms that require a special substrate, cover, or materials to use for clinging, support, case building, or hiding.

Hold cold-water, freshwater organisms between 5 and 15°C. Hold warm-water organisms between 10 and 25°C, depending on season and test objectives.

4. Culturing Test Organisms

The advantage of cultured test organisms over field-collected animals is that the age, life history, and existing conditions are documented so these organisms' responses are more consistent between test lots. For organisms used extensively in effluent biomonitoring programs, EPA has developed a series of test methods[12,13] that are adaptable to most laboratories. Culturing test organisms requires strict adherence to standard protocol, 7-d/week monitoring, and an adequate facility.

a. Facilities, construction materials, and equipment: Construction materials that contact dilution water must not contain leachable substances or adsorb significant amounts of substances from the water. Use tempered glass, fiberglass, or stainless steel (No. 316) and silicone sealant as construction material for freshwater systems. Do not use rubber or plastics containing toxic fillers, additives, stabilizers, plasticizers, etc. Fluorocarbon plastic, nylon, and their equivalents usually are acceptable. Test the toxicity of all materials before purchasing large quantities. Clean, soak, and flush all new tanks, troughs, and similar equipment with dilution water for several days before use. Use a glass or titanium interface between the water and heating elements for marine waters and glass or stainless steel for fresh waters.

Provide adequate space for test organisms, holding facilities, water-storage reservoirs, and water-supply systems. Provide hot- and cold-water distribution and mixing facilities to obtain any desired temperature. Aerate or vigorously mix to prevent gas supersaturation caused by heating dilution water.[11] Use oil-free pumps if possible. If air pumps are not oil-free, they should have

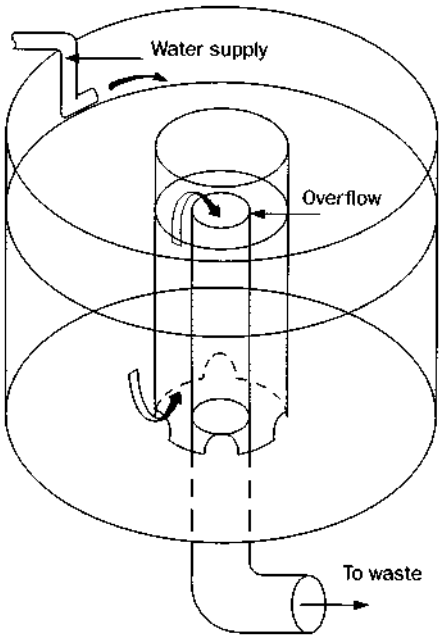


Figure 8010:1. Holding tank design for fish and macroinvertebrates.

water seals and filters to prevent oil from entering air lines and contaminating tanks. When large volumes of air are needed, use low-pressure blowers. Do not locate air intakes in shops or furnace rooms or near outlets from chemical exhaust hoods, chemical laboratories, or vehicle exhausts.

Provide acclimation and culturing tanks with temperature control and aeration. Design holding facilities for ease of cleaning and prevention of bacterial growths. For holding and culturing fish and many macroinvertebrates, preferably use round or oval tanks of at least 1 to 3 m diam (Figure 8010:1). Provide a standpipe drain in the center, threaded below the tank floor so when the standpipe is removed, the opening is flush with the tank bottom. Slope tank bottom gently to center. Use tanks with smooth surfaces to facilitate cleaning, prevent organism injuries, and ensure that no material will collect in corners, cracks, and crevices. Introduce water into a circular tank as a jet along the edge and above the surface to create a circular movement of water around the central standpipe. Fit another pipe, with half-moon cutouts at its base, over the standpipe and screen so outflowing water passes up through the outside pipe, then down the standpipe. This results in a circular current and a certain amount of self-cleaning. Square or rectangular tanks may be used for special purposes, or when space is scarce. Provide standpipes at one end for draining, with threads for securing the pipe on the underside. Ensure that tank corners are rounded and that surfaces are smooth.

b. Water supply: Provide a flowing water system for holding, spawning, and rearing a variety of aquatic organisms. In general, reconstituted freshwater and artificial seawater are not cost-effective for large-scale rearing or for flow-through tests. If available, use natural, unpolluted fresh or salt-water supplies that have low turbidity, high DO, low BOD, and an annual temperature cycle that approximates that of the test organisms. To avoid debris or indigenous organisms that may be confused or compete with test organisms, natural freshwater or seawater must be filtered through a sieve with ≤60 μm mesh. If a flow-through water supply is unavailable, use of reconstituted fresh water or artificial salt water in recirculating culture systems with biological filters may be acceptable.

1) Fresh-water supplies—A good fresh-water supply is constant in quality; provides for adequate survival, growth, and reproduction of test organisms; and does not contain more than 20 mg/L suspended solids, 2 mg/L total organic carbon (TOC), 5 mg/L COD, 20 μg/L un-ionized NH_3, 0.01 mg/L total residual chlorine, 50 ng/L total organophosphorus pesticides,* or 50 ng/L total organochlorine pesticides plus polychlorinated biphenyls (PCBs). Consider water to be of constant quality if the monthly ranges of hardness, alkalinity, conductivity, TOC or COD, and salinity are <10% of average values and the pH range is <0.4 units.

Check municipal water supplies to determine their acceptability from the standpoint of, for example, copper, lead, zinc, fluoride, and free and combined chlorine concentrations; these are typical concerns, but others may be equally relevant. If a satisfactory fresh-water supply is not available or if a standard water is required for comparative toxicity tests, relative sensitivity tests, or tests to determine the effects of hardness, pH, or total alkalinity on the toxicity of various materials, use a reconstituted standard water. Gas supersaturation is common in nearly all water supplies and merits concern. Total dissolved gas levels should not exceed barometric pressure in shallow tanks (≤1 m deep) and should be monitored as described in Section 2810. Supersaturated gas levels can be reduced by aeration,[11] but complete removal requires pretreatment by nitrogen stripping with pure oxygen.

Prepare standard fresh water (Tables 8010:I and 8010:II) by adding reagent-grade chemicals to glass-distilled and/or deionized water. For special studies, determine that the reverse osmosis, distilled and/or deionized water contains less than the indicated constituents:

Conductivity	1 μS/cm
Total organic carbon (TOC)	2 mg/L
or chemical oxygen demand (COD)	5 mg/L
Boron, fluoride	100 μg/L each
Un-ionized ammonia	20 μg/L
Aluminum, arsenic, chromium, cobalt, copper, iron, lead, nickel, zinc	1 μg/L each
Total residual chlorine	<10 μg/L
Total organophosphorus pesticides	50 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs)	50 ng/L

Carbon-filtered deionized water usually is acceptable. Determine conductivity of distilled and/or deionized water for each batch of reconstituted water. Check other constituents periodically. If the water is prepared from a dechlorinated water, test the reconstituted water to determine that first instar daphnids survive for 48 h and can reproduce successfully when mature (Section 8711).[1]

* No individual pesticide should exceed the allowable concentration limit set in EPA's National Water Quality Guidelines, as set in accordance with the Federal Water Pollution Control Act 92–500 as amended in 1972.

Table 8010:I. Recommended Composition for Reconstituted Freshwater

Water Type	Salts Required mg/L				Water Quality		
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	pH*	Hardness *mg $CaCO_3$/L*	Alkalinity *mg $CaCO_3$/L*
Very soft	12	7.5	7.5	0.5	6.4–6.8	10–13	10–13
Soft	48	30	30	2.0	7.2–7.6	40–48	30–35
Moderately hard	96	60	60	4.0	7.4–7.8	80–100	60–70
Hard	192	120	120	8.0	7.6–8.0	160–180	110–120
Very hard	384	240	240	16.0	8.0–8.4	280–320	225–245

* Approximate equilibrium pH after aeration with fish in water.

The pH, alkalinity, and hardness of a receiving water influence the toxicity of some materials, especially metals, so it is desirable to have a supply of both hard and soft waters with suitable pH and alkalinity.

It is advantageous to have water with temperatures between 3 and 12°C during the winter and between 12 and 25°C at peak summer temperatures. For general use, the pH should be in the range of 7 to 8.2 and dissolved CO_2 should be 1 mg/L or less.

2) Marine water supplies—Use unpolluted marine water with low turbidity and settleable solids, and a pH and salinity favorable for the test organism. Ensure that annual salinity variations are not so wide as to be harmful to the organisms. In general, it is preferable to have a source of higher-salinity water (e.g., ocean water) from which brackish water can be prepared via dilution.

If a suitable marine water supply is not available, use artificial seawater for limited culturing and toxicity testing.[14–16] Prepare artificial seawater by adding the compounds in Table 8010:III, in the order listed, to 800 mL glass-distilled or deionized water. Make sure that each salt is dissolved before adding the next. Make up to 1 L with distilled or deionized water. The salinity should be 34 ± 0.5 g/kg and pH 8.0 ± 0.2. Obtain desired salinity at time of use via dilution with deionized water. Alternatively, prepare marine water from commercially available salt mixes that have been shown to provide for adequate survival, growth, and reproduction of test organisms.

To increase the salinity of a natural water, use a strong natural brine prepared by freezing and then partially thawing seawater. Natural brine also may be prepared (not to exceed 100‰ via evaporation of natural seawater with aeration and low heat (maximum of 35°C).[13] This is satisfactory if only limited amounts of water are needed; for larger volumes, use commercial sea salts or a stronger solution of artificial seawater.

When preparing artificial seawater, be sure that the metals concentrations do not become undesirable. Even reagent chemicals contain traces of several metals, and their extensive use can result in a buildup of metals. If large volumes of artificial seawater are not required, remove the metals by passing the seawater through a column containing a cation-exchange resin in the sodium form.[17]

The suitability of any artificial saltwater is enhanced via aging, aeration, and the introduction of nitrogen-fixing bacteria from water in which healthy aquatic organisms have been maintained, or the addition of marine algae.

c. Food and feeding:

1) Culture of microorganisms—Phytoplankton and zooplankton may be cultured as test organisms for studying biostimulation, toxicity, etc. They also may be cultured as food for other

Table 8010:II. Quantities of Reagent-Grade Chemicals to be Added to Aerated Soft Reconstituted Freshwater for Buffering pH[28]

Desired pH*	Quantity of Chemical to Be Added mL/L water		
	1.0*N* NaOH	1.0*M* KH_2PO_4	0.5*M* H_3BO_3
6.0	1.3	80.0	—
6.5	5.0	30.0	—
7.0	19.0	30.0	—
7.5	—	—	—
8.0	19.0	20.0	—
8.5	6.5	—	40.0
9.0	8.8	—	30.0
9.5	11.0	—	20.0
10.0	16.0	—	18.0

* Approximate equilibrium pH. Do not aerate after adding these chemicals.

Table 8010:III. Procedure for Preparing Reconstituted Seawater*[14,16]

Compound in Order of Addition	Final Concentration mg/L
NaF	3
$SrCl_2 \cdot 6H_2O$	20
H_3BO_3	30
KBr	100
KCl	700
$CaCl_2 \cdot 2H_2O$	1 470
Na_2SO_4	4 000
$MgCl_2 \cdot 6H_2O$	10 780
NaCl	23 500
$Na_2SiO_3 \cdot 9H_2O$	20
Na_4EDTA*	1
$NaHCO_3$	200

* Tetrasodium ethylenediaminetetraacetate. Omit when toxicity tests are conducted with metals. Omit when tests are conducted with plankton or larvae. Strip the medium of trace metals.[17]

Table 8010:IV.A. Macronutrient Stock Solution

Compound	Concentration *mg/L*	Element	Resulting Concentration *mg/L*
$NaNO_3$	25.5	N	4.20
		Na	11.0
$NaHCO_3$	15.0	C	2.14
K_2HPO_4	1.04	K	0.469
		P	0.186
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
		Mg	2.90
$MgCl_2$	5.70		
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20

Table 8010:IV.B. Micronutrient Stock Solution

Compound	Concentration mg/L	Element	Resulting Concentration mg/L
H_3BO_3	186	B	32.5
$MnCl_2$	264	Mn	115
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2$	0.780	Co	0.354
$CuCl_2$	0.009	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3$	96.0	Fe	33.0
$Na_2EDTA \cdot 2H_2O$	300	—	—

organisms, such as copepoda, *Daphnia* and other microcrustaceans,[18] the larvae and adults of mollusks, and young and adult fish.

a) Culture medium for freshwater algae—Prepare reconstituted fresh water by adding reagent-grade macro- and micronutrients to glass-distilled and/or deionized water in the concentrations given in Tables 8010:IV.A and B.

Prepare a separate stock solution of each micronutrient salt in 1000 times the specified final concentration in glass-distilled or deionized water. Combine trace metals and ethylenediaminetetraacetic acid (EDTA) in a single micronutrient stock solution in glass-distilled or deionized water at 1000 times the final concentration of each. Note: In toxicity testing of metals, the effect of EDTA must be established and reported as part of test results.

To prepare algal culture medium, add 1 mL of each macronutrient stock solution to 900 mL glass-distilled or deionized water, then add 1 mL micronutrient stock and make up to 1 L with glass-distilled or deionized water. As an alternative to algal culture medium preparation, commercially supplied nutrient media are available.†

To prepare duckweed culture medium, add 10 mL of each micronutrient stock solution to 900 mL glass-distilled or deionized water, then add 1 mL trace metal EDTA mixture and make up to 1 L. Alternatively, mix 10 mL each of three stock solutions (A, B, and C) to 1 L (see Section 8211B.2).

† Fritz Chemical Co., Dallas, TX, or equivalent.

Table 8010:V. Nutrients for Algal Culture Medium in Seawater

Compound	Concentration	Concentration of Nutrient
$NaNO_3$	25.0 mg/L	4.2 mg N/L
K_2HPO_4	1.05 mg/L	0.19 mg P/L
$FeCl_3$	72.6 μg/L	
$MnCl_2$	2.30 μg/L	
$ZnCl_2$	2.10 μg/L	
$Na_2MoO_4 \cdot 2H_2O$	2.50 μg/L	
$CuCl_2$	0.20 μg/L	
Na_2EDTA	300 μg/L	
Vitamins:		
Thiamine	0.100 mg/L	
Biotin	0.50 μg/L	
B_{12}	0.50 μg/L	

Whenever axenic algal cultures are used, or if bacterial growth interferes with the test, prepare media aseptically: To 800 mL glass-distilled or deionized water, add 1 mL of each micronutrient stock solution in the order listed, mixing after each addition. Filter-sterilize by passing through a sterile 0.2-μm-porosity membrane filter (pre-rinsed with 100 mL double-distilled water) into an autoclave-sterilized container. Add 1 mL filter-sterilized micronutrient solution, and make up to 1 L with sterile distilled or deionized water.

Store uninoculated sterile reference medium in the dark to avoid photochemical changes.

When sterility is desired in algal tests, check sterility periodically by adding 1 mL inoculated test culture to tubes of sterile nutrient test medium and incubate in the dark at the test temperature for 2 weeks. The appearance of opalescence in the test medium indicates contamination.

Prepare sterile bacterial nutrient test medium by adding the following quantities of chemicals to 1 L glass-distilled water:

Sodium glutamate	250 mg
Sodium acetate	250 mg
Glycine	250 mg
Sucrose	250 mg
Sodium lactate	250 mg
DL alanine	250 mg
Nutrient agar	50 mg

Bring medium to a boil, dispense to test tubes, and sterilize by autoclaving.

b) Culture medium for marine algae—To artificial seawater (Table 8010:III), add nutrients listed in Table 8010:V to give the indicated concentrations in the algal culture medium.

When an unpolluted seawater is used, prepare the medium by enriching filter-sterilized seawater with micronutrients at one-half the indicated concentrations. If sterile techniques are required, follow procedures for fresh water. When sterilization is performed by autoclaving, add vitamins after autoclaving. When filter-sterilization is used, use a positive pressure of 72 kPa.

2) Mass production of algae as food for other organisms—Rearing zooplankton, various filter-feeders, the larvae of crustaceans, and fish requires large quantities of phytoplankters. These needs must be met with an apparatus capable of producing continuous amounts of desired organisms at high densities. Such

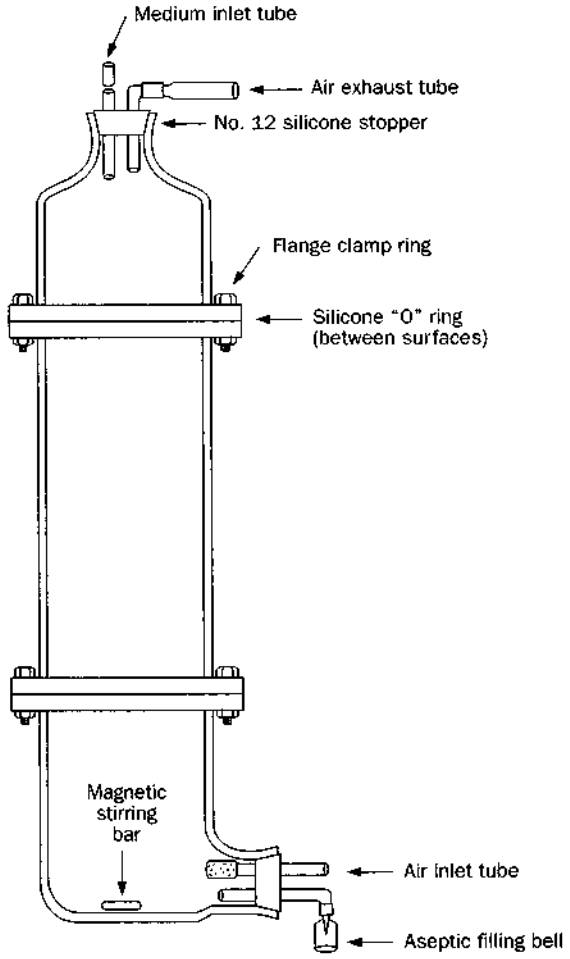


Figure 8010:2. Algal culture units. Cells are withdrawn from unit through the aseptic filling bell.

an apparatus (Figure 8010:2) permits easy assembly, cleaning, sterilization, and efficient use of light energy, and is constructed for continuous use. The main body of the unit is a 60-cm section of 15-cm-diam borosilicate glass drainage pipe. The top section is a 15- to 5-cm concentric reducer, and the bottom section is a 15- to 5-cm ell. Each section accommodates a No. 12 silicone rubber stopper held in place by a carboy or similar clamp. Hold the three sections together by aluminum ring clamps and seal adjoining surfaces by silicone "O" rings made from small-diameter tubing. Use material that is autoclavable and nontoxic.

Use this or a similar device to supply cells on a periodic or continuous basis. As the cells are withdrawn, add more medium. The following species have been grown at the indicated concentrations:

- *Skeletonema costatum,* 4.3×10^6 cells/mL;
- *Dunaliella tertiolecta,* 4.4×10^6 cells/mL;
- *Isochrysis galbana,* 7.0×10^6 cells/mL; and
- *Monochrysis lutheri,* 5.0×10^6 cells/mL.

3) Food for macroinvertebrates and fish—A suitable food is essential for rearing various macroinvertebrates and early life stages of some fishes. Distinguish carnivores from herbivores to supply the correct type of food. Organisms taken as food differ for different life stages of a species. As organisms grow, they require progressively larger food organisms. Many feed on pelagic organisms whose movements should be sufficient to attract the predator but slow enough so they can be caught readily. Use food organisms that are nutritious, easily digested, uncontaminated, and readily obtainable. The nutritional requirements of salt- and fresh-water organisms can differ greatly; check specific requirements of particular species. Distribute zooplankton food in rearing tanks to match distribution of organisms using it. Provide an adequate amount of food with a prey-to-predator ratio varying from 50:1 to 200:1, depending on the cultured species' feeding efficiency. If a small number of organisms is reared in a large tank, provide an acceptable food-organism density to ensure that enough are captured. Some algae and diatoms used for food have a tendency to settle to the bottom. Circular movement of water in the rearing tanks, provided as described in 8010E.4*a*, keeps food materials in suspension.

When using cultured microorganisms as food, be aware of possible environmental changes they may cause. In addition to the possible presence of toxic metabolites, algal blooms may produce excess oxygen and result in supersaturation and gas-bubble disease.[11] Use live food whenever possible. Analyze food for toxicants, especially pesticides and heavy metals. Supplement natural foods with commercially available dried and pelleted foods.‡ (See Section 8910 for fish feeding.) These foods should be attractive to the organisms, supply necessary nutrients and trace elements, and contain binders to ensure pellet stability.[19] Nutritionally deficient diets may cause significant differences in test organisms' sensitivity to toxicants and will affect reproductive performance.

Methods for rearing freshwater organisms have been described.[20] Methods for rearing larvae of marine animals with special reference to their food organisms have been summarized.[21] The literature on laboratory feeding of larvae of marine fish[22] and fresh-water fish[23] has been reviewed. Standard fish hatchery culture facilities and operations and other useful fresh-water and marine aquaculture references are available.[24–26]

d. Cleaning containers and equipment:

1) Cleaning holding, acclimation, testing, and dilution water tanks—Clean test containers and toxicant delivery systems before use. Soak new solvent and acid-resistant containers overnight in tap or dilution water, then wash with laboratory detergent, rinse with 100% acetone, water, acid [e.g., 5% nitric acid (HNO_3) or hydrochloric acid (HCl)], and rinse twice with tap water. After each test, wash the system appropriately [e.g., acid to remove metals and bases; detergent, sodium hypochlorite§ (NaOCl) solution (200 mg/L), organic solvent, or activated carbon to remove organic compounds, etc.] Immediately before testing, rinse again with dilution water.[1]

2) Removal of unused food and wastes—Do not let unused food or fecal material accumulate. Whenever possible, build holding and testing containers with sloping bottoms so food and feces can be removed easily with a siphon. The amount and

‡ Some foods that have been used widely include Glenco Trout Food, Glenco, MN 53336; Biorell and TetraMin, available from local pet shops; Oregon Moist, Warrenton, OR 97146; and Cerophyl®, Agri-Tech, Inc., Kansas City, MO 64112. The latter has been used both for the small forms and as a food for organisms to be eaten by higher species.

§ Do not use acid and hypochlorite together.

frequency of cleaning depends on the organism, ratio of dilution water to weight and volume of organisms, and feeding schedule. Clean holding containers at least once every other day. If growths occur on sides of containers, dislodge and let settle for removal.

5. Parasites and Disease

a. Stress in relation to parasites and infectious disease: Unexpected and often unexplained mortalities in experimental and control animals interfere with acute or chronic test results. While many factors may be responsible for an animal's death, diseases due to specific pathogens are among the most significant. In general, obtain fish and other animals from specific pathogen-free stocks (commercial bioassay organism supplier, specific hatcheries, etc.) rather than stressing populations via parasite and disease controls. Also, optimize laboratory conditions for the individual species to avoid fostering disease conditions.

When large numbers of organisms are retained in a relatively small space, undesirable growths, infectious diseases, and parasites may become a problem. Pathogens and parasites that might be rare in natural waters become potential and ever-present dangers in intensive culture.

Filtration and/or sterilization of water, regular cleaning of holding vessels, strict sanitation practices, and sterilization of equipment are essential for healthy animals. Uniform food distribution, limiting the amount of unused food, and expeditious removal of unused food and waste materials are also important.

Organisms exposed to toxicants become stressed and weakened, and may become more susceptible to parasites and disease. Because other environmental factors contribute to reduced resistance, pay careful attention to nutrition, oxygen supply, and water quality.

b. Control methods: Ultraviolet (UV) light and ozonation have been used successfully to control disease and parasites. Antibiotics can be used in holding tanks to reduce bacterial populations.[27] To reduce mortality and avoid introducing disease into stock tanks, treat with a wide-spectrum antibiotic immediately after collection, during transport, during egg production and hatching, or on arrival at the laboratory. Holding in a tetracycline-based antibiotic‖ (15 mg/L for 24 to 48 h) may be helpful. Other chemotherapeutic agents are available, but apply them with care because some are toxic at low concentrations.[27] Do not use treated organisms for tests for at least 10 d after treatment unless eggs are treated and <48-h-old organisms are required for the test. If contamination is suspected, disinfect tanks and containers with 200 mg NaOCl/L for 1 h.

For larval tests, use strict sanitary measures (e.g., sterilization of utensils and containers, filtration and UV sterilization of water, and removal of metabolic products). If disease signs appear in larval cultures, discard the entire culture.

For tests using adult fish and shellfish, early diagnosis and prompt treatment, when available, can prevent losses.

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‖ Terramycin, or equivalent.

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8010 F. Toxicity Test Systems, Materials, and Procedures

1. Water Supply Systems and Testing Equipment

a. Composition of materials used: Construct all test-system components—including water heating and cooling units, constant-level troughs and head boxes, valves and fittings, diluters, pumps, mixing equipment, tanks, and exposure chambers—from inert materials. Acceptable materials include lead-free glass, perfluorocarbon plastics, silicone sealant and tubing, polyvinyl clear flexible plastic,* polyvinyl chloride (PVC), nylon, fiberglass, and No. 316 stainless steel. Unplasticized plastics (e.g., polyethylene or polypropylene) may be used in the dilution portion, including holding and acclimation tanks. Avoid contact with brass, copper, lead, or rubber. Some neoprene formulations have been acutely toxic, while others have been proven safe for toxicity testing. Cure materials in dilution water at least 48 h before use.

b. Temperature regulation: Obtain the desired temperature of dilution water by mixing constant-temperature hot and cold waters in the correct proportions or by using heat exchangers, water baths, or heaters or coolers in constant-level troughs and head boxes. A heated room or high–low incubator with thermostatic controls usually is suitable for static tests on warm-water organisms. For static tests, hold dilution water in tanks until it reaches ambient temperature. For cold-water species, use a specially insulated constant-temperature room or large water bath equipped with temperature controls and adequate circulating water. A satisfactory design for short-term static tests at a small laboratory has been described.[1–3] Any special facilities required for different groups of organisms are described in Sections 8111–8930.

c. Toxicant-delivery system: In flow-through tests, use metering pumps or other devices to deliver toxicant or test material accurately into dilution water.[1] Most toxicant-delivery systems have been designed for fresh water and may not be applicable to all test substances. Deliver dilution water from constant-head troughs or head boxes via siphons, constricted tubing, nozzles, or pumps. Deliver toxicants via siphons from constant-head reservoirs, pumps, calibrated glass nozzles, solenoid valves, or Mariotte bottles.[4] Mix dilution water and toxicant in tanks with baffles or stirrers, or in mixing troughs.[5] Since the serial diluter was introduced,[6] various methods and types of diluters have been described.[1,7–25]

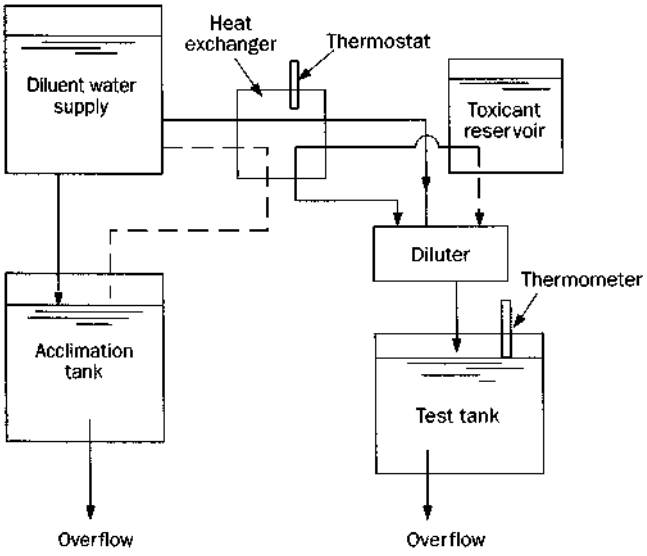


Figure 8010:3. Basic components of flow-through system.

The choice of a toxicant-delivery system for flow-through toxicity tests depends on such factors as dilution, flow rate, quantity of toxicant available, and the presence of suspended solids. The proportional diluter[17] was designed to handle a dilution factor (i.e., the factor by which a concentration is multiplied to calculate the next lower concentration) between 0.50 and 0.90. The serial diluter has been modified to provide a narrow range of concentrations.[18] Other diluters operate well with a dilution factor of 0.50.[1,23] Flow rates through test chambers may vary from 400 mL/min for the proportional diluter[6] to 6000 mL/min.[23] Some stock solutions[1,20,22–24] and other systems have been modified to handle larger volumes of toxicant and high suspended solids concentrations.[1,20] These diluters are most suitable for effluent toxicity tests. Several diluters have been designed for flow-through toxicity tests with embryo–larval stages of aquatic organisms.[1,22] One system was designed to eliminate the air/water interface for tests using volatile compounds and compounds with very low solubility.[22]

The basic components of a flow-through system are shown in Figure 8010:3. The diluent water reservoir is large enough to provide water for at least 5 d. If dilution water is added to this reservoir continuously, a smaller capacity is preferred. Dilution

* Tygon®, or equivalent.

water flows at a constant rate by gravity from this reservoir to a constant-head diluent-supply head box through a nonmetallic float-controlled valve or other device and then to the diluter. Provide head box with heating or cooling equipment and a thermostat to maintain constant temperature. Equip test containers with an overflow system designed to prevent organisms from entering outlets. Clean test containers daily as described in 8010E.4*d*.[25]

The constant-head toxicant supply is a constant-level tank, a Mariotte bottle, or other device. If toxicant is added at greater than a drip rate, adjust toxicant temperature to that of the diluter via a heat exchanger; otherwise, the toxicant heat exchanger in Figure 8010:3 is unnecessary. If the toxicant stock solution is unstable, renew it before it degrades. If metering pumps are used, the toxicant supply system need not maintain a constant head.

A simple valve-control system for regulating dilution-water and toxicant-solution flow rates has been described.[26] For more toxic materials, less toxicant is required and a Mariotte bottle or syringe pump that delivers a very slow but constant flow is useful.[19,20,22,27]

A diluter meters dilution water from the constant-head box and toxicant from the constant-head tank or other containers and mixes them in the proper proportions for each test chamber. After calibrating the diluter properly, make proper concentrations of toxicant stock solutions. Shield the toxicant supply reservoir from light when necessary.

Provide a mixing chamber between diluter and test container for each concentration. If duplicate test containers are used, run separate delivery tubes from the mixing chamber to each duplicate or use specialized glassware that will split the mixed solution equally. Use flow rates through test containers of at least 6 tank water volumes/24 h. Do not let rates through test containers vary temporally or between containers by more than ±10%. Calibrate the toxicant and dilution water volumes used in each portion of the toxicant-delivery system and the flow rate through each test container. Check operation of toxicant-delivery system daily during test.

2. Preparing Test Materials

a. Dilution water: Whenever possible, test effluent toxicity onsite if ample supplies of toxicant and dilution water are available. Onsite testing permits temperature, DO, pH, hardness, salinity, turbidity, and other dilution-water qualities to vary normally with those of the receiving water. That said, consistent overnight delivery service is available virtually anywhere in the United States and elsewhere, and the expense and logistics involved in performing onsite studies may make testing under more stable conditions in permanent laboratories more advantageous.

Convey the effluent sample to testing chambers with as little modification as possible. Do not unnecessarily aerate, heat, cool, or agitate. If the testing facility is remote from the effluent-discharge site, artificial or reconstituted water may be used as the diluent. If the diluted effluent is low in DO, adjust flow-through and loading in the test chambers so DO is not reduced significantly; aerate as a last resort. Hold temperature at or near that of the receiving water.

If the receiving water is DO-deficient and has temperatures above locally applicable water-quality standards, bring these into compliance so allowable levels of a specific waste can be assessed meaningfully. Determine toxicity of test waste compared to other contaminants present in receiving water by taking dilution water from receiving water just outside the area affected by the test waste. This is especially necessary when effluents contain metal salts, cyanide complexes, ammonium compounds, or other materials, the toxicity of which is greatly influenced by changes in pH, hardness, temperature, etc.[28] If there are wide variations in receiving-water-quality characteristics, determine waste toxicity at the upper and lower limits of the range.

Evaluate receiving-water effects on aquatic biota by using two controls: one with the receiving water and another (either natural or synthetic) with an unpolluted water of similar quality. If appropriate to the study, adjust calcium, magnesium, sulfate, alkalinity, pH, and DO for freshwater controls to those of the receiving water before adding wastes to determine if dilution water itself is unfavorable for the more sensitive aquatic species in the area. Conduct chronic tests to detect the most subtle sources of stress.

The dilution water's turbidity is important in determining harmful concentrations of potential toxicants because some toxicants are sorbed on particles. Turbid dilution water may limit visual inspection and photosynthesis of algae, affect the response of test organisms to tested material, form deposits, and clog water systems. When large amounts of settleable solids significantly remove toxicants from the water, determine concentrations of toxicants in bottom sediments and their toxicity by appropriate tests with benthic organisms.

When testing toxicants other than effluent, use for dilution water only a nonpolluted natural or synthetic dilution water of constant and reproducible quality that is favorable for aquatic life. Warm or cool dilution water to test temperature and bring to equilibrium with atmospheric gases (i.e., DO, CO_2) before use.

Use standard water conditions and organisms for comparative toxicity and sensitivity tests. Use reconstituted fresh or marine water [8010E.4*b*1) and 2)] if natural supply is unsuitable. Because water quality affects toxicity, it may be desirable to use both hard and soft water for tests on fresh-water organisms.

Many marine organisms spend part of their life cycles in estuaries. In life-cycle tests, change dilution water in accordance with their requirements at different life stages. If the effects of temperature are not being studied, keep it within a favorable range.

For warm-water (≥ 20°C) species, keep DO > 40% of saturation; for cold-water species, keep DO > 60% of saturation. Some larval forms (e.g., those of marine crustaceans) may require higher DO concentrations.

Determine pH requirements for test organisms. In long-term studies (other than effluent and monitoring tests), keep pH within 0.4 units of the desired value. Avoid rapid changes in temperature, pH, or CO_2 content. A rapid increase in the CO_2 content of marine waters indicates that a significant change has occurred that should be investigated at once. Freshwater organisms are more tolerant of pH changes and accommodate much wider variations than strictly marine forms. Changes in pH drastically alter the toxicity of many materials (e.g., cyanide and NH_3).

When working with estuarine and marine organisms and different life stages that may be marine or estuarine, salinity is of prime importance. Use the natural salinity for each test species and its different developmental stages.

Keep acidity, total alkalinity, and hardness of dilution water constant. Alkalinity and hardness influence some metals' toxicity, and total alkalinity is an important factor in photosynthesis and algal growth.

b. Toxicant solution: Prepare toxicant solution in advance and add immediately to the dilution water for static tests. If a toxicant is unstable, determine its stability and replace as necessary. If possible, measure toxicant concentrations during the test. Prepare all solutions for each series of tests from the same source sample. Disperse undissolved material uniformly by shaking or gently mixing.

If solvents are necessary, use acetone, acetonitrile, dimethylacetamide, dimethylformamide (DMF), ethanol, ethylene glycol, isopropanol, methanol, or triethylene glycol to prepare stock solutions. Certain surfactants may be useful. Use only the minimal amount of solvent necessary to disperse the toxicant. Do not exceed 0.5 mg/L in static and 0.1 mg/L in flow-through test solutions. A solvent control is also necessary when a carrier is used.

If a solvent is used, use two sets of controls: one containing no solvent and the other containing the highest concentration of solvent used during the test.

Some effluents, especially oily wastes, are difficult to distribute evenly. The nature of the test material (e.g., single chemical or solid versus effluent) governs the preparation of test concentrations and frequency of test-medium replacement. Common problems include insolubility, adsorption on exposed surfaces, decomposition, photolysis, loss of volatiles, high BOD and/or COD levels, and bacterial growth. These can change the apparent test concentration and lead to erroneous results. Effluent samples that vary in composition with time may require a series of tests to characterize toxicity.

Store effluent samples at 4 ± 1°C in containers from which all air has been expelled. Do not store samples longer than absolutely necessary because toxicity may change with time. (Choose sample containers to minimize changes in sample-constituent concentrations.)

Thoroughly mix test materials before use. Use material directly as a stock solution of toxicant or prepare a stock solution using filtered dilution water. Make stock solutions with dilution water on a volume-to-volume basis. If the effluent is liquid, designate the percentage waste in each test concentration. If the waste is a solid, dilute on a weight-to-volume basis (e.g., milligrams per liter).

If the waste contains both solids and liquids, mix thoroughly to disperse before using as a stock toxicant, and provide agitation in the stock reservoir and test containers. If larger organisms are tested, a propeller placed under a screen or perforated false bottom may be used to maintain solution consistency. If solids settle out rapidly and do not contact pelagic organisms, test only the liquid portion. After thorough mixing, let settle and decant or drain off the liquid for use as test toxicant. If the solid-waste portion is toxic, set up test chambers with a certain weight-to-volume ratio of bottom material and expose the receiving-water area's benthic and burrowing organisms. Mix wastes and let settle before adding organisms. If waste contains sparingly water-soluble materials, check solubility; if below or at very low toxic concentrations, then solvents, emulsifying agents, or water-miscible solvents may be used to disperse.

c. Test organisms: Select test organisms as described in 8010E.1 and handle as indicated in 8010E.3 and 4. For long-term tests, use only the highest quality test organisms available. At the end of the test, control organisms still should be in good condition.

3. Test Procedures

a. Experimental design: Expose test organisms in at least duplicate containers of each experimental concentration. Using more organisms and replicate test containers for each toxicant concentration enables analysts to evaluate intratest variability. Individual test methods may specify the number of replicates. Statistical methods also may dictate numbers of replicates. Use only true replicates with no water connection between test containers. Typically, each test consists of a minimum of five test concentrations and a control, with an additional control if a solvent is used. Tests of ambient waters may make the typical dose–response test design impractical. To compensate for positional effects, arrange test containers at random in the testing area. If replicates are used, randomize each series of test containers separately. Distribute organisms randomly to test containers—adding one at a time to each container if each container's total will be <11 organisms, or two at a time if each container's total is more. In short-term static tests, place the correct number of organisms into intermediate containers, then add all at once to test chambers containing the toxicant. Minimize volume of liquid transferred with the organisms to each test chamber. Take care to avoid contaminating treatments during organism distribution.

Generally, short-term, acute tests with fish and invertebrates require that 90% or more of controls survive for results to be considered acceptable. If this is not achieved, repeat the test. Acceptability criteria also may be used with other endpoints (e.g., spawning, normal development, growth, reproduction, or general apparent health), but the various levels of control performance depend on the specific test procedures. Lower levels of survival may be acceptable in longer-exposure assays or in tests that use life stages where survival, even under ideal conditions, is limited. Examples of acceptable reduced survival include short-term tests for estimating the chronic toxicity of effluent to freshwater and marine organisms.[2,3] In these tests, acceptable survival for *Americamysis bahia, Cyprinodon variegatus, Ceriodaphnia dubia*, and *Pimephales promelas* is 80%.[2,3]

In short-term static or renewal tests with fish, use 20 or more test organisms in each toxicant concentration. Use of larger numbers of organisms per test concentration for smaller organisms is desirable. The number of organisms exposed in each test concentration is governed by organism size; expected normal mortality; extent of cannibalism; availability of dilution water, toxicant, and test organisms; and desired test precision. Test precision depends on variability of organism response, number of organisms exposed to each concentration, number of replications, differences between concentrations and range of concentrations tested, and toxicant concentration and its variability.

For a given test, increasing the number of test organisms increases confidence in estimates of effect and precision. It is recommended that the 95% confidence interval be less than ±30% of the mean. With test organisms for which culture methods are not available, this precision may be difficult or

TABLE 8010:VI. PERCENTAGE OF AMMONIA UN-IONIZED IN DISTILLED WATER*

Temperature °C	Percentage Un-ionized at Given pH								
	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
5	0.01	0.04	0.11	0.40	1.1	3.6	10	27	54
10	0.02	0.06	0.18	0.57	1.8	5.4	15	36	64
15	0.03	0.08	0.26	0.83	2.6	7.7	21	45	72
20	0.04	0.12	0.37	1.2	3.7	11.7	28	55	80
25	0.05	0.17	0.51	1.7	5.1	14	35	63	84
30	0.07	0.23	0.70	2.3	7.0	19	43	70	88

* Prepared from data given in Sillen and Martell.[30]

impossible to attain. Reference toxicant tests are used to evaluate the test organisms' condition and may be used to evaluate intertest performance of the method (see Section 8020B.2). Make specifically designed intra- or inter-laboratory tests to evaluate precision.

Before the test, determine length and weight of representative organisms to establish loading rates and acceptability with respect to size variation. After acclimation has begun, handle test organisms as little as possible. Increases in weight or growth may be determined by adding more animals than initially required, so some may be removed to make necessary measurements. At the end of test, measure weight and length to determine sublethal impacts for computation of statistical endpoints (see 8010G).

b. Selecting test concentrations: Express liquid-waste concentrations as percent on a volume-to-volume basis. Express concentrations of nonaqueous wastes and of individual chemicals as milligrams or micrograms per liter. Clearly indicate what the weight represents, as part of the solute's weight includes water of hydration (e.g., $CuSO_4 \cdot 5H_2O$). It often is more appropriate to express weight as weight of toxicant (e.g., μg Cu/L). When an impure chemical is tested, especially in a formulation containing added inert ingredients, indicate the chemical composition by weight and whether the EC value is based on the concentration of total material or only active ingredient.

Although test endpoints (e.g., EC or LOEC) may be determined by using any appropriate series of test concentrations, the geometric series of concentration values is simplest to use when the approximate toxicity range is unknown. Multiply the highest and succeeding concentrations by a constant factor (0.3 to 0.5) to obtain concentrations that are evenly spaced on a logarithmic scale. Range-finding tests can help determine the dilution factor to use in subsequent tests.

The magnitude-of-concentration intervals to establish an EC by interpolation depends on the required degree of confidence in the point estimate and on the experimental data. Intervals spaced more tightly around the expected EC will give a more precise estimate of the true EC.

c. Loading: For static tests, do not exceed an organism loading of 0.8 g/L in the test container. In tests with small organisms and tropical forms, decrease loading to as low as 0.1 g/L and accommodate large test organisms by using larger or duplicate test containers. Limit the number of test organisms per volume of test solution so during the test

- DO remains >60% of saturation for cold-water species and >40% of saturation for warm-water species;
- toxicant concentration is not lowered significantly;
- concentrations of metabolic products (e.g., NH_3, CO_2) do not become too high; and
- organisms are not stressed by crowding or increased potential for cannibalism.

Do not let concentration of un-ionized ammonia exceed 20 μg NH_3-N/L (Table 8010:VI).

For flow-through studies, use a flow rate of at least 6 tank volumes/24 h to maintain desirable temperature and DO and safe concentrations of metabolites. If the DO concentration drops below the desired level, increase the turnover rate within the diluter. If this is inadequate, aerating the test chambers may be permissible.

d. Physical and chemical determinations:

1) Dilution water analysis—For freshwater, measure hardness, alkalinity, pH, TOC (or COD), and suspended solids at least once every 30 d and at the beginning and end of the test. If water quality is variable, test more frequently. Tap water can be dechlorinated by active aeration (using air stones) for 24 h, filtration through activated carbon, or use of sodium thiosulfate. If a treated tap water is used, measure residual chlorine by one of the methods given in Section 4500-Cl.[29]

Analyze weekly for pH, alkalinity, and hardness to define test-water variability. If characteristics are affected by the toxicant, test samples from each toxic concentration at least once every other week. For brackish or marine dilution water, measure salinity, pH, DO, and temperature two or three times daily; and suspended solids and TOC at least once every 30 d and at the beginning and end of each test.

2) Toxicant analysis—It may not be necessary to make routine detailed analyses in flow-through life-cycle tests, but test periodically to ensure that exposure tanks maintain the correct ratio of effluent to dilution water.

For studies to determine chemical water-quality criteria, it is desirable to measure the concentration of toxicants in each container at the beginning and at least once during testing, or weekly in longer tests. Chemical measurements should be made in at least one container at the next-to-lowest toxicant concentration (or within the calibration range of analytical method). Whenever a malfunction is detected in any part of the toxicant delivery system, check toxicant concentration in at least one container. For replicate test containers, use a ratio of highest measured concentration to lowest measured concentration of less than 1.15; if this is exceeded, check toxicant delivery system and analyze additional samples from test containers to determine if the sampling or analytical method is sufficiently precise. Do not

accept measured toxicant concentrations differing by more than ±15% from the calculated concentration unless specific reasons justify a greater difference. Precision is important when assessing measurements of nominal toxicant concentrations in chemical test methods, and test methods ideally should be calibrated in a range that includes the nominal toxicant concentration.

Record temperature at least hourly throughout the test (24 h/d) in at least one test container and make additional measurements on dilution water and other test solutions. If test is performed in a laboratory, continuously monitoring the temperature of the testing area (i.e., water bath, environmental chamber, etc.) may be appropriate. Measure DO, pH, and salinity at the beginning of the test and daily thereafter in the control, high, medium, and low toxicant concentrations. Generally, variation should not exceed ±1.0°C.

Take water samples for chemical analysis at the center of the exposure tank; do not include surface scum or material from tank bottom or sides. If analytical results are not affected by storage, collect daily, equal-volume grab samples and composite for a week. Analyze sufficient samples throughout the test to determine whether the toxicant concentration is reasonably constant. If not, analyze enough samples weekly to show the variability of toxicant concentration. If methods are available, determine the toxicant loss in the next-to-lowest concentration. If the loss is >10%, attempt to alleviate by using either a faster flow rate or a lower loading.

When necessary, analyze mature and immature test organisms for toxicant residues. For larger organisms, analyze muscle and liver and possibly gills, blood, brain, bone, kidney, gastrointestinal (GI) tract, gonads, and skin. For large organisms, analysis of whole specimens can be used but does not replace analysis of individual tissues, especially muscle (edible fillet).

e. Biological data and observations: In short-term tests with macroinvertebrates and fish, count number of dead or affected organisms in each container at least daily throughout the test. With certain fast-acting biocides, it may be useful to count number of dead or affected organisms in each container at 1.5, 3, 6, 12, and 24 h after test begins. Remove dead organisms as soon as observed. Often it is more important to obtain data that will define the shape of the toxicity curve than to obtain data at prespecified times.

Death is the adverse effect most often used to reflect acute toxicity. The usual criterion for death is no movement—especially no gill movement in fish—and no reaction to gentle prodding. Death is not easily determined for some invertebrates; cessation of movement of antennae, mouth parts, or other organs may be used. When death cannot be determined, use EC_{50} rather than LC_{50}. The effect usually used for determining EC_{50} with daphnids, midge larvae, copepods, and other organisms is *immobilization* (inability to move, except for minor activity of appendages). Other effects can be used to determine EC_{50}, but always report the effect and its definition. Consistency in defining the effect directly influences toxicity test precision and repeatability. Also report such effects as erratic swimming, loss of reflex, discoloration, changes in behavior, excessive mucus production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism.

In short-term tests, organism reactions during the first few hours may indicate the nature of the toxicant and serve as a guide for further tests.

In long-term partial- or full-life-cycle tests, a photographic method for counting and measuring small test organisms has proven useful.[30] This is rapid and accurate, and does not entail handling the organism. With this method, use exposure tanks with glass bottoms and drains that allow the water level to be drawn down. To count and measure test organisms, draw water down to 2 to 3 cm deep, and transfer tank to a light box with fluorescent lights under a square millimeter grid of adequate size. Photograph aquarium bottom; this shows organisms over the grid. Count and measure organisms on an enlargement of the picture.

f. Photoperiod and artificial light: In long-term studies to determine water quality requirements for those species requiring annual-light-cycle photoperiods, simulate natural seasonal daylight and darkness periods at the locality or some central location.[31] (See Section 8910 for more information on light cycles for fish.)

Use cool white fluorescent tubes or a wide-spectrum lamp as a light source. Some organisms require subdued light, others need a place to hide, and some (e.g., lake trout eggs) require darkness during certain life stages. Base exposure to light on what is normal to, and required by, the species. Measure light intensity at the water surface. In short-term tests, a standard photoperiod of 16 h light:8 h dark is suggested.

g. Exposure chambers: For organisms weighing >0.5 g, use a test solution between 15 and 30 cm deep. In short-term tests, these organisms often are exposed in about 15 L solution in 20-L wide-mouth, soft-glass bottles. Fabricate test containers of other sizes by welding (not soldering) stainless steel, by gluing double-strength or stronger window glass with clear silicone adhesive formulated for aquarium use, or by modifying glass bottles, battery jars, or beakers to provide screened overflow holes or V-notches. Because silicone adhesives absorb some organochlorine and organophosphorus pesticides, expose as little of the adhesive as possible to water. Place extra beads of adhesive for added strength only on the outside of containers. Expose smaller organisms in 30-mL to 2-L beakers that contain 15 to 1500 mL solution. Expose daphnids, midge larvae, copepods, and other small organisms in loosely covered beakers or other containers. Disposable plastic containers may be used for tests involving compounds that do not react with the plastic. Disposable plastic containers are recommended for such tests as the 7-d *Ceriodaphnia* survival and reproduction assay[3] and *Champia parvula* sexual reproduction assay.[2]

With flow-through tests, keep liquid surface area/volume ratio small to reduce loss of volatiles. For various exposure-chamber designs, see Sections 8211–8910.

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8010 G. Calculating, Analyzing, and Reporting Results of Toxicity Tests

This section identifies statistical methods used in analyzing data from acute and chronic toxicity tests, evaluates methods appropriate for each type of data, and discusses advantages and disadvantages of the statistical methods that are used most often. If unfamiliar with basic statistical concepts and methods, consult a statistician when data-analysis questions arise.

A biological test's precision is limited by a number of factors, including the normal biological variation among individuals of a species, robustness of test protocols, and analyst proficiency. Calculation of test endpoints from experimental data, therefore, will reflect the net effect of these test variables.[1] In general, the statistical methods presented in this section are applicable to both acute and chronic methods; however, some techniques traditionally have been used for acute test results and others for chronic ones.

1. Toxicity Data Analysis

Data generated in acute toxicity tests are quantal—"yes" or "no" responses to research questions (e.g., Did exposure cause immobilization? Did exposure cause death?). Continuous measurements recorded in quantitative or graded tests, such as length, weight, or number of young produced, typically are not used as endpoints in an acute toxicity test. Data generated in chronic toxicity tests may be quantal (e.g., long-term survival), continuous (e.g., growth), or count (e.g., number of young produced).

Results from chronic toxicity tests may be analyzed by one or more statistical methods, depending on the purpose of the test. Regression methods—both parametric and nonparametric—may be used to generate ECs, evaluate conventional concentration-response test designs, and provide confidence intervals for point estimates (e.g., EC_{50} or LC_{50}) interpolated from the appropriate model. Hypothesis test methods (both null and alternate) can be used to generate estimates of effect threshold (e.g., NOEC, LOEC) or of equivalence (e.g., "bioequivalence"). The advantages and drawbacks of various statistical applications have been reviewed in detail.[2,3] All of the methods are computationally intense, but computer software applications and descriptive material facilitate their use in most instances. Although more than one statistical method can be used to treat a given data set, the experimental design chosen for the bioassay may affect the robustness of the statistical results. For example, point estimates and confidence intervals derived from regression models benefit from designs with more doses near the effect level of interest, while hypothesis test methods improve (in terms of statistical "power") with more replicates at tested doses. In order to estimate an effect (or the test concentration below a specified effect), regression methods interpolate points on the modelled concentration-response curve while hypothesis test results are constrained to be one of the doses tested. In this sense, both regression and hypothesis test methods benefit from having the selected test concentrations bracket close to the "true" effect level of interest. If the purpose of testing is to compare sources or evaluate changes in toxicity over time, then effect levels from appropriate regression methods are preferred because the results from multiple tests are amenable to normal population statistics, whereas multiple NOECs are not.

EC_P values can be calculated from parametric models [e.g., probit, logit, and generalized linear modeling (GLiM)].[4–6] Choose the model appropriate for the type of data generated in the test. For example, probit analysis is only suitable for quantal data,[7] while GLiM models require the appropriate data link.[5] The nonparametric IC_P or linear interpolation model is also available for evaluating nonquantal data.[8] Carefully evaluate goodness of fit and biological plausibility of the resultant toxicity curves and confidence-interval data with any model results. The model should be able to account for apparent thresholds or low-dose enhancement (nutritive or hormetic effects) in test data. Models that "force" data to fit by averaging adjacent dose–response data ("smoothing") or extrapolating to zero may not be appropriate for use on a given data set.

The NOEC (no-observed-effect concentration) or LOEC (lowest-observed-effect concentration) values calculated from hypothesis test statistics are based on statistical significance (usually at the $P = 0.05$ level): the NOEC is the highest dose tested whose response is not statistically different from control response, and the LOEC is the lowest dose tested whose response is statistically different from control response. The NOEC can be used to analyze any biological endpoint and data type commonly encountered in aquatic toxicity testing. The ChV (sometimes referred to as MATC) is a point estimate determined as the geometric mean of the LOEC and NOEC doses. Hypothesis test results are sensitive to intratest variability, which controls statistical power, and results also must be examined for homogeneity in variance among test groups. Data transformations usually are required (see 8010G.2).

Recently, the alternative hypothesis method (sometimes termed *bioequivalence* or *test for significant toxicity*) has been proposed and discussed[3,9,10] as an adjunct method for comparing the toxic response of a specified sample concentration to a threshold or acceptable level of response. This method is useful when analysts want to determine statistically that a specific sample response is equivalent to, rather than different from, a targeted value. It also is useful in pharmacokinetic applications or other experimental designs where limited numbers of doses can be tested, and when analysts are more concerned about false-negative statistical errors than false-positive ones. In simplest form, it is applied in a two-concentration test design (control and one dose, or two different doses) and is subject to the same concerns over variance and power as the null test.

Either hypothesis test method can be used to compare statistical significance between point estimates derived from regression models. This may be useful for comparing different species' response to toxicants, or different temporal responses.

a. Calculation of LC_{50}: Acute toxicity test results generally are characterized by the LC_{50} when mortality is the endpoint or EC_{50} when a sublethal effect is the endpoint. The LC_{50} is an estimate of the true median lethal concentration of the test material for the entire test species. Therefore, also provide a measure of statistical confidence in the point estimate, such as the 95% confidence interval of the LC_{50}. Values other than 50% can be used to characterize toxicity; however, the precision of test results for a typical sigmoid (or "S"-shaped) cumulative distribution dose–

response curve may be best in the vicinity of the 50% effect level because this is the straightest part of the curve. LC values near the tails of this curve (e.g., LC_{10} or LC_{90}) have wider confidence intervals.

Numerous procedures are available for analyzing quantal toxicity data. LC_{50} calculations include parametric procedures, such as probit analysis,[7,11] logit,[12] and GLiM.[4,5] The most commonly used nonparametric procedures are the Spearman-Karber method and the trimmed Spearman-Karber method,[13] while numerical interpolation techniques include graphical interpolation, moving average interpolation, and the binomial distribution. No single method is most appropriate for all data sets, but graphical interpolation and binomial distribution methods are simple to use. Computer programs facilitate the use of more sophisticated statistical methods and models, which may provide a better fit of the experimental data for many data sets. The statistical procedure cannot always be selected before the test is conducted because the data generated must meet certain model assumptions or minimum criteria before the applicability of various methods can be determined.

Parametric procedures transform dose–response data to a known or expected functional form before the LC_{50} determination (parametric methods). The probit method[11] probably is the most widely used LC_{50} calculation procedure and uses the probit transformation of mortality data in combination with a standard curve-fitting technique. A second parametric procedure uses a logit transformation[12] of mortality data. Common disadvantages of the parametric computational methods are that the distribution properties data must meet the model assumptions or the LC_{50} produced will not be considered appropriate. The probit and logit methods yield symmetric dose–response curves; they are not valid if the true curve is asymmetric. Models (GLiM) have been used to avoid such drawbacks.[4,5] Additionally, unless 0 and 100% mortality data are adjusted, the parametric methods can be used only when at least two partial kills are present in the data set.

The nonparametric Spearman-Karber method and the trimmed Spearman-Karber method[12] do not require that data meet model assumptions and do not depend on the presence of partial kills. These methods estimate the LC_{50} if the dose–response curve is symmetric. However, if the true curve is asymmetric, the Spearman-Karber method estimates the mean of tolerances and the trimmed Spearman-Karber method estimates the trimmed mean of tolerances; the LC_{50} is the median of tolerances. One drawback of the Spearman-Karber method is that the test data must cover the range from 0 to 100% mortality.

The moving average and moving average angle (transformation of mortality to angular values) methods also do not assume that the data distribution fits a predetermined model nor require partial kills for the LC_{50} calculation.[14] Shortcomings of these two methods are that the concentration series of the test must be equally spaced, and the methods cannot be used to calculate an LC value other than the LC_{50}. Median effective time (ET_{50}) for mortality at each concentration is estimated by plotting percentage mortality on a probit scale against time on a logarithmic scale and then using probit analysis techniques similar to those given above.[11,15–17] The procedures are the same, although more frequent observations of mortality may be required.

Single-chemical toxicity tests often use preliminary range-finding tests with a wide range of test concentrations to obtain a toxicity estimate. For subsequent definitive tests, a dilution series with more tightly spaced concentrations is used. For effluent-toxicity tests, range-finding tests may not be feasible, because of stipulated limitations on effluent-sample holding times. In these instances, effluent tests often produce results in which all organisms live at one test concentration, while all organisms die at the next higher concentration (i.e., no partial kills). For this type of data, the binomial test provides an approximate LC_{50} and specifies the concentration that can serve as statistically sound 95% confidence limits. However, the binomial test does not provide a true LC_{50} estimate and should be used only when the other available methods (e.g., probit) cannot be used. A preferred alternative to the binomial distribution method is the Williams test.[18]

Manual techniques for graphical determination of LC_{50} and confidence limits are available, and have been described in past editions of *Standard Methods*. A number of user-friendly computer software packages,* which can be used for parametric and nonparametric methods for treating data and generating confidence limits, also are available. Because each method has limitations with respect to certain data characteristics that can be analyzed (concentration series, number of partial kills, data type, data distribution, etc.) take care in using the appropriate method with the experimental data.

b. Control mortality: Generally, control mortality should not be greater than 10%. More than this is usually unsatisfactory and requires repetition of the test. Sometimes with long-term tests or with some invertebrates that have considerable mortality under the best possible conditions, it is necessary to use Abbott's formula:[19]

$$P = \frac{P^* - C}{1 - C}$$

where:

P and P^* = corrected and observed proportions responding to the experimental stimulus and
C = proportion responding in the control.

This approach does not solve the problem of the toxicant's possible interaction effects with whatever is causing mortality in the control.

Stephan[20] reviewed the methods available for calculating LC_{50}s and recommended using the moving average method and log concentration when one or more partial kills are present in a data set, and using either binomial or moving average when no partial mortality is observed. The latter type of data sets are undesirable when accurate toxicity estimates are required.

A review of LC_{50} calculation methods[21] concluded that if the effect data are normally distributed, then probit was the most efficient method to use for quantal data, whereas the trimmed Spearman-Karber method was preferred when normality did not hold. While other statistical models have been recommended and used[5,8] for continuous and count data, similar evaluations of how

* Such as TOXSTAT®, University of Wyoming, Laramie; TOXCALC®, Tidepool Scientific Software, P.O. Box 2203, McKinleyville, CA; CETIS®, Tidepool Scientific Software, P.O. Box 2203, McKinleyville, CA; Toxdat, Statistical Support Staff, Biological Methods Branch, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH.

the data fit the model assumptions must be made for any regression model. Because no single LC_{50} technique may be best in all situations, analysts must evaluate goodness-of-fit estimates of the model outputs, as well as confidence limits.

c. Hypothesis test methods for analyzing toxicity data: Traditionally, these methods have been used for chronic rather than acute toxicity test results. However, with the development of short-term critical life-stage test methods (many of which have lethality endpoints) the distinction between acute and chronic is sometimes specious, so these statistical methods need not be limited to one type of toxicity test. Determine NOECs and LOECs with available computerized statistical methods for hypothesis testing (Dunnett's Test, Bonferroni's T-Test, Steel's Many-One Ranked Test, or Wilcoxon Rank Sum Test).[22] Parametric statistical methods require that individual test data meet normality assumptions and that the variances of different treatment groups within a test are homogeneous. So before using these methods, check the data to ensure that model assumptions are met. Use the Chi-square or Shapiro-Wilk's tests to test for normality, and Bartlett's test for homogeneity of variance. Statistical software packages (¶ 1*a* above) are suitable for these procedures.

Often it is necessary to transform data to conduct desired statistical analyses. Perform certain transformations routinely before any analysis. One example is percentage data (e.g., percent fertilized, hatch, survival). Adjust percent or proportion data by using the arcsine square-root transformation before using Dunnett's Test; this corrects for the bias in treatment group variances that is expected for percent data. Transformations (e.g., log, square root) of quantitative data, such as number of young or larval weight, sometimes is useful in helping the data meet the assumptions of normality and homogeneity of variance.

d. Regression models for point estimates: EC_P values other than median lethal concentrations are frequently desirable, particularly in chronic tests. Point-estimation techniques for LC, EC, and IC determinations include probit, logit, GLiM, and linear interpolation. Linear interpolation and probit methods are available in the software packages referenced in ¶ 1*a* above. GLiM models also are available in software products.† Probit usually is considered to be effective near median effect levels, and should be used only for quantal data; check to see that normality assumptions are met. Nonmonotonic dose–response curves, especially those with low-dose enhancement or thresholds, may best be analyzed by the appropriate GLiM model with data link,[4,5] or the nonparametric linear interpolation method.[8] The latter is often the most efficient method for calculating point estimates from nonquantal test results. One advantage of using an appropriate regression model is that nominal confidence intervals can be calculated for any given point estimate. This information can be used to help evaluate fit from alternative models, and, more importantly, provides an expression of statistical confidence in the test result. Confidence intervals can be constructed from the entire data set, or just the means of the experimental replicates. Typically, expect better coverage of the nominal confidence interval range from the former. "Bootstrapping" techniques typically are used for constructing confidence intervals, but the user should refer to the specific software provider or available literature for more information on specific methods.

e. Plotting the data: Many anomalies and trends in test results are not obvious unless the data are plotted. Plot chronic toxicity test data before making statistical analysis to help interpret results properly. By plotting the data, answers can be obtained to such important questions as:

- Is the variability among replicates homogeneous across all concentrations?
- What is the pattern of response vs. concentration—is the dose–response curve monotonic?
- Do some concentrations have enhanced response relative to controls?
- Do some data appear to be highly unusual (outliers)?
- Is the effect at the LOEC consistent with the concentrations above and below it, or could it be different just by chance? and
- Do the confidence limits look plausible for the shape of the curve?

f. Outliers: Outliers are data points that are inconsistent with the trends exhibited by the majority of the data. They are detected most often by visual examination of data plots. Standard procedures for statistically detecting outliers are available[22] but should be used with extreme caution. In many instances, outliers are a result of measurement, transcription, or data-entry error. Because apparent outliers are usually mean values computed from replicates, inspection may show that the mean was affected by just one replicate or that the variance among replicates was quite large. If the error can be traced and be corrected justifiably, then it is acceptable to do so. If not, make the analysis with and without the data point in question and report the results of both analyses. Regression methods usually are more robust to the effects of an outlier than are hypothesis test methods, and thus outliers are a more critical concern in the latter.

2. Reporting Results

Report results from toxicity tests as completely as possible so any conclusions can be evaluated independently. Include all of the following that are applicable:

- test organisms used: species, age, life stage, food used in cultures, acclimation, mean length, mean weight, reference toxicant test data for the test population, diseases and treatment, source, and observations on behavior during test;
- tested material: its source, storage, physical and chemical characteristics, and collection method and time;
- dilution water: its source, storage, physical and chemical characteristics, collection method and time, pretreatments, additives, preparation (if applicable), and known contaminants;
- test solution: its physical and chemical properties, especially toxicant concentrations (if applicable), and temperature;
- test method; its endpoint(s), deviations from referenced procedures, data and time of initiation and termination, type and volume of test chambers, volume of test solutions, number of replicate test chambers per treatment, number of

† Such as SAS®, SAS Institute, Cary, NC; JMP®, SAS Institute, Cary, NC; The GLiM System, Release 3.77, Numerical Algorithms Group, Oxford, UK; or S-Plus®, Statistical Sciences, Seattle, WA.

organisms per replicate, toxicant delivery system, and flow rate or frequency of renewal; and
- QA methods used to ensure data integrity.

Present raw data for individual biological endpoints (e.g., mortality) and water-quality measurements. Reference statistical methods and provide tabular summaries on toxic endpoints (e.g., LC_{50} with confidence limits, LOEC, NOEC, chronic value) and physical and chemical data. If applicable (e.g., effluent testing), include QA data, such as results of reference toxicant tests, in tabular form.

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8010 H. Interpreting and Applying Results of Toxicity Tests

1. Interpretation of Results

The 48- and 96-h LC_{50} values produced from standard acute toxicity tests are useful estimates of relative acute lethal toxicity to test organisms under specified conditions. However, these values alone do not necessarily determine whether a water is "safe" or "hazardous" (e.g., exposure analysis). Long-term exposure to much lower concentrations may be lethal to fish and other organisms and/or may cause nonlethal impairment of their function. Similarly, short-term exposure to these or higher concentrations may have no discernible effect. Numerous site-specific factors may influence the test material's effect on the biota of a receiving waterbody.[1,2]

2. Influence of Test Conditions

Toxicity test results depend, in large part, on the conditions and nature of exposure; they are the product of operationally defined procedures. Therefore, it is important to select testing procedures carefully to provide appropriate conditions and ensure that the results are applicable to the water quality problem at hand. At the outset, define the problem carefully and succinctly and establish how toxicity test results will help solve the problem. The type and species of test organism, life stage, test response, test duration, and physical and chemical testing conditions are key factors in obtaining useful, interpretable toxicity test results.

Although not always possible, it usually is desirable to prescribe toxicity-test conditions that are as close as possible to the natural environmental conditions. For some variables (e.g., pH, temperature, and contaminants), even small differences between laboratory-test conditions and those in the natural environment can affect organisms substantially, making the results less useful. Some situations dictate that analysts run both "standard" toxicity tests (i.e., incorporating a standard set of test conditions, such as 96-h exposure, standard chemical and physical conditions, and standard test organisms) and tests whose conditions more nearly mimic the environment of concern. This practice provides results that could be compared with those reported in the literature, as well as a potential route for adapting or interpreting results of other standard toxicity tests. In some instances, in-stream toxicity tests with caged organisms or laboratory tests using receiving-stream waters are a more reliable means of evaluating ambient toxicity. Such procedures can test for the effect of a complex variety of contaminants.

When interpreting toxicity-test results to predict in-stream effects,[3] consider such factors as expected dilution rate, aquatic chemistry, bioavailability, and duration and pattern of organism exposure in the environment. In addition, consider the test organisms' function and sensitivity, compared to the resident species of concern. Apply hazard assessment approach[4] to interpret more accurately the impact on aquatic organisms or designated beneficial uses of the water.

In recent years, aquatic toxicity testing has been applied to a variety of different regulatory and scientific purposes, including toxicity testing of municipal and industrial effluents as part of monitoring/permit compliance,[2–3,5–7] the derivation of national and site-specific water quality criteria for individual chemicals,[1,8,9] product-safety evaluations,[10] chemical-persistence studies,[5,11] effluent-interaction studies, testing of leachates and sediments, and studies included in TRE programs to identify constituents causing toxicity in effluents.[2,12,13] These diverse applications have broadened the utility of toxicity testing and made the judicious interpretation of results more important.

3. Statistical Interpretation

It is crucial in conducting or interpreting the results of a toxicity test to understand clearly that statistically significant differences between control and test organisms, upstream and downstream populations, seasonal variations, etc., do not necessarily have ecological impact. Conversely, trends or other changes that appear to have biological/beneficial use significance may not be statistically demonstrable because of sample size or other limitations.

While the application and interpretation of toxicity tests in water-quality management programs may appear to be more difficult than the more frequently used chemical-only tests performed in clean lab water, they offer certain advantages: they directly address bioavailability and the complex interaction of multiple chemicals, they yield a single integrated measurement of organism response to a chemically complex sample, and they may be less expensive and easier to interpret than a series of chemical measurements.

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8010 I. Selected Toxicological Literature

Toxicity testing has become an integral part of evaluating the effects of waste discharges on the aquatic environment. Listed below are the principal scientific and engineering journals that publish the results of original toxicological research. Textbooks and manuals that summarize and synthesize such results also are listed.

1. Journals

Aquatic Ecosystem Health and Management
Aquatic Toxicology
Archives of Experimental Contamination and Toxicology
Archives of Toxicology
Biomarkers
Bulletin of Environmental Contamination and Toxicology
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8020 QUALITY ASSURANCE AND QUALITY CONTROL IN LABORATORY TOXICITY TESTS*

8020 A. General Discussion

Quality assurance and quality control (QA/QC) are essential elements of laboratory toxicity test procedures. A good QA/QC program provides a framework and criteria for assessing data quality, including a well-defined chain of responsibility, explicit data-quality indicators (DQIs), test procedures, protocols, and a mechanism for identifying and correcting potential problems. Elements to be included in a quality assurance plan (QAP) are outlined in Section 1020A; other resources for developing a comprehensive QAP for laboratory toxicity-testing programs are available.[1–7] At a minimum, QAPs for laboratories performing aquatic toxicity testing should provide specific guidance on DQIs, test procedures, sample handling, data management, internal QC, and corrective actions.

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8020 B. Elements of QA/QC

1. Data Quality Indicators

Data quality indicators (DQIs) are either qualitative or quantitative statements describing the overall acceptable uncertainty in results or decisions derived from environmental data. Such objectives for evaluating toxicity must ensure that the information obtained will provide an accurate and precise determination of environmental effects. They identify the types of measurements to be made, the allowable bias, and desired precision of measurements.

Accuracy is the degree of agreement between an observed value and the true value or an accepted reference value. For water-quality parameters, a measurement of accuracy might include calibration against a known standard. *Precision* is the degree of agreement among repeated measurements collected under identical conditions; it usually is described by a measure of variance (e.g., variance, standard deviation, or coefficient of variation). For toxicity testing, precision of organism response can be described in a control chart of responses to a reference toxicant (i.e., repeatedly exposing test organisms to set concentrations of a known toxicant).

If the response (e.g., survival) of test organisms exposed to a sediment/water sample is significantly different from the response to a reference or control treatment, then the organism has been affected by the sample. Traditionally, decisions of statistical significance are made at $\alpha = 0.05$. This means that the probability of a false-positive result (detecting a difference when none exists) must remain $<5\%$. Data quality indicators must set precision and accuracy limits to ensure that statistical significance is not affected by measurement error.

Minimum DQIs should be provided for water-quality measurements in the test chamber (e.g., temperature, salinity, alkalinity, hardness, dissolved oxygen, pH, and ammonia), including frequency and acceptable limits; minimum control survival; sensitivity of test organisms (e.g., reference toxicant testing); and frequency and number of observations.

Limits for bias and desired precision generally are not stipulated in standardized test protocols described herein, but should be specified in the laboratory's standard operating procedures (SOPs). Performance criteria (e.g., acceptable levels for control survival or water-quality measurements) for most of these categories may be found in the test protocols for the organism of interest.

2. Test Procedures

Test procedures describe how to make all routine measurements associated with toxicity testing and related QA/QC activities. Follow these procedures to ensure the integrity and quality of data.

Use SOPs and standardized data forms to ensure the quality and consistency of toxicological testing and reporting. Write SOPs for all routine laboratory activities and periodically review

and update them. Examples of QC checklists, project schedule lists, procedural checklists, and test and reference toxicant procedures are available.[1–5]

Steps taken in the laboratory to reduce the potential for bias include blind testing, random assignment of organisms to test chambers, statistical designs (e.g., the randomized block), procedures to prevent cross-contamination, confirmation and witnessing of recorded observations, use of reference toxicant tests, and control charting.

Blind testing, in which the experimental treatment is unknown to the analyst, prevents the analyst from potentially applying biases to treatments due to preconceived expectations.

Use randomized designs to eliminate bias due to test-chamber position in the test array. The completely randomized block, in which treatments are allocated to experimental units at random, is the simplest form of the design. Each unit has an equal chance of receiving a particular treatment. In addition, the experimental units should be processed in a random order at all subsequent stages of a test in which the order could affect results. For example, random assignment of test containers to positions within a water bath under a light source helps ensure that potential variations in lighting or temperature in the water bath don't affect results. Discussions of randomized block design, completely randomized block design, and other statistical aspects of experiment design are available.[6–10]

Any material in contact with the samples, solutions, control water, organisms, or food must be nontoxic. While setting up and conducting toxicity tests, it is critical to prevent contamination from any external source and/or cross-contamination between treatments. Preventive measures include cleaning equipment between contact with treatments, proper conditioning of laboratory test apparatus to minimize leaching, and covering test chambers to minimize loss of volatiles and evaporation and prevent introduction of extraneous contamination. It is also generally recommended to periodically (at least annually) analyze food, dilution water, and control water/sediment for contamination.

Periodic double checks of observations, data entry, and calculations, and witnessing of all raw data sheets (i.e., having a coworker review and sign each raw data sheet) are good preventive steps to identify and correct errors early. Important preventive procedures should include counting animals twice to ensure accuracy before adding them to the test chamber and periodically confirming calibration and measurements, particularly if environmental factors seem to be out of range.

Use reference toxicant tests to assess sensitivity of test organisms (for frequently used or in-house cultured organisms, monthly reference toxicant tests are recommended). Plot results from reference toxicant tests on control charts (Section 1020B.13) to determine whether the test organisms' sensitivity to a given reference toxicant is within a predetermined range of acceptability. Construct control charts by plotting successive values (e.g., LC_{50}s) for a reference toxicant, and evaluate temporal changes in sensitivity. Recalculate the mean and standard deviation with each plot until the statistics stabilize. Evaluate individual values in relation to the mean and standard deviation. Procedures for developing and using control charts are described in detail in Section 1020B.13 and elsewhere.[11]

3. Sample Handling, Storage, and Shipment

Consistency in sample handling and tracking is most important when testing samples with possible legal ramifications. To make technically sound decisions that withstand legal review, analysts must handle samples appropriately and be able to trace the sample to its point of origin. Key components of this QA/QC element include established chain-of-custody procedures, as well as procedures for sieving, subdividing, homogenizing, compositing, shipping/transporting, storing, and monitoring samples.

Chain-of-custody procedures require an unbroken record of sample possession from point of collection through subsequent handling, storage, shipment/transfer for analysis or testing, disposal, and possibly up to and during a court proceeding.[12] The goals of chain-of-custody are twofold: to ensure that the collected sample was the one tested and to ensure that the collected sample has not been tampered with, or altered in any way. Chain-of-custody can be accomplished via use of custody seals and sample tracking forms. Examples of such forms are available.[1,12]

a. Water and wastewater: Guidance for handling effluent samples under the National Pollutant Discharge Elimination System (NPDES) program dictates that samples are to be stored at 4°C and that ≤36 h should elapse between sample collection and test initiation.[2] However, holding times may be adjusted depending on study objectives and other specific logistical considerations (e.g., shipment of samples from remote areas). If water samples are to be stored, it is generally recommended that headspace be eliminated or at least minimized to the extent practical depending on nature of sample, study objectives, etc. Record sample characteristics (e.g., pH, temperature, salinity, conductivity, dissolved oxygen, and residual chlorine) upon receipt and before use. Ideally, samples should be dechlorinated in the field before transport to the lab (except when residual chlorine is the potential toxicant of concern) and should be checked for residual chlorine by the lab if collected and transported by outside personnel. If residual chlorine is detected and dechlorination is appropriate, samples should be dechlorinated before storage or test initiation. If samples are dechlorinated (e.g. using anhydrous sodium thiosulfate), the testing program must include appropriate dechlorination controls (e.g., thiosulfate). Before storage, debris may be removed by carefully pouring water samples through a 2- to 4-mm mesh sieve. If there is a possibility of interference due to the presence of indigenous organisms that show predation, competition, etc., pass samples through a 60-μm mesh sieve.[2] If volatile contaminants are of concern, take care to minimize aeration during collection, handling, storage, and testing.

b. Sediment: Sediment samples may require sieving before testing. Sieving decisions are driven by the presence of debris, such as twigs or leaves, that may affect recovery of test animals at test termination and/or the presence of indigenous species in the sample that may serve as food for, compete with, or prey on the test organism. If sieving is required, press-sieve all sediments without adding water (including reference and control sediments) before testing. In most cases, a 0.5-mm screen size is sufficient for removing predators, and larger sieves may be used to remove debris. Recommendations about sieving test material usually are found in specific standardized test protocols. Also, consider which toxicants are likely to be present in the sample

and whether sieving would likely change sediment conditions, such as pH and redox potential, that may alter the toxicant. This is particularly of concern for metals whose toxicity is valence-state dependent.

Depending on test objectives, samples may be composited, homogenized, and/or subdivided before testing. Use clean, non-contaminating containers and implements to handle and store samples. Suggested materials are stainless steel, TFE, Lexan®, high-density polyethylene, and glass. Other appropriate materials may be specified, providing that they do not affect sample toxicity. Homogenize sediments to a consistent color and texture. Samples may be homogenized by hand with a spatula made of non-contaminating materials, or by mechanical mixing. Verify efficiency of homogenization by chemical analysis.

Sediments frequently are stored before testing. Current guidance for dredged material evaluations permits pre-test storage of sediment samples for up to 8 weeks from time of collection.[13] Preferably store sediment samples at 4°C with zero headspace or under an inert gas, such as argon. Re-homogenize samples just before testing. Maximum time limits for sediment storage before testing are of concern; test samples as soon after collection as possible.

4. Data Recording, Reduction, Validation, and Reporting

Quality control of data recording, reduction, validation, and reporting is necessary to produce complete, scientifically defensible reports. Issues to be considered include maintenance of laboratory notebooks, data management, reporting and validation procedures, identification and handling of unacceptable data and outliers, measurements of completeness and comparability, and procedures for data archival.

Standardization of data recording facilitates electronic transfer and data manipulation. At a minimum, standardize procedures for intra-laboratory data entry. Identify "no-data" entries with a mark ("—") to indicate that data were not omitted. Use abbreviations for names of personnel and routine laboratory observations to reduce data recording and entry time; standardize these whenever possible. Attach a list of definitions and code descriptions to data sheets and project files. Record data in indelible ink; make corrections by drawing a single line through the mistake, correcting the mistake, dating and initialing the correction, and writing an initialed explanation for the lined-out data in a footnote on the data sheet. More detailed guidance on maintaining laboratory notebooks can be found elsewhere.[14]

Validate all original data at each level of transcription (e.g., entering data from bound laboratory notebooks into computer databases). Arrange for an independent QA/QC review on a minimum of 10% of the data. Review laboratory records daily for outliers or unusual observations so any necessary corrective action can be taken.

Criteria for establishing outlier values are program-specific. Toxicity-endpoint outliers (e.g., survival, growth, or reproduction) may be more important than water-quality outliers. Depending on program requirements, identify outliers and either accept them as "real" or reject and selectively remove before analysis. If outliers are removed from a data set, note this and clearly justify the reason. For example, an outlier for mortality in a given replicate might be reasonably excluded from a data set when it is clearly related to spurious low dissolved oxygen levels. If there is no rational explanation for the outlier, it must be assumed that the value is real and representative of the test system's variability.

Completeness and comparability are measures of data quality. Completeness is a measure of the amount of data obtained versus the amount originally specified for collection. Generally, 80 to 90% is an acceptable level of completeness for water-quality data. However, endpoint data (e.g., survival or reproduction) should be 100% complete; otherwise the test's statistical power may be compromised. If the data are less than 80% complete, use professional judgment to assess the data's usefulness for decision-making. *Comparability* is the confidence with which one data set can be compared to another. Comparability can be enhanced through inter-laboratory calibration, including use of reference toxicants and control charts.

5. Internal Quality-Control Checks

Internal QC checks are "in-house" procedures implemented by the laboratory to ensure high-quality data. They include reviewing documentation to determine that all samples are tested, sample holding times are not exceeded, holding conditions are acceptable, test protocols are followed, instruments are calibrated and maintained, and control survival and water-quality conditions are within acceptable ranges. Other important issues are verification of the taxonomy and viability of test organisms.

Document source and culture history of test organisms. Identify organisms to species, record, and include taxonomic references used. If possible, preserve a subsample of the test organisms for future identification in the event of aberrant toxicity. The age, size, and/or maturity of the test organisms usually are specified in the test protocol; verify these. Organisms must be healthy, exhibiting good survival (e.g., $>80\%$) preceding testing. Specify appropriate holding time and acclimation procedures either in the test protocols or the laboratory's SOPs; ensure that resulting documentation is available for audit. If animals are provided by a supplier, request documentation from the supplier. For cultured organisms, documentation should include culture history [original source, conditions under which they are reared, reference toxicant data, analysis of food and water supplies, and (for bioaccumulation test species) tissue residue analysis]. For organisms collected in the wild, request collection-related data (e.g., date, location, and water quality at time of collection), acclimatization, and shipping procedures. Confirm species by taxonomic identification before use.

Two widely accepted ways to assess test-organism viability are the use of test-validation controls (or "negative controls") and reference-toxicant tests. A *test-validation control* is a group of organisms that, except for the treatment factor, are handled identically to the other organisms in the test. Test-validation controls for most acute lethality tests limit acceptable mortality levels to $\leq 10\%$ (i.e., survival $\geq 90\%$). If $<90\%$ of the test-validation control survive, the test is considered invalid and must be repeated. For chronic sublethal tests, the test-validation control may also include acceptable limits for other endpoint data, such as growth and reproduction. Reference toxicant tests are designed to assess sensitivity to a specific contaminant. In a *reference toxicant test*, organisms are exposed to a range of concentrations of a single contaminant or contaminant mixture in water-only exposures, and an LC_{50} (usually 48 or 96 h) is

TABLE 8020:I. SUMMARY OF TYPICAL TEST DEVIATIONS AND NEED FOR RETESTING

Deviation	Need for Retesting	
	Required	Possible*
Lack of test array randomization		✓
Testing not blind		✓
Required references, controls not tested	✓	
Test chambers not identical		✓
Test containers broken or misplaced		✓
Mean control mortality exceeds acceptable limits	✓	
Excessive control mortality in a single replicate		✓
Test organisms not randomly assigned to test chambers		✓
Test organisms not from the same population		✓
Test organisms not all the same species or species complex	✓	
Test organism holding time exceeded		✓
Water-quality parameters consistently out of range	✓	
Brief episodes of out-of-range water-quality parameters		✓
Test monitoring documentation incomplete		✓
Sample holding times exceeded	✓†	
Sample storage conditions outside acceptable ranges		✓†
Reference toxicant test results outside of control limits		✓

* If not retested, data may have to be qualified depending on study objectives.
† Unless evidence provided to clearly show that sample quality (physico-chemistry and contaminant levels) has not been affected.

calculated. Evaluate results of reference toxicant tests in a laboratory control chart (see 8020B.2).

Before testing, develop guidance for defining deviations, deficiencies, and appropriate corrective action. Corrective action may be required when a deficiency or deviation from planning documents or procedures is discovered or when there are deviations from established DQIs.

Deviations are data outside the range specified in the DQIs. Out-of-compliance data may be due to deviations in test protocols or deficiencies associated with toxicological tests. Examples of deviations from DQIs in toxicity tests include excessive control mortality, out-of-range water-quality conditions, lack of randomization, lack of required reference, control, and/or out-of-range reference-toxicant results.

Poor control survival, loss of control over exposure conditions, major mechanical errors, or mishandling of test organisms may result in a decision to retest. However, brief episodes of out-of-range water-quality conditions or incomplete test monitoring information may require only that data be flagged and qualified. A number of typical test deviations and suggested corrective actions are summarized in Table 8020:I. T1

Corrective actions may include, but are not limited to, reviewing the data and calculations, identifying and qualifying suspicious data, and retesting. Review all "out-of-limit" events as soon as data are tabulated and validated.

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8030 MUTAGENESIS*

8030 A. Introduction

1. Significance

Mutagenesis is the induction of a heritable change in an organism's genetic material. Studies of human cancer have established that a mutagenic event is very likely the initiating factor in some kinds of cancers. Most known carcinogenic chemicals and ionizing radiation also are mutagenic, so demonstration of mutagenic activity suggests that the substance may be carcinogenic. The common association between mutagenic activity and carcinogenicity is the basis for using short-term mutagenesis tests with bacteria or cultured cells to detect potential carcinogens.

It is also known that naturally occurring mutagens are ubiquitous in the environment.[1] Therefore, the relationship between an environmental sample's mutagenic activity and its chemical pollutants must be examined very carefully in order to draw conclusions about the source of mutagenic activity.

2. Method Selection

There are many mutagenicity-detection tests.[2] Studies were reviewed on the genotoxicity of wastewaters and natural waters in the United States, Canada, Asia, and Europe conducted between 1977 and 1988[3] and on mutagens in surface waters in North America, South America, Asia, Europe, and Africa conducted between 1990 and 2004.[4] According to these studies, numerous industrial water, groundwater, and surface water samples (e.g., river, lake, stream, basin, bay, and ocean) were mutagenic.[3–6] Reports of drinking water containing mutagens have been reviewed.[7] Meanwhile, other studies have detected mutagenic activity in organic wastewater concentrates and their neutral and basic fractions.[8] Some raw water samples were mutagenic, but most samples had to be concentrated; the reviews summarized various sample-preparation processes.

The most common mutagenicity-detection tests are those using bacteria—particularly the *Salmonella* microsomal mutagenicity (Ames) test.[9–12] The Ames test uses nonvirulent "tester strains" of *Salmonella typhimurium.*[13–15]† Mammalian enzyme preparations can be added to metabolize some chemicals to mutagenic forms if they are not direct-acting on the tester strains. The Ames test provides a tool for recognition that our environment is replete with mutagens.[16]

In 2005, the World Health Organization's International Organization for Standardization (ISO) established a standardized Ames test for determining the genotoxicity of water and wastewater (ISO 16240). The test is simple, inexpensive, and sensitive; gives results in 2 d; and shows good correlation between mutagenicity/nonmutagenicity and carcinogenicity/noncarcinogenicity in rodents. However, it will not detect some mutagens active in mammalian cells, it will not detect carcinogens that are not also mutagens (e.g., asbestos), and the relative potency of the mutagens does not necessarily correlate with their carcinogenic potency in mammals. Also, the Ames test provides *qualitative* information about mutagens, but its *quantitative* results vary greatly among laboratories. It is best used as a preliminary screening test.

Together, bioassays and chemical analysis have effectively identified and characterized hazardous waste sites (e.g., former ammunition sites), leading to the reduction of human health and ecotoxic potential.[17,18] The Ames test is useful in bioassay-directed chemical analysis to identify mutagenic chemical species and in designing processes to remove mutagenic activity from treated water.[3,4,19,20]

The Ames test detects mutagenicity in complex environmental samples (e.g., drinking water, wastewater, groundwater, and surface water) and of combinations of chemicals in such samples.[7,16,21] For example, it can detect mutagens generated via disinfection (e.g., chlorination and ozonation of drinking water and wastewater)[19] even though various disinfectants (chlorine, chlorine oxide, ozone, etc.) influence the mutagenicity of urban wastewater differently.[22] MX [3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanome], one of the most potent mutagenic chlorination byproducts identified by the Ames test, later proved to be an animal carcinogen.[23] The test also is useful in studying the structure and mutagenicity of disinfection by-products in chlorinated drinking water,[23]and in evaluating the genotoxic responses of source water samples treated with alternate disinfectants (e.g., UV[24]or peracetic acid[25]). However, it has shown limited use in evaluating synergism and antagonism among chemicals in mixtures.[26]

Because environmental samples are complex mixtures, no standard application of the Ames test is possible.[27,28] Individual test designs will depend on the sample, circumstances, and desired information about mutagenic activity. For example, a specific chemical extraction procedure may be necessary to determine whether a particular mutagen is present. If a concentration step is used, it must be considered in reporting results. To determine whether a particular treatment process (e.g., chlorination or ozonation) leads to mutagen production, samples should be assayed before and after treatment. Sample preparation depends on the nature of the materials in raw water.[28,29]

The methods described here are for the Ames plate incorporation test. Generally, they are applicable to samples soluble in aqueous or organic solvents. Within limits, they will provide preliminary data on the presence of mutagenic materials. For definitive information, tailor the Ames test and sample preparation to the specific situation. When reporting results, state the sample-preparation method used.

3. Sample Collection, Storage, and Preparation

Because of variability among samples, a single sample-preparation procedure cannot be provided, but general principles

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† NOTE: The nomenclature and taxonomy of the genus *Salmonella* has been revised as *Salmonella enterica* serovar Typhimurium, as proposed by the Judicial Commission of the International Committee on Systematics of Prokaryotes in 2005 and referenced by others.[12–14]

apply.[27–31] For general guidance on dealing with environmental samples, see the quality assurance principles in Part 1000. However, complete stability for every constituent can never be achieved. In general, environmental samples may be kept in refrigerated storage and protected from light for up to 14 d before organic extraction. Conduct tests as soon as possible after sample collection. Delays will be accompanied by a progressive loss in mutagenic activity, no matter how the sample is stored. To minimize loss of mutagenic activity, store prepared samples (e.g., extracts) at or below –20°C, under an inert atmosphere (N_2, argon), and protected from light. Many mutagens, particularly polycyclic aromatic hydrocarbons (PAHs), are readily photooxidized to nonmutagenic, but still toxic, compounds. Add no preservatives.

When exposing *Salmonella* tester strains, the sample must be in a solvent compatible with the aqueous suspension of the bacteria. Dimethylsulfoxide (DMSO) is used frequently. If the sample is in a volatile solvent [e.g., dichloromethane (DCM) or hexane], add DMSO and remove the organic solvent with a stream of N_2, leaving behind the relatively less volatile DMSO. This is called *solvent-exchanging*. After the sample has been transferred to DMSO, analyze immediately.[32]

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8030 B. *Salmonella* Microsomal Mutagenicity Test

1. General Discussion

a. Principle: Tester strains of *S. typhimurium* require the amino acid histidine for growth. Reversion of the tester strains from histidine dependence to independence is evidence of mutagenicity. The bacteria can be cultivated on simple media and show reproducible responses to test mutagens. The bacteria are exposed to the sample, with or without additional activating enzymes, and are plated on minimal agar containing a trace amount of histidine. Mutants (i.e., revertants to histidine independence) can grow and form macroscopic colonies. The dose response can be quantified by varying sample concentration and counting revertant colonies per plate at each concentration. The number of revertants per unit dose of sample is calculated using statistical methods.

b. Tester strains: The tester strains currently most widely used in testing environmental samples are TA98 and TA100. Strains TA97a and TA102 can be used but are found to give high and variable rates of background mutation. Because different strains are reverted by different classes of mutagens, using multiple strains provides information on the nature of the mutagenic chemical(s) present. These strains also contain the R-factor plasmid pKM101, which confers resistance to ampicillin. Other commonly used strains include TA1535 and TA1538, which do not contain the R-factor plasmid. Details of the tester strain mutations and other available strains have been published.[1–4] Quality assurance requirements and procedures,[5,6] as well as data production and analysis methods,[7,8] are available.

Tester strains are available from various universities (e.g., Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley, CA 94720) and some research or testing laboratories of federal agencies, such as the U.S. Environmental Protection Agency or National Toxicology Program (NTP).

A number of related tester strains have been developed and validated for detecting specific classes of mutagenic compounds. Tester strains TA98NR (nitroreductase) and TA98/1,8-DNP_6 (1,8-dinitropyrene) have been widely used to detect the presence of nitro-substituted compounds. Tester strain TA98NR is deficient in "classical" nitroreductase and responds less to 2-nitrofluorene and 1-nitropyrene, for example, than the activity detected in tester strain TA98. Tester strain TA98/1,8 DNP_6 is deficient in acetyl-CoA (acetylcholin-esterase): *N*-hydroxyarylamine *O*-acetyltransferase, an enzyme important for the metabolic activity of such compounds as 2-nitrofluorene and 1,8-dinitropyrene.

To detect aromatic amines and nitro-PAHs, investigators[9,10] have developed *Salmonella* tester strains (derived from the standard tester strains) that have elevated levels of enzymes important for metabolically activating nitro-substituted compounds. For example, tester strains YG1021 and YG1024—derived from tester strains TA98—have elevated levels of the enzyme nitroreductase and OAT (*O*-acetyltransferase) activity.[10,11] Tester strains YG 1026 and YG 1029—derived from tester strain TA100—have elevated nitroreductase and OAT enzymes, respectively. Tester strains carrying both nitro-reductase and OAT have also been developed and are sensitive to nitro-aromatic compounds.[12] The YG tester strains have been used in investigations of toxic compounds in water; the samples were more mutagenic with YG1024 than TA98.[13] The protocol for growing YG strains incorporates overnight incubation with antibiotics.

The investigators who developed the YG tester strains also added antibiotics to the overnight broth culture; they added antibiotics to TA98 and TA100 cultures as well. For tester strains YG1021, YG1026, YG1024, and YG1029, ampicillin (25 μg/mL) and tetracycline (12.5 μg/mL) were added. For tester strains YG1041 and YG1042, ampicillin (25 μg/mL) and kanamycin (25 μg/mL) were added. These antibiotics also are used to test for strain characterization.

2. Apparatus

a. Autoclave: See Section 9030B.3.
b. Water bath, reciprocating, for use at 37°C.*
c. Water bath for use at 45 to 50°C.*
d. Incubator for use at 37°C.*
e. Refrigerator or cold room.
f. Freezer, −80°C* (or liquid nitrogen refrigerator).
g. pH meter.
h. Centrifuge, capable of 10 000 × *g*.
i. Vortex mixer.
j. Magnetic stir-plate and stirring bars.
k. Hot plate.
l. Colony counter, manual and automatic (optional).
m. Microscope, dissecting or light.
n. Micropipettor, 10 μL, 100 μL, 500 μL, and 5 mL.

3. Media and Reagents

a. Nutrient broth: Use dehydrated nutrient broth prepared in accordance with manufacturer's directions.†

b. Nutrient agar plates: These are used to test for tester strain characteristics, including crystal violet sensitivity. Add 15 g agar/L

* Thermal flexibility of ±2°C is acceptable.
† Oxoid No. 2, Oxoid Ltd., Basingstoke, Hants, England, available in the United States from KC Biological, Inc., Lenexa, KS, or equivalent.

nutrient broth before sterilizing.‡ Mix well and autoclave at 121°C for 15 min with slow exhaust. Remove from autoclave and let cool to about 50°C. Pour 25 to 30 mL into 100-mm Petri plates and let harden on a level surface. To evaporate excess moisture, hold covered plates in a clean, draft-free environment overnight. Store prepared plates in a tightly covered container in the refrigerator.

c. Vogel–Bonner medium E (50X): This is an inorganic salt medium[2] used in the preparation of minimal agar. To prepare 1 L of 50X concentrate, heat 670 mL water to 45°C and add the following in order (making sure each salt is completely dissolved before adding the next): 10 g magnesium sulfate heptahydrate, $MgSO_4 \cdot 7H_2O$; 100 g citric acid monohydrate; 500 g potassium phosphate, dibasic, K_2HPO_4 (anhydrous); and 175 g sodium ammonium phosphate, $NaNH_4HPO_4 \cdot 4H_2O$. Make up to 1 L with water in a loosely capped 2-L flask or bottle and sterilize by autoclaving for 20 min at 121°C. After cooling, tighten cap and store at room temperature.

d. Glucose solution, 40%: To 600 mL water, add 400 g D-glucose. Stir and make up to 1 L with water. Mix well and sterilize in a loosely capped flask by autoclaving for 20 min at 121°C (slow exhaust). Alternatively, dispense to 250- or 500-mL bottles with rubber-lined screw caps before autoclaving. Leave caps loose during autoclaving. Tighten after solutions have cooled to room temperature.

e. Minimal agar plates for use in the mutagenicity test: To 930 mL water in a 2-L flask, add 15 g agar and a magnetic stirring bar. Mix, cap loosely, and autoclave for 20 min at 121°C, with slow exhaust. Remove from autoclave, cool slightly, and add the following sterile solutions slowly with continuous stirring: 20 mL 50X Vogel-Bonner medium E and 50 mL 40% glucose. Mixing is facilitated if the salts and glucose solutions are first warmed to 45°C.

Place agar in a 45°C water bath or dry temperature block and pour approximately 25 to 30 mL into 15-mm × 100-mm Petri plates. Let agar harden on a level surface and cool to room temperature. To let excess moisture evaporate, hold plates covered in a clean, draft-free area overnight or up to 2 d. A convenient method of plate storage is to return them to the plastic bags in which they were originally packaged and seal the bags securely with tape. Long-term storage at room temperature is acceptable. If plates are stored under refrigeration, let them come to room temperature before use. Autoclave and discard any plates showing contamination.

f. Histidine-biotin solution, 0.5 m*M:* This solution is added to the top agar in the proportion of 10 mL to 100 mL top agar. (It provides a necessary trace of histidine to permit bacteria to undergo a few cell divisions. The tester strains also are biotin-dependent, but because this requirement is the result of a gene deletion, it cannot be reverted.) Add 12.4 mg D-biotin to 100 mL water. Dissolve by heating to the boiling point and add 9.6 mg L-histidine · HCl. Sterilize by filtering through a 0.22-μm-pore-diam filter or by autoclaving for 20 min at 121°C, with slow exhaust.

g. Top agar:

Agar	6	g
Sodium chloride (NaCl)	5 or 6	g§[10,11]
Distilled water	1	L

‡ Bacteriological agar, BBL Select, Oxoid No. L28, or equivalent.
§ Either amount works.

Add magnetic stirring bar, mix, and autoclave for 20 min at 121°C, with slow exhaust. While agar is still melted, mix thoroughly and dispense 100-mL portions into sterile screw-capped bottles of a convenient size (100 to 250 mL). Alternatively, make top agar in 100-mL amounts (0.6 g agar, 0.5 or 0.6 g§[10,11] NaCl, 100 mL H_2O) and autoclave in loosely capped bottles. Cool to room temperature, tighten caps, and store at 4°C. Before use, remelt top agar in a boiling water bath or microwave oven and add 10 mL sterile 0.5 m*M* histidine-biotin solution. Hold top agar in a 45°C water bath or dry heat device.

h. Phosphate-buffered saline (PBS)[14] for washing bacteria in the microsuspension mutagenicity test method.[15] To 900 mL distilled water, add 8.0 g sodium chloride, NaCl; 0.2 g potassium chloride, KCl; 0.2 g potassium phosphate, monobasic, KH_2PO_4; 0.1 g magnesium chloride hexahydrate, $MgCl_2 \cdot 6H_2O$; and 1.15 g sodium phosphate, dibasic, Na_2HPO_4.

Dissolve completely and add 0.10 g calcium chloride, $CaCl_2$, dissolved in a little water. Adjust to pH 7.4 with either HCl or NaOH, as appropriate. Make up to 1 L. Sterilize by filtration through a 0.22-μm-pore-diam filter or equivalent.

i. Sodium phosphate buffer, pH 7.4, used in S9 mix (see ¶ *m* below). Prepare stock solutions:

1) *Sodium phosphate,* monobasic, monohydrate, $NaH_2PO_4 \cdot H_2O$, 13.8 g/500 mL water.

2) *Sodium phosphate,* dibasic, anhydrous, Na_2HPO_4, 14.2 g/500 mL water.

Mix 60 mL Solution 1) with 440 mL Solution 2). Check pH and adjust if necessary to pH 7.4 by adding more of one of the stock solutions. To lower pH, add Solution 1); to raise it, add Solution 2). Sterilize by autoclaving for 20 min at 121°C with slow exhaust.

j. Ampicillin stock solution, 8 mg/mL: Make a solution of 0.80 g ampicillin trihydrate in 100 mL 0.02*N* NaOH. Sterilize by filtering through a 0.22-μm-pore-diam membrane filter. Store in capped glass bottle at 4°C. Add 3.15 mL/L master plate agar solution and pour plates as usual.

k. Master plate agar: To a 2-L flask, add 15 g agar, 914 mL water, and a magnetic stirring bar. Mix and autoclave for 20 min at 121°C with slow exhaust. Remove from autoclave and add, with stirring, 20 mL 50X Vogel-Bonner medium E salts, 50 mL 40% glucose, 10 mL sterile L-histidine · HCl solution (0.5 g/100 mL), 10 mL sterile D-biotin solution (12.2 mg/100 mL), and 3.15 mL ampicillin stock solution for ampicillin-fortified plates. Eliminate ampicillin if growing tester strains TA1535 or 1537 that do not have the pKM101 plasmid. (NOTE: The final concentration of histidine in the master plates is approximately fivefold greater than in top agar. Clearly label plates to distinguish them from minimal agar plates. For strains without the R-factor plasmid, omit ampicillin.)

l. Crystal violet solution, 0.1%: Dissolve 0.1 g in 100 mL water. Mix well and store at 4°C in screw-cap glass bottle in the dark. Use to confirm presence of the *rfa* mutation.

m. S9 mix: S9, a cell-free fraction prepared by homogenization and centrifugation of rat liver (or other tissue) at 9000 × *g* for 10 min, is added when metabolic activation is required. Prepare S9 from the liver of rats pretreated with polychlorinated biphenyls (PCBs, Aroclor 1254) to increase activity of liver enzymes.[2] Unless animal facilities are available, preferably obtain S9

commercially.‖ Commercial S9 preparation should be tested for sterility and enzyme activity. Store commercial S9 preparation per the supplier's instructions. Because S9 contains temperature-sensitive enzymes, store frozen at –80°C or below and thaw only for immediate use. Each mutagen may have an optimum concentration of S9 for maximal mutagenic activity. Because this optimum cannot be specified in advance and the amount of sample often is limited, standardize on an S9 concentration. Between 20 and 40 mg protein/mL S9 is common, but consistency is essential.

Standardize S9 according to protein content. Determine protein content of a small portion of undiluted S9.[16] Freshly prepared S9 has a protein content of about 40 mg/mL.[2] Adjust protein concentration immediately before use to the desired concentration with 0.1*M* sodium phosphate buffer, pH 7.2 to 7.4, or with 0.15*M* KCl. Check for sterility by spreading 0.1 mL on a minimal agar plate containing histidine and biotin and incubate for 2 d at 37°C. Discard contaminated S9 (more than 10 colonies/0.1 mL).

In an assay, add cofactors to provide necessary reducing activity for the cytochrome enzymes. Add reduced nicotinamide adenine dinucleotide phosphate (NADPH) or a NADPH generating system consisting of nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), and $MgCl_2$. The combination of S9 and cofactors is termed "S9 mix." Ideally, select the amount of S9 in the mix to give optimum mutagenic response with the sample. Practically, use either 4 or 10 mL S9/100 mL S9 mix (i.e., 4% or 10% S9, equivalent to approximately 1.6 or 4.0 mg protein/mL S9 mix, respectively).[2] For consistency, adjust the protein concentration in S9 mix to these concentrations. Once prepared, keep S9 mix on ice and use immediately; do not refreeze.

Use the following stock solutions for preparing S9 mix:

1) *Potassium chloride* (KCl), 1.65*M*—Dissolve 12.3 g KCl in 80 mL water and make up to 100 mL. Autoclave for 20 min at 121°C and store at room temperature.

2) *Magnesium chloride hexahydrate* ($MgCl_2 \cdot 6H_2O$), 0.4*M:* Dissolve 8.13 g $MgCl_2 \cdot 6H_2O$ in 80 mL distilled water and make up to 100 mL. Autoclave for 20 min at 121°C and store at room temperature.

3) *Sodium phosphate buffer*—See ¶ *i* above.

4) *Nicotine adenine dinucleotide phosphate* (NADP), 0.1*M*—Dissolve 743 mg in 80 mL sterile water and make up to 100 mL with sterile water. Sterilize by filtering through a 0.22-μm-pore-diam membrane filter. Store 5- or 10-mL portions at –20°C for up to 6 months. (NOTE: The weight given here is the formula weight of anhydrous free acid. The sodium salt typically is used and it may have associated water, so the amount required will vary from lot to lot. Most suppliers provide a specification sheet with the calculated formula weight. Use this value in preparing the solution. Typical values range from 750 to 825.)

5) *Glucose-6-phosphate,* 1*M*—Dissolve 6.5 g in 20 mL sterile water and make up to 25 mL. Sterilize by filtering through a 0.22-μm-pore-diam membrane filter. Store 5- or 10-mL portions at –20°C for up to 6 months.

To prepare 50 mL S9 mix, add the following (in order) to a vessel in an ice bath: 18.75 or 15.75 mL sterile water; 25 mL 0.2*M* sodium phosphate buffer; 2.0 mL 0.1*M* NADP; 0.25 mL 1.0*M* glucose-6-phosphate; 1.0 mL 1.65*M* KCl; 1.0 mL 0.4*M* $MgCl_2 \cdot 6H_2O$; and 2.0 or 5.0 mL rat liver S9. [NOTE: The amount of S9 can be varied to obtain desired protein concentration. Preferably use either 2 mL (4%) or 5 mL (10%) of rat liver S9/50 mL S9 mix.[2] Adjust water volume to maintain concentrations of other reagents.]

Prepare S9 mix immediately before use. Discard any unused portion. Do not refreeze.

4. Tester Strain Stock Cultures

a. Preparation of stock cultures: Tester bacteria are available (see ¶ 1*b* below) as small paper disks saturated with the *Salmonella* culture, sealed in small sterile plastic bags with a little agar. On receipt, aseptically remove disks and make subcultures by wiping the disk across an agar master plate and then placing disk in sterile nutrient broth. Adjust volume of nutrient broth, depending on how many frozen stock vials are to be produced. To a culture grown overnight in nutrient broth, add dimethylsulfoxide (DMSO spectrophotometric grade, 0.09 mL DMSO/mL culture). Mix well and dispense aseptically into sterile 1.5-mL cryotubes, filling each tube almost to the top. Label, freeze in crushed dry ice, and store at –80°C or in liquid nitrogen refrigerator. If frozen cultures will be used repeatedly, do not allow them to thaw because this tends to increase the spontaneous background mutation rate and the chance of contamination. If freezer facilities are unavailable, less preferably maintain strains by repeated subculture and confirm strain characteristics at each mutagenicity test.[2] To prepare bacteria for a test, remove a small amount of the frozen culture with a sterile spatula and inoculate a broth culture. Cultures also can be preserved via lyophilization.[2]

As an alternative to repeatedly sampling frozen stocks, use the "master plate" method.[2] Streak a drop of a broth culture or a small piece of the frozen culture on an ampicillin–master agar plate (for tester strains with the pKM101 plasmid), or streak on a His/Bio–master agar plate (His/Bio only) for strains without plasmids. Minimal agar plates also can be used if subsequently fortified with histidine, biotin, and—if required—ampicillin.[17] The plate may be stored in a refrigerator for up to 2 months except TA102, which is due after 2 weeks. Use well-separated colonies from this plate to initiate broth cultures. Prepare new master plates from broth cultures initiated from the frozen stocks.

b. Characterization of tester strains: Characterize tester strains immediately on receipt and preferably as a part of each mutagenesis assay. The procedures described herein are for strains TA98 and TA100; procedures for other strains are available.[2–4] Strains TA98 and TA100 require histidine and biotin for growth. In addition, they contain the *rfa* mutation affecting the cell wall, the *uvrB* deletion leading to reduced DNA repair capacity, and the R-factor plasmid pKM101 conferring resistance to ampicillin.

Confirm the histidine requirement by streaking a sample of broth culture on a minimal agar plate containing biotin, but lacking histidine. The biotin requirement is the result of a dele-

‖ AMC Cancer Research Center and Hospital, c/o Dr. Elias Balbinder, 6401 W. Colfax Ave., Lakewood, CO 80214; Litron Laboratories, 1351 Mt. Hope Ave., Suite 207, Rochester NY 14620; Microbiological Associates, c/o Dr. Steve Haworth, 5221 River Road, Bethesda, MD 20816; MolTox, 335 Paint Branch Drive, College Park, MD, 20742; or Organon Teknika, 1 Technology Court, Malvern, PA 19355.

TABLE 8030:I. DIAGNOSTIC MUTAGENS FOR TESTER STRAINS TA98 AND TA100

Chemical	Amount per Plate	S9	Histidine Revertants per Plate TA98	Histidine Revertants per Plate TA100	Animal Toxicity
Sodium azide	1.5 μg	–	3.0	3000	Highly toxic
Daunomycin	6.0 μg	–	3123	47	Toxic
Methyl methanesulfonate	1.0 μL	–	23	2730	Carcinogen
2-Aminofluorene	10 μg	+	6194	3026	Carcinogen
Benzo(a)pyrene	1.0 μg	+	143	937	Carcinogen

tion and cannot revert. No bacterial growth should be seen on the histidine-deficient plate.

The *rfa* mutation renders the cell wall permeable to large molecules; growth of the tester strains is inhibited by crystal violet. To test for this inhibition, add 0.1 mL broth culture to 2 mL melted top agar, and spread the mixture on a nutrient agar plate. Place a sterile 6.4-mm-diam (¼-in.-diam) disk of filter paper# in center of plate and add 10 μL 0.1% crystal violet solution to the filter paper. The crystal violet will diffuse into the agar; sensitive strains show a clear zone around the paper disk, indicating growth inhibition. The *uvrB* deletion confers increased sensitivity to ultraviolet light, which can be demonstrated by comparison with the wild-type strain.[17] The presence of the R-factor plasmid allows tester strains to grow on agar containing ampicillin.

The strains are reverted by different mechanisms, and their response to chemical mutagens depends on the chemical's mode of interaction with bacterial DNA. Strain TA98 is reverted by frame-shift mutagens that generally are large molecules (e.g., polycyclic aromatic hydrocarbons). The reading frame of the DNA is shifted by the deletion or addition of a base pair. Strain TA100 is reverted by mutagens that cause base-pair substitutions in the bacterial DNA. In this mutation, an adenine-thymine base pair is replaced by a guanine-cytosine pair, or vice-versa. For strains TA98 and TA100, diagnostic mutagens are recommended;[2] these are relatively more mutagenic for one strain than for the other (see Table 8030:I). In using diagnostic mutagens, relative mutagenic activity is important; absolute activity varies among laboratories. When a test is conducted with diagnostic mutagens, a dose–response curve is generated in the same manner as with samples.

c. Safety precautions: The tester strains were derived from *S. typhimurium* type LT2, which is of low virulence. They have been modified further to increase their sensitivity to mutagens but incidentally to decrease their virulence. Aside from deliberate ingestion, the tester strains do not present a health hazard to workers. Nevertheless, good laboratory practice dictates certain precautions. Never pipet by mouth. Do not eat, drink, or smoke in the laboratory. Autoclave all live cultures and culture plates with colonies before disposal.

Handle plates or cultures or materials containing positive control mutagens or carcinogens as hazardous materials. Treat all test plates (except for negative controls) and other materials prepared for testing as potentially hazardous, and dispose of them accordingly.

5. Procedure

a. Without metabolic activation: Conduct all operations in subdued light or under dim incandescent, nonactinic lighting. Yellow "bug-lights" or "gold" fluorescent tubes that emit very little ultraviolet radiation are satisfactory.

1) Culture preparation—Prepare overnight broth cultures of tester strains via inoculation with either frozen stock or master plate colonies. Each test plate requires 0.1 mL of broth culture (approximately 1 to 2×10^8 bacteria). Prepare the volume of broth culture accordingly. Incubate cultures overnight (10 to 12 h) at 37 ± 2°C, with shaking at approximately 210 rpm to ensure adequate aeration.

2) Test material preparation—Most mutagens are soluble in the recommended DMSO. Other relatively nontoxic, nonmutagenic solvents (e.g., acetonitrile, ethanol, or acetone) may be used if excessive toxicity is absent.[18] Because many compounds are water-soluble, use water whenever possible. When using a volatile solvent, take precautions to prevent solvent evaporation before adding it to the top agar or incubation mixture. Keep solutions with volatile solvents tightly capped and on ice. The final concentration of solvent in the top agar should not exceed 5%, except when the solvent is water. Dissolve test material at the highest concentration to be tested and prepare appropriate dilutions. Use a concentration range of at least three logs, with test concentrations at half-log intervals (e.g., 1, 3, 10, 30, 100, 300, and 1000 μg/plate). It may be more convenient to specify dose levels for water samples in terms of original water volume equivalents per plate. In the latter case, mutagenic activity, if present, has been detected in the range of 0.05- to 2.0-L equivalents.[19] However, each laboratory should evaluate the literature and conduct appropriate pilot studies to find the best dose range for each sample type. If negative results are obtained at the highest concentration and no toxicity to the bacteria is apparent, increase concentration of test material to 5 or even 10 mg/plate,[5,7] unless limited by toxicity or precipitation. Prepare sample solutions immediately before use; do not store.

3) Plate incorporation test—Prepare at least three plates for each concentration of test material with minimal base agar. Remove melted top agar tubes from water bath or dry temperature block (43 to 45°C) and add to 2 mL of melted top agar: 0.1 mL bacteria, 0.1 mL or less (usually 0.05 mL) test compound or solvent control, and either 0.5 mL S9 mix for with-S9 testing, or 0.5 mL phosphate buffer for minus-S9 testing. It may be convenient to place the required number of tubes containing melted top agar in the 45°C water bath in advance and to add bacteria and test material just before pouring test plates. Briefly mix with a vortex mixer, pour on minimal agar plates, and immediately tilt and rotate plates so the top agar forms a uniform layer before hardening. Place plates on a level surface and let top agar solidify. (Use a carpenter's level to confirm that the surface is level.) Work quickly. Negative controls receive solvent (e.g., DMSO) or no addition to the mixture of bacteria and top agar. When the top agar has solidified, invert and incubate plates in the dark at 37°C.

Schleicher & Schuell No. 740-E, or equivalent.

4) Scoring plates—Count revertant (histidine-independent) colonies after 48 to 72 h. Longer incubation will increase the number of revertant colonies but also increase the chance of contamination. Hold incubation time constant in any test series. Because *Salmonella* strains are motile, colony size depends on whether their bacteria were at the surface or embedded in the top agar. Surface colonies tend to be large (up to 2 mm), circular, and flat, while embedded colonies are small (<1 mm) and dense. All colonies are a uniform light cream color, slightly translucent, with a smooth surface and a regular smooth edge to the surface colonies. The appearance of colonies with differing morphology indicates contamination; discard plate and check stock culture for purity. Colony size is inversely proportional to the number of revertants per plate because of nutrient exhaustion. A trace of histidine in the top agar enables the bacteria to undergo several replications before exhausting the histidine; it effectively increases the number of bacteria at risk for mutagenesis. This replication (if absent) also indicates toxicity. Replication of nonrevertant bacteria gives a hazy appearance to the top agar between the revertant colonies. If this "background lawn" replication is absent, it indicates excessive toxicity to the bacteria and an unreliable mutation test. The number of revertant colonies usually varies by about 10 to 15% among replicate plates; the percentage tends to shrink as analyst experience grows.

Plates with bacteria only show a characteristic number of spontaneous revertant colonies. Among a group of eight laboratories, the spontaneous revertant range for strain TA98 was 15 to 75 colonies/plate; for strain TA100, it was between 60 and 220 colonies/plate.[7] Within a given laboratory, these values should remain relatively constant. If marked variation occurs, investigate immediately by recharacterizing the strains and confirming media formulation. These numbers may be slightly different on plates with S9 mix. Plates with bacteria exposed to the solvent only may have slightly higher numbers of spontaneous revertants.

b. With metabolic activation:

1) Without preincubation—Except for adding S9 mix to the standard plate test to convert certain mutagenic chemicals to their active forms, the general procedure is the same as that described above.

Add 0.5 mL S9 mix to the top agar, test material, and bacteria as described above. Incubate and score plates as above. Include a positive-control mutagen when using S9 to demonstrate that the preparation is active and to detect possible variations between batches of S9. Recommended control compounds requiring metabolic activation include 2-aminofluorene;[2] 7,12-dimethylbenz(a)-anthracene; and 2-acetylaminofluorene.[7]

2) With preincubation—To enhance test sensitivity for materials or mixtures containing low levels of mutagenic activity, preincubate the bacteria with the test material, with or without S9 mix.[20] To a 13- × 100-mm tube containing the test material in 0.1 mL sodium phosphate buffer, add 0.5 mL S9 mix and 0.1 mL of desired tester strain (approximately 1 to 2 × 10^8 cells). If S9 mix is not used, replace it with 0.1 mL buffer. Incubate at room temperature or at 37°C for 20 min. Add 2 mL melted top agar and pour mixture on a minimal agar base plate as above. The temperature and length of preincubation affect the yield of revertant colonies and should be optimized and standardized for the material being tested. The S9 mix remains metabolically active for 30 to 45 min; avoid prolonged incubation.

3) Microsuspension modification—When the amount of material available for testing is small or the mutagenic activity is very weak, the microsuspension assay may be used.[21,22] Prepare overnight bacterial cultures as described above. Harvest the bacteria via centrifugation (5000 × *g*, 10 min, 4°C) and resuspend pellet in a volume that is one-tenth the original volume, using phosphate buffered saline (PBS) or sodium phosphate buffer. Add the following ingredients, in order, to a 12- × 75-mm sterile glass tube: 0.1 mL bacterial suspension, 0.1 mL S9 mix or PBS, and 0.005 mL test compound dissolved in a compatible solvent. After incubation for up to 90 min, add top agar, mix, and pour plates. Sensitivity is increased up to tenfold or more because the bacteria are in contact with higher concentrations of the test material or mutagenic metabolites during preincubation. However, the toxic effects of the material also may be increased. Make standard assay to provide a reference. Optimize incubation conditions for the particular material being tested.

6. Data Presentation and Analysis

a. Data presentation: Preferably report both raw data (i.e., revertant colonies per plate, including control plates) and interpreted data.[7,8] If this is not feasible, indicate means of replicate plates, number of replicates, number of experiments, and a measure of the variability (e.g., standard deviation). In cases with a clearly positive result, present data graphically to facilitate comparisons. Direct comparisons of different samples (e.g., extracts from different waters) usually are made in one experiment that subsequently is replicated. Presentation of data transformed to revertants per weight of test material or water volume equivalents or to ratios of treated to control cultures is acceptable only if the original data also are available. Report any pretreatment used on the sample.

b. Data analysis: Usually, it will be apparent if the test material is mutagenic, so involved statistical analysis of the results is not required. However, when dealing with weakly mutagenic materials, preferably establish objective criteria for deciding whether a material is mutagenic or contains mutagenic substances. An important first criterion is that a reproducible dose–response relationship can be demonstrated (i.e., the number of revertants per plate is proportional to the amount of test material added per plate over some part of the range of amounts tested). As a guide, use the *modified twofold increase rule,*[1,5] which states that a test is considered positive if two consecutive dose levels, or the highest nontoxic dose level, produced a response at least twice that of the solvent control *and* at least two of these consecutive doses showed a dose–response relationship. If results are in doubt, further testing is indicated, perhaps with test modifications.

It may be desirable, for quantitative comparison purposes, to express mutagenic activity in terms of *Salmonella* revertants per microgram of material or per water volume equivalent, but often the dose–response curve is nonlinear. With low amounts of test material, the number of revertants per plate may increase with increasing amount of material, but beyond a certain amount, the number of revertants per plate no longer increases and even may decline. The reasons for this nonlinearity include toxicity of the test material to the bacteria and limited solubility of the test material in the aqueous medium. Therefore, mutagenic activity of an unknown compound or mixture often is described in terms of the slope of the linear portion of the dose–response curve with

low amounts of test material. Statistical methods have been described for deciding which points on these nonlinear curves to use in defining the sample's specific mutagenic activity.[14,23]

Failure to demonstrate mutagenic activity with one or more *Salmonella* tester strains does not amount to proof that the sample contains no mutagenic material. The individual strains have certain mutagens to which they are sensitive, the concentration of S9 may be critical, and some mutagens and carcinogens are not mutagenic for any commonly used strain. Therefore, qualify conclusions about mutagenic activity based on tester-strain characteristics and test conditions.[2–4,8]

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8050 BACTERIAL BIOLUMINESCENCE*

8050 A. Introduction

A *bacterial bioluminescence test* (BBT) is a metabolic inhibition test that uses luminescent bacteria to measure a substance's toxicity. This method provides a rapid, reliable, and convenient means of determining acute toxicity. The BBT has been validated for various environmental applications (e.g., effluent monitoring; groundwater, drinking water, sediment[1–2], and hazardous waste testing; bioremediation-efficiency assessments; and general biomonitoring).

Luminescent bacteria possess several attributes useful for toxicity testing. Certain strains of luminescent bacteria divert up to 10% of their respiratory energy into the luciferase metabolic pathway that converts chemical energy into visible light by enzymatic catalysis of a chemical substrate. This pathway is intrinsically tied to respiration; any change in cellular respiration or disruption of cell structures changes respiration and, therefore, the amount of bioluminescence.

In bacteria, many metabolic pathways—including those related to respiration, oxidative phosphorylation, osmotic stabilization, and transport of chemicals and protons into and out of the cell—are in or near the cell membrane. The luciferase enzymes, which shunt electrons directly to oxygen at the level of reduced flavin mononucleotide, are also in the cell membrane.[3] Because luminescent bacteria are small (<1 μm diam), have a relatively simple morphology, and have no membrane-sided compartmentalization of internal functions, they provide many target sites at or near the cell membrane for toxicants to exploit.

Also, bacterial respiration is 10 to 100 times greater than that of mammalian cells, resulting in a dynamic metabolic system that can be quantified easily and accurately by measuring the amount of light output from a bacterial suspension. Such suspensions typically contain approximately 10^6 individual organisms. The light intensity is substantial (well within the operating range of common light sensors), and the large number of organisms statistically compensates for variations among individuals.

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* Approved by Standard Methods Committee, 2014.
Joint Task Group: Ruth M. Sofield.

8050 B. Bacterial Bioluminescence Test

1. General Discussion

In a BBT, analysts expose living bioluminescent bacteria [*Vibrio fischeri* (formerly *Photobacterium phosphoreum*)] to a sample and then measure the sample's effects on the bacteria. Both the bacteria and the testing equipment are available commercially.* Manufacturer-recommended procedures and information are available for software and common testing methods.[1–3] Data reported in the literature were produced in tests using standard, commercially available, freeze-dried strains of the bacterium, so test results from different laboratories can be compared directly.[4–8] Analysts measure the rehydrated lyophilized bacteria's light output after exposure to a specified dilution series of a sample and then compare that data to the light output of a control blank (*Vibrio fischeri* suspended only in diluent). The assay's premise is that a decrease in light production is due to metabolic inhibition of the bacteria, which is proportional to the toxicant dose. Analysts determine a concentration–response curve based on the test's dilution series, which the instrument software then uses to estimate the half-maximal inhibitory concentration (IC_{50}). Positive controls of either standard phenol or zinc (Zn^{2+}) prepared from zinc sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$), solutions are used to assess ongoing method performance. Sample handling and preparation (e.g., pH and salinity adjustments) may alter the sample toxicity, as can naturally occurring toxicants (e.g., sulfides).[9] The effects of naturally occurring and artificially produced toxicity on test results should be considered when analyzing data.

2. Apparatus

a. Photometer† *and computer with appropriate software*.‡
b. Glass cuvettes, 12 × 50 mm, disposable, with flat bottoms.
c. Pipettor,§ adjustable (200- to 1000-μL).
d. Pipettor,‖ with disposable 0.5-mL syringe tips.

* Modern Water Plc, Guildford, U.K.

† Microtox® Model 500 Analyzer, available from Modern Water plc, Guildford, U.K. [formerly Strategic Diagnostics, Inc. (SDIX)] or Azur Environmental, Carlsbad, CA, or equivalent.
‡ Microtox Omni software, or equivalent.
§ Eppendorf, or equivalent.
‖ Eppendorf Repeater®, or equivalent.

e. Laboratory glassware, including sample bottles, 30- to 250-mL beakers, and volumetric flasks.

f. Spatulas, stainless steel.

g. Weigh boats, plastic.

h. Water bottles, 250- to 500-mL, glass, polyethylene, or polypropylene.

i. Incubator.

j. Freezer for storage of Bacterial Reagent (not self-defrosting).

k. pH meter and probe.

l. Refractometer.

3. Reagents and Materials

a. Bacteria, *Vibrio fischeri*, freeze-dried. This is referred to as the *Bacterial Reagent* and the glass vial that contains the Bacterial Reagent is the *Bacterial Reagent vial*.

b. Water, ultrapure.#

c. Sodium chloride (NaCl), solid, reagent-grade.

d. Diluent: Make up a 2% w/v NaCl solution with ultrapure water. Use to dilute sample and Bacterial Reagent as necessary.

e. Acetic acid (1 + 1).

f. Sodium analysed stock solution: Dissolve 1.575 g Na_2SO_3 in 1000 mL ultrapure water. Prepare daily.

g. Phenol, reagent-grade, crystalline.

h. Zinc analysed ($ZnSO_4 \cdot 7H_2O$), reagent-grade.

i. Hydrochloric acid (HCl) and sodium hydroxide (NaOH), 1*M* each, for pH adjustment.

j. Potassium iodide (KI). Dissolve 10 g KI in 100 mL ultrapure water.

k. Starch indicator solution. Either use commercial product or prepare as follows: to 5 g starch (potato, arrowroot, or soluble), add a little cold water and grind to a thin paste in a mortar. Pour into 1 L boiling water, stir, and let settle overnight. Use clear supernate, preserving with 1.25 g salicylic acid and 4 g zinc chloride, or a combination of 4 g sodium proprionate and 2 g sodium azide per L.

*l. Plastic film.***

m. Disposable wipes.††

n. Dissolved oxygen (DO) meter.

o. Oil-free compressed air and disposable glass pipet: May be required based on DO and BOD of sample.

4. Procedure

a. Sample collection, handling, and treatment: This method is suitable for measuring the toxicity of chemicals, receiving waters, effluents, elutriates, and leachates. Collect 250 to 500 mL sample in glass, polyethylene, or polypropylene jars or bottles, leaving no airspace. Test sample as soon as possible, preferably within 24 h of collection. Before use, store sample in the dark at 4 ± 2°C. Prompt testing avoids possible changes in sample toxicity.

If significant turbidity is present, results may be affected by light scattering. Suspended matter may be reduced by settling for a few hours in storage conditions or by centrifugation. Transfer cleared sample to a new container. (NOTE: The material causing turbidity may also have toxic effects.) If turbid samples must be analysed as received, other tests are available (i.e., solid-phase testing procedures[3]) or a correction can be applied.[10] Color may also interfere with measurements by absorbing light. If osmotically adjusted sample has visible color, conduct the BBT as described; once the IC_{50} is calculated, determine if the concentration equivalent to the IC_{50} has visible color. If so, a color-correction procedure is needed.[10]

Luminescent bacteria are adversely affected by pH <6.0 or >8.0, so adjust sample pH to between 6.0 and 8.0 with either NaOH or HCl, as needed. If over-titration occurs, discard sample and start over.

If salt content is <2%, osmotically adjust samples to a final concentration of 2.0% (M/M) NaCl. If sample has a higher salt content, make a Diluent Substitute to match the sample's salinity with ultrapure water and NaCl. The Diluent Substitute is used in place of the Diluent. If the sample has 0% NaCl, pour 50 g of well-mixed sample into a 100-mL beaker. Tare out the sample-filled beaker and add 1.00 g solid analytical reagent (AR)-grade NaCl crystals to the beaker. Stir with a disposable glass pipet until all crystals have completely dissolved.

Luminescent bacteria are sensitive to chlorine, so sample collection before chlorination is preferred. If it is not possible, dechlorinate samples with sodium sulfite. Determine amount of sulfite solution required on a 100- to 1000-mL portion of neutralized sample by adding 10 mL 1 + 1 acetic acid, 10 mL KI solution (10 g/100 mL) per 1000-mL sample, and titrating with Na_2SO_3 solution to starch-iodide endpoint (blue color is discharged). Add to neutralized sample the proportional volume of Na_2SO_3 solution determined by the above residual test, mix, and check for residual chlorine after 10 to 20 min.

b. Prepare positive control: As part of the basic test protocol, include positive controls of either phenol or Zn^{2+} and analyze them at least once a day (frequency depends on the number of samples to be tested). Phenol ordinarily has an IC_{50} between 13 and 26 mg/L after 5 min exposure, while Zn^{2+} has an IC_{50} between 0.6 and 2.2 mg/L after 15 min exposure.‡‡ Prepare a 100-mg/L stock solution of either reagent-grade phenol or $ZnSO_4 \cdot 7H_2O$ by accurately weighing and transferring 100 mg of chemical into a clean, rinsed (with dilution water), 1000-mL volumetric flask. Protect the phenol standard from light and prepare in an amber-colored or aluminum-foil-wrapped flask. Although both standards will last for 3 to 4 months when stored at 2 to 8°C, preferably prepare standards on each day of testing. Alternatively, analyze standards on testing day to determine actual concentration.

c. Prepare analyzer and bacteria (Vibrio fischeri): Turn on the analyzer at least 30 min before testing begins to allow the unit to bring the reagent well (where Bacterial Reagent is held), incubator wells (where samples are held), and the read well (where photometric measurements are taken) to their preset temperatures. Follow manufacturer's instructions for calibrating and operating instrument.

Place clean, unused cuvettes in the reagent well (maintained at 5.5 ± 1°C) and incubator wells (maintained at 15 ± 0.5°C)—as

Lacking microscopic impurities.

** Parafilm®, or equivalent.

†† Kimwipes®, or equivalent.

‡‡ Reagent Certificates of Performance, which report IC_{50} values for phenol and Zn^{2+}, are available from Modern Water (formerly Strategic Diagnostics, Inc., and Azur Environmental) for each lot number of bacteria.

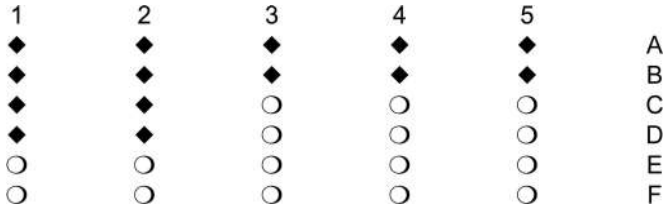


Figure 8050:1. Incubator diagram for acute toxicity testing of one sample at multiple dilutions. ◆ = Incubator well with added sample, diluent, and Bacterial Reagent; which are present depends on the specific incubator well. ❍ = empty well.

many as required for the number of samples to be analyzed. When handling cuvettes, hold them near the open end so light measurements are not affected by finger- or handprints. To set up analyzer for acute toxicity testing of one sample at multiple dilutions, follow the incubator diagram (Figure 8050:1). During testing, take care to maintain samples at the test temperature (15°C) when not in the incubator wells.

Place 1000 μL reconstitution solution (ultrapure water) into the cuvette in the reagent well. Allow 10 min for solution to reach the temperature in the well. Take Bacterial Reagent from the freezer, tap the pellet to bottom of vial, and remove seal.

Take water-filled cuvette from reagent well, and place cuvette lip on top of Bacterial Reagent vial. Quickly pour ultrapure water into Bacterial Reagent vial (slow reconstitution causes bacteria lysing and low Bacterial Reagent light levels). Swirl Bacterial Reagent vial only three or four times (over-mixing will warm Bacterial Reagent), pour mixture back into cuvette, and place cuvette back in reagent well.

Use the 500-μL pipettor to mix reconstituted Bacterial Reagent by filling and dispensing the pipettor 20 times for uniform dispersal of Bacterial Reagent. Allow 20 min for reconstituted Bacterial Reagent to stabilize.

d. Prepare samples: Though in general it is believed that as long as DO is >1 mg/L (10% saturation at the test temperature of 15°C) there should be no effect on test results,[11] there is some concern that samples high in biological or chemical oxygen demand could strip the oxygen fast enough to affect results.§§ Therefore, if DO is <40% saturation once the sample is at test temperature, side-by-side tests with and without aeration are recommended, especially if high oxygen demand is suspected. The sample must be aerated if DO is <10% at test temperature. To aerate samples, dispense oil-free compressed air through a glass pipet. A minimal flow rate should be used to obtain effective aeration. Aeration should be performed until 40% saturation is obtained in ≤20 min.

Pipet 1000 μL diluent into each cuvette in incubator wells A1, A2, A3, A4, A5, and C1. The cuvette in Well C2 receives no diluent. Pipet 500 μL diluent into each cuvette in incubator wells B1, B2, B3, B4, B5, D1, and D2. Then, pipet 1000 μL osmotically adjusted sample into the cuvette in incubator well C2 (which is empty) and into the cuvette in incubator well C1 (which contains diluent). Mix cuvette in incubator well C1 using pipettor.

§§ Peter Adolphson, Senior Environmental Toxicologist, Washington State Department of Ecology. Personal communication, Nov. 10, 2014.

To prepare the series of 1:2 dilutions, transfer 1000 μL from C1 to A5 and mix using pipettor. Then, transfer 1000 μL from A5 to A4 and mix using pipettor. Transfer 1000 μL from A4 to A3 and mix using pipettor. Then, transfer 1000 μL from A3 to A2 and mix using pipettor. Withdraw 1000 μL from cuvette in A2 and discard. The cuvette in A1, which only contains diluent, is the control blank (negative control). Wait 5 min for temperature equilibration.

The sample concentrations (%) in the cuvettes are now as follows:

1	2	3	4	5	Row	Test Volumes
0.00	3.125	6.25	12.5	25.0	A	Each cuvette in Row A contains 1000 μL (sample and/or diluent).
0.00	0.00	0.00	0.00	0.00	B	Each cuvette in Row B contains 500 μL (diluent only).
50.0	100.0				C	Each cuvette in Row C contains 1000 μL (sample or sample with diluent).
0.00	0.00				D	Each cuvette in Row D contains 500 μL (diluent only).

e. Add Bacterial Reagent: Using the repeat pipettor, add 10 μL reconstituted and mixed Bacterial Reagent from the reagent well (about 10^6 bacteria) into each cuvette in incubator wells B1, B2, B3, B4, B5, D1, and D2. These cuvettes already contain 500 μL diluent. When dispensing Bacterial Reagent into cuvettes, do not remove either Bacterial Reagent or sample cuvettes from the incubator and ensure that Bacterial Reagent does not dispense onto sides of the cuvette.

After adding Bacterial Reagent to each cuvette, mix each dilution by gently removing each cuvette and gently tapping the bottom two to three times. Wait 15 min to let Bacterial Reagent stabilize. Meanwhile, set up the recording software (per manufacturer instructions).

f. Measure light emission: Once Bacterial Reagent has stabilized, calibrate analyzer by measuring the initial light level (I_0) for the cuvette in Well B1 (the Bacterial Reagent control) according to the instrument manufacturer's instructions. All subsequent readings will be expressed as values on a comparative scale, based on this reading (i.e., the Bacterial Reagent's actual performance). Then, measure I_0 for each cuvette in incubator wells in rows B and D (B1, B2, B3, B4, B5, D1, and D2), carefully moving and replacing each cuvette in turn. After all I_0 readings have been acquired, prompt the software timer to track sample additions to each Bacterial Reagent cuvette (rows B and D) and perform the following steps immediately.

g. Sample-to-reagent transfer: Using a pipettor adjusted to deliver 500 μL, transfer 500 μL from cuvette in incubator well A1 to cuvette in incubator well B1 and gently mix by repeatedly drawing up and delivering the mixture with the pipettor. Repeat this process for A2 to B2, A3 to B3, A4 to B4, A5 to B5, C1 to D1, and C2 to D2.

Immediately after the last transfer, prompt the computer (per manufacturer instructions) to note the time elapsed since the last I_0 reading. This is the transfer time, which the system uses to schedule I_t measurements (where $t = 5$ or 15 min) to ensure that all cuvettes' exposure times are equal.

At this point in the test, the cuvette sample concentrations (%) are:

1	2	3	4	5	Row	Test Volumes
0.00	3.125	6.25	12.5	25.0	A	Each cuvette in row A contains 500 µL (sample and/or diluent).
0.00	1.563	3.125	6.25	12.5	B	Each cuvette in row B contains 1010 µL (diluent, sample, and Bacterial Reagent).
50.0	100.0				C	Each cuvette in row C contains 500 µL (sample or sample with diluent).
25.0	50.0				D	Each cuvette in row D contains 1010 µL (diluent, sample, and Bacterial Reagent).

When the computer timer sounds, measure I_5 for each cuvette. When the computer timer sounds again, measure I_{15} for each cuvette. Then, complete the test as prompted by the computer.

5. Data Reduction, Reporting, and Interpretation

Once testing is complete, the data for each cuvette include sample dilution (as a percentage), I_0, I_5, I_{15}, and gamma (Γ). *Gamma* is the ratio of light lost to light remaining after the Bacterial Reagent was exposed to the sample. It is calculated for each concentration as follows:

$$\Gamma_t = [(CR \times I_0) \div I_t] - 1$$

where CR is the control ratio (CR = control $I_t \div$ control I_0), which corrects for natural light loss over time [as indicated by the data for the blank cuvette (in Well B1)].

Using these data, the system attempts to estimate the IC_{50} with a 95% confidence range. A narrow confidence range means more accurate results. If an IC_{50} value can be calculated from the data, it is displayed in the units specified when test parameters were outlined. By definition, IC_{50} = a Γ value of 1. Not every concentration necessarily produces a valid Γ.

Collectively, the cuvette data should show a concentration–response relationship, meaning that Γ should increase as sample concentration increases (unless the sample does not inhibit bioluminescence at any concentration). At least three valid Γ data points are needed to make final calculations. If all light output data in B2 through D2 = 0, then retest sample at lower concentrations. If all light output data are similar (i.e., no significant light decrease), flag all data points with an asterisk.

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8070 P450 REPORTER GENE RESPONSE TO DIOXIN-LIKE ORGANIC COMPOUNDS*

8070 A. Introduction

1. General Discussion

A cell culture of human liver cancer cells can be used to detect the presence of toxic and/or carcinogenic organic compounds in environmental samples. A sample of water, soil, aquatic sediment, or tissue is extracted with a solvent to remove semivolatile organic compounds, and a small amount of the extract is applied to a culture well containing cells attached to the bottom of the well in medium. After exposure, the cells are rinsed and lysed, the cell fragments are removed via centrifugation, and the extract is tested for luminescence. The cells produce a luminescent enzyme (luciferase) if the extract contained dioxin-like compounds because a reporter gene (plasmid) from the firefly has been attached to the human chromosome at the site induced by dioxin and other planar compounds (at the CYPlAl gene). The amount of light produced, which is quantified by a luminometer, is a function of the concentrations and induction potency of the organic compounds in the extract. Dioxin [2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD)] has the strongest affinity for the receptor on the cell membrane (Ah-receptor), and therefore will be detected at the lowest concentration.

This assay is both a detection system and a meaningful biological response to the toxicants in environmental samples. It can be used to screen environmental samples for some of the most toxic and carcinogenic compounds. Only compounds that are dioxin-like and attach to the Ah-receptor will induce the CYPlAl gene and result in the production of luciferase. Such induction would occur in humans or wildlife, including aquatic species, if these compounds came in contact with their tissues. Inducing the CYPlAl gene is one of the key factors used in designating a compound a carcinogen. High levels of such induction in fish (P450 measurements) have been shown to correlate with histological damage and reduced reproductive capacity.

Another procedure based on these principles is available.[1]

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8070 B. The P450 RGS Test

1. Principle and Significance

The P450 Reporter Gene System (RGS) is a biomarker test for detecting toxic and/or carcinogenic organic compounds, using a transgenic cell line (101L)* derived from the human hepatoma cell line (HepG2). Under appropriate test conditions, induction of the CYPlAl gene in mammalian cells normally results in the production of the enzyme P450lAl. This response is evidence that the cells have been exposed to one or more xenobiotic organic compounds, including dioxins, furans, coplanar polychlorinated biphenyls (PCBs), and several polycyclic aromatic hydrocarbons (PAHs). Detection of induction has been made rapid and inexpensive by the stable integration of a firefly plasmid, such that Ah-receptor binding and subsequent transcription results in the production of luciferase. This RGS test has shown concentration–response relationships using dilutions of TCDD, 2,3,7,8-TCDF, five coplanar PCBs, and eight PAHs, and has responded to application of extracts from environmental samples.[1–4] Environmental samples (water, tissue, soil, or aquatic sediments) may be extracted via established methods, and the extracts applied to the test system to detect toxic and carcinogenic organic compounds.[5]

The organic compounds that induce the CYPlAl site on the chromosome are toxic, often carcinogenic, and several have been shown to bioconcentrate and biomagnify. Various birds, mammals, and fish exposed to these compounds have exhibited physiological, reproductive, and histopathological effects.[6–7] Millions of dollars are spent annually on chemical analyses of water, food, wildlife, soil, and aquatic sediment because of concern for contamination from these compounds. Using a screening tool, such as RGS, will permit selection of a subset for further chemical characterization.

2. Test Summary

Details of the culture and testing methods have been published.[8–10] For most environmental samples, dichloromethane (DCM) is used as the extraction solvent, but for extraction of ash samples for dioxins/furans, toluene is used. Initial dry weight (determined on a separate subsample) of extracted sample, final volume of solvent containing the extracted material (0.5 or 1.0 mL), and amount applied to the cells are recorded. Regardless of the extraction solvent or system, DMSO, iso-octane, or another low-toxicity solvent is the final solvent applied to the cells. Extracts of environmental samples are added to individual wells (six-well plates) containing approximately 1 million cells that have been growing for 3 d. Usual exposure time is 16 h. To

* Cells are available from Columbia Analytical Services, Vista, CA 92083.

examine the relationship between chlorinated hydrocarbons and PAHs in an extract, measure response at 6 and 16 h, because PAHs reach peak induction at 6 h and PCBs and dioxins reach peak induction at 16 h.[11] If information is required on only the chlorinated inducers (dioxins, furans, coplaner PCBs) first clean the extracts of PAHs with silica gel columns. Application of 20 μL solvent produces a low background (control) induction when applied to cells in 2 mL of culture medium. The luminescence [in relative light units (RLU)] of the combined cytoplasm from the cells in each well is compared to that of other replicate wells, the solvent control, and reference toxicants (TCDD and five concentrations of a dioxin/furan mixture) using a 96-well luminometer. The mean RLU of the control wells is set to unity. Mean RLUs of samples are first recorded as fold induction (times background), which is derived by dividing by the mean RLU of the solvent (background control). A dioxin/furan standard curve [toxic equivalents (TEQ) vs. fold induction] is prepared from the results of each test, and the slope is used to adjust fold induction values to TEQs (in ng/g dry weight). The RGS response represents the integrated CYPlAl induction from all dioxin-like compounds in the extract. Because standard curves have been prepared for the response to benzo(a)pyrene and a mixture of dioxins and furans, the assay results may be expressed as micrograms of benzo(a)pyrene equivalents, or nanograms of TCDD TEC per gram dry weight or per liter.

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8071 COMET/SINGLE-CELL GEL ELECTROPHORESIS ASSAY FOR DETECTION OF DNA DAMAGE*

8071 A. Introduction

1. Significance

Many pathological conditions are initiated by an increased incidence of DNA damage.[1] The most common example is carcinogenesis initiated by mutagens resulting from DNA damage. Changes in an organism's normal function due to contaminant exposure begin at the cellular and molecular levels.[1–3] DNA damage may be manifest in the form of base alterations, adduct formation, strand breaks, and cross-linkages.[4] Of these, the most prevalent type of genetic damage is the DNA single-strand break; many waterborne contaminants have been shown to cause dose-dependent incidences of strand breaks.[1,4–11] Strand breaks may be introduced directly by genotoxic compounds, through the induction of apoptosis or necrosis, secondarily through the interaction with oxygen radicals or other reactive intermediates, or as a consequence of excision repair enzymes.[11–13] In addition to a linkage with cancer, studies have demonstrated that increases in cellular DNA damage precede or correspond with reduced growth, abnormal development, and reduced survival of adults, embryos, and larvae.[14–16]

The comet assay is a simple, sensitive, and versatile method detecting DNA damage in individual cells. The assay can be applied to cells collected from virtually any eukaryotic organism, and can be used to detect DNA damage in vitro, in vivo, and in situ resulting from exposure to a broad spectrum of environmental contaminants.

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8071 B. Comet/Single-Cell Gel Electrophoresis Assay

1. Principle and Application

Under alkaline conditions, the comet assay facilitates the detection of DNA single-strand breaks and alkaline labile sites in individual cells, and can determine their abundance relative to control or reference cells.[1–3] A small number of cells (as few as 5000 or so) are immobilized in an agarose gel by suspending the cells in liquid agarose, which is then placed on a microscope slide and allowed to solidify at low temperature. The cells are lysed in a buffer containing detergents and high concentrations of salt; the immobilized DNA is then denatured under alkaline conditions and subjected to electrophoresis. DNA strand breaks cause localized relaxation and fragmentation of the tightly coiled DNA molecule. During electrophoresis, the relaxed and broken strands of negatively charged DNA migrate away from the nucleus toward the anode. When stained with a fluorescent DNA stain and viewed through a fluorescence microscope, each nucleus and associated tail of damaged strands of DNA resembles a comet. Cells with increased DNA damage resulting from strand breaks have a greater fraction of the total DNA migrating away from the immobilized nuclear DNA; cells with increased damage resulting from crosslinks exhibit reduced DNA migration. The migration of DNA away from the nucleus (i.e., comet tail length) can be measured by eye with an ocular micrometer. Alternatively, the comets can be classified into different categories associated with increased migration based on appearance. Comet tail length, percentage of migrated DNA, tail moment (tail length multiplied by fraction of DNA in the tail), and other DNA migration values can be calculated with the use of image-analysis software.

Electrophoresis under alkaline conditions (pH>13) allows for the detection of single-strand breaks and alkaline-labile lesions, while neutral pH conditions facilitate the detection of double-strand breaks.[4] Various sample treatments can be used to identify specific types of DNA damage or to preserve damage at sites of DNA repair.[5] Nuclease digestion steps can be used to introduce strand breaks at sites of specific lesions. With this approach, oxidative base damage can be detected by the use of endonuclease III or formamido pyrimidine glycosylase,[6] as well as DNA modifications resulting from exposure to ultraviolet (UV) light through the use of T4 endonuclease V.[7] The variation used depends on the type of cell being examined, the types of DNA damage the researcher wishes to detect, and the imaging/analysis capabilities of the laboratory conducting the assay.

When analyzing DNA damage, this assay offers a number of advantages: it measures DNA damage in individual cells, only very small numbers of cells need to be sampled, the assay can be performed on practically any eukaryotic cell type, and it has been shown in comparative studies to be very sensitive.[8,9]

This method has detected significantly elevated levels of DNA damage in cells collected from organisms in polluted sites (compared to reference sites).[10–12] These studies have shown that increases in cellular DNA damage correspond to decreased growth, survival, and development, and correlate with significant increases in contaminant body burdens or highly elevated metabolic demands.

Because the method is simple, cost-effective, and sensitive, it has been used to screen the genotoxicity of various compounds on cells *in vitro*, cells collected from *in vivo* exposures of whole organisms, field-deployed organisms, and organisms collected in the wild, as well as to evaluate the dose-dependent antioxidant (protective) properties of various compounds.[1–6,9,13,14,15]

2. Apparatus

a. Water bath(s), set at temperatures of 35 to 40°C and 70°C.

b. Centrifuge, capable of exerting a 600-*g* force, handling, 0.4 to 1.5-mL microcentrifuge tubes.

c. Microwave oven, small, low-energy model.

d. Microscope slides, 7.6 cm × 2.5 cm × 1 mm, clear glass with frosted label area or hydrophilic plastic support media.

e. Electrophoresis chamber, submarine. Larger chambers with platforms approximately 20 × 20 cm allow more gels to be processed at one time.

f. Electrophoresis power supply, generally capable of delivering DC constant current of 300 mA and a voltage gradient of 0.4 to 1.3 V/cm.

g. Fluorescence microscope, with excitation and emission filters appropriate for the fluorescent stain being used (e.g., ethidium bromide, 510- to 560-nm excitation filter and 590-nm barrier emission filter).

h. Ocular micrometer, for measuring DNA migration distance by eye if an image system is not available.

i. Image analysis system (optional), consisting of a CCD camera attached to the microscope and connected to a computer loaded with the appropriate image-analysis software. Commercial systems are available specifically for comet assay applications.

3. Reagents and Materials

a. Slide preparation (only required when using glass slides): electrophoresis-grade, for slide base coat dissolved in TAE working buffer, to a final concentration of 1.0%. Store melted and solidified agarose in sealed glassware at room temperature, for up to several months if necessary. Before use, re-melt in a 70°C water bath or a microwave oven.

b. TAE buffer stock solution, 50×: Combine 242 g Tris [tris(hydroxymethyl) aminomethane] base, 57.1 mL glacial acetic acid, 100 mL 0.5*M* EDTA, pH 8.0, and enough distilled water to produce a final volume of 1 L. Store in refrigerator.

c. TAE working buffer solution, 1×: Dilute 10 mL stock TAE in 490 mL distilled water. Make as needed.

d. Cell suspension agarose (CSA), with low melting point (~30°C), dissolved to 0.5 to 1.0% in buffer solution (¶s *e* and *f* below) suitable for cells being studied. Solidified agarose can be stored in sealed vials at room temperature for months and re-melted in a 70°C water bath or microwave oven, then transferred to a 35 to 40°C water bath before cell preparation.

e. Kenny's salt solution (for marine invertebrate cells):[16,17,19] To 900 mL distilled water, add 23.5 g NaCl , 0.7 g KCl, 0.1 g K_2HPO_4, and 0.2 g $NaHCO_3$. Adjust pH to 7.5 with NaOH, bring

to final volume of 1 L, and filter through a 0.22-μm filter (filter-sterilize). Store at 4°C (usable for several weeks).

f. Phosphate-buffered saline (PBS) stock solution (for many vertebrate cell applications),[3,18] 10×: To 900 mL distilled water, add 1.361 g KH_2PO_4, 14.2 g Na_2HPO_4, 80.1 g NaCl, and 20.1 g KCl. Adjust to pH 7.0, bring to final volume of 1 L, filter-sterilize, and store at 4°C.

g. PBS working solution: Dilute PBS stock solution using 10 mL 10× PBS and 90 mL distilled water. After dilution, pH should be ~7.4.

h. Stock lysis solution: Combine 146.4 g NaCl, 37.2 g EDTA, and 1.2 g Tris-HCl in 500 mL distilled water; while stirring slowly, add 8 to 12 g NaOH to facilitate dissolution of EDTA and adjust pH to 10. Adjust volume to 1 L, filter-sterilize, and store at 4°C.

i. Working lysis solution: Combine 89 mL lysis stock solution, 10 mL DMSO, and 1.0 mL polyethylene glycol *p*-isooctylphenyl ether.* Bring to final volume of 100 mL. Make fresh on day of use; refrigerate at 4°C before use.

j. Electrophoresis/unwinding solution (pH>13): To 968 mL distilled water, add 30 mL 10*N* NaOH and 2 mL 0.5*M* EDTA (pH 8.0). Bring final volume to 1 L. Make fresh on day of use.

k. Tris neutralization buffer, 0.4*M*, pH 7.5: Mix 200 mL 1*M* Tris (pH 7.5) with 300 mL distilled water, for 500 mL final volume.

l. Tris buffer, 1*M*, pH 7.5: Dissolve 121.1 g Tris base in 800 mL distilled water, add 63 mL conc HCl, adjust pH to 7.5, and bring to final volume of 1 L.

m. Ethidium bromide stock solution: (**CAUTION: Use extreme care when handling; ethidium bromide is a powerful mutagen. Wear gloves and other appropriate safety protection. Dispose of contaminated items and waste properly.**) Dissolve 10 mg ethidium bromide (EtBr) in 1 mL distilled water, and store in light-protected container at 4°C. The solution is stable for months.

n. Ethidium bromide working solution: Add 10 μL EtBr stock solution to 5 mL distilled water. Store in light-protected container at room temperature; this solution is stable for weeks.

o. Ethanol, reagent-grade, 95%: Store in freezer at −20°C; this reagent is stable for months.

p. Cell preparation, suspension of selected cells in 50 to 1000 μL maintenance medium. Use maintenance medium most likely to reduce stress on cell types used.

4. Procedure

a. Slide preparation: (NOTE: Wear gloves whenever handling slides and slide covers; skin oils reduce adherence of agarose to microscope slides.) Prepare 100 mL of melted slide coating agarose. Solid agarose preparations can be liquefied in a microwave oven with alternating short pulses of microwaves followed by gentle swirling. Grasp each slide (8071B.2*d*) at label area, dip into melted agarose, and leave immersed for at least 10 s. Remove from agarose, wipe excess agarose off back of slide, place on a level surface, and let dry for 2 h at room temperature or for 30 min in a drying oven at 37°C. Store dry slides in a moisture-free slide storage box at room temperature. Slides prepared in this manner may be stored almost indefinitely and require no further preparation before sample application.

b. Sample preparation: Place cells suspended in maintenance medium (8071B.3*p*) in a 1.5-mL centrifuge tube and centrifuge (e.g., at 600 *g* for 2 min) to form pellet. Carefully draw off supernatant and discard. In most cases, a cell pellet about 1 to 2 mm in diameter is more than sufficient to yield cell densities of 5 to 20 cells per microscopic field of view at 200×, when resuspended in 50 to 400 μL cell suspension agarose. Experience in estimating and adjusting cell densities will reduce the number of overlapping and superimposed nuclei encountered during scoring. Melt CSA (8071B.3*d*) and place in a 35 to 40°C water bath; let temperature stabilize. Gently resuspend pellet in appropriate volume of CSA at 35 to 40°C, as determined by the researcher. Before the agarose solidifies, transfer a portion of the cell suspension (e.g., 50 to 75 μL) to an agarose-coated slide. Spread sample uniformly over slide by placing a clean glass cover slip on top of the still-liquefied sample. Place slide on a level, ice-cold metal or glass surface until agarose has solidified. Then add a topcoat of 50 to 75 μL CSA by slipping cover slip off slide, applying agarose, and replacing cover slip on top. Let agarose solidify as before, remove cover slip, and place slide in a glass slide jar filled with ice-cold working lysis solution (8071B.3*i*), so solution completely immerses slide area covered by sample. Minimum lysis period is usually 1 h, with no apparent maximum. However, gels can become more fragile with extended lysis times.

c. Unwinding and electrophoresis conditions: After lysis, rinse slides in distilled water or neutralizing buffer to remove lysis salts and detergents by immersing slides for 2 min and replacing with fresh distilled water/neutralizing buffer three times. Place slides in electrophoresis chamber and fill chamber with electrophoresis/unwinding solution to a depth of 3 to 4 mm above the slides. Set slides side by side and in contact, if necessary forming multiple rows on the chamber platform. Leave slides undisturbed in electrophoresis/unwinding solution for 15 to 60 min. [Optimum unwinding and electrophoresis times can be determined by comparing the extent of migration in untreated control target cells and target cells exposed to a DNA damaging agent (e.g., gamma radiation, hydrogen peroxide, methylmethane sulphonate). In order for historical data to be useful, the negative control cells should exhibit some level of DNA migration.] Set power supply to run for 5 to 60 min at 300 mA constant current, with a voltage gradient that may range from 0.4 to 1.3 V/cm. After electrophoresis, turn power off, remove slides from chamber, and neutralize alkali by three 2-min rinses in neutralization buffer (8071B.3*k*). Fix DNA in agarose gel by soaking for 5 min in ice-cold 95% ethanol. Dry slides at room temperature or in a 37°C oven. Dried slides can be stored indefinitely in slide boxes until stained and scored.

d. Staining: **CAUTION: Wear gloves throughout these procedures, and whenever the slides are handled subsequently.** Place 10 to 40 μL EtBr† working stain solution (¶ 8071B.3*n*) on each slide, place a cover slip on top, and remove excess stain with an absorbent tissue.

* Or equivalent nonionic surfactant.

† Many fluorescent DNA stains are commercially available. While EtBr is referred to in this procedure, propidium iodide and YOYO also have been used successfully.[10,18]

e. Scoring: Examine stained slides with epifluorescence microscope, optimally between 200 and 400× magnification. (Optimal magnification depends on the size of cells being assessed.) Ethidium bromide bound to double-stranded DNA has fluorescence excitation and emission maxima of 518 and 605 nm, respectively. Use excitation and barrier filters specific for this fluorescent dye. Slides viewed in this way reveal stained comets as bright fluorescent orange balls 10 to 40 μm in diameter. Conduct scoring with either an ocular micrometer (8071B.2*h*) or an image-analysis system (8071B.2*i*). With an image-analysis system, it is possible to measure fluorescence intensity and DNA distribution throughout the comet.[18] In this way, the percentage of DNA in the comet tail, the tail length, tail moment (which is the product of the fraction of DNA in the tail and tail length), and numerous other measures can be determined.

Regardless of scoring method, always determine the number of comets per slide. Generally, score 50 to 100 cells per sample.[11,12] Fewer cells would eliminate the ability to identify subpopulations of cells with altered migration among a larger population of cells whose migration patterns match those in the control sample. Because of inherent variability within and across electrophoresis runs, preferably score two slides per sample, with 50% of the data obtained from each replicate slide. Score comets in different sectors of each slide. Avoid scoring comets near edge of slide and do not score slides with high background levels of staining. Once the field of view is randomly moved to a sector, use a systematic method of scoring (e.g., scoring comets from left to right starting in the upper left-hand corner) until a predetermined number of comets has been counted. Do not count overlapping and superimposed comets. Ensure that slide scoring is "blind" (i.e., that the sample identity of the slide is not known during scoring).

After scoring, remove cover slips of scored slides, dry slides, and store as permanent records that can be restained and viewed at a later date.

5. Data Analysis

a. Interpretation of data: Depending on scoring method, different types of data may be gathered, (e.g., ocular measurement of comet image or tail lengths, the fraction of cells with different migration patterns, image-analysis-based comet tail lengths, percentage of migrated DNA, tail moment). The distribution of migration patterns can be expressed graphically in histograms by plotting frequency of comets (*Y* axis) and corresponding DNA damage measurement gathered for those comets (*X* axis). In addition, dose–response plots can be constructed showing DNA damage (mean and standard deviation of data, *Y* axis) and test compound concentration (*X* axis).

b. Acceptability of data: Compare DNA damage levels in controls and (if study design allows) damage resulting from reference toxicants to previously gathered data to determine acceptability. If studying cells from organisms or cell lines on which it is possible to perform laboratory exposure tests, design experiments to incorporate positive controls and construct control charts to evaluate test performance (see Section 1020B.13). Prepare control chart for each combination of reference toxicant and test organism, and include the most current data in each control chart. Endpoints from five tests are adequate for establishing a chart. Use control charts to evaluate the cumulative trend of results from a series of samples. Recalculate mean and upper and lower control limits (±2 standard deviations) with each successive result.

If the value from a given test with the reference toxicant falls more than 2 standard deviations outside the expected range, the organisms' sensitivity and the test system's overall credibility may be suspect. In this case, examine test procedure for defects and repeat with a different batch of test organisms.

c. Statistics: Establish statistical methods to be used and data requirements for those methods during initial experiment design. The unit of exposure for *in vitro* studies is the culture, while for *in vivo* studies it is the animal. Thus, multiple cultures or multiple animals are needed per dose group to provide data for an appropriate statistical analysis. Examine the homogeneity of variance between treatments to determine whether parametric or nonparametric analysis is appropriate. Transformation of nonhomogeneous data also can be explored. If homogeneity is not achieved using transformed data, use nonparametric procedures. Linear regression analysis can be used to establish dose–response relationships, while pairwise comparisons of each treatment group against the concurrent control can be conducted. Use two-tailed statistical test if both an increase and decrease in DNA are being tested; a one-tailed test if only one is being tested.

d. Evaluation and interpretation of results: If a positive comet assay response is obtained, assess the possibility that the increase in migration is not associated with genotoxicity. Information on the extent of cytotoxicity associated with each positive dose group, the nature of the dose–response curve, the intercellular distribution of comet response at each dose, and the presence or absence of necrotic or apoptotic cells in the treated cell population may be useful. Common determinations of cytotoxicity rely on simple dye exclusion on vital staining assays. If a negative comet assay response is obtained, assess the validity of the assay and the dose-selection procedure. Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgment about the activity of the test substance. Reproducibility in independent experiments is considered the strongest evidence for a positive or negative call. However, results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

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8080 SEDIMENT POREWATER TESTING*

8080 A. Introduction

1. Applications

The standard approach for assessing the quality or potential toxicity of marine or estuarine sediments has been to expose macrobenthic organisms directly to whole sediments for a specified time, after which the survival of the test species was determined. Whole-sediment methods[1,2] have limitations, including use of adult macrobenthic organisms and the use of mortality as the primary endpoint. In addition, the standard amphipod test protocol[1,2] underestimates the potential toxicity of contaminated sediments because the pore water is flushed out and replaced with fresh overlying water before exposure begins.

The porewater toxicity test approach offers several advantages over the standard whole-sediment method. Sensitive life stages of sensitive species can be used in tests using sublethal endpoints. There are no artifacts produced by sediment texture. A dilution series test design can be used easily for better differentiation among highly toxic samples. Whereas whole sediment preferably should be tested within 2 weeks of collection, studies indicate that pore water can be stored in the frozen state for extended periods without any change in toxicity.[3] Most of the studies on porewater testing have focused on marine and estuarine species.

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* Approved by Standard Methods Committee, 1997. Editorial revisions, 2009. Joint Task Group: 20th Edition—Robert S. Carr (chair), Marion Nipper, Donald J. Reish.

8080 B. Sediment Collection and Storage

Sediment collection methods vary considerably with specific study objectives. Remove as much overlying water as possible from the sample before placing it in the sample container or adding it to a composite sample. Nearly fill sample container to minimize headspace, allowing some room for sample to be rehomogenized in its original container. Extract pore water as soon as possible after sample collection. If the sediment sample cannot be processed immediately, store on ice or refrigerate at 4°C. The toxicity of pore water extracted from refrigerated sediments can change considerably after weeks or even days.

8080 C. Extraction of Sediment Pore Water

Methods that have been used to obtain sediment pore (interstitial) water include centrifugation,[1–3] pressurized (pneumatic or mechanical "squeezing") extraction,[4–8] vacuum (suction) methods,[9,10] and equilibration methods using dialysis membranes or fritted glass samplers.[4,11,12] Studies comparing recovery efficiencies of trace metals and organics for different extraction methods indicate that substantial losses of nonpolar contaminants (e.g., fluoranthene and *p,p′*-DDE) can occur with all methods.[13] Toxicity tests with echinoderm gametes and embryos have been conducted to compare the toxicity of pore water obtained by various extraction techniques.[14] These studies suggest that centrifugation minimizes loss of nonpolar contaminants. Loss of metals is comparable among the various extraction methods. Centrifugation is preferable to filtration for removing

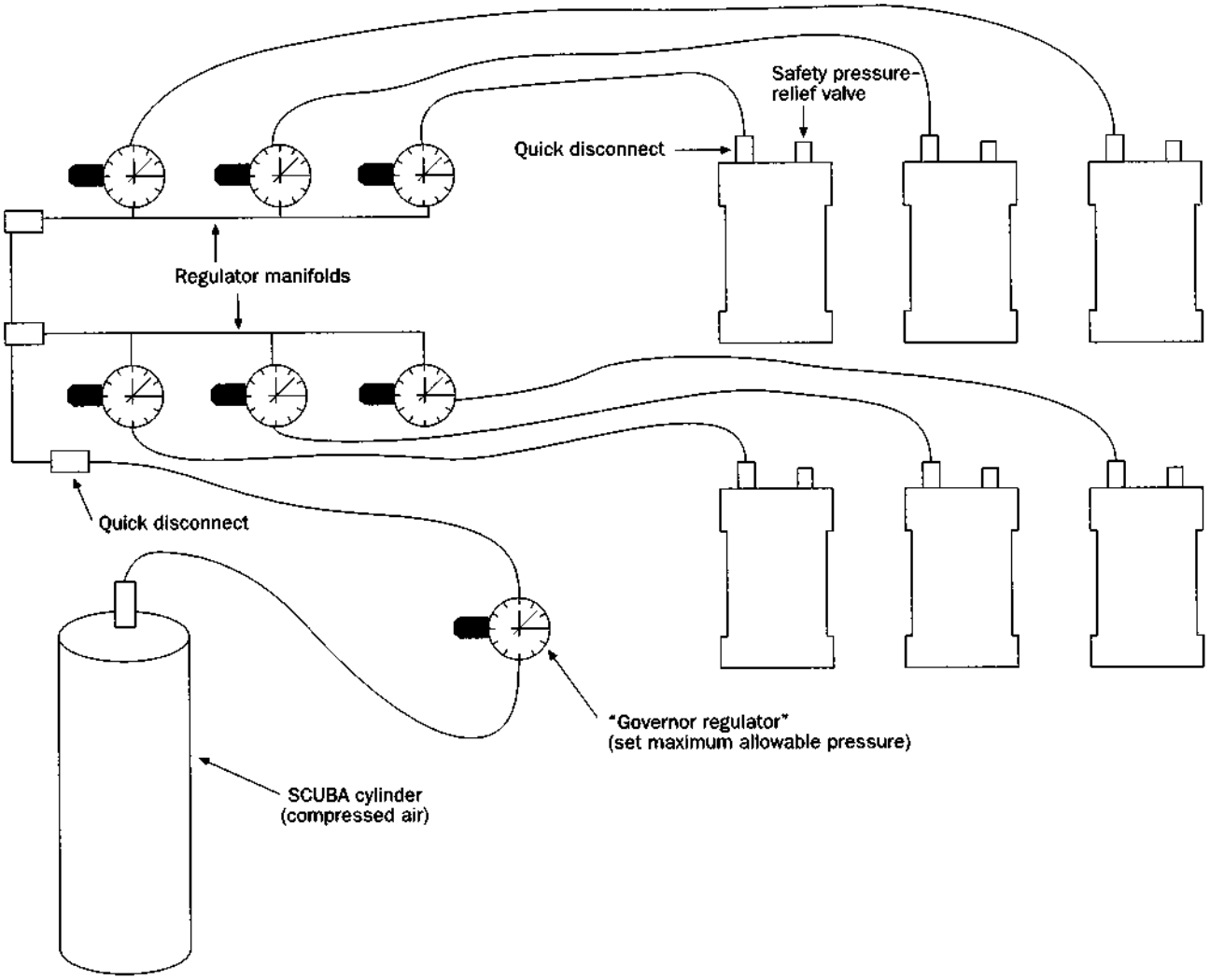


Figure 8080:1. Pneumatic system for porewater extraction. Source: CARR, R.S. & D.C. CHAPMAN. 1995. Comparison of methods for conducting marine and estuarine sediment porewater toxicity tests. I. Extraction, storage and handling techniques. *Arch. Environ. Contam. Toxicol.* 28:29.

particulates because it minimizes adsorptive loss of contaminants.

Sandy sediments do not compact appreciably during centrifugation, making pore water recovery difficult; however, the pneumatic extraction method (Figures 8080:1 and 2) is particularly effective.[11] The vacuum method is least expensive for small-scale projects.

Regardless of the method used for initial extraction, centrifuge the extracted pore water to remove suspended particulates for fertilization and embryo development assays with echinoderms and mollusks.

1. Centrifugation

Use a centrifuge equipped with a swinging bucket-type rotor capable of spinning 100- to 1000-mL bottles at 10 000 × *g*. Use glass or polycarbonate tubes or bottles to minimize adsorption of soluble contaminants on container wall. For some sediments, it may be possible to decant supernatant without disturbing the pellet; however, for most sediments, use a pipet to transfer the supernatant to a separate container.

2. Pressurized Squeeze Extraction

The most common squeeze extraction devices use compressed air (or nitrogen) to pressurize a cylinder containing the sediment. Normally, use a filter in the bottom of the cylinder so minimal sediment is introduced into the porewater sample. Some filters (e.g., glass fiber filters) can adsorb a high percentage of nonpolar contaminants from solution.[13,14] Other filter materials (e.g., polyester and nylon) are preferable. Before use, test any part of the extraction device that contacts pore water during extraction for toxicity. Fill extraction device with a small volume of test dilution water and, after a minimum of 8 h, test dilution water for toxicity. Soak new filters in deionized water or test dilution water, with several exchanges for at least 24 h to remove any residual contami-

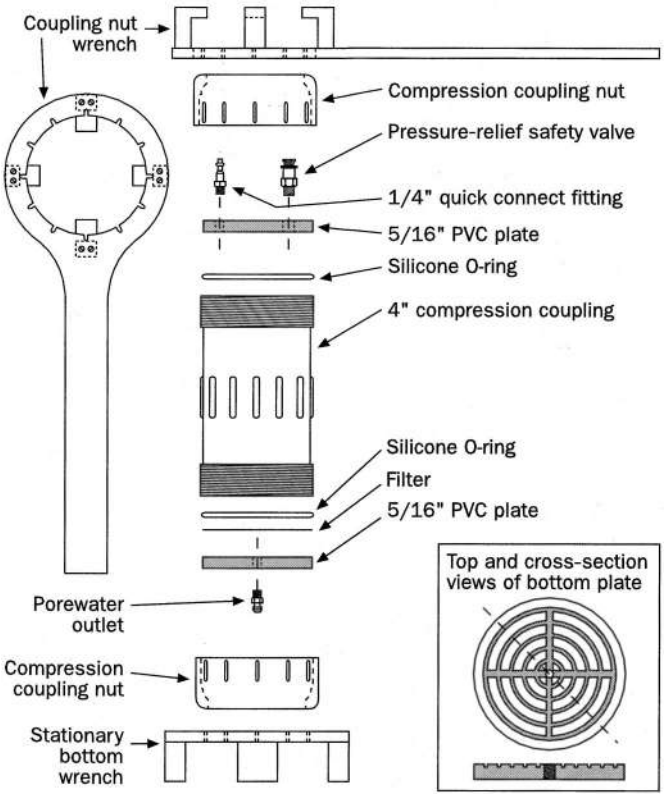


Figure 8080:2. Detail of porewater extraction cylinder. (For dimensions in centimeters, multiply dimensions in inches by 2.54.) Source: CARR, R.S. & D.C. CHAPMAN. 1995. Comparison of methods for conducting marine and estuarine sediment porewater toxicity tests. I. Extraction, storage and handling techniques. *Arch. Environ. Contam. Toxicol.* 28:29.

nants before use. Between samples, acid-wash the parts of the extraction devices that come in contact with the sample.

3. Vacuum Extraction

The simplest vacuum extraction system is a fused-glass air stone attached with aquarium air-line tubing to a polypropylene syringe. Apply vacuum by bracing the syringe plunger or using a vacuum pump. Modify the system with TFE* tubing and a glass syringe when loss of contaminants due to adsorption is a concern. This method is inexpensive and may retain more volatile compounds than centrifugation or pressurized extraction. Vacuum methods may be more time-consuming than other extraction methods when large (>1 L) volumes are needed, particularly for fine-grained sediments. Thoroughly rinse all system components before use to remove residual toxicants.[15] Determine effectiveness of the rinsing procedure by testing the toxicity of test dilution water held in the vacuum extraction system for a minimum of 8 h. Pore water extracted via vacuum methods from sandy sediments has a higher particulate content than pore water obtained by the other methods; if the suspended particulates are not removed before testing, they may produce a response in fertilization and embryo development toxicity tests.

4. Equilibration Methods

The most commonly used equilibration technique for collecting pore water involves a small-volume vessel with a membrane placed in the sediment and allowed to equilibrate with the surrounding interstitial water.[11,16,17] The limitations to this technique are that only milliliter volumes can be obtained within a reasonable time (days). Test toxicity of components used to construct the equilibration device by soaking device in clean test dilution water or clean sediment for at least the same length of time as the longest equilibration period to be used.

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* Teflon®, or equivalent.

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8080 D. Toxicity Testing Procedures

1. General Procedures

Because of the difficulty in obtaining large volumes of pore water, organisms and life stages that only require small volumes are most amenable to testing with pore water. For tests requiring more than 7 d to complete, preferably use a static renewal test design to ensure acceptable water quality. Short-term toxicity tests have been used most frequently with pore water. Much of the general guidance provided in Section 8010 is applicable to testing with pore water. More specific guidance can be found in sections for particular species or groups of organisms (e.g., Sections 8510, 8610, and 8710).

2. Exposure Chambers

The type of exposure chamber used depends on the test. Most porewater tests are conducted in relatively small volumes (i.e., ≤10 mL). Preferably cover test chambers to minimize evaporation and resulting salinity increases during the exposure period. Scintillation vials (20 mL) with polyethylene or polypropylene cap liners are ideal inexpensive disposable test chambers for many species. Avoid caps with urea-formaldehyde liners because these can be toxic. Stender dishes with ground-glass lids (20-mL capacity with 10 mL of exposure media) make excellent exposure chambers for tests that require microscopic examination of the test organisms without transferring them to another container (e.g., the *Dinophilus gyrociliatus* life-cycle test).[1]

3. Organisms

Many types of organisms have been used in porewater tests. Minute species or larval forms are preferable not only for their small volume requirements, but also because they tend to be the most sensitive. Most of the studies on porewater testing have focused on marine and estuarine species.

a. Marine and estuarine species: A commercially available test system,* which detects changes in the photoluminescence of the marine bacterium *Photobacterium phosphoreum* as an endpoint, has been used more frequently in freshwater porewater studies[2–4] than in marine or estuarine pore waters. Although the small sample size required is well suited for limited sample sizes, the sensitivity of the standard assay of this type for pore water from freshwater, estuarine, or marine sediments is low compared to those of other toxicity tests.

Algal studies with *Ulva fasciata* and *Ulva lactuca* suggest that a zoospore germination endpoint is as sensitive as some of the most sensitive embryological development assays used in porewater testing. This test appears to be particularly resistant to ammonia toxicity. Many algal species used in microplate procedures could easily be adapted for use with porewater samples.

Porewater toxicity testing has been conducted with the polychaete *Dinophilus gyrociliatus*.[1,5,6] Other minute polychaetes, such as *Ctenodrilus serratus* or *Ophryotocha* spp.,[7,8] can be tested in small volumes.

The mollusk tests used most successfully with pore water are fertilization and embryological development tests with the abalone *Haliotes refugens*. Other more common embryological development tests with oysters[9] and clams[10] could be adapted for use with porewater samples.

Most of the toxicity testing with marine and estuarine pore water has been conducted with sea urchin gametes and embryos.[6,11,12] The species most commonly used is the sea urchin *Arbacia punctulata* but other species of sea urchin (e.g., *Strongylocentrotus* spp. and *Lytechinus* spp.), as well as the sand dollar (e.g., *Dendraster* spp.), also have been used successfully. Types of tests include fertilization tests, embryological development tests, and cytogenetic assay.[13]

Fish embryos and larvae of red drum *Sciaenops ocellatus* also have been used successfully in porewater testing.[14]

b. Freshwater species: Only a limited number of species have been used in porewater studies with fresh water. A number of studies with a commercially available system* have been reported.[2,3,15] The freshwater amphipod *Hyalella azteca* has been used to test the toxicity of pore water from freshwater sediments.[3,15] *Ceriodaphnia dubia* also has been used in life-cycle tests with pore water.

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* Microtox®, or equivalent.

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8110 ALGAE*

Algae are unicellular to multicellular plants that occur in fresh water, marine water, and damp terrestrial environments. All algae posses chlorophyll, the green pigment essential for photosynthesis. Algae may contain additional pigments, such as fuco-xanthin (brown) or phycoerythrin (red), which can mask the green color of chlorophyll. The life cycle of algae may be simple, involving cell division, or complex, involving alternation of generations. Algae are primary producers of the organic matter upon which animals depend either directly or indirectly through the food chain.

Test procedures using algae are valuable for determining the primary productivity of a water and for testing the toxicity of chemicals present in a water. Biostimulation (algal productivity) (Section 8111) measures the response of a cultured species of algae to the nutritional condition of the water.

Phytoplankton (Section 8112) measures the response of an algal species to materials that interfere with its normal metabolism.

Together, the tests allow the assessment of the effects of point or nonpoint discharges in fresh and marine waters.

A third algal test, marine macroalgae (Section 8113), measures fertilization and growth in the giant brown kelp *Macrocystis pyrifera*, which is useful in monitoring the effects of contaminants or discharges into the marine waters of the eastern Pacific Ocean.

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8111 BIOSTIMULATION (ALGAL PRODUCTIVITY)*

8111 A. General Principles

1. Characteristics of Algal Assays

Algal assays consist of three steps:

a. selection and measurement of appropriate factors or conditions during the assay (e.g., biomass indicators, such as measured or calculated dry weight);
b. presentation and statistical analysis of measurements; and
c. interpretation of results.

Interpretation of results involves assessing receiving water to determine its nutritional status and its potential sensitivity to change, effects of chemical constituents on algal growth in receiving waters, effects of changes in waste treatment processes on algal growth in receiving waters, impact of nutrients in tributary waters on algal growth in lakes and confluent receiving waters, and effects of such measures as those used in lake restoration and advanced waste treatment.

The maximum standing crop and the maximum specific growth rate are responses that can be estimated from growth measurements. The maximum standing crop described in this method is proportional to the initial amount of limiting nutrient available. The maximum specific growth rate is related to the concentration of rate-limiting nutrient present.

The algal test procedure for determining a water sample's primary productivity is based on Liebig's "Law of the Minimum," which states that growth is limited by the substance that is present in minimal quantity in respect to the organism's need. *Biostimulants* are substances that increase algal growth or the potential for algal growth.

Algal species used in biostimulation tests are selected to allow for a standardized test of growth response using a well-characterized organism under standard laboratory conditions. See Sections 10010, 10200, and 10300 for methods appropriate to field studies.

Effects of various substances on maximum crop of selected algal species cultured under specified conditions are measured in this text. Results are assessed by comparing growth in the presence of selected nutrient and chelator additions to growth in controls. Experimental designs must incorporate sufficient replication to permit statistical evaluation of results.

A method for growth inhibition tests with algae has been published.[1]

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8111 B. Planning and Evaluating Algal Assays

1. Sampling

Because water quality may vary greatly with time and point of collection, establish sampling programs to obtain representative and comparable data.

Consider all pertinent environmental factors in planning an assay, to ensure that valid results and conclusions are obtained. In a stratified lake or impoundment, collect only depth-integrated (composite) euphotic zone samples. In most cases, the *euphotic zone* is the depth to which at least 1% of the surface light is available. For euphotic zone depths of more than 8 m, subsample at least at the surface and at each 3-m depth interval. Likewise, for euphotic zones of less than 8 m, sample at least at the surface and at 2-m intervals. Composite equal-volume depth samples in a suitable nonmetallic container, mix thoroughly, and subsample for algal assay and chemical and biological analysis, including indigenous algal biomass and identification.[1]

Transect lines are helpful in sampling. Samples from a transect can be taken from predetermined euphotic zones. Representative river samples can be identified by specific conductance measurements that show the sampling transect's homogeneity. In rivers and streams, useful information may be obtained by taking samples upstream and downstream from suspected pollutant sources or confluent tributaries.[1]

The nutrient content of natural waters and wastewaters often varies greatly with time; variation may be seasonal or even hourly in wastewaters. When sampling, consider and minimize the effects of these variations.

2. Test Variables

Deficiency of any essential nutrient may limit algal growth, but tests are made for those few nutrients most likely to be growth-limiting (nitrogen, phosphorus, trace elements). Measuring the water's algal growth potential distinguishes between the nutrients in the sample (as determined by chemical analysis) and nutrient forms actually available for algal growth.[1]

To evaluate a substance's potential effect on receiving waters, consider the following factors: amount and distribution, chemical and/or physical nature, fate and persistence, pathways by which it will reach the receiving water, dilution by the receiving body, and selection of appropriate test water.[1,2]

When the algal assay is used to measure stimulation of growth by a given effluent, include the following in the overall evaluation: effluent quality, growth measurements and test organisms, concentration of growth-limiting nutrient, and potential nutrient concentration and changes in availability.

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8111 C. Apparatus

1. Sampling and Sample Preparation

a. Sampler, nonmetallic.

b. Sample bottles, borosilicate glass, linear polyethylene, polycarbonate, or polypropylene, capable of being autoclaved.

c. Membrane filter apparatus, for use with 47- or 104-mm prefilters (e.g., glass fiber filter) and 0.45-μm-porosity filters.

d. Autoclave or pressure cooker, capable of producing 108 kPa at 121°C.

2. Culturing and Incubation

a. Culture vessels: Use Erlenmeyer flasks of good-quality borosilicate glass. When trace nutrients are being studied, use special glassware made of high-silica glass or polycarbonate. While flask size is not critical, the surface-to-volume ratios of the growth medium are, because of CO_2 limitation. Use the following:

- 25 mL sample in 125-mL flask
- 50 mL sample in 250-mL flask
- 100 mL sample in 500-mL flask

b. Culture closures: Use demonstrably nontoxic foam plugs,* loose-fitting aluminum foil, or inverted beakers to permit some gas exchange and prevent contamination. Determine for each batch of closures whether that batch has any significant effect on maximum specific growth rate and/or maximum standing crop.

c. Constant-temperature room: Provide constant-temperature room, or equivalent incubator, capable of maintaining temperature of 18 ± 2°C (marine) to 24 ± 2°C (freshwater).

d. Illumination: Use "cool-white" fluorescent lighting to provide 4304 lux ± 10% or 2152 lux ± 10% measured adjacent to the flask at the liquid level with closure in place.

* Gaymar white, polyurethane foam plugs, VWR Scientific or Gaymar Industries, Inc., 701 Seneca St., Buffalo, NY 14210, or *demonstrably nontoxic* equivalent.

e. Light measurement device: Calibrate device against a standard light source or light meter.

3. Other Apparatus[1]

a. Analytical balance capable of weighing 100 g with a precision of ±0.1 mg.

b. Electronic particle (cell) counter.

c. Fluorometer, suitable for chlorophyll *a.*

d. Microscope and illuminator, good quality, general purpose.

e. Hemocytometer or plankton counting slide.

f. Shaker table, capable of 100 oscillations/min.

g. pH meter to measure to ±0.1 pH unit.

h. Dry-heat oven capable of operating at up to 120°C.

i. Centrifuge capable of a relative centrifugal force of at least 1000 × *g.*

j. Desiccator.

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8111 D. Sample Handling

1. Sampling Procedure

Use a nonmetallic water sampler and autoclavable storage container. Leave a minimum of air space in the transport container and keep it in the dark at 0 to 4°C.

2. Removal of Indigenous Algae

To use unialgal test species, "remove" indigenous algae before assay by autoclaving and filtering. Always prepare sample as soon as possible (within 24 h) after collection.

Use autoclaving followed by filtration to determine amount of algal biomass that can be grown from all bioavailable nutrients in the water, including those contained in filterable organisms. Autoclave freshwater samples at 108 kPa and 121°C for 30 min or 10 min/L of sample, whichever is longer. Pasteurize marine or estuarine samples for 4 h at 60°C. After autoclaving and cooling to room temperature, equilibrate sample by bubbling with a 1% mixture of carbon dioxide in air for at least 2 min/L. This will restore carbon dioxide lost during autoclaving and lower pH to its original level (usually it will rise on autoclaving). In some instances, waters with total hardness greater than 150 mg/L will lose calcium and phosphorus during autoclaving. The precipitate may be resistant to resolubilization by the addition of carbon dioxide and air. In waters containing high levels of hardness and alkalinity, the pH may not increase during autoclaving. Filter carbon-dioxide-equilibrated sample through pre-filter, if necessary, followed by a 0.45-μm membrane filter.[1]

3. Storage

Changes occur in water samples during storage regardless of storage conditions. The extent and nature of these changes is not well known. Therefore, keep storage duration to a minimum after sample preparation. Store samples in full containers with no air space. Before sample preparation, store samples in the dark at 0 to 4°C. If prolonged storage is anticipated, prepare sample first and then store in the dark at 0 to 4°C.

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8111 E. Synthetic Algal Culture Medium

See Section 8010E.4*c*1).

8111 F. Inoculum

1. Recommended Test Algae

The following selected species are used primarily in the United States, Canada, and northern Europe. The tests are probably valid for other species worldwide but would require validation testing. If diatoms are the selected test species, silica must be added to the synthetic algal culture medium.

a. Freshwater algae:

Selenastrum capricornutum Printz (see Section 10900, Plate 1A:G).

b. Marine algae:

1) *Dunaliella tertiolecta* Butcher (DUN Clone) Woods Hole Oceanographic Institution.

2) *Thalassiosira pseudonana* (Hasle and Heimdal) (CN Clone) (old *Cyclotella nana*) Univ. Rhode Island. Do not shake. (See Section 10900, Plate 1B:T; Plates 30, Figure C-6.)

3) *Skeletonema costatum* (Greville) Cleve. (See Section 10900, Plate 1B:W.)

2. Sources of Test Algae

Obtain algal cultures from recognized sources.* After receipt of cultures, check identity and purity.

3. Maintaining Stock Cultures

a. Medium: See Section 8010E.4*c*1).

b. Incubation conditions:

1) Freshwater species—Temperature 24 ± 2°C under continuous cool-white fluorescent lighting at 4304 lux ± 10% for *S. capricornutum;* shake at 110 oscillations/min.

2) Marine species—Temperature 18 ± 2°C under continuous cool-white fluorescent lighting at 4304 lux ± 10% for *D. tertiolecta* (shake at 110 oscillations/min) and for *T. pseudonana* (do *not* shake but swirl daily). Higher temperatures (up to 24°C) may be justified for appropriate test species used in the Gulf of Mexico and other warm-water marine systems. If other species are used, always relate growth of those species to *D. tertiolecta* to ensure comparability.

* American Type Culture Collection (sales@atcc.org); UTEX Culture Collection of Algae, Department of Botany, University of Texas at Austin (www.botany.utexas.edu); Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) (ccmp@bigelow.org).

c. First stock transfer: On receipt of inoculum species, transfer a portion to the algal culture medium. (Example: 1 mL of inoculum in 50 mL in a 125-mL Erlenmeyer flask).

d. Subsequent stock transfers: Make a new stock transfer, using aseptic technique, as the first operation on opening a stock culture. The volume transferred is not critical so long as enough cells are included to overcome significant growth lag. Make weekly stock transfers to provide a continuing supply of "healthy" cells. Check algal cultures microscopically to ensure that the stock cultures remain unialgal.

e. Age of inoculum: Use cultures 1 to 3 weeks old as a source of inoculum. For *Selenastrum* and *Dunaliella,* a 5- to 7-d incubation often is sufficient to provide enough cells.

4. Preparing Inoculum

Centrifuge stock culture and discard supernatant. Resuspend sedimented cells in an appropriate volume of glass-distilled water containing 15 mg $NaHCO_3$/L for freshwater species and artificial seawater minus nutrients for marine species [Section 8010E.4*c*1); Table 8010:II] diluted to appropriate salinity, and again centrifuge. Resuspend sedimented algae in the proper solution and use as the inoculum.

5. Amount of Inoculum

Count cells suspended in the prepared inoculum and pipet into the test water to give a starting cell concentration as follows:

S. capricornutum	10^3 cells/mL
D. tertiolecta	10^3 cells/mL

Calculate volume of transfer to result in the above concentrations in the test flasks (e.g., for *S. capricornutum,* if there are 5×10^5 cells/mL in stock culture, transfer 0.2 mL/100 mL test water).

8111 G. Test Conditions and Procedures

1. Temperature

Keep temperature at 18 ± 2°C for marine species and 24 ± 2°C for freshwater species.

2. Illumination

See 8111F.3*b.* Measure light intensity adjacent to the flask at the liquid level.

3. Procedure

a. Preparation of glassware: Wash all glassware with detergent (nonphosphate or sodium carbonate) and rinse thoroughly with tap water. Then rinse with a warm 10% (v/v) solution of reagent-grade HCl. Fill vials and centrifuge tubes with 10% HCl. Fill all containers to about one-tenth capacity with HCl solution and swirl to bathe entire inner surface. After HCl rinse, rinse glassware five times with tap water, then five times with deionized water. An automatic laboratory glassware washer may be used and is the preferred method. The acid-washed glassware should be neutralized with a saturated solution of Na_2CO_3 before washing in an automatic washer.

If an electronic particle counter will be used, add a final rinse of deionized water that has been filtered through a 0.22-μm filter.

Dry clean glassware at 105°C in an oven and store either in closed cabinets or on open shelves with tops covered with aluminum foil.

Before use, autoclave culture flasks covered with aluminum foil at 108 kPa for 15 min. After autoclaving, prerinse flasks with culture medium and invert on absorbent paper for 20 to 30 min to drain. Close culture flasks with foam plugs.

Use disposable pipets to minimize possibility of contamination.

b. pH control: To ensure the availability of CO_2, keep the pH below 8.5 by using optimum surface-to-volume ratios (8111C.2), continuously shaking the flask (approximately 100 oscillations/min).

c. Growth measurement: Describe the growth of a test alga in the bottle test by maximum standing crop. Generally, these measurements can be made on Days 3, 5, 7, 10, 12, and 14 if a growth curve is desired. If determination of maximum standing crop is the goal, count only on Days 12 and 14.

The *maximum standing crop* in any flask is the maximum algal biomass achieved during incubation. For practical purposes, it may be assumed that the maximum standing crop has been achieved when the increase in biomass is less than 5%/d. The maximum standing crop usually is achieved in the algal assay test after 12 to 14 d of incubation.

After attaining the maximum standing crop, determine the biomass and relate to the dry weight gravimetrically. Although not described here, an alternative growth measurement, the maximum specific growth rate, can be measured early in the bottle test. The *maximum specific growth rate* (μ_{max}) for an individual flask is the largest specific growth rate (μ) occurring at any time during incubation.

The *specific growth rate*, μ, is defined by:

$$\mu = \frac{\ln (X_2/X_1)}{t_2 - t_1}$$

where:

X_2 = biomass concentration at end of selected time interval,
X_1 = biomass concentration at beginning of selected time interval, and
$t_2 - t_1$ = time interval of selected time period, d.

4. Biomass Monitoring

Several methods may be used, but the selected measurements should be related to dry weight.

a. Dry weight: Use either the aluminum dish or membrane filter method. To use the first, centrifuge a suitable portion of algal suspension, wash sedimented cells three times in distilled water, transfer to tared crucibles or aluminum cups, dry overnight in a hot-air oven at 105°C, cool to room temperature in a desiccator jar, and weigh.

For the membrane filter method, rinse each filter with 50 mL deionized water and place in folded sheets of paper or on an aluminum weighing dish on which identification codes have been written. Dry overnight in a hot-air oven at 60°C, cool to room temperature in a desiccator jar, and determine tare weight. Filter a suitable measured portion of algal suspension through a tared 0.45-μm-pore-diameter membrane filter under a vacuum of 51 kPa. Use $\leq$50 mL as the cell density dictates. Rinse filter funnel with 50 mL deionized water using a wash bottle and let rinsings pass through filter. Dry in an oven for several hours at 60°C, cool in a desiccator, and weigh.

b. Electronic particle counting: Suspend *S. capricornutum* cells in a 1% NaCl electrolyte solution in a ratio of 1.0 mL cell suspension to 9 mL of 0.22-μm-filtered saline (10:1 dilution). Pass the resulting suspension through a 100-μm-diam aperture. Each cell that passes through the aperture causes a voltage drop proportional to its displaced electrolyte volume, which is recorded as a count. A knowledge of both the number of particles (cells) counted per unit volume of sample (usually 0.5 mL) and the mean particle (cell) volume displaced allows changes in cell biomass (in microliters per liter) to be calculated. Equations that can accurately relate volume to dry weight must be developed by each laboratory.

c. Chlorophyll: All algae contain chlorophyll and measuring this pigment can yield some insight into the relative amount of algal biomass present. To measure chlorophyll by fluorescence, swirl test flask to suspend cells. Pipet a portion of cell suspension (5 to 6 mL minimum) into a cuvette and read fluorescence. Zero fluorometer with a distilled water blank before each sample reading.

d. Direct microscopic counting: Use a hemocytometer or plankton counting cell (Section 10200F.2). For filamentous algae, break up the algal filaments by using a syringe, an ultrasonic bath, a high-speed blender, or vigorous stirring with glass beads. Each of these techniques has drawbacks, but expelling the sample forcefully through a syringe against the inside of the flask is most satisfactory. Other methods of biomass measurement, such as dry weight, absorbance, or chlorophyll fluorescence, are more precise than cell counts for assessing growth of filamentous algae.

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8111 H. Effect of Additions

1. Procedures

The quantity of cells produced in a given medium is limited by the concentration of nutrient present in the lowest relative quantity with respect to that required by the organism. If a quantity of the limiting nutrient is added to the medium, cell production increases until this additional supply is depleted or until some other nutrient becomes limiting. Additions of substances other than that which is limiting would yield no increase in cell production. Nutrient and chelator additions may be made singly or in combination and the growth response compared to that of untreated controls to identify those substances that limit growth. The selection of additives (e.g., nitrogen, phosphorus, iron, EDTA, wastewater effluents) will depend on the requirements of the test.

In all cases, keep volume of added nutrient or chelator solution as small as possible, but make it large enough to yield a potentially measurable response. Relate the concentrations of additions to nutrient levels in the sample. To assess the effect of nutrient and chelator additions, compare treated sample to an untreated control. For highly productive controls, flask-to-flask

variations may be high and might mask the effect of small additions of the limiting nutrient.

It is sometimes necessary to check the test water for the presence of toxic constituents. To do this, treat the sample with an appropriate dilution of the complete synthetic medium. If no growth, or less than expected growth, occurs, toxic materials are suspected. In some situations, sample dilution or addition of a chelating agent will eliminate toxic effects.

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8111 I. Data Analysis and Interpretation

1. Reporting Requirements

The fundamental measure of biostimulation in the algal assay is the amount of suspended solids (dry weight) produced and determined gravimetrically. Other biomass indicators may be used, but all results must include experimentally determined conversion factors and the dry weight of suspended solids. Use several biomass indicators whenever possible, because biomass indicators respond differently to any given nutrient-limiting condition.

Report results of addition assays with results from two types of reference samples: the assay reference medium and untreated water samples. Report results of assays as maximum standing crop (with time at which it was reached).

Determine the available concentration of growth-limiting nutrient by comparing maximum standing crop in an untreated sample with a maximum standing crop in reference medium.

To determine the nutrients that limit standing crop by single-nutrient additions, treat a number of replicate flasks with single nutrients, determine the maximum standing crop for each flask, and compare the averages by Student's *t* test or other appropriate statistical tests.

To identify standing-crop-limiting nutrients by multiple-nutrient tests, make analysis-of-variance calculations. Account for possible interaction between difference nutrients by using factorial analysis.[1]

Report maximum standing crop with confidence interval. Base the calculation of confidence interval for the average values on at least five samples. Consequently, make a minimum of five replications when an unfamiliar source water is first analyzed. Use these results to calculate the standard deviation. For subsequent samples from the same source, use only three replicates and report with the confidence interval established for that source water.

The overall evaluation of assay results consists of first determining whether a result is significant when considered as a laboratory measurement. Several methods are available, such as the Student's *t* test and analysis-of-variance techniques.

The second part of the evaluation is the correlation of laboratory assay results to effects observed or predicted in the field. Specific guidelines are not yet available, but note the general considerations in 8111B.

2. Reference

1. EUTROPHICATION AND LAKE RESTORATION BRANCH. 1974. Marine Algal Assay Procedure: Bottle Test. U.S. Environmental Protection Agency, Pacific Northwest Environmental Research Lab., Corvallis, Ore.

8112 PHYTOPLANKTON*

8112 A. Introduction

Phytoplankton are primary producers in the aquatic community and, as such, are at the base of aquatic food chains. Because of this, they must be tested in bioassays that predict and determine the potential effects of a substance on the aquatic environment. The same general principles and techniques used in determining biostimulation (Section 8111) are used to determine toxicity to phytoplankton. The procedure applies to fresh water, estuarine, and marine phytoplankton. See Sections 10200D–H and 10300D for more information on phytoplankton.

* Approved by Standard Methods Committee, 2000.

8112 B. Inoculum

In addition to the marine or estuarine algae listed in Section 8111F.1*b*, *Monochrysis lutheri* Droop may be used. Maintain the test species in full-strength media [Section 8010E.4*c*1) and Tables 8010:III and 8010:IV.A and B]. Test species must be in the logarithmic growth phase; therefore, transfer them to fresh culture medium every 4 to 5 d.

8112 C. Test Conditions and Procedures

1. Maximum Specific Growth Rate

The *maximum specific growth rate* (μ_{max}) is the greatest specific growth rate (μ) occurring at any time during an incubation. The μ_{max} can be estimated from growth measurements. It is related to the concentration of rate-limiting nutrient available. The *specific growth rate* is defined by:

$$\mu = \frac{\ln (X_2/X_1)}{t_2 - t_1}$$

where:

X_2 = biomass concentration at end of selected time interval,
X_1 = biomass concentration at beginning of selected time interval, and
$t_2 - t_1$ = time interval of selected time period, d.

Add test material to test vessels to give desired concentrations. Prepare triplicate vessels for each concentration. Use dilutions of culture medium to simulate chemical conditions of specific receiving waters. For optimum surface-to-volume ratios, see Section 8111C.2*a*.

The μ_{max} occurs during the logarithmic phase of growth, usually between Day 0 and Day 5. Therefore, measure biomass at least daily during the first 5 d of incubation. Indirect measurements of biomass, such as chlorophyll *a* or cell numbers, usually will be required because accurate gravimetric measurements at low cell densities are difficult. See Sections 8111G.3*c* and 8111G.4 for methods.

Test a geometric series of concentrations initially (see Section 8010F.3*b*). After this preliminary test, progressively bisect intervals on a logarithmic scale. Narrow the range of test concentrations to determine the concentration that reduces μ_{max} to 50% of the control. This requires that two of the concentrations tested fall on each side of the concentration that inhibited μ_{max} to 50% (see Section 8010G).

Compare μ_{max} to that obtained in the synthetic freshwater or artificial seawater culture medium. Regional and seasonal variations in quality make natural waters unsuitable as standard test media for comparative toxicity tests. Therefore, use a synthetic freshwater medium and/or artificial seawater. Add various concentrations of toxicants to the culture medium in triplicate and inoculate with test species.

2. Other Tests

For other types of tests, such as those to determine effluent requirements or compliance with water quality standards, take dilution water from the receiving body near the outfall but outside its influence. Remove undesirable organisms before making growth rate tests with selected sensitive species (Section 8111D). Determine μ_{max} in test vessels and compare with controls and EC_{50}s based on percent of growth reduction. An alternative approach that provides a number that may relate to natural conditions should be reviewed.[1]

3. Reference

1. Miller, W.E., J.C. Greene & T. Shiroyama. 1978. The *Selenastrum capricornutum* Printz Algal Assay Bottle Test; U.S. Environmental Protection Agency Rep. EPA-600/9-78-018. National Technical Information Serv., U.S. Dept. Commerce, Springfield, Va.

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8113 MARINE MACROALGAE*

8113 A. Introduction

1. Occurrence and Importance

Marine macroalgae are important biological components of Earth's oceans and are found in coastal and intertidal zones as deep as light penetration is sufficient for photosynthesis. They are classified in one of three phyla, which are named according to the color derived from major photosynthetic pigments present in their members. Members of Chlorophyta (green algae) contain chlorophyll *a* and *b* and β-carotene. The red algae (Rhodophyta) contain phycoerythrin, an accessory pigment that provides the reddish color observed in most species of this phylum. The brown algae (Phaeophyta) contain the brown accessory pigment fucoxanthin. Within this phylum are the most structurally complex of all algae, the laminarians (Order Laminariales). Laminarians include the large, canopy-forming kelps of the world, such as the giant kelp *Macrocystis pyrifera*.

Macrocystis pyrifera (see Section 10900, Plate 2A:E) forms extensive coastal submarine forests that provide food, habitat, and shelter for numerous species.[1] The giant kelp also is an important economic resource that is harvested commercially to obtain alginic acid, a thickening agent used in foods, cosmetics, culture media, and other products.

Therefore, it is an attractive test species for monitoring coastal water quality. Indeed, test methods using the early life stages of giant kelp have been developed and found to be sensitive to many toxicants found in the coastal environment.[2–4] The *Macrocystis pyrifera* method has been used successfully in National Pollution Discharge Elimination System (NPDES) monitoring and compliance programs in California and other regions along the Pacific Coast of North America since 1990.[5]

* Approved by Standard Methods Committee, 2015.
Joint Task Group: Lan C. Wiborg.

2. References

1. North, W.J., ed. 1971. The biology of giant kelp beds *(Macrocystis)* in California. *Nova Hedwigia* 32:1.
2. James, D.E., S.L. Manley, M.C. Carter & W.J. North. 1987. Effects of PCBs and hydrazine in life processes in microscopic stages of selected brown seaweeds. *Hydrobiologia* 151/152:411.
3. Anderson, B.S. & J.W. Hunt. 1988. Bioassay methods for evaluating the toxicity of heavy metals, biocides, and sewage effluent using microscopic stages of giant kelp *Macrocystis pyrifera* (Agardh): A preliminary report. *Mar. Environ. Res.* 26:113.
4. Singer, M.M., D.L. Smalheer, R.S. Tjeerdema & M. Martin. 1990. Toxicity of an oil dispersant to the early life stages of four marine species. *Environ. Toxicol. Chem.* 9:1389.
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8113 B. Selecting and Preparing *Macrocystis pyrifera* Sporophylls

1. Life Cycle

The life cycle of *Macrocystis pyrifera*, like that of all laminarians, alternates between a diploid sporophyte and a haploid gametophyte generation (Figure 8113:1). The conspicuous adult sporophyte produces haploid, dioecious, biflagellate zoospores in dark patches (sori) found on reproductive blades (sporophylls) near the base of the plant throughout the year. The zoospores are released and settle on an appropriate substrate, where they germinate and develop into microscopic gametophytes. Flagellated male gametes are produced within the antheridia of the male gametophytes and released to fertilize eggs produced within oogonia on the female gametophyte. The fertilized eggs develop into young sporophytes in 12 to 15 d.

2. Occurrence

Macrocystis pyrifera forests are found subtidally along the Pacific Coast of North America from Central California south to Baja California. Substantial populations also are found along the west coast of South America from Peru south and off the coasts of New Zealand, Tasmania, and the islands of the Subantarctic.[1]

3. Collection and Maintenance of Test Organisms

Zoospores of *Macrocystis pyrifera* are obtained via subtidal collection (usually by SCUBA divers) of reproductive blades called *sporophylls*. Sporophylls are found near the base of the plant, usually lack air bladders, are thinner than vegetative blades, and have sori that extend to near the sporophyll margin. Sori contain both mature and developing zoospores. Unless contrary to the purpose of the study, collect sporophylls from several plants in a region relatively free from point and nonpoint sources of contaminants and below the thermocline, if present. Sporophylls should not be collected immediately following rain events, when storm-water runoff can significantly increase the pollutant concentrations in coastal waters.[2] Check local regulations and collection permit requirements before obtaining sporophylls.

Once sporophylls have been collected, keep them damp but avoid immersion in seawater because this will cause the zoo-

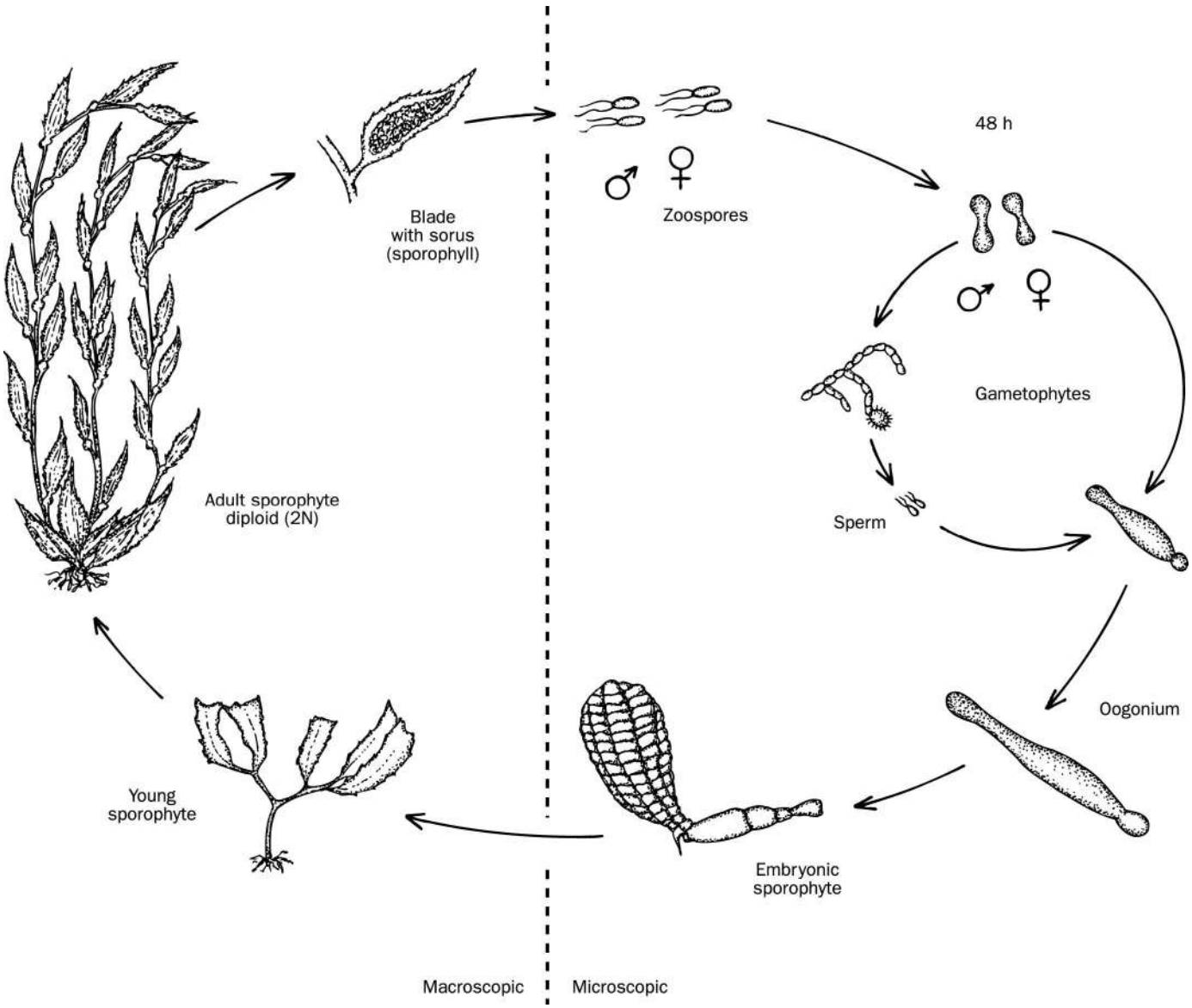


Figure 8113:1. The life cycle of the giant kelp, *Macrocystis pyrifera.*

spores to be released. Keep blades out of direct sunlight to avoid damage to the zoospores from ultraviolet radiation. Clean the sporophylls by gently rubbing them with fingers or a soft-bristle brush and rinse with seawater filtered through a 0.22-μm filter. Layer sporophylls between damp paper towels to prevent any blades from overlapping. Keep sporophyll temperature between 9 and 12°C during transport and/or shipment to test facility using newspaper-insulated chemical ice packs and a cooler. Stored in this manner, the sporophylls can be used up to 24 h after collection. However, sporophylls collected at one southern California kelp bed were more sensitive to copper after storage.[3]

4. Predators, Parasites, and Diseases

Giant kelp is a valuable food source for many herbivores in the coastal environment, including the opaleye *(Girella nigicans),* the half-moon *(Medialuna californiensis),* and other fishes. Many gastropods also feed on *Macrocystis* sp., including abalone *(Haliotis* sp.*),* the black turban *(Tegula funebralis)*, and the sea hare *(Aplysia californica*). However, the most notorious grazers are sea urchins *(Strongylocentrotus purpuratus, S. franciscanus)*, which can cause extensive damage to giant kelp forests when the population explodes due to a lack of predation.[4]

Certain bryozoans, particularly *Membranipora membranacea*, attach themselves to blades of giant kelp; do not use encrusted sporophylls for toxicity tests. Morphological deformities associated with endophytic algae[5] and epidemic diebacks from infection by virus-like particles[6] have been documented in other species of kelp. When collecting samples for use in toxicity tests, take them only from plants that appear healthy and free from deformities.

5. References

1. Dring, M.J. 1982. The Biology of Marine Plants. Edward Arnold Limited, London, U.K.
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8113 C. Toxicity Test Procedures

1. General Procedures

Expose zoospores from mature sporophylls of the giant kelp, *Macrocystis pyrifera*, to several concentrations of a standard toxicant ($CuCl_2$) and the test sample for 48 h at 15°C under a specified light intensity and cycle without solution renewal (static). At the conclusion of the test, determine the proportion of germinated zoospores and the length of the resulting germ tube microscopically.

2. Water Supply

a. Artificial seawater: The use of artificial seawater in this test has not been evaluated and so cannot be recommended at this time.

b. Natural seawater: Collect natural seawater with a salinity of 33 ± 3 g/kg from a location relatively free from point and nonpoint sources of waste discharge (unless the study warrants the use of a seawater from a specific location). After collection, filter using a 0.45-μm membrane filter and store in the dark at 4 ± 1°C until used. Seawater held in this manner may be used up to 14 d after collection. Filtered seawater may be held at 15 ± 1°C for up to 24 h before the test begins. During a test, the salinity of all test solutions should be 33 ± 3 g/kg and not vary by more than ± 2 g/kg among test concentrations. Hypersaline brine produced from natural seawater may be used to adjust the salinity of test solutions that are below the recommended range, but extra controls and statistical procedures must be employed in the test design and analysis. Guidance on using brine to adjust salinity can be found elsewhere.[1]

3. Exposure Chambers

Use Petri dishes (100 × 20 mm) filled with 50 mL test solution as test chambers. The composition of the exposure chamber depends on sample type and study objectives. Preferably use acid-cleaned glass Petri dishes; dilution-seawater-leached disposable polystyrene Petri dishes, plastic chambers, plastic food containers, and glass beakers also have been used successfully. Place glass microscope slides (approximately 7.6 × 2.5 cm) in test chamber before adding test solution and spores. The slides serve as a substrate for spore settlement and gametophyte development that can be removed and analyzed microscopically at the end of the test. This testing system is not suitable for volatile compounds.

4. Conducting the Toxicity Test

a. Preparation of test chambers for inoculation: Label test chambers in a way that will facilitate blind analysis of biological endpoints at the conclusion of exposure. Place test chambers randomly in testing area to account for subtle differences in light intensity and temperature. Measure light intensity at each test chamber using a planar light meter and adjust light fluence rate and/or position of chambers as required to obtain a fluence rate of 50 ± 10 μEinsteins/m^2/s (2000 to 3010 lux; 186 to 280 ft-c). Set the light cycle in testing area for 16 h light:8 h dark for test duration.

Prepare volumes of test solution needed to provide solution for test chambers and water quality/chemistry measurements. Use minimum of five replicates at each test concentration. Selection of sample test concentrations depends on the nature and intent of the study; for guidance, see Sections 8010D and F. Seek a concentration series that causes a partial response at several concentrations because such a series provides valuable information about the nature and precision of the concentration–response curve. However, in some studies it may be more important to select concentrations around specific exposure conditions of interest. The concentration series used in the copper (II) chloride ($CuCl_2$) standard toxicity test is fixed at 0 (control), 5.6, 10, 18, 32, 100, and 180 μg/L of copper ion (nominal).[1]

Measure pH, dissolved oxygen (DO), salinity, and temperature of each test sample and copper concentration. Acceptable salinity values range from 30 to 36 g/kg; higher salinity values have been associated with reduced germination and growth, so they are not recommended. Ensure that solution temperature is 15 ± 1°C before inoculation with spores. The solutions may be aerated according to established procedures if DO is <4 mg/L.

When target water-quality measurements have been obtained, fill each test chamber with the appropriate test solution and volume (100 × 20 mm Petri dishes = 50 mL).

b. Spore release and inoculation of test chambers: Rinse approximately 100 g wet weight of sporophylls (approximately 30 blades) with filtered seawater. Rub blades with fingers or gently scrub them with a soft-bristle brush to remove debris and epiphytic organisms. Rinse with filtered seawater and lay on paper towels so no blades overlap. Blot dry using paper towels and place in a room at 15 ± 1°C for 1 to 24 h. Maintain 15 ± 1°C temperature for spore release and inoculation procedure. Desiccation of sporophylls in this manner will cause spores to be released upon exposure to seawater.

Fill a clean glass 2-L beaker with 1 L filtered seawater. After desiccation, place sporophylls into seawater for 1 h. The water should become discolored as the sori release the spores. After 1 h, remove sporophylls and allow spore suspension to settle for 30 min so inactive spores and debris will collect on the bottom of the beaker. Midway through settling procedure, microscopically examine a small sample of the spore suspension (collected from the upper 2 cm) to ensure that the spores are motile. After settling, decant 250 mL of spore solution into a clean 250-mL graduated cylinder or beaker.

Collect a sample of the inoculation suspension and determine spore density using a hemocytometer. The target density of spores in test chambers is 7500 spores/mL. Calculate volume of inoculation suspension required to obtain target spore density using the equation:

$$V_i = (7500 \text{ spores/mL} \times V_1)/D_i$$

where:

V_i = volume of inoculation added to test chamber,
V_1 = volume of test suspension in test chamber, and
D_i = spore density of inoculation suspension.

Typically, the volume of inoculant required to obtain 7500 spores/mL is <1% of test volume. If greater volumes are required, the spore quality and consequent test results should be suspect. Inoculate each test chamber with spore suspension using a cut or wide-mouth pipet. Note the time; this denotes the start of the test.

c. Duration and type of test: The exposure of spores to test solution is static, and the duration is 48 ± 3 h. Continuous temperature monitoring of a surrogate test chamber in the testing area is recommended to ensure that proper temperature was maintained during the test.

d. Test termination and endpoint examination procedures:

1) Slide preparation—Prepare slides for microscopic analysis by carefully removing slide from Petri dish with forceps. Save remaining test solution and store it in testing area until final water-quality measurements (pH, DO, temperature, and salinity) of each test-solution concentration are made. Wipe excess water from bottom and drain top by placing edge of slide on a paper towel. Carefully place a cover slip (24 × 50 mm) on top of slide, wipe excess water from around cover slip, and label each slide with test-chamber identification. Do not prepare more slides than can be read in 30 min to avoid potential inaccuracies in length determination due to drying gametophytes.

2) Percent-germination endpoint determination—Place prepared slides on a microscope set at a magnification of approximately 400×. Focus microscope on the plane where the majority of gametophytes and spores are clearly visible. Using a laboratory counter with at least two counting levers, move across the slide counting both germinated and nongerminated spores until at least 100 spores have been scored. Record number of germinated and nongerminated spores and calculate proportion of germinated spores for the replicate.

A spore is considered germinated if the germ tube is equal to or longer than the width of the spore case (about 3 μm). Typically, the germ tube has an irregular shape and cellular contents, while the spore case usually is circular and devoid of cellular material (Figure 8113:2). If an object cannot be easily identified as a spore or gametophyte, do not count it.

3) Germ-tube-length endpoint determination—Place prepared slide on a microscope at approximately 400× magnification; the microscope must be equipped with a calibrated micrometer that can measure ≤2.0 μm/micrometer unit. Micrometers with conversion factors greater than 2.0 are not recommended due to their negative effect on test sensitivity. See Section 10200E for micrometer-calibration procedures. Blindly move to a random location on the slide and locate the spore case of the germinated spore that is closest to the micrometer. The selected germ tube should be straight and in focus for its entire length. Move slide and micrometer as necessary to overlay micrometer on germ tube. The *germ tube length* is the distance from the tip of the germ tube to the edge of spore case at the tube's base (Figure 8113:2). Measure and record germ-tube length to nearest whole micrometer unit. Once germ-tube length has been recorded, blindly move to another field of view and repeat selection and measuring procedures until 10 germ tubes have been measured. If germination percentage is low (<10%), the slide may be scanned for germinated spores, measuring the first 10 germ tubes identified. Calculate mean germ-tube length in micrometer units and convert mean value into microns using the conversion factor derived from the calibration procedure. When calculating the mean germ-tube length, follow instructions in Section 1050B and carry out all calculations; only round the final result. Record this value as the mean germ-tube length for the replicate.

5. Reference

1. Chapman, G.A., D.L. Denton & J.M. Lazorchak, eds. 1995. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms; EPA/600/R-95/136. Off. Research & Development, U.S. Environmental Protection Agency, Washington, D.C.

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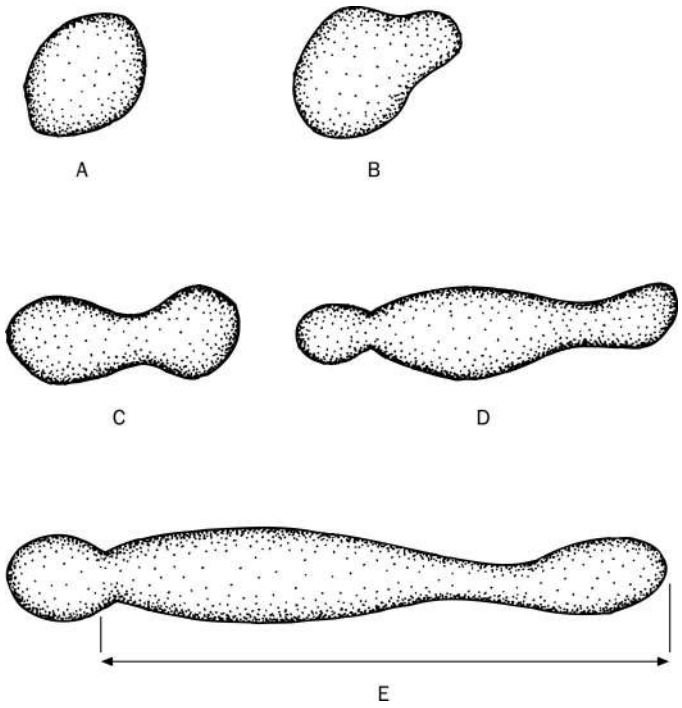


Figure 8113:2. Examples of nongerminated (A and B) and germinated (C and D) giant kelp zoospores and germ-tube-length measurement of germinated zoospores (E). Zoospores are considered germinated when the germ tube length is equal to or greater than the width of the spore case. The length of the germ tube is measured from the edge of the spore case to the tip of the germ tube.

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8113 D. Data Evaluation

1. Statistical Analysis

The statistical analysis selected to evaluate sample toxicity or the reference toxicant depends on the study's objectives (see Section 8010G).

To evaluate the quality of a reference toxicant test, calculate the mean germination percentage and germ-tube length in seawater controls for each test and the EC_{25}/IC_{25} and minimum significant difference (MSD) for that test. If brine was added to any test concentrations, recalculate toxicant concentrations and compare response of brine controls with natural seawater controls using a *t*-test. If control responses were statistically different (two-tailed; $\propto = 0.05$), the data may become difficult to interpret and the test may need to be repeated. If the data are used, perform separate statistical analyses for unaltered and brine-enriched treatments using the corresponding control data.

2. Toxicity Test Evaluation

a. General considerations: The following test-acceptability criteria are intended to help ensure that healthy kelp sporophylls and spores and adequate dilution water were used and that the test was performed in a manner consistent with the method described above. Data derived from tests that do not meet these criteria should be considered suspect and may warrant repeating the test.

b. Negative control minimums: The purpose of negative control minimum criteria is to ensure that the spores germinated and grew to a minimal level consistent with those seen in tests using healthy sporophylls, adequate dilution seawater, prescribed procedures, and qualified staff. The minimum mean germination percentage and germ-tube length of the control replicates in the reference toxicant and sample tests should be 70% and 10 μm, respectively.

c. Positive control response ranges: The purpose of positive control response range criteria is to ensure that the spores' response to a standard toxicant (copper) is consistent with that observed in tests using healthy sporophylls, adequate dilution seawater, prescribed procedures, and qualified staff. The EC_{25} for the germination endpoint of the reference toxicant test should be between 18 and 158 μg Cu/L. The IC_{25} in the germ-tube-length endpoint of the reference toxicant test should be between 13 and 106 μg Cu/L.

d. Within-test variability maximums: The purpose of within-test variability maximum criteria is to ensure that the variability of the spores' response, particularly among treatment replicates, does not exceed the variability observed in tests using healthy sporophylls, adequate dilution seawater, prescribed procedures, and qualified staff. The minimum significant difference (%MSD) relative to the control must be less than 20 for all parameters in the reference toxicant test.[1]

3. Reference

1. Chapman, G.A., D.L. Denton & J.M. Lazorchak, eds. 1995. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms; EPA/600/R-95/136. Off. Research & Development, U.S. Environmental Protection Agency, Washington, D.C.

8200 AQUATIC FLOWERING PLANTS*

Aquatic flowering plants belong to the phylum Spermatophyta, characterized by possession of true roots, stems, and leaves, and production of seeds from flowers. The phylum contains most of the conspicuous land plants of the world. Aquatic flowering plants are almost exclusively fresh-water inhabitants. Test procedures are described for duckweed (Section 8211), which floats on the surface of the water, and aquatic emergent plants (Section 8220), which have roots extending into the sediment.

* Joint Task Group: 20th Edition—Donald J. Reish.

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8211 DUCKWEED*

8211 A. Introduction

1. Organism Characteristics

F1 *Lemna minor* L. (Figure 8211:1) (also known as common duckweed) is a small flowering aquatic macrophyte (a monocot) widely distributed in quiescent fresh water and estuaries ranging from tropical to temperate zones.[1] It is the most common species of the family Lemnaceae in the United States and many other parts of the world. It is morphologically simple, consisting only of frond and root. The frond size is approximately 2 to 4 mm and root length is up to 50 mm. The plant is colonial (up to 8 fronds), multiplies sexually and asexually, and has a maximum growth rate far exceeding those of other flowering plants. Duckweed is a food for waterfowl and small animals, and provides food, shelter, and shade for fish and other aquatic organisms. It also serves as a habitat for various invertebrates.

2. Test Applications

Duckweed is an ideal organism for testing the aquatic phytotoxicity of herbicides, industrial and municipal wastewaters, and leachates or slurries of waste and soil and other contaminants.[2] Because many ambient waters and effluents are colored and/or turbid, they are difficult to test directly for toxicity via most algal tests without filtering, which can alter sample integrity. However, duckweed is a floating plant that can be used to test unfiltered colored or turbid samples. In addition, some samples may contain labile, volatile, or sorptive materials and require either renewal or flow-through methods. Algal testing may be inappropriate for these types of tests, but the duckweed toxicity test described herein can be easily modified to apply in either method.

The duckweed toxicity test is useful, especially for determining phytotoxicity at the air–water interface, where surface-active substances, oil and grease, and toxic organic compounds may be concentrated. The test also is useful for determining the toxicity of metals,[3] organic compounds,[4,5] industrial and municipal effluents,[6–9] and herbicides.[10] As a higher plant, duckweed has a sensitivity pattern nearer to most vascular plants than to algae. However, as monocots, duckweeds are insensitive to auxin-stimulating herbicides (e.g., 2,4-D). The test is generally described as fast, simple, sensitive, and cost-effective.[2] This is essentially true when compared to growth tests with higher plants in soil.

However, because common duckweed is a floating plant, the test may underestimate the toxicity of a substance that adsorbs on particulates and precipitates during a static test. Also, substances that concentrate at the air–water interface tend to affect duckweed more than other aquatic plants (e.g., algae or submerged plants). Gently shaking or stirring test vessels to increase mixing may overcome or lessen these problems. Alternatively, renewal or flow-through methods can be used.

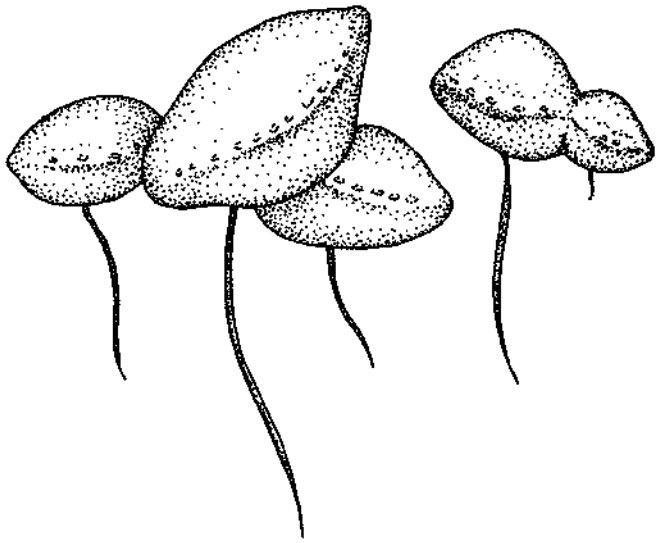


Figure 8211:1. Common duckweed: *Lemna minor.* Each plant consists of frond and root; together, plants form a colony.

Several standard tests using duckweed are available in the literature.[11–13]

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8211 B. Selecting and Preparing Test Organisms

1. Test Species

The procedure is designed for use with *Lemna minor*. The organism can be obtained from commercial sources, testing laboratories, or the field. It must be identified and confirmed taxonomically before use.[1] If available, use *L. minor* ST from culture collections especially for substance testing or for legal purposes, because this clone is well characterized and widely used, and behaves very well in permanent lab cultures. Other duckweed species (e.g., *L. gibba, L. perpusilla, L. paucicostata,* and *L. polyrrhiza*) have been used successfully with modified procedures.[2,3]

2. Culturing Test Organisms

Acclimate a new duckweed culture to the test environment (e.g., lighting, temperature, and nutrient conditions) for at least 2 weeks before a test—at least up to the stage of demonstrating growth validation criterion. This culture grows vigorously and provides a nearly inexhaustible supply for testing under proper conditions. Grow duckweed in a culture vessel (e.g., an aquarium or stainless steel basin). To prepare a 10-L culture solution, add 100 mL of each stock nutrient solution A, B, and C (Table 8211:I) to deionized or other suitable water. Do not use tap water. Use a water depth of at least 40 mm and provide continuous (24 h/24 h) cool-white fluorescent light at 2150 to 4300 lux at the water surface. Maintain a temperature of 24 ± 2°C. Change nutrient solution weekly and avoid overcrowding (more than one layer of duckweed). Axenic culturing is unnecessary but covering the vessel loosely helps avoid algal and fungal contamination.

3. Diseases and Predators

Diseases, phytophagous insects, and other pests normally are not problems for a duckweed culture. If the culture appears unhealthy (e.g., chlorotic or necrotic) or contaminated by fungi or algae, destroy it and start a new one. It is good practice to maintain several cultures isolated from each other. For long-term storage or as a backup, use axenic stock cultures on test medium solidified by 1% agar with 1% glucose added to erlenmeyer flasks. Autoclave the solidified medium with cotton or foam plugs. Add 2 to 3 plants axenically after agar has cooled. Cover the cotton plug with aluminum foil to minimize evaporation, and store in a cool place with low light conditions for some months.

Table 8211:I. Duckweed Nutrient Solution

Solution	Stock Solution Concentration	Element	Final Concentration
A:			
$NaNO_3$	25.5 g/L	N	42.0 mg/L
		Na	110.0 mg/L
$NaHCO_3$	15.0 g/L	C	21.4 mg/L
K_2HPO_4	1.04 g/L	K	4.69 mg/L
		P	1.86 mg/L
B:			
$CaCl_2 \cdot 2H_2O$	4.41 g/L	Ca	12.0 mg/L
$MgCl_2$	5.7 g/L	Mg	29.0 mg/L
$FeCl_3$	0.096 g/L	Fe	0.33 mg/L
$Na_2EDTA \cdot 2H_2O$	0.3 g/L		
$MnCl_2$	0.264 g/L	Mn	1.15 mg/L
C:			
$MgSO_4 \cdot 7H_2O$	14.7 g/L	S	19.1 mg/L
H_3BO_3	0.186 g/L	B	325 μg/L
$Na_2MoO_4 \cdot 2H_2O$	7.26 mg/L	Mo	28.8 μg/L
$ZnCl_2$	3.27 mg/L	Zn	15.7 μg/L
$CoCl_2$	0.78 mg/L	Co	3.54 μg/L
$CuCl_2$	0.009 mg/L	Cu	0.04 μg/L

Notes:
1. Omit $Na_2EDTA \cdot 2H_2O$ in Solution B if test samples contain toxic metals. In that case, acidify Solution B to pH 2 to prevent precipitation.
2. To prepare the duckweed nutrient solution, add 1 mL of each stock solution to 100 mL deionized water. Adjust to pH 7.5–8.0.

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8211 C. Toxicity Test Procedure

1. General Considerations

Use static, or where necessary, renewal methods.[1,2] As a general rule, if a test solution is stable (e.g., a solution with low microbial population, high toxicity, or low volatility), use a static test. If samples are unstable, use repeated renewal methods (see Section 8010D).

2. Preparing Test Materials

As used herein, dilution water and control water are identical to duckweed nutrient solution. To prepare this solution, see Table 8211:I.

To prepare toxicant solutions, see Section 8010F.2*b*.

3. Test Procedures and Conditions

Screening tests use a predetermined concentration (e.g., 100% effluent) to determine if a sample is toxic in comparison with the control water. If the sample is toxic, test it further using range-finding and definitive tests. In the range-finding test, examine a series of concentrations, usually at ratios of 10 (10%, 1%, 0.1%, etc.).

Range-finding-test results are used as a basis for designing definitive tests. For definitive tests, use at least five concentrations of sample in a constant ratio (geometric series). For example, for environmental samples using a recommended ratio of two, concentrations should be 90% environmental sample (adding tenfold concentrated medium to sample, thus not reaching 100%), 50%, 25%,12.5%, 6.25%, 3.12%, 1.56%, 0.78%, 0.39%, etc. Ideally, choose a ratio to prepare a series of solutions in which all effect concentrations (EC values) to be assessed are bracketed by at least one higher and one lower concentration. Alternatively, select the highest and lowest concentrations to produce approximately 80% or higher and 10% or lower inhibitory effects.

Use three replicates for each treatment. Include a negative control: six replicates containing only duckweed nutrient solution. If any solvents were used for pure chemicals, add an additional solvent control with six replicates. Prepare a positive dose–response test with 3,5-dichlorophenol [EC_{50} around 2 mg/L test range (0.54 mg/L)]. Preferably use cylindrical vessels (beakers or petri dishes that are at least 60 mm diam and hold at least 150 mL). (Although the dish may be shallower than root length, duckweed growth is not adversely affected.[3]) Vessels should be inert to expected chemicals and not adsorb them significantly. This generally leads to a preference for glass vessels. However, cationic substances (e.g., metals) may significantly adsorb to borosilicate glass, so specific plastics may be preferred for such samples. Discard vessels if contamination is suspected.

Add the same amount of nutrients to all control and test samples (e.g., 1 to 10 mL of concentrated nutrient solution to make 100 mL sample). Prepare at least 100 mL portions of test solution (or control sample). Select duckweed specimens from stock cultures that have been grown under the same conditions. Use only unblemished colonies (green and healthy looking; not chlorotic, necrotic, or irregularly shaped) containing two or three fronds of approximately equal size per colony. Place 12 duckweed fronds (identical numbers of colonies per vessel) in uniform glass plates or hourglasses.

Illuminate from above with continuous (24-h) cool-white fluorescent light (2150 to 4300 lux at water surface). If no appropriate light meter is available, check light field via a set of control samples. If all control samples surpass a growth rate of 0.275/d, then there is enough light (this is generally a more reliable indication of sufficient light than any physical measurement). To avoid dose-dependent shadowing and reflection effects of colored or turbid solutions, place a black bottom under all test vessels (very important) and incubate at 24 ± 2°C.

Test duration is 168 h. Sometimes shorter test durations may be possible, but this can significantly reduce test sensitivity. If a shorter test is necessary, do not reduce the number of observations; instead, eliminate the first 1 or 2 days of growth if this seems to be an adaptation phase (delayed reaction) in which even highly contaminated samples will still grow near to control values. Because results are calculated based on growth rate, such a reduction is scientifically permitted but should always be noted in the results [e.g., EC_{50} (2–5 d)].

When testing effluent toxicity in a receiving water, renew with fresh effluent if sample seems to be unstable, and use receiving water as diluent if necessary for the test goal. Otherwise, use a standard water as diluent (see Table 8010:III). Complete frond counts at least every 72 h (at the beginning and end of the test, and at least two intermediate points) to determine any intermediate toxic effects.

4. Test Results

For visual observations, place test vessels on a white background or apply direct light from the side or bottom into the vessel. Observe duckweed plants for symptoms, including chlorosis (loss of pigment/yellowing), necrosis (localized dead tissue), colony breakup, root destruction, loss of buoyancy, and gibbosity (humpback or swelling). Compare affected fronds with duckweed specimens in the control. Count frond increase daily to provide a quantal value directly reflecting duckweed growth. To measure frond increase, count every visible, protruding bud. This nondestructive method allows repeated observations of the same test vessel.

Using projected lead area measurement based on image processing (combining automatic leaf count and area measurement*) provides another nondestructive observational parameter to help compensate for variability caused by colonies with different start sizes. It also serves as a good estimate for biomass production. Leaf area measurement is generally at least as sensitive as frond measurement because many toxicants not only reduce frond number but also average frond size. Use this additional parameter at least at the beginning and end of the test (where measurement is destructive); take six additional randomly selected replicates for initial measurement to calculate mean start value.

* LemnaTec Scanalyzer, or equivalent.

Although other scoring methods have been used, including ^{14}C uptake, chlorophyll content, dry weight, frond area, plant colony counts, total root number, and root length,[4,5] only dry weight measurement is recommended as an alternative here to produce reliable results. Sometimes duckweed may exhibit delayed toxic effects (2 to 3 d), when newly developed frond is affected but not those already developed in the pouches. Lag phases (delayed start of growth) found in controls after a change of medium are a clear sign of change-of-growth conditions at test inception that should be avoided. Stimulatory effects may occur in some instances due to hormesis. Stimulatory effects due to extra nutrients are widely minimized by the optimized nutrient Steinberg medium.

Report this effect if observed, even if you cannot include it in EC-value calculations.

5. Statistical Analysis

Follow general procedures described in Section 8010G. In control samples, duckweed growth conforms more to exponential than linear kinetics, so percent inhibition values must be calculated from growth rates, not measurement values (which imply a linear growth pattern).[6] Using growth rate eliminates the mathematical difference among EC values due to differences in test durations and the controls' absolute growth. It also allows for biologically relevant comparisons of sensitivity between different species (e.g., algae) and the use of segmented growth rates (e.g., day 3 to 5 or 7, as described above). First, calculate average growth rate for each parameter X (e.g., frond number, frond area) separately for each vessel over part or all of the test period using intermediate values to identify time-dependent changes:

$$r = \frac{\ln x_{t2} - \ln x_{t1}}{t_2 - t_1}$$

where:

r = growth rate per day,
x_{t1} = value of observation parameter at t_1 days,
x_{t2} = value of observation parameter at t_2 days, and
$t_2 - t_1$ = time period between t_1 and t_2, d.

Differences are measured in days. A graph that plots the log of the measured parameter versus time (in which slope = growth rate) also is instructive.

Second, calculate mean values of average growth rate for controls and treatments to express percent toxicity (or stimulation) relative to the control:

$$\% \ I = 100 \ (C - T)/C$$

where C and T are mean average growth rates of the measured parameter in control and test samples, respectively.

Inhibition/stimulation values of definitive tests may be graphed using linear, semi-log, or log–log plots. Typically, the concentration–effect relationship is sigmoidal. Determine inhibition values (the concentration causing X% inhibitory effect; X = 5, 10, 20, 25, 50) via graphical or statistical methods. Each effective reproduction concentration (*ErC*) value should be either near or bracketed by inhibition values. Whenever possible, report *ErC* value with confidence intervals, growth rate, and parameter used [e.g., ErC_{10} (frond area)]. Only such comprehensive reporting in databases allows scientifically reliable comparison between actual and literature values.

In older studies, the calculation method was often not reported; it may be scientifically outdated or have biased final biomass or yield values [effective biomass concentration (*EbC*)] due to exponential growth and different test duration. There is no retrospective way to recalculate *ErC* values from such EC values because the conversion factor depends on the controls' absolute growth rate and the slope of the dose–response relationship (which generally was not reported). As a general estimate, an EbC_{50} value corresponding to ErC_{20} values can be used for duckweed tests. Although they seem less sensitive, growth rate values are scientifically correct, while *EbC* values are not. The slope of dose–response relationship is toxicant-specific and thus can be valuable information; it should be reported as different ErC_X values for each parameter.

6. Quality Control

Negative controls are used to ensure that growth effects are reasonable based on the test population's typical growth rates. A test is unacceptable if the duckweed's doubling time is less than 2 d, or if the control sample yields less than a sevenfold increase in fronds in 168 h. If the coefficient of variation for the controls' growth rate is more than 10%, conditions should be controlled and optimized, but the test is still considered valid. In most cases, it is sufficient to avoid placing test vessels at sites with unsuitable growth conditions.

Use a reference chemical at one specified concentration as a positive control. Perform a positive control dose–response test with 3,5-dichlorophenol regularly [ErC_{50} around 2 mg/L test range (0.5–4 mg/L)]. Chromate ion can also be used as a reference toxicant.[6,7]

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8220 AQUATIC EMERGENT PLANTS*

8220 A. Introduction

1. Organism Characteristics

Emergent plants are important components of aquatic and wetland ecosystems. They are among the primary producers, providing oxygen, food, and habitat for periphyton and many other organisms, including invertebrates, fish, amphibians, birds, and mammals. These plants function in nutrient cycling and in stabilizing sediments of near-shore environments.

2. Test Applications

This method is designed to evaluate the phytotoxicity of chemical substances in water on emergent plants. The method uses several measurement endpoints of toxic response, including seed germination, root elongation, root dry weight, and dry weight of the seedling shoot. Seed germination and seedling growth represent the first phase of plant development. Significant inhibition of this developmental phase may diminish the ability of affected plants to compete and survive in their environment.

The seed germination and seedling growth tests are simple, versatile, and useful for screening toxicity in water.[1–3] The tests are useful for evaluating toxicity of metals,[4–6] organic compounds,[5,7] and complex effluents.[2,3] They can be conducted in fresh, sea, or brackish water with the use of appropriate plant species. They are applicable to turbid or discolored aqueous samples.[3] Sediments can be screened for toxicity by testing aqueous extracts, pore water, or whole sediment.[2,8]

One advantage of seed germination and seedling growth tests is that seeds can be obtained in bulk and stored for extended periods with minimal maintenance costs. Stored plant seeds are quiescent and resistant to environmental stress. Under favorable germination conditions, seeds undergo rapid changes and become highly sensitive to the environment.[1] Standard water (Section 8010E.4*b*1) can be used as dilution water and control solution (see 8220C.2). The tests are highly desirable to complement other plant tests (e.g., algal or duckweed tests), where nutrients and adurants could interact.

The tests can be conducted in darkness or light with the species suggested. When both dark- and light-phase experiments are conducted, one can evaluate the effects of photodegradation on the availability and toxicity of toxicants.

The tests can be performed with a relatively small volume of test solution (30 mL/vessel or less) compared to aquatic animal testing (100 to 200 mL/vessel). A small volume may be used to lower the cost of sample collection and storage; however, as the volume of test medium is lowered, the capacity to detect phytotoxic effects may be diminished.

Other toxicity tests for aquatic emergent plants are available.[9,10]

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2010. Joint Task Group: 21st Edition—Wuncheng Wang (chair), James F. Fairchild, L. Gordon Goldsborough, Robert W. Holst, Lawrence A. Kapustka, Jerry C. Smrchek, Jane Staveley, David E. Weber.

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8220 B. Selecting and Preparing Test Organisms

1. Test Species

Criteria for selecting test species include availability, cost, similarity of test species to species of interest, consistent performance, and high germination percentage.[1] There are many emergent species[2] but only a few are readily available.[3,4]

The following freshwater plant species have been used successfully in toxicity testing and are available from commercial vendors:*

Echinochloa crusgalli (Figure 8220:1)	Japanese (duck) millet
Leersia oryzoides	Rice cutgrass
Nelumbo lutea	American lotus
Oryza sativa	Rice
Rorippa nasturtium-aquaticum	Watercress
Zizania aquatica	Wild rice

The germination of American lotus seeds requires scarification that may affect toxicity test results.[5]

Obtain species variety (cultivar) and any certification information, such as germination percentage and date collected. Field-collected seeds can be used. Remove weed seed if present.

* For example, Wildlife Nurseries, P.O. Box 2724, Oshkosh, WI 54903; Environmental Concern, P.O. Box P, St. Michaels, MD 21663; Kester's Wild Game Food Nurseries, Inc., Omro, WI 54963; or Mangelsdorf Seed Co., 1415 13th Street, St. Louis, MO 63106.



Figure 8220:1. *Echinochloa crusgalli* (Japanese millet or duck millet). Left: entire plant; right: spikelet (top), spikelet (center), and floret seed (bottom).

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Generally, avoid plant seeds treated with fungicides, repellents, or micronutrients (e.g., boron, manganese), etc. If it is desired to use such seeds, ensure that bias does not result.

2. Preparing Test Organisms

Obtain sufficient seeds for 1 year of testing. Store fresh seeds at −10°C (or 4°C if seeds cannot tolerate lower temperature). Test seeds regularly during storage for percent germination. Some species can be stored longer than 1 year without a decrease in percent germination.

Use seeds from the same storage lot and year or season of collection. Before beginning toxicity testing, separate seeds into size classes using standard seed dockage sieves. Discard damaged seeds. Use the size class that gives the highest, uniform germination rate exclusively for the test.

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8220 C. Toxicity Test Procedure

1. General Considerations

Seed germination and seedling growth tests may be conducted using either static, renewal, or flow-through methods. As a general rule, if test samples are not highly volatile or degradable, use a static test. Otherwise, use a renewal (daily renewal) or flow-through method with fresh sample (see Section 8010D).

2. Preparing Test Materials

Use reconstituted fresh water (Table 8010:I) as dilution water and control water. For experiments limited to seed germination and initial root growth, use deionized or distilled water with amendments for the control and add the pollutant to test samples. For growth studies, use the culture medium (Table 8010:IV.A and B) and buffer to desired pH levels (hardness also can be adjusted) for the controls and the test media.

For preparation of toxicant solutions, see Section 8010F.2*b*.

3. Test Procedures and Conditions

Use these procedures in screening, range-finding, or definitive tests.

In a screening test, use a predetermined concentration (e.g., 100% effluent) to determine if a sample is inhibitory in comparison with the control. If a sample is inhibitory by more than 10%, test it further by using range-finding and definitive tests. In the range-finding test, examine at least three concentrations, usually at ratios of 0.1 (e.g., 10, 1, and 0.1%) plus the control water.

Devise a definitive test on the basis of range-finding test results. Use at least five concentrations of toxicant solutions in a ratio of 0.5 (e.g., 10, 5, 2.5%, etc). Ideally, prepare a series of solutions in which the midpoint concentration produces an inhibitory effect of approximately 50% and the highest and lowest concentrations produce approximately 90 and 10% inhibition. Stimulatory effects may occur in some instances.

Use four replicates of each test treatment and control. Include control water in each test. Use a 100- × 15-mm or 47-mm culture dish, test tube, or equivalent, as the test vessel. Use only one kind of vessel through each test. Do not use filter paper or seed pack growth pouch because it may cause erratic results.[1–3] Pipet 8 or 10 mL test solution into each test vessel. Seed trays may be used, requiring 30 mL test solution.[3]

Place 10 to 15 seeds in each test vessel. Seeds should not contact each other or the sides of culture dishes. Place test vessels in a seed germinator or other growth facility. Use a randomized complete block design with blocks delineated within the growth facility; if blocking is not feasible, totally randomize vessels in the growth facility.

Test duration, temperature, and light regimes will depend on experimental design and test species. Typically, incubate seeds until the expected percentage of control seed germination has been attained (8220C.5) and control seed root elongation reaches at least 20 mm.[4]

Table 8220:I provides an example of appropriate test conditions.

If fungi or other microorganisms interfere with germination, pretreat test seeds with a reagent-grade sodium hypochlorite solution (3.33 g OCl^-/L) or 10% household bleach for 20 min. Rinse pretreated seeds eight to ten times with deionized or distilled water. Remove excess solution with tissue paper. Omit pretreatment step if this affects germination of the control by more than 10%.

Conduct the renewal test, if necessary, by transferring seeds or seedlings into clean vessels containing fresh toxicant or control water daily. Alternatively, modify a proportional diluter for flow-through testing.

TABLE 8220:I. EXAMPLE OF SEED GERMINATION AND SEEDLING GROWTH TEST CONDITIONS

Test Variable	Condition or Value in Example Test
Test species	*Echinochloa crusgalli* (Japanese millet)
Pretreatment	20 min, hypochlorite solution (3.33 g OCl^-/L)
Test type	Static or renewal
Temperature	25 ± 1°C
Light quality	Dark or light
Test vessel	100- × 15-mm culture dish
Test solution	8 mL/vessel
Specimens	15 seeds/vessel
Replicates	4
Control solution and dilution water	Standard water
Test duration	120 h
Indicators	Seed germination (radicle 5 mm or longer) Root elongation Root dry biomass Shoot dry biomass Abnormal appearance

4. Test Results

A seed is counted as having germinated if the radicle reaches a length of 5 mm or longer. Record all root and shoot elongation (to 1 mm) of each germinated seed in each dish.

Measure length of each primary root from the transition point of the hypocotyl to the tip of the root. Use a digitizer interfaced with a computer, if available.

Additionally, cut primary roots in each dish, combine, dry to constant weights at 70°C, and weigh. Likewise, determine shoot biomass. Report abnormal appearance, such as discoloration, stunted growth, and chlorosis.

At low concentration, some substances stimulate, rather than inhibit, seed germination or root elongation. Report this effect if it is observed.

5. Statistical Analysis

Follow general procedure described in Section 8010G.

Express sample toxicity in percent inhibition relative to the control.[5,6]

$$\% I = 100 (C - T)/C$$

where C and T are mean seed germination percentages in control water and test solutions, respectively, if seed germination is used as the test indicator. If root elongation is used as the test indicator, C and T are root length (in mm) in control and test solutions, respectively.

Determine IC_{50} value (the concentration causing 50% inhibitory effect) by statistical curve-fitting methods. Report concentration–effect relationship and confidence limit of test results.

6. Quality Control

The negative control sample is needed for quality control and effective measurement. Derive empirical performance criteria (e.g., germination percent, mean and standard deviation of root elongation of the negative control sample, and test duration for primary root reaching 20 mm) and statistical confidence limits for each species for 3 months. Anytime the performance of a test in the next 3 months falls below confidence limits, repeat tests; if two consecutive tests fall below the criterion, replace the seed lot and discard the data. Use a positive control [10 mg/L Cr (III)] in every test as an additional, long-term quality control. Develop statistical confidence limits of the positive control.

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8310 CILIATED PROTOZOA*

8310 A. Introduction

1. General Discussion

Ciliated protozoa are ubiquitous unicellular eukaryotes that inhabit freshwater and marine aquatic environments, soils, and sediments. Ciliates are important organisms in the transfer and transformation of nutrients in ecological food chains.[1,2] In the aquatic environment, ciliates are an integral part of the zooplankton and periphyton communities, feed predominantly on bacteria and small algae,[3–5] and mediate the transfer of energy from the microbial food web to the larger metazoan.[1] In soils and sediments, ciliates feed primarily on bacteria and organic detritus.[6] The prevalence of ciliates and their importance in trophic processes make them particularly appropriate as organisms used to assess water quality.[7]

Recent advances in environmental toxicity assessments have focused on microscale testing, more rapid bioassessment techniques, and more sensitive indicators of water quality (i.e., sublethal versus lethal effects).[8,9] The potential for using ciliates to evaluate water quality was recognized some time ago.[10–12] More recently, investigators have focused increasingly on ciliates as test and/or indicator organisms for assessing eutrophic and contaminated media because they represent a neglected trophic level in most toxicity-test batteries and are sensitive to a broad range of toxicants in the natural environment.[11,12] A comprehensive review of this field can be found elsewhere.[13,14]

2. Selection of Method

This section includes three standardized toxicity test methods using ciliated protozoa as test organisms. The first two use ciliates common in fresh water and can be used for whole-water testing with effluents and pure chemicals. The third uses a soil ciliate; this test is most appropriate as an elutriate test, where contaminated soils and mine tailings could be implicated.

* Approved by Standard Methods Committee, 2015.
Joint Task Group: James R. Pratt (chair), Sharon G. Berk, Guy L. Gilron.

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8310 B. Growth Inhibition Test with Freshwater Ciliate *Dexiostoma* (syn. *Colpidium*) *campylum*

1. Background

The short generation time of ciliates and the usefulness of growth as a sensitive biological characteristic have made growth inhibition a widely used endpoint for toxicity tests. Such tests measure the population growth rate of the test ciliate species in response to a series of test concentrations.

This method, a short-term toxicity test,[1–4] is based on a change in the population growth of *Dexiostoma* (syn. *Colpidium*) *campylum* (Stokes) Jankowski (Figure 8310:1) over a 24-h period. The number of cells produced during 24 h in the presence of the toxicant is compared to the growth in a control culture. The toxicity test has a broad range of applications for single toxicants and contaminant mixtures, such as effluents. Inter-

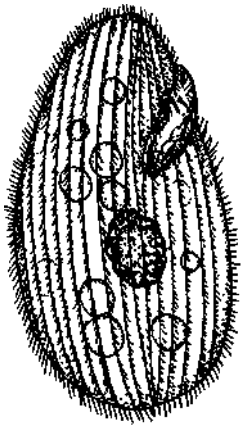


Figure 8310:1. ***Colpidium campylum.***

calibration data and technical review[5] support its usefulness as a standard method.

Other examples of test methods that have used growth inhibition as a test endpoint for aquatic assessments are described elsewhere.[6,7]

2. Source of Test Organisms

Cultures of *Dexiostoma* (syn. *Colpidium*) *campylum* (ATCC 50414) can be obtained from the American Type Culture Collection.*

3. Holding and Culturing Test Organisms

a. Culture maintenance: Culture *Dexiostoma* (syn. *Colpidium*) *campylum* axenically in Proteose Peptone Yeast Extract and Serum (PPYS) medium[8] enriched with bovine serum albumin.[9]† Incubate cultures at 28°C in the dark; subculture each week.

b. Preparing organisms for testing: Acclimate organisms to monoxenic cultivation. Grow them with commercially available lyophilized *Escherichia coli*, strain ATCC 11303,‡ strain ATCC 9637,§ or strain K12.‖

Prepare Minimal Medium (MM) used for the test as follows:

$CaCl_2 \cdot 2H_2O$	107 mg
NaCl	14.5 mg
$NaNO_3$	4.5 mg
$MgSO_4 \cdot 7H_2O$	75.7 mg
Na_2SO_4	39.5 mg
$NaHCO_3$	135 mg
Reagent water	1 L

Mix thoroughly. The medium's pH should be 8.15 ± 0.02. Filter through a 0.45-μm membrane filter and store at 4°C.

* ATCC, Rockville, MD.
† A4503, Sigma Chemical Co., St. Louis, MO, or equivalent.
‡ Sigma EC11303.
§ Sigma EC9637.
‖ Sigma EC1.

TABLE 8310:I. SUMMARY OF ECOLOGICAL AND TESTING CONDITIONS FOR THE FRESHWATER CILIATE *DEXIOSTOMA (SYN. COLPIDIUM) CAMPYLUM*

Condition	Description
Geographical distribution	Cosmopolitan
Habitat	Littoral and benthic zone of ponds and lakes
Length of life cycle	Cell cycle: 2–5 h
Type/duration of test	Growth rate; 24 h EC_{50}
Test temperature	28 ± 1°C
Light cycle	Regular ambient lighting
Validity criteria	None provided
Endpoints	Growth inhibition, EC_{50}
Reference toxicant	Potassium dichromate

Inoculate two 125-mL sterile borosilicate Erlenmeyer or sterile cell culture bottles# containing 10 mL MM medium and 0.4 mL *E. coli* suspension (2.5 mg/mL in MM medium) with 2 drops of axenic *D. campylum* culture.

After 48 h incubation at 28°C, count the cells. Count ciliate cells either electronically or manually. If electronically, dilute sample with a 1% NaCl electrolyte solution, filter it through a 0.45-μm membrane filter, and then use a particle counter fitted with a 200-μm aperture probe. If manually, use a microscope and a counting chamber (e.g., a hemocytometer, Palmer cell, or Sedgwick-Rafter cell). Prepare the definitive inoculum in 500-mL sterile borosilicate Erlenmeyer or cell culture bottles** by inoculating 50 mL MM medium and 2 mL *E. coli* (2.5 mg/mL in MM medium) with 1000 cells/mL. After 48 h growth at 28°C, the inoculum can be used for the toxicity test.

4. Test Conditions and Procedures

A summary of ecological and prescribed test conditions for *Dexiostoma* (syn. *Colpidium*) *campylum* is given in Table 8310:I.

a. Test vessels: Perform test in 30-mL crystal polystyrene screw-capped vials. Alternatively, if using electronic particle counting, use counter cuvettes directly. If products tested can adsorb on plastic or alter it, use borosilicate glass or TFE vials.

b. Test initiation: To each vial, add in the given order:

Toxicant solution in MM (1.25 final concentration in the vial)	4 mL
E. coli, suspension 2.5 mg/mL (in MM)	0.25 mL
D. campylum dilution (3333 cells/mL)	0.75 mL

Start test timing when the ciliates are added. The final volume is 5 mL with an initial cell concentration of 500 cells/mL. For each test on a substance, use one vial per concentration and three control vials (i.e., without toxicant).

To verify true value of the inoculum (500 cells/mL in theory), distribute 0.75-mL portions of the 3333-cells/mL dilution in three separate vials, add 0.225 mL MM, and fix with 1 mL commercially prepared 2.5% glutaraldehyde solution. Count samples and calculate densities. The mean of the three values is

Nunclon, 50 mL, 25 cm^2, or equivalent.
** Corning, 270 mL, 75 cm^2, or equivalent.

considered the initial concentration (N_0). Incubate vials in the dark at 28°C for 24 h.

At the same time, initiate a reference toxicant test (with potassium dichromate) with an appropriate range of concentrations to verify the sensitivity of the biological material. An EC_{50} of 10 to 15 mg potassium dichromate/L indicates acceptable test-system quality control.[1]

c. Counting and calculation: At end of incubation period, fix each vial with 1 mL commercially prepared 5% glutaraldehyde solution and count as described in 8310B.3*b*. Compute the number of cells produced (*CP*) as follows:

$$CP = N - N_o$$

where:

N = final counted population, and
N_o = initial concentration, (see ¶ *b* above).

5. Evaluating and Reporting Test Results

The test's standard statistical endpoint is the EC_{50}, but other endpoints may be used (NOEC, EC_{25} , etc.). The cells produced in each concentration of toxicant are estimated as percentage of the control (mean of the three vials). Determine the EC_{50} via a computer program, such as the Stephan LC_{50} program[10] (see Section 8010G).

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8310 C. Chemotactic Test with Freshwater Ciliate *Tetrahymena thermophila*

1. Background

The movement of ciliates toward or away from chemicals (i.e., chemosensory behavior) is a well-studied physiological response in ciliates.[1,2] There are several tests that measure chemosensory behavior or its inhibition as the biological endpoint.[3–5]

Chemotaxis-inhibition toxicity tests have a broad range of applications for single toxicants and contaminant mixtures, such as effluents. The T-maze toxitactic assay (TMTA),[6] upon which this method is based, has undergone species-comparison validation, technical refinement, and interlaboratory calibration.

2. Source of Test Organisms

Cultures of *Tetrahymena thermophila* (Figure 8310:2) can be obtained from the American Type Culture Collection.*

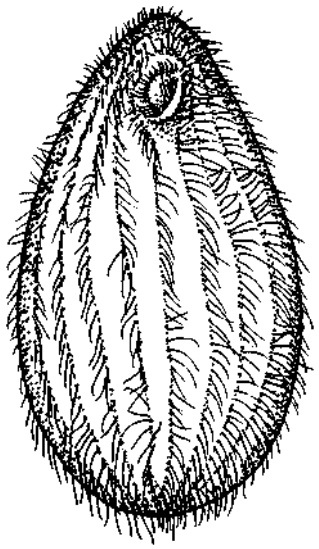


Figure 8310:2. ***Tetrahymena thermophila.***

* 30382 Strain B–18684 (1975) or 30383 Strain B–18686 (1975), ATCC, Rockville, MD.

3. Holding and Culturing Test Organisms

a. Culture medium preparation: Prepare proteose peptone yeast extract (PPYE) medium as follows (depending on culture medium requirements):

Dextrose	0.5 g
Proteose peptone†	2.0 g
Yeast extract†	2.0 g
Distilled water	400.0 mL

Heat distilled water in a beaker over a Bunsen burner. Add dextrose and stir. Add proteose peptone and mix. Add yeast extract, but *do not* stir. Heat solution until yeast extract is dissolved, but do not let solution boil. Dispense 10-mL portions into culture (test) tubes (20 × 150 mm or 15 × 150 mm). Cap tubes and autoclave for 20 min at 103 kP. The culture medium's shelf life is 1 month if it is refrigerated and covered with plastic film.‡

b. Culture transfer and maintenance: Using sterile technique, transfer culture every 2 weeks. Keep cultures at room temperature with regular ambient lighting.

c. Preparation of cultures: Ensure that all solutions used in the toxicity test are at room temperature (20 ± 2°C).

Using sterile technique, inoculate 10 mL sterile PPYE with about 1 mL stock *Tetrahymena thermophila* culture. After 48 h, aseptically transfer the 10-mL culture to 50 mL sterile PPYE in a 250-mL Erlenmeyer flask. At this time, soak the corks for the mazes in dilution water (spring water).

After 24 h, harvest 50 mL PPYE culture by centrifuging in centrifuge tubes (preferably use conical 12- to 15-mL tubes) at 1200 rpm for 3 min. With a Pasteur pipet, remove the supernatant, ensuring that the pellet of cells is not disturbed.

Gently and completely resuspend the pellets into a centrifuge tube, and add 12 to 15 mL dilution water. Centrifuge at 1200 rpm for 3 min. Repeat resuspension, centrifugation, and dilution twice more, removing supernatant each time. Gently and completely resuspend the pellet in a small amount of dilution water. Transfer cells with a Pasteur pipet to 50 mL dilution water in a 250-mL Erlenmeyer flask. Leave for 18 h under ambient temperature and lighting conditions to starve the culture.

Harvest cells by centrifuging at 1200 rpm for 3 min and carefully removing supernatant with Pasteur pipet. Gently and completely resuspend pellets into one centrifuge tube. Using dilution water, adjust cell density to approximately 400 000 cells/mL (±10%).

4. Test Conditions and Procedures

A summary of ecological and prescribed test conditions for *Tetrahymena thermophila* is given in Table 8310:II.

a. Test apparatus and design: A schematic diagram of the apparatus used in the TMTA procedure is presented in Figure 8310:3. The test design comprises at least three replicate glass T-mazes (30 cm in longest dimension) for each concentration, with five concentrations in a serial dilution and a control. Before running the definitive test, perform a preliminary motility test to ensure that cells are motile in the test medium. For a full test, set up five concentrations and a control, with three replicate T-mazes for each concentration (total of 18 mazes).

Table 8310:II. Summary of Ecological and Test Conditions for the Freshwater Ciliate *Tetrahymena thermophila*

Condition	Description
Geographical distribution	Cosmopolitan
Habitat	Littoral and benthic zone of ponds and lakes
Length of life cycle	Cell cycle: 2–5 h
Type/duration of test	Chemotaxis; 20-min IC_{50}
Test temperature	20 ± 2°C
Light cycle	Regular ambient lighting
Validity criteria	Test is invalid if mean control I_{tox} value is outside the range 0.43–0.57; total cell counts for each replicate are ≥ 200.
Endpoints	Chemotaxis, LOEC, IC_{50}
Reference toxicant	Sodium chloride

b. Test exposure: Turn stopcock for each maze so the bore is in line with the third (upright) arm. Label maze arms "test" and "control" with tape and/or marker. Using Pasteur pipets (14.6-cm/5.75-in.), fill arms of each T-maze apparatus, one at a time, with the respective solutions [one test (toxicant solution) and one control, in that order]. Stop each arm with a rubber cork. Ensure that no air bubbles are caught in the arms, particularly around the stopcocks. Holding one arm upwards at a 45° angle, shake out all air bubbles by firmly hitting the T-maze apparatus on the palm of the hand; repeat for second arm. Recork, if necessary, to release any air bubbles.

Using a 23-cm Pasteur pipet, transfer the cells from a homogeneous suspension into each stopcock barrel (the solution is filled above the level of the bore). Gently tap bottom of each T-maze apparatus to remove any initial air bubbles. Remove air bubbles from all stopcock barrels before commencing test for all mazes. (NOTE: Removing air bubbles is crucial to conducting the assay properly because bubbles will prevent organisms from migrating into the arms.)

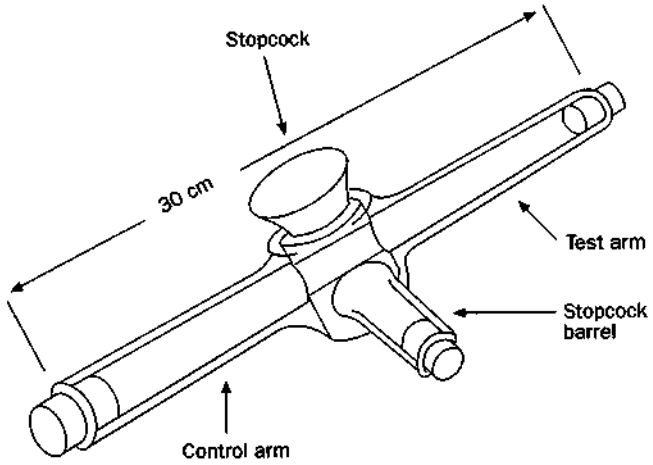


Figure 8310:3. Test apparatus for T-maze chemotactic test.

† Difco, or equivalent.
‡ Parafilm™, or equivalent.

After all T-mazes are filled completely, begin 20-min exposure period by turning the stopcock so cells can migrate freely through the arms. Grease stopcocks sparingly with high-vacuum grease before use. Ensure that the stopcock barrel is completely aligned with the stopcock arms. After 20-min exposure period, turn stopcocks to a closed position to terminate the test.

In parallel with each run of the T-maze tests, perform a standard reference toxicant test (using sodium chloride) with an appropriate range of concentrations to verify the biological material's sensitivity. A lowest-observed-effect concentration (LOEC) of 2000 to 3000 mg/L indicates acceptable test-system quality control.[6]

c. Test termination and enumeration: Immediately after the test is completed, empty arms of T-maze into counting tubes (e.g., test tubes, Coulter counter cuvettes). Using a 14.6-cm Pasteur pipet, rinse each arm with the test solution from that arm to ensure that all cells have been removed. Enumerate cells under 400× magnification. Evenly disperse cells in counting tubes by inverting tubes or using a vortex mixer. Take five 10-μL samples from each counting tube and add this to five wells of a polystyrene 96-well microplate with flat wells. Add 20 μL dilution water and 10 μL Lugol's iodine solution§ [see Section 10200B.2)*a*)] to each of the five wells. Count no fewer than three of the five wells per arm. If necessary, count a smaller or larger portion, depending on cell density. As a guideline, the densest arm (where accumulation/attraction has occurred) should have at least 100 cells/well or 10 000 cells/mL. Record replicate counts and average results.

5. Evaluating and Reporting Test Results

Follow general procedures described in Section 8010G.

The test's statistical endpoints are the LOEC and the IC_{50}. They are determined by calculating the I_{tox} values defined below for the concentration series, plotting them graphically, and applying statistical analysis.

Calculate a "toxitactic" index (I_{tox}) for each T-maze as follows:

$$I_{tox} = \frac{T}{T + C}$$

where:

T = mean number of cells in test arm, and
C = mean number of cells in control arm.

To determine LOEC value, plot I_{tox} values for concentrations tested and a control (y-axis) against the toxicant concentration (x-axis). When there is a response, an increase in I_{tox} with increasing concentration denotes attraction, and a decrease in I_{tox} with increasing concentration denotes repulsion.

Conduct an analysis of variance (ANOVA) and a multivariate test (e.g., William's or Dunnett's tests)[7] on all data for a given test in order to determine the lowest concentration at which the I_{tox} value is statistically, significantly different from the control I_{tox}. That concentration is the LOEC.

Calculate the IC_{50} via the linear interpolation method[8] with a software package (e.g., ICPIN).‖

6. References

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§ Corning No. 25880-96, or equivalent.

‖ BOOTSTRP, available from U.S. Environmental Protection Agency, Cincinnati, Ohio.

8310 D. Growth Inhibition Test with the Soil Ciliate *Colpoda inflata*

1. Background

This toxicity test measures the population growth rate of the test ciliate species in response to a gradient of test concentrations. It is similar to that used for algal growth-inhibition tests.[1,2] Growth, in this case, may be inhibited by direct effects on the ciliate cell maintenance or by effects that suppress energy intake (i.e., feeding).

The description below is based on a test method[3] for evaluating solid-phase media (i.e., soils and soil elutriates) using the soil ciliate *Colpoda inflata* Stokes (Section 10900, Plate 6:D). The number of cells produced during a 24-h period in the

presence of a toxicant is compared to that produced in a control culture. The method also has been applied successfully to mining effluents,[4] and it has been modified for alternative food sources and endpoint detection.[5]

2. Source of Test Organisms

Dry cysts of *Colpoda inflata* (ATCC 30917) can be obtained from the American Type Culture Collection.*

3. Holding and Culturing Test Organisms

a. Holding organisms: Dry cysts can be held at room temperature on filter paper for extended periods (1 to 2 years). Cysts in spent cultures can be stored wet for months without loss of viability. Grow these cultures or dry cysts as needed by adding culture medium as described below.

b. Maintaining cultures: Maintain cultures developed from stored cysts for 2 to 7 d in 10% Sonneborn's *Paramecium* medium, prepared as follows: Boil 2.5 g cereal grass leaves† for 5 min in 1 L distilled, deionized water; filter,‡ adjust volume to 1 L with distilled water, and add 0.5 g Na_2HPO_4. Dilute full-strength medium before use with distilled, deionized water and autoclave in 50-mL portions. Add to cultures a food bacterium, such as nonpathogenic *Klebsiella pneumoniae*,§ as recommended by ATCC.

4. Test Conditions and Procedures

A summary of ecological and prescribed test conditions for *Colpoda inflata* is given in Table 8310:III.

a. Test vessels: Conduct tests in sterile, 24-well, polystyrene tissue culture plates,‖ using either 10% Sonneborn's medium (8310D.3*b*) or a minimal salts medium consisting of 6 mg KCl, 4 mg $CaHPO_4$, and 2 mg $MgSO_4$ in 1 L sterile distilled, deionized water. Test volume is 2 mL per well.

b. Test initiation: Dispense sterile medium into wells and amend with toxicant from stock solutions of reagent-grade chemicals prepared in sterile distilled, deionized water. Alternatively, conduct tests using percentage dilutions of complex mixtures (e.g., wastewater effluents, soil extracts[4,5]). For mixture tests, prepare minimal salts medium in concentrated form (10×) and dilute with sterile distilled water.

After medium and toxicant are dispensed into test wells, add ciliates from log-phase cultures (48 to 96 h old), along with prey bacteria. The volume of well-mixed culture added should ensure that equal numbers of ciliates (approximately 100 cells/mL) are added to each well.

At the same time, perform a reference toxicant test (using copper sulfate) with an appropriate range of concentrations to verify the biological material's sensitivity. A toxicant concentration between 25 and 70 μg/L that corresponds to a 50% inhibition of growth relative to controls (IG_{50}) indicates acceptable test-system quality control.

Table 8310:III. Summary of Ecological and Test Conditions for the Soil Ciliate *Colpoda inflata*

Condition	Description
Geographical distribution	Cosmopolitan
Habitat	Soil, vegetation litter
Length of life cycle	Cell cycle: 2–5 h
Type/duration of test	Growth; 24-h IG_{50}
Test temperature	20 ± 2°C
Light cycle	Regular ambient lighting
Validity criteria	IG_{50} 25–70 μg/L for reference toxicant test
Endpoints	Growth inhibition (IG_{50})
Reference toxicant	Copper sulfate

c. Counting and calculation: After 24 h, remove subsamples from each test well and enumerate using a direct counting technique as follows: Thoroughly mix each well with a micropipettor and transfer a 20-μL subsample to a clean microscope slide as 3 or 4 drops. Scan all drops immediately at low magnification (40×) on a stereomicroscope to search for active cells. Active cells are always moving and can be distinguished easily from bacterial aggregates or cysts. For a given well, repeat subsampling at least three times to ensure that an estimate of the well's population is accurate. If repeat counts of subsamples vary by >30%, continue subsampling until population estimates stabilize. Repeat this procedure for each well, and compute the mean of subsample estimates for a given replicate. Alternatively, use electronic particle-counting techniques (i.e., Coulter counter) to enumerate cells.

5. Evaluating and Reporting Test Results

Follow general procedures described in Section 8010G.

Estimate IG_{50} by regressing cell number on log dose and then using inverse prediction[6] to estimate IG_{50} from the control response (mean = 100%).

6. References

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* ATCC, Rockville, MD.
† Cerophyl®, Agri-Tech, Inc., Kansas City, MO 64112, or equivalent.
‡ Whatman No. 1 filter paper, or equivalent.
§ ATCC 27889
‖ Costar, Inc., or equivalent.

8420 ROTIFERS*

8420 A. Introduction

1. Ecological Significance

Rotifers are classified in the Phylum Rotifera , one of several phyla of lower invertebrates. There are approximately 2000 rotifer species named; they are divided into two classes: Digononta and Monogononta.[1,2] Monogononts reproduce parthenogenetically; however, in response to specific environmental cues, they reproduce sexually, yielding dormant embryos called *cysts* (resting eggs), which have been used in toxicity testing.[3] The use of rotifer cysts for toxicity testing has been discussed in the literature.[3] Most rotifer species inhabit fresh water,[4] but there are some genera, like *Synchaeta*, in which most species are marine.[5] In coastal marine habitats, rotifers sometimes are the dominant portion of the biomass.[6] They also are abundant in marine interstitial habitats, interstitial water of soils,[7] and water clinging to mosses, liverworts, and lichens.[8] In freshwater lake plankton[9] and in river sediments,[10] rotifers often are abundant, with high species diversity.

Rotifers play an important role in the ecological processes of many aquatic communities.[11] As suspension feeders, planktonic rotifers influence algal species composition through selective grazing.[12–14] Rotifers often compete with cladocera and copepods for phytoplankton in the 2- to 18-μm size range.[15] Along with crustaceans, rotifers contribute substantially to nutrient recycling.[16] Rotifers are food for many fish larvae.[17]

2. Types of Toxicity Tests

The procedures in this section serve as guidelines for using rotifers to estimate sublethal toxicity, with asexual population growth rate as endpoint. These procedures have been adapted for examining surface water, effluents and sediment pore water. Several other types of rotifer tests described are based on such endpoints as mortality,[18] ingestion,[19] swimming,[20] enzyme activity,[21] endocrine disruption,[22] and gene expression.[23]

3. References

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* Approved by Standard Methods Committee, 1997. Editorial revisions, 2013. Joint Task Group: 20th Edition—Terry W. Snell (chair), David B. Dusenbery, Colin R. Janssen, Michael C. Newman, Guido Persoone.

8420 B. Selecting and Preparing Test Organisms

1. Selecting Test Organisms

The rotifers recommended for use were chosen because of the existence of published reports describing protocols, a database of responses to pure toxicants, and the availability of cysts. In *Brachionus calyciflorus* and *B. plicatilis*, for which standardized tests exist, the recommended strain also is indicated. A summary of ecological and test conditions to be considered in tests with these organisms is given in Table 8420:I. Substantial differences in sensitivity to toxicants have been reported among rotifer strains of different geographic origin.[1] In accord with the criteria listed in Section 8010E.1, the recommended test species include (but are not restricted to) the following:

a. Freshwater rotifers:

Class: Monogononta

Brachionus calyciflorus (Gainesville strain)[2]

Brachionus rubens[3]

Brachionus patulus[4]

Asplanchna brightwelli[5]

Class: Digononta

Philodina roseola[6]

Philodina acutiocornis[7]

See Section 10900, Plate 8, for drawings of several freshwater rotifer species.

b. Marine rotifers:

Brachionus plicatilis (Russian strain)[1]

2. Obtaining Test Organisms

a. Rotifer cysts: B. calyciflorus in fresh water and *B. plicatilis* in marine waters are hatched from cysts. Rotifer cysts hatch synchronously, providing test animals of similar age in uniform physiological condition. Detailed descriptions of rotifer cyst hatching are available.[1,2]

b. Cyst hatching: To initiate *B. calyciflorus* hatching, place about 40 mL standard fresh water in a glass Petri dish or tissue-culture-grade polystyrene dish. Incubate rotifer cysts at 25°C in light of 3000 to 4000 lux. Hatching should start after about 15 to 16 h; within 2 h of hatching, remove dish from incubator to transfer rotifers to test tubes. Cooler temperatures, low or high pH, and elevated hardness and alkalinity can delay hatching. If hatching is delayed, check cysts hourly to ensure that test animals are collected within 2 h of hatching.

Hatch *B. plicatilis* cysts by a similar procedure in standard synthetic seawater. *B. plicatilis* cysts usually begin hatching after 24 to 26 h at 25°C in light of 3000 to 4000 lux.

c. Food and feeding: See 8420C.3.

d. Rotifer reproduction: Rotifers hatched from resting cysts reproduce asexually via ameiotic parthenogenesis.[8] Monogononts also can reproduce sexually, but this capacity usually is not used in routine toxicity tests (with certain exceptions[9]). Asexual rotifer reproduction allows simple sublethal toxicity tests to be conducted using population growth as an endpoint.

Table 8420:I. Summary of Ecological and Test Conditions That Should Be Considered When Conducting Toxicity Tests with *B. calyciflorus* (BC) or *B. plicatilis* (BP) Rotifers

Condition	Comment
Geographical	Pan-global distribution
Habitat	Pelagic zooplankter
Life cycle	Parthenogenetic and sexual reproduction
Lifespan	5–7 d at 25°C
Temperature	10–32°C
Salinity	BC = 0–5 ppt, BP = 1–60 ppt
Nutrition	Suspension feeders on microalgae
Photoperiod	No special requirements
Control mortality	Not to exceed 10%
Reproductive test	Water, pore water[1]

3. Parasites and Diseases

Fungal parasites on rotifers have been reported in a few natural populations, but never in laboratory populations used for toxicity testing. No known diseases affect the use of brachionid rotifers in toxicity tests.

4. References

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8420 C. Toxicity Test Procedures

1. General Procedures

Use exploratory tests[1] to determine the toxicant concentrations for short-term tests (see Section 8010D). Prepare control and test solutions in standard synthetic fresh water or seawater and introduce them into test containers as described in Section 8010F.

2. Water Supplies

a. Artificial fresh water: See Section 8010E.4*b*1) and Table 8010:I for preparation of a moderately hard water. Adjust to pH 7.5 with 10*M* KOH or HCl.

b. Artificial seawater: Prepare standard synthetic seawater[2] with a salinity of 15 by adding 11.31 g NaCl, 0.36 g KCl, 0.54 g $CaCl_2$, 1.97 g $MgCl_2 \cdot 6H_2O$, 2.39 g $MgSO_4 \cdot 7H_2O$, and 0.17 g $NaHCO_3$ to 1 L deionized or distilled water. Mix well on a magnetic stirrer and adjust pH to 8.0 with 10*M* KOH or HCl.

c. Deionized water: Prepare all media with high-quality deionized or distilled water (see Section 1080). Water from certain commercially available systems* is suitable.

3. Food and Feeding

The following procedures are recommended for growth of food organisms. However, other diets, such as a mixture of *Selenastrum capricornutum* and *Chlorella vulgaris* grown on Bold's Basal Medium, have been used successfully.[3]

a. Nannochloris oculata (food for *Brachionus calyciflorus*): Maintain unialgal stock cultures of the green alga *Nannochloris* cells† in sterile test tube cultures containing 20 mL Bold's basal medium (BBM), prepared as follows: To 1 L water (8420C.2*c*), add 250 mg $NaNO_3$, 75 mg $MgSO_4 \cdot 7H_2O$, 175 mg KH_2PO_4, 25 mg $CaCl_2 \cdot 2H_2O$, 75 mg K_2HPO_4, 25 mg NaCl, 2 mL vitamin stock, and 2 mL trace metal stock.

To prepare 500 mL trace metal stock water, add 2.5 g NaFeEDTA, 11 mg $ZnSO_4 \cdot 7H_2O$, 90 mg $MnCl_2 \cdot 4H_2O$, 5 mg $CoCl_2 \cdot 6H_2O$, 5 mg $CuSO_4 \cdot 5H_2O$, and 3.2 mg $NaMoO_4 \cdot 2H_2O$ to water (8420C.2*c*).

To prepare 500 mL vitamin stock water, add 100 mg thiamine, 5 mg biotin, and 5 mg B_{12} to water (8420C.2*c*).

Propagate cultures by serial transfer using sterile technique. To inoculate a large *Nannochloris* culture, pour contents of a dark green 20-mL test tube culture into 2 L BBM; this will yield an initial density of about 10^3 cells/mL. Aerate this culture with filtered air and maintain at 25°C in light for 3 to 6 d until the cells reach log-phase growth, at which point they have the highest nutritional quality. Harvest and concentrate algal cells by centrifugation at 5000 × *g* for 10 min. Concentrated algal cells can be stored in the refrigerator for 3 to 4 d without loss of nutritional quality. Quantify algal cell density using a Neubauer slide hemacytometer according to the manufacturer's protocol. Add smallest volume of algae stock necessary to each test solution to make a suspension of 10^6 cells/mL. Pour 100 mL of each test solution into a 150-mL beaker and stir gently on a magnetic stirrer (approximately 120 rpm) using a small stir bar (about 1.5 cm). Use test solutions promptly; do not stir for more than 30 min. Pipet 12 mL control solution into each of seven replicate test tubes. Repeat for each test solution.

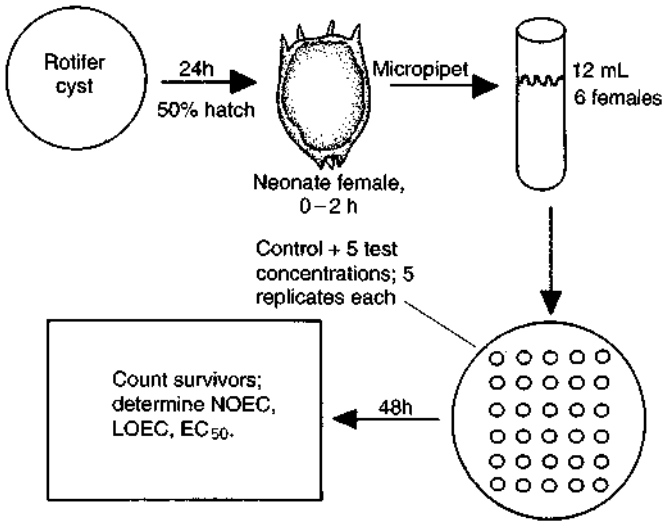


Figure 8420:1. Schematic diagram of rotifer static life-cycle toxicity tests. Test conditions: Generations—2; endpoint—reproductive rate $r = (\ln N_t - \ln N_o)/T$; temperature—25°C; photoperiod—darkness; medium—synthetic freshwater; food—*Nannochloris*.

b. Nannochloropsis sp. (food for *Brachionus plicatilis*): Maintain unialgal stock cultures of the Eustigmatophycean alga *Nannochloropsis*‡ cells in test tubes containing 20 mL of sterile ASPM algal growth medium prepared as follows: To 1 L synthetic seawater (8420C.2*b*), add 150 mg $NaNO_3$, 10 mg K_2HPO_4, 2 mL trace metal stock (¶ *a* above), and 2 mL vitamin stock (¶ *a* above). Propagate cultures by serial transfer using sterile technique. Follow procedures given in ¶ *a* above for culturing, concentrating, and dispensing to test tubes.

4. Exposure Chambers

Use standard, disposable 16- ×150-mm borosilicate glass test tubes as exposure chambers.

5. Conducting the Test

An overview of the test is shown in Figure 8420:1.

a. Adding test animals: To begin test, transfer six newly hatched rotifers (neonates) into each test tube. *B. calyciflorus* rotifers are approximately 250 μm in length, about 1/4 the size of newborn *Daphnia*. Their small size and slow swimming speed have some advantages for capture and manipulation. Newly

* Nanopure II system with one pretreatment, one high-capacity, and two ultrapure cartridges, or equivalent.

† University of Texas at Austin, Culture Collection of Algae, LB 1998.

‡ University of Texas at Austin, Culture Collection of Algae, LB 2164.

TABLE 8420:II. SAMPLE TEST RESULTS

Species	Toxicant	24-h LC_{50} *mg/L*	48-h Population Growth Rate NOEC *mg/L*	EC_{50} *mg/L*	CV *%*
B. calyciflorus	Copper	90.03	0.02	0.03	44
	Cadmium	1.3	0.04	0.07	2
	Pentachlorophenol	1.2	0.11	0.27	29
B. plicatilis	Copper	0.06	0.01	—	—
	Cadmium	39	1.0	—	—
	Pentachlorophenol	1.9	0.5	—	—

hatched rotifers are white, so they are most visible on a dark background at about 10× magnification. The best type of illumination is a darkfield setting. Because they are moderately phototactic, rotifers tend to congregate around the edges of the hatching dish. Squeeze the transfer micropipet gently to provide the right amount of suction. Practice to develop a feel for the right pressure. Confirm that each tube receives exactly six rotifers by watching their entry into the tube under the microscope. Cap and immediately place tubes on a wheel rotator in a 25°C incubator in darkness. Rotation rate should be 10 to 120 revolutions per hour to maintain the algae in suspension. Do not use a shaker because it will damage the animals. Repeat until all remaining test solutions have been inoculated with rotifers. Record time at which neonates are placed in control treatment as the beginning of the 48-h incubation period.

b. Duration and type of test: The test uses rotifer asexual reproduction to estimate sublethal toxicity. A typical schedule of reproduction is presented in Figure 8420:2. The 48-h population growth rate is calculated and its decline with increasing toxicity is quantified.

c. Scoring the test: Remove test tubes from rotator after 48 h. Empty contents of one tube into a Petri dish and count number of animals per tube, discriminating between live and dead individuals. Repeat until all tubes have been counted. Calculate *r*, the population growth rate for each tube, as follows:

$$r = \frac{\ln N_t - \ln N_o}{T}$$

where:

N_t = number of live rotifers in tube after 2 d,
N_o = initial number of rotifers in tube (6), and
T = incubation period (2 d).

Typically, *r* ranges from 0.7 to 1.2 offspring per female per day. This corresponds to a total of 20 to 70 animals at the end of the control test, when the test begins with 6 rotifers. Appropriate statistical procedures to analyze these data include dose–response statistics (EC_{20} and EC_{50})[4,5] and analysis of variance followed by a multiple-comparison test [e.g., Dunnett's test, no-observed-effect concentration (NOEC), and lowest-observed-effect concentration (LOEC)] (see Table 8420:II).

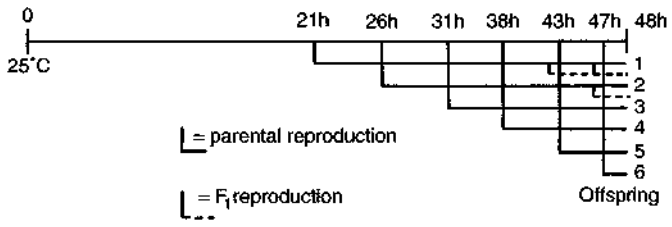


Figure 8420:2. Schedule of reproduction.

d. Reference toxicant test: Perform a reference toxicant test (positive control) with every fifth test. This verifies that the animals will respond to any toxicity present. Perform reference tests according to the protocol described above. Cadmium chloride, expressed as cadmium, is commonly used as a reference toxicant. Other metal chlorides or organic compounds may be used.

6. Data Evaluation

The data obtained from this test can be considered valid if the control's *r* is at least 0.70 (the minimum acceptable population growth rate). Lower values suggest that there is an unidentified problem with the dilution water, algae, or rotifers. Often when low population growth rates are observed, there is a problem with algae quality. For additional guidance in data analysis and other statistical considerations, see Sections 8010G and H.

7. References

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8510 ANNELIDS*

8510 A. Introduction

1. Characteristics and Ecology

The phylum Annelida includes three classes: Polychaeta, Oligochaeta, and Hirudinea. Polychaetes are an important, often predominant, component of marine and estuarine biota.[1] In subtidal benthic environments, they comprise about 30 to 75% of macroinvertebrate species and individuals. They include a variety of feeding types, with the majority being either filter or detritus feeders. Polychaetes affect surface sediments by their burrowing and irrigating habits. They are important food for snails, large crustaceans, fish, and birds. Many species have short life cycles.

Oligochaetes are among the most common benthic invertebrates in all types of aquatic environments. Particular species assemblages are recognized indicators of environmental quality. In grossly polluted freshwater habitats, oligochaetes dominate benthic fauna, while in estuarine areas, they and polychaete worms are often the most common benthic organisms. They feed mainly on bacteria, although other feeding types occur.[1] They affect surface sediments, as do polychaetes. Oligochaetes are an important primary or alternate food for leeches, crustaceans, fish, and birds.

Hirudinea are leeches, either free-living or parasitic, that inhabit either fresh or marine waters. They have not been used for toxicity tests.

2. Test Applications

The following procedures are intended to serve as guidelines for using polychaetes and oligochaetes in various toxicity tests. These procedures also can be, and have been, adapted to testing sediments.

Other toxicity tests for annelids have been published.[2,3]

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* Approved by Standard Methods Committee, 2014.
Joint Task Group: Donald J. Reish (chair), Mary Ann Rempel-Hester, J. Daniel Farrar

8510 B. Selecting and Preparing Test Organisms

1. Selecting Test Organisms

In accordance with the criteria listed in Section 8010E.1, the recommended test species include (but are not restricted to) the following:

a. Marine polychaetes:

1) Family Nereidae
   Neanthes arenaceodentata (*Neanthes acuminata* and *Neanthes caudata* of some authors) (New England, Florida, California coasts, Europe) (Figure 8510:1A).
   Neanthes succinea (all U.S. coasts).
   Neanthes virens (east U.S. coast).
2) Family Spionadae
   Polydora cornuta (*Polydora ligni* of some authors) (Atlantic, Pacific, and Gulf of Mexico) (Figures 8510:1B and C).
3) Family Capitellidae
   Capitella capitata (cosmopolitan) (Figures 8510:1D and E).
4) Family Dinophilidae
   Dinophilus gyrociliatus (cosmopolitan) (Figure 8510:2A).
5) Family Dorvilleidae
   Ophryotrocha diadema (west U.S. coast) (Figure 8510:2B).

Other species used in toxicity tests but not discussed include *Nereis diversicolor, Arenicola cristata, Abarenicola pacifica,* and *Armandia brevis.*

b. Freshwater oligochaetes:

1) Family Tubificidae
   Limnodrilus hoffmeisteri (cosmopolitan).
   Tubifex tubifex (cosmopolitan) (Figure 8510:3A).
   Branchiura sowerbyi (cosmopolitan) (Figure 8510:3B).
2) Family Lumbriculidae
   Stylodrilus heringianus (holarctic) (Figure 8510:3C).
   Lumbriculus variegatus (holarctic).

c. Marine oligochaetes:

Family Tubificidae
Monopylephorus cuticulatus (N.E. Pacific).
Tubificoides "fraseri" (all North American coasts).
Tectichilus verrucosus (all North American coasts).

d. Other freshwater and marine oligochaetes used in toxicity tests but not discussed include *Quistadrilus multisetosus* (Figure 8510:3D), *Spirosperma ferox, Spirosperma nikolskyi, Rhyacodrilus montana, Varichaetadrilus pacifica, Ilyodrilus frantzi, Nais communis, Paranais frici,* and *Paranais litoralis.*

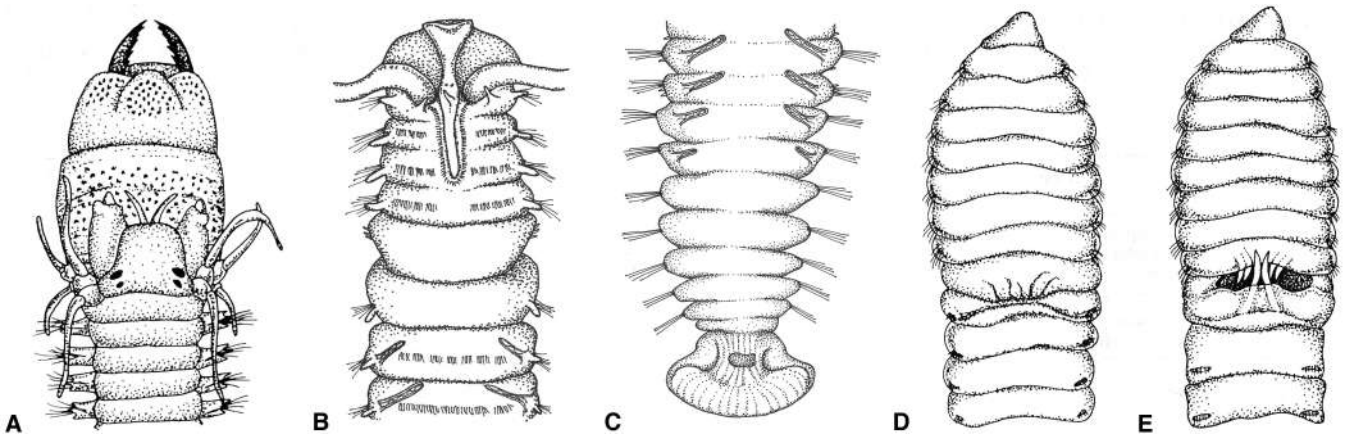


Figure 8510:1. Marine polychaetes. A—*Neanthes arenaceodentata*, anterior end, dorsal view; B—*Polydora cornuta*, anterior end, dorsal view; C—*Polydora cornuta*, posterior end, dorsal view; D—*Capitella capitata*, female, anterior end, dorsal view; E—*Capitella capitata*, male, anterior end, dorsal view.

2. Collecting Test Organisms

a. Marine polychaetes:

1) *Neanthes arenaceodentata, Neanthes succinea,* and *Capitella capitata* inhabit intertidal and subtidal mud flats in estuarine areas and the fouling communities on pilings, boat floats, or submerged objects. Subtidal collections can be made using one of the sampling devices described in Section 10500B. Worms can be separated from sediment as directed in Section 10500C. To obtain worms, bring substrate or fouling material into laboratory, place in white enameled pans, and cover with seawater. After a period of time, the worms come to the surface; remove them with a fine brush and transfer to Petri dishes containing seawater. Examine each specimen under a dissecting microscope, and discard all injured worms. Transfer uninjured specimens to 4-L aquariums or shallow trays.

2) *Polydora cornuta* inhabits intertidal sand-mud flats or subtidal waters. It lives in tubes constructed of fine sediments. Intertidal worms can be collected with a shovel and washed through a 1.0-mm sieve. Tubes can be transferred to a Petri dish so the species can be verified and its condition examined under a dissecting microscope.

3) *Ophryotrocha* and *Dinophilus* occur on fouling organisms attached to floats and pilings. Because these species are minute, use a dissecting microscope to look for them. Because only a small number can be collected at once, establish a laboratory colony [8510B.3*a*3)].

b. Freshwater and marine oligochaetes: Limnodrilus hoffmeisteri and *Tubifex tubifex* inhabit muddy sediments and are particularly common in areas of gross organic pollution. To positively identify this species, analysts usually must examine preserved specimens and compare them. In live culture, these species can be separated based on the presence (*T. tubifex*) or absence (*L. hoffmeisteri*) of hair setae. *Branchiura sowerbyi* is a larger worm with gills on posterior segments; it is common in muddy, warm-water areas. *Stylodrilus heringianus* is common in areas of clean, fine sand and is identified by the presence of extra rings and nonretractable penes.

The marine species *Monopylephorus cuticulatus, Tubificoides fraseri,* and *Tectidrilus verrucosus* are found in muddy sediments and are separable based on size (*M. cuticulatus* > *T. fraseri* > *T. verrucosus*), setae (*M. cuticulatus* has occasional irregular hair setae), and color (*T. verrucosus* has a papillate skin and greenish tinge; the others are dark red). To positively identify this species, analysts must examine preserved specimens and compare them.

To obtain worms, sieve sediments through a 0.5-mm sieve and sort them under a dissecting microscope. Discard damaged worms. Transfer uninjured specimens to aerated aquariums or shallow trays for holding and feeding. Ensure that worms are collected from an uncontaminated area because they can develop rapid resistance to some toxicants.[1]

3. Culturing

a. Marine polychaetes:

1) Condition of animals—Discard animals injured during collection. Some species (e.g., *Neanthes arenaceodentata*) can regenerate a tail, so it is not always necessary to discard worms missing tails when establishing a culture. Save worms with gametes in the coelom for starting cultures, but normally do not use them for toxicity tests.

2) Food and feeding—Cultures of the polychaete species mentioned here can be maintained without sediment; therefore, the worms must be fed, as should worms in long-term experiments (see 8510C.4*a*). Cultures of larger species (e.g., *N. arenaceodentata*) have better survival, growth, and tube production if fed a mixed diet that consists of a macroalga for tube construction and a commercially prepared invertebrate, rabbit, or fish diet for nutrition. Green alga [*Enteromorpha* (syn. *Ulva*) sp.] is convenient because it grows abundantly in most estuarine areas of North America. Collect in quantity, wash with seawater, freeze, and store indefinitely. Before use, soak the alga in seawater and knead to separate individual filaments. Other macroalgae (e.g., cultured brown *Ectocarpus siliculosus*) produce excellent results in polychaete cultures but are less convenient to use. Place sufficient macroalga in culture containers to allow

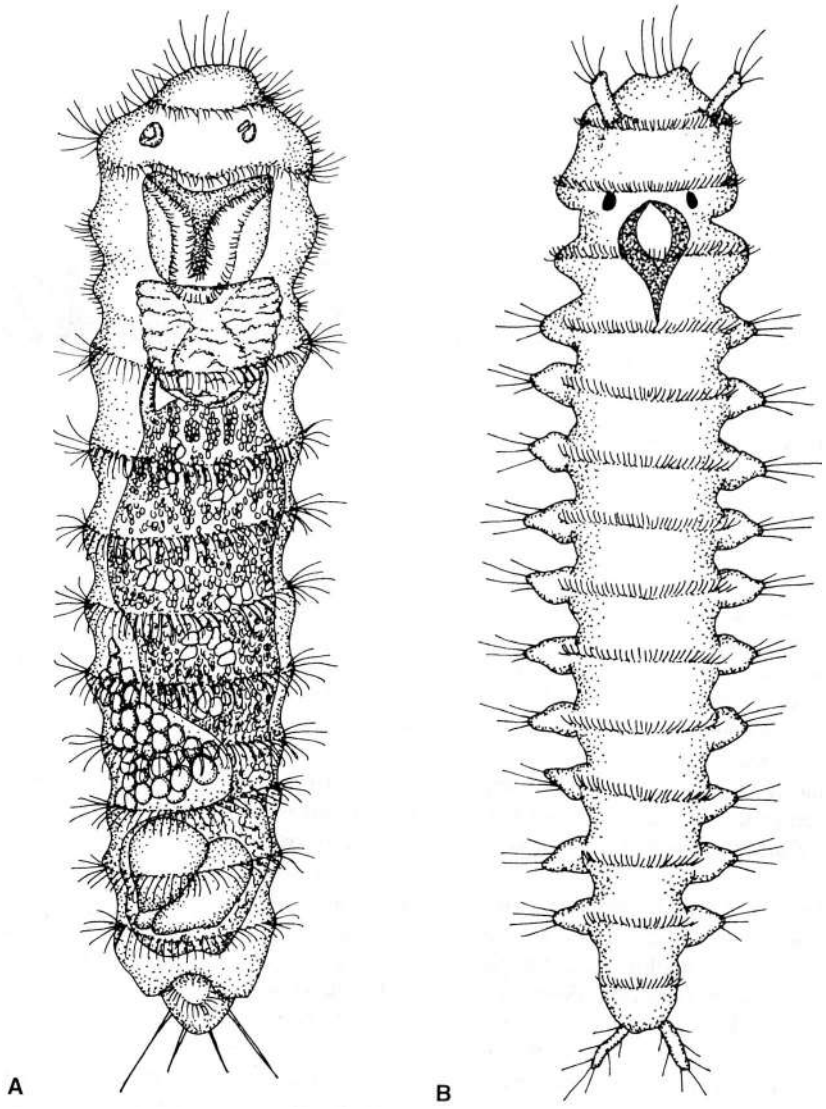


Figure 8510:2. Marine polychaetes. A—*Dinophilus gyrociliatus*, adult; B—*Ophryotrocha diadema*, adult.

worms to construct tubes. Add commercially prepared diet* to worm cultures three times weekly. Vigorously mix flakes with a small amount of seawater to moisten, and break them up before adding them to the cultures. To minimize overfeeding, examine each culture container before adding the commercial diet. If most of the food is uneaten, do not add more, and add less food at subsequent feedings.

A powdered diet is suitable for small species (e.g., *Capitella capitata*, *Ophryotrocha diadema*, and *Dinophilus gyrociliatus*) and the larvae of *N. arenaceodentata*. Prepare a fine powder from dried *Enteromorpha* sp. or one of the commercial diets by grinding the dry material in a blender and sieving it when <0.061 mm.

Feed living *Dunaliella* sp. to larval *N. succinea* until the larvae settle. For culturing instructions, see Section 8010E.4*c*1)b). Feed *Dunaliella* sp. at least 2 million cells/L of worm culture or at a rate large enough to maintain a green color in the seawater. After larvae settle, feed *Enteromorpha* sp. until the swimming reproductive epitoke stage is reached.

3) Producing test organisms

a) *Capitella capitata*—Laboratory-cultured specimens begin to mature about 15 to 25 d after hatching. A mature female develops white masses of eggs in the coelom from about segment 10 posteriorly, and a mature male develops specialized setae on the dorsal surface of segments 8 and 9. The female lays fertilized eggs along the inside lining of her tube, where larval development continues until the trochophore larvae emerge 4 to 6 d later (Figure 8510:4). To obtain free-swimming trochophore larvae, examine tubes under a dissecting microscope to detect those containing eggs or larvae. Recently fertilized eggs appear white, but as they mature they become grey-green and can be seen moving about. Place tubes containing larvae in a Petri dish.

* Prawn Flakes, Plankton Flakes, TetraMarin®, or equivalent.

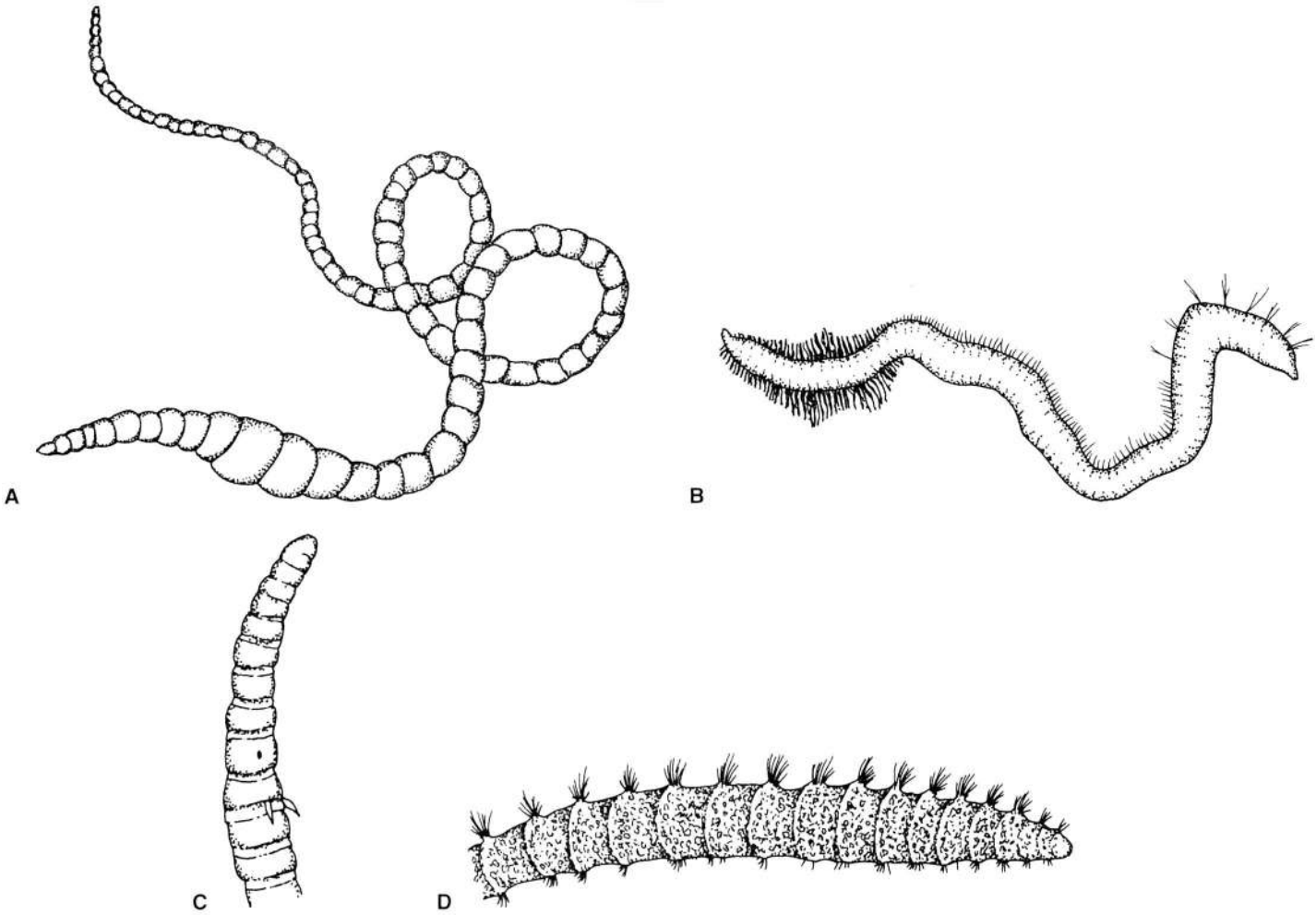


Figure 8510:3. Freshwater oligochaetes. A—*Tubifex tubifex*, adult; B—*Branchiura sowerbyi*, adult; C—*Stylodrilus heringianus*, adult; D—*Quistradrilus multisetosus*.

Under a dissecting microscope, open the tubes to free trochophores. One female provides 200 to 300 trochophores. Remove and discard the female and the tube containing any larvae that did not swim free. Use free-swimming larvae in tests or let them develop for later use.

Sibling species of *Capitella capitata* have been described;[2] however, the taxonomic status of this species complex is still in question.

b) *Neanthes succinea*—Take nearly mature epitokes from the field or laboratory colony and hold until they complete sexual metamorphosis. Mature epitokes swim to the water surface and release gametes. If fertilization is successful, separate zygotes into several 4-L jars containing aerated water and let them develop to the three-setiger stage (about 1 week). These larvae are ready for use in tests. One fertilization provides more than 2000 larvae.

c) *Neanthes arenaceodentata*—Before spawning, either the male or female enters the tube or burrow of another worm. If the worms are of different sex, they remain together and spawn in the tube. The female dies within 1 d after spawning, and the male incubates the eggs for about 3 weeks, when they have 18 to 21 setigerous segments. Then the young worms leave the tube, begin feeding, and construct their own tubes. Feed them *Enteromorpha* [as indicated in ¶*a*2) above]. Under laboratory conditions (20°C), sexual maturity is reached in 3 to 4 months. It is impossible to distinguish the sex of immature forms morphologically. Distinguish by observing whether they fight when placed together (Figure 8510:5). Like sexes fight; unlike sexes do not. Use a female with maturing eggs in her coelom as a known individual to identify the sex of immature worms. The most convenient time to obtain young juveniles is shortly after they have left the parent's tube and have begun to feed.

d) *Dinophilus gyrociliatus* completes its life cycle in 7 to 10 d at 20°C. The female lays two to five eggs in a capsule. The larger eggs develop into females and the smaller ones into males. The male mates with the female before hatching from the capsule (Figure 8510:6A), then dies. Large colonies can be maintained with minimal care.

e) *Ophryotrocha diadema* (Figure 8510:2B) completes its life cycle in 30 to 40 d at 20°C. This species is hermaphroditic, with Segments 3 and 4 containing the male elements. The remaining segments posterior to Segment 4 are female. Ten to 14 eggs are

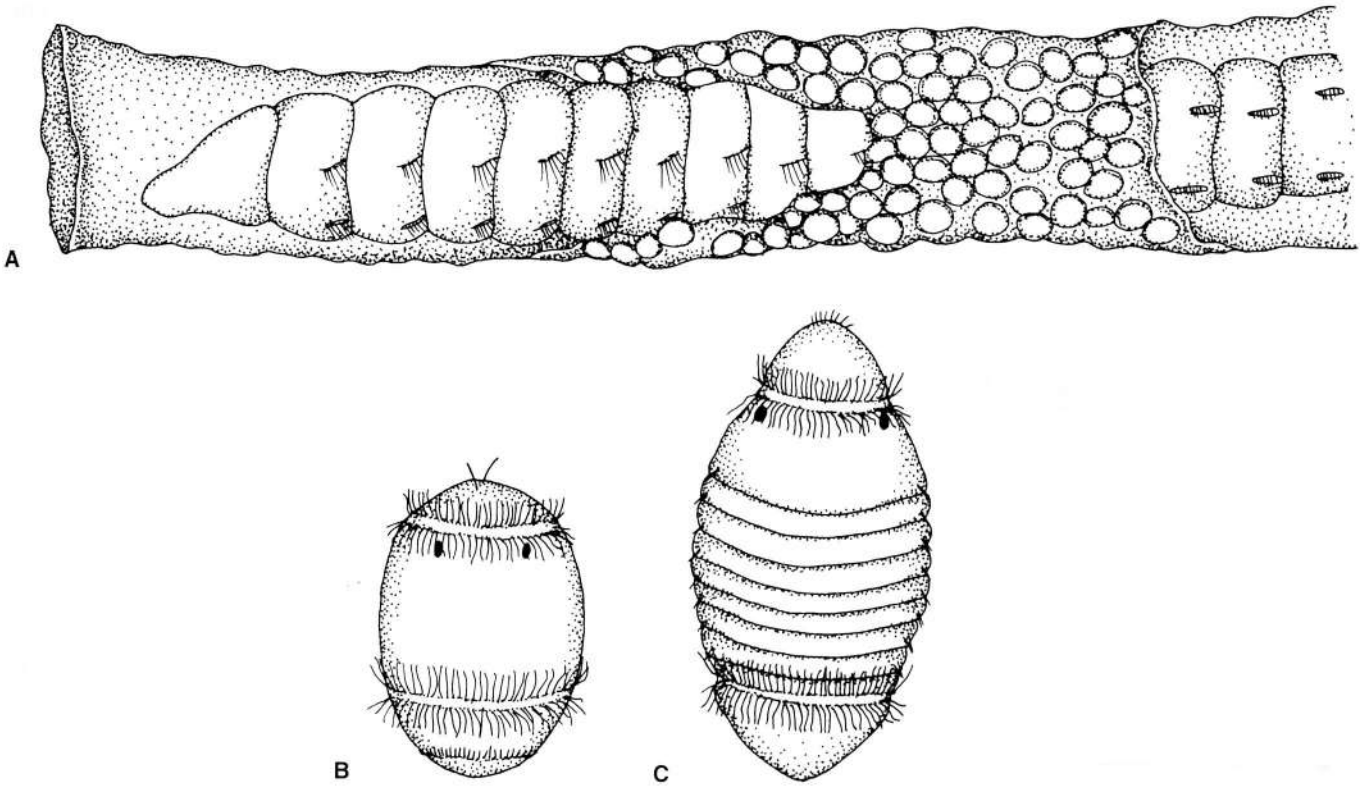


Figure 8510:4. Life stages of *Capitella capitata*. A—Female incubating developing embryos; B—Recently hatched trochophore larva; C—Metatrochophore stage, ready to settle.

laid in a loose jelly capsule (Figure 8510:6B); they hatch from the capsule in 8 d as a four-segmented larva (Figure 8510:6C) and begin feeding the next day. Large colonies can be maintained with minimal care; however, subcultures should be established every 5 to 6 weeks in clean containers.

f) Feed *Dunaliella* sp. or a 1:1 mixture of ground TetraMarin® and powered *Enteromorpha* sp. to both larvae and adult *Polydora cornuta.* Feed several times a week at the rate of 2 mg per worm. Larvae will settle and begin tube construction, using food particles for material.

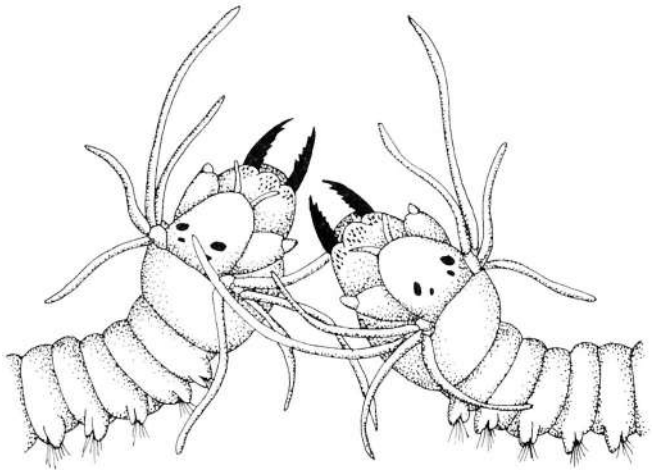


Figure 8510:5. *Neanthes arenaceodentata.* Adults of same sex in a fighting position.

g) *Polydora cornuta* (Figure 8510:1C) completes its life cycle in 28 d under laboratory conditions at 20 ± 2°C.[1–2] The sexes are separate, and the sperm (contained in a spermatophore) are transferred to the female. Fertilized eggs are laid in capsules in the female's tube (Figure 8510:7A), which occur between Segments 14 and 21. The number of eggs in a capsule vary from 35 to 63 under laboratory conditions. Larvae develop in the capsule and are expelled in 4 d. Swimming larvae have at least three segments at the time of release (Figure 8510:7B). The larvae begin tube construction within a week. The life cycle is completed in 1 month. Sperm are stored in the female, and she produces more than one string of capsules.

b. Freshwater and marine oligochaetes:

1) Condition of animals—Oligochaetes show great regenerative abilities, so it is not always necessary to discard injured specimens. Mature individuals with well-developed clitellar regions are particularly important for culture establishment. Keep cultures in the dark or under natural light/dark regimes.

2) Food and feeding—Oligochaetes feed mainly on bacteria in sediments, so in experiments with natural sediments, additional feeding is unnecessary. Short-term experiments do not require feeding; for long-term experiments (>10 d), provide sediment.

Condition sterile sediments by preparing an inoculum of *Enteromorpha* (for marine worms) or lettuce (for freshwater

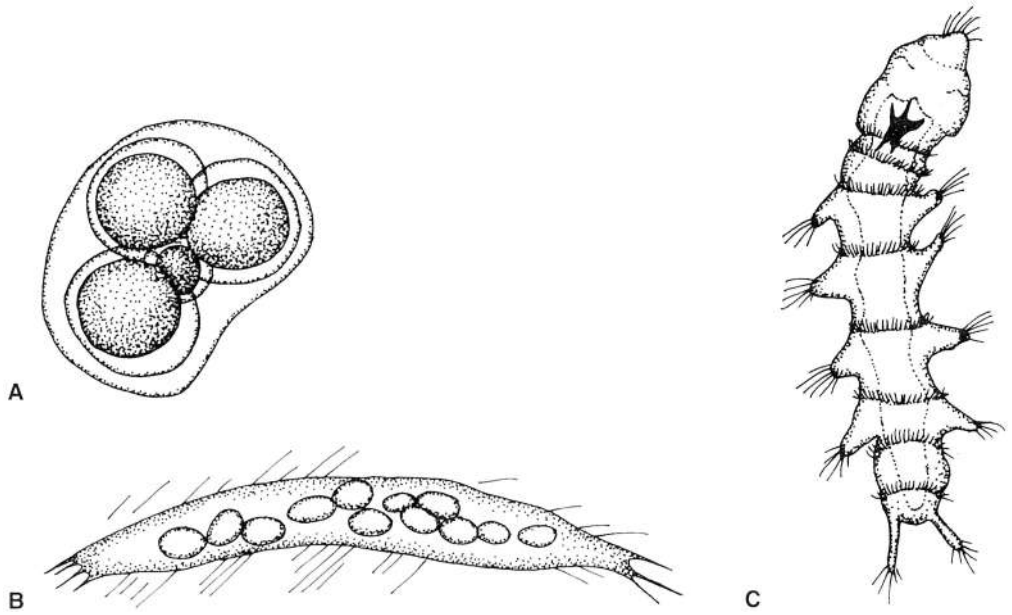


Figure 8510:6. Life stages of selected marine polychaetes. A—*Dinophilus gyrociliatus*, three female and one male (small) embryos in a developing capsule; B—*Ophryotrocha diadema*, developing embryos in egg capsule; C—*Ophryotrocha diadema*, larva recently emerged from egg capsule.

worms) consisting of the aqueous material remaining after plant fibers decay in diluent water. Add inoculum directly to culture containers in a volume not to exceed 10% of the total. Preferably use sediments of fine sand with some silt content, rather than more muddy sediments in which worms are difficult to find. Check cultures periodically for spoilage; if this occurs, clean cultures and restart. Oligochaetes have no larval stage. No separate feeding regime is required for juveniles.

3) Producing test organisms—Gravid worms in culture lay eggs that hatch in 3 to 14 d, depending on species and temperature. Newly hatched worms lack the full component of adult setae but rapidly develop these.

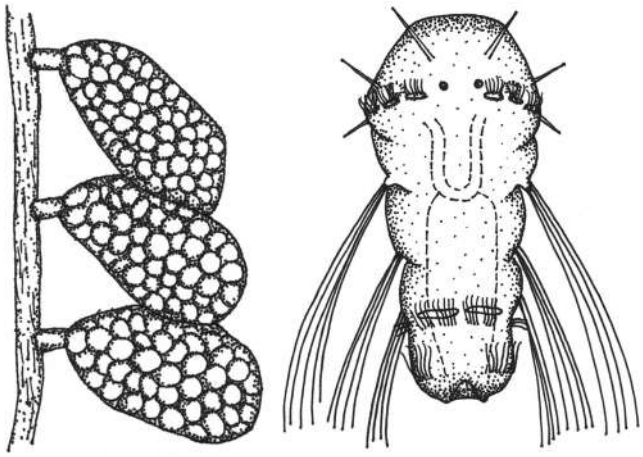


Figure 8510:7. Life stages of *Polydora cornuta*. A—Egg capsules taken from tube of female; B—Pelagic larvae.

Freshwater species generally grow better in mixed culture. The following combinations are recommended: *L. hoffmeisteri* and *T. tubifex, L. hoffmeisteri* and *B. sowerbyi, T. tubifex* and *B. sowerbyi,* and *S. heringianus* and *L. hoffmeisteri*. *T. fraseri* is a parthenogenic species that is particularly amenable to culturing; *T. verrucosus, L. variegatus*, and *M. cuticulatus* can be cultured as pure species.

4. Parasites and Diseases

Microbial growth can result from overfeeding, improperly conditioned food, or insufficient dissolved oxygen (DO). Prevent fungal growths by providing sufficient aeration. To minimize overfeeding, examine each aquarium before feeding. Do not add more food if most of the diet is uneaten, and add less food at subsequent feedings. Generally, there is adequate DO in 4-L aquariums; however, aeration can be increased to correct for any deficiency. The internal protozoan parasitic gregarines have been observed to reduce the vitality of some species of laboratory populations of polychaetes. Gregarines are common in polychaetes and oligochaetes, but it is not known if they cause similar problems in these species.

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8510 C. Toxicity Test Procedures

1. General Procedures

Use exploratory tests (see Section 8010D) to determine toxicant concentrations for short-term tests. Prepare dilution water and toxicant solutions and introduce them into test containers (as described in Section 8010F).

2. Water Supply

a. Artificial seawater: See Section 8010E.4*b*2). Use a salinity of approximately 35.5 g/kg and a pH of about 7.8 for marine populations; use lower salinity for estuarine worms.

b. Natural seawater: Determine and report quality routinely. Maintain dilution-water salinity at or near selected or normal concentration. During a test, do not allow salinity to vary by more than ±3 g/kg. Filter seawater through a 0.45-μm membrane filter.

c. Distilled or tap water: Determine quality (hardness, alkalinity, chemical constituents) and report routinely. Use a near-neutral (pH 7.0) water.

3. Exposure Chambers

a. Marine polychaetes: Use 4-L aquariums or glass jars for short-term and intermediate static and renewal tests, and for long-term tests where flow-through facilities are inappropriate. Cover aquariums to keep foreign materials out. Do not add more than 2.5 L test solution to each 4-L aquarium. Use 500-mL Erlenmeyer flasks (containing 100 mL seawater) for either short-term or long-term experiments when only one organism is placed in each flask. Close the flask with a No. 7 TFE stopper fitted with a glass tube for aeration. Use 30-mL stender dishes for larval tests. For flow-through tests, use exposure chambers [described in Section 8010F.1*c*)]. In the case of cannibalistic species [e.g., *Neanthes arenaceodentata* (Figure 8510:5)], isolate individuals during testing. Container size depends on biomass; maintain loading densities <0.5 g/L for static conditions and <0.5 g/L/d for flow-through tests at 20°C. For tests with sediment, use glass crystallizing dishes of the appropriate size for species tested and number of individuals per dish. Fill dishes with sediment 1 to 4 cm deep. Let clean seawater flow over top of sediment.

b. Freshwater and marine oligochaetes: Conduct test similar to that described for polychaete larval tests. Use shallow disposable polyethylene Petri dishes with covers for static or replacement tests. Container size depends on biomass; maintain loading densities <0.5 g/L, and preferably <0.2 g/L. Place 10 worms in each container per test concentration plus controls. Run duplicate tests. Worms can be tested individually, with 20 individuals per test concentration. For flow-through tests, consider using or adapting exposure chambers [described in Section 8010F.1*c*)].

4. Conducting Toxicity Tests

a. Test chamber setup: For static and renewal tests, set up as described in Section 8010D. In short-term tests, do not clean exposure containers. In long-term tests in which the organisms are fed, remove unused food and other materials as described in Section 8010E.4*d*2). It is unnecessary to provide a bottom substrate for any but long-term oligochaete toxicity tests. Photoperiod and light intensity do not appear to be factors in polychaete tests; however, test oligochaetes either in the dark or with a natural light/dark simulation.[1] Keep temperatures within ±2°C of the natural habitat unless testing the effect of temperature on worms.

1) Marine polychaetes—Use at least 20 worms for each test concentration. For cannibalistic species (e.g., *N. arenaceodentata*), use one worm per container. For other species, place 2, 5, or 10 worms in each container (depending on biomass and container size; see Section 8010F.3*c*). For tests with sediments, use at least three replicate dishes per sediment concentration and at least five worms per dish. Individuals of cannibalistic species do not have to be separated when placed in dishes containing sediment. Table 8510:I summarizes the ecological and test conditions for *Neanthes arenaceodentata.*

2) Freshwater and marine oligochaetes—Use at least 20 worms for each test concentration, preferably in two replicates of 10 worms each. Although the worms will intertwine when healthy, toxified individuals remain separate and toxic

TABLE 8510:I. SUMMARY OF ECOLOGICAL AND TEST CONDITIONS FOR *NEANTHES ARENACEODENTATA*

Condition	Emergent Juvenile Sediment Toxicity Test	Early Life Stage Sediment Toxicity Test[2]
Geographical distribution	North America, Europe, South Pacific Ocean[3]	
Habitat	Burrows in intertidal and subtidal sands and silts[3]	
Length of life cycle	3–4 months in laboratory[4]	
Duration of test:	20 d	28 d
Age of animal at start of test	2–3 weeks post emergence, 0.25–1 mg/individual (0.5–1 mg preferred)	≤7 days post emergence
Test temperature	20 ± 2°C average	20 ± 2°C average 20 ± 3°C instantaneous
Salinity	28–35 ppt, not varying more than ±3 ppt	30 ± 2 ppt average 30 ± 3 ppt instantaneous
pH	7–9	7–9
Dissolved oxygen	≥60% saturation	≥50% saturation
Feeding	40 mg Tetramarin® per chamber every other day	2 mg Tetramarin® per chamber on Tuesdays and 2 mg Tetramarin® and 1 mg of alfalfa per chamber on Fridays
Light cycle	16:8 or 12:12 L:D	12:12 L:D
Test chamber size	1 L	300 mL
Sediment depth (volume)	2 cm	2 cm (75 mL)
Overlying water volume	750 mL	125 mL
Number of organisms per chamber	5	1
Number of replicates	5	10
Renewal of overlying water	50% renewal every 3rd day	50% renewal once weekly
Aeration	Trickle flow (<100 bubbles/min); >50% saturation	Trickle flow (<100 bubbles/min); >50% saturation
Endpoints	Survival and growth rate (ash-free dry weight growth rate optional)	Survival and growth rate
Control acceptability criteria		
Survival	≥90%	≥80%
Growth	0.38 mg/individual/d	Positive growth
Reference toxicant ($CdCl_2$)	9.4 ± 4.4 mg/L Cd 96-h LC_{50}	5.7 ± 0.75 mg/L Cd 96-h LC_{50}

effects (e.g., the progressive disintegration of posterior segments) will be manifest.

b. Duration and type of test:

1) Short-term tests—The length of short-term or acute tests depends on the organism's life cycle (see Section 8010F.3*a*). Short-term tests may be conducted under static conditions (recommended for tests <4 d) or with periodic renewals (for tests >4 d).

2) Intermediate tests—Use intermediate tests to determine adult polychaete survival. For most species, conduct these renewal or flow-through tests for 20 to 28 d.[5]

3) Long-term tests—Long-term tests are either partial or full life-cycle tests. Partial life-cycle tests begin with the polychaete trochophore larval or oligochaete egg-case stage and end with sexual maturity. Full-life-cycle tests also begin with the larvae or egg-case stage but continue through reproduction and the offspring's subsequent egg production or larval settlement. Because of the long duration, conduct these tests using either periodic renewal or flow-through conditions.

Select and prepare test concentrations as described in Section 8010F.2*b*. Measure and mix dilution water and stock toxicant solutions via proportional diluters and deliver to exposure chambers as described in Section 8010F.1. Make tests in flow-through exposure chambers similar to those described in Sections 8740C.3 and 8010F.1. Renewal tests using up to 4-L exposure chambers may be necessary if flowing water is unavailable.

The duration of long-term tests depends on the organism's life-cycle. For example, for polychaetes it varies from about 1 month (*C. capitata* and *P. cornuta*) to 3 or more months (*N. succinea, N. virens,* and *N. arenaceodentata*).

c. Test organisms: See 8510B.

d. Testing procedure:

1) Short-term tests—Set up and conduct renewal tests as described in Section 8010D.2. Determine survival of adults by checking exposure chambers at 1, 2, 4, 8, 18, and 24 h, then once or twice daily thereafter. Dead specimens generally are pale and swollen and lie on the bottom; live specimens usually respond to physical stimulation. If the tests last more than 4 d, renew solutions, preferably daily but at least every 4th day. In short-term tests with polychaete larvae, determine survival after 96 h by microscopic examination. The absence of larvae generally indicates death because small larvae rapidly decompose.

2) Intermediate tests—Set up test chambers described in ¶ *a* above to determine adult lethality (LC_{50} or incipient LC_{50}). Examine test containers daily to determine survival. If no organisms are killed after a certain exposure period, report the period beyond which there is no further kill and the percentage killed in each test concentration. For contaminated sediment tests, sieve contents of each replicate dish and count number of survivors. If a graded series of mixed sediments has been used, calculate LC_{50} based on percentage of contaminated sediment.

3) Polychaete life-cycle tests beginning with the trochophore larval stages—Set up as described previously in this section. Conduct test through sexual maturity, with test periods varying from 3 to 4 weeks (*C. capitata*) or from 2 to 3 months or longer (*N. arenaceodentata, N. succinea*, and *N. virens*). Feed larvae as

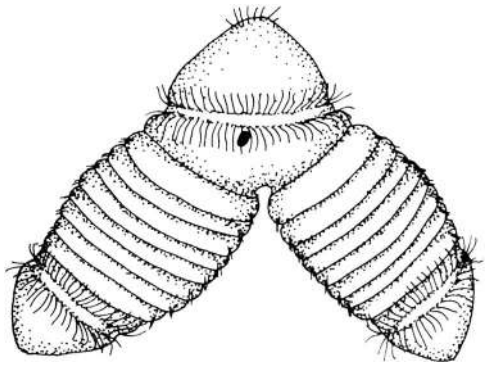


Figure 8510:8. ***Capitella capitata.*** Abnormal larva.

described in 8510B.3*a*2). Determine survival at least twice weekly for *C. capitata* and once a week for *Neanthes*. During the early part of the study, count organisms on bottom of exposure chambers. If a renewal test is being conducted, decant supernatant fluid, examine under a dissecting microscope, and replace fluid with fresh test solution. For flow-through tests, remove chambers from exposure box, count organisms, and replace chamber. If no organisms are observed by the third examination, terminate that test chamber.

When *C. capitata* is the test organism, remove test chambers after about 15 to 16 d and every 2 d thereafter to check for eggs in the coelom, and later, zygotes along the sides of the tube (use a dissecting microscope for these observations). Remove females when developing eggs are in the trochophore stage and count the larvae. Discard females and larvae after counting larvae, and record the number of dead and deformed larvae. Continue to examine each exposure chamber every 2 d to detect females incubating larvae until all females have been removed and the total number of larvae recorded.

Abnormal larvae of *C. capitata* (Figure 8510:8) have been observed during life-cycle tests when exposed to sublethal concentrations of chromium, zinc, or detergents.[6]

For *N. succinea*, set up exposure chambers as described in ¶ *a* above, with 25 larvae in each 1-L exposure chamber or 10 larvae in each flow-through exposure chamber. Use 10 chambers per concentration tested. Because these worms fight and are cannibalistic when crowded, prepare additional exposure chambers or reduce each test-chamber population to five individuals after the first month. Continue tests until animals reach the epitoke stage, then determine individual lengths and total weights and compare with those in the control.

4) Polychaete life-cycle tests beginning with the newly settled larval stage—These tests will vary in duration from about 1 month (*C. capitata*) to 3 months or more (*N. arenaceodentata*). Set up tests as described previously with newly settled larvae. Use a minimum of at least two specimens per flask and 10 flasks per concentration. As tests progress, count organisms as above. Examine for survival once or twice per week as in ¶ *d*3) above.

For *N. arenaceodentata*, use recently emerged juveniles with approximately 18 to 21 setigerous segments. If a renewal test is conducted, place four worms in each of five 4-L exposure chambers with 2.5 L test solution. Set up five containers for each test concentration and control. For flow-through tests, place two larvae in each of 10 exposure chambers for each test concentration and the controls. At 25 d, look through the container to examine worms for eggs in the coelom. If necessary, move mature worms among the replicates of a given treatment to pair males with females. Mature eggs reach 450 μm diam and are yellowish-orange. Examine at 5-d intervals until eggs are noted and then at 2- to 3-d intervals to determine whether eggs are being laid. The females die within 1 d of laying eggs, and the males incubate them for about 3 weeks. The life cycle is complete when juvenile worms emerge from the parental tube. Remove males and count larvae.

5) Oligochaete life-cycle tests—Set up as described above. Test duration depends on test conditions and endpoints chosen. Use procedures similar to those for polychaetes.

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8510 D. Sediment Test Procedures Using the Marine Polychaete *Neanthes arenaceodentata*

1. General Procedures

Two chronic sediment test procedures with the polychaete *Neanthes arenaceodentata* are detailed below. The 20-Day Emergent Juvenile Sediment Test measures survival and growth effects on 2- to 3-week post-emergence juvenile worms, and has been used routinely for a number of years in parts of the United States, Canada, and Hong Kong.[1] The U.S. Army Corps of Engineers has more recently developed the 28-Day Early Life Stage Sediment Test, which measures survival and growth effects on ≤7 d post-emergence worms.[2] While the test is longer, the use of younger worms and one worm per chamber increases sensitivity and decreases food competition, increasing the potential for detecting effects on growth.[2,3] Before conducting a definitive sediment test with *N. arenaceodentata*, conduct preliminary tests to become familiar with the test procedures below. See Table 8510:I for a summary of ecological and test conditions for this species.

2. Water Supply

a. Artificial seawater: See Section 8010E.4*b*2). Use a salinity of approximately 28 to 35 g/kg, not varying more than ±3 g/kg from target salinity, and a pH of 7 to 9 for the 20-d test. Use an average salinity of 30 ± 2 g/kg and a pH of 7 to 9 for the 28-d test.

b. Natural seawater: Determine and report salinity routinely. Salinity and pH requirements are the same as described for artificial seawater. During the test, do not let salinity vary more than ±3 g/kg (instantaneous) for either test.

3. Sediment

a. Collection: Collect sediment with a benthic grab (e.g., a van Veen bottom sampler) [see Section 10500B.3*a*3)]. Collect multiple samples at a site to obtain at least 4 L of sediment. Press sediments (using no water) through a 1.0-mm sieve to remove macroscopic invertebrates, if necessary. Place all sieved sediments in a plastic bucket and mix later in the laboratory. Hold sediments in the dark at 4°C until start of test; use within 2 weeks of collection, if possible. Collect uncontaminated or reference sediments from an area with similar-sized particles free from contaminants.

b. Sediment chemistry: Analyze sediments for grain size, total organic carbon, metals, organic compounds, total Kjeldahl nitrogen, interstitial salinity, pH, porewater sulfide, and total ammonia concentrations.

4. Exposure Chambers

For the 20-Day Emergent Juvenile Sediment Test, use as a test chamber a 1-L glass beaker with an internal diameter of 10 cm. For the 28-Day Early Life Stage Sediment Test, use a tall-form 300-mL glass beaker. Cover beaker with a watch glass or other device to minimize evaporation and reduce contamination. Aerate each chamber through a 1-mm-opening glass pipet that extends between the beaker spout and the watch glass cover to a depth of not closer than 2 cm from the sediment surface. See Figure 8510:9.

5. Conducting the 20-Day Emergent Juvenile Sediment Toxicity Test

a. Test chamber setup: Use five replicates with each sediment sample. Add sufficient sediment to each beaker to make a 2.0-cm layer. Carefully add approximately 750 mL seawater to each beaker with minimal physical disturbance of sediment. Aerate at the rate of about 1 to 2 bubbles/s. Prepare sediments and overlying water the day before starting the test to provide time for sediment and seawater to adjust to test temperature.

b. Environmental conditions: Although photoperiod is not critical, usually perform sediment tests with *Neanthes arenaceodentata* at either 16 h light/8 h dark or 12 h light and dark. Perform tests at 20 ± 2°C. Porewater ammonia from the bulk sediment should be ≤115 mg/kg, and overlying (water immediately above sediment layer) ammonia at test initiation should be ≤10 mg/L. Overlying sulfide levels at test initiation should be ≤3.4 mg/L. If ammonia or sulfide levels are higher, it may be necessary to purge with up to 2 water renewals daily until levels are below those listed above to ensure ammonia and sulfide levels are not a source of toxicity.[4]

c. Test animals: The male *N. arenaceodentata* incubates the developing embryos in his tube for approximately 3 weeks after fertilization. Embryos do not feed during this time; they subsist on the yolk in the embryo. At 21 to 24 d, juvenile worms leave the male parent's tube and begin feeding. In the sediment test, use juvenile worms (Figure 8510:10) that are

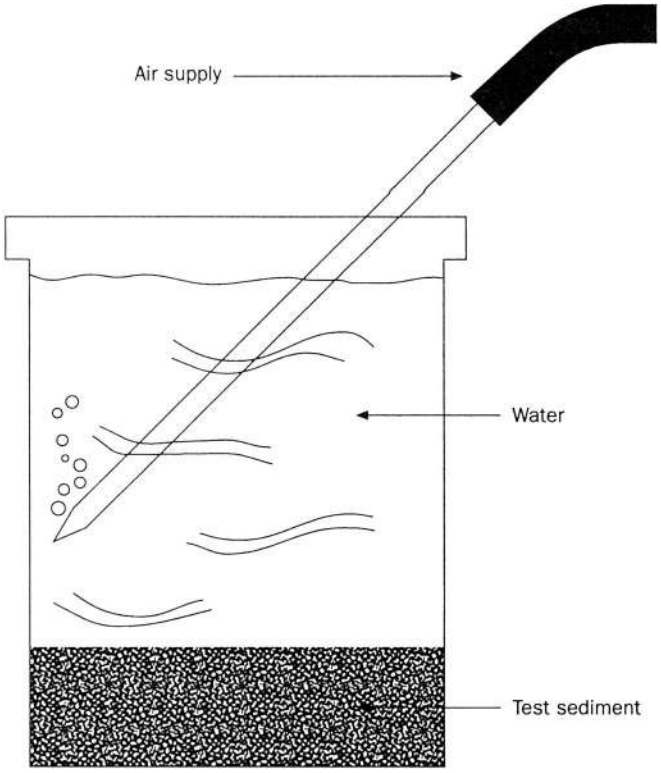


Figure 8510:9. Experimental setup for sediment testing.

2 to 3 weeks post-emergent (approximately 5 to 6 weeks after fertilization) and 0.25 to 1.0 mg/worm dry weight (0.5 to 1.0 mg/worm preferred).[5] [See 8510B.3*a*3)c).] Place all juvenile worms to be tested in a white, enamel pan together and select uniform-sized worms. Generally, place 20 to 30% more worms than needed in the pan and discard the larger and smaller ones. Place five worms in a Petri dish with seawater; the number of Petri dishes equal the total number of replicates to be tested plus five additional dishes, each containing five worms. Choose five dishes at random and weigh the five worms together to obtain initial dry weight (see ¶ *e* below). Select additional worms for the reference toxicant test (see ¶ *f* below).

Randomly distribute worms to the beakers, making sure that all five worms are removed from the Petri dish. The test begins when worms are added to the sediment.

Add 1 mL food slurry solution to each test container every other day. To make this solution, grind food* into a fine powder and mix with seawater at a ratio of 1.0 g food to 25 mL seawater. When feeding and water changes occur on the same day, add food after the water change.

d. Test monitoring: During the test, examine beakers daily to ensure that aeration is adequate. On Days 3, 6, 9, 12, 15, and 18 for the 20-d test, replace one half of the seawater in each beaker with clean seawater. Measure DO, temperature, pH, and salinity on first days, last days, and water-change days (before the change). Measure overlying ammonia on first day, Day 3 before water change, and last day, and measure overlying sulfides on first and last days.

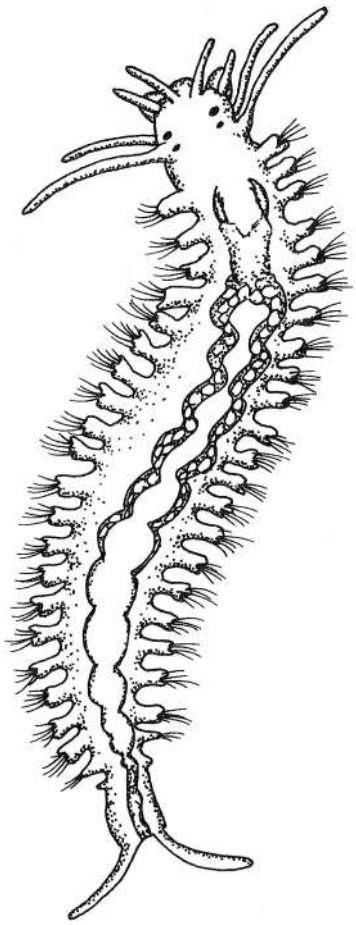


Figure 8510:10. ***Neanthes arenaceodentata.*** Larva recently emerged from male parent's tube.

e. Termination of the test: On Day 20, remove worms from each test container, count, place in a small, clean Petri dish, and wash in distilled water. Place worms from each replicate in a pre-weighed aluminum pan and dry at 60°C until constant weight (overnight); record dry weight of worms in each pan. Record as the dry weight per worm per day as calculated using the formula:

$$G = (W_t - W_i)/T$$

where:

G = estimated individual growth rate, mg dry weight/d,
W_t = mean estimated individual dry weight, mg, at termination of test,
W_i = mean estimated individual dry weight, mg, at start of test, and
T = exposure time, d.

Using the mean individual growth rate per worm per day facilitates comparison of results between different sediments tested and with other experiments. Because each test is not

* TetraMarin®, or equivalent; widely available in aquatic pet supply stores.

started with exactly the same size worm (weight), expressing growth as a rate function rather than absolute mass per worm normalizes results.

There has been some indication that the grain size, and thus the density, of the sediment in the gut at test termination can skew growth rate results.[6] If the test is being performed on sediments with large variations in grain size, it may be beneficial to record ash-free dry weight (AFDW) growth rate, which removes the weight of gut sediment, in addition to standard dry-weight growth rate. Before test termination, place empty weigh pans into a muffle furnace set at 550°C for 2 h. Carefully remove the pans and place into a desiccator until the temperature equilibrates, then weigh. Terminate the test as described in the paragraph above. Once the dry weight has been determined, pinch the pans closed to prevent loss of ashes. Place the pans in a muffle furnace set at 550°C for 2 h. Carefully remove the pans and place into a desiccator until the temperature equilibrates. Weigh the pans. The AFDW is the difference between the weight of dried worms plus pan and the weight of ashed worms plus pan. Divide the AFDW by the number of worms in the pan then proceed to calculate the mean individual growth rate, as described above. NOTE: In order to determine AFDW mean individual growth rate, the worms used to determine initial weight on Day 0 will also need to be ashed.

f. Reference toxicant test: Make a reference toxicant test (positive control) concurrently with the sediment test and use it to check the test animals' health. Use reference test animals from the same batch as those used in the sediment test. Cadmium chloride (expressed as *cadmium*) is commonly used as the reference toxicant; however, other chlorides of metals or organic compounds may be used. The reference toxicant test is a standard 96-h test without sediment.

g. Control acceptability criteria: Survival in the control must be ≥ 90%. The mean individual growth rate in the control must be ≥0.38 mg/ind/d for the test to be considered valid. Ideally, the growth rate will be ≥0.72 mg/ind/d.[5]

6. Conducting the 28-Day Early Life Stage Sediment Test[2]

a. Test chamber setup: Use ten replicates with each sediment sample. Set up two additional surrogate chambers per sample to measure porewater ammonia on Day 0 and Day 28. Add sufficient sediment (~75 mL) to each beaker to a depth of 2 cm. Carefully add 125 mL seawater to each beaker with minimal physical disturbance of sediment. Aerate at the rate of about 1 to 2 bubbles/s. Prepare sediments and overlying water the day before starting the test to provide time for sediment and seawater to adjust to test temperature.

b. Environmental conditions: Maintain photoperiod at 12 h light/12 h dark. Perform tests at a pH of 7 to 9, DO >50% saturation, an average temperature of 20 ± 2°C (20 ± 3°C instantaneous) and an average salinity of 30 ± 2 ppt (30 ± 3 ppt instantaneous). The total ammonia concentration in the porewater from the Day 0 surrogate chamber must be <20 mg/L for each sample before test initiation. If ammonia is not a contaminant of concern (e.g., dredged sediment testing), perform up to 2 water exchanges daily until the desired ammonia level is achieved in all test treatments, then proceed with test initiation. It may be necessary to set up more surrogate chambers to monitor porewater ammonia reduction if ammonia levels are high.

c. Test animals: Use worms that are ≤7 day post-emergent larvae (approximately 3 to 4 weeks after fertilization). Place more juveniles than necessary in a white pan then place one randomly selected worm into a 50-mL beaker. The number of beakers required will equal the total number of test chambers required. Randomly place 5 worms each into 5 additional beakers and set aside for initial weight determination. After all the worms are added to all beakers, observe each to determine that all worms are present and healthy. Then, add worms to the test chamber by gently pouring the contents of beaker into the test chamber. Test animals may be trapped in the surface tension of the water. Animals can be freed of surface tension by gently dropping water from a pipet onto the animal. The test begins when worms are added to the sediment. The worms from the five beakers set aside for pre-weight determination are washed in deionized water, placed on pans, dried in an oven at 60°C for 24 h, and then weighed to determine individual dry weight for calculation of growth rate.

d. Test monitoring: Each organism is fed 2 mg of Tetramarin® on Tuesdays and Fridays and 1 mg of ground alfalfa on Fridays. Water renewals (50%) are conducted once weekly prior to feeding. Measure DO, pH, temperature, ammonia, and salinity before water change on three rotating replicates per treatment. Observe each chamber twice a week for animal activity and condition of sediment.

e. Termination of the test: On Day 28, measure DO, pH, temperature, and salinity on 3 replicates per treatment. Additionally, measure porewater ammonia in one surrogate chamber per treatment. Sediment from each chamber is gently poured through a 425-μm sieve and organisms recovered. Determine survival by gently prodding the animal and observing presence or absence of movement. Calculate growth rate following the procedures in 8510D.5*e*.

f. Reference toxicant test: Follow instructions in 8510D.5*f*.

g. Control acceptability criteria: Control survival must be ≥80%, and growth rate must be positive.

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8510 E. Sediment Test Procedures Using the Marine Polychaete *Polydora cornuta*

1. General Procedures

The following procedures are for a short-term (14-d) test. Before conducting a definitive sediment test with the polychaete *Polydora cornuta,* conduct preliminary tests to become familiar with the test procedure. See Table 8510:II for a summary of the ecological and test conditions for this species.

2. Water Supply

See 8510D.2.

3. Sediment

See 8510D.3.

4. Exposure Chambers

As a test chamber, use a 300-mL glass beaker covered with a watch glass to minimize evaporation. Continuously aerate each chamber overnight via a 1.0-mm opening glass pipet, at the rate of 2 to 3 bubbles per second. The pipet should not be closer than 2.0 cm from the sediment surface. See Figure 8510:9 for a similar setup.

5. Conducting the Sediment Toxicity Test

a. Test chamber setup: Use at least five replicates for each sediment sample. Add approximately 50 mL sediment to make a 2.0-cm layer.

b. Duration and type of test: 14 d.

TABLE 8510:II. SUMMARY OF ECOLOGICAL AND SEDIMENT TEST CONDITIONS FOR CONDUCTING TESTS WITH *POLYDORA CORNUTA*[1]

Condition	Description
Geographical distribution	North America, Europe
Habitat	Conducts silt tubes in intertidal or subtidal estuarine environments
Type of test:	
Sediment	
Chronic	14 d
Life cycle	3–4 weeks, capsule number; post-emergence larval count; not a standard test
Test temperature	20–22°C
Salinity	30 ± 4 ppt
Feeding	1:1 mixture of ground fish food flakes (Tetramin™) and powdered *Enteromorpha* sp. at the rate of 2 mg/worm 3 times/week
Light cycle	No special requirements
Control mortality	Not to exceed 20%
Endpoints	
14-d test	Death
Life cycle	Number of young

c. Test organism: Animals used in the test should be 3 to 4 weeks post release from the capsule (Figure 8510:7A) and have established a mucoid tube. Take worms from the stock colony and gently wash the sediment through 1.0-mm sieve. Tubes retained on the sieve should be gently transferred with a fine brush to a shallow pan containing seawater. Worms will either emerge from their tubes or remain within them. Gently

probe the tube with a fine brush to determine the worm's health; discard any injured or unhealthy worms. Test worms should be of similar size.

d. Testing procedure: Place five worms in a small dish and randomly assign them to a test chamber. Transfer worms to the test chamber via a pipet. Verify the number of worms in each chamber. The air supply should be turned off during the transfer. Raise the water level in each container to 250 cm. Resume aeration and cover the dish. Set aside three groups of five worms each to determine the initial dry weight for a reference (see 8510D.5*c*).

e. Test monitoring: Examine each chamber frequently—daily if possible. Look for worms emerging from their tubes and lying on the sediment surface. The water should be changed once during the experiment. Worms should be fed according to the procedure in 8510B.3*a*2).

f. Termination of the test: End the test on Day 14 and follow the procedures outlined in 8510D.5*e*.

g. Reference toxicant test: See 8510D.5*f*.

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8510 F. Sediment Test Procedures Using the Freshwater and Marine Oligochaetes *Pristina leidyi, Tubifex tubifex,* and *Lumbriculus variegatus*

1. General Procedure

The following procedures comprise short-term acute tests. Oligochaetes also can be used to measure reproductive effects[1,2] and in combined toxicity and bioaccumulation tests.[3]

Before conducting a definitive sediment test with the oligochaetes, *Pristina leidyi, Tubifex tubifex,* or *Lumbriculus variegatus,* conduct preliminary tests to become familiar with the test procedures given below.

2. Water Supply

Use fresh water for all tests. It may be moderately hard synthetic water prepared with a commercially available system,* deionized water and reagent-grade chemicals, receiving water, or synthetic water modified to reflect receiving water hardness.

3. Sediment

Collect sediment with a benthic grab (e.g., an van Veen or Ekman sampler). See Sections 10500B.3*a*3) and 6), and 8510D.3.

4. Exposure Chambers

Use a 250-mL beaker with a 6-cm internal diameter as a test chamber. Cover beakers with a watch glass to minimize evaporation and reduce contamination. Aeration is unnecessary.

5. Conducting the Sediment Toxicity Test

a. Test chamber setup: See 8510D.5*a*, but add only about 100 mL water to each beaker.

b. Environmental conditions: Set lighting for a 16 h light/8 h dark photoperiod at an intensity of 550 to 1050 lux (50 to 100 ft-c). Make tests with *P. leidyi* at 24 ± 1°C and with *T. tubifex* and *L. variegatus* at 20 to 25°C.

c. Test animals: Use specimens of mixed age in these tests. Place worms in a white enamel pan and select healthy-appearing specimens. Generally place 20 to 30% more worms than needed in the pan. Use five worms of *P. leidyi* and *T. tubifex* per replicate and 10 of *L. variegatus*. Place worms for each replicate in a Petri dish with water, with the number of Petri dishes equaling the total number of replicates to be used. Select additional worms for the reference toxicant test. See 8510D.5*f*.

Randomly distribute worms to beakers, making sure that all worms are removed from the Petri dish. The test begins when the worms are added to sediment.

Do not feed animals during the test.

d. Test monitoring: Measure DO concentration and temperature on the first and last days, and on any day when water changes are made.

e. Termination of the test: On Day 10, remove worms from each test container and count the number of survivors. Record separately the number of survivors from each replicate.

f. Reference toxicant test: See 8510D.5*f*.

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* Millipore Milli, or equivalent.

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8510 G. Data Evaluation

1. Short-Term and Intermediate Adult Survival Studies

Determine the LC_{50} values for each exposure period (as described in Section 8010G.1*a*). A useful supplementary measure is to determine LT_{50} (time to 50% mortality) in comparative exposures to single toxicant, effluent, water, or sediment concentrations. LT_{50}s also provide useful ancillary information for LC_{50} studies.

2. Polychaete Life-Cycle Studies Beginning with the Trochophore and Settled Larval Stages

The number of females forming and laying eggs, and the number of offspring produced are inversely related to sublethal toxicant concentrations at levels below the LC_{50}. They provide a more subtle measure of effects than the LC_{50}. Record life-cycle data for each concentration of toxicant as follows: number of females forming eggs, number of females laying eggs, and number of eggs and live offspring produced. Compare these data (expressed as percentages) for all test concentrations with those obtained from the controls. Use statistical and reporting techniques described in Sections 8010G and H.

8610 MOLLUSKS*

8610 A. Introduction

1. Characteristics and Ecology

The Phylum Mollusca, the second largest phylum of the animal kingdom, is made up of such forms as clams, mussels, oysters, snails, slugs, octopuses, and squids. The life cycle of mollusks varies from about 1 to more than 300 years.[1] In numbers of species (about 100 000 living and 35 000 fossil), mollusks are second only to the arthropods. Their ecology also is very diversified: they have been able to live successfully in nearly all terrestrial, freshwater, and marine habitats, from the greatest depths of the ocean to the highest altitudes recorded for animal life.[2,3] Mollusks are important ecologically and are a source of food for human beings around the world.

2. Types of Tests

Mollusks, particularly marine gastropods (snails and slugs) and bivalves (clams, mussels, oysters, etc.), have been used extensively as bioassay test species throughout the United States. Two types of larval development toxicity tests are described in this section. The marine bivalve larval development test is suitable for water toxicity testing for all coasts and uses oysters (*Crassostrea* spp.), mussels (*Mytilus* spp.), or quahog clams (*Mercenaria mercenaria*). The marine gastropod larval development test with red abalone (*Haliotus rufescens*) is used in bioassays on the Pacific Coast. These aqueous-phase larval development tests are conducted on varying types of matrices, including ambient environmental samples, effluents, receiving waters, chemically spiked aqueous exposures, and sediment elutriates (dredge material characterization). A modification to this testing procedure has also been employed to evaluate the toxicity of potential contaminants released from the sediment–water interface or from the interaction of developing larvae in direct contact with sediments.[4]

To determine the potential for chemical bioaccumulation, there is a test that exposes adult bivalves, such as bent-nose clams (*Macoma nasuta*), to sediments in the laboratory. At test termination, tissues can be excised and analyzed chemically for pollutants (e.g., pesticides and heavy metals) to determine whether animals have accumulated toxic substances above background or ecologically significant levels. Based on the purpose of these tests, the organisms may be allowed to purge gut contents so uptake-versus-gut-content assessments can be made.[5]

A field test (*in situ*) consists of placing freshwater or marine species of bivalves in cages and suspending them in the water column or placing them in direct contact with sediment for 28 d or longer. Endpoints may be survival, growth, or accumulation of toxic substances above target threshold levels in tissues excised for chemical analysis.

Other procedures for toxicity tests using mollusks have been published.[6–10]

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* Approved by Standard Methods Committee, 2016.
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8610 B. Selecting and Preparing Test Organisms

1. Selecting Test Organisms

In accordance with the criteria listed in Section 8010E.1, the recommended test species include (but are not restricted to) the following:

a. Marine gastropods:
Family Haliotidae:
Haliotus rufescens (U.S. west coast)

b. Marine bivalves:
Family Ostreidae (Section 10900, Plate 21:N):
Crassostrea gigas (U.S. west coast)
Crassostrea virginica (U.S. east coast)
Family Veneridae:
Mercenaria mercenaria (all of U.S. coasts) (Section 10900, Plate 21:J)
Family Tellinidae:
Macoma balthica (Atlantic Ocean)
Macoma nasuta (U.S. west coast)
Family Mytilidae:
Mytilus spp. (cosmopolitan) (Section 10900, Plate 21:L)
(There are three species of the genus in North America: *Mytlus edulis*, eastern North America and Oregon north; *M. trossulus,* central and northern California; *M. galloprovincialis,* southern California and aquacultured organisms in Washington and Oregon.)

c. Freshwater bivalves:
Family Unionidae
Ellipstio complanata
Pygenodon grandis
Family Corbiculidae
Corbicula fluminae
Family Sphaeriidae
Sphaerium simile (Section 10900, Plate 21:H)
Musculium transversum
Family Dreissinidae
Dreissena polymorpha (Section 10900, Plate 21:F)

d. Other freshwater and marine mollusks: Also used in toxicity tests, but not discussed, are the freshwater *Anodonta imbecillis* (Section 10900, Plate 21:E), and the marine species *Ostrea lurida*, *Ostrea edulis, Argopecten irradians irradians*, *Spisula solidissima*, *Mulinia lateralis*, *Perna* spp., and *Rangia cuneata* (Section 10900, Plate 21:M).

2. Collecting and Conditioning Test Organisms

a. Marine bivalves:

1) *Crassostrea gigas* (Pacific oysters), *Crassostrea virginica* (Eastern oysters), *Mercenaria mercenaria* (quahog clams), and *Mytilus* spp. (mussels) may be collected in the field, but those purchased from commercial suppliers who deal in bioassay organisms usually will spawn more consistently. Identify all field-caught bivalves to species.[1]

2) *Macoma nasuta* (bent-nose clams) commonly inhabit intertidal areas, such as bays and harbors. They reside in the upper few cm of sediment, which may be collected by hand and sieved to acquire specimens. Identify species of field-caught organisms before using in testing procedures. Wild-stocks of these animals also are commercially available. Hold clams as briefly as possible and introduce into test sediments soon after collection.

b. Marine gastropods: Collect *Haliotus rufescens* (red abalone) adults from the field or purchase from commercial dealers. Mature red abalone can be collected on rocky substrates from the intertidal zone to depths exceeding 30 m. They are most commonly found in crevices where there is an abundance of macroalgae. State collection permits are always required for collecting abalone. While abalone captured in the wild can be induced to spawn, those grown or conditioned in culturing facilities have been more dependable. Commercial mariculture facilities can supply ripe organisms (sexually mature abalones are usually about 70 mm or more in shell length). In any case, obtain brood stock from sources free of contamination by toxic substances to avoid genetic or physiological preadaptation to pollutants. Brood stock also need to be obtained from areas free of diseases (e.g., withering foot). In the laboratory, identify the organism's sex by inspecting the gonads (under the right posterior edge of the shell). An abalone placed upside down on a flat surface will soon relax and begin moving the foot trying to right itself. During this movement, bend the foot away from the gonad area and determine the sex. The ovary is jade green, the testes are cream-colored. When the gonad fully envelopes the dark blue-gray conical digestive gland and is bulky along its entire length, the abalone is ready for spawning.[2] Ripe spawners have a distinct color difference between the gray digestive gland and the green or cream-colored gonad. Less developed gonads appear gray (in females) or brown (in males).

c. Freshwater bivalves:

1) Zebra mussels (*Dreissena polymorpha*) are the only freshwater bivalves that attach permanently to substrates with byssal threads. They were introduced into the Great Lakes and have spread to other freshwater localities. Because they attach themselves to substrate, they can be collected in large numbers. There are restrictions for their import into areas where they have not been established.

2) Asian clams (*Corbicula fluminea)*, also introduced into North American waters, occur in large numbers and are easily collected.

3) Fingernail clams (*Musculium transversum)* are small and are convenient test animals because of their size.

3. Culturing

a. Marine bivalves: Maintain adult bivalves in glass aquaria or fiberglass tanks and continuously supply with high-quality, fresh seawater (salinity 18 to 34 g/kg, depending on location and species). If a continuous flow-through system is not available, use natural seawater or if demonstrated to be effective, commercially available sea-salt preparations made with deionized water. Change water on a daily basis and use a recirculating system. Incoming water may require activated charcoal or other type of filtration to remove excess nutrients or suspended solid materials and to maintain high water quality. Observe animals daily, and discard any obviously unhealthy animals. Before spawning or direct use in a toxicity test, brush or gently scrape animals to remove barnacles or other encrusting organisms. If conditioning bivalves to spawn, hold for 1 to 8 weeks at 20°C for oysters and quahogs and

15 to 18°C for mussels. During extended holding and conditioning, provide animals with an adequate supply of natural or cultivated phytoplankton to ensure adequate nutrition. Alternatively, some suppliers will precondition animals before shipping, but the health of the test organisms should be determined by examining survival success for the first few days if possible. However, if the organisms are extremely ripe they may spawn immediately upon transfer to water and it may be necessary to begin toxicity testing the same day that the animals are received.

b. Marine gastropods: Keep abalone separated in aerated tanks with flowing seawater. Ideal maintenance temperature is 15 ± 1°C, the toxicity test temperature. If brood stock will be held for longer than 5 d at the testing facility, feed brood stock blades of the giant kelp, *Macrocystis* (Section 10900, Plate 2A:E). Feed to slight excess; large amounts of uneaten algae will foul culture water. If *Macrocystis* is unavailable, substitute other brown algae [*Nereocystis* (Section 10900, Plate 2A:G), *Egregia, Eisenia*] or any fleshy red alga.[2] For brood stock, preferably use abalone 7 to 10 cm in shell length. They are easier to handle than larger ones, and can be spawned more often (about every 4 months under suitable culture conditions).[3]

4. References

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8610 C. Short-Term Test Procedures Using Marine Mollusk Larvae

1. General Procedures

Test conditions are summarized in Table 8610:I for marine bivalves and Table 8610:II for marine gastropods. Use exploratory range finding tests (see Section 8010D) to determine toxicant concentrations for short-term tests. Prepare dilution water and toxicant solutions and introduce them into test containers as described in Section 8010F.

2. Water Supply

a. Marine bivalve larvae: Use high-quality natural seawater, preferably filtered to 20 μm. Test salinity (18 to 34 g/kg) is dependent on the location and species selected for the test, but regardless of selected salinity, do not vary the salinity by more than ±1 among treatments.

b. Marine gastropod larvae: Tests require a marine laboratory with a supply of clean, 20-μm-filtered seawater, with salinity 34 ± 2 g/kg.

3. Exposure Chambers

a. Marine bivalve larvae: Use 30-mL borosilicate glass vials, five chambers per concentration.

b. Marine gastropod larvae: Use 30- to 600-mL borosilicate glass vials or beakers, five chambers per concentration. Tissue culture flasks may also be used. For both tests, cover chambers during the test with glass plates or individual caps to avoid contamination from air and excessive evaporation of test solutions. Soak new test chambers in dilution water overnight.

4. Conducting the Toxicity Tests

a. Spawning and fertilization: If mature brood stock are shipped by a supplier, if possible allow 2 d or more for laboratory acclimation to test salinity and temperature conditions before spawning induction; this should increase the probability of successfully spawning viable gametes. If the animals are very ripe this may not be possible as they will spawn immediately upon transfer to water. Always bring brood stock up to acclimation temperature slowly to avoid premature spawning. Before beginning the spawning-induction process, be sure that test solutions will be mixed, sampled, and temperature-equilibrated in time to receive the newly fertilized eggs. Spawning induction generally takes about 3 h, but if embryos are ready before the test solutions are at the proper temperature, the delay may allow embryos to develop past the one-cell stage before transfer to the toxicant. Transfer can then damage the embryos, leading to unacceptable tests results.

Table 8610:I. Summary of Test Conditions for the Marine Bivalve Larval Toxicity Test

Parameter	Conditions
Test species	Mussels (*Mytilus* spp.) Pacific oysters (*Crassostrea gigas*) Eastern oysters (*Crassostrea virginica*) Quahog clams (*Mercenaria mercenaria*)
Test chamber	30-mL glass vials
Test solution volume	10 mL minimum
Test type	Static non-renewal
Number of replicates	5
Number of larvae per replicate	15–30 per mL, minimum average of 150 per replicate
Salinity	Mussels: 18–34 g/kg Pacific oysters: 18–34 g/kg Eastern oysters: 18–32 g/kg Quahog clams: 18–32 g/kg Maintained at ±1 g/kg of chosen salinity for all species
Temperature	Mussels: 15 ± 1°C Oysters and clams: 20 ± 1°C
Dissolved oxygen (DO)	≥4 mg/L
Aeration	Provide gentle aeration if DO drops <4 mg/L
Light cycle	16 h light:8 h dark
Light quality	Ambient
Test duration	48 h
Observations during the test	Temperature, salinity, DO and pH daily in surrogate chamber for each test concentration
Endpoints	Survival and normal development
Acceptability criteria	Survival in the controls: ≥70% for all species Normal shell development in the controls: Mussels and oysters: ≥70% Clams: ≥60%

Table 8610:II. Summary of Test Conditions for the Marine Gastropod Larval Toxicity Test

Parameter	Conditions
Test species	Red abalone (*Haliotis rufescens*)
Test chamber	30- to 600-mL glass vials, beakers, or tissue culture flasks
Test solution volume	20 mL minimum in 30-mL chamber, up to 200 mL in 600-mL chamber
Test type	Static non-renewal
Number of replicates	5
Number of larvae per replicate	5–10 per mL, minimum average of 200 per replicate
Salinity	34 ± 2 g/kg
Temperature	15 ± 1°C
Dissolved oxygen (DO)	≥4 mg/L
Aeration	Provide gentle aeration if DO drops below 4 mg/L
Light cycle	16 h light:8 h dark
Light quality	Ambient
Test duration	48 h
Observations during the test	Temperature, salinity, DO, and pH daily in surrogate chamber for each test concentration
Endpoint	Normal shell development Survival is an optional endpoint
Acceptability criterion	80% normal shell development in the controls

1) Marine bivalve larvae—Remove at least a dozen bivalves from conditioning chamber and place them in a container filled with seawater at about 20°C for oysters and quahogs and 15°C for mussels. They should resume pumping within about 30 min. Over a 15- to 20-min period, slowly increase the temperature; do not exceed 32°C for oysters and quahogs, and 20°C for mussels. If animals do not spawn within 30 min, return them to water at the original temperature, and, after about 15 min, raise temperature again. Other methods have been used successfully to induce bivalves to spawn. Adding algae into the water may work for all bivalves. The injection of the posterior adductor muscle with 0.5*M* potassium chloride has been used successfully for mussels, and the addition of heat-killed sperm has worked for oysters. Bivalves that are difficult to spawn are generally not in ripe condition and the tests initiated with developing embryos from these individuals are likely compromised.

When individuals are observed to be shedding gametes, place each spawning bivalve in an individual chamber. The containers should contain test solutions that are already salinity adjusted (if required) maintained at 20°C for oysters and clams and 15°C for mussels. Examine a small sample of gametes from each spawning individual. Although it is highly desirable to pool populations of sperm and eggs, it is more important that they are of high quality. Use only sperm that are highly motile and eggs that are not vacuolated, small, or abnormally shaped. Combine the best sperm and filter through a 36-μm screen* to remove extraneous material or clumped gametes. Filter the eggs through a 75-μm nylon screen into a 1-L beaker and dilute to a density of 5000 to 8000 eggs/mL. Use a Sedgwick-Rafter slide to verify density. While using a perforated plunger to continually suspend eggs, remove three 100-mL portions of the egg suspension and fertilize these with 1, 3, and 10 mL of the sperm suspension. After 1.5 to 2.5 h, inspect each portion microscopically. Use the zygote suspension with the lowest amount of sperm giving normal cell division (i.e., ≥90%). Each suspension contains enough embryos to perform multiple tests. The number of tests, however, will depend on the initial density of the egg solution, the test chamber volume, and the final density of embryos in the test chambers.

2) Marine gastropod larvae—Ripe abalone can be induced to spawn by stimulating the synthesis of prostaglandin-endoperoxide in the reproductive tissues.[1] This can be done by adding hydrogen peroxide to seawater buffered with trishydroxymethylaminomethane (Tris)[1] or by irradiating seawater with ultraviolet light.[2] Select four ripe male and four ripe female abalone. Place organisms of each sex in separate, clean containers filled with 6 L of 0.22-μm-filtered seawater. Aerate the water and keep at 15°C. Prepare buffer by placing 12.1 g Tris in a 125-mL Erlenmeyer glass flask and adding 50 mL

* Nitex, or equivalent.

deionized water. Mix solution thoroughly until the buffer reagent is completely dissolved. Then, mix 12 mL of 30% H_2O_2 with 38 mL deionized water in a flask. Use H_2O_2 less than 1 year old from purchase date, refrigerated, and opened for no more than 30 d.

Add 25 mL buffer solution to each bucket followed by 25 mL H_2O_2 solution; mix thoroughly. Keep buckets covered and undisturbed for next 2.5 h of exposure. After exposure, empty, rinse, and refill buckets with 6 L 0.22-μm-filtered seawater. Continue moderately heavy aeration until spawning begins. Gently siphon eggs into 2 L of seawater in a wide-bottom fertilization container until there is a single layer of eggs on the bottom of the container. If there are plenty of eggs available, siphon more eggs into a second fertilization container and hold in case there is a problem with the fertilization of the first container of eggs. Collect the freshest sperm possible into a beaker, preferably collected directly from the respiratory pore, and dilute until there is just enough sperm in the water to give a slightly cloudy appearance. Fertilize eggs within 1 h of release by adding 200 mL sperm-laden water. Use a gentle flow of filtered seawater poured along the edge of fertilization container to roil the eggs, mixing them with sperm. Let fertilized eggs settle for 15 min before siphoning off sperm-laden water. Refill container with filtered seawater and settle eggs again. After 15 min, siphon fertilized eggs into a 1-L beaker for counting. Evenly mix the embryos in the 1-L beaker by gentle vertical stirring with a clean perforated plunger.[3] Never let embryos settle densely in the bottom of the beaker, and take care not to crush embryos while stirring. Using a 1-mL wide-bore graduated pipet, take five samples of evenly suspended embryos. Empty contents of pipet onto a Sedgwick-Rafter slide and count embryos using a dissecting microscope at approximately 10 to 25×. Take the mean of five samples to estimate number of embryos. Number of embryos in the beaker should be between 200 and 300 embryos/mL. Dilute if concentration is too high, let embryos settle and pour off excess water if concentration is too low. While determining the density of the embryos, also note the number of sperm around a few representative eggs. The number should be between one and 100. If there are so many sperm that the egg appears fuzzy, there is a higher chance of polyspermy, which will lead to abnormal development. If this occurs, start the fertilization process again with fresh eggs and sperm, if available.

b. Setting up test chambers:

1) Marine bivalve larvae—Conduct assay at 20 ± 1°C for oysters and clams and 15 ± 1°C for mussels. Maintain photoperiod at 16 h light and 8 h dark; ambient laboratory lighting between 550 and 1050 lux is sufficient. Prepare test chambers as described in Section 8010F. Prepare sufficient water volume for five replicate chambers plus a surrogate water-quality chamber per dilution and control, and an additional five chambers for determination of zero time stocking density of larvae, with a minimum of 10 mL water in each. Measure dissolved oxygen (DO), salinity, pH, and temperature in surrogate chamber from each concentration. Do not aerate chambers unless DO falls below 4.0 mg/L. If aeration is required, do not exceed that necessary to maintain an acceptable minimum oxygen level. Calculate volume of fertilized egg suspension necessary to provide 15 to 30 larvae/mL and add to each replicate and each of the five zero time stocking density chambers. Immediately add 0.5 mL buffered formalin in a 5% solution (alternatively, use glutaraldehyde in a 0.5% solution) to each zero-time vial to preserve the fertilized eggs. Count the number of fertilized eggs in each zero-time vial and calculate the average. This is the initial stocking density, to be used for determining survival at the end of the test.

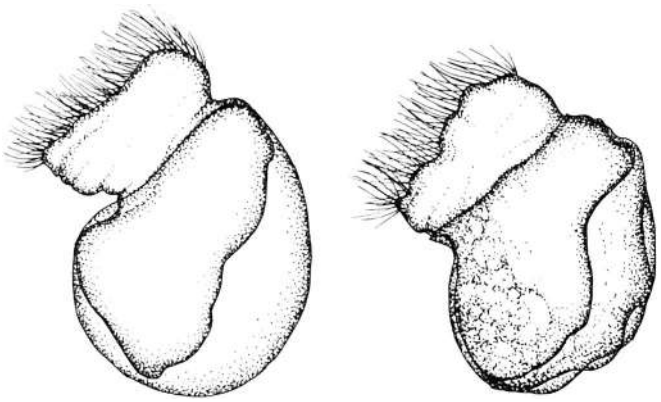


Figure 8610:1. Abalone: (left) normal veliger; (right) abnormal veliger.

2) Marine gastropod larvae—Conduct assay at 15°C and then maintain within ±1°C of that temperature. For photoperiod, lighting, test chamber preparation, and DO control, see ¶ *b*1) above. Prepare sufficient water volume to initiate five replicate chambers per test concentration and control with a minimum of 20 mL of water in each replicate. Inoculate each test chamber to provide a minimum of 200 embryos from the adjusted stock per replicate (5–10 embryos per mL).

c. Performing tests:

1) Marine bivalve larvae—Incubate for 48 h at 20 ± 1°C for oysters and clams and 15 ± 1°C for mussels. Measure DO, pH, salinity, and temperature in surrogate chamber daily. At 48 h, add 0.5 mL buffered formalin in a 5% solution (alternatively, use glutaraldehyde in a 0.5% solution) to each vial to preserve the larvae. Using an inverted microscope at 40–100× magnification, count the total number of normal and abnormally developed larvae in each replicate. If an inverted microscope is not available, the larvae may be transferred to a Sedgwick–Rafter counting slide for examination under a standard compound microscope. Normal development is larvae that have fully reached the prodissoconch I D-hinge shell stage with meat inside the shell. Malformed shells, or larvae in earlier development stages are considered abnormal. To calculate the percent survival, divide the total number of larvae (normal and abnormal combined) at the end of the exposure by the average stocking density of fertilized eggs determined at time zero. To calculate the percent normal larvae, divide the number of normal larvae by the total number of larvae (normal and abnormal combined) at the end of the exposure. An acceptable test will have an average of at least 70% survival in the controls for all species, and an average of at least 70% normality in the surviving controls for mussels and oysters, and at least 60% normality in the surviving controls for clams. Further details and test variability are available.[3,4]

2) Marine gastropod larvae—Incubate for 48 h at 15 ± 1°C. Measure DO, pH, salinity, and temperature in one surrogate chamber daily. At 48 h, add 0.5 mL buffered formalin in a 5%

solution (alternatively, use glutaraldehyde in a 0.5% solution) to each vial to preserve the larvae. Either use an inverted microscope, or pipet a sample from each vial onto a Sedgwick-Rafter counting slide, and examine 100 larvae from each replicate at a magnification of 40–100×. Count number of normal and abnormal larvae. Normal development is larvae that have fully reached the veliger larval stage and produced smooth, meat-filled shells (Figure 8610:1). Arrested egg development and abnormal-shaped larvae are considered abnormal. To determine the percent normal development, divide the number of normal larvae by 100. An acceptable test will have at least 80% normal development in the controls. Optionally, a survival endpoint may also be determined following the procedures described above for marine bivalve larvae. Further details and test variability are available.[5]

5. Statistical Analysis

Calculate and report results in accordance with Section 8010G. Computer methods for data processing and analysis are available.[3–5]

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8610 D. Sediment Bioaccumulation Test Procedures Using Marine Bivalves

1. General Procedures

Before conducting a definitive sediment bioaccumulation test with the bivalve *Macoma nasuta*, conduct preliminary tests to become familiar with the test procedures given below. Test conditions are summarized in Table 8610:III.

2. Water Supply

Use seawater with a salinity of approximately 35 g/kg and a pH of about 7.8. A minimum salinity of 25 g/kg is required for the test but any salinity within the range 25 to 35 that reflects site-specific conditions is appropriate. Filter seawater to 20 μm or less in quantities sufficient to support this testing scheme for its duration. Routinely analyze and document source seawater for metals, especially heavy metals, and other potential contaminants.

3. Sediment

Collect sediment from the site with a benthic sampling device (see Section 10500B). Multiple samples may be required from each individual site to acquire sufficient material. Collect control sediment at the same time and in the same general location as the collection site for the test organisms. Collect reference sediment from a site known to be generally free of contaminants. Sediments may be dry sieved through a 1.0- or 2.0-mm screen to remove resident organisms. Store sediment at 4°C until required for test initiation; recommended holding times for sediment range from 2 to 8 weeks.[1] Begin tests within 8 weeks of sediment collection.

Table 8610:III. Summary of Test Conditions for the Sediment Bioaccumulation Test Using Marine Bivalves

Parameter	Conditions
Test species	*Macoma nasuta*
Test chamber	20–40 L aquaria
Test solution volume	5–8 cm sediment depth
Test type	Flow through (min. 1–2 exchanges daily) or daily static renewal
Number of replicates	5
Number of organisms per replicate	≥15 in 20-L aquaria or ≥30 in 40-L aquaria, depending on analyte requirements
Loading rate	≥50 g (100–200 g preferred) sediment per gram wet flesh (without shell)
Salinity	25–35 g/kg, depending on site salinity, 35 g/kg preferred, varying by no more than ±2 g/kg of chosen salinity
Temperature	12–16°C, maintained at ±2°C of chosen temperature
Dissolved oxygen (DO)	≥4 mg/L
pH	7.8 ± 1.0
Aeration	Trickle flow, if necessary to maintain DO >4 mg/L
Light cycle	16:8, 14:10, or 12:12 light:dark
Light quality	Ambient
Test duration	28 d exposure, plus 1 d depuration
Observations during the test	Temp, DO, pH, salinity daily in 1 rep, overlying ammonia on Day 0, 5, 10, 15, 20, 25 and 28 in 1 rep, mortality in all reps daily
Maximum sediment holding time	8 weeks

4. Exposure Chambers

Use 20- to 40-L aquaria for long-term flow-through or static-renewal exposures of *Macoma nasuta*; these will generate sufficient quantities of tissue for the chemical analyses necessary to determine bioaccumulated tissue concentrations of virtually any analyte. The duration of bioaccumulation tests (28 d, plus a depuration period of 1 d) requires the use of relatively large sediment volumes. A 40-L aquarium should contain a 5- to 8-cm depth of sediment with overlying water reaching 4 to 6 cm below aquarium height. Maintain ratio of sediment to seawater for smaller aquaria to ensure that there is enough material to support the animals throughout the 28-d exposure and 1-d depuration period. Approximately 50 g wet weight sediment per gram of wet flesh (without shell) is an appropriate minimum; however, consider the final volume of tissue needed for the analytes of concern when choosing the number of animals per replicate and ultimately the amount of sediment and water required to support them. Varying guidance on recommended loading rates range from 100 to 200 g of sediment per gram of wet flesh (without shell) exposed.[1–3] Preferably, use flow-through facilities, if available; clean seawater should flow over the sediment surface at the bottom of the tanks at a rate of at least 1 to 2 exchanges of overlying water per day and may include a greater exchange rate of 5 to 10, depending on program objectives. If static-renewal procedures are used, siphon 80% of overlying water in each aquarium and replace with clean seawater every day at minimum. Cover aquaria to prevent introduction of foreign materials.

Sediment tests can be conducted for longer periods of time but require the addition of sediment aliquots after the first 3 weeks of exposure. This is to provide sufficient food for test organisms whose source is the sediment being tested.

5. Conducting the Bioaccumulation Tests

a. Setting up test chambers: Conduct assay at a chosen temperature between 12 and 16°C and maintain within ±2°C of that temperature. Although photoperiod does not appear to be a critical parameter for sediment testing with clams, maintain and report a 16:8, 14:10, or 12:12 light:dark period. Ambient laboratory lighting is sufficient. Prepare test chambers as described in Section 8010F. Prepare sufficient aquaria to initiate five replicate chambers per site, as well as five replicates each for control and reference sediments. Each site is an individual test material; typically there is no dilution series or sediment concentration associated with bioaccumulation testing with clams, although such a design is possible. Label each chamber with an identifier. Place an appropriate volume of sediment (as described in 8610D.4) at bottom of each test chamber. A total volume of about 60 L sediment per site (before sieving) is sufficient for testing in 40-L aquaria. Adjust volume to reflect test chamber size and number of animals used per replicate. Choose a test temperature between 12 and 16°C, but maintain within ±2°C of the chosen temperature.

b. Performing tests: Set up test chambers as described above and in a randomized position within the laboratory test area. After equilibration, add mollusks to each tank. Thirty clams will be well supported by 5 to 8 cm of sediment in a 40-L aquarium with overlying seawater. This number of animals is generally sufficient to ensure adequate tissue for the chemical analyses typically associated with this type of testing (100 g of wet tissue mass). This assay is often used to expose more than one organism type simultaneously (interspecies relationships need to be considered). Should additional animals or other species be included in the analysis, ensure adequate sediment and water for their health. Before adding animals, record initial temperature, DO, pH, and salinity, and confirm that values are within the appropriate range for testing. Remove a water sample from each tank and hold for ammonia analysis. If ammonia analysis is not performed within a day, provide acidification to preserve the ammonia levels. The duration of exposure for determining bioaccumulation is 28 d with another 1-d exposure to clean seawater for tissue depuration. This exposure time can be extended to longer periods of time (45–90 d), but sediment needs to be replenished for these extended periods. Examine each replicate daily and monitor for mortality. Remove dead organisms immediately to preserve water-quality. Note removal of dead organisms on water-quality observation sheet. Record daily monitoring of salinity, DO, pH, and temperature on a water-quality data sheet. On the first day, every fifth day during the test period, and at test termination, remove a subsample from one replicate of each sediment type (i.e., site, control, or reference) for ammonia analysis in the overlying water. Adjust flow rate of the water to maintain replacement volumes of 1–2 times per day (minimum). If DO falls below 4 mg/L add trickle flow aeration to maintain DO above 4 mg/L.

On Day 28 of the assay, label equivalent size test chambers with the same identifiers and fill with clean seawater. Transfer animals to the clean seawater to depurate for 24 h. After the depuration period, remove test organisms and scrub outer surface clean of debris, rinsing the scrubbed shells in clean seawater. In preparation for test termination, label pre-cleaned glass containers with species name and replicate identifiers. After shells are cleaned and depurated excise tissue from the shells using non-contaminating tools (e.g., titanium scalpel blades and forceps), transfer tissue to corresponding labeled clean glass containers, and freeze samples for subsequent transfer to an analytical chemistry laboratory.

6. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

7. References

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8610 E. Field Test Procedures Using Freshwater and Marine Bivalves

1. General Procedures

Conduct controlled experiments with caged bivalves under field conditions to characterize chemical exposure and associated biological effects in the same organism. Obtain test specimens, place them into cages, and position in the study areas. Exposure can be characterized by measuring the accumulation of chemicals in tissues or by measuring biochemical changes. Effects can be assessed by measuring endpoints (e.g., survival, growth, and reproduction).

Although it is possible to measure chemicals in individual bivalves, this method assumes that, for cost-effectiveness, tissues will be pooled for chemical analyses. The number of individuals necessary for the composite depends on amount of tissue required for analysis.

2. Water Supply

The water where bivalves are transplanted should be sufficient for growth or, at a minimum, maintenance of tissue mass, so available chemicals will accumulate in their tissues. Consult published information[1] on normal temperature and salinity ranges for commonly transplanted marine and freshwater species.

3. Exposure Chambers

a. Types of cages: Although it is possible to mark individuals, cages with individual compartments are recommended for field studies with caged bivalves to minimize interferences, standardize procedures, minimize handling individuals, and deter predation. The cage is designed to maximize water flow to the test animals while containing them within the cage to ensure that each individual has the same water exposure. Cages can be flexible material with compartments attached to a rigid frame (e.g., mesh bags attached to a PVC frame; Figure 8610:2) or rigid with fixed compartments (e.g., plastic trays or wire baskets with internal divisions). Cages without compartments can be used, but this is not recommended because of the potential for clumping of individuals and uneven exposure conditions. Compartmentalization facilitates tracking individuals throughout the test and eliminates the need to mark them. Although there are techniques for numbering individuals,[2,3] this approach is time-consuming if large numbers of animals are used. Using glues or epoxies for marking may introduce potentially toxic chemicals.

Rigid cages with fixed compartments have been used in freshwater[4,5] and marine[6] environments. PVC frames supporting flexible mesh material have been used in freshwater, estuarine, and marine habitats.[7,8] Different cage designs, including rigid cages with and without compartments, corrals that limit the movement of sediment-dwelling bivalves, and leashes (monofilament lines glued to each bivalve shell), have been compared with natural conditions.[9]

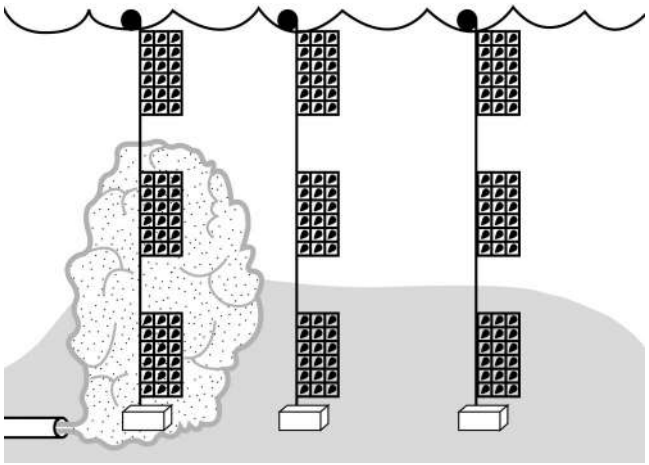


Figure 8610:2. Schematic layout of cages, consisting of mesh bags attached to PVC frames, suspended from a line attached to a buoy at water surface and anchor at bottom.

b. Flexible mesh and PVC cage construction: The final dimensions of the PVC frames and the mesh bags depend on the species, size of organism at the start of the test, expected growth, and number of organisms per cage. Oyster culch netting, used in bivalve aquaculture, is the recommended material for the mesh bags; tubular oyster culch netting is available in many diameters and mesh sizes. A 15-cm-diameter of the mesh material is recommended for most species (e.g., mussels and clams) because there is less excess mesh at the point of constriction. Note: Use larger-diameter mesh for larger, rough, and irregular shells (e.g., oysters). Create individual compartments by separating bivalves within mesh bags with a plastic cable tie. Provide sufficient space in each compartment for test animal growth during test. The mesh bag should be long enough to accommodate the desired number of bivalves per bag and attach to the frame. Frames can be constructed from aluminum, stainless steel, or plexiglass, but PVC pipe (1.25 or 1.9 cm in diam) is preferred (consider the purpose of the test, the potential chemicals of concern and the potential for the frames to provide contaminating materials that might interfere with test results). Frame sizes can vary but establish size of the frame to minimize crowding and possible damage to shells by choosing frame dimensions that

are 5 cm longer and wider than the space occupied by the bivalves after they have been placed in the bag. Soak frames in flowing fresh or seawater for at least 24 h after construction, to remove any volatile chemicals.

Drill holes in PVC materials about 0.6 cm in diam at intervals of about 24 cm to allow water to enter the pipe and remove trapped air, to obtain neutral buoyancy and prevent floating of the frame. To maintain frame strength, do not drill at the corners.

Compartmentalized cages for holding bivalves can be constructed from high-density polyethylene, polycarbonate, or fluorocarbon plastic; glass; Type 316 stainless steel (fresh water only); or nylon to minimize dissolution, leaching, and sorption. Do not use plastic cable ties containing metal stops, since corrosion may occur. Consider the chemicals of concern that are being evaluated before selecting cage materials.

4. Conducting Tests

a. Preparation for distribution: Label mesh bags with colored identification tags made of durable plastic or other inert material and labeled with indelible ink. Attach tags to bags with plastic cable ties. Preferably knot bag approximately 30 cm from the end and attach tag near knot. Indicate both cage number and bag number on tag. Colored beads can be used as a backup labeling system to identify bag number on the PVC frame if it becomes dislodged.

Separate bags into groups according to bag number because the distribution is based on bag number (with all bags of a common number filled at a given time). For example, in a study with ten cages and each cage holding three bags of bivalves, the following sequence of labels would be prepared (the first number indicates cage number and the second, bag number):

1-1, 2-1, 3-1, 4-1, 5-1, 6-1, 7-1, 8-1, 9-1, 10-1 (Bag 1 for the 10 cages).
1-2, 2-2, 3-2, 4-2, 5-2, 6-2, 7-2, 8-2, 9-2, 10-2 (Bag 2 for the 10 cages).
1-3, 2-3, 3-3, 4-3, 5-3, 6-3, 7-3, 8-3, 9-3, 10-3 (Bag 3 for the 10 cages).

After labeling, gather all bags with the same bag number designation; there should be one for each cage number. Fill bags in groups, starting with the -1 group first. Stretch the mesh material by hand to approximately $6 \times 6 \times 12$ cm so the bivalve falls into the bag. Attach bags to frame (as if hanging stockings on a clothes line) starting with the -1 bags, so the bags are in cage number sequence.[1]

b. Presorting: To minimize size differences among cages at the start of test, presort bivalves into size groups. Do not use specimens with holes or broken shells. Base sorting on either shell length (as determined with vernier calipers) or whole-animal wet-weight. After presorting, determine the number of bivalves in each size category. Initiate test using the minimum size range, with a target range of 5 to 10 mm (i.e., 20 to 25 mm, 33 to 38 mm, 36 to 46 mm) that contains the maximum number of individuals. Keep animals moist and cool during presorting, as described below. Keep all bivalves originally collected until the test is initiated to ensure there is a sufficient number of individuals. Randomize the placement of the sorted groups of bivalves into compartments.

Handle specimens as carefully, gently, and quickly as possible to minimize stress. When bivalves are transported a long distance, do not keep them submerged in water, but keep them moist and cool by placing them in an ice chest with either wet ice or frozen gel packs on the bottom; place newspaper, paper toweling, or cloth toweling between specimens and ice to prevent direct contact. Wet towels also can be placed over the specimens to provide additional moisture.

Keep organisms out of water before the test to eliminate exposure to oxygen-deficient conditions. Do not expose specimens to direct sunlight and keep them moist as described above. If the air temperature is high, keep specimens in an ice chest with wet ice as described above.

c. Preparing bivalves for distribution to mesh bags: Once the size range has been determined, make more precise shell length and/or whole-animal wet-weight measurements. It may not be possible to measure shell length in irregularly shaped specimens (e.g., oysters). Place all bivalves within the size group in a tray containing water. Maintain specimens in water during the measurement and distribution process to eliminate entrapment of air between valves. Monitor water temperature and keep it within approximately ± 5°C of field temperatures. Plastic bags containing wet ice may be placed in the tub with the bivalves to maintain desired water temperature. A rapid change in temperature may induce spawning of gametes in mature animals, which would add another variable to the test. Keep bivalves completely submerged and flat on the bottom before measurement. Bivalves that float or are positioned upright on the bottom may have air trapped between their valves; do not use these individuals because the trapped air may be an indication of an unhealthy individual. Transfer these individuals to a separate container where they may purge the trapped air within 5 to 10 min; if this occurs, these animals can be used.

d. Measuring and distribution to mesh bags: Once bivalves have been placed in a tray filled with water, the specimens will begin to respire and their shells will open slightly ($\sim$1 mm). Most species close their shells on physical contact. If any specimens do not completely close their shells upon movement, physical contact, or water agitation, do not use them. Randomly select one specimen from tray, blot excess water from surface, measure and record its length and weight. Place specimen into the first mesh bag on the distribution rack. Affix a 10-cm cable tie around mesh material above this specimen, and adjust so it is tight enough to prevent the animal from passing through but loose enough to be removed if necessary. Do not constrict the surrounding mesh to the point that it might restrict valves from opening or growing. Randomly select a second specimen from the tray and repeat the process until each mesh bag contains desired number of specimens. When all bags on a distribution rack have been filled, remove bags and knot or cable-tie the open end, leaving a tail length of approximately 30 cm. Place completed bags in a cooler lined with ice and moist paper towels. Repeat the above process until all mesh bags are filled. This process ensures that each cage will have approximately the same number of individuals from each size group.

Once the bivalves have been distributed to all mesh bags, sort bags by cage number (i.e., the first number on the label). Either attach bags to the PVC frames, as described below, or place them, secured together with a large plastic cable tie, into ice chests for transporting to a holding area in the laboratory or field

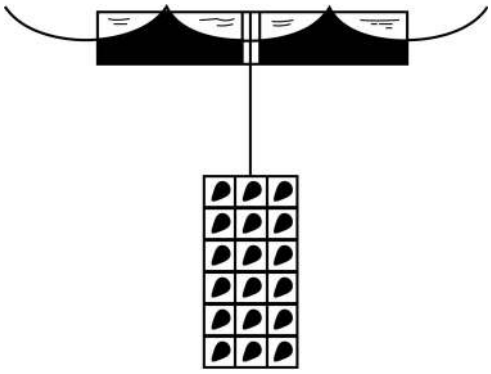


Figure 8610:3. Cage suspended from a fixed mooring.

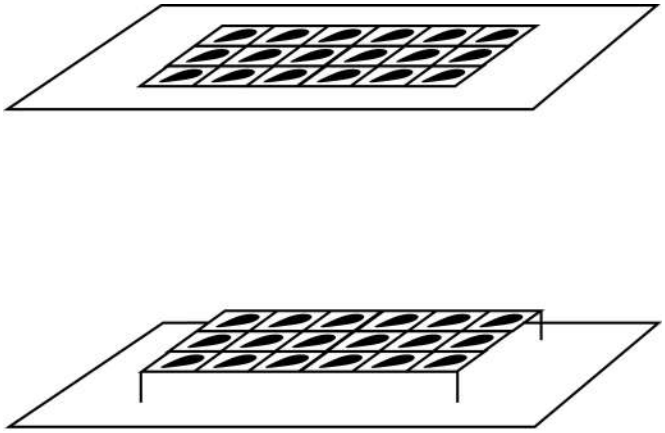


Figure 8610:4. Cages placed directly on sediment (above) and on attached legs a fixed distance above sediment (below).

with uncontaminated flowing water. Hold in water overnight or until ready to attach bags to the PVC cages.

e. Measuring and distributing bivalves to be used for baseline (beginning-of-test) tissue chemistry: During the measurement and distribution of test specimens, identify bivalves to be used for baseline tissue chemistry, to ensure similar sizes. This can be accomplished by randomly assigning a cage number for each baseline tissue chemistry sample. However, the bivalves can be distributed to compartmentalized boxes instead of mesh bags. Using compartmentalized boxes eliminates the need to remove individuals from the mesh bags. Place the individuals into the compartmentalized boxes in numerical order. At the end of the distribution process, remove tissues for chemical analysis according to the procedures in ¶ *k* below.

f. Attaching mesh bags to PVC frame: A set of mesh bags attached to a PVC frame constitutes a cage (Figure 8610:2). To attach mesh bags to the frame, knot tail ends of mesh directly to PVC or use cable ties. Allow sufficient slack in the mesh bag after it is attached for the bivalves to open properly, but not so much slack that the bivalves are suspended below the plane of the cage. If the mesh is stretched too tightly, it will restrict valve opening, filtration, and growth. If the bag is too loose, it will tend to become tangled in the deployment array. If necessary, slide cable ties to increase or decrease the space between individuals to ensure adequate space for respiration and growth. A temperature-recording device may be attached to the frame at this time.

g. Water column suspension: Cages can be suspended in the water column either from a fixed mooring (Figure 8610:3), such as a floating pier or piling, or from a line with an anchor on one end and a buoy attached to the other end (Figure 8610:2). Factors to consider during the placement of cages in the water column include tidal fluctuations, bottom topography, and boating and recreational activity in the vicinity. Water-column deployments are used primarily to assess chemicals in the water column. However, if the cage is suspended near the bottom, the test also can be used to assess chemicals released or associated with sediments.

h. Fixed bottom deployment: Cages can be placed directly on the sediments or set at a fixed distance from the bottom by attaching legs to the cage and pushing the legs into the sediments to hold the cage in place (Figure 8610:4). Bivalves of certain species, caged and placed directly on the sediments, can be used to assess chemicals associated with both sediments and the water column. If the goal is to assess subsurface sediments, place cages directly on top of sediments and allow bivalves to bury themselves. The bivalves are exposed to chemicals in the sediments as the sediments infiltrate the mesh material or the foot rakes the sediment surface. This approach is generally less stressful than forced burial. Forcing cages into the sediments or digging out sediments to bury the cages may disturb the integrity of sediments and alter natural biogeochemical processes.

Anchors attached to the sides of the cage with line or rebar strapped to the side of cage can be used as weights to ensure that the cages remain upright. Rebar also can be bent into a "U" and pushed over the cage into the sediment to secure the cage in position. Iron rebar should have a rubberized coating or be covered by plastic bags to prevent water exposure to the metal.

When placing cages on sediments, consider presence of natural vegetation, type of substrate, boating traffic, and recreational activity. More detail on deployment hardware and configurations is provided elsewhere.[1,10,11]

i. Deployment period: Preferably let test run for 60 to 90 d; 30 d is the recommended minimum. Consider the chemical of concern and the testing's purpose to determine the exposure duration. If the chemicals of concern are low-molecular-weight organic compounds and a chemical signature of exposure is the required experiment (e.g., some PAHs), then a shorter test period can be used. Equilibrium for most chemicals (e.g., metals or high-molecular-weight organic compounds) usually is achieved in bivalves in less than 60 to 90 d.[8,12–20]

j. Retrieval and end-of-test measurements: After retrieving caged bivalves, remove any foreign material, if present, by dipping the cage into water. Remove any growth from exterior of the shells. If the bags are removed from the PVC frames, use a separate ice chest lined with wet ice and moist paper towels to transport bivalves to the laboratory. Cover top and bottom of cages with a tarp or other protective covering to reduce evaporation and prevent temperature elevation and contamination.

Process bivalves from all bags constituting a cage as a unit; retain numerical order so initial and terminal measurements of each specimen are recorded. Remove bivalves from mesh bags

and place them in a compartmentalized tray to facilitate end-of-test measurements, starting with Bag 1 and ending with Bag N. Begin at end of the bag with plastic label, since this end was filled first. Remove bivalves and place them, in sequential order, into a compartmentalized plastic tray filled with water. After all individuals from Bag 1 have been transferred to the compartmentalized tray, repeat process with remaining bags, maintaining the sequence. If an individual bivalve is dead or missing, place a marker in the specific compartment to maintain numerical order of individuals.

Place the tray(s) containing bivalves from a cage into a container with clean water. Some individuals may float, indicating that air is trapped between the two valves. Ensure that a floating specimen remains in its compartment. Usually floating bivalves will release the air within 5 to 10 min and sink to the bottom.

Measure and weigh specimens. If tissues will be analyzed chemically, retain numerical order; if not, tissues may be placed together. In either case, it is no longer necessary to keep them in water.

k. Collection and preparation of tissues for chemical analysis: All bivalves need their outer surfaces scrubbed clean of fouling materials and debris before shucking. Scrape the shells with a stiff fingernail-cleaning brush and then rinse in fresh seawater. Consider the test's purpose to determine whether depuration should be initiated with the test organism before shucking. Depuration's purpose is to purge the gut contents so uptake to more permanent storage of contaminants can be determined. Shucking knives, cutting boards, trays, and weighing pans are then needed for tissue preparation. Use tools made of non-contaminating material (e.g., titanium, resistant stainless steel, anodized aluminum, or borosilicate glass). Before each use, wash equipment with a residue-free cleaning solution, rinse in hot tap water, and rinse with deionized water. An acetone or 95% ethanol rinse can follow the last water rinse if required by the analytical laboratory. Let equipment air-dry to prevent contaminating tissues with water. Non-contaminating gloves should be worn; keep them powder-free and replace after processing a cage of mussels.

Retain order of specimens during tissue extraction; record tissue weights separately. To remove tissue, slide a thin knife blade between the two shells and sever the anterior and posterior adductor muscles. **(CAUTION: *Danger of injury.*)** It may be necessary to notch a clam or oyster shell so the knife can be inserted. Drain excess water from the animal. Spread the two valves, exposing tissue. Remove tissue with knife blade and place on holding tray.

Weigh tissues individually, using a clean weighing pan each time. Tare between each weight measurement. Place tissues within a cage group in a sample jar, seal, and store according to laboratory specifications. For procedures for analyzing tissues, see Part 3000 (metals) and Part 6000 (organic compounds).

5. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

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8710 ARTHROPODS*

Phylum Arthropoda is the largest group of animals; it comprises more than one million species, the majority of which are insects. Other arthropods include crustaceans, spiders, ticks, mites, and other less-known species. Arthropods are found in all environments, including both fresh and marine waters. Two classes of arthropods are used extensively in toxicity testing: crustaceans and insects. Test procedures are described for several crustacean groups, including *Daphnia* (Section 8711), *Ceriodaphnia* (Section 8712), mysids (Section 8714), and decapods (Section 8740). Representatives of the insect orders belonging to stoneflies, mayflies, caddisflies, and dipterans are the most commonly used groups in aquatic testing (Section 8750).

* Joint Task Group: 20th Edition—Donald J. Reish.

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8711 *DAPHNIA**

8711 A. Introduction

Daphnia sp. are small freshwater crustaceans (Figure 8711:1). They have been used for many years to assess the acute and chronic effects of single chemicals and complex environmental mixtures, such as wastewater effluents. Several methods have been published.[1–5]

Daphnia are valuable as test organisms because of their sensitivity to toxic substances, ease of identification and handling, and ubiquitous distribution; they are used extensively in toxicity testing. *Daphnia* are fecund and reproduce parthenogenically, which allows for the establishment of clones with little genetic variability and with reproducible testing results.

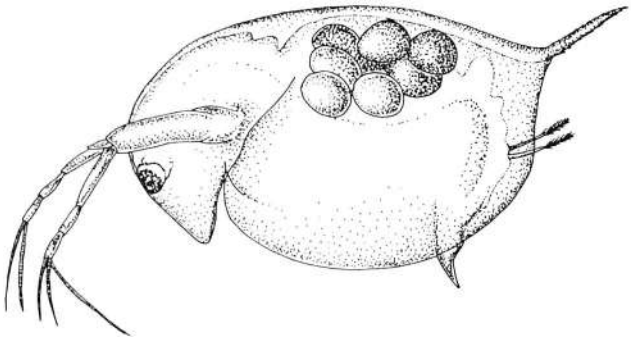


Figure 8711:1. *Daphnia* sp., adult female.

1. Life History

Two commonly used species are *Daphnia pulex* and *Daphnia magna. Daphnia pulex* attains a maximum length of approximately 3.5 mm, while *D. magna's* maximum length is 5 to 6 mm. The best way to differentiate these species with certainty is by determining the size and number of spines on the postabdominal claws via a dissecting or compound microscope (see Figures 8711:2 and 3) .[2,6]

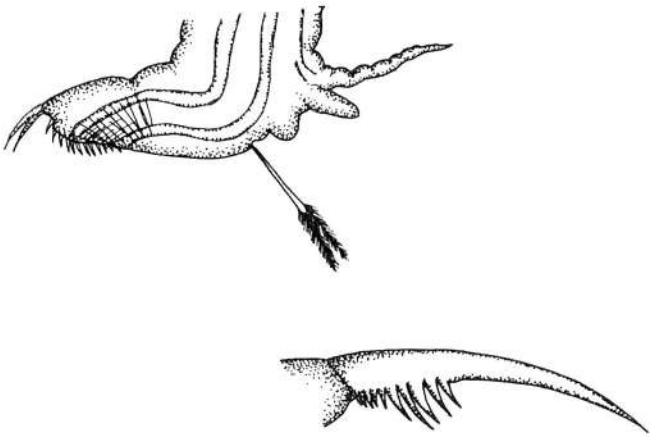


Figure 8711:2. *Daphnia pulex:* (above) postabdomen; (below) postabdominal claw.

The lifespan of *Daphnia* varies greatly, depending on species and environmental conditions.[2] Temperature is an important factor and is typically inversely related to lifespan. For *D. magna*, it is about 40 d at 25°C and about 56 d at 20°C. *Daphnia pulex*'s lifespan is approximately 50 d at 20°C. Four distinct life-cycle periods are recognized: egg, juvenile, adolescent, and adult.

Under laboratory conditions, *Daphnia* typically release a clutch of 6 to 10 eggs into the brood chamber, although some have released up to 57 eggs. The maximum number of eggs occurs in the 5th and 10th adult instar for *D. magna* and *D. pulex*, respectively. The eggs morph into juveniles who are released from the brood chamber into the environment during the adult molt, which occurs approximately every 2 d under favorable conditions. Each juvenile instar terminates with a molt followed immediately by growth. The growth rate is greatest during juvenile stages (early instars). The time required to reach sexual maturity varies from 6 to 10 d, depending on temperature. The adolescent period is a single instar between the last juvenile instar and the first adult instar; during this instar, the first clutch of eggs reaches full development in the ovary. In adults, each instar terminates with the release of juveniles from the brood chamber, molting, growth, and release of eggs into the brood chamber. *Daphnia pulex* has three to four juvenile instars and 18 to 25 adult instars. *Daphnia magna* has three to five juvenile instars and 6 to 22 adult instars.

In the environment, *Daphnia* populations consist almost exclusively of females most of the year; males are abundant only in spring or autumn. *Daphnia pulex* and *D. magna* typically reproduce via cyclic parthenogenesis (in which sexual reproduction follows a series of parthenogenic populations) and via obligate parthenogenesis (in *D. pulex*, where only females produce young). Males are smaller than females and have larger attennules. The males' first legs have a stout hook (used in clasping); males also have a modified postabdomen. Temperature extremes can induce production of males, as can high population densities and subsequent accumulations of excretory products and/or a decrease in available food. These conditions also may prompt the production of sexual (resting) eggs in cases (ephippia), which are cast off during the next molt. The parent's metabolic rate is related to the shift toward male and sexual egg production. As a rule, males and ephippia will not occur unless stock cultures are neglected or the culture is stressed.

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Joint Task Group: Ruth M. Sofield (chair) and Sandra Valeria Buratini.

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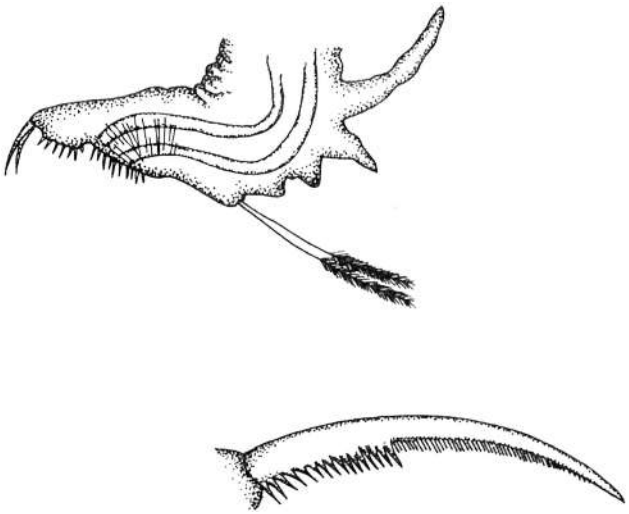


Figure 8711:3. ***Daphnia magna:*** **(above) postabdomen; (below) postabdominal claw.**

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8711 B. Selecting and Preparing Test Organisms

1. Obtaining and Selecting Test Species

Daphnia are widely available from many laboratories and commercial biological supply houses. Only 20 to 30 organisms are needed to start a culture. Some biologists prefer *D. pulex* to *D. magna* because it is more widely distributed and easier to culture. However, *D. magna* neonates (first instar) are larger and somewhat easier to use. Verify species by microscopic examination.

2. Culturing Organisms

a. Water supply: Although *Daphnia* cultures can be maintained successfully in some natural waters, preferably use a synthetic (reconstituted) water medium. Reconstituted water is easily prepared, is of known standardized quality, produces predictable results, and permits adequate growth and reproduction. Because daphnids are very sensitive to media hardness, reconstituted hard water (160 to 180 mg $CaCO_3$/L) is recommended for *D. magna*, and reconstituted moderately hard water (80 to 90 mg $CaCO_3$/L) is recommended for *D. pulex*.[1] See Table 8010:I for materials needed to prepare reconstituted water.

Dissolve salts in distilled or deionized water and aerate vigorously for several hours before use. Initial pH is approximately 8.0, but it will rise as much as 0.5 unit as the *Daphnia* population increases. Although *Daphnia* can survive over a wide pH range, the optimum is 7.0 to 8.6.[2] However, pH generally doesn't need to be monitored or adjusted during cultivation because it usually remains within this range.

b. Food and feeding: Feed *Daphnia* either a mixture of green algae or a suspension of trout chow, alfalfa, and yeast.

1) Algae mixture—Food consisting of several species of algae is preferable.[3] For example, use three algae: *Ankistrodesmus falcatus, Pseudokirchneriella subcapitata (Selenastrum capricornutum),* and either *Chlamydomonas reinhardi* or *Chlorella* sp. To prepare the algal mixture, centrifuge algae, wash in filter-sterilized culture water (water passed through 0.22-μm filter), and centrifuge again. Transfer *Daphnia* to fresh culture water and feed using a sterile Pasteur pipet by adding 2 drops of each alga per beaker containing *Daphnia* culture $\leq$9 to 10 d old, or 1 drop of each alga per 2 adults in each beaker containing *Daphnia* culture $>$9 to 10 d old, rounding up when there is an odd number of adults.

At the end of a work week (e.g., Friday), add 1 extra drop of each alga per *Daphnia* culture beaker. Adjust algae feed so the algae are almost cleared before *Daphnia* are transferred to fresh

culture beakers. If only 2 of the 3 algae are available, add proportionately more of the two algae.

2) Trout chow-yeast-alfalfa suspension—Mix 5 g trout-chow pellets with 1 L deionized water in a blender. Pour the mixture into a 2-L separatory funnel and aerate from the bottom for 1 week at ambient laboratory temperatures. Replace any water lost to evaporation. After 1 week, let settle in a refrigerator for 2 h. Decant the supernatant and save. If necessary, filter the trout-chow supernatant through a fine mesh screen. Mix 5 g dried yeast* with 1 L deionized water on a magnetic stir plate for 1 h; maintain the yeast suspension before combining with trout chow and alfalfa leaves by shaking or mixing the sample. Mix 5 g dried alfalfa leaves† or dried cereal leaves with 1 L deionized water on a magnetic stir plate for 24 h. Let mixture settle 1 h; decant supernatant and save. Combine equal parts of trout-chow supernatant with suspended yeast and alfalfa supernatant. Freeze 30- to 50-mL portions in 50- to 100-mL polyethylene bottles with screw caps. Thaw portions as needed. After thawing, refrigerate but do not hold for longer than 1 week.

Feed 1.5 mL prepared food per 1000 mL of medium, three times per week. There may be excess food at this feeding rate, but if medium is aerated continuously and replaced each week, no problems should result.

3) *Pseudokirchneriella subcapitata (Selenastrum capricornutum)*—The green alga *Pseudokirchneriella subcapitata (Selenastrum capricornutum)* can be used as a *Daphnia* food source. Combinations of other green algae are also suitable as an alternative [see ¶ *b*1) above]. The *P. subcapitata* culture procedure produces 7-d-old cultures containing 4 to 5 million algal cells/mL and 2- to 4-d-old cultures containing 1 to 3 million cells/mL. Prepare algal food and feed it three times per week to *Daphnia* as follows:

Combine volumes of 7-d-old and 3-d-old algal cultures in a ratio of two volumes to one, respectively. Centrifuge algal cells and resuspend in a volume of reconstituted moderately hard or hard water calculated to yield a combined algal culture containing approximately 10 million cells/mL. Add sufficient volume of cell suspension to stock cultures daily to provide approximately 300 000 algal cells/mL of culture (e.g., add approximately 30 mL cell suspension to 1 L *Daphnia* stock culture).

c. Temperature: Protect *Daphnia* from sudden changes in temperature that may cause death or induce ephippia (sexual egg) production. Optimal temperature range is approximately 18 to 26°C. If laboratory temperatures are 20 ± 2°C, normal growth and reproduction of *Daphnia* can be maintained.

d. Lighting: Variations in ambient light intensities (538 to 1076 lux) and prevailing day/night cycles in most laboratories do not affect *Daphnia* growth and reproduction significantly. Provide a minimum of 16 h of light/d.

e. Culture vessels: Use culture vessels of clear glass or plastic to allow easy observation. A practical culture vessel is a 3-L glass beaker filled with approximately 2.75 L of medium. Vessels must be covered to avoid dust and evaporation.

Maintain at least five culture vessels to ensure backup cultures. A 3-L vessel stocked with 30 *Daphnia* will provide approximately 300 young/week.

Wash all culture vessels before use. After culture is established, clean each chamber weekly with distilled or deionized water and wipe with a clean sponge to remove accumulated food and dead *Daphnia*. Monthly, wash each vessel with detergent during medium replacement. After washing, rinse three times with tap water and then with culture medium to remove all traces of detergent.

f. Air supply: Daphnia can survive when the dissolved oxygen concentration is as low as 3 mg/L but grow better when the concentration is above 6 mg/L. Gently but continuously aerate each culture vessel via an aquarium air pump or a general laboratory compressed air supply (oil-free).

g. Culture maintenance: Replace medium in each stock culture vessel weekly. If large tanks (100 L) are used, weekly replacement may be unnecessary.

Cull *Daphnia* populations weekly to about 30 adults per stock culture vessel to prevent overcrowding, preferably during medium replacement. Transfer *Daphnia* with 15-mm-diam glass pipet (with fire-polished end) or disposable plastic pipet.

3. Selecting Test Organisms

Use *D. magna* or *D. pulex* neonates (first instar ≤24 h old)—preferably from the second or third brood—to initiate tests. To obtain young for a test, remove females bearing embryos from the stock cultures 24 h before starting the test and place them in 400-mL beakers containing 300 mL medium and either 0.5 mL trout chow–yeast–alfalfa suspension [see 8711B.2*b*2)] or 10 mL cultured algae. Use the young found in the beakers within 24 h. Five beakers, each containing 10 adults, usually will supply enough first instars for one toxicity test.

Because the appearance of ephippia is indicative of unfavorable conditions, do not use *Daphnia* from cultures producing ephippia. Do not use neonates from cultures with >20% mortality.

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* Fleishmann's, or equivalent.
† Obtainable at most health food stores.

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8711 C. Toxicity Test Procedures

1. Short-Term Tests

a. Preparation of test materials and medium: Prepare test materials and concentrations, dilution water, and toxicant solutions as described in Section 8010F. Make up test solutions and controls in 100-mL quantities in 125-mL wide-mouth flint-glass bottles or equivalent vessels (Table 8711:I).

b. Performing tests: Run tests in replicates of at least three. Prepare at least five concentrations and a control (dilution water only).[1] Segregate neonates that have been released from the mothers' brood chambers during the preceding 24 h at 20 or 25°C and collect in one vessel (use neonates cultured at the test temperature). Introduce the same number of neonates (at least 10) into each test vessel and control. Use either a plastic, disposable pipet or a glass bulb pipet with a 5-mm bore to collect and transfer neonates.

Introduce neonates to test solutions by releasing them below the solution surface. Observe animals regularly—ideally after 1 and 4 h—and daily thereafter. A 48-h exposure is generally accepted for a *Daphnia* acute toxicity test.[1] Record number of motile animals in each test vessel. Consider an animal nonmotile if it shows no independent movement even after gentle squirting with test solution from a pipet (nonmotile animals are not necessarily dead). At threshold concentrations of such substances as ethanol, acetone, and chlorobutanol, animals may show no movement and their hearts may have ceased to beat, but they will recover when transferred to dilution water. If kept in the test medium, however, such animals will die. In addition to immobilization, note behaviors and features (e.g., the number of *Daphnia* that are on the bottom, lethargic, swimming, caught on the bottom or on debris, floating on the surface, swimming erratically, or have a flared carapace).

Record the medium's conditions (e.g., whether it is cloudy or if any particulate, precipitate, undissolved material, or film is present). Continue observations for at least 48 h or as long as the control mortality is 10% or less.

TABLE 8711:I. SUMMARY OF SHORT-TERM AND LONG-TERM TOXICITY TESTS WITH *DAPHNIA* SPP.[1]

Parameter	Test Conditions	
	Short-Term	Long-Term
Species	*D. magna, D. pulex*	*D. magna, D. pulex*
Age of test animal	≤24 h	≤24 h
Test vessel type and size	125–mL wide-mouth flint-glass bottle	Glass or plastic container
Volume of test solution	100 mL	100 mL
Number of animals per test chamber	10	1
Test temperature	20 or 25°C	20 or 25°C
Photoperiod	16 h light/8 h dark	16 h light/8 h dark
Feeding regime	Do not feed	Feed daily
Media change	Do not change	Three times a week for test duration
Number of replicates	Minimum of 3	Minimum of 10
Test duration	Maximum 48 h	21 d at 25°C for *D. magna* for a set number of broods in control replicates
Endpoints	Mortality or immobility upon stimulation	First-generation survival, number of young produced, first appearance of broods, number of broods, dry weight of survivors/replicate

Do not feed animals during tests. Longer-term tests require modifications of standard conditions.

c. Criteria for test acceptability: An acceptable test will have no more than 10% mortality in the control. A clear dose response must be apparent from a sample plot of the data. Ideally, one concentration will have no effect (no more mortality than control), and the EC_{50} will be bracketed by concentrations producing mortality. Use a scatter plot to visualize possible outliers; use statistically sound methods to verify outliers in the 48-h data. When outliers exist, examine records for clerical or experimental errors.

2. Long-Term Tests

a. Determine toxicity effect(s) on survival, growth, and reproduction: Sublethal effects may occur at lower toxicant concentrations than those causing acute toxicity. Precede long-term tests by acute (48-h) toxicity tests to establish the maximum concentration to be used.

b. Prepare test medium: Prepare test medium as regular culture medium, but use water representative of the effluent receiving water, or the dilution water used to culture daphnids when testing chemicals. Prepare a series of six to ten 1-L quantities of medium to which graded amounts of effluents, mixtures, or chemicals have been added. Dilute each successive concentration in a consistent progression (e.g., geometric). Use a reference toxicant concentration range that will put the 48-h LC_{50} at the approximate midpoint of the dilution series. Use test dilution water as a control. Dispense each liter of test medium in 100-mL quantities to each of 10 glass or plastic chambers. Run tests in replicates of at least 10.

c. Perform tests: Preferably conduct test according to Good Laboratory Practice standards/regulations.[2–4] Segregate and collect 24-h-old neonate *Daphnia* that have been cultured at the test temperature. Introduce one neonate into each chamber randomly. Cover all test chambers loosely with transparent covers to minimize evaporation. Add an appropriate amount of food (see 8711B.2*b*) per animal per day. First-generation *Daphnia* (the animals used to begin the test) may be transferred to new media as necessary, but at least three times weekly. Make daily observations and note dead or immobilized animals. As animals grow and reproduce, count and remove young; record the number. Continue test for 21 d at 20 or 25°C (there may be regulatory preferences in different regions). Handle animals as in the individual cultures of stock animals. This test design may be inappropriate for highly volatile chemicals because of possible evaporation.

At the end of the exposure period, analyze results and test for statistically significant differences in the number of young produced, first-generation *Daphnia* survival, and if appropriate, the dry weights of surviving animals in each treatment. Note appearance of first broods and number of broods.

d. Criteria for test acceptability: A test is invalid if control mortality or male development exceeds 20% during exposure. The controls should produce ≥60 young, on average, over the duration of the test. If ephippia are produced in the controls, the test is invalid.

3. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

4. Quality Assurance/Quality Control

Quality assurance (QA) practices for toxicity tests address all aspects of the test that affect data quality,[5–7] including sampling and handling, source and condition of test organisms, condition of equipment, test conditions, instrument calibration, replication, use of reference toxicants, record keeping, and data evaluation.

Prepare a control chart for the reference toxicant. Plot toxicity values (LC_{50}) from at least five tests and calculate the mean (x) and upper and lower control limits ($\pm 2s$).[1] Examine each new LC_{50} to determine whether the results are within prescribed limits. In this technique, a running plot is maintained for the toxicity values from successive tests with the reference toxicant.

Run reference toxicant tests periodically. Suggested reference toxicants are sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride ($CdCl_2$), or sodium dodecyl sulfate (SDS). Intra-laboratory studies demonstrated a 20°C, 48-h LC_{50} for cadmium of 121 μg/L with a 49% coefficient of variation (CV) for *D. magna* and of 42 μg/L (CV 45%) for *D. pulex*.[1] The 20°C, 48-h LC_{50} for SDS was 20.9 mg/L (CV 28%) for *D. magna* and 12.6 mg/L (CV 32%) for *D. pulex*.[1] If the LC_{50} from a test using a reference toxicant does not fall in the expected range for *Daphnia* based on the reference-toxicant control chart, the organisms' sensitivity and the test system's overall credibility are suspect. In this case, examine test procedure for defects and repeat with a different batch of *Daphnia*.

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8712 *CERIODAPHNIA**

8712 A. Introduction

Unlike other cladocerans, members of the genus *Ceriodaphnia* (family Daphniidae) lack a rostrum and tail spine. *Ceriodaphnia* are amenable to laboratory culture due to their smaller size and shorter generation time than *Daphnia,* their closely related and morphologically similar counterpart. Under optimal conditions, *Ceriodaphnia* produce three, sometimes four broods within one week of hatching, while the larger *Daphnia* do not reproduce until the fourth to sixth instar stage after hatching.[1,2]

A life-cycle test using *Ceriodaphnia* was developed in the early 1980s[3] and subsequently introduced for use in effluent and ambient water evaluation.[4] Several methods have since been published.[5–7]

1. Life History

Ceriodaphnia have a life history similar to those of other daphnids and are believed to occur in limnetic areas all over the world.[2] *Ceriodaphnia* are pond- and lake-dwelling species that are usually common among the vegetation in littoral areas. The *Ceriodaphnia* life cycle has four distinct periods:

1) egg,
2) juvenile,
3) adolescent, and
4) adult.

The growth rate is faster during juvenile stages.[1] The adolescent period consists of a single instar, when the first clutch of eggs reaches full development in the ovary.[1] The lifespan of *Ceriodaphnia* from release of the egg into the brood chamber until death varies; it depends on temperature and other environmental conditions. At 25 and 20°C, the average lifespan for *Ceriodaphnia dubia* is 30 and 50 d, respectively.[8] The longer lifespan in colder water is attributed to a lower metabolic activity.

The organism is called a *neonate* during its first instar stage (when it is still <24 h old). The time to sexual maturity for *Ceriodaphnia dubia* varies from 3 to 5 d and probably depends on body size and environmental conditions—particularly temperature, water quality, and food quality and availability. The eggs hatch in the brood chamber and the neonates are released in about 38 h, just before the adult female molts.[1] The number of young per brood is highly variable and depends on environmental conditions. Typically, *C. dubia* produces 6 to 10 young per brood, but broods with as many as 25 young occur. The organism's growth rate is greatest during the early instar stages, and body size may double during each instar. Each instar stage is terminated by a molt. Growth occurs immediately after each molt while the new carapace is still elastic.

Ceriodaphnia populations consist almost exclusively of parthenogenic females during most of the year. Males appear primarily in the autumn and late spring. Males resemble immature females in size, and in cultures are recognizable by their elongate shape, rapid and erratic swimming habits; absence of a brood pouch; denser coloration; and extended antennules and claspers.[1] The factors responsible for the appearance of males are not fully understood.[1–2] Scientists partially attribute the production of eggs that hatch into male neonates to stress conditions (e.g., overcrowded females, less available food, and lower water temperature).[1–2] If continued, these conditions appear to induce the production of sexual eggs. Gametogenic females are morphologically similar to parthenogenic females; however, sexual females produce only a few "resting eggs" and can copulate with males. When the fertilized eggs of these females pass into the brood chamber, the chamber walls become dark and thick and form an ephippium. *Ephippia* are embryos encased in a tough covering that are resistant to drying. The development of ephippia among cladocerans is an adaptation to adverse environmental conditions and allows populations to survive both drought and freezing conditions.

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* Approved by Standard Methods Committee, 2016.
Joint Task Group: Sandra Valeria Buratini (chair), Adrienne Cibor.

8712 B. Selecting and Preparing Test Organisms

1. Obtaining and Selecting Test Species

Effluent toxicity tests currently use only *Ceriodaphnia dubia* and *Ceriodaphnia dubia*, toothed-pecten variety (a morphological variant) (see Figures 8712:1–3 for distinguishing morphological characteristics).

Ceriodaphnia are available from government and private laboratories and commercial biological supply houses. When starting cultures with organisms from an outside source, species taxonomy must be confirmed microscopically by a qualified taxonomist.[1] A culture can be started with 10 to 20 organisms. To ensure an adequate supply, start organism cultures at least 2 weeks before neonates will be needed for testing.

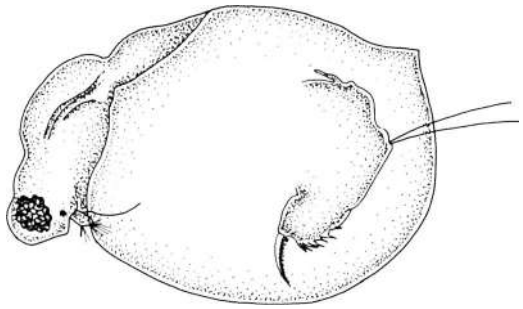


Figure 8712:1. ***Ceriodaphnia dubia.***

2. Culturing Organisms

Ceriodaphnia should be cultured in an incubator or a constant-temperature room. The culture area should be well ventilated with an air supply free of odors and dust.

a. Water supply and renewal: Culture water for *Ceriodaphnia* can be an uncontaminated surface water, well water, dechlorinated municipal drinking water, or a synthetic (reconstituted) water medium.[1,2] Synthetic water usually is recommended because its quality is known and standardized, it is easily prepared, and it yields reproducible results. *Ceriodaphnia dubia* can be cultured successfully in hard, moderately hard, or soft reconstituted water, as needed for the toxicity test.

Prepare soft reconstituted water with 50 g/L stock solutions of sodium bicarbonate ($NaHCO_3$), magnesium sulfate ($MgSO_4$), and potassium chloride (KCl) to provide 48.0 mg $NaHCO_3$/L, 30.0 mg $MgSO_4$/L, and 2.0 mg KCl/L. Add gypsum ($CaSO_4 \cdot 2H_2O$) in powdered form to provide a concentration of 30.0 mg/L. Double these concentrations to prepare moderately hard reconstituted water. Use reagent-grade chemicals and deionized water. (See Section 8010 for further details.) Aerate water for 24 to 48 h before use. Pass air through a water filter containing granular activated charcoal. After confirming water-quality parameters (hardness, pH, etc.), analysts can add sodium selenate (Na_2SeO_4) and vitamin B_{12}, as recommended, to provide 2 μg selenium and 1 to 2 μg vitamin B_{12} per liter of final culture water.[1,2]

Reconstituted waters have been associated with culture and toxicity test performance problems due to contamination by unidentified and toxic components in the deionized water used to prepare the media[3] or due to a nutritional deficiency caused by the absence of a full complement of trace elements (synthetic waters contain four salts).[4] Carbon-treated distilled water is best for preparing synthetic culture waters.

Renewal frequency also can affect the survival and reproductive success of *C. dubia*. Thus, the organisms must be transferred to fresh medium at least three times a week (typically on Monday, Wednesday, and Friday).

b. Food and feeding: Ceriodaphnia dubia must be fed daily during culturing and testing. Cladocerans are filter feeders that are most abundant in eutrophic lakes during summer phytoplankton blooms. Researchers found algae, bacteria, and detritus when examining *Ceriodaphnia* gut contents,[1] so to provide suitable nutrition, use both a combination food (known as YCT) and unicellular algae (most frequently *Pseudokirchneriella subcapitata*, formerly *Selenastrum capricornutum*). YCT consists of yeast, dried and powdered cereal or alfafa leaves,* and commercially available fish flake food† or trout chow. *Pseudokirchneriella subcapitata* cultures can be established and maintained by following the algal culture procedures in Section 8111F. Use a concentrate of 3 to 3.5 $\times$ 10^7 cells/mL. Prepare digested trout chow or fish flake food as follows: add 5.0 g of fish flake food or trout chow pellets in a blender containing 1 L of deionized (Milli-Q™ or equivalent) water. Blend at high speed for 5 min and pour into a 2-L separatory funnel. Digest by aerating continuously through spigot opening for 1 week at ambient laboratory temperature. Water lost due to evaporation must be replaced during digestion. At the end of the digestion period, place mixture in a refrigerator and let it settle for 1 h. Filter the supernatant through a fine mesh screen (e.g., NITEX™, 110 mesh) and discard the sediment. The supernatant can be used fresh, or frozen until use.

Prepare dried, powdered cereal leaves as follows: place 5.0 g of dried, finely ground cereal or alfafa leaves in 1 L of deionized (Milli-Q™ or equivalent) water in a blender. Mix at high speed for 5 min. Place in a refrigerator overnight to settle. Pour supernatant into a clean bottle and discard the remaining solids.

Prepare yeast as follows: place 5.0 g of dry yeast (e.g., Fleischmann's® Yeast) in 1 L of deionized (Milli-Q™ or equivalent) water. Stir with a magnetic stirrer or mix in a blender at low speed until the yeast is well dispersed. Combine this suspension immediately (do not allow to settle) with trout-chow and cereal-leaf supernatants as follows.

To prepare combined YCT food, mix equal (approximately 300-mL) volumes of trout-chow supernatant, cereal-leaf supernatant, and yeast. Determine dry weight (i.e., total solids) concentration of each batch; the YCT mixture should contain 1.7 to 1.9 g total solids/L. Place 30- to 50-mL portions in small polyethylene bottles with screw caps. YCT food can be used immediately or frozen until needed. Fresh or thawed food must be stored in the refrigerator between feedings and used within 2 weeks.

Feed *Ceriodaphnia* mass cultures daily at the rate of 7 mL YCT and 7 mL algae concentrate/L culture. Feed individual cultures and

* Such as Cereal Grass Medium, Carolina Biological Supply Co., Burlington, NC 27215, or equivalent.
† Tetramin®, or equivalent.

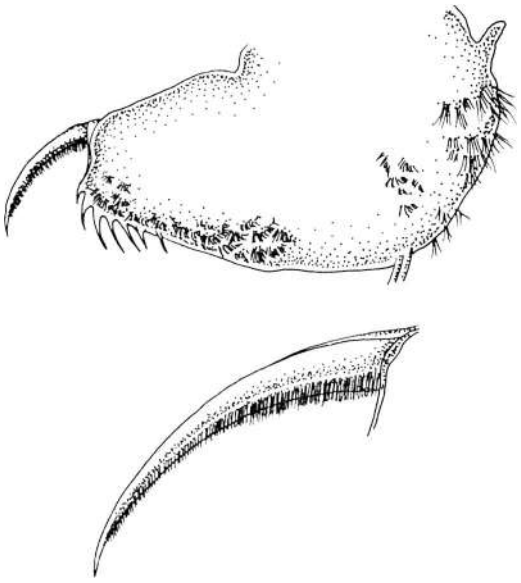


Figure 8712:2. ***Ceriodaphnia dubia:*** (above) postabdomen; (below) postabdominal claw.

test chambers daily at the rate of 0.1 mL YCT and 0.1 mL algae. The quality of each new batch of *Ceriodaphnia* food can be determined in a 7-d reproduction test with control water. Culture grid records also can be used to evaluate food quality.[1]

c. Temperature: Ceriodaphnia dubia should be cultured at 25 ± 1°C. Avoid sudden sharp changes in temperature, which may stress or kill organisms.

d. Lighting: Variations in ambient light intensities and prevailing day/night cycles in most laboratories do not affect *Ceriodaphnia* reproduction significantly. Light intensity should be 50 to 100 ft-c. A photoperiod of 16 h light and 8 h darkness is recommended.

e. Culture vessels and maintenance: Ceriodaphnia dubia may be maintained in mass cultures as a reserve source of organisms to initiate individual cultures and replace lost ones. In 2-L glass beakers or crystallization dishes, add 10 to 20 neonates per liter of culture water. Feed cultures daily and transfer organisms to new culture water at least twice a week for 2 weeks; then discard adults and restart culture with neonates. It is advisable to maintain at least four mass cultures of different ages (0 to 2 weeks) in separate vessels.

Individual cultures are required as a source of neonates for toxicity tests. Culture individual adults in 30-mL beakers or plastic cups containing 15 to 20 mL culture water. Construct styrofoam (or similar rigid material) culture boards consisting of slots that hold the beakers or cups. Maintain at least two boards (60 individuals per board) to ensure backup cultures. Feed organisms daily and transfer them to new culture media at least three times per week (typically on Monday, Wednesday, and Friday). During each transfer, record the number of young produced for a minimum of 10 females. Adults can be used as sources of neonates until 14 d old. Start new boards weekly, using neonates from adults that produce at least eight young in their third or fourth brood. Do not use cultures exceeding 20% adult mortality or <20 young per female (on average) over 7 d to supply neonates used in toxicity tests.

f. Air supply: Ceriodaphnia can survive when the dissolved oxygen (DO) concentration is as low as 2 mg/L but reproduce better when the concentration is >6 mg/L. As long as the culture water source is properly aerated, individual culture boards of *Ceriodaphnia* do not require aeration. Maintain DO levels within criteria for warm-water aquatic life (i.e., a minimum of 2 mg/L).[5]



Figure 8712:3. ***Ceriodaphnia dubia*, toothed-pecten variety:** (above) postabdomen; (below) postabdominal claw.

3. Selecting Test Organisms

For the acute test detailed below, use first instar neonate *Ceriodaphnia* <24 h old. To obtain neonates for a test, use a disposable, wide-mouth (approximately 4 mm) pipet to transfer adult females into a new culture vessel containing fresh culture water up to 24 h before initiation. Keep pipet tip under the water surface and gently release into the test chamber to prevent air from being trapped under the organism's carapace. On the morning that organisms are needed, remove neonates from these vessels and group them together until the group consists of twice as many as needed to begin testing. Acute tests typically receive 5 (or more) organisms per test chamber.

For the chronic test, use first-instar neonate *Ceriodaphnia* <24 h old and obtained within an 8-h window from females that produce at least eight young in their third and subsequent broods. The day before test initiation, transfer females from one culture board into fresh media. On test-initiation day, select 10 appropriately populated cups from the culture board.

4. References

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8712 C. Toxicity Test Procedures

Because the number of replicates and number of organisms per replicate are different in acute and chronic testing, it may not be appropriate to derive acute survival data from a chronic test for compliance purposes, depending on permit requirements (e.g., if using the EPA Test of Significant Toxicity statistical method).

1. Acute Toxicity Tests

Prepare test materials, dilution water, and toxicant solutions as described in Section 8010F. Make at least 100 mL of each test solution and control (see Table 8712:I). Adjust temperature of test solutions to either 20 or 25 ± 1°C. DO should remain ≥4.0 mg/L; if necessary, aerate test solutions to ≥6.0 mg/L before test initiation. Measure temperature, DO, conductivity, and pH in all test concentrations, and measure alkalinity and hardness in the control and highest concentration before test initiation.

The test consists of a control and at least five sample concentrations. Prepare a minimum of four replicates of ≥15 mL solution per test concentration and control. Place test chambers in random order. After preparing test solutions, segregate neonates that have been released from the mothers' brood chambers during the preceding 24 h, and collect in one vessel containing culture water at the appropriate test temperature. Use neonates cultured at the test temperature. Feed animals at least 2 h before test initiation because they are not fed during most short-term tests.[1] Randomly distribute five neonates (<24 h old) to each replicate of the test solutions and control. Use a glass or plastic disposable pipet with a 2-mm bore to collect and transfer neonates; keep the pipet tip just above the water surface when releasing neonates to prevent cross-contamination.

A 48-h exposure is generally accepted for a *Ceriodaphnia* acute toxicity test.[1] However, depending on the study objectives, tests may be conducted for 24, 48, or 96 h. If the test is conducted for 96 h, feed organisms 2 h before renewing test solutions at 48 h. At each 24-h interval during the test, record either the number of surviving animals or the number of motile animals in each test vessel. Consider an animal non-motile if it shows no independent movement even after gentle squirting with test solution from a pipet (non-motile animals are not necessarily dead). This endpoint should be verified under a dissecting microscope. At 24-h intervals, record the temperature, DO, conductivity, and pH in one surrogate chamber per concentration. Cover all test chambers loosely with plate glass or equivalent to minimize evaporation without affecting oxygen transfer. If DO drops below acceptable limits, either aerate all test chambers very gently or place all test chambers in a sealed container and pump sufficient oxygen into the headspace. Up to 5% carbon dioxide (CO_2) may be added to the chamber to control pH drift.[2]

If lethality is the test endpoint, an LC_{50} value can be generated. If immobility is the test endpoint, an EC_{50} value can be generated. The LC_{50} value represents the concentration of test solution or chemical at which 50% of exposed animals have died. The EC_{50} value represents the concentration of test solution or chemical at which 50% of exposed animals are immobilized. Test results usually are considered acceptable as long as there is ≤10% control mortality.

2. Short-Term Chronic Toxicity Tests

a. Prepare test medium: Prepare via methods used to prepare regular culture medium, but use water representative of the receiving waste effluent or dilution water used to culture *C. dubia* when testing chemicals. For effluents, mixtures, or chemicals, prepare a series of at least five test concentrations with at least 250 mL per test concentration and control. Tests conducted with effluents generally use a 0.5 serial dilution so the effluent concentrations tested are 6.25%, 12.5%, 25%, 50%, and 100% effluent. Use test dilution water as a negative control (i.e., 0% effluent).[3] Adjust temperature to 25 ± 1°C. Measure temperature, DO, pH, and conductivity in all test concentrations, and hardness and alkalinity in the control and highest concentration. Maintain DO ≥4.0 mg/L; aerate to ≥6.0 mg/L if necessary before introducing organisms. For each test concentration, pre-

TABLE 8712:I. SUMMARY OF ECOLOGICAL AND TOXICOLOGICAL TEST CONDITIONS USING *CERIODAPHNIA DUBIA*

Ecology/Test Condition	Acute Test	Chronic Test
Habitat	Fresh water	Fresh water
Duration	24, 48, or 96 h	6–8 d, when 60% of surviving control females have had at least 3 broods
Life stage tested	<24 h old	<24 h old
Test temperature	20 or 25 ± 1°C	25 ± 1°C
Size of test chamber	≥30 mL	30 mL
Volume of test solution	≥15 mL	15 mL
Number of animals per container	5	1
Number of replicates	4	10
Dilution water	Moderately hard water; other waters determined by purpose of test. NOTE: If test organisms were cultured in water different from the test dilution water, then a second set of controls using culture water should be included in the test.	
Renewal of test solutions	After 48 h	Daily
Nutrition	Feed YCT and algae suspension while holding before test; add 0.1 mL each YCT and algae suspension 2 h before test-solution renewal at 48 h*	Feed 0.1 mL algae and 0.1 mL YCT daily after test-solution renewal
Photoperiod	16 h light:8 h dark	16 h light:8 h dark
Dissolved oxygen	≥4.0 mg/L	≥4.0 mg/L
pH	6–9	6–9
Control acceptability criteria	Mortality not to exceed 10%	Mortality not to exceed 20%; at least 60% of surviving control females have 3 or more broods by Day 8; ≥15 neonates per surviving female.
Endpoint(s)	Survival	Adult survival and reproduction

* Algal concentration of 3 to 3.5 × 10^7 cells/mL; YCT (yeast, cereal leaves, and trout chow mix).

pare 10 replicates in 30-mL glass beakers or plastic cups. Dispense each test solution in 15-mL volumes.

b. Perform tests: Preferably conduct test according to good laboratory practice standards/regulations.[4–8] Select and distribute <24-h-old neonate *Ceriodaphnia* that have been cultured at the test temperature, in accordance with 8712B.3. All neonates used for a single test should be hatched within 8 h of each other to prevent inconsistent young production among replicates.

Initiate the test using block randomization; identify 10 different parent organisms with an adequate number of neonates to initiate one replicate from each test concentration. Using only neonates from a single parent organism, introduce one neonate into Replicate 1 of each control and test concentration. Select another parent organism and repeat for Replicate 2, then again for Replicate 3, etc. This is known as *blocking by known parentage*. Initiating each replicate with known parentage ensures that if there is a problem with the viability of neonates from a particular brood, it will affect only one replicate per concentration and all concentrations will be affected equally.

Add 0.1 mL YCT and 0.1 mL algal suspension to each test vessel. Cover all test chambers loosely with plate glass or equivalent to minimize evaporation without affecting oxygen transfer.

At 24-h intervals, transfer first-generation organisms (i.e., *Ceriodaphnia* used to begin the test) into fresh test solutions and add an appropriate amount of food to each test chamber (see Section 8711B.2*b*). Under some chronic test protocols, *Ceriodaphnia* may be transferred to new test media less frequently (at 48-h intervals), but food still must added to each test chamber daily. Make daily observations, and note number of dead parent animals. Measure temperature, DO, pH, and conductivity daily in both old and new test solutions.

If necessary, aerate test solutions before transfer into test chambers. Do not aerate test chambers. If aerating test solutions does not sufficiently maintain DO until the next water exchange, then place all test chambers into an oxygenated environment in accordance with 8712C.1.

With each water renewal, remove the young and record the number of live young. If not transferring first-generation organisms to fresh test solution daily, then remove young on days without water renewal and record the number of live neonates produced. Continue test for 6 to 8 d at 25°C until at least 60% of the control females have produced at least three broods. Terminate test within 2 h of original start time. Handle test animals as in the individual cultures of stock animals.

At end of exposure period, analyze results and test for statistically significant differences in first-generation *Ceriodaphnia* survival and reproduction (i.e., number of live neonates produced) in each treatment. Note appearance of first broods and number of broods. Animals not producing young should be examined to determine if they are males. If males are present, or if all replicates from one of the initial females have had unusually poor reproduction or survival rates, they may be culled from reproduction data before analysis.

3. Criteria for Test Acceptability

a. Acute: An acceptable test will have ≥90% mean survival in the controls.

b. Chronic: An acceptable test will have ≥80% mean survival in the controls, regardless of whether they are male or female, and 60% of surviving females in the control replicates must produce three broods. The average live young produced over the course of the test must be ≥15 per surviving female. If <6 replicates in the control remain after excluding males, the test is invalid and must be repeated with a newly collected sample.[3]

4. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

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8714 MYSIDS*

8714 A. Introduction

1. Suitability for Toxicity Tests

Mysids are an important component of both the pelagic and epibenthic communities. They are preyed upon by many species of fish, birds, and larger invertebrate species, and they are predators of smaller crustaceans and larval stages of invertebrates. In some cases, they feed on algae. Mysids are sensitive to both organic and inorganic toxicants. The ecological importance of mysids, their wide geographical distribution, ability to be cultured in the laboratory, and sensitivity to contaminants make them appropriate toxicity test organisms.[1–8] Juvenile mysids used in these tests are taken from cultures shortly after release from the brood and exposed to varying concentrations of a toxicant in static or flow-through conditions. These procedures will be useful for conducting toxicity tests with other species of mysids, although modifications may be necessary. The tests are applicable to most chemicals, either individually or in formulations, commercial products, and known or unknown mixtures, and with appropriate modifications can be used to conduct tests on such factors as temperature, salinity, and dissolved oxygen. These methods also can be used to assess the toxicity of potentially toxic discharges, such as municipal wastes, oil-drilling fluids, produced water from oil well production, and other types of industrial wastes.

* Approved by Standard Methods Committee, 1997. Editorial revisions, 2010. Joint Task Group: 20th Edition—Brian J. Finlayson and Suzanne M. Lussier (co-chairs), Donald J. Reish, Morris H. Roberts.

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8714 B. Selecting and Preparing Test Organisms

1. Selection of Test Species

Test species may be designated by a particular regulation (e.g., Federal Insecticide, Fungicide and Rodenticide Act; Toxic Substances Control Act). If the toxicity test is for nonregulatory purposes, any number of species can be tested if test conditions are suitable for culturing the species. If it is desirable to use mysids that are not cultured routinely, it may be necessary to collect them from a single field source.

Select test species that meet the following criteria:

- The species preferably occurs, or is closely related to a species that occurs, in the receiving water being studied;
- the species is available in unbiased (i.e., not prescreened for resistant individuals by prior exposure to adverse conditions) numbers sufficient for the tests;
- the species can be held in the laboratory in a healthy condition (i.e., active, feeding, free of lesion, etc.); and
- the species represents an important trophic link or economic resource in habitats similar to that of the receiving water.

If the data are available when selecting species, consider relative sensitivities of different species and life stages.

In accordance with the criteria listed in Section 8010E.1, the recommended test species include (but are not restricted to) the following:

a. Estuarine and freshwater mysids:

Neomysis mercedis (Figure 8714:l)

Americamysis almyra[1]* (Figure 8714:2)

* *Americamysis almyra, A. bahia,* and *A. bigelowi* are three of six species forming the group *Americamysis,* confined to the northwestern Atlantic and endemic to estuarine waters along the east coasts from New England to Colombia, and now distinguished from the group *Mysidopsis.* In previously published literature, they may be referred to as *Mysidopsis almyra, M. Bahia, and M. bigelowi*, respectively.

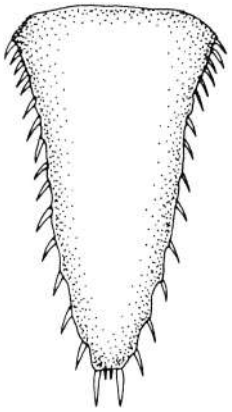


Figure 8714:1. ***Neomysis mercedis:*** telson.

b. Marine mysids:

Holmesimysis costata[2] [= *Acanthomysis sculpta*]† (Figure 8714:3)

Americamysis bahia[1]* (Figure 8714:4)

Americamysis bigelowi[1]* (Figure 8714:5)

2. Collecting and Handling Test Organisms

Collecting equipment and methods are described in Sections 8010E.2, 10200B, and 10500B. Handling and holding are discussed in Sections 8010E.3 and 4. NOTE: Avoid subjecting mysids to unnecessary stress, such as inappropriate capture and transport, temperature shock, or water quality change.

a. Estuarine and freshwater mysids: Neomysis mercedis (Figure 8714:1) is a Pacific coast species living in fresh and estuarine water to 18 g/kg salinity estuarine water; its temperature range is 6 to 22°C.[3] *N. mercedis* ranges from Prince William Sound, Alaska, to south of Point Conception, California.

Collect *N. mercedis* by hand dip nets or plankton tows in rivers and estuaries. Collection with a dip net (0.5- to 1.0-mm mesh) at night results in minimal mechanical damage[4] and yields many specimens in good condition and with little accompanying debris. Transfer specimens to a 100-L (30-gal) plastic container filled with site water and transport to the laboratory. Aerate with a portable air pump. Separate *N. mercedis* from other organisms and discard any specimen that is injured or does not appear to be in good condition. Specimens can be picked up via a bulb pipet with a 5-mm bore or via a plastic spoon. Alternatively, collect organisms by towing a plankton net (0.5-mm mesh) from a boat in open water. This technique can result in high mysid mortality and much accompanying detritus. *N. mercedis* are abundant between February and July but scarce during the remainder of the year.[5]

Americamysis almyra[1] (Figure 8714:2) is an East Coast species often living sympatrically with *A. bahia,* but preferring lower salinities ranging from 10 to 20 g/kg at temperatures over 20°C.[6] It is found in inshore waters along the entire coast of the Gulf of Mexico and northward along the Atlantic coast to Patapsco River, Maryland.[1] Collect *A. almyra* with hand dip nets (350-μm mesh) or a 1.5-m beam trawl with a 0.9-mm mesh size pulled by hand in shallow areas of estuaries.[7] The dip net method yields many specimens in good condition and with little accompanying debris. Remove possible predator species, such as ctenophores, immediately. Transfer specimens to an insulated 4-L (1-gal) or larger plastic container filled with site water and transport to the laboratory. Aerate with a portable air pump. Separate *A. almyra* from other organisms and discard any specimen that is injured or does not appear to be in good condition. Specimens can be picked up via a bulb pipet with a 5-mm bore. Alternatively, collect organisms by

† *Holmesimysis costata* is one of five species of the genus *Holmesimysis*, which is present in the North Pacific Ocean. Confusion has existed about the genus in which to place the mysid used in toxicity tests in California. Up to 1988, all authors referred to this mysid species as *Acanthomysis sculpta* (Tattersall). All known species of the genus *Acanthomysis* from the Pacific Coast of North America were placed in the new genus *Holmesimysis.*

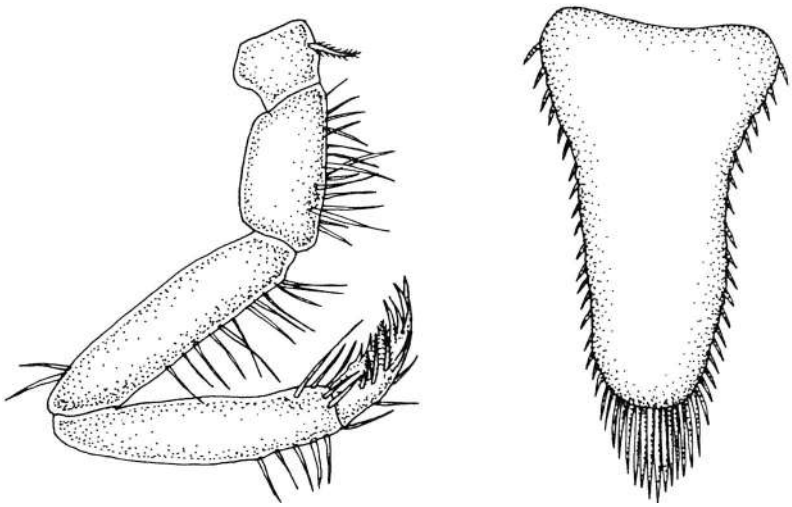


Figure 8714:2. ***Americamysis almyra:*** (left) endopod of thoracic leg 2; (right) telson.

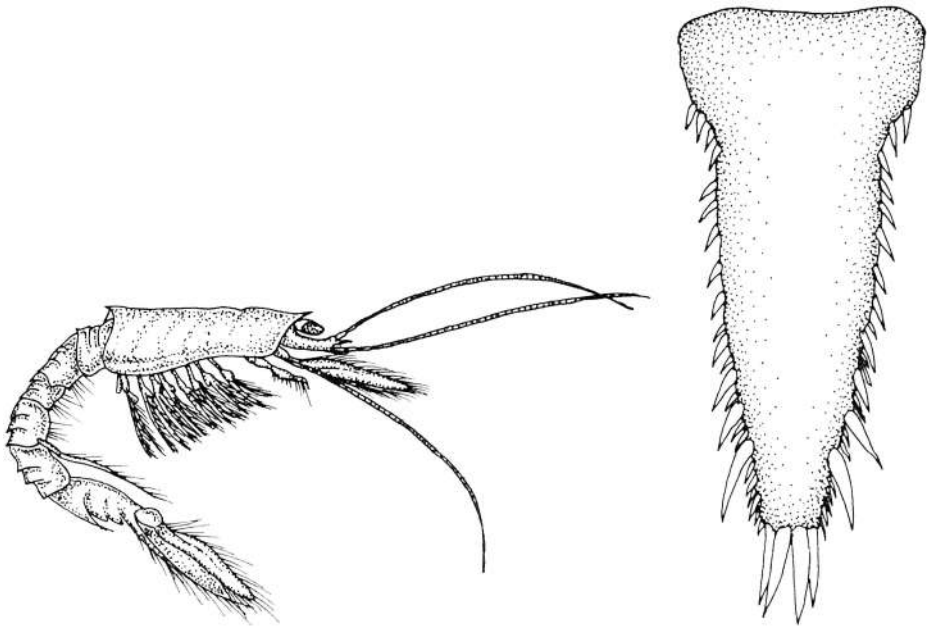


Figure 8714:3. ***Holmesimysis costata:*** (left) entire animal; (right) telson.

towing a plankton net (350-μm mesh) from a boat in open water at night. However, this technique can result in high mysid mortality and much accompanying detritus. *A. almyra* are abundant throughout the year in the southern latitudes.

b. Marine mysids: Holmesimysis costata (*Acanthomysis sculpta*)[2]† (Figure 8714:3) is the principal species of the genus in California marine waters. *H. costata* occurs abundantly offshore among the fronds of giant kelp, especially during summer months.[8] Collect *H. costata* from a boat by passing a hand net (0.5- to 1.0-mm mesh) through the kelp canopy. Transfer specimens to a 20-L (5-gal) bucket filled with seawater and transport to the laboratory. Pour contents of the bucket into one or more pans and separate *H. costata* from other organisms. Discard any specimen that is injured or does not appear to be in good condition. Some specimens might be parasitized externally by a marine leech; do not use these specimens or place them in the laboratory stock colony. Mysids can be picked up via a bulb pipet with a 5-mm diam.

Americamysis bahia[1] (Figure 8714:4), often sympatric with *A. bigelowi* and *A. almyra* at temperatures over 20°C[6] but preferring salinities ranging from 10 to 30 g/kg[9] is found in inshore waters along the coast of the Gulf of Mexico and northward along the Atlantic coast to Narragansett, R.I.[1] *Americamysis bigelowi*[1] (Figure 8714:5), is found on the Atlantic coast from Massachusetts (Georges Bank) southward to Florida. It often occurs sympatrically with *A. bahia*, with a salinity range from 30 to 35 g/kg[9] and in water temperatures from 2 to 30°C.

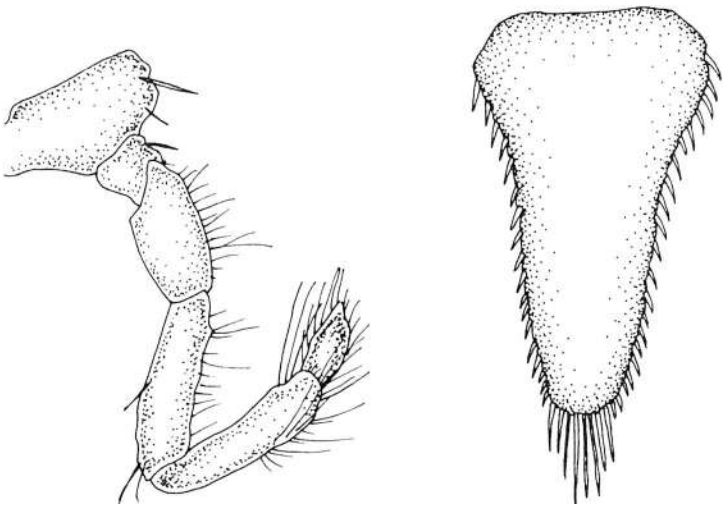


Figure 8714:4. ***Americamysis bahia:*** (left) endopod of thoracic leg 2; (right) telson.

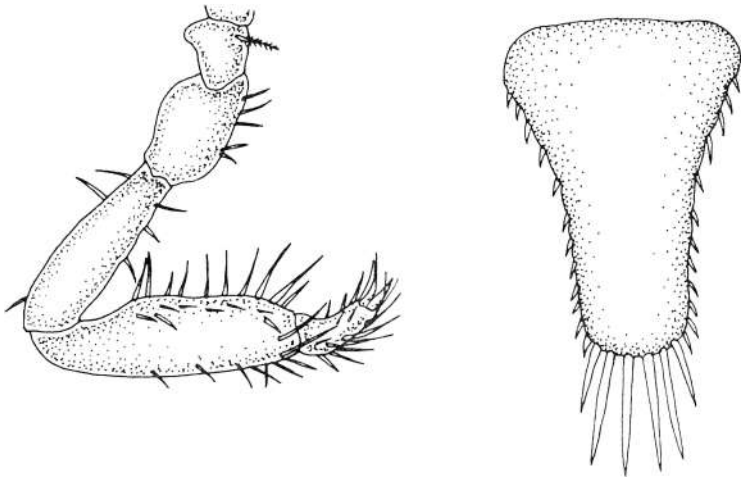


Figure 8714:5. ***Americamysis bigelowi:*** (left) endopod of thoracic leg 2; (right) telson.

Collect *A. bahia* and *A. bigelowi* by using hand dip nets (350-μm mesh) in shallow areas of salt ponds and estuaries.[10] This method yields many specimens in good condition and with little accompanying debris. Remove possible predator species, such as ctenophores, immediately. Transfer specimens to an insulated 4-L (1-gal) or larger plastic container filled with site water and transport to the laboratory. Aerate with a portable air pump. Separate *A. bahia* and *A. bigelowi* from other organisms and discard any specimen that is injured or does not appear to be in good condition. Specimens can be picked up via a bulb pipet with a 5-mm bore. Alternatively, collect by towing a plankton net (350-μm mesh) from a boat in open water at night. However, this technique can result in high mysid mortality and much accompanying detritus. *A. bahia* and *A. bigelowi* are abundant throughout the year in the southern latitudes, and from June through September in temperate latitudes in shallow water with a temperature above 20°C.

3. Holding, Acclimating, and Culturing Organisms

See Sections 8010E.3 and 4.

Keep mysids in tanks, aquariums, or screened enclosure depending on size and number. Use good quality dilution water (see Section 8010E.4*b*) for acclimation. Feed mysids live brine shrimp nauplii daily during acclimation. At least once daily, feed live brine shrimp nauplii in excess to mysids in brood stock tanks and in test chambers, to maintain live nauplii in the chambers at all times to prevent cannibalism and to support adequate survival, growth, and reproduction in the brood stock. Adjust ration in accordance with the number of mysids in the stock colony. A ration of 150 nauplii per mysid per day has been used successfully.[8] A regime of 75 nauplii per mysid twice a day or 50 nauplii three times a day might improve growth and reproduction in the brood stock. Use diets that are certified toxicant-free or test for toxic substances before use.

a. Estuarine and freshwater mysids:

1) *N. mercedis*—Culture in either a static or a flow-through system. For static system, use 75- to 114-L aquariums supplied with aeration and a subsurface filter of 3- to 5-cm-thick dolomite. *N. mercedis* is extremely sensitive to nitrogenous wastes; clean aquariums daily to remove excess food. For a flow-through system, supply sufficient water for a minimum of two tank volumes per day. Successful cultures have been maintained at temperatures between 15 and 19°C (optimum 17°C), hard fresh water (150 to 200 mg $CaCO_3$/L hardness and alkalinity), and additional natural seawater or reconstituted seawater (see Section 8010E.4*b*) to salinity of 1 to 3 g/kg (optimum 2 g/kg).[4,8] Feed *Artemia salina* nauplii[11] to mysids three times a day at the rate of 50 nauplii/mysid/feeding (a total of 150 nauplii/ mysid/d) and add an artificial food supplement containing vitamins and minerals (0.02 to 0.06 mg/mysid) every other day. Preferably supplement diet with commercially available food‡ having micronutrients and vitamins; supplements of rotifers and algae also may be beneficial.

Under laboratory conditions and water temperatures of 15 to 19°C, a life cycle for *N. mercedis* is completed in approximately 3 months. A gravid female will carry an average of 20 embryos in a brood, of which an average of seven will be released.[4]

To collect young *N. mercedis* for acute static or flow-through toxicity testing, place females carrying embryos, which are in the eye-development stage, in brood chambers, 7 to 14 d before starting the test. For brood chambers, use cages covered with 0.25- to 0.50-mm nylon mesh, which allows the neonates to escape into the main body of the aquarium but retains the adults. Remove neonates each day from the aquarium with a fine (0.5-mm) mesh dip net and transfer to a dish. Remove healthy specimens for testing. Pool the young released over a 2- to 3-d period and transfer them to a holding vessel until a sufficient number are obtained for a test.

2) *A. almyra*—Preferably culture in a flow-through system supplied with sufficient water for a minimum of two tank volumes per day for best control of salinity and nitrogenous wastes.[9] Alternatively, culture in static 76-L (20-gal) or larger aquariums supplied with aeration and a subsurface filter of 3- to 5-cm-thick dolomite. Successful cultures have been maintained at a temperature of 25 to 27°C (optimum 26°C), in natural seawater or reconstituted seawater (see Section 8010E.4*b*) at salinity of 10 to

‡ TetraMarin®, or equivalent.

20 g/kg. Feed *Artemia* nauplii[11] to mysids twice a day at the rate of 75 nauplii/mysid/feeding (a total of 150 nauplii/mysid/d). Preferably supplement *Artemia* cultures with an enhancement product§ to ensure the nauplii's amino acid content.[11] Supplementing the diet of *A. almyra* with rotifers and algae also may be beneficial. There is little information regarding the life cycle and brood size for *A. almyra*, but growth, respiration, and energetics studies have been conducted.[7]

Juveniles for acute static or flow-through toxicity testing can be collected in several ways, depending on the frequency of tests and number of animals needed. If juveniles of the same age are required intermittently, place females carrying embryos (in the eye-development stage) in brood chambers or aerated finger bowls 1 d before starting the test. The brood chambers should be either 4-L beakers with netted (1-mm) bottoms placed within wide-mouth separatory funnels, or cages covered with l-mm mesh‖ placed in an aquarium, allowing neonates to escape into the main body of the aquarium but retaining adults. Remove neonates the next day from the separatory funnel, aquarium, or bowl with a 0.5-mm mesh dip net and transfer to a dish. Remove healthy specimens for testing.[9] When age-standardized mysid juveniles are not required, place adults collected from cultures into large mesh containers within separate aquariums, allowing adults to be lifted out into new aquariums every few days and leaving juveniles to be siphoned or netted out as needed. Pool young released over a 2- to 3-d period and transfer to a holding vessel until enough are obtained for a test. When juveniles are needed almost daily for testing, use a siphon entrapment system ("mysid generator"). In this system, juveniles are continuously siphoned out of an aquarium (adults are excluded by a 750-μm screen) into a collection vessel. Juveniles are collected daily and can be used either for testing or for starting up new culture tanks.[12]

b. Marine mysids:

1) *H. costata*—The organisms can be picked up via a bulb pipet with a 5-mm diam. For acclimation, place *H. costata* in an aerated aquarium at a density of approximately 10 to 20 specimens/L seawater. Change the water if it becomes cloudy. Animals can be cultured in the laboratory on a diet of *Artemia* nauplii larvae, powdered fish flake food, and fresh fronds of giant kelp (*Macrocystis*). Preferably add a fresh, carefully washed frond of *Macrocystis* to the brood stock to provide additional substrate and food for mysids.

H. costata can complete three or four life cycles a year under laboratory conditions. Females will produce more than one brood set under laboratory conditions. To obtain young mysids, place adult *H. costata* in a cage within an aquarium. Use a cage covered with 0.25-mm-mesh nylon screening, which allows the newborn to escape into the main body of the aquarium but retains adults. Remove newborn from the aquarium with a fine dip net or glass pipet and transfer to a dish where specimens can be observed and removed for testing.

2) *A. bahia* and *A. bigelowi*—Follow culturing instructions for *A. almyra* [¶ *a*2) above], but use salinity of 25 g/kg.

Under laboratory conditions with water temperatures of 25°C, a life cycle of *A. bahia* and *A. bigelowi* is completed in 1 month or less, at which time the first eggs are laid. A gravid female will carry an average of eight embryos in a brood, all of which are normally released as healthy postlarvae. The first brood is released after 14 to 18 d, and succeeding broods are released every 7 d. Productivity gradually declines in the last third of the 3- to 5-month lifespan.[9]

Collect juveniles for acute static or flow-through toxicity testing as directed in ¶ *a*2) above.

4. Parasites and Diseases

For general problems and control procedures, see Section 8010E.5.

Unexpected and often unexplained mortalities in experimental and control animals interfere with test results and interpretations. Optimize laboratory conditions for each species to prevent the development of disease. Maintain salinity and temperature appropriate for particular species and consistent with specified test conditions.

Reproduction will be depressed when culture density is high. This phenomenon has not occurred when cultures are maintained at densities of 10 mysids/L or less. Therefore, when cultures are not being used to supply test organisms, remove enough adults at least every 2 weeks to stimulate reproduction. Preferably keep neonates, juveniles, and adults of mysids in separate tanks. Keep brood stock tanks free of other animals, such as amphipods, hydroids, and worms. If an outbreak of such animals occurs, remove all mysids and clean tank thoroughly. Examine mysids thoroughly before replacement, and discard any with hydroids attached. Clean tanks with hot water and a 5% solution of hydrochloric or nitric acid.[9] Wash, dry, and autoclave substrate (i.e., dolomite or oyster shells), or discard it.

Handle mysids as little as possible. When handling is necessary, proceed gently, carefully, and quickly to reduce stress. Dip nets are best for removing gravid female mysids from brood-stock tanks. Such nets are commercially available or can be made from 350-μm mesh nylon netting, silk bolting cloth, plankton netting, or similar knotless material. Discard mysids that touch dry surfaces or are dropped or injured. Sterilize equipment used to handle mysids between uses by autoclaving. Wash new equipment with detergent and rinse with water, a water-miscible organic solvent, water, acid (e.g., as 10% conc HCl), and at least twice with deionized, distilled, or dilution water. At the end of a test, clean all equipment via a procedure appropriate for removing the test material (e.g., acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic chemicals) and rinse at least twice with deionized, distilled, or dilution water.[13]

5. References

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§ SELCO, or equivalent.
‖ Nytex®, or equivalent.

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8714 C. Toxicity Test Procedures

1. Short-Term Procedures

a. General test procedures: Short-term testing can be used to determine relative toxicity of substances. Tests are made to determine LC_{50} or EC_{50} values and to estimate toxicant concentrations for intermediate- and long-term tests. Basic requirements for toxicity tests are described in Section 8010C.

Short-term tests may be static, static with renewal, or flow-through, depending on the objective of the test and the character of the toxicant or effluent (see Section 8010D). Acute static, static with renewal, or flow-through toxicity tests are conducted preferably with young mysids in accordance with other studies with this group.[1–3]

Collect young mysids of nearly uniform size in accordance with instructions given in 8714B.3. *H. costata* used in acute toxicity tests should be 3 to 7 d post-release from the brood sac, and *N. mercedis*, 1 to 5 d post-release. *Americamysis* species should be less than 24 h post-release from the brood sac. Use 10 to 20 mysids per toxicant concentration. Transfer mysids to each test chamber via a glass pipet. Feed mysids with brine shrimp larvae three times a day at the rate of 30 nauplii/mysid (a total of 90 nauplii/mysid/d) during the test period. Examine test chambers daily, record mortality, and remove all dead specimens and debris. Generally it is not necessary to consider mysid weight/L test solution given the low weight of mysids, but in flow-through tests, use less than 10 g mysids/L test solution if temperatures are at or below 17°C and 5 g mysids/L test solution at higher temperatures. For static testing, do not load more than 0.8 g mysids/L at 17°C or less and 0.5 g mysids/L at temperatures above 20°C. Limit loading to ensure that concentrations of dissolved oxygen and test material do not fall below acceptable limits, concentrations of metabolic products do not exceed acceptable levels, and test mysids are not stressed because of cannibalism, aggression, or crowding.

b. Specific test procedures:

1) Freshwater and estuarine mysids

a) Equipment and physical conditions—Ensure that equipment and facilities that contact stock solutions, test solutions, or any water into which test organisms will be placed do not contain substances that can be leached or dissolved by aqueous

solutions in amounts that adversely affect mysids. General requirements for toxicity test systems and materials are described in Section 8010F.1. In addition, choose equipment and facilities that contact stock or test solutions to minimize sorption of test materials from water. Use glass, Type 316 stainless steel, nylon, and fluorocarbon plastics to minimize dissolution, leaching, and sorption, but do not use stainless steel in tests on metals with salt water. Do not use cast-iron pipe with salt water, and avoid its use in a fresh water-supply filter system because colloidal iron will be added to the dilution water and strainers will be needed to remove rust particles. Do not let brass, copper, lead, galvanized metal, or natural rubber contact dilution water, stock solutions, or test solutions before or during the test. Avoid items made of neoprene rubber or other materials not mentioned previously unless it has been shown that their use will not adversely affect mysid survival, growth, or reproduction.[1,2]

Use reagent-grade test material or better unless a test of formulation, commercial product, technical-grade, or use-grade material is specifically needed. Before a test is begun, note the following about the test material: identities and concentrations of major ingredients and impurities; solubility and stability in dilution water; the analytical method's precision and bias at the test material's planned concentration(s); and estimate of toxicity to humans.

Select temperature appropriate for the species being tested, and hold test temperature within ±2°C of mean test temperature during a 96-h test, or ±1°C for any 48-h period. Conduct tests with *Americamysis almyra* at 26 to 28°C,[2] and tests with *Neomysis mercedis* at 15 to 19°C.[2] Keep salinity within the selected species' tolerance range. The optimum salinity for *A. almyra* is 10 to 20 g/kg and for *N. mercedis* is 1 to 3 g/kg.[2] If a test salinity other than the optimum is used, set up another control at the optimum salinity. Use ambient laboratory lighting with a photoperiod of 16 h light/8 h dark, preferably with 15- to 30-min dusk/dawn transition period to acclimate mysids to the test photoperiod.

Use dilution water from a surface source, well, or spring, or use reconstituted water (see Section 8010E.4*b*). *N. mercedis* cultures have not been reported for media of reconstituted fresh water. Do not use chlorinated water as, or in the preparation of, dilution water, because chlorine-produced oxidants are toxic to mysids.[3] Establish a supply of dilution water that is available in adequate quantities, acceptable to test organisms, uniform in quality, and not likely to affect test results unnecessarily. An acceptable dilution water is one in which the test species will survive, grow, and reproduce satisfactorily. Maintain uniform quality of the dilution water so the test organisms are cultured or acclimated, and the test is conducted in water of the same quality.[1,2,4]

Use at least two test chambers (in which containers may be placed) for each concentration; these can consist of standard 57-L aquariums or can be constructed by gluing strong window glass with clear silicone adhesive. Because adhesives can sorb some organochlorine or organophosphorus pesticides, apply as little adhesive as possible. Finger bowls can be used for static acute toxicity test containers; for a 2-L bowl, use 20 animals; for a 350-mL bowl, 10 animals. Each bowl constitutes a replicate. Flow-through toxicity tests can be conducted in a 2.5-L wide-mouth glass jar with a central standpipe. The test solution enters the compartment directly and flows through the standpipe into a drain. Cover standpipe with a 200- to 235-μm mesh nylon screen to avoid escape of the young mysids.[1,2,4]

For information pertaining to species selection, collection, holding, acclimation, disease control, and culturing, see Sections 8010E and 8714B.

b) Test procedure—Range-finding test: If the test material's approximate toxicity is unknown, conduct an abbreviated range-finding test to determine the concentrations to use in definitive tests. Use three to five widely spaced toxicant concentrations (e.g., a decade test with concentrations a factor of ten from each other). Static tests may be acceptable, as would use of fewer mysids (e.g., five per container). Run this test for 24 to 96 h.

Definitive test: To determine LC_{50} or EC_{50} values, use a 96-h test period with a minimum of five toxicant concentrations (according to the results of the range-finding test) and a control. In some cases, the test solution can be added directly to the dilution water, but usually it is dissolved in a solvent to form a stock solution that is then added to dilution water. If a stock solution is used, determine the concentration and stability of its test material and dilution water before beginning the test. If the test material is subject to photolysis, shield stock solution from light. Use a solvent control if dosing solutions are prepared in an organic solvent. Acceptable solvents are dimethylformamide, ethanol, methanol, acetone, and triethylene glycol (see Section 8010F.2*b*). Limit solvent concentrations to 0.1 mL/L of test solution. If a solvent other than water is used and the solvent concentration is the same in all test solutions that contain test material, include at least one solvent control containing the same concentration of solvent and using solvent from the same batch used to make the stock solution, and also include a dilution water control. If a solvent other than water is used and the solvent concentration is not the same in all test solutions that contain test material, include both a solvent control (containing the highest concentration of solvent present in any other treatment, using solvent from the same batch used to make the stock solution) and a dilution water control. The percentage of organisms that show signs of disease or stress, such as discoloration, unusual behavior, or death, must be 10% or less in the solvent control and in the dilution water control.

To establish definitive test concentrations, prepare solutions using a dilution ratio of 1.5 to 2 between successive concentrations (see Section 8010F.3*b*).

c) Test initiation—On day of test, remove enough mysids from the holding facility at once to provide about one-third more animals than are needed. Select a set of test chambers [one test chamber for each test concentration plus control(s)] to be processed together to avoid possible selective bias during loading. Transfer mysids with a wide-bore (larger than the largest mysid) glass pipet with a smooth tip. Begin static tests by placing test organisms in the chambers within 30 min after test material was added to dilution water. Begin flow-through tests by placing test organisms in the chambers after test solutions have been flowing through them long enough for test material concentrations to have reached steady state.

d) Biological observations—Monitor survival by daily inspection (see Section 8010F.3*e*). The criteria for mysid death are opaque white coloration, immobility (especially absence of movement of respiratory and feeding appendages), and lack of reaction to gentle prodding. Count, record, and remove dead mysids daily. Count live animals at the beginning of the exper-

iment and daily to account for cannibalism or death resulting from impingement on the sides of test compartments. Record missing or dead impinged animals. Do not stress live test organisms in an attempt to determine whether they are dead, immobilized, or otherwise affected. Prodding of organisms and movement of test chambers during test should be done gently. Some organisms exposed to some organophosphorus compounds seem to be sensitive to sudden changes in light intensity.

e) Chemical data recording—Analyze water in control and test chambers daily for pH, dissolved oxygen, salinity (for marine or estuarine species), and temperature (see Section 8010F.3*d*). Maintain DO concentrations at ≥60% saturation. When testing volatile substances, do not aerate test solution. However, take care that chemical substances that create a DO demand do not result in conditions inconsistent with the test's DO criterion or mysid health. As a last resort to maintain DO above the criterion, use aeration. If aeration is used, make frequent measurements to confirm test chemical concentrations and aerate all test chambers, including controls.

f) Verification of exposure—Before and during the test, verify exposure concentrations of the test chemical (see Section 8010F.3*d*). In static and renewal tests, measure the test material concentration, if possible, in at least the control and high, medium, and low concentrations of test material at the beginning and end of test. Measurement of degradation products may be desirable. Measure concentration of test material in flow-through test chambers as often as practical during test. Measure in all chambers concurrently at least once during test, preferably near the beginning. Except for the control treatment, measure each test chamber (especially those concentrations closest to LC_{50}) at least one additional time during the test on a schedule designed to give reasonable confidence in the material's concentration in the test chambers during the entire exposure period, taking into account the flow rate and number of independent metering devices; and measure at least one appropriate chamber whenever a malfunction is detected in any part of the metering system.

2) Marine mysids—The test procedures for marine mysids are essentially identical to those for freshwater and estuarine mysids except for the noted differences in biological and environmental requirements. Conduct tests with *H. costata*[2] at 17 ± 2°C for south of Point Conception, California, and 15 ± 2°C for north of Point Conception, and with 30 to 35 g/kg salinity. *H. costata* cultures have not been reported for media of reconstituted seawater. Conduct tests with *A. bahia* and *A. bigelowi*[1] at 27 ± 1°C and 20 to 30 g/kg salinity.

2. Life-Cycle Test Procedures for Marine and Estuarine Mysids

a. General test procedures: Life-cycle testing can be used to determine relative long-term toxicity of substances. Tests are conducted to determine changes in numbers and weights of individuals resulting from effects of test material on survival, growth, and reproduction. Results may be used to predict long-term effects in field situations, compare chronic sensitivities of different species, and chronic toxicities of different materials.

Life-cycle toxicity tests are flow-through (see Sections 8010D.1 and 4) with a flow rate through each test container of at least 5 to 10 volume additions per 24 h. Start tests with young mysids in accordance with other studies with this group.[1,2,4] Mysids of *Americamysis* species used in life-cycle toxicity tests should be less than 24 h post-release from the brood sac; collect in accordance with instructions given in 8714B.3. Start test with two containers of 15 or three containers of 10 mysids each, in at least four to eight true replicate chambers per concentration. Transfer mysids to each test chamber with a glass pipet. Feed mysids with brine shrimp larvae three times a day at the rate of 50 nauplii/mysid (a total of 150 nauplii/mysid/d) during test.[5] Examine test chambers daily, record mortality, and remove all dead specimens and debris. Generally it is not necessary to consider mysid weight/L test solution given the low weight of mysids, but in flow-through tests, use less than 5 g of mysids/L of test solution at temperatures above 20°C. Limit loading to ensure that concentrations of DO and test material do not fall below acceptable limits, concentrations of metabolic products do not exceed acceptable levels, and test mysids are not stressed because of cannibalism, aggression, or crowding.[6]

b. Specific test procedures: Conduct *Americamysis bahia, A. bigelowi*, and *A. almyra* tests at 27°C for approximately 28 d. Conduct life-cycle toxicity tests by two general methods: pairing and non-pairing.[4] The pairing method may make it easier to collect population data for life table analysis. If the pairing method will be used, start the test with at least two containers of 15 randomly selected mysids each, in at least two true replicate chambers per concentration [see ¶ *b*3) below]. Pair mysids at sexual maturity (Day 12 to 14), with one female and one male in each test container. Preferably use at least 20 randomly selected pairs per treatment; transfer them between containers within a test chamber (but not from one test chamber to another) to create as many pairs as possible. Pair all mysids on the same day of the test. If the non-pairing method will be used, start test with at least three containers of ten randomly selected mysids each, in at least four true replicate chambers per concentration[6] [see ¶ *b*3) below]. Keep mysids in these containers throughout the test.

1) Equipment and physical conditions—For general guidance on equipment and materials, see 8714C.1*b*1)a). For the test material, in addition to the items noted in 8714C.1*b*1)a), ascertain acute toxicity to test species, measurement or estimate of chronic toxicity to test species, and recommended handling procedures.

Select temperature appropriate for the species being tested, and hold test temperature within ±1°C of mean test temperature. Conduct tests with *Americamysis* species between 26 and 28°C. Keep salinity within the selected species' tolerance range. The optimum salinity for *A. bahia* and *A. bigelowi* is 20 to 30 g/kg; for *A. almyra*, it is 10 to 20 g/kg (see 8714B.2). If a test salinity other than the optimum is used, set up another control at the optimum salinity. Use ambient laboratory lighting with a photoperiod of 16 h light/8 h dark, preferably with 15- to 30-min dusk/dawn transition period to acclimate mysids to the test photoperiod.

For dilution water requirements, see 8714C.1*b*1)a).

Calculate minimum number of test chambers, test containers, and pairs of mysids per treatment, expected variance within test chambers, expected variance between test chambers within a treatment, and either the maximum acceptable confidence interval on a point estimate or the minimum detectable difference using hypothesis testing.[4,6]

Test solution can flow from one container to another but not from one chamber to another. Test chambers can be constructed

by gluing strong window glass with clear silicone adhesive. Because adhesives can sorb some organochlorine or organophosphorus pesticides, apply as little adhesive as possible. Cover chambers to prevent contamination and reduce evaporation. Test solution may enter the container directly, containers may be oscillated in the test solution, or the water level in the test chamber may be varied via a self-starting siphon. Test containers used successfully include 250-mL glass beakers with holes drilled in the sides and covered with 250-μm mesh*, 90- or 140-mm-ID glass Petri dish bottoms with collars made of 210- or 250-μm screen,[7] and 110- $\times$ 180- $\times$ 200-mm-deep glass rectangular chambers partitioned into six containers with a 65-mm-high, 330-μm mesh nylon collar. Provide metering system that will accommodate type and concentration(s) of test material and necessary flow rates of test solutions. Mix test material with dilution water immediately before entrance to test chambers, and supply selected concentration(s) of test material reproducibly.[1] Ensure that mysids remain submerged and are not stressed by crowding or turbulence in exposure system. Use test containers that provide a surface area of at least 25 cm^2/mysid and a solution depth of at least 25 mm at all times.[4,6]

For information pertaining to species selection, collection, holding, acclimation, disease control, and culturing, see Sections 8010E and 8714B.

2) Test procedure—For general information on test procedures, see Section 8010F.3. If a life-cycle test is intended to allow calculation of an endpoint, include one or more control treatments and a geometric series of at least five concentrations of test material, each of which is at least 50% of the next higher one. Use results from range-finder or definitive tests to determine the appropriate range of concentrations. To determine whether a specific concentration reduces survival, growth, or reproduction, only that concentration and control(s) are necessary; however, two additional concentrations at about one-half and twice the specific concentration of concern are preferable.

While test solution sometimes can be added directly to dilution water, preferably dissolve it in a solvent (reagent-grade or better) to form a stock solution and add stock solution to dilution water in the metering system. If a stock solution is used, determine the test material's concentration and stability in it before beginning the test. If test material is subject to photolysis, shield stock solution from light.

Use a solvent control if dosing solutions are prepared in an organic solvent. Acceptable solvents are triethylene glycol, methanol, ethanol, and acetone.[4] Limit concentrations of solvent to 0.1 mL/L test solution. Do not use surfactant in preparation of a stock solution. If a solvent other than water is used and solvent concentration is the same in all test solutions that contain test material, include at least one solvent control containing the same concentration of solvent (using solvent from the same batch used to make the stock solution) and a dilution water control. If a solvent other than water is used and its concentration is not the same in all test solutions that contain test material, conduct a solvent test to determine whether survival, growth, or reproduction of the test species is related to the solvent concentration over the range used in the toxicity test (if such a solvent test with the same dilution water and test species has not already been conducted). A life-cycle test is unacceptable if any treatment contains a solvent concentration in the range of effect. If no effect on the test species using the same dilution water is found at the test concentration of solvent, a life-cycle test may be conducted using solvent concentrations within the tested range, but include in such tests a solvent control containing the highest concentration of solvent present in any other treatment (using solvent from the same batch used to make the stock solution) and a dilution water control.

The percentage of organisms that show signs of disease or stress, such as discoloration, unusual behavior, or death, must be 30% or less in both solvent and dilution-water controls.

To establish test concentrations, set highest concentration of life-cycle test to be equal to the lowest concentration that caused adverse effects in a comparable definitive acute test. Use a dilution ratio of 1.5 to 2 between successive concentrations.[1] For more information on experimental design, see Section 8010F.3*a*.

3) Test initiation—On the day that the toxicity test begins, remove enough mysids from the holding facility at once to provide about one-third more animals than are needed. Transfer mysids gently with a wide-bore (larger than the largest mysid) glass pipet with a smooth tip. Begin flow-through life-cycle tests by placing test organisms into randomly selected containers (after test solutions have been flowing through the test chambers long enough for the concentrations of test material to have reached steady state).

4) Biological observations—See 8714C.1*b*1)d). Also count, record, and remove live young in each container daily. Record day of brood release.

Determine dry weight (dried at 60°C for 72 to 96 h or to constant weight) of each individual first-generation mysid alive at end of test to nearest microgram. Rinse mysids with deionized water to remove salt before drying. Weigh males and females separately to determine sex-specific effects.[8] Remove brine shrimp nauplii caught in female brood sacs before drying. Total body length (total midline body length from anterior tip of carapace to posterior margin of endopod of uropod, excluding setae) may be determined for mysids alive at the end of the test, but not for preserved mysids because of body curvature. Note any abnormal development or aberrant behavior for first- and second-generation mysids.

5) Chemical data—Analyze water in control and test chambers daily for salinity and temperature. Measure pH and DO in the control at beginning, end, and at least weekly during the test, and measure pH at least once in the highest test concentration. Also measure DO whenever there is an interruption of test solution flow. For DO maintenance requirements, see 8714C.1*b*1)e).

6) Verification of exposure—Before and during the test, verify exposure concentrations of test chemical. Measure concentration of test material in the flow-through test chambers at least weekly in each treatment, including the control(s) during the test. Measurement of degradation products may be desirable. If a malfunction that could alter the test material concentration is detected in the metering system, take water samples immediately from affected test chambers and analyze as soon as possible.

* Nytex®, or equivalent.

3. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

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8740 DECAPODS*

8740 A. Introduction

Decapod crustaceans are among the most commercially important invertebrates.

Larval, postlarval, or adult stages of several species of decapods may be found in large numbers in estuaries and rocky intertidal habitats near the shore, where they are vulnerable to various types of discharges. Because of their phylogenetic relationship to insects, and because pesticides often are applied in watersheds draining to estuaries, using decapods in pesticide toxicity testing is particularly relevant. Postlarvae of penaeid shrimp use the estuaries as nursery grounds until they are large enough to migrate offshore. Early life stages are particularly vulnerable.

There is considerable diversity among decapods, with the basic separation of adult animals into the more active swimmers (Natantia), including shrimp and lobsters, and the more sedentary crabs (Reptantia). While there are exceptions and variations, shrimp are generally planktivores, while lobsters and crabs are predators and scavengers. Decapods are much more similar at the larval and postlarval stages, when zooplankton is the primary food. Laboratory holding and testing is easier at these early stages because brine shrimp (*Artemia salina*) nauplii usually are an appropriate food.

* Approved by Standard Methods Committee, 1999. Editorial revisions, 2010.

8740 B. Selecting and Preparing Test Organisms

Some species used frequently in toxicity studies are penaeid shrimp (*Penaeus* sp.) larvae, postlarvae, and juveniles,[1–3] which have high commercial value on the Gulf of Mexico coast, and larvae of the American lobster,[4] an equally important commercial species along the northeast coast of the United States. Grass shrimp, *Palaemonetes* sp., is another species that has been used in many toxicity investigations[5–12] because of the ease of collecting and holding, abundance in marshes and estuaries along the Gulf of Mexico and southeastern U.S. coast, and sensitivity, especially to pesticides.

1. Selecting Test Organisms

Many of the species used previously in toxicity testing are listed below. Consult specific references to determine which life stages were tested and the methods used to collect, hold, feed, and expose the animals. For information on selecting test organisms, as well as the handling, holding, and conditioning them, see Section 8010E.1–3. Regional references to location and identification of decapods are given in Section 10900.

a. Marine and estuarine decapods:

1) Suborder Natantia

a) Section Penaeidea—Species of *Penaeus* are prominent commercial shrimp harvested in the Gulf of Mexico.

Penaeus setiferus
Penaeus aztecus
Penaeus duorarum

b) Section Caridea

Crangon—cosmopolitan
Palaemonetes pugio—southeast U.S. coast, and Gulf of Mexico.
Palaemonetes vulgaris
Palaemonetes intermedius
Pandalus danae—Pacific Northwest, including Alaska.
Pandalus hypsinotus—Pacific Northwest, including Alaska.

2) Suborder Reptantia

Section Macrura

Panulirus (spiny lobster)—Point Conception south along coasts of southern California and Baja California.
Homarus americanus—northeast U.S. coast
Petrolisthes—U.S. coast of Gulf of Mexico.
Rithropanopeus harrisii—U.S. coast of Gulf of Mexico.
Panopeus herbstii—U.S. coast of Gulf of Mexico.
Menippe mercenaria—rock or reef areas of Florida.
Cancer productus—Pacific U.S. coast
Cancer oregonensis—Pacific U.S. coast
Cancer magister (see Section 10900, Plate 12:M)—Pacific U.S. coast
Callinectes sapidus (see Section 10900, Plate 12:N)—southeast and Gulf of Mexico coasts of the United States
Uca pugilator—southeast U.S. coast

b. Freshwater decapods:

Cambarus—43 species, between Blue Ridge Mountains and Mississippi River (see Section 10900, Plate 12:H).
Procambarus—97 species, New England and Great Lakes to Mexico.
Orconectes—59 species, Maine to Texas, most in Central Basin.
Macrobrachium ohione—Atlantic coastal plain from North Carolina to Georgia, along Mississippi River from St. Louis southward, and Texas.

https://doi.org/10.2105/SMWW.2882.172

Palaemonetes kadiakensis—west of the Alleghenies from southern Ontario and Great Lakes to Gulf coast of northeastern Mexico.

2. Collecting Test Organisms

a. Marine and estuarine decapods: In shallow estuarine environments and tidal flats, collect juvenile or adult decapods with seine nets. Hold the collection within the net in shallow water while gently dipping individuals from the water to a water-filled ice chest. Cool the water to be used for transporting the animals to slow activity and to increase the initial levels of dissolved oxygen. Air pumps with battery packs may be necessary to provide sufficient oxygen during transport, particularly if animal density is high. This collection method is also applicable to gravid adults of some species (blue crabs, *Palaemonetes*, etc.), to hatch the larvae in the laboratory and conduct larval toxicity testing.

Lobsters and blue crabs (*Callinectes*) are best obtained from traps, but a scientific collecting permit may be required to capture and retain gravid females. Penaeid shrimp, *Pandalus* species, and other decapods are best obtained by using 10-min otter trawls, so the animals are subjected to less stress from crowding in the cod end. Rapidly transfer animals into cool, well-aerated water in an ice chest. Avoid excessive crowding during holding on shipboard and during transportation to the laboratory. Provide aeration during transport.

Some crab species (*Rithropanopeus, Petrolisthes, Menippe, Cancer,* etc.) are best collected by hand at low tide, but it will be difficult to obtain enough individuals to conduct tests with adults. These species are best suited for larvae tests.

b. Freshwater decapods: While adults may be tested when a sufficient supply of individuals is obtained, preferably collect gravid females and use the offspring in testing.

3. Holding, Acclimating, and Culturing Test Organisms

Guidelines for culturing test organisms, including decapods, are found in Section 8010E.4. The following sections provide guidelines for collecting, handling, and obtaining larvae from crayfish, crabs (e.g., *Cancer magister*), American lobster (*Homarus americanus*), and shrimp (*Penaeus* and *Palaemonetes*).

a. Water supply: See Section 8010E.4*b*.

b. Acclimating, holding, and maintaining stock cultures: See Sections 8010E.3 and 4. Risks in handling most adult crustaceans usually are not great because of their rigid exoskeleton and general durability. Both larval and adult forms of many species are cannibalistic and readily attack each other in the soft-shell stage. Hold juveniles and adults in individual compartments in long troughs or divided tanks. Form the compartments with perforated separators that slide into slots on the sides. Use stainless steel for freshwater forms and glass, acrylic, plastic, or plywood covered with fiberglass for marine forms. Provide rigid, transparent covers to prevent loss of the highly motile specimens. Use perforated separators to ensure a flow of water through each compartment to remove metabolic products and provide DO. The crustacean growth process, which involves a periodic ecdysis or sloughing of the rigid exoskeleton, imposes a lack of uniformity in test animals that is not readily detectable in advance. In the pre-ecdysis stage and during ecdysis, animals are heavily stressed and more sensitive to unsatisfactory environmental conditions and toxicants.

1) Crayfish—Collect specimens from their natural habitat by trapping, seining, or by hand (see Section 8010E.2). General procedures for holding and acclimating are described in Sections 8010E.3 and 4. Because crayfish are cannibalistic, hold all but the young stages in separate compartments. Suitable holding, acclimating, and culturing chambers are stainless steel, glass, fiberglass-covered wood, or plastic troughs, 180 cm long, 30 cm wide, and 20 cm deep, with a divider down the center to make two long troughs. Make shallow channels on the sides and central divider every 15 cm into which separators can be slipped to make 12 compartments on each side, each approximately 15 $\times$ 15 cm square and 20 cm deep. This size is suitable for crayfish. The number and size of compartments depend on the size and number of organisms to be tested. To hold a large number of small crayfish, remove the separators to make a tank of the desired length. Provide separators with a large number of perforations so they operate as screens. Control water depth in test chambers by a standpipe in the last compartment of the trough. When cleaning separators, temporarily raise them a short distance from the bottom to allow excess food and wastes to be washed out and remove the standpipe in the last compartment to ensure strong flows. Clean routinely with a siphon and a brush to loosen materials from compartments, screens, walls, and bottoms. Supply water adjusted to the desired temperature and DO to the two head compartments via a siphon from a constant-head box. Use a minimum flow of 10 trough volumes/d. Adjust volume to maintain favorable water quality in each compartment. Required water depth depends on size of organisms; 15 cm is preferred. Provide each set of troughs with a transparent lid. For life-cycle studies beginning with eggs or newly hatched young, collect ovigerous females and place in flow-through troughs under natural water conditions. Begin acclimation to different conditions after 2 d. Hold animals in troughs until young hatch. Remove compartment dividers to provide freedom of movement of young. Clean as described in Section 8010E.4*d*. Use macerated fish food for juveniles and adults. Alternatively use prepared dry fish food. Use finely divided pieces of fish and commercial fish food pellets as food for the newly hatched.

2) Crabs—Static culture of brachyuran crab larvae has been achieved for several species of Atlantic coast crabs.[13–16] Long-term static or renewal bioassays with these species have been performed. Culture of dungeness crab (*Cancer magister*) larvae has been reported.[17] Culturing crab larvae requires a favorable water supply and control of competitors, predators, and disease. Filter water and disinfect it via UV light treatment. For unpolluted open ocean water, little or no treatment is required. If the supply is from an estuary receiving organic wastes, purify before use. Filter seawater for the flow-through system via gravity flow through a coarse, quartz sand filter and adjust to desired salinity (approximately 25 to 30 g/kg) by adding fresh water. To remove other organisms, refilter under pressure through sequential layers of 40/60-mesh garnet, 20/30-mesh silica sand, and 0.3-cm hard charcoal. Follow by filtration through a polishing filter and treat with UV light. Use constant-level head boxes equipped with heating, cooling, and stirring devices to deliver constant mea-

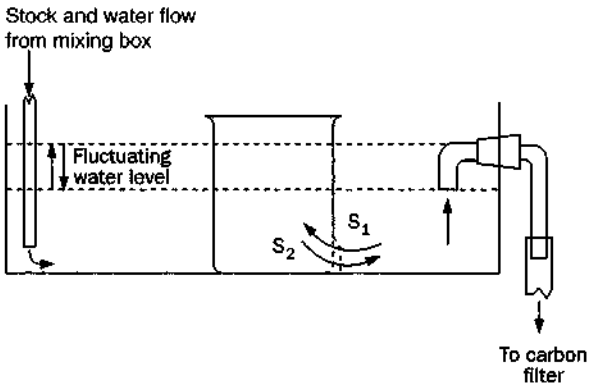


Figure 8740:1. Rearing and exposure beaker and automatic siphon for dungeness crab larvae. After BUCHANAN, D.V, M.J. MYERS & R.S. CALDWELL. 1975. An improved flowing water apparatus for culture of brachyuran crab larvae (unpublished).

sured flows by siphons, selected nozzles, or constant and accurate delivery pumps.

Collect ovigerous females or purchase from fishermen and place in holding tanks or in flow-through troughs similar to, but larger than, those described for crayfish. Acclimate and condition as described in Section 8010E.3. When eggs are ready to hatch, transfer females to static tanks containing aerated and UV-disinfected water at 30 g/kg and 13°C. As eggs hatch, dip out swimming first-stage larvae with beakers and transfer to culture beakers with large-bore pipets. Many crab larvae are positively phototactic. Collection of crab larvae may be simplified by applying an intense light source to one side of the hatching container.

Dungeness crab larvae have long delicate spines that make their culture in flowing systems difficult. Culture larvae to the fourth and fifth stage in 250-mL beakers that have a 15-mm-diam hole blown through their sides near the bottom. Using silicone cement, fasten a plastic screen with 360-μm openings over this hole on the inside of the beaker and a plastic screen with 210-μm openings over the hole on the outside. Because of the lip created by blowing the glass, the two screens are 3 to 4 mm apart. The larger-mesh screen on the inside is less likely to catch and damage spines of larval crabs while the smaller-mesh screen on the outside does not come in contact with larvae but does retain food organisms, brine shrimp nauplii. Set the 250-mL beakers in glass trays or aquariums large enough to accommodate 10 beakers and provide a depth of at least 10 cm. Supply trays with a constant flow of water via a tube that discharges near the tray bottom. Provide an automatic siphon at the outlet so there is continual filling and drawdown (Figure 8740:1). Construct the automatic siphon so when the water reaches the high point and the siphon is activated, the beakers contain approximately 200 mL, and when the siphon is broken, the beakers contain about 150 mL. The automatic siphon consists of a silicone rubber stopper drilled to receive an 8-mm-ID right-angle glass tube on one end and a 5-mm-ID right-angle glass tube on the other end. In a 1.3-cm-diam hole blown through side of tray, insert stopper with 8-mm hole on inside of tray. Insert tubes into stopper (as shown in Figure 8740:1). Placement of hole inside of tray controls water level in beakers at 200 mL. The distance between the top of the inside hole in the stopper and the bottom of the inside siphon leg is equal to the difference in depth between 200 and 150 mL. Make siphon intake perfectly flat and smooth to prevent air from being drawn into the siphon. Adjust tube diameters to give a 15-min cycle: 10-min filling and 5-min drawdown.

When culture chambers are set up and functioning, place 10 first-stage larvae in each beaker with a smooth large-bore pipet. The larvae can be fed nonliving food, but preferably feed first-stage brine shrimp nauplii at the rate of 70 per larva three times/week through the third stage, then 100 brine shrimp per larva. The nutritional quality of brine shrimp will vary depending on source. This will affect the larvae's sensitivity and the test results. Keep density of crab larvae low and food organism density high to minimize crab larvae contacts that may result in cannibalism. Before feeding, transfer larvae to clean, chlorine-disinfected, and rinsed beakers. Maintain at 12 to 13°C, pH 8, and 25 to 30 g/kg salinity. Adjust photoperiod to correspond with natural conditions or, if the cycle is off-season, to correspond to the normal annual cycle of light and dark. Exclude natural light and use fluorescent light (see Section 8010F.3*f*). Under these conditions, survival of 80 to 90% through the fourth zoeal stage has been attained. Larvae usually begin molting into the fifth zoeal stage by the 45th day. Mortalities then increase.

Juvenile and adult dungeness crabs are much less susceptible to disease than larvae. Older life stages are much less sensitive than larvae and will be more tolerant of conditions. With strict sanitation and unpolluted open-sea water, sand filtration alone provides sufficient water quality control. Hold juvenile and adult crabs in trough compartments similar to, but larger than, those used for crayfish. To allow sufficient space for each juvenile crab, use compartments 15 × 15 cm and 15 to 20 cm deep. For adult crabs, use 30 × 30-cm or 40 × 40-cm compartments that are about 30 cm deep. For large specimens, use deeper water. For ease of supplying water, arrange troughs on stands having three shelves with space on each for two troughs. Feed cut-up or macerated fresh fish, clams, or mussels, or commercial dried fish foods to juveniles and adults. Remove unused food within 24 h to reduce fouling. Routinely clean sides and bottoms of compartments and remove wastes with vacuum or siphon cleaners. Raise screen separators a few millimeters and flush as suggested for crayfish troughs.

3) American lobster, *Homarus americanus*—Obtain adult lobsters via trapping or purchase from lobster fishermen. Ovigerous females can be obtained most readily in early spring from lobster fishermen who have permits. Select females with brownish eggs (because these eggs will hatch within a few weeks to a few months, depending in part on water temperature). Place each ovigerous female in a separate holding tank measuring at least 30 × 45 × 30 cm. The tank floor should have a raised grate that allows wastes to fall below the lobster; this helps prevent bacterial contamination of the egg mass. Fasten claws with elastic bands but not wooden pegs. Pass uncontaminated seawater continuously through tank at a rate that maintains DO at or above 80% saturation. Maintain salinity between 30 g/kg and that normal to seawater. Maintain temperature at 15°C. Feed brood lobsters once a week, preferably with crab or large krill, which are available frozen and provide the essential protein-binding carotenoid pigments. Other foods, such as commercial dry pel-

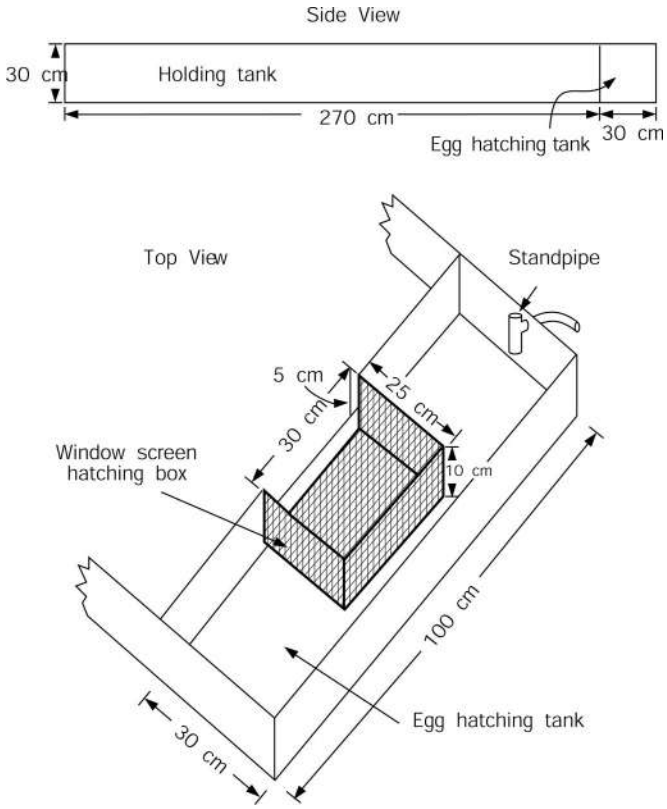


Figure 8740:2. Egg-hatching tank for lobsters.

leted food, can be used for short periods. Use brine shrimp only for larvae and young juveniles.

When eggs are about to hatch, place ovigerous female in a hatching tank measuring 30 × 100 cm (Figure 8740:2). Place a partition across the lower end of the holding tank 30 cm from the end. Locate standpipe at one side of this box to maintain desired water level. Remove a piece 5 × 30 cm long from central portion of partition for outflow from hatching tank. Fit a screen box, 25 cm wide, 15 cm deep, and 39 cm long with a notch 5 by 30 cm in top of one side of the frame, against the notch in the partition. Use plastic window screen with 2-mm-square openings for the screen box. Supply with flowing seawater at a rate sufficient to maintain DO above 80% saturation. Maintain water temperature at 19 to 20°C.[18]

The larval development stages of *H. americanus* have been described.[19–22] The larval period extends from hatching to the fifth molt or attainment of the fifth stage. The duration of this period depends somewhat on water temperature. During the first three stages, larvae are free-swimming, move toward light, and remain near the water surface. At the fourth stage, the larvae are still photophilic but occasionally make excursions to the bottom and back. At the end of this stage, the larvae become photophobic bottom-dwellers.

To rear larvae to various stages, construct from molded fiberglass a special 40-L culture tank with a combined water circulator and overflow device at the center (Figure 8740:3).[23] Only use flowing natural filtered seawater to culture and test lobsters.

The maximum concentration of lobster larvae is about 45/L. At higher concentrations, there are more lobster-to-lobster contacts and cannibalism. As eggs begin to hatch, wash first-stage larvae over the 5- × 30-cm notch in the partition into the screen box. Dip larvae from this box with a small beaker and place in modified Hughes larvae-rearing chamber by submerging the beaker and gently removing it.[22] Stock with less than 225 larvae. Because lobster larvae are cannibalistic, keep dispersed by currents and by providing many food particles for each larva. Best results are obtained by feeding newly hatched nauplii of brine shrimp under slow flows to lobster larvae held in individual containers. An automatic brine shrimp feeder has been described.[24] Techniques for rearing lobster to sexual maturity have been described;[25] such rearing is expensive and not recommended for routine toxicity testing.

A survival of 80 to 90% can be attained by feeding lobster larvae live adult brine shrimp. When lobster larvae reach the fifth stage, place in individual compartments formed by placing separators in a trough (as described for crayfish). For the fifth stage and juveniles, use 15- × 15- × 15-cm compartments. As lobsters grow, use larger tanks. For those weighing 460 g or more, use a 60- × 45- × 30-cm compartment. Feed as recommended for ovigerous females. Ground, whole crabs improve coloration. Feed daily during spring, summer, and fall; during winter, feed once a week. After 24 h, remove all unused food. Clean sides and bottom; siphon and flush out tanks. Growth rate depends not only on food and water quality but also on holding tank size. Long before the lobster is physically restrained, it reduces its growth in response to holding compartment size. During the first calendar year of life, the lobster has an average of 10 molts. In nature, larval molting actively reaches a peak in the 15 to 20°C range; it seldom occurs below 5°C. Lobsters usually reach maturity at a weight of about 460 g. For mating, place a male in a compartment with a female immediately after she has molted and is in the soft stage. Mating success decreases with time after molting. When temperatures of 22 to 24°C are maintained year-round, lobsters reach maturity in 2 years. The rate of egg development depends in part on temperature. For extrusion of eggs, place females in a deeper tank because they need at least 45 cm of water over them. Provide the egg-laying tank with a rough or nonslip bottom that allows female to assume and remain in the egg-laying position until all eggs are laid and attached to the nonplumose hairs of the swimmerets. With stable temperatures, it should be possible to maintain larval cultures year-round. Hold nonovigerous females and those bearing green eggs collected in the fall at low temperatures to retard development. Before eggs are needed, remove and gradually acclimate some females to egg-laying temperatures. Even when eggs have reached the brown stage, hatching can be spread out via different temperature regimes. Another method of producing larvae is to rear and mate lobsters in the laboratory at different times and under different temperature regimes. Although culturing in the laboratory is expensive, it has certain advantages: larvae are produced year-round; larvae are of a known genetic constituency, which can reduce experimental variability; and complete-life-cycle tests can be conducted. A method is available to determine beforehand when lobster eggs will hatch. Once the eye pigment has been formed, monitor the course and rate of embryo development by measuring the eye periodically.

4) Shrimp (Natantia)—Obtain via collection or purchase from bait dealers. Seine shrimp of the genera *Penaeus, Palaemonetes*, and *Crangon* from estuaries. Check animals for parasites, disease, and general condition. For general instructions on collect-

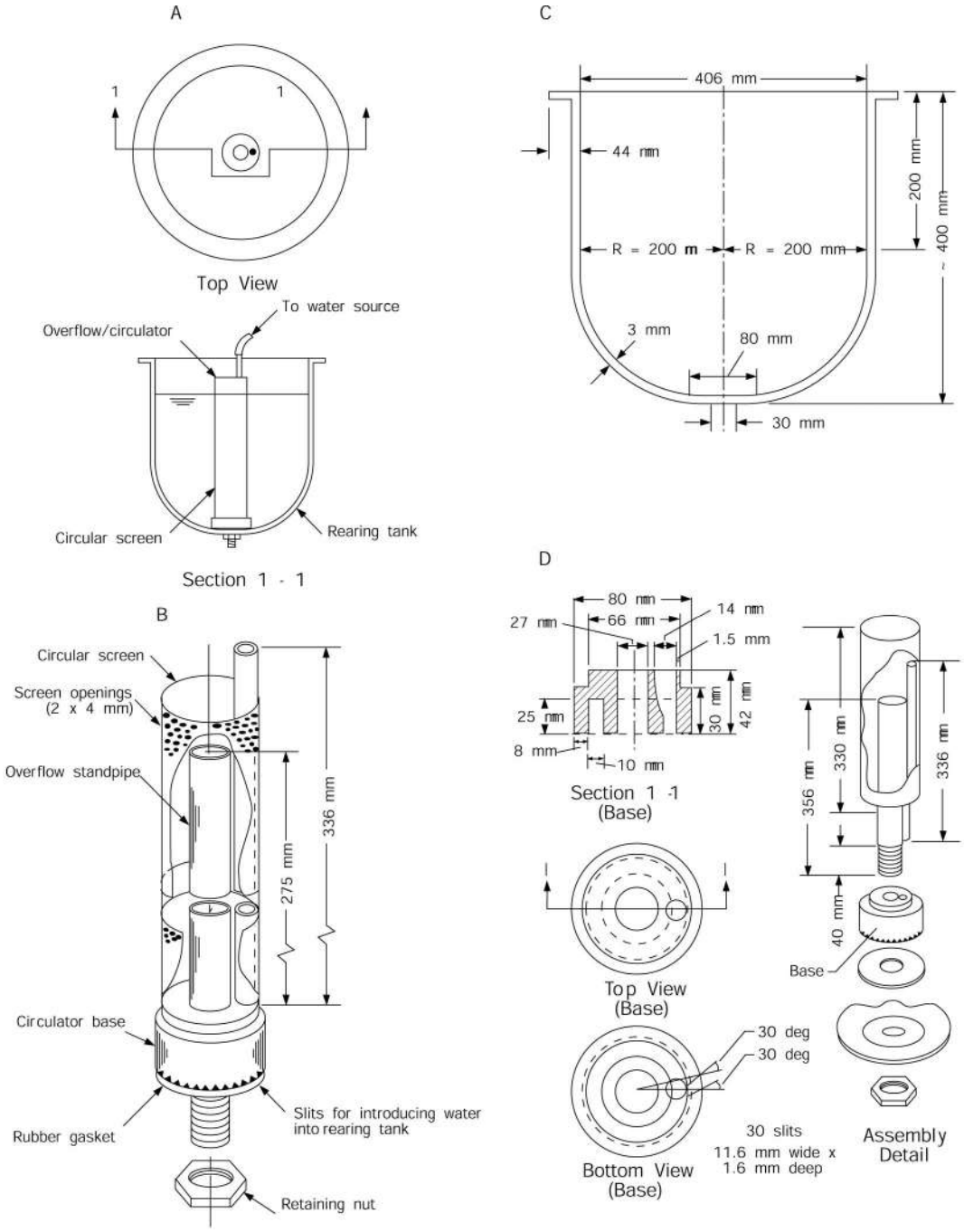


Figure 8740:3. Hughes lobster-rearing tank.[23] A—general views; B—views of overflow-circulator; C—details of rearing tank construction; D—construction and assembly details for rearing tank and overflow/circulator. This is a 40-L tank. For bioassays, scale to 5-L volume.

ing, handling, transferring, holding, acclimating, and culturing, see Section 8010E.

a) *Palaemonetes*—Several marine and freshwater species of the genus *Palaemonetes* have been reared through metamorphosis.[26–32] They are suitable for life-cycle studies and can be brought from the field for direct use or for laboratory rearing. Place field-collected adult shrimp in suitable flow-through aquarium water. Feed freshwater species macerated parts of local fishes; feed marine forms macerated mollusks or fish. Examine shrimp periodically to detect ovigerous females. When eggs are nearly ready to hatch, remove desired number of females from tank and put into individual containers. Keep females in these containers, preferably with flow-through water, until eggs begin to hatch. During this period, feed macerated fish or other suitable food. After eggs hatch, remove female and feed prelarvae or prezoeae 1-d-old *Artemia salina* nauplii. The rearing procedure for larvae is similar for all six species. Use equipment and procedures similar to those for the dungeness crab, but with 1-L

rearing chambers set in a deeper tray. Place 10 larvae in each beaker and feed with newly hatched brine shrimp nauplii and ideally maintain at 25°C. Filter and disinfect water. During the larval period, provide 14 h light and 10 h dark cycle (see Section 8010F.3*f*). Inspect larvae and feed daily. If sediments or wastes tend to collect, remove them daily with a siphon. At 25°C the larval period lasts 16 to 24 d. The average length of larval life is between 19 and 20 d. To rear through entire life cycle, immediately place females that have laid and hatched their eggs in an aquarium with males. Mating takes place, producing a second batch of fertilized eggs. The egg incubation period depends on temperature; usually 24 to 28 d are required. The number of eggs laid varies. There are six larval stages: the first is protozoea, the seventh is postlarval or juvenile shrimp, which marks the end of metamorphosis. Keep larvae of marine species in seawater adjusted to 25 g/kg salinity. Feed with newly hatched *Artemia salina* nauplii. Rear at temperature between 23 and 27°C and dissolved oxygen above 60% of saturation, using procedures similar to those used for freshwater species. Larval development has been described.[26–31] Remove chelipeds of ovigerous females with fine surgical scissors to prevent egg removal. When rearing larvae to a particular age, maintain a 10 to 15% surplus to compensate for mortality and to provide for other uses.

b) *Penaeus*—The rearing and culturing of larvae of this genus have been described in several studies.[33–39] Hold shrimp in glass tanks of at least 30-L capacity. Provide each tank with flow-through water, 2 to 3 cm of sand over the bottom, and a screen over the top to prevent shrimp from jumping out. Avoid overloading. Keep no more than 22 to 24 animals in a 30-L tank. For *Penaeus* spp., use a minimum flow of 7.5 L/g/d. Flows up to 22 L/g/d may be desirable to ensure DO above 60% of saturation and the removal of metabolic products. Acclimate to laboratory test conditions for about 2 weeks. For short- or medium-term tests with adults and juveniles, shrimp can be field-collected. Cut-up fish is a satisfactory food. Cut a fillet from mullet, grouper, or other abundant species into 1- to 2-cm pieces. Feed one piece per shrimp each 2 to 3 d, depending on size of shrimp. Remove uneaten food daily. For larval tests or life-cycle studies, collect gravid females offshore, let them spawn, and rear larvae at least to the postlarval stage. Penaeid shrimp can be reared from egg to postlarvae in the laboratory. Use the diatom *Skeletonema* as food for protozoeal stages. When diatoms are used as food, use air-lift pumps to prevent accumulation of diatoms. Feed freshly hatched brine shrimp, *Artemia*, to the mysis and postlarval stages.

The protozoeal stages, I through III, of the penaeid shrimp require algae as food. Because larval shrimp are pelagic and unable to search for food during the early part of their life cycle, maintain the required density of the phytoplankton *Skeletonema costatum* and *Tetraselmis* sp. Add these to larval culture chambers according to stage of development, number of larvae present, and volume of water:

Protozoeal I—*Skeletonema*, 50 000 cells/mL
Protozoeal II—*Skeletonema*, 150 000 cells/mL
Protozoeal III—*Tetraselmis*, 20 000 cells/mL
Mysis I—*Artemia* nauplii, 3/mL
Mysis II—*Artemia* nauplii, 3/mL
Mysis III—*Artemia* nauplii, 3/mL
Postlarvae I–IV—*Artemia* nauplii, 3/mL

Maintain phytoplankton in continuous culture or harvest and freeze to use later. Algal culture production units shown in Figure 8010:2 and Section 8010E.4*c*2), will produce daily 7.5 L of culture containing 4.3×10^6 *Skeletonema costatum*/mL or 7.0×10^6 *Isochrysis galbana*/mL and several other species of algae at similar concentrations. Add algae as food for larval shrimp as either a fresh or frozen concentrate. Concentrate algae by centrifuging and discard growth medium. Use a temperature of 28 to 30°C and a salinity of 27 to 35 g/kg. Omit antibiotics from the larval culture medium if EDTA (disodium salt) is substituted at a concentration of 10 mg/L seawater. Feed juvenile shrimp with fresh pieces of fish, clams, or mussels or with prepared dried foods.

4. Parasites, Diseases, and Harmful Growths

Consult Section 8010E.5 for general problems of parasites, infectious diseases, and control procedures. Parasites and diseases of crustaceans (including specific infections known to occur in decapods), include bacteria, phycomycetes, and fungi.[31–46] Parasites have been found in many species of crustaceans, and their presence can influence results. In *Uca*, an ectoparasitic isopod is found on the gills, nematodes in the gut, and metacercaria in the green glands. Species of *Lagenidium* (similar to the one that occurs in shrimp) occur in other marine crustaceans. *L. callinectes* occurs in eggs and larvae of the blue crab. The blue crab has a barnacle (*Octolasmus lowei*) living in association with its gills and gill chamber, metacercariae in various organs, and the sacculinid *Loxothylacus taxanas* beneath its abdomen. *Saprolegia parasitica* attacks larvae of the shrimp *Palaemonetes kadiakensis*.

Adult shellfish in recirculated or flow-through systems are susceptible to biotoxins and pathogens. Remove metabolites and dead individuals from recirculating systems. Juvenile and adult lobsters, crabs, and shrimps are subject to bacterial and fungal infections. *Gaffkya*, a bacterial pathogen, is particularly prevalent in tank-held lobsters, while *Vibrio* disease occurs in tank-held postlarval adult shrimp. Most captive crustaceans are subject to "shell disease," produced by chitin-destroying bacteria. A systemic fungal disease has been described in European prawns, and several fungal infections occur in wild shrimp populations. The larval stages of the lobster and several other crustaceans are prone to infections of the ubiquitous marine bacterium *Leucothrix mucor*, which has produced mortalities of more than 90% in larval cultures. The exuvia and the new exoskeleton after molting become entangled in the bacteria's long dense filaments, and the larvae are unable to swim or feed adequately. This organism also can produce high mortalities by causing pelagic eggs to sink and by interfering with the filtering apparatus of larval forms and the functioning of gills. In some instances, it may be necessary to culture larvae in artificial seawater to avoid *L. mucor* infection. Place ovigerous females in a bath of malachite green (5 mg/L) for 1 min only or rinse them several times in artificial seawater of the correct salinity that contains streptomycin, 2 mL/L, from a stock solution containing 2 g antibiotic/L.

Maintaining a 1-mg/L concentration of antibiotic throughout the larval culture period prevents infections. Twice daily cleaning also is a good preventive method. Seawater, filtered and

exposed to UV radiation, should be nearly bacteria-free. A disease of lobster larvae tentatively has been associated with the phycomycete *Haliphtorus*. It appears as a scab on the first segment of the thoracic appendages up to and surrounding the first row of gills. Thorough cleaning and UV treatment of the water supply is the only known treatment. In most cases, these scabs adhere to both old and new carapaces and thus cause a mechanical impediment to molting. Mortality appears to be restricted to larvae and young juveniles. No deaths of specimens with a carapace length over 27 mm have been observed. The fungus *Lagenidium* sp. causes serious problems in rearing larval shrimp. The disease first becomes apparent in the second protozoeal stage and disappears as the shrimp reach the first mysis stage. Shrimp become immobilized by replacement of muscle tissue by fungal mycelium.

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8740 C. Toxicity Test Procedures

A basic discussion of toxicity testing, including terminology and basic procedures, is presented in Section 8010C. The types of tests are described in Section 8010D and the general recommendations for the type of facilities, equipment, water, and food are discussed in Section 8010E. The appropriate materials for constructing a test system are described in Section 8010F. Also important in this section is a discussion of the toxicant measurements necessary to ensure that organisms are exposed to the calculated concentrations. Section 8020B describes the need for a reference toxicant as part of the test procedure. A reference toxicant is valuable for determining both the health of the test organisms and the analyst's ability to reproduce the results of previous reference toxicity tests with the same species and conditions. Section 8020B also discusses the importance of controlling exposure concentrations during toxicity testing.

Specific procedures for conducting toxicity tests with crayfish, crab larvae, adult, juvenile, or larval lobster, and adult or larval shrimp are described below.

1. Toxicity Test Procedures Using Larvae or Postlarvae of Crabs or Shrimp

a. General procedures: Read and understand the basic procedures and concepts described in Section 8010 before initiating tests. Conduct preliminary tests to become familiar with the specific organisms and test procedures given below.

1) Collecting adult decapods—Depending on the species, gravid adult decapods may be collected either by trap, otter trawl, seine net, or by hand at low tide (see 8740B.2). Small crab species may be found under rocks, while larger crabs may hide in dense marine grasses. Females with eggs (sponge) may be observed with yellow to brown egg masses in a brood pouch (shrimp), or extending from the undersurface (crabs and lobsters). Because there are hundreds to millions of eggs per female, only a few gravid animals are needed to conduct a toxicity test. Careful handling and lower temperatures will decrease the possibility of females releasing eggs during transport, before the exposures are ready.

2) Collecting larval or postlarval stages—Before attempting to raise larval decapods to postlarval stages, determine if this or a related species is being reared at a commercial or government facility for mariculture/aquaculture. Staff members at these facilities have the appropriate expertise to answer questions, and it is often possible to purchase or obtain the needed test organisms from such facilities. See 8740B.3 for procedures related to acclimating, holding, and maintaining stock cultures.

2. Static, Short-Term, Early-Life-Stage Test

As an example of a test system that may be used with several species, the following is a toxicity test procedure that has been used with embryos of the blue crab *Callinectes sapidus.*[1]

a. General procedures: Conduct preliminary tests to become familiar with test procedures.

1) Collecting adult female *Callinectes sapidus* with sponge (embryos)—Collect *Callinectes sapidus* with sponge in crab traps or buy from local fishermen. They are available from March through October in the southeastern United States. Bright yellow sponges are preferred (Stage 3 embryos). Later stages include orange (Stage 6) and red-brown (Stage 7) sponges. Each sponge has 2 to 3 million embryos.

2) Collecting embryos from the sponge of *Callinectes sapidus*—Using forceps, remove pieces of sponge from a sponge-carrying female and shake pieces gently in a beaker of seawater (salinity between 18 and 33 parts per thousand). Take up embryos (Figure 8740:4) shaken from the sponge with a pipet and transfer to culture plates containing natural seawater. Embryos that are Stage 6 or younger can be kept in seawater at 3°C in the refrigerator for up to 1 month. When needed for toxicity tests, bring embryos to room temperature (20°C).

3) Exposure chambers—Use sterile 24-well polystyrene culture plates (16-mm well diameter).

b. Conducting toxicity tests: Add 10 embryos to each well. Add toxicants dissolved in water or 1 μL solvent (ethanol or acetone) but do not exceed aqueous solubility. For solvent controls, use 1 μL solvent. Use five replicates for each concentration. Add 2 mL seawater to each well. Incubate plates at 28°C in the dark and examine each day until zoea (hatching stage) emerge from the egg sacs. Determine hatching by checking embryos each day under a dissecting microscope. Stage 3 em-

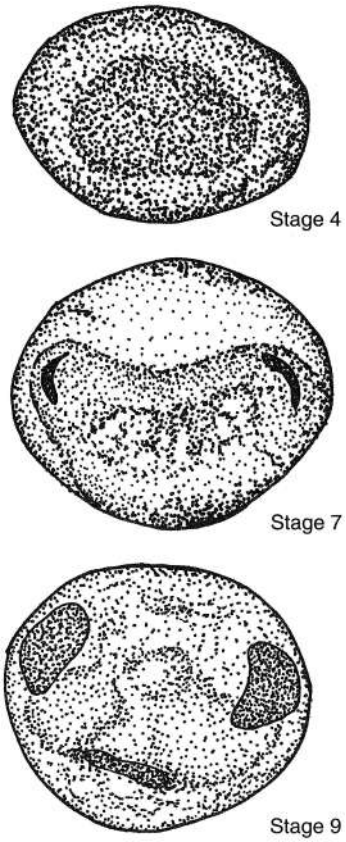


Figure 8740:4. Crustacean embryos.

bryos take approximately 7 d at 28°C to hatch. Stage 6 embryos take about 4 d to hatch. The zoea (Figure 8740:5) emerge over a 12-h period from Stage 9.

c. Interpreting results: After counting the number of emerged zoea in control and toxicant-treated wells, calculate EC_{50} values using probit analysis procedures. Hypothesis testing procedures (Dunnett's Test, etc.—see Section 8010G) may be used to estimate NOEC and LOEC. Another endpoint that may be of value is the concentration that produces a 25% inhibition in normal survival (IC_{25}).

3. Long-Term Tests in Flowing Exposure Systems

a. General procedures: Become familiar with the test species and procedures described below before conducting a test with an effluent or specific toxicant.

b. Collecting and holding animals: See 8740B.2–4 for information on collecting and holding the organism (crab, shrimp, lobster) selected for testing. Static systems (closed aquariums) can be used to hold decapods either before testing or to obtain larvae, but the water must be changed frequently, depending on the number and size of individuals in each aquarium. Monitor ammonia levels in water to determine when to renew water. Avoid excess feeding, which leads to water cloudiness due to bacterial growth. A flowing clean seawater system, where the water is completely replaced in 24 h (about 7 tank volumes/d), is ideal for holding decapods.

c. Preparation of exposure system: Figure 8740:1 shows apparatus that is also appropriate for use in the flowing-water exposure of fresh water or marine decapod larvae or postlarvae. These trays, which fill and empty as a function of flow rate and the high and low levels of the automatic siphon, may be constructed to hold several partitions or several beakers with nylon mesh over drilled holes. One tray can hold all the replicate exposure chambers of a given exposure concentration, or if space permits, there can be two trays per concentration, representing true replicates of a specific concentration. Include five toxicant concentrations plus a control in a test, as well as a solvent control if a carrier solvent is used. The flow to a specific tray, therefore, must contain a given concentration of the test substance produced by mixing clean seawater and the high concentration of toxicant (or full-strength effluent). Each tray (or set of replicate trays) must receive one of the six concentrations of test material (including control), and the same total flow rate of water.

Two types of exposure systems have been used frequently. In a simple system, a Mariotte bottle of toxicant is placed above a head-box receiving a flow of clean water.[2] The head-box has an overflow tube to keep the head of water and flow rate stable, and

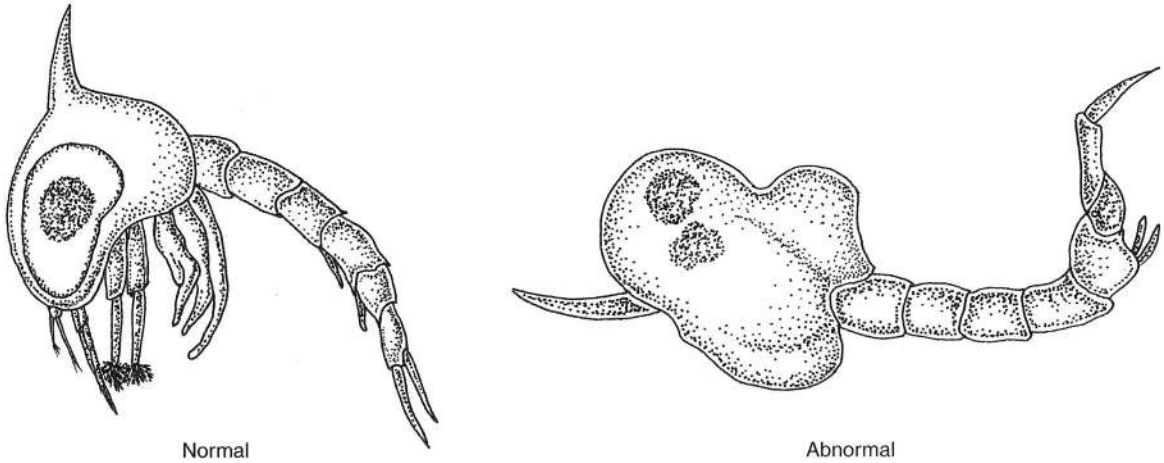


Figure 8740:5. Crustacean larvae.

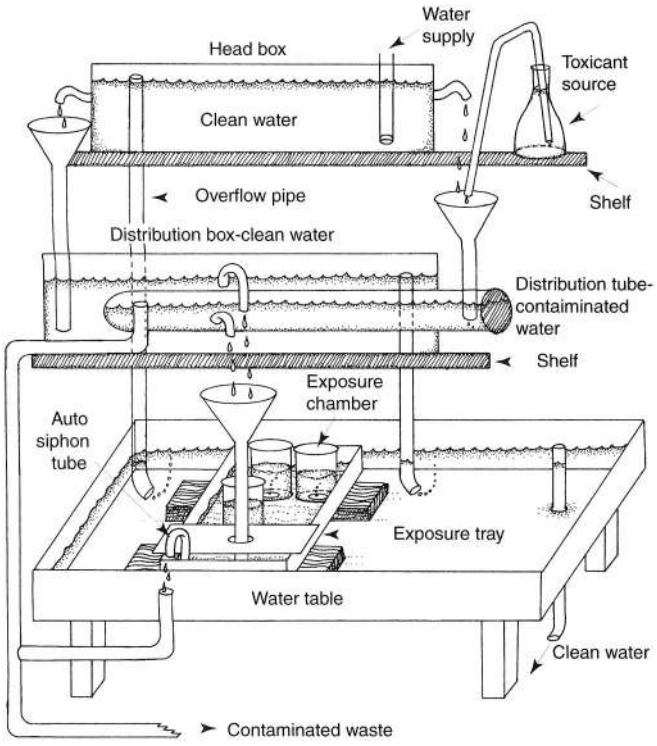


Figure 8740:6. Water table.

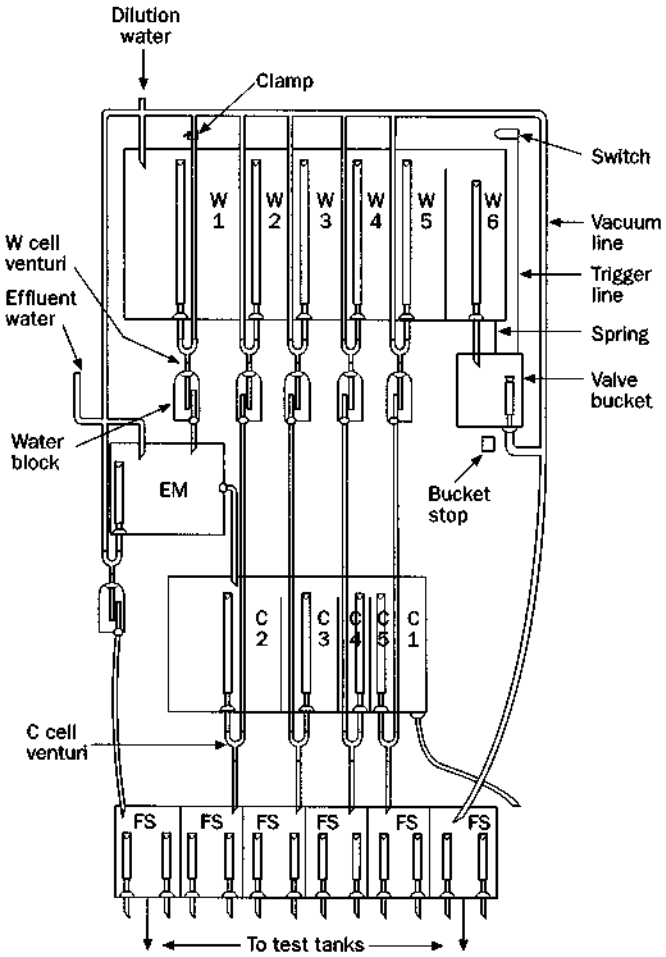


Figure 8740:7. Proportional diluter. Source: LEMKE, A.E., W.R. BRUNGS & B.J. HALLIGAN. 1978. Manual for Construction and Operation of Toxicity-Testing Proportional Diluters; EPA-600/3-78-072. U.S. Environmental Protection Agency, Duluth, Minn.

the main flow from the box goes to the delivery box. As Figure 8740:6 shows, the slow drip from the bottle enters the flow of clean water so mixing will occur both before and while this water enters the delivery box. This long, narrow delivery box or tube receiving the high concentration of toxicant can be fitted with stoppers and glass tubing that exit the stopper and then make a 90° angle, paralleling the tube. The outer portion of tubing then is bent to curve downward. When all stoppers and tubes are in place and the delivery box is receiving test solution, the tubes can be bent downward so water begins to flow out the end. A funnel held in place over the exposure tray receives this water at a given rate from the test's contaminated water supply, and a similar system (with larger tubing and higher flow rates) can be used to introduce clean water to the same funnel. The flow rates of both systems can be regulated by the size of the tubing and the downward direction of its end. The total flow rate in each tray must be the same (±15%), and the concentrations of the exposure dilutions should vary by a factor of approximately 0.5 (100%, 50%, 25%, etc.).

A second exposure system is the Brungs–Mount or Mount–Brungs diluter (Figure 8740:7). A description and another diagram of the system are found elsewhere.[3] Once constructed, this system will deliver six concentrations of contaminant to two replicate tanks, as long as needed.

d. Conducting the test: Expose postlarval decapods in beakers that are placed on trays, which receive the combined flow of clean and contaminated water. The total flow may be about 1 L/min, but the flow into and out of the beakers depends on the rise and fall of the water level in the tray. Each beaker will have a hole drilled by a diamond-hole drill near the bottom, with a nylon-mesh cemented (by silicone) over the hole. Use a glass tube bent to form an automatic siphon, placed in the front of the tray, where the effluent can be captured and sent to the waste treatment system. Adjust tube length to govern the upper and lower levels of water in the tray and beakers; incoming total flow rate (funnel) will determine the number of fluctuations per day. For a standard test, use two replicates (trays) of each inflowing concentration and use at least four concentrations (preferably five) plus a control. NOTE: The beakers in a tray are not true replicates. If 10 or 12 trays will be used, they must be relatively narrow and preferably hold only about 10 beakers. Depending on the size of the eggs, larvae, postlarvae, or juvenile, there may be 1 to 5 animals per beaker. Use at least 10 animals per replicate to conduct a toxicity test. With this type of exposure system, the primary concerns are checking and regulating the flow rates of clean and contaminated water about twice per day, and ensuring that the inflow of toxicant to the contaminated water is stable. Use these same time periods to count living and dead organisms and to observe any behavioral abnormalities. This type of test can proceed for 30 d or more if, for example, the purpose is to determine effects of a toxicant on the hatch, growth, and survival of larvae. The Mount-Brungs diluter system is another exposure

method that can be run for 10 to more than 30 d, given the supply of clean water and volume of toxicant required. Procedures for more difficult conditions, such as studies of petroleum hydrocarbons, are available in the literature.[4–7]

4. Toxicity Test Procedures Using Larvae and Postlarvae of the American Lobster

General procedures: Conduct preliminary tests to become familiar with the test procedures given below.

Never hold lobster larvae and juveniles communally during a toxicity test. Cannibalism and wounds will affect test results. Hold larvae and juveniles in individual glass* or inert plastic beakers or culture dishes for static or static/renewal tests. Choice of test container material will depend on the chemical makeup of the substance to be tested. Generally, glass is used for testing organics and inert plastic for inorganics. Use 100 mL seawater or more for each container at 28 to 32 g/kg salinity. Pre-dose containers with toxicant before adding test animals.[8] Keep test temperature within ±1°C of the temperature of the culture vessel seawater in which test animals were reared or held. Ideal temperature is 20°C, which is the optimum seawater surface temperature for larval hatching and growth in nature. If necessary, hold individual test containers in an incubator or circulating water bath to maintain ideal temperature.

For continuous-flow testing, hold larvae and juveniles in individual nylon-screened† containers or perforated plastic tube containers in larger dosing boxes.[9] Use a testing system similar to that described elsewhere[9] for mysids. Inject toxicant from dosing pumps into the seawater lines or use a system of siphon diluters (Figures 8740:6,7). Use same temperature and salinity range recommended for static testing.

For testing, choose only vigorous animals that swim near the surface in the strong current of the culture vessel. For all test methods, transfer test animals from culture vessel to test containers with a large-bore capture pipet to prevent the animals' gills from contacting air.[8] When transfer of larvae is complete, add 1 mL concentrated freshly hatched brine shrimp nauplii (±500) daily to the static and static-renewal tests. Test animals generally feed less than control animals, so adjust feeding according to the visual observation of remaining nauplii in the test chambers. Such observations and adjustments will alleviate ammonia buildup or dissolved oxygen reductions in test containers. For continuous-flow tests, feed larvae and juvenile lobsters with thawed frozen brine at a rate of approximately 5 to 20/d, depending on the larval/juvenile state.[10] Observations of remaining or quickly consumed food will help maintain healthy animals and a valid test. In continuous-flow tests, preferably pipet uneaten food from the chambers each day before adding more food.

Use no less than 10 animals per concentration, with four or five toxicant concentrations plus control. Repeat tests at least three times, using animals from new brood lobsters each time to ensure correct results.

* Pyrex, or equivalent.
† Nytex, or equivalent.

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8740 D. Data Evaluation

1. Calculating Results

Section 8010G describes methods for calculating, analyzing, and reporting results of toxicity tests. In acute toxicity tests, the LC_{50} or EC_{50} values may be determined by probit, linear interpolation, or even graphical methods, which also may provide the 25% effect concentration (EC_{25}). Computer programs* not only provide these values, but present the 95% confidence limits. Chronic tests may produce EC_{25} and EC_{50} values; also use hypothesis testing (Dunnett's, etc.) to produce NOEC and LOEC values. Most available computer programs will first test the data for assumptions of normality, and also test that the variances of the different treatment groups are homogenous. These criteria should be met before conducting hypothesis testing to determine NOEC and LOEC values. Transform percentage data by arcsine square-root transformation before using Dunnett's test. Transformations of quantitative data (log, square-root, etc.) as number of larvae, or weight of larvae often are useful in helping the data meet assumptions of normality and homogeneity of variance.

2. Reporting Results

See Sections 8010G.2 and 8010H.

* Such as ToxCalc, TOXSTAT, and EPA programs.

8750 AQUATIC INSECTS*

8750 A. Introduction

1. Ecological Importance

Aquatic insects are important components of lake and stream biota.[1–3] In trout streams, for example, they may comprise 50 to 90% of the macroinvertebrate species. Mayflies, stoneflies, caddisflies, midges, and other aquatic insects are major food items for many species of fish,[1,4] and many insect species play key functional roles in aquatic systems, including decomposition, organic matter processing, and nutrient spiralling.[5]

2. Suitability for Toxicity Tests

Aquatic insects are useful test animals because of their wide variety, abundance in unpolluted streams, and sensitivity to low concentrations of pollutants. Indeed, aquatic insects are often more sensitive to certain pollutants than are fish.[6,7] Toxicants may interfere with their survival, growth, reproduction, emergence, and metabolism. Also, many species are easy to maintain under laboratory conditions.

Researchers have developed procedures for determining acceptable environmental conditions or toxicant concentrations for aquatic insects.[2,8] Most studies have been short-term, but long-term test procedures are available. In fact, long-term flow-through tests are recommended because the effects of chronic exposure to sublethal concentrations of toxicants may be more relevant than the effects of acute exposure to higher concentrations.

Historically, acute and chronic toxicity tests relied heavily on aquatic insects, but now standard tests for regulatory and monitoring purposes use many different animals.[9–11] The change not only reflects the difficulties associated with maintaining continuous laboratory cultures but also the desire to include a broader array of invertebrates, such as Crustacea (Sections 8710–8740), in environmental assessments to better capture the potential range of sensitivity.

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Joint Task Group: Paul Sibley.

8750 B. Selecting and Preparing Test Organisms

1. Species Selection

Numerous aquatic insect species have been used for toxicity testing during the past 60 years [Section 10900, Plate 13, A–D (stoneflies) and E–I (mayflies); Plate 15, H–K (caddisflies)], although much of this testing occurred before the 1990s. In the majority of cases, tested insects were not cultured in the laboratory; instead, they typically were collected from a local source (stream or lake) a few days before testing. While useful information on contaminant toxicity can be gained via field-collected organisms, their response variability and relative sensitivity may be greater than that of laboratory-cultured organisms during testing because of the organisms' different ages, unknown health (due to sampling effects, parasites, disease, or contaminants), a

dilution medium different from their collection-site environment, etc.[1] For testing purposes, therefore, cultured insects are preferred because their source and history are known.

In recent years, toxicity testing involving aquatic insects has tended to focus on a few key species that are amenable to culturing and can be used consistently in toxicity tests. In North America, the primary aquatic test species for both water and sediment testing include the midges *Chironomus dilutus* (previously *C. tentans*) and *C. riparius,*[2–4] and the mayfly *Hexagenia* spp.[5,6] Only *C. dilutus* and *C. riparius* are currently the subject of standardized test protocols.[2,3]

If non-standardized aquatic insects must be used for toxicity testing, use insects that are important food for fishes, readily available and abundant, relatively easy to keep and culture in the laboratory, and sensitive to the materials under investigation.

2. Collecting Test Animals

When cultured animals are unavailable, collect test specimens from clean, natural waters rich in aquatic insects (see Section 10500B). Collect larger stream species from riffle areas of clean, well-aerated gravel rubble streams using hand screens, dip nets, or bottom samplers. Agitate the bottom and let the current carry dislodged insects downstream into the net.

Immediately after collection, gently place net contents in a 15- to 20-L insulated container partly filled with stream water. Remove and discard larger rocks after it has been determined that they are free of insects.

Alternatively, collect insects by gently picking up rocks, rubble, or gravel; carefully washing or picking; and then placing desired insects in insulated containers for transport to the laboratory. Providing a small amount of plant material (e.g., moss or macrophytes) can help minimize stress and/or damage during transport. If sorting is not conducted in the field, transport the contents to the laboratory. If transportation time exceeds 30 min, provide aeration and temperature control.

In the laboratory, swirl water in containers and then pour through a screen-bottom container (mesh sized to retain insects but minimum 500 μm) held partly submerged in a tank of collection-site or dechlorinated tap water. Wash screenings into a holding tank. If insects must be separated, wash into a large white enamel pan containing 3 to 5 cm of water. Remove desired species with a large-bore pipet or small spoon-shaped screen and place in separate holding tanks. For riffle insects, use oval or round flow-through tanks[1] with rocks for cover and paddle wheels to provide a current in dilution water.[7]

To obtain benthic insects from deeper waters (e.g., lake bottoms), use a grab sampler (e.g., Ekman, Petersen, or Ponar dredge, or Box corer). Empty dredge contents into a large pail, add water, and swirl by hand. Partly submerge an appropriately sized mesh washing screen, pour a portion of swirling sample into it, and wash by moving up and down in the water. Place washed insects in an insulated container and continue until enough insects have been collected. Chironomids probably will be the dominant insect species in and on silt substrates. However, other important immature insects (e.g., dragonflies, damselflies, several species of Diptera, beetles, and mayflies) may be found in and on silt bottoms, especially in near-shore areas. The mayfly *Hexagenia limbata* is a large species often occurring in great abundance in soft, unpolluted muds rich in organic matter that occur in deep pools, ponds, lakes, and reservoirs. Obtain these by collecting the top 8 cm of mud and washing as described previously.

3. Holding, Acclimating, and Culturing

a. General considerations: Examine insects for injury as soon after collection as possible. Place all uninjured specimens in holding chambers, feed them (see ¶ *b* below), and hold for at least 1 week for observation and acclimation to desired temperature. Acclimate stream species in flowing water. Keep in stainless steel wire cages in running water or in oval troughs with a water current and flat stones covered with attached algae (which serve as both cover and food for herbivorous species).[7] If the insects construct cases (e.g., caddisflies) or tubes (e.g., *Chironomus* spp.), supply the appropriate materials. For caddisflies, use sand grains, small pieces of wood, and plant materials retained by a 16-mesh screen. For chironomid larvae, use the highly organic mud that overlies the collection-site bottom, shredded paper towels, or silica sand.[2,8] For burrowing benthic species (e.g., *Hexagenia*), use aquariums with a 3- to 5-cm layer of unsterilized mud from the collection site or another substrate that has been demonstrated to support their survival and growth.[9] Provide water, dissolved oxygen (DO), and other conditions described in Sections 8010E and F and other guides.[2,3,4] Maintain final holding temperature within 3°C of collection temperature. For long holding periods, maintain natural seasonal temperatures. If aquatic insects are collected in winter at water temperatures of 1°C or lower, acclimate them slowly to higher temperatures if they will be used in short-term tests (Section 8010E.3). Different species require different light intensities. Stoneflies and mayflies often require stones under which they can hide from direct light. Fix light cycle at a certain day length, or vary it seasonally to correspond with natural annual photoperiod. For *Chironomus* spp., use a 16:8-h light:dark photoperiod. Lamps and fixtures are described in Section 8010F.3*f*.

b. Food and feeding: The food supplied to collected insects must reflect the feeding trophy of the insect. Guidance on the trophic relationships of aquatic insects is available.[10] Predators (e.g., *Acroneuria*, *Paragnetina*) require live food and should be fed to excess with small midges, blackfly larvae, mosquitoes, or small caddisfly larvae from an unpolluted environment.[9] For shredding species (e.g., *Pteronarcys*), feed to excess with coarse-chopped maple, birch, or aspen leaves that have fallen naturally and been dried and then soaked in test water for at least 2 weeks before feeding to allow colonization of leaves by microorganisms. For collecting/gathering, filtering, and burrowing species (e.g., *Hexagenia, Hydropsyche,* and *Arctopsyche*), feed finely ground leaves and fish-food pellets. If the substrate is rich in organic matter, additional food may not be required for *Hexagenia*. Avoid overfeeding with fish food because this can deplete DO. The larvae of some Trichoptera (e.g., *Hydropsychidae*) are carnivorous and cannibalistic; keep them well-fed with plankton, microcrustacea, blackfly larvae, and other organisms collected from fish hatcheries, ponds, lakes, and streams with a net of No. 20 bolting silk. Numerous species of aquatic *Diptera*, especially those within the *Chironomidae*, have been successfully cultured (Table 8750:I) and standard methods for culturing *Chironomus riparius* and *C. dilutus* are available.[3] Keep in jars supplied with algal culture medium [Section 8010E.4*c*1)a)] in-

TABLE 8750:I. SUMMARY OF TEST CONDITIONS FOR THE ONTARIO MINISTRY OF THE ENVIRONMENT'S *HEXAGENIA SPP*. SURVIVAL AND GROWTH TEST*

Parameter	Conditions
Test duration and type	21-d static, non-renewal (with evaporated water replaced by DI or RO water)
Overlying water	Dechlorinated tap water, or as requested by customer; 90–100% oxygen saturation
Laboratory control sediment	Clean sediment known to support *Hexagenia* growth and survival
Test organisms	Within range of 5 mg/individual (~2–4 months old)
Test chamber	1.8-L glass jars
Sediment volume	325 mL
Overlying water volume	1.3 L (1:4 sediment-to-water ratio)
Replicates	3 minimum
Number of organisms per replicate	10
Temperature	23 ± 1°C daily average; ±3°C instantaneous
Photoperiod/light intensity	16:8 h light:dark; 100–1000 lux full-spectrum fluorescent
Aeration	Continuous, minimal
Feeding	Not fed during test
Observations/measurements	Test initiation: pH, temperature, DO, conductivity, ammonia, (hardness & alkalinity if requested) Daily: temperature Three times (~Days 0, 10, and 21): DO, pH, conductivity, ammonia, if requested Test termination: pH, temperature, DO, conductivity, ammonia (hardness & alkalinity if requested).
Allowable holding time of sediment before testing	6 weeks maximum
Endpoints	Mortality; growth (mean wet weight)
Test validity (in the control)	Mean mortality ≤20%; weight ≥2× initial weight

* Based on Ontario Ministry of the Environment (2008) and Bedard *et al.* (1992).[5,6]
DI = de-ionized.
DO = dissolved oxygen.
RO = reverse osmosis.

oculated with algae-including diatoms. Alternatively, use a mixture of 5 g fish food plus 1 g powdered dried cereal grass blended in 1 L of water. Add about 100 mL of this suspension to each culture per feeding. If there is no flow-through, remove 100 mL test solution before feeding. Use 10-L culture jars/aquaria containing ≤8 L medium with a screen cover to retain adults.[11,12] Keep in a constant-temperature room at 21 to 24°C. For long-term studies, follow natural temperature cycle of water from which chironomids were taken. Because the jars have a mud substrate, do not clean them or overfeed organisms. Collect emerging adults for breeding in wire screen cylinders placed over culture jars.

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8750 C. Toxicity Test Procedures

1. General Procedures

The following is general guidance for aquatic insects that lack standard test protocols. Conduct tests as described in Section 8010D. Use early instar larvae or nymphs when possible, as they tend to be more sensitive than older animals. If emergence is an endpoint of interest, late instars can be used. If possible, use at least 10 specimens for each toxicant-concentration replicate and another 10 animals/replicate for growth studies. General experimental design considerations for toxicity tests are available.[1] Two species may be tested in the same tank if precautions are taken to avoid predation. Do not use static testing on stream insects unless air stones or water movement can simulate natural water conditions, and do not use static tests on certain lake or reservoir species unless required DO levels are maintained. For long-term tests, see Sections 8010D.1 and 4.

a. Test tanks: For quiet-water species, use 8- or 20-L glass and stainless steel aquariums. For stream species, use round or oval stainless steel or epoxy-painted troughs 1–3 (90 cm × 15 cm × 15 cm deep) in which natural stream flow is simulated. Set tanks side by side so paddle wheels on one long shaft can circulate water in them all.[2] Alternatively, jetted inflow from the diluter can maintain adequate flow. Recirculating systems[3] also can be used to hold and expose aquatic insects.

b. Flow rate: To simulate stream flow, set each tank's inflow at no less than 6 to 10 tank volumes/24 h. In aquariums without water-circulating devices, use much higher flows. In oval test tanks, use velocities near 0.5 cm/s. For quiet-water species, such as *Hexagenia* and *Chironomus*, do not disturb the substrate with water flow.

c. Aeration: Aeration in flowing systems is usually unnecessary. In static tests or those involving feedings (where DO is more susceptible to declines over the exposure period), aeration may be necessary to ensure compliance with DO acceptability criteria[1] or control water movement. Aeration should not be used with volatile toxicants.

d. Cleaning: See Section 8010E.4*d*. In long-term tests, siphon detritus from tank bottom weekly. If a mud substrate is used, no cleaning is necessary. Avoid overfeeding.

e. Substrate: For all stream riffle species, use fine-mesh stainless steel screens formed into cylinders or cubes that provide 10 to 15 cm^2/insect. Place cages in oval troughs or glass cylinders.[2] For 30- to 90-d adult-emergence tests, obtain clean 5- to 10-cm-diam rocks (one for every three insects) from a clean collection site for a substrate, provide fine screening or sticks that protrude above the water surface, and place netting (mesh size <300 μm) or a screened container over the top of the aquarium to retain emerged adults.[4]

f. Light and photoperiod: See Section 8010F.3*f*. A light:dark ratio of 16 hours of light and 8 hours of dark is the standard photoperiod for most toxicity test procedures. Alternatively, use the natural photoperiod for the locality for which the test is conducted.

g. Temperature: See Section 8010F.1*b*. For trout stream insects, use 10°C as a winter temperature and near 15°C as a summer temperature, increasing temperature by 1°C each week in adult-emergence tests (up to a maximum of 5°C above initial temperature). For warm-water stream or lake insects, follow the natural temperature cycle. For tests involving *Chironomus* spp. and *Hexagenia*, for example, a constant temperature of 23 ± 1°C is commonly used.

h. Time of year: Under natural conditions, many species emerge as adults in spring or early summer, so adult-emergence tests should be initiated no later than around March 1 to June 1, depending on latitude. The exceptions are *Hexagenia* spp., and multivoltine species, such as Chironomidae and Culicidae, which emerge throughout the summer in most localities, so related adult-emergence tests can be initiated at any time during the summer. Species that can be continuously cultured (e.g., *Chironomus*, *Tanytarsus*) can be used throughout the year in adult-emergence tests.

2. Toxicant Preparation

See Sections 8010F.1*c* and 2*b*.

3. Test Procedures for *Hexagenia*

Hexagenia has been used in short- (4- to 7-d), medium- (7- to 60-d), and long-term (90- to 120-d) sediment tests to assess acute and chronic toxicity via survival, growth, or adult emergence as endpoints. A standard test protocol for *Hexagenia* developed by the Ontario Ministry of the Environment (OMOE)[5] is summarized in Table 8750:I. Annex A3 of ASTM Standard 1706-05 also provides guidance on *Hexagenia* sediment testing.[1]

Following is general guidance for conducting *Hexagenia* tests. Use at least 20 organisms per ≥8-L aquarium. Make the water depth 8 to 20 cm, and provide a 4- to 5-cm-deep fine organic ooze substrate that is as similar as possible to that where larval stages occur naturally. If the test begins with newly hatched *Hexagenia*, then use 50/tank. If starting with *Hexagenia* eggs, then pipet the eggs into petri dishes (about 200/dish) with 200 mL test water at about 20°C, and let hatch. For mud substrates, determine survival by either:

- counting the number of dead animals that have left their burrows and/or by counting the number of new burrows formed after disturbing the mud surface sufficiently to destroy entrances to old burrows (if counts do not agree, use the latter); or

- sieving the mud at the end of testing and counting recovered larvae.

For growth or emergence tests, set up additional exposure containers so larvae can be removed periodically for measurement. Remove 10 larvae from their burrows after 20 to 60 d to determine growth. Do not remove more than 50% of surviving animals before testing concludes, and keep a record of the total number removed. Use these animals to provide additional data on growth and emergence [e.g., body length, head-capsule width, and live (wet) weight]. In acute toxicity tests, determine survival visually after 1.5, 3, 6, and 12 h, and twice daily thereafter. *Death* is defined as failure of specimens to move when gently probed or illuminated with a flashlight.

In longer-term studies, check tanks daily to remove and record dead animals and larval exuviae, which indicate successful molting. For growth studies, determine initial range and mean of one or more characteristics (total length, head-capsule width, and weight) of specimens in the holding tank. Kill all animals in warm water (40 to 50°C) before measuring. Take measurements twice during testing, using animals to be discarded. Obtain final measurements for all survivors. Make two counts: number of adults and cast skins (if different, use cast skins because some adults may have escaped).

Determine and record percentage of adults that emerge, sex, incidences of incomplete emergence (half-out of nymphal skin, wings unsuccessfully unfolded, etc.), adult length, weight, and head-capsule width.

4. Test Procedures for *Chironomus*

Chironomus spp. have been used extensively in short- (4- to 7-d) and long-term (10- to 60-d) sediment tests to assess acute and chronic toxicity via survival, growth, emergence, and reproduction endpoints. The most common test species are *C. dilutus* (formerly *C. tentans*)[6–8] and *C. riparius*.[9–11] Standard test methods for *C. dilutus* have been published.[1,12] Guidance for conducting tests with *C. riparius* is provided in Annex 1 of ASTM Standard 1706-05[1] and elsewhere.[13,14] Information pertaining to test conditions for *C. dilutus* and *C. riparius* is presented in Table 8750:II; test conditions for the *C. dilutus* life-cycle test are presented in Table 8750:III.

Following are general test conditions for *Chironomus* spp. and midge species that lack standard test protocols. Follow procedures described in Section 8010F. For each concentration, use duplicate 20-L aquariums, or a minimum of three 300-mL beakers[1] to which mud, fine sand, or powdered dried cereal grass is added as a substrate. If emergence will be assessed, or the test is initiated with later instars, add screen covers to each container to retain adults. If using a mud substrate, one similar to that used for *Hexagenia* is appropriate. Maintain water flow to each test container at a rate of approximately 2 L/h or ensure at least two volume changes per day.[12] Use lighting and photoperiod as described in Section 8010F.3*f* and elsewhere.[1] Feed daily during tests using guidance on feeding rates from ASTM Standard E1706-05.[1] For short-term tests, add second- to third-instar larvae to each test container at a rate of 10 to 12/beaker or 50/aquarium. For long-term tests, use either first-instar larvae (about 1.5 mm long and less than 24 h old)[4] or 7-day old larvae (the latter being easier to handle). For <24-hr old larvae, transfer only those that have recently hatched. Use a pipet or similar device to transfer larvae.

In short-term tests, remove larvae at 10 d to assess survival and/or growth.[1] In long-term (life-cycle) tests, remove a subset of larvae at 20 d for survival and growth determinations and use the remaining larvae to determine the number of emerging adults and reproduction.[4] For emergence, count both adults and pupal cases. If counts differ, use pupal case count. At 23 ± 1°C, emergence takes between 15 and 30 days (emergence generally takes longer in *C. dilutus* than in *C. riparius*). To assess reproduction, count the number of eggs in at least five rings along the length of each egg mass (count eggs only in the first egg mass per female), and multiply by the total number of rings to estimate the total number of eggs.[4] To determine fertilization success, incubate egg masses in separate petri dishes containing 15 mL control water and determine the percent hatchability by counting the number of unhatched eggs after 6 days. Repeat complete test at least once.

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TABLE 8750:II COMPARATIVE TEST CONDITIONS AND ACCEPTABILITY CRITERIA FOR SHORT-TERM (10-d) SEDIMENT AND WATER TOXICITY TESTS WITH THE MIDGES *CHIRONOMUS DILUTUS* AND *CHIRONOMUS RIPARIUS**

Parameter	Conditions	
	C. dilutus	*C. riparius*
Test duration and type	Whole sediment or water-only test with renewal of overlying water	Whole sediment or water-only test with or without renewal of overlying water
Temperature	23 ± 1°C	20–23°C
Light quality, illuminance & photoperiod	Wide-spectrum fluorescent light at 100–1000 lux, set to 16:8 light:dark photoperiod	Wide-spectrum fluorescent light at 100–1000 lux, set to 16:8 light:dark photoperiod
Test chamber	300-mL high-form lipless beakers	300-mL or 1-L beakers
Sediment/sand volume	100 mL	100 mL, 200 mL
Overlying water volume	175 mL	175 mL, 800 mL
Renewal of overlying water	2 volume additions/d; continuous or intermittent (e.g., one volume addition every 12 h)	Static or water renewal
Age of organisms at test start	Second- to third-instar larvae	First- (<3 d old) or second-instar larvae
Number of organisms per chamber	10	10, 20
Number of replicate chambers per treatment	Depends on test objectives; 8 chambers are recommended; 3 chambers minimum	Depends on test objectives; 8 chambers are recommended; 3 chambers minimum
Feeding	Tetrafin goldfish food fed at 1.5 mL/d/test chamber (1.5 mL contains 6.0 mg dry solids)	Fish food flakes, trout chow, cereal leaves, algae, dog treats
Aeration	None, unless DO in overlying water drops below 2.5 mg/L	None, unless DO in overlying water drops below 2.5 mg/L
Overlying water	Culture, surface, site, well, or reconstituted water [e.g., ASTM E 1706-05 (ASTM 2009)]	Culture, surface, site, well, or reconstituted water {e.g., ASTM E 1706-05 [ASTM (2009)]}
Test chamber cleaning	When using beakers, periodic light brushing if screens become clogged	When using beakers, periodic light brushing if screens become clogged
Overlying water quality	Hardness, alkalinity, ammonia, pH, and conductivity at the beginning and end of a test and on Day 20; temperature and DO daily	Hardness, alkalinity, ammonia, pH, and conductivity at the beginning and end of a test and on Day 20; temperature and DO daily
Test duration	10 d (see Table 8750:III for life-cycle test duration)	10–14 d (up to 30 d if emergence is monitored)
Endpoints	Survival and growth (as AFDW)	Survival and growth (as AFDW), head-capsule width, and emergence
Test acceptability criteria (controls @ 20 days)		
Survival	≥70%	≥70%
Growth	≥0.48 mg (AFDW)	Not specified

* Modified from ASTM (2009).[1]
AFDW = ash-free dry weight.
DO = dissolved oxygen.

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TABLE 8750:III. COMPARATIVE TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR LONG-TERM SEDIMENT AND WATER TOXICITY TESTS WITH THE MIDGE *CHIRONOMUS DILUTUS**

Parameter	Condition
Test duration and type	Whole sediment or water-only test with renewal of overlying water
Temperature	23 ± 1°C
Light quality, illuminance & photoperiod	Wide-spectrum fluorescent light at 100–1000 lux, set to 16:8 light:dark photoperiod
Test chamber	300-mL high-form lipless beakers
Sediment/sand volume	100 mL
Overlying water volume	175 mL
Renewal of overlying water	2 volume additions/d; continuous or intermittent (e.g., one volume addition every 12 h)
Age of organisms at test start	<24 h old
Number of organisms per chamber	12
Number of replicate chambers per treatment	16 (12 at Day −1, and 4 for auxiliary males on Day 10)
Feeding	Tetrafin goldfish food fed at 1.5 mL/d/test chamber (1.5 mL contains 6.0 mg dry solids) starting at Day −1
Aeration	None, unless dissolved oxygen in overlying water drops below 2.5 mg/L
Overlying water	Culture, surface, site, well, or reconstituted water {e.g., ASTM E 729-98 [ASTM (2009)]}
Test chamber cleaning	When using beakers, periodic light brushing on the outside of screens if they become clogged
Overlying water quality	Hardness, alkalinity, ammonia, pH, and conductivity at the beginning and end of a test and on Day 20; temperature and dissolved oxygen daily
Test duration	About 40–50 d; each treatment is ended separately when no emergence has been recorded for seven consecutive days
Endpoints	20-d survival and growth (as ash-free dry weight); percent emergence and adult mortality; number of eggs per female; number of eggs hatched
Test acceptability criteria (controls @ 20 d)†	
Survival (must)	≥70%
Growth (must)	≥0.60 as dry weight or 0.48 mg as ash-free dry weight
Emergence (should)	≥50%
Mean eggs per egg case (should)	≥800
Percent hatch (should)	≥80%

* Modified from ASTM (2009)[1]

† Additional endpoints and criteria can be found in ASTM (2009).

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8750 D. Data Evaluation

Analyze, evaluate, and report data from various tests, as described in Section 8010G.

8810 ECHINODERM FERTILIZATION AND DEVELOPMENT*

8810 A. Introduction

1. Background

The Phylum Echinodermata encompasses a widely distributed and diverse group of marine animals. The class Echinoidea includes sand dollars and sea urchins, organisms that are common inhabitants of rocky shores and ocean bottoms over all depth ranges. Many echinoid species are maintained easily in the laboratory and are responsive to simple methods of spawning inducement. Gametes and embryos are easily obtained and reared in the laboratory and have been the subject of scientific research for more than 100 years.[1] Numerous species have been used, and laboratory techniques have been developed that use various stages of the organism's life cycle.[2–6]

Toxicity tests involving short-term exposure of gametes or embryos are of comparable or greater sensitivity to many contaminants than tests with other marine species and life stages.[7–11] Echinoid toxicity tests can be performed on small volumes (≥2 mL) over short periods (1 to 96 h), and under static conditions without feeding. These tests have been used successfully to evaluate the toxicity of effluents, receiving waters, chemicals, and sediments if the test samples' salinity is near typical ocean levels (28 to 34 g/kg). Recent adaptations of these test methods have expanded applications to include evaluation of genotoxic effects,[12] interstitial water,[13] and toxicity identification evaluation (TIE) studies.[14] Methods similar to these have been proposed or recommended as components of regulation programs.[2,15–17]

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8810 B. Selecting and Preparing Test Organisms

1. Selecting Test Organisms

In accordance with the criteria listed in Section 8010E.1, the recommended test species include (but are not restricted to) the following:

Scientific Name	Common Name	Location	Approximate Spawning Season
Arbacia punctulata	Atlantic sea urchin	Atlantic coast Gulf coast	Summer Winter
Strongylocentrotus droebachiensis	Green sea urchin	Northern Atlantic and Pacific coasts	Winter
Strongylocentrotus purpuratus	Pacific purple sea urchin	Pacific coast	Fall–spring
Dendraster excentricus	Pacific eccentric sand dollar	Pacific coast	Spring–summer
Tripneustes gratilla	Collector urchin	Indo-pacific region	Year-round

The use of the above species is encouraged to increase the comparability of results from different laboratories. Successful toxicity tests can be conducted with other species (e.g., the Hawaiian sea urchin *Echinometra* spp.[1] or *Lytechinus* spp.).[2] Using alternate species may be advantageous in certain regions, but modifications to the test method may be necessary and results may not be comparable.

2. Collecting Broodstock

Obtain test organisms (gametes or embryos) from broodstock collected from the field during their natural spawning season and held in the laboratory until needed. Collect in areas away from obvious sources of pollution, with water quality similar to that used for holding and testing. Organisms obtained from a commercial supplier may be used. *Dendraster excentricus* forms dense aggregations in intertidal or subtidal sandy areas; collect individuals by hand at low tide, by diving, or by dredge. Regular sea urchin species inhabit rocky or sandy areas of the intertidal and subtidal zones. Gamete viability of *Tripneustes gratilla* can vary depending on locality. It is best to collect urchins from different areas for each round of tests. Collect individuals by hand, either at low tide or by diving.

Discard any individuals damaged during collection or subsequent handling. Avoid sudden or extreme variations in temperature, salinity, pressure, or other environmental factors during collection and transport because they may induce premature spawning. Animals may be shipped by overnight mail service in insulated containers containing an ice substitute. Take precautions to avoid oxygen-depletion stress during shipping to avoid premature spawning. Sea urchins have been successfully shipped in supersaturated oxygenated water or by wrapping animals in seaweed or towels soaked in seawater to maintain high humidity.

3. Culture Techniques

Hold sea urchins and sand dollars in aquariums with either a flow-through seawater supply or recirculating filter system.[3] Using freshly collected animals in spawning condition is recommended. The spawning season can usually be extended to year-round by holding the organisms in the dark at constant temperature. Feed sea urchins *ad libitum* brown macroalgae (e.g., *Macrocystis* spp. or *Egregia* spp.). Substitute romaine lettuce[4] or commercial fish feed if fresh seaweed is unavailable. Rehydrated seaweed purchased from food markets or dried overnight at 100°C also has been used successfully. In addition, a formulated diet based on a combination of eggs, carrots, seawater, and agar has been used with success.* Using vegetables from organic food sources is recommended to reduce the possibility of introducing pesticides to the holding system. *Tripneustes gratilla* will feed on sea grasses (e.g., *Thalassia* spp. and macroalgae), with the exception of *Sargassum* spp.[5] Aquariums containing sand dollars should contain several centimeters of sand if animals will be held more than a few days. Sand dollars feed on suspended or benthic materials (e.g., detritus or plankton); provide a source of unfiltered natural seawater or prepared food (e.g., powdered fish feed) if the animals will be held for long periods.

Holding temperature varies with the species and should be similar to that at the collection site. Recommended temperatures are 15 to 18°C for *A. punctulata*, 12 to 16°C for *D. excentricus*, 8 to 15°C for *S. purpuratus*, 8 to 12°C for *S. droebachiensis,* and 20 to 25°C for *T. gratilla*. Hold animals at 28 to 34 g/kg salinity.

4. Parasites and Diseases

A variety of commensal organisms (e.g., annelids and crustaceans) are often associated with sea urchins and sand dollars. These organisms are neither harmful nor essential to the survival of the broodstock.

Excessive microbial growth can result from the accumulation of feces in aquariums. These growths produce metabolites (e.g., hydrogen sulfide) that may be toxic or stress the animals. Clean aquariums several times per week.

5. Gamete Preparation

Induce sea urchins or sand dollars to spawn just before the beginning of a test. Females and males of most echinoid species usually can be induced to spawn via injection of 0.5*M* potassium chloride (KCl). *T. gratilla* can also be induced to spawn by vigorously shaking, which is effective at stimulating spawning with or without KCl. Use a hypodermic syringe (20-gauge needle) to pierce the peristomial membrane surrounding the mouth and inject approximately 0.5 mL into the coelomic cavity (another 0.5 mL may be injected if necessary). Use sterile needles and KCl to guard against disease if the animals will be returned to laboratory aquariums. Usually, two injections of KCl are made on opposite sides of the mouth. Place sea urchins upright (oral side down) and observe for

* Sea Urchin Embryology: Urchin Cookie Recipe. http://www.stanford.edu/group/Urchin/cookie.html. Accessed February 2017.

evidence of gamete release through the genital pores, located around the anus on the aboral surface. Sperm are milky white in color, while eggs are orange to red, depending on species.

Electrical stimulation is another spawning method that has been used with success on some species (primarily *A. punctulata*). Place electrodes from a 12-V DC power source on either side of the urchin's anal pore, and spawning will occur until the electrodes are removed.[4] This method has the advantage of permitting a check of gamete type and quality by applying the electrodes briefly. Neither spawning method kills the animal, which may be respawned in 30 to 60 d if held under appropriate conditions.

If spawned animals are returned to the culture system, they should be isolated from unspawned individuals. The presence of recently spawned animals may stimulate unintended spawning by other sea urchins.

Invert females, releasing eggs (oral side up) and place on a beaker filled to the brim with seawater at the appropriate temperature. The eggs will fall to the bottom of the beaker after extrusion. Collect sperm in the "dry" condition (without contacting seawater, which activates the sperm). Remove sea urchin sperm from the gonopore area with a glass transfer pipet or automatic pipet (with enlarged tip) and place in a small conical test tube. Dry sperm collection is impractical for sand dollars; the sperm should be collected via minimal mixing with seawater until needed. Collect sand dollar sperm by inverting males over 5- to 10-mL beakers of seawater. The sand dollar sperm fall to the bottom with little dilution and can be removed easily via pipet. Use care to avoid transferring fecal material with gametes.

Gametes for most species may be stored for several hours in an ice bath or refrigerator. Do not allow gametes to freeze. Keep eggs from each female separate until evaluated for quality. Store *A. punctulata* and *T. gratilla* eggs at the culture temperature. Examine subsamples of sperm and eggs from each animal under a compound microscope to evaluate their quality. Eggs should be round, and a germinal vesicle (clear spot) should not be visible. A germinal vesicle indicates immature eggs. Viability also can be checked by removing a subsample, adding sperm, and determining fertilization. Use eggs from a more mature female if more than a few percent of immature eggs are present. Evaluate sperm quality by diluting a subsample in seawater containing eggs to check for motility and fertilization.

Good quality gametes increase the test's sensitivity and success rate. Individual batches of gametes should be evaluated for quality using a pretest in which several combinations of sperm and egg batches are evaluated at various sperm:egg ratios. The combination yielding the most viable gamete combination at the optimal sperm:egg ratio to maximize test sensitivity is selected. Alternatively, if it is infeasible to evaluate batches individually, gametes from several individuals should be pooled to minimize the influence of variable gamete quality.

Store sperm in an ice bath and avoid additional dilution until just before testing. Activate sperm via dilution in seawater and use within 30 min. Sperm have limited energy reserves and their viability declines exponentially within minutes of activation; carefully monitor holding times of activated sperm and preferably standardize in the laboratory. Longer holding times (e.g., 60 min) can be used successfully if sperm are chilled on ice and higher sperm densities are used in the test. The final sperm density needed for the test varies according to test type, species, and maturation stage. Determine this value from trial fertilization tests (see 8810C.4*d*) or previous experience.

Gently wash eggs once by allowing them to settle in the spawning beaker and decanting off excess seawater. Gently resuspend eggs in fresh seawater and let them settle again. Use care when washing sand dollar eggs; they are surrounded by a thick jelly coat that is easily disturbed (with adverse fertilization effects).

Prepare a working stock solution of known egg density (dependent on test volume and test type). Check density by mixing the stock solution well, removing a small subsample, diluting it 1:100× with seawater, and counting number of eggs in a known volume on a Sedgwick–Rafter cell. Using a perforated plunger (plastic disk containing numerous holes, attached to a plastic rod) is strongly recommended to provide a homogeneous suspension of gametes and embryos for this and other steps of the procedure. Adjust density by adding or removing seawater. Store the working stock at or below the exposure temperature (depending on species) and use within 2 h if possible.

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8810 C. Echinoderm Fertilization Test

1. General Procedures

Conduct exploratory tests (see Section 8010D) first if the concentration range to be tested is unknown. Prepare dilution water and toxicant solutions and introduce them into test containers as described in Section 8010F.

Observe the following general precautions in procedures that involve handling gametes. First, take stringent measures to avoid

cross-contamination of egg and sperm solutions (e.g., use separate pipets and glassware for each sex). Seemingly minute amounts of sperm are sufficient to fertilize an egg stock prematurely and invalidate an entire test. Second, enlarge the opening of disposable pipet tips used to dispense gametes. Trim tip with a razor blade to produce a 1- to 2-mm opening when transferring eggs. Preferably also enlarge pipet tips used for concentrated sperm solutions to facilitate transfer of this highly viscous suspension. Modified pipet tips are not required for sand dollar sperm shed into seawater. Finally, avoid inadvertently warming gamete or embryo solutions during preparation steps because this may greatly hasten degradation during storage. Use a temperature-controlled room or chilled water baths to ensure that all preparatory steps are conducted at or below test temperature.

2. Water Supplies

Maintain dilution water salinity within 2 g/kg of holding salinity and at a pH that is within the species' tolerance range. Dilution water quality should be sufficient to produce ≥70% fertilization in control samples.

a. Artificial seawater: See Section 8010E.4*b*2). Avoid commercial sea salt mixes because they are often toxic to echinoid gametes and embryos. However, some seawater formulations based on reagent-grade chemicals have been used successfully.[1,2] Conduct preliminary tests to determine the suitability of each batch of artificial salts before use.

b. Natural seawater: Choose a source of natural seawater that is free of contamination and of uniform quality. Pass the water through a filter with an effective pore size ≤1.0 μm to remove sediment and organisms that might interfere with the test. Additional treatment (e.g., aeration, additional filtration, sterilization, or activated carbon treatment) may be needed to obtain acceptable water quality, especially during storage. Avoid prolonged storage of seawater (e.g., >4 days) and store in the dark at 4°C, if possible, to minimize water quality degradation. Seawater stored for extended periods should be filtered to ≤5.0 μm, continuously circulated, aerated, and held in the dark.

c. Salinity adjustment: Echinoids have limited osmoregulation ability. Adjust salinity of test samples that deviate by more than 2 g/kg from the culture environment to eliminate potential interferences. Use hypersaline brine (HSB) or an artificial sea salt mixture to adjust salinity. Dry reagent-grade sea salts may be added directly to sample when necessary to avoid diluting sample due to brine addition. Exercise caution in selecting the material used to adjust salinity so sample toxicity is not altered by the introduction of such chemicals as chelators (e.g., EDTA) or toxic contaminants (e.g., metals).

Slow evaporation is an effective method of preparing HSB in sufficient quantities for fertilization or embryo development tests.[3] Freezing and partially thawing seawater is also a convenient method of preparing HSB.[3] Freezing (−10 to −20°C) one or two 4-L containers (glass or plastic) overnight for 6 to 12 h will provide sufficient 80 to 100 g/kg brine for most tests. Filter HSB through a filter with an effective pore size of >1.0 μm to remove any particulates that may interfere with the test. Either HSB preparation method could introduce toxicity into the experiment via the use of inappropriate materials or methods; preliminary experiments should be conducted to verify HSB quality before using it in experiments. HSB salinity should not exceed 100 g/kg.

Use the following formula to determine the volume of brine (V_B) to be added to the sample:

$$V_B = V_S \times (S_T - S_S)/(S_B - S_T)$$

where:

V_S = volume of test sample to be added (mL),
S_T = desired test salinity after adjustment,
S_S = initial salinity of sample, and
S_B = salinity of brine.

Check pH of adjusted samples because some brine-preparation methods may alter pH. Allow samples to equilibrate with the atmosphere to stabilize pH. Because of the risk of chemical contamination, only add dilute hydrochloric acid or sodium hydroxide to adjust pH when necessary to correct deviations likely to confound test results.

3. Exposure Chambers

Conduct tests in glass culture tubes or vials with 10 to 20 mL of capacity. Cover chambers loosely to prevent contamination during test. Sealed chambers may be used if acceptable control performance is obtained. Disposable glass tubes, or scintillation or shell vials make convenient exposure chambers that can be discarded after test. Ensure that all test chambers and equipment used to prepare test solutions are clean and noncontaminating. Disposable test chambers should be rinsed or soaked in distilled water or seawater before use. Avoid using detergent and hypochlorite solutions to clean other equipment because they could be toxic to test organisms. In multipurpose laboratories, use glassware dedicated solely to toxicity tests.

4. Conducting the Test

a. Setting up test chambers: Set up the test as described in Section 8010D. Prepare all solutions and equilibrate to test temperature before beginning to spawn animals. Prepare at least four replicates of each solution in order to facilitate statistical analysis, such as determining the NOEC or LOEC (see Section 8010B).

b. Duration and type of test: In the fertilization test, add a predetermined number of sperm to test solution and expose for 20 min (60 min for *T. gratilla*). Other times ranging from 5 to 120 min have been used. Then add eggs to produce a specific sperm-to-egg ratio and allow 20 min for fertilization to occur. Preserve samples by adding buffered formalin and examine under a compound microscope. Toxic effects are manifested by sperm's impaired ability to fertilize eggs, indicated by lack of an obvious fertilization membrane around the egg.

c. Test organisms: Fertilization tests can be conducted with all recommended species.

d. Performing the test:

1) Preparation—Arrange replicate 5-mL samples of test solution in random order by assigning random numbers to individual test chamber numbers (i.e., replicate chambers of the first treatment group will have unrelated numbers, such as 16, 31, and 4,

instead of sequential numbers); then arrange chambers in a rack in numerical order.

Other test volumes may be used if preferred (e.g., 2 mL or 10 mL), but adjustments to the following instructions will be necessary to maintain the desired number of eggs and sperm in test chambers. Measure water quality of each test substance concentration on additional samples of test material. A single initial measurement is sufficient for most parameters unless the test material is highly unstable. Using the proper sperm-to-egg ratio is critical to obtaining good test sensitivity and precision. Because a fixed number of eggs is used in the test, sperm-to-egg ratios are altered by varying the number of sperm added to test chambers. The proper number of sperm is the least amount that produces ≥80 to ≤95% fertilization. Although controls outside this range do not automatically disqualify a test, particularly if a valuable dose response is generated, test sensitivity is reduced by fertilization rates exceeding 95% and good dose responses may be difficult to obtain with less than 80% fertilization in controls. Density of sperm solution or sperm:egg ratios should be determined with this goal in mind.

2) Sperm density measurement—Use a portion of the concentrated pooled sperm to determine the density (be sure to reserve sufficient sperm to conduct the test and a possible trial fertilization). Use the following procedures to aid in accurately pipetting the highly viscous concentrated sperm: Enlarge pipet tip opening to about 2 mm, wipe off any sperm adhering to the outside of the pipet tip before delivering sample (take care not to wick away sperm from inside the tip), and repeatedly rinse pipet tip with dilution water after sample delivery until all sperm inside has been removed.

Add 0.025 mL sperm to approximately 180 mL seawater in a graduated cylinder. Bring mixture up to 200 mL using 10% acetic acid (kills sperm) to produce an 8000× dilution. For *T. gratilla,* use a 0.1% acetic acid solution to kill the sperm; using a more concentrated solution will cause sperm to clump. Cover the cylinder, mix well by inversion, and let bubbles dissipate. Add a sample of the mixture to each side of a hemocytometer. Alternate dilution volumes (e.g., 1 mL sperm solution in 100 mL) may be more suitable if a less dense sperm solution is used.

Let sperm settle for about 10 min. Examine a sufficient number of small squares on the slide so about 100 sperm are counted. Examine the same number of squares on the opposite side of the hemocytometer. If the two counts are within 20%, use the mean to calculate the density according to the equation below. Reload hemocytometer and repeat counts if variability exceeds 20%.

$$\text{sperm/mL} = \frac{(\text{dilution factor})\ (\text{count})\ (4000\ \text{squares/mm}^3)\ (1000\ \text{mm}^3\text{/mL})}{\text{No. squares counted}}$$

Alternatively, use a ratio turbidimeter with a 1-cm-diam cuvette to determine sperm density rapidly.[4] The relationship between turbidity and sperm density is linear but may vary with species, individual, season, or type of instrument. Use hemocytometer counts for initial calibration of turbidimeter, regression equation, and verification of method suitability.

3) Sperm-to-egg ratio selection—The sperm-to-egg ratio for the test usually is based on the control's performance in previous tests. The correct value may vary, depending on species and time of year. Conduct a trial fertilization test (just before the actual test) if adequate data are not available to determine the sperm-to-egg ratio [see ¶ *d*5) below]. For purple sea urchins, use sperm-to-egg ratios ≤500:1 if fertilization in controls is acceptable. Higher ratios may reduce test sensitivity and should only be used when prior experiments or trial fertilization results indicate that the ratio is needed to obtain acceptable control fertilization (≥70%). Sperm-to-egg ratios above 3000:1 indicate unacceptable quality of purple sea urchin sperm; use additional animals or a different species (in better spawning condition). Optimum sperm-to-egg ratios are species-specific.[1,5,6]

4) Egg stock preparation—Add a sufficient volume of washed eggs to seawater to make 100 to 500 mL of a stock solution containing 4000 to 5000 eggs/mL. Determine density of eggs as directed in 8810B.5. Adjust density to proper range by adding or removing seawater. Verify that stock volume is sufficient for number and size of test chambers.

5) Trial fertilization test—Although a trial fertilization test is optional, it allows for the evaluation of sperm condition and viability. To conduct a trial fertilization test, determine density of pooled sperm as directed in ¶ *d*2) above. Prepare egg stock solution as directed in ¶ *d*4) above. Calculate volume of seawater needed to dilute 0.025 mL pooled sperm and produce a trial stock solution such that a sperm-to-egg ratio of 3000:1 will result when 0.05 mL of stock is added to test chamber (e.g., if 1000 eggs will be in each chamber and 0.05 mL sperm stock is added to the test sample, then a sperm stock density of 6.0×10^7 sperm/mL is needed). Prepare duplicate test chambers for each sperm-to-egg ratio to be tested (including 3000:1), each containing 5 mL seawater at correct temperature. Prepare trial sperm stock solution. Prepare several dilutions of stock that will produce desired range of sperm-to-egg ratios in test chambers.

A suggested dilution series is as follows:

Sperm-to-Egg Ratio	Trial Stock *mL*	Seawater *mL*
3000:1	No dilution	—
1304:1	5	6.5
545:1	2	9.0
231:1	1	12.0
100:1	0.5	14.5

Add 0.05 mL trial stock or dilution to appropriate test chambers. After 20 min, add 1000 eggs. Add formalin preservative after 20 additional min. Determine percent fertilized in a subsample of 100 eggs from each replicate. Select lowest sperm-to-egg ratio producing ≥80 to ≤95% fertilization. A reference toxicant dilution series may also be included in the pretest to verify test sensitivity at various sperm densities. If using this approach, then the sperm dilution selected for use corresponds to the lowest sperm-to-egg ratio producing ≥80 to ≤95% fertilization and a reference toxicant EC_{50} within the laboratory control chart confidence limits. It may be necessary to determine the final sperm-to-egg ratio by interpolation. Sperm viability for some species may decline between the trial fertilization test and actual test, necessitating the use of a higher sperm-to-egg ratio to obtain the desired control fertilization value. Verify sperm den-

sity in stock dilution corresponding to the chosen sperm-to-egg ratio via a hemocytometer count.

6) Sperm stock preparation—Calculate sperm stock solution density required to produce desired sperm-to-egg ratio in the test chambers (e.g., a stock containing 5.0×10^6 sperm/mL is needed to produce a sperm-to-egg ratio of 250:1 when 1000 eggs are present). Remove 0.025 mL pooled sperm and dilute with seawater to produce a stock solution of the desired density. Mix the solution well and use within 30 min.

7) Sperm addition—Use an automatic pipet to add 0.05 mL sperm stock to each test chamber. Mix stock frequently during inoculations. Add sperm in a steady rhythm, with each addition at intervals of about 5 s.

8) Egg addition—Add eggs after a 20-min exposure period (other sperm exposure times of 5 to 60 min can be used for comparability with other laboratories). Use egg stock to add 1000 eggs (e.g., 0.20 mL stock) to each chamber using the same order and rhythm as for sperm. Use a perforated plunger or equivalent device to gently mix egg stock thoroughly during additions.

9) Test termination—Stop test by adding buffered formalin [pH>7.0; see Section 10200B.2*a*2)c)] to each tube 20 min after egg addition to produce a 5% final concentration. Glutaraldehyde may be used as a preservative instead of formalin. Lugol's solution [Section 10200B.2*a*2)a)] also is an effective preservative and has the advantage of being less toxic to analysts. However, formalin and more concentrated glutaraldehyde negatively affect fertilization membranes of *T. gratilla*. Use only a 0.002% final concentration of glutaraldehyde to stop *T. gratilla* tests. Cap test chambers securely, store at room temperature, and determine fertilization within 48 h, or as soon as practical. The samples can be stored indefinitely, but appearance of egg or fertilization membrane may change during storage, making the endpoint more difficult to detect. Be extremely careful not to contaminate test equipment or laboratory furniture with preservative.

10) Sample evaluation—Examine eggs in exposure vial using an inverted compound microscope or transfer a representative subsample to a Sedgwick-Rafter cell for use with a conventional microscope. It is often convenient to concentrate the eggs before transfer by removing most of the overlying water with a pipet. Mix remaining sample well before transfer to counting chamber. If membranes are difficult to discern, adding one or two drops of HSB to the slide just before observation shrinks the eggs momentarily, resulting in more discernable membranes. Discard formalin-contaminated exposure chambers promptly (do not reuse chambers). To protect analysts from exposure to toxic chemicals, provide adequate ventilation when transferring or examining samples containing formalin or glutaraldehyde in unsealed containers.

Examine at least 100 eggs (40 to 100× magnification) from each replicate and score for presence or absence of an elevated fertilization membrane. Avoid bias by counting all eggs in subsamples transferred to counting chambers. Newly fertilized eggs usually have a completely elevated membrane around the egg (Figure 8810:1, A and B). The fertilization membranes of *T. gratilla* eggs are not as elevated as in other species and should be examined under a phase contrast microscope. The fertilization membrane may change in appearance with prolonged storage, partially collapsing or touching a portion of the egg. Consequently, count eggs showing any elevation of the fertilization membrane as fertilized. Exclude unusually small, immature, or abnormally shaped eggs from counts.

Calculate percentage of fertilized eggs in each sample.

5. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

6. Quality Assurance

Continued success in conducting this toxicity test depends on an overall effort to maintain and improve laboratory techniques and equipment.[3] Accurate background information regarding sample characteristics and test organism condition is necessary to enable correct interpretation of test results. Sea urchin gametes and embryos are relatively sensitive to ammonia, pH, dissolved organic carbon, and unmeasured variations in these and other water characteristics may confound test results.[7] Measure basic water quality parameters (e.g., pH, dissolved oxygen, salinity, temperature) on representative samples of controls and test materials. Because ammonia can be highly toxic to marine organisms, measure total ammonia with a sensitive method (e.g., 4500-NH_3.E) and report concentration of un-ionized ammonia (NH_3). Additional controls or adjustments may be needed to avoid confounding effects of variations in water quality characteristics (e.g., pH). For example, reduced fertilization and embryo development in *S. purpuratus* has been observed at pH<7.5 or pH>8.3.[8]

Include additional controls or blanks in the experimental design to verify that special treatments (e.g., storage, centrifugation, pH adjustment, carrier solvent addition, salinity adjustment) do not produce unanticipated effects. A "no sperm" control—consisting of several exposure chambers containing just the test sample and eggs—should be included to account for artificial parthenogenesis and verify that the eggs were not accidentally fertilized before conducting the test. This control is recommended when conducting tests with unknown substances.[7]

Use reference toxicant tests to provide a measure of test precision and possible organism condition.[3] The reference toxicant test usually consists of replicate exposures to three to five concentrations of a stable chemical (e.g., Cu, Cd, sodium dodecyl sulfate) that are sufficient to calculate a point estimate of effect (e.g., EC_{50}). Preferably include a reference toxicant test with each experiment, or test at least monthly. Plot cumulative mean and confidence limits on a control chart to identify outlier values. Outliers indicate potential problems with the technique or test organisms. It is recommended that test organisms be replaced if repeated reference toxicant EC_{50}s fall outside the laboratory quality control confidence limits.

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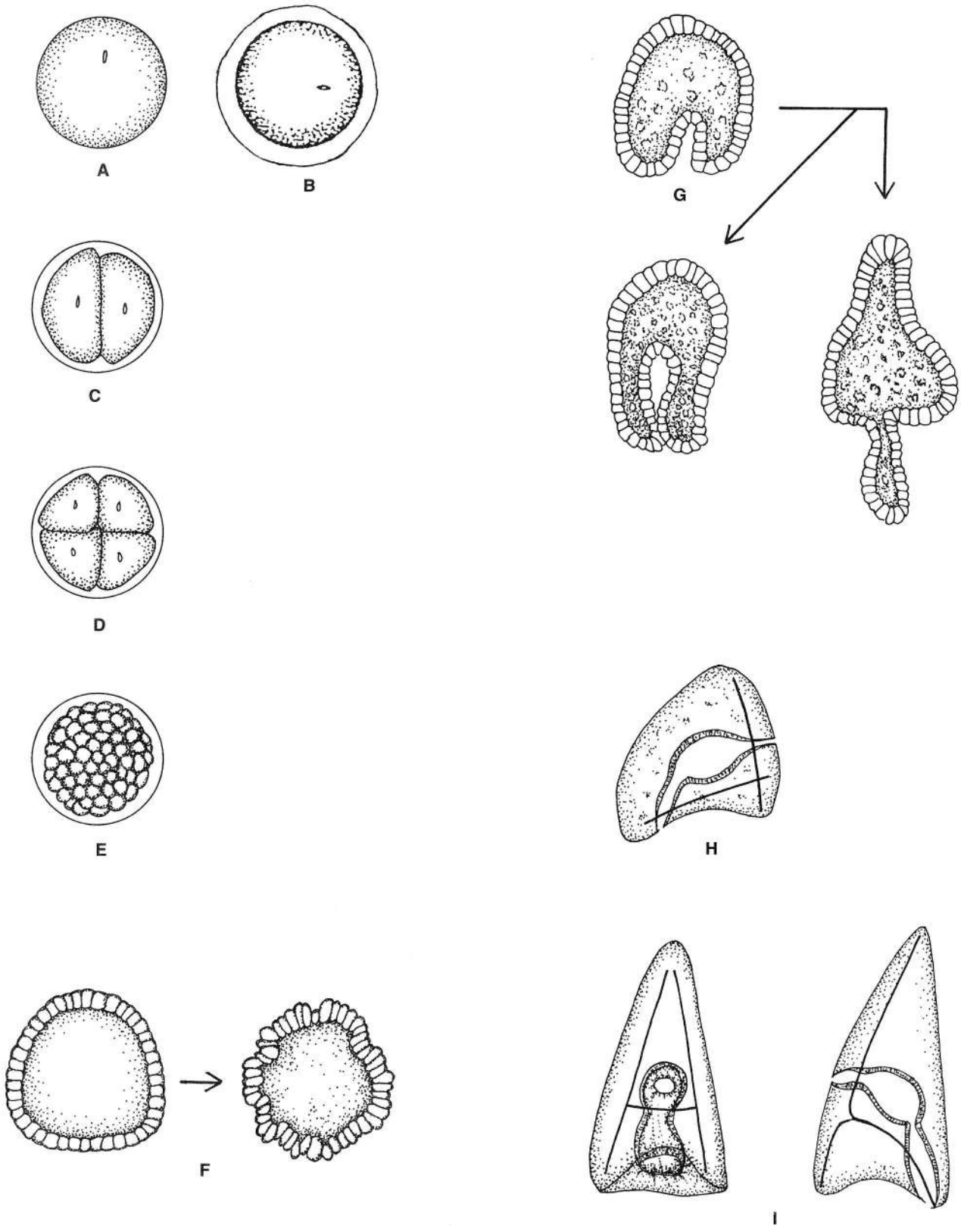


Figure 8810:1. Early development stages of sea urchins and sand dollars. A—unfertilized egg; B —fertilized egg; C, D, E—early cleavage; F—blastula with arrow indicating abnormal example; G—gastrula with arrows indicating abnormal examples; H—prism; I—frontal and lateral views of normal pluteus.

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8810 D. Echinoderm Embryo Development Test

1. General Procedures

Conduct exploratory tests (see Section 8010D) first if the concentration range to be tested is unknown. Prepare dilution water and toxicant solutions and introduce them into test containers as described in Section 8010F. Take precautions to avoid gamete temperature stress (see 8810C.1). The procedure described below uses many of the same techniques described for the fertilization test (8810C) but has been optimized for use with embryos. Some laboratories conduct both tests on a sample to gain more information. The fertilization test also may be extended into an embryo development test by including additional replicate chambers. Such an approach requires modification of the test methods and may reduce precision of embryo development results because of variable fertilization rates. An embryo development test has not yet been developed for *T. gratilla*.

2. Water Supplies

Maintain dilution-water salinity within 2 g/kg of the holding salinity and at a pH that is within the species' tolerance range. Dilution water quality should be sufficient to produce ≥70% normal survival (relative to initial number of embryos) in control samples.

a. Artificial seawater: See Sections 8010E.4*b*2) and 8810C.2. Avoid commercial sea salt mixes because they often are toxic to echinoid gametes and embryos.

b. Natural seawater: Choose a source of natural seawater that is free of contamination and of uniform quality. Pass water through a filter with an effective pore size ≤1.0 μm to remove sediment and organisms that might interfere with the test. Additional treatment (e.g., aeration, activated carbon treatment) may be needed to obtain acceptable water quality.

c. Salinity adjustment: The sea urchin development test usually is more sensitive to deviations in salinity than is the fertilization test. Use the methods described in 8810C.2*c* to adjust salinity of samples that deviate by more than 2 g/kg.

3. Exposure Chambers

Preferably, use glass chambers with a capacity between 10 mL and 1 L. Maintain recommended density of test organisms, regardless of volume. Cover chambers loosely to prevent contamination and reduce evaporation during the test. Sealed chambers may be used if control performance is acceptable. Scintillation or shell vials make convenient exposure chambers that can be discarded after the test. Clean all equipment for preparing test solutions before use. Clean test chambers by soaking in fresh water or seawater. Avoid detergent or hypochlorite solutions because they could be toxic to test organisms.

4. Conducting the Test

a. Setting up test chambers: See 8810C.4*a*.

b. Duration and type of test: Various volumes of test solution (5 to 1000 mL) may be used. Add embryos to test solution and let develop under static conditions for 48 to 96 h until the pluteus stage is reached. Preserve subsamples (or entire sample if vials are used) with formalin and examine under the microscope. Toxic effects are indicated by embryo mortality or abnormal development.

c. Test organisms: Embryo development tests can be conducted with all the recommended species.

d. Performing tests:

1) Preparation—Test preparation is the same as for the fertilization test [8810C.4*d*1)], with two exceptions. First, the test may be conducted in larger volumes (up to 1000 mL) if desired. Using large volumes provides no distinct advantage in test sensitivity or precision, but does allow exposure chamber to be subsampled to measure water quality or determine effects at various times or developmental stages. Second, measure both initial and final water quality for each treatment group. Include one additional replicate test chamber in each treatment group for final water quality measurements when tests are conducted in small volumes (e.g., 10 mL).

Quantify toxic response by either complete count or relative count. For complete count, calculate percentage of normal pluteus larvae at the end of exposure, based on counts of all preserved test organisms and number of embryos added at the start. This method provides the most comprehensive assessment of effects because all instances of embryo mortality and aberrant development are included in the percentage. A relative count requires counts of only a subsample of organisms at the end of the test. Both embryo mortality and aberrant development are reflected in this method as well, but toxic effects may be underestimated if the test solution causes rapid decomposition of dead embryos and consequent failure to detect them during microscopic examination. Penicillin-G in a concentration of 100 units/mL has been used in the embryo stock solution to retard bacterial degradation of dead or dying embryos during the test, thus allowing for more complete counts upon termination. Choose the evaluation method before the test is started so all required information will be obtained.

2) Egg density adjustment—Add a sufficient volume of washed eggs to seawater to make 100 to 500 mL of a stock solution containing 1000 eggs/mL. It may be more convenient to prepare a more concentrated solution (e.g., 10 000 eggs/mL) if test volumes larger than 50 mL are used. Determine density of eggs as directed in 8810B.5. Adjust density to desired value by adding or removing seawater. Verify that stock volume is more than sufficient (approximately 50% greater) for the number and size of test chambers used.

3) Sperm stock preparation—Prepare a sperm stock by adding about 0.025 mL dry sperm to 50 mL seawater. If volume of stock solution needed to fertilize eggs is not known from prior experience, determine density of sperm stock with a hemocytometer (see 8810C.4*d*).

4) Egg fertilization—Add a sufficient volume of sperm stock to egg stock to produce a sperm-to-egg ratio of 200 to 1000:1. Mix well and examine a subsample after about 10 min to assess fertilization percentage. Add more sperm if less than 90% of eggs are fertilized. A fertilization rate of less than 90% after the second addition of sperm indicates poor quality gametes. Spawn additional animals to obtain better gametes if possible. Add embryos to test containers as soon as possible (generally within 2 h but no later than 4 h after fertilization).

5) Embryo addition—Use an automatic pipet to add sufficient embryo stock solution to each test chamber to result in a count of at least 100 embryos in the sample at the end of the test. Typically, a density of 25 to 400 embryos/mL is used, depending on test volume and method used to evaluate embryos. It is important that the same number of embryos is added to each test chamber. Use a perforated plunger to mix the stock solution thoroughly during embryo addition.

If using the complete count method to evaluate toxic effects, add embryos to at least five additional test chambers containing control water. Intersperse these chambers throughout the experimental array and add embryos in the same manner used for the other chambers. Examine these additional chambers promptly to estimate actual number of embryos added.

6) Exposure—Loosely cover test chambers and leave undisturbed for 48 to 96 h under static conditions. The optimum exposure time varies with species and test temperature. Exposure time should be long enough to allow embryos to develop to the pluteus stage, yet short enough (≤96 h) that internal food reserves are not exhausted. The following exposure conditions are recommended to provide consistency with results from other laboratories: *A. punctulata*, 48 h at 20°C; *D. excentricus*, 72 h at 15°C; *S. purpuratus*, 72 h at 15°C or 96 h at 12°C; and *S. droebachiensis*, 96 h at 12°C. These are target times; a few extra hours may be allowed to help ensure that most control larvae (>90%) have attained the normal pluteus stage. Ambient laboratory light levels and photoperiods are adequate for all species.

7) Test termination—Preserve organisms for later microscopic examination by adding sufficient buffered formalin [pH>7.0, see Section 10200B.2*a*2)c)] to produce a 5% concentration. Add formalin directly to test chamber if disposable vials or culture tubes are used. Otherwise, thoroughly mix test cha161mber contents, transfer a 10-mL subsample to a vial, and add formalin. Glutaraldehyde (e.g., 0.02% final concentration) may also be used as a preservative.

8) Test evaluation—Examine preserved embryos and larvae with a compound microscope at a magnification of 100×. Concentrate test organisms by removing overlying water from the storage vial and transfer to a Sedgwick-Rafter counting chamber for examination with a conventional microscope. Alternatively, use an inverted microscope to examine organisms in the storage vial and eliminate losses due to transfer.

Normally developing embryos develop synchronously through a series of characteristic stages, including early cleavage, blastula, gastrula, prism, and pluteus (Figure 8810:1, B–I). The appearance of pluteus larvae varies with species, but all normal plutei should have the following features: a pyramid shape supported by a framework of skeletal rods, an internal gut that is attached to the body wall at both ends and consists of three distinctive regions, and at least one pair of post-oral arms (Figure 8810:1). Compare sample larvae to the control to determine abnormal development. The length of the post-oral arms varies with species. Count as abnormal all grossly deformed pluteus larvae, deformed embryos, mostly normal-appearing embryos that have not attained the pluteus stage (inhibited development), and uncleaved fertilized eggs. Do not count unfertilized eggs.

Determine percentage of normal pluteus larvae for each replicate using either the complete or relative count method (below).

a) Complete count method—Count all embryos in preserved sample. Preferably use an inverted microscope to minimize counting errors. If a conventional compound microscope is used, use a consistent and efficient method to transfer embryos to counting chamber because lost embryos (remaining in vial or stuck to transfer pipet) are assumed to have died. Variability in recovering larvae from the storage vial may introduce experi-

mental error that reduces the ability to detect statistically significant effects. Calculate percentage of embryos developing to normal pluteus larvae, P_n, as follows:

$$P_n = 100(E_n/E_i)$$

where:

E_n = number of normal larvae at end of test, and
E_i = number of embryos at start of test.

b) Relative count method—It is easier and usually just as effective to determine percentage of normal development in a representative sample of at least 100 embryos and larvae at the end of the test. To use the relative count method when using an inverted microscope, start at one side of the vertical center of the vial and move across to the opposite site, scoring eggs as normal or abnormal. Counting is complete if at least 100 embryos have been scored. If fewer have been counted, move the stage down one field of view and score all embryos encountered in a second pass across the vial. Repeat this process until at least 100 embryos have been scored. Calculate the percentage of normally developed embryos as follows:

$$P_n = 100[E_n/(E_n + E_a)]$$

where:

E_a= number of abnormal embryos/larvae, and
other terms are as defined above.

5. Statistical Analysis

See Section 8010G.2 for general information.

Control performance may vary among tests because of such factors as variations in test temperature and gamete condition. Normalize response data to the control performance before statistical evaluation (e.g., EC_{50}) and to facilitate comparisons among tests as follows:

$$P_{adj} = 100(P_n/M)$$

where:

P_{adj} = normalized value,
P_n = percent normal or fertilized for the sample, and
M = mean percent normal or fertilized for controls.

6. Quality Assurance

See 8810C.6.

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8910 FISH*

8910 A. Introduction

1. Background

Fish are considered a good test species for assessing aquatic toxicity because of their ecological and economic importance. While many fish species may be used in toxicity studies, the choice depends on the test objective, the species' availability, and the ease of culturing and handling individuals. This section provides guidance for selecting, culturing, and testing fish in aquatic toxicity studies.

Other toxicity test methods for fish are available.[1–6]

2. References

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* Approved by Standard Methods Committee, 2015.
Joint Task Group: Gabriela Rodríguez-Fuentes (co-chair), Xin Deng (co-chair).

8910 B. Fish Selection and Culture Procedures

1. Selection of Test Species

General guidelines for selecting test organisms are outlined in Section 8010E.1. A prime consideration when selecting a fish species is the purpose of the test. In tests for regulatory purposes, species selection may be limited by particular regulations [e.g., the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) or the Toxic Substances Control Act (TSCA)]. In tests for nonregulatory purposes, any species may be selected so long as the lab can maintain the fishes' optimal environmental requirements. If it is desirable to use a fish that is not routinely cultured, the species may need to be collected from a single field source.

Select test species according to the following criteria:

- the species should be available in unbiased numbers (i.e., not prescreened for resistant individuals via prior exposure to adverse conditions) sufficient for the tests; and
- the species should be capable of living in laboratory conditions with normal swimming behavior, feeding activities, and health for at least 1 month.

Consider the relative sensitivities of different species and life stages (if data are available) when selecting species.

2. Collecting and Handling Test Fish

Collection equipment and methods are described in Sections 8010E.2 and 10600B. Handling and holding are discussed in Section 8010E.3. It is extremely important to avoid subjecting fish to unnecessary stress, such as inappropriate handling and transport, temperature shock, or water quality change.

a. Freshwater fish: Whenever possible, obtain routinely cultured species. Salmonid fish usually are available from private, state, and federal hatcheries. Obtain certified pathogen-free trout, if possible. When fish cannot be obtained from hatcheries, appropriate field collection is acceptable. State agencies usually require collection permits. Avoid fish from bait dealers or fishermen because information on source, handling, holding time, etc., usually is not available.

b. Marine and estuarine fish: Various life stages of marine fish may be collected from the field for laboratory tests. Vertical movement of early larval stages may necessitate nighttime collection. Many marine fish and most marine fish larvae are extremely fragile; handle carefully during collection, sorting, and transfer. To sort and transfer larvae during and after collection, use a pipet appropriate to the size of the larval fish. Whenever possible, transfer larger larvae, juvenile, and adult fish by dipping or gently pouring. Fine-mesh dip nets also are suitable if transfers are made gently.

3. Holding and Acclimating

Keep fish stocks in tanks, small ponds, live boxes, or screen pens, depending on fish size and number. Use good-quality dilution water (see Section 8010E.4*b*) for acclimation. Feed fish natural or commercially prepared foods daily during acclimation. Detailed information on handling, holding, caring for, and feeding fish is available.[1–4] Because food requirements vary with the species and size of fish, select an appropriate diet for the species. Fish obtained from a hatchery should initially be provided with

food to which they are accustomed. Many fish can be maintained on dried food for long periods, but live food supplements may be desirable. Do not overfeed. Diets should be certified toxicant-free or tested for toxic substances before use.

While maintaining fish during holding and acclimation, watch carefully for signs of disease, stress, physical damage, and mortality. Remove dead and abnormal individuals immediately. If the mortality rate exceeds 10%, discard the entire stock.

Handle fish carefully and as quickly as possible.[1,2] For extensive handling (e.g., weighing, measuring, or taking other data), anesthetize fish.[3]

Fish anesthetic protocols vary depending on the species and the anesthetic agent. In general, the following guidelines apply:

- fast fish 12 to 24 h before anesthesia in order to maintain water quality,
- monitor water-quality parameters during the process,
- administer anesthesia in water, and
- test anesthetic concentration in a small batch if no information is reported.

A list of recommended anesthetic agents is available.[5–7]

For short-term tests, use fish of similar size and source. The longest fish should not be more than 1.5 times longer than the shortest. Acclimate fish to laboratory conditions before the test. Standard acclimation periods range from 48 h to 14 d. However, this may not be possible when using early-life-stage fish in short-term tests.

See Sections 8010E and F for more discussion.

4. Parasites and Disease

a. Stress in relation to parasites and disease: Unexpected and often unexplained mortalities in experimental and control animals interfere with test results and interpretations. Optimize laboratory conditions for each species to prevent the development of disease.

When large numbers of organisms are retained in a relatively small space, microbial diseases or parasites may become epidemic. Pathogens and parasites that might be rare in natural waters may become epidemic in intensive culture. Disease also may arise if the water is enriched with organic materials or if toxic substances are present. Uneaten food and fecal material are potential sources of bacteria, parasites, and toxic products. If the water is unpolluted and nutrient-poor, disease often can be prevented by strict sanitation. The first lines of defense are securing disease-free fish, sterilizing equipment, filtering and/or sterilizing water, feeding fish adequately, and cleaning holding vessels regularly.

Organisms exposed to toxicants may become more susceptible to parasites and diseases. Because various environmental factors may contribute to reduced resistance, pay careful attention to nutrition, oxygen supply, and water quality. To minimize accumulation of fecal material [and hence dissolved oxygen (DO) demand], do not feed fish during the 2 d before initiating short-term tests for cold-water species or 1 d for warm-water species. Siphon any fecal material or uneaten food daily from the holding tank. When testing young fish, feeding live brine shrimp nauplii may be desirable if starvation is a possibility during the test.

b. Control methods:

1) General—Ultraviolet light and ozonation have been used to control diseases and parasites in dilution and/or culture water. Antibiotics used in holding tanks reduce bacterial populations. To reduce mortality and avoid introducing diseases into stock tanks, treat fish with a broad-spectrum antibiotic immediately after collection, during transport, or on arrival at the laboratory. Do not place treated organisms into holding tanks within 4 d of treatment. Do not use treated organisms for tests until at least 14 d after treatment. Clean and disinfect tanks and containers with 200 mg sodium hypochlorite (NaOCl)/L for 1 h after removing diseased fish. Dechlorinate tanks with sodium thiosulfate and rinse with clear water before reuse.

2) Recommended disease therapy—Treat freshwater fish to cure or prevent diseases by the methods in ¶ *b*1) above and Table 8910:I. These methods have been found dependable, but their efficacy may be altered by temperature or water quality. If fish are severely diseased, destroy the entire stock. A number of good reviews of fish diseases and parasites, and methods for controlling them, have been published.[8–14]

Published information on related topics include a summary of problems in marine fish larval culture,[15] a description of a larval culture system,[16] and a discussion of disinfecting water supplies.[17]

5. Culturing Test Fish

a. Freshwater fish: More than 30 species of freshwater fish have been reared for stocking. The culture methods can be adapted to laboratory-scale to produce various life stages of fish.[18–28] Methods are given below for three freshwater species commonly used in toxicity tests: rainbow trout (*Oncorhynchus mykiss*), bluegill sunfish (*Lepomis macrochirus*), and channel catfish (*Ictalurus punctatus*). For specifics on the fathead minnow (*Pimephales promelas*), see Section 8921. These species are representative of test organisms that can be found in various freshwater habitats, ranging from free-flowing streams and rivers to ponds and lakes.

1) Rainbow trout—Rainbow trout of various ages can be purchased from certified specific pathogen-free hatcheries. Life stages range from unfertilized eggs and sperm (fertilization can be performed in the testing laboratory) to juvenile fish. Preferably obtain eyed embryos and grow these to testing size unless spawning, fertilization, and early embryonic stages are important for the test.

Overnight courier shipment of hatchery-raised eyed trout embryos in special insulated cartons is standard. These cartons are adequate to maintain cold temperatures during shipping. Upon receipt, measure the egg mass' ambient temperature and, if necessary, slowly temper the eggs (±3°C/h) to testing temperatures or culture conditions. Maintain embryos at temperatures ±2°C in a range from 8 to 12°C.[26]

To fertilize eggs in the laboratory, obtain gametes in plastic bags from the supplier within 24 h of removal from adults. Hold gametes in unopened bags and slowly acclimate to test or culture temperatures. When eggs and milt are at the desired temperature, mix together with either ovarian fluid or a small volume of 0.75% saline solution. Gently stir and let stand for about 1 min while fertilization occurs. Pour off excess water and milt, and replace with fresh dilution water. Repeat several times until the embryos are in clear water. It may be useful to let eggs rest for 1.5 to 2 h to harden before transferring to incubation cups or culture systems.

Place fertilized eggs (embryos) in incubation chambers until hatching. For small-scale cultures [e.g., those that may be ma-

TABLE 8910:I. RECOMMENDED PROPHYLACTIC AND THERAPEUTIC TREATMENTS FOR FRESHWATER FISH TO BE USED FOR EXPERIMENTAL PURPOSES

Disease	Chemical	Concentration *mg/L*	Application
External bacteria	Hyamine 1662® or 3500®*	1–2 AI†	30–60 min in flow-through system‡
	Nitrofurazone (water mix)	3–5 AI	30–60 min in flow-through system‡
	Neomycin sulfate	25	30–60 min in flow-through system‡
	Oxytetracycline hydrochloride (water-soluble)	25 AI	30–60 min in flow-through system‡
Monogenetic trematodes, fungi, and external protozoa§	Formalin *plus* zinc-free malachite green oxalate	25 ± 0.1	1–2 h in static systems, 30–60 min in flow-through system‡
	Formalin	150–250	
	$KMnO_4$	2–6	1–2 h in static system, 30–60 min in flow-through system‡
	NaCl	14 000–30 000	5–10 min dip
		2000–4000	24 h minimum, but may be continued indefinitely
	Para-dimethyl aminobenzenediazo sodium sulfonate (35% AI)‖	20	30–60 min in flow-through system‡
Parasitic copepods	Trichlorfon#	0.25 AI	Weekly for up to 4 weeks if necessary in static or flow-through systems. Do not use at >27°C.

* Benzalkonium chloride.
† AI = active ingredient.
‡ Add concentrated stock solution to inflowing water by a drip system or by the technique of Brungs and Mount.[8]
§ One treatment usually is sufficient, except for *Ichtyophthirius*, which must be treated daily or every other day until no sign of the protozoans remains. This may take 4 to 5 weeks at 10°C and 11 to 13 d at 15–21°C. A temperature of 32°C is lethal to *Ichthyophthirius* in 1 week.
‖ Dexon®, or equivalent.
Masoten®, or equivalent.

nipulated experimentally during an early-life-stage toxicity test (8910C.2)], incubation cups may be constructed from 8-cm sections of 5-cm-outer diameter (OD) plastic or glass tubing with nylon or stainless steel screening cemented over one end to retain embryos. During incubation, supply a flow of fresh, high-quality, well-oxygenated water over the embryos. Oscillate incubation cups (containing <250 embryos) in holding tanks via a rocker arm using a 2-rpm electric motor to supply the necessary flow. Incubation chambers for mass culture of trout generally consist of wire-screen trays with rectangular or oblong mesh (15- × 3.5-mm openings) stacked vertically in deep flow-through troughs or spaced along horizontal troughs.[26,27]Agitate or shock embryos periodically. Remove dead embryos that turn white every 2 d. Maintain incubation chambers in the dark because exposure to light may result in premature hatching or death.[26] The hatching rate is determined by the temperature regime; the optimum range (7 to 10°C) produces hatching in 44 to 68 d.

After development to the free-swimming fry stage, provide at least one complete exchange of water per hour and use a 20-gal (75-L) aquarium per 100 fry. Maintain water pH between 6.5 and 8.5, DO levels >5 mg/L, total dissolved solids >50 mg/L, and ensure that water is free of pollutants. Feed fry slightly to excess, as often as 10 times/d.[26,27] Daily rations generally average 7 to 9% body weight. Commercial dry food has proven successful in hatchery production; for laboratory culture, live brine shrimp nauplii are preferred for early stages.

When trout reach the fingerling stage, reduce quantity of fish per tank to approximately 1 g fish/L flow/d.[26] Feed fish at a rate equivalent to 4 to 5% of body weight.[26,27] As fish grow, grade and sort them into separate tanks according to size to reduce size-dependent adverse hierarchical feeding dominance.

2) Bluegill sunfish—Bluegills may be bred and cultivated in a variety of ponds or tanks.[26–28] Adult bluegills generally can spawn at 1 year old, with body weight ranging from 100 to 150 g and total length ranging from 12 to 18 cm. The breeding period is May to August, and a given individual can spawn more than once during the season.

Stock spawning ponds with a 1:1 or, preferably, a 2:3 ratio of male to female adults. Although bluegills do not require environments as highly controlled as trout do, they do need adequate DO and water-quality conditions (see Section 8010E.4*b*). Bluegills adapt readily to a wide variety of commercial feeds; feed to satiation.[28]

Provide spawning ponds with small piles of pea gravel in the shallow water (0.5 to 1.0 m deep) around the edges.[27] Male bluegills will use this material to build nesting areas. Space gravel piles at least 1 m apart to reduce aggression between males guarding territories.

If dense spawning for mass fry production is desired, place spawning stalls side by side around the perimeter of the ponds.[27] Make stalls 1 m long and enclose on three sides with wood or concrete, place gravel on the bottom, and orient the open side toward the pond center. Hatching should occur within 5 d. To capture fry after hatching but before they have dispersed, slip a screen over the open end of the stall after the females leave the nest. Stock fry in growing ponds at a density of 40 fry/m^2.

It is not recommended to rear fry in spawning ponds with adults, but if this is necessary, reduce stocking densities of brood fish to limit losses due to predation by adults. For fairly high densities of fry (40/m^2), maintain only two or three pairs of spawning adults per 4000 m^2. If large fry are desired for harvest, use a lower stock density: one breeding pair per 4000 m^2 to

produce 10 fry/m^2. Under intensive culture, periodically sort juvenile bluegills according to size in order to facilitate uniformity of specimens for toxicity tests.

3) Channel catfish—To establish breeding stock, collect adult catfish 3 or more years old, preferred size 1 to 4.5 kg. Segregate adults by sex in holding ponds until spawning is desired. Feed adult catfish commercially available pellets supplemented with fresh or frozen cut fish and live minnows. Maintain a daily ration of dry feed at approximately 3% of stock weight.[26,27]

When spawning season begins (April or May), increase the daily ration to 4% of stock weight. Methods have been developed for spawning in ponds and pens,[26] but aquarium spawning is most efficient in terms of space and success of spawning rates.[26] In this method, pair catfish according to size in 23- to 240-L troughs and provide with flowing water and a spawning compartment (e.g., stainless steel milk can or similar structure). Inject females intraperitoneally with hormones: three doses of 10 mg acetone-dried fish pituitary material/kg female at intervals ranging from 6 h in warm water to 24 h in cold water, or else a single dose of 2200 IU/kg body weight of human chorionic gonadotropin.[26] Most fish will spawn within 16 to 24 h after the last injection. Fertilized eggs adhere to each other in oval gelatinous masses. New eggs are golden and turn pink as embryos develop. After spawning, remove spawners and eggs. Use troughs for additional spawning.

Incubate embryos either in hatching troughs or open-mouth hatchery jars. Hatching troughs may be of any convenient size but at least 25 cm deep and supplied with running water. Retain the egg mass in a wire-mesh basket suspended in the hatching trough, and place a paddle-like agitator driven by an electric motor alongside each basket to ensure mixing of eggs. If (6- to 8-L) hatchery jars are used, introduce a gentle flow of water over the mass via a rubber tube to simulate the agitation naturally provided by the male's fanning activities.

Catfish embryos hatch in 8 to 10 d at 24°C. The fry have light-colored bodies with pink yolk sacs. Remove fry from hatching troughs or hatchery jars near the time when the yolk sac disappears, but before complete absorption (approximately 3 to 5 d after hatching). Transfer fry by siphoning through a large-bore glass tube into rearing troughs.

Catfish fry can be reared according to the methods established for trout, except that warmer temperatures (24 to 28°C) are necessary. For the first 4 to 5 d in the rearing troughs, feed fry sparingly with finely ground fish food 10 times/d. Siphon off uneaten food after 2 h. Increase daily rations gradually to approximately 4 to 5% of total body weight.

b. Marine and estuarine fish: Culture methods for marine and estuarine fish species are less developed than those for freshwater species. General information about marine fish culture methods can be found in several sources.[16,17,29–41] For any species, maintain adult brood stock, eggs, and larvae under conditions approximate to those in the natural environment.

A method is described below for culturing sheepshead minnow (*Cyprinodon variegatus*), which is routinely cultured and used in egg-to-embryo or embryo-larval toxicity tests.

Sheepshead minnows thrive over a wide range of salinities and temperatures. Acclimate adult fish >27 mm standard length to laboratory conditions for at least 2 weeks at a salinity of at least 10 to 20 g/kg [parts per thousand (‰)] and a temperature of 30°C. Hold a 12-h-light/12-h-dark photoperiod. During this period, feed liberally with fresh or frozen adult brine shrimp. Eggs from natural spawning may be obtained by placing a pair of adult fish in a spawning chamber about 12 cm wide × 18 cm long × 10 cm high. Place spawning trays (2 cm deep, formed by 0.5-mm nylon screen attached to a frame and covered with 2-mm nylon screen) in the spawning chambers. Larger spawning tanks with several spawning pairs may be used so long as they provide each male enough space to establish a territory. As embryos are deposited, they fall through the screen into the trays, thus preventing them from predation by adult fish and allowing easy removal. Each pair may spawn a maximum of 10 to 30 embryos/d, but average production is about 8 embryos/d.

Alternatively, sheepshead minnows may be induced to produce eggs via hormone injection.[35] Inject each female intraperitoneally with 50 IU human chorionic gonadotropic hormone. Repeat after 2 d. On the third day, most females can be readily stripped to obtain ripe eggs. Strip or dissect eggs into filtered seawater in a beaker and add macerated testes. The number of eggs produced per female by this method is 100 to 200, depending on fish size. This method has the advantage of producing eggs at specified times; however, the fish typically are sacrificed to obtain eggs and sperm, thus reducing brood stocks.

Fertilized eggs may be hatched in flowing or static water systems. In a flowing water system, place embryos in a hatching chamber formed by gluing a 9-cm-high collar of 0.5-mm mesh nylon screen around a Petri dish. Suspend hatching chambers in flow-through seawater aquariums with self-starting siphons. As the water level in the aquarium changes, water in the hatching baskets is exchanged gently. Alternatively, place embryos in separator funnels and aerate gently.[40,41] Sheepshead minnow fry hatch after 5 d at 30°C at a salinity between 15 and 20 g/kg. As embryos hatch, transfer to a rearing aquarium and immediately feed newly hatched brine shrimp (live or frozen) or a dry diet. Occasionally supplement the dry diet with live organisms. Juveniles become sexually distinguishable at about 24 mm standard length, and females may produce eggs within 3 months of hatching.

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8910 C. Toxicity Test Procedures

1. Short-Term Tests

a. General test procedures: Short-term testing can be used to determine relative toxicity of substances. Tests are made to determine LC_{50} or EC_{50}, and to estimate toxicant concentrations for intermediate- and long-term tests.

Short-term tests may be static, static with renewal, flow-through, or recirculating, depending on the test objective, the life stage being tested, and the character of the toxicant or effluent (see Section 8010D.1 and 2 and Section 8921).

Although any life stage may be used, short-term tests are performed most frequently with small species (<5 g body

weight) or juvenile forms of large fish species (see 8910B.1). The life stage selected depends on test purposes, availability, and laboratory facilities (see 8910B.1). If culturing is necessary to obtain a particular life stage, see 8910B.5.

Select fish of near uniform size, with the longest no more than 1.5 times longer than the shortest. Use 10 to 20 fish per replicate for each toxicant concentration. Additional replicates may be used to increase the number of test fish at each concentration. For juvenile and adult fish, terminate feeding 48 h before initiating tests. For all tests, limit fish weight/L test solution. This practice minimizes oxygen depletion, metabolic waste accumulation, and crowding-induced stress. In flow-through tests, use less than 10 g of fish/L of test solution for tests at or below 17°C, or 5 g of fish/L at higher temperatures. For static testing, do not load >0.8 g/L at 17°C or less, and 0.5 g/L at >20°C.

b. Specific test procedures:

1) Freshwater fish

a) Equipment and physical conditions—Use test equipment made of glass, No. 316 stainless steel, or perfluorocarbon plastics.* Use unplasticized plastics (e.g., polyethylene, polypropylene, and polyvinyl chloride†) or silastic in the water-delivery system. In static and flow-through systems with low exchange rates, avoid certain types of TFE stoppers; pretest stoppers to ensure absence of toxicity.

Select temperature appropriate for the species being tested. Hold test temperature within ±2°C of mean test temperature during a 96-h test, or ±1°C during any 48 h. The photoperiod at the test site can be ambient laboratory lighting with a photoperiod of 16 h light/8 h dark. A 15- to 30-min dusk/dawn transition period is desirable to acclimate test fish to the photoperiod.[1,2]

Water used for dilution may be clean surface water, ground water, dechlorinated tap water, or reconstituted synthetic water.[3] If the source could be contaminated with pathogens, irradiate with ultraviolet (UV) light before using. Analyze water in control and assay chambers daily for pH, DO, and temperature. Maintain DO concentration at ≥60% saturation. When testing volatile substances, do not aerate test solutions. However, take care that chemical substances that create a DO demand do not result in conditions inconsistent with the test's DO criterion (or the fish's health). As a last resort to maintain DO above the criterion, use aeration. If aeration is used, it may be desirable to make frequent analytical measurements to confirm test chemical concentrations.

The length of a short-term test generally varies from 24 to 96 h. Longer test periods may be used, depending on waste characteristics and variability, and the purpose of the test. Dilution factors in the receiving stream provide guidance in determining effluent concentrations for range-finding tests.

For information pertaining to species selection, collection, holding, acclimation, disease control, and culturing, see Section 8010.

b) Test procedure

(1) Range-finding test—If the approximate toxicity of test material is unknown, conduct an abbreviated range-finding test to determine the concentrations that should be used in the definitive tests. Use three to five widely spaced toxicant concentrations (e.g., a decade test, with concentrations a factor of ten from each other). For these tests, static tests may be acceptable, as would using fewer fish (e.g., five per chamber). Run this test for 24 to 96 h.

(2) Definitive test—To determine LC_{50} or EC_{50}, use a 96-h test with a minimum of five toxicant concentrations and a control according to the results of the range-finding test. Use at least two replicate exposure chambers per concentration, including control; at least three replicates are preferred. Use a carrier control if dosing solutions are prepared in an organic solvent. Acceptable carriers are dimethylformamide, ethanol, methanol, acetone, dimethyl sulfoxide, and triethylene glycol. Limit carrier concentrations to 0.1 mL/L of test solution. Carrier controls should not result in increased mortality. If >10% of fish in a control system die, repeat the test. To establish definitive test concentrations, prepare solutions using a dilution ratio of 1.5 to 2 between successive concentrations.

2) Marine fish—The test procedure for marine species is essentially identical to that for freshwater fish. Additionally, determine salinity daily. Conduct toxicity tests requiring saline dilution water using natural or reconstituted seawater with a salinity appropriate to the test fish's requirements. Run stenohaline species in seawater with a salinity of 30 to 34‰ and euryhaline species at 10 to 25‰ (±1 to 2‰) salinity.

Be aware that effluent testing often requires exposing fish to 50 to 100% effluent. This exposure may create a salinity-induced stress on stenohaline organisms that could invalidate test results unless salinity is controlled by adding appropriate amounts of sea salt or saline brine (produced by evaporating natural seawater). Remember to include a control group that would have the same amount of sea salt or saline brine as the highest concentration of salt or brine used in the tested effluent.

2. Early-Life-Stage Toxicity Tests

a. General test procedures: Start fish early-life-stage toxicity tests with newly fertilized eggs and expose them through their developmental stages to an early juvenile age.[4] Testing procedures for the freshwater rainbow trout and marine/estuarine sheepshead minnow are discussed below. For fathead minnow, see Section 8921. Other species can be used if the fish's specific environmental requirements can be approximated in the laboratory.

Common to all these tests are such endpoints as time-to-hatch, survival during different life stages, and growth. Also observe behavior to determine behavioral effects of the test compound. Histological, physiological, morphological, or biochemical endpoints relevant to study objectives also can be measured.

1) Equipment and physical conditions—For a description of suitable diluter systems, see Section 8010F. Set up a minimum of two (at least three is preferred) replicate exposure chambers per test concentration. Construct egg hatching cups of glass tubing (8 cm diam × 10 cm long) with 40 mesh stainless steel or equivalent nylon‡ screen glued at one end with clear silicone sealant. Suspend egg cups from a rocker arm assembly and low-speed motor designed to oscillate the egg cups slowly up and down approximately 2 to 3 cm. Use a flow rate through the

* Teflon®, or equivalent.
† Tygon®, or equivalent.
‡ Nitex, or equivalent.

exposure chambers sufficient to replace 90% of water in 8 to 12 h. A self-starting siphon tube in each exposure chamber can be substituted for the rocker-arm system to ensure test-solution exchange.

Provide approximately 400 to 800 lux of light over the chambers and establish a 16-h-light/8-h-dark photoperiod. Preferably provide a 15- to 30-min dusk/dawn transition period.

2) Chemical data recording—Periodically measure DO, pH, conductivity, temperature, hardness, and alkalinity. Typically measure hardness, alkalinity, and conductivity at least weekly. Record temperature at least daily or continuously in one chamber that is centrally located in the row of exposure chambers. Measure DO and pH at least weekly in each exposure chamber containing surviving fish.

3) Verification of exposure concentrations—Before and during the test, verify exposure concentrations of the test chemical. This will confirm actual versus nominal concentrations.

Initially, analyze concentration in each replicate test chamber. If consistent concentrations are observed among replicates at each concentration, make subsequent measurements on composite samples from each replicate. However, make other checks of diluter function to ensure that it is operating properly.

It is not necessary that measured concentrations be within any specific percentage of nominal concentrations. Indeed, such characteristics as hydrolysis rate and volatility may make it impossible. It is important to maintain consistent concentrations during exposure and to confirm concentrations via chemical measurements. Pre-exposure monitoring will confirm that the diluter system is operating properly and that fish exposure may begin. Fluctuating exposure concentrations may indicate an improperly operating diluter system, while slowing concentration increases may indicate that system equilibration (e.g., volatility, adsorption to chamber surfaces, hydrolysis) has not been achieved.

Once concentrations have stabilized, start exposure. Measure exposure concentrations at least weekly until the test ends.

Dichotomous data have endpoints that fall within two categories, such as mortality (alive–dead) and hatch (hatched–not hatched). These data can be analyzed with 2 × 2 contingency tables, logit, probit, and the chi-square statistic. Continuous data have endpoints that can take any value within a range (e.g., weight, length). These data can be analyzed with the appropriate analysis of variance, followed by the appropriate multiple comparison test.[5]

b. Specific test procedures:

1) Rainbow trout—The rainbow trout early-life-stage test is conducted for 60 d post-hatch at 12 ± 2°C.

a) General considerations—Run the rainbow trout early-life-stage test beginning with newly fertilized embryos or eyed embryos. Commercial suppliers of eggs and sperm make it convenient to begin with male and female gametes and fertilize eggs in the laboratory. Study designs may expose gametes before, during, and/or immediately after fertilization. Selection may depend on available time because using newly fertilized eggs adds approximately 1 month to the test duration.

b) Equipment and physical condition—For a description of suitable diluter systems, see Section 8010F. Use an exposure system similar to that described in ¶ *a*1) above with the following changes. On bottom of the egg cup, use 16 mesh stainless steel or nylon screen§. Incubate under little or no light. When eggs hatch, provide approximately 400 to 800 lux of light over the chambers on a 16-h-light/8-h-dark photoperiod. Preferably include 15- to 30-min dusk/dawn transition. Hold at 12 ± 2°C.

c) Test initiation—The test begins with distribution of newly fertilized or eyed eggs to each incubation cup. Select number of embryos based on the desired discriminating power for the test and the number of replicates. At a minimum, apportion 60 embryos per experimental group among four incubation cups and place two cups in each of two replicate test chambers. At least three replicates of 30 embryos each is preferred.

d) Biological observations—Monitor survival by daily inspecting embryos or hatchlings. Record observations of fish behavior, noting normal and abnormal individuals along with characteristics of any abnormalities. Behavioral changes may include loss of equilibrium, hyperventilation, atypical feeding, and quiescence. Morphological changes may include the presence of edemas, vertebral column malformations, and asymmetry.

During embryo incubation, remove dead eggs to prevent fungal infections. Dead eggs can be distinguished from living eggs by their white color.

When hatching is more than 95% complete, count post-hatch alevins and release them to their respective growth chambers. For approximately 2 weeks following hatching, alevins will feed from their yolk sacs. When the first few alevins begin to swim up, feed fry with brine shrimp nauplii combined with a standard commercial fish food at least three times per day as needed.

Because hatching may occur over a 3- to 6-d period, use the time to obtain at least 95% hatch of control to establish the 60-d post-hatch growth period. Determine growth at 30 d post-hatch (midpoint of growth period) and 60 d post-hatch (end of test). Use a measurement method that least stresses the fish. The photographic method[6] has been used successfully to estimate weight and lengths when fish are not to be sacrificed.

e) Chemical data recording—See ¶ *a*2) above.

f) Verification of exposure concentrations—See ¶ *a*3) above.

2) Sheepshead minnow—The sheepshead minnow early-life-stage test is conducted for 35 d post-hatch at 25 ± 2°C.

a) Equipment and physical conditions—For a description of suitable diluter systems, see Section 8010F. Use an exposure system similar to that described in ¶ *a*1) above, except with seawater salinity of 10 to 20‰.

b) Test initiation—See 8910B.5*b* for egg collection. The test begins with distribution of newly fertilized eggs to each embryo cup. Select number of embryos based on the desired discriminating power for the test and the number of replicates. At a minimum, distribute 60 embryos per experimental group among four incubation cups and place two cups in each of two replicate test chambers. At least three replicates of 30 embryos each is preferred. Sheepshead minnows hatch in about 7 d at 25°C.[5,7] Release larvae from hatching cup to the test chambers and start feeding. Initially feed live brine shrimp nauplii, then shift to brine shrimp mixed with commercial food after 7 to 10 d.[7]

c) Biological observations—See ¶ *b*1)d) above.

§ Nitex, or equivalent.

d) Chemical data recording—In addition to the water quality factors described in ¶ *a*2) above, measure dilution water salinity at least weekly.

e) Verification of exposure concentrations—See ¶ *a*3) above.

3. Reproductive Toxicity Tests

a. General test procedures: Use newly spawned eggs, newly hatched larvae, juveniles, or sexually immature fish to start a test. The life stage selected depends on species, laboratory space and facilities, availability of life stages, and test objective.

Expose enough fish to each concentration of toxicant to ensure adequate numbers of each sex at maturity while avoiding stress due to crowding. Additional fish may be introduced to tanks with similar treatment to provide specimens for histological examination, residue analysis, or selected physiological measures of condition. The exact number of fish required depends on life stage at start of test and test species (see ¶ *b* below).

At start of test, measure total length and weight of all fish. Repeat measurement for any fish that die during the test. To prevent injury, anesthetize‖ large fish before handling. Larvae and small fish may be measured via a photographic method.[6] At the end of a test, record length, weight, and, if possible, sex and gonadal condition of each fish.

For viability and hatchability tests, incubate eggs from each spawning at an optimum temperature in control water. Count live and dead eggs, and remove dead eggs daily. Evaluate egg viability for all spawnings by incubating eggs until development clearly is observed [some defined stage of embryogenesis (e.g., eyeing) is reached]. Determine hatchability for all spawnings in all exposure chambers or from a predetermined number of spawnings if the tested species spawns continuously (many times per season). Count number of dead, deformed, and normal larvae hatched daily, using a dissecting microscope if necessary.

To evaluate larval growth and survival at each toxicant concentration, collect a uniform number of larvae (usually 20 to 50) at random from two or more successful hatches and place in chambers for that toxicant concentration. Determine length and number of larvae upon transfer to growth chambers, preferably via the photographic method. Determine total length of larvae at selected intervals and at end of test. Count, measure, and remove dead larvae daily.

For methods of toxicant mixing and delivery, see Section 8010F.1*c*.

Use spawning tanks, exposure tanks, and growth chambers appropriate for the test species. Design each growth chamber so test solutions can be drained down to 2.5 to 3 cm and the chamber transferred to a fluorescent light box provided with a millimeter grid for photographing fish.[6]

Monitor fish and embryos maintained for physiological, biochemical, and histological tests carefully. As a minimum, report all pertinent data for each test container at the beginning, about a third of the way through, and at end of test. Include number and weight of individuals, number of spawnings, number of embryos, and total lengths of normal, deformed, and injured mature and immature males and females. Count and record all survivors and mortalities. Calculate mean incubation time for median spawning and hatch dates if known. The hatchability, fry survival, growth, and percent deformities also may be determined.

Measure toxicant concentration in all tanks at each concentration weekly. Composites of equal-volume daily grab samples for 1 week may be used if it has been shown that analytical results for the test compound are unaffected by storage. Include samples for assessing recovery (i.e., known additions) and blanks. Analyze enough samples throughout the test to determine whether toxicant concentrations are constant. If this is not possible, analyze enough samples weekly to establish variability of toxicant concentration (see Section 8010F.3*d*).

Record temperature continuously in a centrally located tank. Measure oxygen levels periodically in each tank. Analyze water from the control and one exposure tank at least weekly for pH, hardness, alkalinity, and conductivity in freshwater systems, and pH and salinity in marine systems. If any characteristic is affected by the toxicant, analyze that characteristic at least 5 d/week, rotating among tanks so each is analyzed once every other week.

When possible, analyze mature fish and/or eggs, larvae, and juveniles for toxicant residues.

b. Specific test procedures: Procedures used for reproduction tests are described below for a fresh-water and a marine species.

1) Brook trout, *Salvelinus fontinalis* (fresh-water)—This test procedure only extends over part of the life cycle because of the longevity of brook trout. It follows the life cycle from the yearling stage through spawning, egg hatching, and development for 90 d.

a) Equipment and physical conditions—For a description of suitable diluter systems, see Section 8010F. Set up duplicate tanks for each test concentration and control. Premix each concentration before delivery to duplicate spawning tanks and growth chambers.

Construct 18 × 15 × 18 cm alevin-to-juvenile growth chambers using glass or stainless steel with a glass bottom. Maintain water depth at about 13 cm. Design each chamber so water can be drained down to 2 to 3 cm deep to allow the chamber to be placed over a millimeter grid on a fluorescent light box so analysts can photograph fish for length measurements.

Construct 80 × 30 × 40 cm spawning tanks of No. 316 stainless steel. Use a 30-cm water depth. Place a spawning substrate or nest[7,8] in spawning tanks at the appropriate time. The 28 × 33 × 7.5 cm spawning nest should be made of double-strength glass or stainless steel. Large fish may require a larger nest. Drill three 2.5-cm holes in each end, 2.5 cm from the bottom, and cover with 10-mesh stainless steel wire to allow water in the box to drain to 2.5 cm deep when the box is removed from the spawning chamber. Place a bottomless screen egg retainer (27 × 32 × 1.3 cm with 2.5-cm square compartments, constructed from 1.3-cm-wide strips of 7-mesh stainless steel screen) in the spawning box. Place 27 × 32 cm 2-mesh stainless steel screen, to which 1.3- to 2.5-cm gravel is attached with silicone adhesive, on top of the screen egg retainer.[8] Use smooth gravel to prevent injury to active, spawning fish. This spawning box is readily removed from the spawning tanks to collect eggs for transfer to incubation cups. For spawning stocks, select yearling fish that will not grow too large for the spawning box. Fish weighing ≤50 to 70 g at time of selection and 150 g at

‖ 3-aminobenzoic acid ethyl ester, or equivalent.

spawning are appropriate. If fish weigh >150 g, use a larger spawning box.

Provide a daylight period conducive to the spawning of brook trout. Ideally use 15- to 30-min dawn/dusk transition times and provide conditions described above in 8910C.1.

Provide water flow of 6 to 10 tank volumes/d to maintain oxygen levels >60% saturation. Remove uneaten food and wastes from growth chambers daily. Brush interior surfaces to remove attached growths as needed.

b) Exposure procedures—To begin the test, collect juveniles from the field no later than March 1 and acclimate for at least 1 month, or use cultured stock of equivalent age. Judge fish's suitability for testing on the basis of acceptance of food, apparent lack of disease, <2% mortality during acclimation, and no mortality during the 2 weeks before the test.

Begin exposure by placing at least 12 acclimated yearling brook trout in each replicate tank at each test concentration and suitable controls using a stratified random assignment (see Section 8010F.3*a*). This allows about a 4-month exposure to toxicant before the onset of secondary or rapid-growth phase of the gonads. Extra test animals may be included at the beginning so fish can be removed periodically for special examination or chemical analysis.

Use a particulate or pelleted trout food. Feed fish the largest particle or pellet they will take, at least twice daily. Base amount on a reliable hatchery feeding schedule.[9] Analyze each batch of food for pesticides.

Record mortalities daily and measure total length and weight of fish directly at initiation of tests and every 3 months thereafter. Stop feeding fish 24 h before weighing. Lightly anaesthetize them to facilitate measuring.

When secondary sexual characteristics are well-developed (approximately 2 weeks before spawning), separate males, females, and undeveloped fish in each tank and randomly reduce number of sexually mature fish to two males and four females per tank. Record number of mature, immature, deformed, and injured males and females in each tank, and number from each category to be discarded. Thoroughly clean, sterilize, and rinse the spawning substrates and place one for each male in each spawning tank. As soon as spawning begins, set up incubation cups [as described in 8910B.5*a*1)] or a suitable alternate system to receive embryos for hatching. Remove embryos from the substrate at a fixed time each day, preferably so fish are not disturbed during early part of the light period.

Randomly select 50 embryos from the first eight spawnings of 50 embryos or more in each replicate spawning chamber and place in an embryo incubator cup. Count remaining embryos from the first eight spawnings and all embryos from subsequent spawnings and place them in separate incubator cups to determine viability, as evidenced by development to a specific stage (e.g., formation of neural keel after 11 to 12 d at 9°C or eyeing). Remove and record number of dead embryos from each spawn. Never place >250 embryos in one incubator cup. Incubate all embryos to determine viability and discard after reaching some clearly distinguishable stage (development of neural keel or eyeing). Discarded embryos may be analyzed chemically or used for other measurements.

Obtain more information on hatchability and alevin survival by transferring embryos from control tanks immediately after spawning (*a*) to tanks with test concentrations where spawning is reduced or absent and (*b*) to tanks where an effect is seen on survival of embryos or alevins, and by transferring embryos from those test concentrations to control tanks. Always reserve two growth chambers in each replicate spawning tank for embryos produced in that tank.

Remove dead embryos daily from incubator cups. When hatching begins, record number of alevins hatching daily in each cup. On completion of hatching in any cup, transfer fish to a culture dish and randomly sample 25 alevins. Count dead or deformed alevins. Transfer 25 selected alevins to a growth chamber and place it over the light box to measure via the photographic method. After photographing, return alevins to incubator cup. Never net alevins, but transfer via gentle pouring or large-bore pipets. Transport in growth chambers containing enough test solution to limit harsh contact with bottom screening. Preserve unused alevins in formalin for subsequent histological examination. Record length and weight of discarded alevins separately from the data for fish kept for continued exposure.

For 90-d growth and survival exposures, randomly select 20 alevins from each replicate incubator cup for each test concentration and control. Because embryos from one spawn may hatch over a 3- to 6-d period, use the median hatch date to establish the start time of the 90-d growth and survival period. For growth tests, select a minimum of two (at least three preferred) groups of 20 alevins that are <3 weeks apart in age. Use any remaining groups only for hatchability testing. After photographing to determine length, preserve for weight determination. To equalize effects of incubator cups on growth, keep all groups selected for 90-d exposure in the incubator cups for 3 weeks after the median hatch date, then release into growth chambers. Begin feeding immediately. Keep the two (at least three preferred) groups from the same exposure chambers separate for replication of each test concentration. Record mortalities daily, total lengths at 30 to 60 d after hatching (via the photographic method), and total length and weight at 90 d after hatching. At the end of the test, cease feeding juveniles for 24 h and then weigh. Terminate survival and growth studies after 3 months, at which time fish may be used for chemical analysis of tissue and physiological measurements of toxicant-related effects.

End exposure of all parental fish after 3 weeks in which no spawning occurs in any tank. Record mortality and weight, measure total length of parental fish, and check sex and condition of gonads (e.g., reabsorption, degree of maturation, spent ovaries).

For each tank in a partial-life-cycle test, record number and individual weights and total lengths of immature males and females at initiation of test, after 3 months, at reduction in numbers, and at end of test. Report individual weights and total lengths of normal, deformed, and injured fish, number maturing, number dying during test, number of spawnings and eggs, hatchability and fry survival, growth, and deformities. Calculate a mean incubation time based on spawning date and median hatch dates.

For more information on the life cycle of brook trout, consult other sources.[8–17]

2) Fathead minnow, *Pimephales promelas* (fresh-water)—See Section 8921.

3) Reproduction tests with other fresh-water species—Partial-life-cycle toxicity tests have been performed with the bluegill,

Lepomis macrochirus, *Oryzias latipes*, and the flagfish, *Jordanella floridae*.[18–23] When culture techniques are available, other native fresh-water species may be used as appropriate.

4) Sheepshead minnow, *Cyprinodon variegatus* (euryhaline)

a) Equipment and physical system—Use test apparatus similar to that used for fresh-water fish, with spawning aquariums at least 30 × 18 × 20 cm. Place a spawning chamber in each aquarium, as described in 8910B.5*b*.

b) Exposure procedures—Begin with adult fish or preferably with embryos. Secure embryos via either natural or hormone-induced spawning (as described in 8910B.5*b*). Keep water temperature >22°C, preferably at 30°C, with salinities >15 mg/kg. When starting with adults, set up five or six spawning aquariums for each test concentration and controls. Use breeding fish all from the same stock, which have been kept in holding tanks for at least 2 weeks, during which <2% mortality occurred. Feed fish a combination of frozen adult brine shrimp and dry trout food. Maintain water flow through spawning aquariums at 6 to 10 tank volume/d. Use natural seawater filtered to remove planktonic larvae 15 μm and larger.

When embryos are produced, remove them from spawning chambers and evenly distribute to a minimum of 2 (at least 3 preferred) replicate hatching chambers for each concentration being tested, as well as the controls. Start toxicant dosing in exposure chambers before the hatching chambers are placed inside them. Construct hatching chambers by cementing a 9-cm-wide strip of 500-μm nylon screen around a Petri dish. Place the hatching chambers in 90- × 30- × 30-cm exposure chambers in 7 cm of water with flow-through of the toxicant. As embryos hatch, feed fry with newly hatched brine shrimp nauplii. Clean screens on incubation cups and chambers daily. Check and record daily survival of embryos and fry, which constitute the first filial (F_1) generation.

On the first day after hatching, remove each chamber and count and measure fry photographically. During the first 2 weeks, feed with newly hatched brine shrimp nauplii. During the following 2 weeks, supplement this diet with dry trout pellets or dry mollie flakes. After 4 weeks, count and measure fish via the photographic method and reduce the number to 25 per replicate for each test concentration and control. Record length, weight, condition, and number of living, deformed, and dead fish remaining. Determine percent mortality and abnormality in each test concentration and control. Preserve specimens for future tests or discard. Place the 25 selected fish in each of 2 replicate (at least 3 preferred) growth chambers with a glass bottom and provisions for drawing the water level down to 1 to 2 cm, preserving the replication from the hatching chambers. Feed a mixed diet of brine shrimp and dry food twice daily and examine daily for dead specimens. At 8 weeks, measure again via the photographic method. Twice daily, feed dry food supplemented with frozen adult brine shrimp until maturity. Check each batch of food for pesticides, PCBs, or other contaminants of concern. Clean all exposure aquariums and spawning and hatching chambers two to three times per week. Siphon out all wastes.

As fish approach sexual maturity, place separate pairs in spawning chambers, five pairs from each replicate exposure chamber [i.e., 10 pairs (minimum) for each test concentration and controls] and continue exposure. Count, measure, and weigh all unused fish from each replicate exposure chamber. Record number deformed and dead in each test concentration and controls, condition of fish, and other pertinent data. Preserve some fish for whole-body tissue residue analysis. As fertilized eggs are produced, remove at a specified time daily, count, and place 25 F_2 fish in a hatching chamber (as for the F_1 generation). Record the total number of embryos produced in each chamber, time required to hatch, hatching success, and survival of embryos. Test those not placed in hatching chambers for fertility and record percent of fertile females.

Keep pairs in each spawning chamber until all needed embryos have been obtained. At termination, measure and weigh spawning pairs and record all other pertinent data. Preserve for toxicant analyses, if desired.

Expose embryos of F_2 generation in hatching chambers in their respective replicate exposure chambers for each test concentration and control as before. Count and measure via photographic method,[6] as for the F_1 generation. Feed fish and record results. At the end of 4 weeks, terminate the test. Weigh and measure all fish; record number of deformed fish and determine number that died. Preserve for histological examination and tissue analyses. Determine effects of each test concentration and calculate safe levels. During tests, record temperature daily, and oxygen concentration, pH, and salinity at least weekly. If possible, chemically analyze test water for toxicant at the beginning, at regular intervals (e.g., weekly) during exposure, and on completion of tests. Analyze lots of 10 fish from highest and lowest exposure concentrations and from controls for toxicant accumulation. Analyze dilution water for toxicant at beginning and end of test.

For more information about the life cycle of *Cyprinodon variegatus* and testing procedures, consult other sources.[22–26]

5) Life-cycle tests with other marine fishes—Life-cycle tests may be performed with other marine fishes, such as *Fundulus heteroclitus* and *Menidia menidia*. For information on life cycle and culture of these species, consult other sources.[27–29]

4. Statistical Analysis

Analyze, handle, and report data as in Section 8010G.

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8921 FATHEAD MINNOW*

8921 A. Introduction

The fathead minnow (*Pimephales promelas* rafinesque) is a small, common, and widely distributed freshwater fish of the family Cyprinidae. This minnow is maintained easily in the laboratory and can be spawned year-round. These attributes have led to its widespread use in aquatic toxicology studies, particularly those using early life stages (i.e., embryos and larvae), such as the short-term tests for measuring the chronic toxicity of effluents.

Other procedures for testing larval growth of fathead minnows are available.[1]

1. Description

Adult fathead minnows typically range in size from 43 to 102 mm, averaging about 51 mm (2 in.) in total length.[1,2] Young and nonbreeding adults are light in color with a distinct lateral band from caudal peduncle to head; males and females at this stage are difficult to differentiate, except that males are typically larger. In breeding condition (Figure 8921:1), males are distinguished from females by the presence of nuptial tubercles on the snout and by coloration. Mature males are dark in overall coloration with a saddle-like pattern behind the head, whereas females are quite drab.

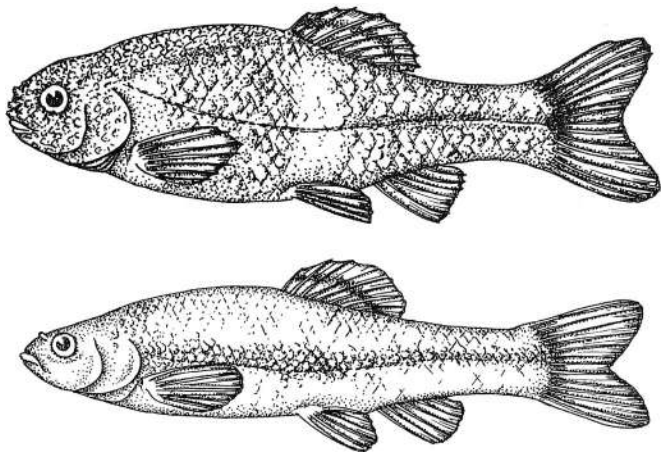


Figure 8921:1. Adult fathead minnows in breeding condition: (above) male; (below) female.

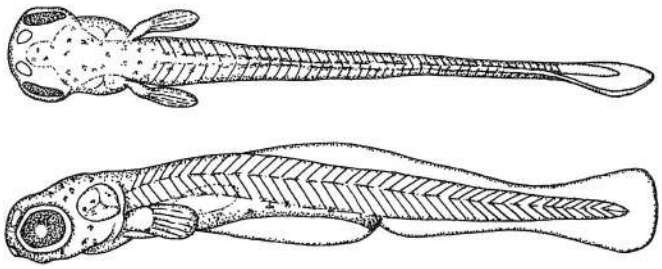


Figure 8921:2. Newly hatched fathead minnow larvae: (above) top view; (below) lateral view.

2. Distribution, Biology, and Life History

The fathead minnow tolerates adverse conditions, including high temperature, turbidity, and low oxygen concentrations,[2] so it is found in diverse habitats and is widely distributed throughout central North America from Canada to northern Mexico.[2,3] It is most abundant in muddy streams, brooks, ponds, and small lakes.[2] This species is a popular bait fish and has been introduced to areas both within and outside of its native range because of the relative ease with which it is maintained and propagated.

The fathead minnow rarely lives beyond 2 years old. In warm, food-rich waters, it grows rapidly and may reach adult size and begin spawning in as little as 3 months. In cold waters, it may take a year to reach maturity.[3] It is omnivorous, with a diet consisting at times of algae, organic detritus, aquatic insects, worms, small crustaceans, and planktonic organisms. Because it is highly prolific, can use many foods, and is widely preyed upon by other fish and fish-eating birds, the fathead minnow is considered an ideal forage fish and important bait species.

In the wild, fathead minnows begin spawning in the spring and often continue to spawn throughout the summer. Spawning typically begins when the water temperature reaches about 16 to 18°C, although this temperature may vary with the population and latitude.[2] Spawning usually occurs in the early morning in shallow water less than 1 m deep. The male selects a suitable substrate (e.g., underside of a log, branch, root, large rock, board) and herds a receptive female into position. Using her ovipositor, the female deposits her adhesive eggs (from 100 to 500 per spawn) on the substrate and is then driven off by the territorial male. The male aggressively guards the nest and often seeks out additional females to spawn in the nest. Time to egg hatching depends on water temperature. For example, eggs hatch in about 1 week at 22°C and between 4 and 5 d at 25°C. Newly hatched larvae are approximately 5 mm long, are opaque white in color, and have large black eyes (Figure 8921:2).[2,4]

3. References

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* Approved by Standard Methods Committee, 2016.
Joint Task Group: Mary Ann Rempel-Hester (chair), Catherine A. Curran.

2. U.S. Environmental Protection Agency. 2002. Appendix A, Distribution, life cycle, taxonomy, and culture methods A.5. (Fathead minnow *Pimephales promelas*). *In* Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, 5th ed.; EPA-821-R-02-012. Off. Water, Washington, DC.
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4. Bibliography

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8921 B. Culture and Maintenance of Test Organisms

1. Obtaining Test Organisms

Organisms of various ages may be obtained for toxicity testing from commercial breeders, biological supply houses, or an in-house culture facility. An in-house breeding facility is recommended, and may be required if testing is to be conducted with early life stages or fish of a specific age. Ensure that fish, particularly those from outside sources, are certified as to their identity, age, and freedom from disease. Preferably use sources that supply reference toxicant data with their shipments. In general, except as sources of "new" genes, avoid using organisms from bait shops, hatcheries, or field populations because their suitability for use and disease status cannot be ensured.

2. Culturing and Care of Test Organisms

Fathead minnows (all life stages from egg to adult) may be successfully cultured in the laboratory using static, recirculating, or flow-through systems. Basic information on establishing and maintaining a culture facility is presented below. For more information, see Sections 8010E.4 and 8910B, and other sources.[1–3]

a. Water supply and culture system: Supply cultures with good-quality water; reconstituted (synthetic) water, dechlorinated municipal water, and natural water (see Section 8010E.4*b*) are all acceptable. Natural water is preferred so long as its quality is relatively constant and it meets minimum acceptability criteria. Reconstituted water usually is recommended to be of moderate hardness (see Table 8010:I). Analyze all water supplies periodically for chlorine (free and combined), ammonia, toxic metals (e.g., Cr, Cd, Cu, Pb), organic compounds [e.g., pesticides, polychlorinated biphenyls (PCBs)], and basic water quality factors [e.g., pH, dissolved oxygen (DO), conductivity, hardness, alkalinity].

Choose a spawning unit designed to simulate natural spawning conditions (e.g., light, temperature). Typically, fish are bred in small (60- to 120-L) tanks or aquaria. Flow-through culture systems are recommended; however, static or recirculating systems may be used if adequate water quality is maintained via activated carbon filtration and/or other treatments. See Section 8010E.4*a* for a discussion of appropriate construction materials and equipment. For spawning, hold water temperature at 25 ± 2°C. Aerate water as necessary to maintain DO concentration at or near saturation. Establish a controlled photoperiod of 16 h light (e.g., 5:00 a.m. to 9:00 p.m.) and 8 h dark.

b. Establishment of breeding: Establish breeding units with 15 to 20 mature (>6-month-old) adults and two or three spawning substrates per tank. Construct spawning substrates from inverted halves (semicircular sections) of Schedule 40 polyvinyl chloride (PVC) pipe [7.5 cm inner diameter (ID) × 7.5 cm length] or other nontoxic material (e.g., glass, stainless steel). If the inverted surface is smooth, roughen it to aid embryo adhesion. At first it may be difficult to differentiate the sex of fish, but this process becomes easier over the next week as organisms develop their secondary sexual characteristics (Figure 8921:1). Remove excess males to maintain a sex ratio of approximately six females per male and no more than two males per tank.[1,2] As an alternative, pair females and males in divided tanks.

c. Embryo collection and incubation: Check spawning substrates daily in the late morning or early afternoon. Record the number of eggs per substrate and incubate embryos using one of the techniques described below.[1,2] Examine embryos daily and remove all that are dead (milky and opaque) or show fungal growth. Embryos maintained at 22 to 25°C will hatch in about 4 to 7 d.

1) Incubation of embryos on substrate—Place several substrates on end in a circular pattern (embryos on the inside) around a source of gentle aeration. Maintain a constant temperature and sufficient water depth to cover substrates.

2) Incubation of embryos in a separatory funnel—Remove embryos from substrates with a gentle rolling action of the index finger.[4] Incubate embryos in a 2-L separatory funnel containing approximately 1.5 L water. Hold separatory funnel in a constant-temperature bath and maintain constant gentle aeration from bottom of funnel.

3) Incubation of embryos in incubation cups—Remove embryos from substrates with a gentle rolling action of the index finger.[4] Place embryos in incubation cups attached to a rocker-arm assembly[5] that maintains constant water movement over the embryos.

d. Rearing of larvae and juveniles: Each day, transfer newly hatched larvae to small (10- to 60-L) rearing tanks. Use large-bore pipets or other methods that do not require direct handling of larvae. Do not use nets until fish are approximately 30 d old. Establish initial density at or below 150 larvae/L. Reduce density proportionally as fish grow larger by thinning fish or moving them to larger tanks. Hold juveniles at a density of ≤1 fish/L, typically in tanks of 200 L or more. Maintain aeration and a relatively constant temper-

ature (20 to 25°C). Keep tanks holding replacement spawners (brood stock) at or near spawning temperature (25 ± 2°C).

e. Food and feeding: Feed larvae up to 30 d old two to three times a day with newly hatched brine shrimp (*Artemia salina*). Culturing of brine shrimp is described elsewhere.[6] Supplement live food once or twice a day with commercial fish starter food. Feed older fish with frozen adult brine shrimp and commercial fish starter or tropical fish flake food.[1,2] Fish may be fed *ad libitum* but avoid overfeeding because it increases tank maintenance, decreases water quality, and may increase stress and susceptibility to disease.

f. Parasite and disease control: Observe fish daily for disease and abnormal behavior. Parasites and disease will rarely be a problem if proper water quality and aeration are maintained in rearing tanks. If necessary, provide treatment as described in Section 8910B.4. Clean and disinfect tanks and related equipment regularly and, in particular, before new fish are added or after any disease outbreaks. Avoid spread of disease by disinfecting dip nets before use.

3. Acclimating and Holding Test Organisms

When possible, quarantine and acclimate (preferably for at least 48 h) test organisms obtained from outside sources; however, no acclimation is necessary for tests initiated with fish early-life stages. During acclimation, protect fish from large changes in temperature or water quality (e.g., pH, hardness) and minimize handling. Avoid overcrowding and maintain sufficient DO concentrations. During acclimation, change water from 100% holding water to 100% dilution water. Keep all organisms in 100% dilution water for at least 48 h before use.

Observe fish daily for signs of stress and disease; remove dead or abnormal organisms promptly. Mortality of 5 to 10% is not unusual during the first 48 h of acclimation; however, do not use organisms in tests if mortality exceeds 5% in the 48-h period preceding test initiation. See Sections 8010E.3 and 8910B.3 for more information.

4. References

1. U.S. Environmental Protection Agency. 2002. Appendix A, Distribution, life cycle, taxonomy, and culture methods A.5. (Fathead minnow *Pimephales promelas*). *In* Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, 5th ed.; EPA-821-R-02-012. Off. Water, Washington, DC.
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6. U.S. Environmental Protection Agency. Appendix A, Distribution, life cycle, taxonomy, and culture methods A.4. (Brine shrimp *Artemia salina*). *In* Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, 5th ed.; EPA-821-R-02-012. Off. Water, Washington D.C.

8921 C. Toxicity Test Procedures

1. Short-Term (Acute) Test

Test procedures and conditions common to short-term tests on fathead minnows are summarized in Table 8921:I; additional procedures and conditions for specific short-term tests are shown in Table 8921:II.

a. Scope and application: Short-term tests are conducted with the fathead minnow to determine the toxicity of pure compounds, formulations and mixtures, effluents, and receiving waters. Test populations are usually mixed sex. Mortality is the primary test endpoint (see Table 8921:II); other endpoints, such as loss of equilibrium, should be noted and a median effect concentration (EC_{50}) value determined. Test results may be used to compare toxicity among chemicals to determine sensitivity of different species, for regulatory purposes, or for ecological risk assessments.

b. General test procedures: Short-term (acute) test procedures applicable to the fathead minnow are described in several sources.[1–5] For test duration and types, see Table 8921:II. Choose type based on availability of test compound, mixture, or effluent; toxicant characteristics, such as volatility and solubility; and age/size of minnows used for testing (see Sections 8010D.1 and 2). Early life stages (i.e., larvae) or juveniles are preferred over adults for use as test organisms because they typically are more sensitive and require less test solution. Selection of larvae or juveniles depends on test purpose, availability, and laboratory facilities (see Section 8910B.1). Testing of effluents and receiving waters requires use of early life stages[1] (see 8921B.2).

Initially determine the test material's approximate toxicity in a range-finding test of 24 to 96 h. Use three to five widely spaced concentrations (e.g., dilution factor of 10) and, optionally, fewer organisms per concentration than normally recommended for definitive tests. Use results of the range-finding test to determine appropriate concentrations for use in the subsequent definitive test.

c. Specific test procedures:

1) Equipment and physical conditions—Always use materials that minimize sorption and leaching of toxic substances, such as tempered glass, perfluorocarbon plastics,* and No. 316 stainless steel (see Section 8010F.1). Clean and rinse all equipment, including new glassware, before use (see Section 8010E.4*d*). Flow-through tests may require use of a diluter system (see Section 8010F.1*c*) or continuous-flow, low-volume (e.g., peristaltic) pumps. Conduct testing in well-ventilated, temperature-controlled facility. Maintain test temperature and light

* Teflon®, or equivalent.

Table 8921:I. Test Conditions Common to Various Fathead Minnow Short-Term Tests

Test Condition	Type or Value
Light quality and intensity	Ambient laboratory levels; 550–1050 lux (50–100 ft-c)
pH	6.0–9.0 (if outside this range, adjust to pH 7.0 and perform a parallel test without pH adjustment—see text)
Dilution water	High-quality fresh water. May consist of natural water, receiving water, moderately hard reconstituted fresh water, or dechlorinated municipal water [see Section 8010E.4*b*1) and 8010F.2*a*]
Test concentrations	≥5 plus a control; factor of ≥0.5 between concentrations preferred
Carrier solvent	When testing low-solubility compounds, use ≤0.1 mL/L of a suitable solvent (acetone, dimethylformamide, ethanol, methanol, isopropanol, acetonitrile, or ethylene glycol) (see Section 8010F.2*b*)
Test solution aeration	Not needed unless DO concentration drops below 4.0 mg/L; avoid supersaturation

conditions as directed in Tables 8921:I and II. Cover test vessels with clear plastic or glass allowing air circulation.

For dilution water requirements, see Table 8921:I. Prepare test solutions as described in Section 8010F.2*b*. Use a dilution factor of ≥0.5 to determine test concentrations in the definitive test. To test compounds of low solubility, follow carrier-solvent recommendation in Table 8921:I and use a solvent control. Analyze water in control and test chambers daily for pH, DO, and temperature. For renewal tests, make these measurements in new solutions and before renewing solutions, to see the range of conditions. Avoid aeration, particularly for volatile compounds, except to maintain DO concentrations at a minimum of 4 mg/L.

Outside pH range 6.0 to 9.0, the toxicity of metals and organics may be masked by the toxic effects of low or high pH. In such cases, preferably conduct two parallel tests: one with the pH adjusted to 7.0 and one without an adjusted pH. Adjust sample pH by adding 1*N* NaOH or 1*N* HCl dropwise, as required, being careful to avoid overadjustment.

2) Test initiation—Obtain suitable fathead minnow larvae (1 to 14 d old) or juveniles (30 to 60 d old) from an in-house culture or outside supplier. Larvae are often the most sensitive fish life stage and usually are required for compliance testing of effluents.[1] Distribute test organisms randomly among replicate test chambers containing test solutions. Use at least two replicates with 10 organisms each. If sufficient numbers of test organisms are available, preferably test 80 to 100 organisms at each concentration (preferably four replicates each with 20 to 25 organisms). For static and static-renewal tests, use live weight loading in the test solutions <0.65 g/L (20°C) or <0.40 g/L (25°C). For flow-through tests, use live weight loading <5.0 g/L (20°C) or <2.5 g/L (25°C).

3) Solution renewal—Unless the supply of test material or effluent is limited, renew test solutions daily or every other day. Periodic renewal is especially important in tests of volatile compounds, chemicals with low solubilities, and compounds that degrade rapidly.

4) Feeding—See Table 8921:II. Avoid overfeeding because it may reduce toxicant concentrations and DO levels. Culturing *Artemia* is discussed elsewhere.[1,6]

5) Biological data and observations—Monitor and record mortality daily starting approximately 24 h after test initiation; remove dead organisms. Criteria for establishing death include lack of movement and no reaction to gentle prodding. Record general observations of fish appearance (coloration, deformities) and behavior (lethargy, lack of schooling, loss of equilibrium).

6) Chemical data recording—Measure hardness and alkalinity at test initiation and termination in the highest concentration of test solution and in the dilution water. Measure pH, DO, conductivity, and temperature at test initiation and daily thereafter in all test concentrations. In static-renewal tests, take measurements in both freshly prepared and 24-h-old solutions.

7) Verification of exposures—If resources are available for analyses, verify exposures by measuring test-chemical concentrations in exposure solutions at test initiation and termination. Base statistical analyses and results [e.g., LC_{50}, no-observed-effect concentration (NOEC)] on measured concentrations rather than nominal concentrations.

8) Test termination—End test after 24, 48, or 96 h. Before termination, record number of dead and abnormal fish in each test chamber.

2. Short-Term Methods for Estimating Chronic Toxicity

Several test methods are available for estimating the long-term (chronic) effects of a toxicant or effluent after a relatively short (7-d) exposure period (see Section 8010D.5). Two of these tests—the larval survival and growth test and the embryo–larval survival and teratogenicity test—use fathead minnows as test organisms and are described below. These short-term tests were developed as cost-effective alternatives to long-term early-life-stage and life-cycle tests. They were designed primarily to evaluate effluent toxicity[7–10] and are often included as biomonitoring requirements in discharge permits. They also have been used successfully to estimate the potential chronic toxicity of pure compounds.[9,11–13] In addition, because embryos and larvae often are the most sensitive stages in a fish's life cycle,[14,15] studies with embryo–larval stages have been used to investigate teratogenesis and identify developmental toxicants.[16–19]

a. Larval survival and growth test: This method estimates the chronic toxicity of effluents and chemicals using newly hatched fathead minnow larvae in a 7-d test. Test results are based on larvae survival and weight. Test conditions and procedures are given in Tables 8921:I and II. For testing effluents and receiving waters, see available literature.[7]

1) Equipment and physical conditions—For effluents and most chemicals, static-renewal exposures are typically used. Other types of exposure (e.g., static, flow-through) may be used if test-material supplies are limited or if test compounds are volatile or degrade rapidly. Suitable test chambers include 500-mL or 1-L beakers made of borosilicate glass or nontoxic disposable plastic. For other requirements, see 8921C.1*c*.

2) Test initiation—Obtain organisms and set up test chambers according to the requirements of Table 8921:II. Begin tests with effluents as soon as possible, preferably within 24 h of sample

TABLE 8921:II. TEST CONDITIONS SPECIFIC TO VARIOUS FATHEAD MINNOW SHORT-TERM TESTS

Test Condition	Acute Test	Survival and Growth Test	Embryo-Larval Survival and Teratogenicity Test
Test type	Static, static-renewal, recirculating, flow-through	Static-renewal	Static-renewal
Duration	24, 48, or 96 h (typically)	7 d	7 or 8 d
Temperature	20 ± 1°C or 25 ± 1°C	25 ± 1°C	25 ± 1°C
Test chamber size	≥250 mL	≥500 mL	≥150 mL; ≥250 mL (preferred)
Test solution volume	≥200 mL	≥250 mL	≥70 mL; ≥200 mL (preferred)
Test solution renewal	After 48 h (minimum); after 24 h (preferred)	Daily	Daily
Age of test organisms			
Effluents and receiving waters:	1–14 d (post hatching), ≤24-h range in age	<24-h-old larvae (post hatch); if larvae are not obtained from in-house cultures they should be <48 h old (<24-h range in age)	≤36-h-old embryos, ≤24-h range in age (maximum of 48-h if shipped)
Pure compounds and mixtures:	1–14 d (post hatching), ≤24-h range in age or 30–60 d (post hatching); ≤24-h range in age		
Organisms per test chamber	≥10; 20–25 (preferred)	≥10; 15–25 (preferred)	≥10; 15–25 (preferred)
Replicate chambers per concentrations	≥2; 4 (preferred)	≥3; 4 (preferred)	≥3; 4 (preferred)
Organisms per concentration	≥20; 80–100 (preferred)	≥30; 60–100 (preferred)	≥30; 60–100 (preferred)
Feeding			
Larvae (<24 h–14 d old at test initiation):	0.2 mL *Artemia* (brine shrimp) nauplii concentrate before test initiation and 2 h before test-solution renewal at 48 h	0.1 g newly hatched *Artemia* nauplii (<24 h old) three times daily at 4-h intervals, or at least 0.15 g twice daily with 6 h between feedings. No feeding during final 12 h.	Not required
Juveniles (30–60 d old at test initiation)	Feed before test initiation; do not feed during test.	N/A	N/A
Cleaning of test chambers	As required. Generally not required if test is conducted flow-through or solutions are renewed after 24 or 48 h.	Siphon daily, immediately before test solution renewal	Not required
Test endpoints	Mortality (normally LC_{50} and NOEC), behavior (activity, swimming, buoyancy, feeding, etc.)	Mortality and growth (weight) or biomass	Mortality and teratogenicity (deformed larvae)
Test acceptability	Mortality of control organisms ≤10%	Mortality of control organisms ≤20%; average dry weight per surviving organism in control chambers of ≥0.25 mg	Mortality of control organisms ≤20%

collection. From a pool of larvae, randomly select and distribute one or two larvae at a time until each test chamber contains a minimum of 10, but preferably 15, larvae. Use a large-bore pipet or similar device to transfer larvae; do not use a dip net. During transfer, avoid adding excess water to test chambers because this will dilute exposure concentrations.

3) Solution renewal—Unless the supply of test material or effluent is limited or flow-through procedures are used, renew test solutions daily. Before renewal, remove uneaten and dead *Artemia*, dead larvae, and other debris by siphon. Take care not to accidentally remove or injure larvae. Using a light box will enhance larval visibility and simplify this task. Add new test solutions to test chambers after removing approximately 80 to 90% of old solutions. Add solutions slowly down the side of the test chamber to avoid injuring larvae.

4) Feeding—See Table 8921:II. Rinse *Artemia* with fresh water before feeding. Avoid overfeeding because this may reduce toxicant concentrations and DO levels. Do not feed during final 12 h of test. Refer to the literature for information on culturing *Artemia*.[1,6]

5) Biological data and observations—See 8921C.1*c*5).

6) Chemical data recording—See 8921C.1*c*6).

7) Verification of exposures—See 8921C.1*c*7).

8) Test termination—Terminate test after 7 d. Before termination, record number of dead and abnormal fish in each test chamber (replicate). Prepare fish in each replicate for dry-weight

determination. If necessary, preserve larvae in 70% ethanol or 4% formalin for up to 7 d before drying and weighing. Rinse each group of larvae with deionized water, transfer to a labeled and tared weighing boat, and dry at 60°C for 24 h (or at 100°C for 6 h). Let cool in a desiccator, weigh to nearest 0.01 mg, and record.

9) Endpoints—Mortality, growth, and/or biomass can be used as endpoints for the chronic test. For mortality, divide the number of dead organisms per replicate at test termination by the number per replicate at test initiation to determine the proportion dead. Remove from analysis any fish that were killed accidentally during the test.

For growth, determine mean individual dry weight per surviving fish in each replicate to the nearest 0.001 mg.

Biomass is a combined endpoint that includes both survival and growth effects, and is potentially more sensitive than the standard growth endpoint. For each replicate, divide the dry weight by the number of larvae at test initiation and report to the nearest 0.001 mg. If all fish in a replicate died, the biomass equals zero. Remove from analysis any fish that were killed accidentally during the test.

10) Test acceptability criteria—The test is considered acceptable if control survival is ≥80% and average dry weight per surviving fish larvae in the control replicates is ≥0.25 mg.

11) Statistical analysis—Assemble, analyze, evaluate, and report data as described in Section 8010G and other references.[1,7] Calculate LC_{50} by a point estimation technique, such as regression analysis. Obtain lowest-observed-effect concentration (LOEC) and NOEC values for survival and growth and/or biomass by using hypothesis-testing techniques, such as Dunnett's Test or Steel's Many-one Rank Test.

b. Embryo–larval survival and teratogenicity test: This method estimates the chronic toxicity of effluents and chemicals by exposing fathead minnow embryo–larval stages in a 7- or 8-d test. Tests are initiated with recently fertilized embryos. Exposure is continued for several days after larvae hatch (approximately 4 to 5 d after fertilization at 25°C), depending on age of embryos at test initiation. Test results are based on total frequency of both mortality and gross morphological deformities (terata). Examples of abnormally developed fathead minnow larvae are shown in Figure 8921:3. The test is useful in screening for teratogens (agents that produce terata) because organisms are exposed during early embryonic development, when they are most susceptible. General test conditions and procedures are given in Tables 8921:I and II. For testing effluents and receiving waters, see available literature.[7]

1) Equipment and physical conditions—For effluents and most chemicals, static-renewal exposures are typically used. Other types of exposure (e.g., static, flow-through) may be used if test-material supplies are limited or if test compounds are volatile or degrade rapidly. Suitable test chambers include 250- or 500-mL beakers made of borosilicate glass or nontoxic disposable plastic. Chambers as small as 150 mL may be used when small solution volumes are used. For other requirements, see 8921C.1*c*.

2) Test initiation—Use embryos, preferably <36 h old, to initiate tests. Obtain embryos from an in-house culture (see 8921B.1) or commercial supplier. Remove embryos from spawning substrates within 12 h of spawning. If organisms must be shipped from a supplier, embryos up to 48 h old may be used, provided all embryos are the same approximate age. Set up test chambers according to the requirements of Table 8921:II.

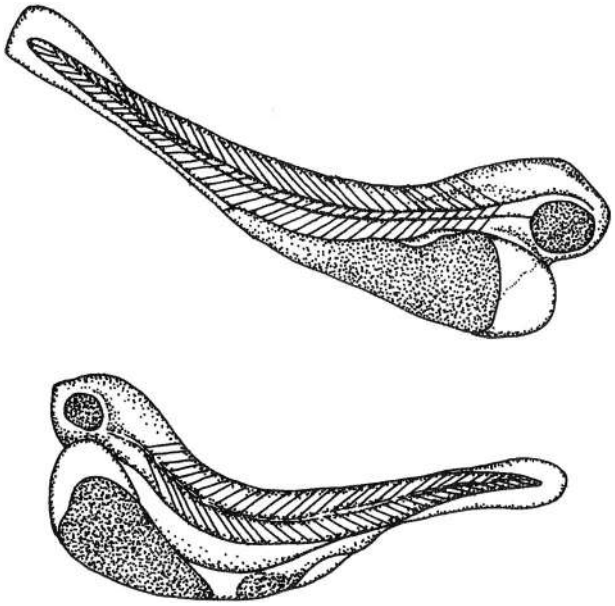


Figure 8921:3. Examples of abnormal fathead minnow larvae. Compare to Figure 8921:2.

Preferably use larger volumes for testing compounds that are rapidly degraded, are volatile, or have low water solubility. Begin tests with effluents as soon as possible, preferably within 24 h of sample collection.

From a pool of embryos from three or more spawns, randomly select and distribute several at a time until each test chamber contains at least 10, but preferably 15 to 25, embryos. Exclude abnormal and nonviable (milky-colored and opaque) embryos, as well as any showing signs of fungal infection. A light box and stereoscopic microscope are recommended for examining and counting embryos. Use a large-bore pipet or similar device to transfer embryos. During transfer, avoid adding excess water to test chambers because this will dilute exposure concentrations.

3) Solution renewal—Unless the supply of test material or effluent is limited, or flow-through procedures are used, renew test solutions daily. Before renewal, remove dead embryos or larvae. Take care not to accidentally remove or injure embryos or larvae. A light box enhances fish visibility, simplifying this task. Add new test solutions to test chambers after removal of approximately 80 to 90% of old solutions. Add solutions slowly down the side of the test chamber to avoid injury to embryos and larvae.

4) Feeding—No feeding is required.

5) Biological data and observations—Each day, approximately 24 h after test initiation and before solution change, record number of dead (milky and opaque) and live embryos in each test chamber. After hatching begins, record the number of hatched, dead, live, and deformed larvae each day. Deformed larvae are those with gross morphological abnormalities (Figure 8921:3) or other characteristics that preclude survival (Figure 8921:2 shows normal larvae). See other sources for detailed information on the embryology and development of the fathead

minnow and identification of abnormalities.[14–20] NOTE: Larvae typically do not become active for 1 to 2 d after hatching and may remain relatively immobile on the bottom of the test chamber during this time.

6) Chemical data recording—See 8921C.1*c*6).

7) Verification of exposures—See 8921C.1*c*7).

8) Test termination—Terminate test after 7 or 8 d. Before termination, record number of surviving, dead, and abnormal larvae in each test chamber (replicate).

9) Test acceptability criteria—The test is considered acceptable if control survival is ≥80%.

3. Early-Life-Stage Test

In this test, fathead minnow early life-stages, beginning with newly fertilized eggs, are exposed through embryo–larval development to an early juvenile age.[20–23] This test is run at 25°C for approximately 33 d (28 d post hatch) under flow-through or static-renewal conditions. Test endpoints include time-to-hatch, percent hatch, survival during different life stages, and growth. The intent is to determine the test substance's lowest-effect and highest no-effect concentrations.

a. Equipment and physical condition: For a description of suitable diluter systems, see Section 8010F. Construct egg-hatching cups (8 cm diam × 10 cm long) of glass tubing or other acceptable materials, such as 316 stainless steel or tetrafluoroethylene (TFE). Use clear silicone sealant to glue a 40-mesh nylon or stainless steel screen to one end of the cup. Suspend egg cups from a rocker-arm assembly and low-speed motor designed to oscillate the egg cups slowly up and down approximately 2 to 3 cm. Use a low rate through the exposure chambers sufficient to replace 90% of the water in 8 to 12 h. A self-starting siphon tube in each exposure chamber can be substituted for the rocker-arm system to ensure test solution exchange.

Provide light intensities over the chambers of approximately 400 to 800 lux and establish a 16-h light and 8-h dark photoperiod, preferably with a 15- to 30-min dusk–dawn transition period. Hold temperature at 25 ± 2°C.

For dilution water requirements, see Table 8921:I. Prepare test solutions as described in Section 8010F.2*b*. To test low-solubility compounds, follow a carrier-solvent recommendation in Table 8921:I and use a solvent control.

b. Test organisms: Use embryos, preferably <48-h old, to initiate tests. Obtain embryos from an in-house culture (see 8921B.1) or commercial supplier.

c. Test procedures: Use a minimum of five exposure concentrations and one control, and a dilution factor for determining test concentrations of ≥0.5. Set up at least two (preferably four) replicate exposure chambers per test concentration. Use a range-finding test of 4 to 10 d conducted with juveniles to determine the test concentrations for the definitive study.

1) Test initiation—From a pool of embryos from three or more spawns, randomly select and distribute several at a time until there are 60 embryos per test concentration, divided among the replicate embryo-incubation cups suspended in each test chamber. Exclude abnormal and nonviable (milky-colored and opaque) embryos, as well as any showing signs of fungal infection. A light box and stereoscopic microscope are recommended for examining and counting embryos. Use a large-bore pipet or similar device to transfer them.

2) Feeding—Provide live brine shrimp (≤24 h old) three times daily during the first 5 d following hatching.[6] At about Day 7, supplement brine shrimp diet with a fine grade of commercial fish meal (fish starter). A slightly larger grade may be substituted as the fish grow.

3) Biological data and observations—Monitor survival via daily inspection. After hatching is complete, record number of live larvae, live embryos, dead embryos, and unaccounted-for embryos per incubation cup, then release the larval fish from the incubation cup into the test chamber. Each day, record observations of fish behavior, noting normal and abnormal individuals as well as characteristics of any abnormalities. At test termination, measure standard length (to nearest 0.1 mm) and weigh fish, after blotting dry, to nearest 0.01 g.

4) Chemical data recording—Periodically measure DO, pH, conductivity, temperature, hardness, and alkalinity. Typically measure hardness, alkalinity, and conductivity at least weekly. Record temperature at least daily or continuously in one chamber that is centrally located in the row of exposure chambers. Measure DO and pH daily in random containers, including a control and treatment; DO and pH must be measured in each exposure chamber at least once every 7 d.

5) Verification of exposures—Before and during test, verify the test chemical's exposure concentrations to confirm actual versus nominal concentrations.

Initially, analyze concentration in each replicate test chamber. If concentrations among each dilution's replicates are consistent, then make subsequent measurements during the test on composite samples of replicates for each dilution. However, check diluter function independently to ensure proper operating conditions. Although desirable, it is not necessary that measured concentrations be within any specific percentage of the nominal concentrations (e.g., ±20%); certain characteristics (e.g., hydrolysis rate and volatility) may make that impossible. Measure exposure at least weekly until test ends. Use chemical measurements to help maintain consistent concentrations for each dilution throughout the test. Base statistical analyses and results (LOECs, NOECs) on measured rather than nominal concentrations.

6) Test termination—Terminate test 28 d post-hatching.

7) Test acceptability—The test is acceptable if the survival rate of all control fish at test termination is ≥80% (based on initial egg count) and ≥70% in any one control replicate.

4. Life-Cycle Reproductive Toxicity Test

This type of test starts with newly spawned eggs or newly hatched larvae, continues through fish maturation and reproduction, and ends ≥28 d after the second generation hatches. See Section 8910C.3 for a description of general procedures for this test and other sources for more details.[24–26]

a. Equipment and physical conditions: See Sections 8010F and 8910C.3. The physical systems are similar to those described for testing brook trout [Section 8910C.3*b*1)].

Use one of the following two arrangements of test tanks (made of glass or stainless steel with viewing windows):

- Use duplicate spawning tanks for each of the five or more test concentrations and controls, measuring 30 × 30 × 90 cm with a 30-cm^2 portion at one end, screened off and divided in half to form two larval chambers for the progeny;

deliver test water separately to the larval and spawning chambers of each tank, with about one-third of the water volume going to each larval chamber.

- Alternatively, use duplicate progeny tanks measuring 30 × 30 × 60 cm plus duplicate progeny tanks for each spawning tank. Use a larval tank with minimum dimensions of 30 × 30 × 30 cm, divided to form two separate larval chambers with separate standpipes, or separate 30 × 15 × 30 cm tanks. Supply test solutions and water for controls as in Sections 8010F.1 and 2. Maintain a water depth of 15 cm in all tanks.

Flow rate, oxygen requirements, aeration, cleaning, and operation are as described for the brook trout in Section 8910C.3*b*1).

Fathead minnows deposit eggs on the underside of submerged objects. For spawning substrates, use inverted semicircular sections of ceramic drain tile or PVC pipe (7.5 cm ID, 7 to 10 cm long), or equivalent (see 8921B.2*b*). If the inverted surface of the substrate is smooth, roughen it to aid embryo adhesion. Place substrate parallel to the long axis of the spawning tank so each end is readily accessible to the fish. Fasten incubation cups, such as those described in 8921C.3*a*, to a rocker arm with a vertical travel distance of 3 to 5 cm. For illumination, see Section 8010F.3*f*.

Use a 16-h light/8-h dark photoperiod, preferably with a 15- to 30-min dawn–dusk transition time.

Maintain temperature at 25 ± 2°C and record continuously.

b. Test initiation: Initiate tests with embryos or larvae from at least three females. Begin the life-cycle test by randomly selecting and distributing embryos or 1- to 5-d old larvae to each duplicate spawning tank for each test concentration. Extra fish may be added at the beginning so some can be removed periodically for special examinations.

Exclude abnormal and nonviable (milky-colored and opaque) embryos, as well as any showing signs of fungal infection. A light box and stereoscopic microscope are recommended for examining and counting embryos. Use a large-bore pipet or similar device to transfer embryos.

c. Feeding: Feed newly hatched larvae minimal amounts of live brine shrimp nauplii.[6] Avoid overfeeding. Continue feeding larval and juvenile fish twice daily with live brine shrimp nauplii for 30 to 60 d. Thereafter, frozen adult brine shrimp may be supplemented by pelleted and/or flake food. Feed quantitatively among all test groups.

d. Thinning and preparation for spawning: When test fish are 60 ± 2 d old, discard injured or deformed individuals and randomly reduce the number in each tank to 15. Record number, length, and weight of discarded and deformed fish. To obtain 15 fish per tank, it may be necessary to transfer or combine fish from duplicate tanks. Continue routine feeding and cleaning until fish mature and are almost ready to spawn. Place five spawning tiles in each duplicate spawning tank, separated fairly widely to reduce fighting among the territorial male fish. Place tiles so their undersides and guard males can be seen from the tank end. When fish are fully mature (i.e., have well-defined secondary sexual characteristics—see 8921A and Figure 8921:1) and spawning is imminent, reduce the number of males to no more than four per tank. Reserve the fifth tile as cover for females. Do not remove males with established territories under tiles where a recent spawn has occurred.

e. Spawning and embryo incubation: Each day, check spawning tiles and remove those with newly deposited embryos, beginning about 6 h after start of the light period. Loosen embryos from spawning tiles and at the same time separate them from one another by lightly placing a finger on the egg mass and moving it in a circular pattern with increasing pressure until the embryos begin to roll. Wash groups of embryos into separate containers and return to spawning tanks. Count embryos, select those needed for incubation, and discard remainder. Check all embryos for different stages of development.[27] If more than one distinct stage is present, consider each stage as one spawning and handle separately as described below.

Each day, randomly select 50 unbroken embryos from a single spawn and place in an incubator cup to determine viability and hatchability. Count, record, and discard remaining embryos. Determine viability and hatchability on each spawn of ≥50 embryos until number of spawns in each tank equals number of females in that tank. Subsequently, test for hatchability only on subsamples from every third spawning of ≥50 embryos. Remove spawns from tiles, count and record embryos, and discard.

If no spawning occurs for a week, cease testing of parental fish. Record total length and weight, sex, and gonadal condition of parental fish (F_0), then discard.

Each day, record live and dead embryos in incubator cups, remove dead embryos, and clean cup screens. After larvae begin to hatch (about 4 to 5 d), cease handling or removing them from cups until all have hatched. Then if enough larvae are still alive, select 40 at random and transfer immediately to a larval growth chamber to determine survival and growth of the second (F_1) generation. Count and discard incubation groups not used for survival and growth studies.

f. F_1 generation larval–juvenile survival and growth: Select larvae for 30- and 60-d growth and survival exposures from early spawned embryos in each duplicate tank. Plan their distribution for hatchability tests so a new group of larvae is ready to be tested as soon as possible after the previously tested group is removed from the larval chambers. Record mortality and larval lengths at 30 and 60 d after hatching. Weigh juveniles when exposures are terminated (60 d). Do not feed fish (larvae, juveniles, or adults) for 24 h before weighing.

g. Extended testing: Normally, testing is concluded with the F_1 generation larval–juvenile exposures; however, an extended life-cycle test may be conducted through another generation (F_2), if desired. In this case, transfer 50 of the 60-d post-hatch F_1 fish from each growth chamber to the corresponding spawning chamber. Follow procedures used for the F_1 generation to determine survival of embryos, larvae, and juveniles of the F_2 generation. Cease testing adult fish when spawning is complete. Continue post-hatch study to 60 d.

h. Biological data and observations: Record the following data for each tank and the controls:

- total number and length of normal and deformed individuals at the end of 30 and 60 d for each generation;
- total length, weight, and number of each sex, both normal and deformed, at the end of the tests;
- mortality during tests;
- number of spawns and embryos produced in each tank and total embryo production by each generation;
- percentage of surviving larvae;
- juvenile growth based on weight and/or length; and
- percentage of juveniles with deformities.

Use fish and embryos obtained from the test for physiological, biochemical, histological, and other tests for toxicant-produced effects, as necessary.

i. Chemical data recording: Periodically measure DO, pH, conductivity, temperature, hardness, and alkalinity. See 8921C.3*c*4 for more information.

j. Verification of exposures: Before and during the test, verify exposure concentrations of the test chemical; see 8921C.3*c*5. Base statistical analyses and results (LOECs, NOECs) on measured rather than nominal concentrations.

5. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

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8930 AMPHIBIANS (PROPOSED)*

8930 A. Introduction

1. Significance

Amphibians are important organisms in freshwater ecosystems, and many of these species' populations have been declining for several years.[1–3] Because some of the declines likely result from exposure to aquatic toxic materials,[1,4,5] more focus on amphibians in aquatic toxicology and standard experimental protocols is imperative. The following summary outlines protocols for anuran amphibians (Order Anura: frogs and toads). Anurans are the best avenue for standardizing methods because they have been the most extensively studied. Salamanders (Order Caudata) have been used less frequently in toxicology studies, so they will not be specifically addressed. However, the following methods can be modified for salamanders as necessary.

2. Test Organism Characteristics

Two genera of anurans have been studied extensively in aquatic toxicology: *Xenopus* and *Rana*. Members of the genus *Xenopus* (family Pipidae) are not native to North America or other temperate zones, but they have been developed as a model for early-life-stage tests because they can be easily cultured and bred in the laboratory. However, *Xenopus* species have significantly different life histories and larval stages than temperate-zone species.

Members of the genus *Rana* (family Ranidae) are tested when a native species is desired (see Section 10900, Plate 25:B). The distribution, life history, and biology of these amphibians can be found in the literature.[6–18]

The African clawed frogs *Xenopus laevis* Daudin and *X.* (*Silurana*) *tropicali*s Gray share generally similar morphology and natural history.[6–8] Adults are fully aquatic, with long, muscular hindlimbs, dorsally located eyes, and a dorso-ventrally flattened body. The hind toes terminate in keratinized claws. The dorsal surface is olive or greenish-grey with darker mottling or spots, while the ventral surface is paler. Females are usually larger than males and have papillae around the cloaca. Males in breeding condition develop dark, sticky pads on the digits and forelimbs. They eat any live or dead animal material in water that will fit in their mouths, and olfaction is an important sense. Larvae are transparent and feed on fine particles suspended in the water column.

The two species differ in size and karyotype.[14] *X. laevis* has an oligotetraploid genome and typically ranges from 70 to 125 mm (snout to vent) when mature. In contrast, *X. tropicalis* ranges from 28 to 40 mm and has a diploid genome. *X. laevis* historically has been more frequently used in toxicological assays, but *X. tropicalis* recently gained favor because of its smaller size, larger clutch size, diploid genome, and shorter generation time.[14–18]

Members of the genus *Rana* have long muscular hind limbs, narrow waists, and large, laterally placed eyes. The most frequently used species in toxicology are the Northern leopard frog (*R. pipiens* Schreber), the Southern leopard frog (*R. sphenocephala* Cope), and the American bullfrog (*R. catesbeiana* Shaw). Adults are semi-aquatic and use their vision to locate living prey. Adult *R. pipiens* and *R. sphenocephala* range from 50 to 90 mm (snout to vent) and are marked with large dorsal spots on a green or brown background and a pair of dorso-lateral ridges. Sexes are difficult to distinguish, although females are usually larger. Adult *R. catesbeiana* range from 90 to 150 mm (snout to vent) and can be green, brown, or heavily mottled dorsally. Males have a bright yellow throat and an external tympanic membrane that is larger in diameter than the eye. All three species' larvae have a keratinized oral disc, which is used to macerate plant or animal material. Features of the oral disk, coloration, and size are used to identify larvae of a particular species.[9–13]

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8930 B. Culture and Maintenance of Test Organisms

1. Obtaining Test Organisms

Xenopus laevis and *X. tropicalis* of various ages can be obtained for toxicity testing from commercial breeders, biological supply houses, or an in-house culture facility. An in-house breeding facility is recommended and may be required because most assays involve amphibians in early life stages. Ensure that specimens—particularly those from outside sources—are certified as to their identity, age, and freedom from disease. Examine animals on arrival for skin lesions or red patches on ventral surfaces. Quarantine new arrivals from the resident population for at least 2 weeks.[1–4]

Rana specimens of various ages can be obtained from biological supply houses, hatcheries, and bait shops. Such specimens probably were field-collected, so in general, avoid them due to inaccurate species identity, the potential for disease (from shipping), and lack of locality data. Preferably obtain specimens of *Rana* sp. by collecting them from habitats with known toxicant-exposure history. Such collections are subject to breeding-season constraints, but during this time a variety of life stages and sizes, as needed, can be gathered easily and inexpensively. Quarantine specimens before use in experiments. In-house breeding cultures are difficult to establish for *Rana* sp. because adults require live food and eggs must be artificially fertilized.[5]

If laboratory gloves are worn while handling specimens or cleaning containers, be cautious when using latex gloves. Amphibian larvae are extremely sensitive to direct and indirect contact with latex. Vinyl and nitrile gloves are less toxic, but specimens should be monitored for increased mortality not associated with chemical exposure.[6–9]

2. Culturing and Care of Test Organisms

All stages of *Xenopus* sp.—from egg to adult—may be cultured successfully in the laboratory through several generations. Basic information on establishing and maintaining a culture facility has been developed for *X. laevis* and is presented below.[1] Requirements are similar for *X. tropicalis,* with adjustments for their smaller size and higher preferred temperatures.[2–4] Separate life stages of *Rana* sp. can be cultured easily, but maintaining a continuous viable breeding facility for successive generations can be difficult for several reasons.

a. Water supply and culture system: Natural water is preferred for maintaining adult *Xenopus* sp. Supplies from wells or springs usually are more uniform in quality than those from surface waters. Dechlorinated tap water can be used if residual chlorine is monitored and at low levels (dechlorination can be incomplete; preferably dechlorinate with sodium bisulfite). The salt solution used for the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) test and for breeding adults and culturing early stages also can be used for maintenance, but is only suitable for small colonies because of cost and formulation time. Maintain water temperature at 20 ± 2°C for *X. laevis* (26 ± 0.5°C for *X. tropicalis*) before adding adults. Provide aeration, if needed, to maintain dissolved oxygen (DO) levels. Take quarterly measurements of pH, total dissolved solids (TDS), total organic carbon (TOC), organophosphate pesticides, organic chlorine [or organochlorine pesticides plus polychlorinated biphenyls (PCBs)], chlorinated phenoxy herbicides, perchlorate, chlorate, ammonia, bromide, beryllium, cadmium, chlorine, chloramine, chromium, copper, fluoride, iodide, iron, lead, manganese, mercury, nickel, selenium, silver, and zinc. The water's physical and chemical limits include pH 6.5 to 8.5, TOC <10 mg/L, and alkalinity and hardness between 16 and 400 mg/L as $CaCO_3$.[1–4,8,9] Water requirements for *Rana* sp. are similar to those for *X. laevis*.

The FETAX assay (8930C.1) requires the following salt solution for all experiments with *X. laevis*:

Sodium chloride (NaCl)	625 mg
Sodium bicarbonate ($NaHCO_3$)	96 mg
Potassium chloride (KCl)	30 mg
Calcium chloride ($CaCl_2$)	15 mg
Calcium sulfate ($CaSO_4 \cdot 2H_2O$)	60 mg
Magnesium sulfate ($MgSO_4$)	75 mg
Deionized or distilled water	1 L

The final solution's pH should be between 7.6 and 7.9. Use this solution for breeding, static or renewal assays, and flow-through experiments whenever possible. Other water sources must allow for embryonic growth at the same rate as this solu-

tion, and there should be acceptable mortality and malformation rates.[1] Some types of test solutions will require modifying the above formulation by decreasing or increasing certain salts, or using a more concentrated or more dilute solution.[10]

Maintain adult specimens of *Xenopus* sp. in glass aquariums or fiberglass or stainless steel raceways at densities of 4 to 6/1800 cm^3. The water should be 7 to 14 cm deep. Use opaque tanks or shield tank sides (e.g., with black plastic) so specimens are not disturbed by outside activity. Use a photoperiod of 12 h day/12 h night.[1–4] For adult *Rana* sp., adjust tanks to provide a dry area because these species are not completely aquatic. This dry area can be created by tipping the tanks at one end so water pools at the opposite side, or by adding platforms above the water level so specimens can climb out of the water. Keep adult *Xenopus* sp. and *Rana* sp. in same-size groups to decrease cannabalism and competition for food.[1–5]

b. Establish breeding colonies: To establish an *X. laevis* breeding colony, use males that are at least 2 years old and 75 to 100 cm long (snout to vent), and females that are at least 2 years old and 100 to 125 cm long.[1] To establish an *X. tropicalis* breeding colony, use adults that are at least 4 months old.[2–4] Breed males and female *Xenopus* sp. as single pairs. Females of both species should have visible cloacal papillae and swollen sides indicative of full ovaries (sides of the abdomen appear swollen from dorsal view). Males should have darkened nuptial pads on the palms of their forelimbs and light mating strips on the underside of their arms.[1–4]

Breeding can be conducted in any container with a mesh false bottom. Fit a 20- or 40-L (5- or 10-gal) glass aquarium with a 1-cm mesh (nylon or plastic only) suspended about 3 cm from the bottom of the aquarium so deposited eggs can lie undisturbed on the bottom. Shield sides of breeding aquarium (e.g., with black paper) and add an aerator if desired. Cover the top of aquarium with an opaque porous material (e.g., a fiberglass furnace filter).[1]

Plastic containers can be used by stacking two dish pans on top of one another. Perforate the uppermost pan with enough holes to create the mesh false bottom.[1–4]A useful alternative to glass aquaria or plastic dish pans is a sifting cat litter box. These boxes come equipped with an outer base and an inner perforated sifting grate, which functions as the mesh false bottom. The boxes are opaque and have a base area of 1260 cm^2.[11]

Breed adult *Xenopus* sp. in the same dilution water in which the test will be conducted. Hold water temperature at 20 ± 2°C for *X. laevis* (26 ± 0.5°C for *X. tropicalis*). To induce breeding, inject male with 250 to 500 IU of human chorionic gonadotropin into the dorsal lymph sac, and female with 500 to 1000 IU. The specific amount injected depends on time of year and condition of adults. The hormone concentration should be 1000 IU/mL in sterile 0.9% NaCl. Use a 1-mL tuberculin syringe fitted with a 1.3-cm (0.5-in.) long, 26-gauge needle. Amplexus normally occurs within 2 to 6 h and egg deposition about 9 to 12 h after injection. The fertility rate should be >75%. Do not use eggs laid in "strings" or not perfectly round, because they develop abnormally.[1–4]

Eggs of *Rana* sp. and other North American anurans can only be collected during the breeding season, either directly from the field or from reproductively active adults. Obtain proper permits to collect eggs or adults. Collect fertilized egg masses during breeding season and treat as in ¶ *c* below. Reproduction in adult *Rana* sp. can only be induced in animals collected during breeding season. Collect pairs that are in amplexus and place them in an undisturbed holding tank with water; fertilized eggs can be collected in 24 h. Alternatively, artificially induce ovulation in gravid females collected from the field. The standard induction method involves intraperitoneal injection of pituitaries from several adults into a gravid female. Extrude ova into a Petri dish 24 to 48 h after injection by applying pressure to the female. Inseminate the ova by mincing testes (previously removed from a male) in buffer and applying the sperm suspension over the extruded ova.[5,12,13] Sperm and ova might also be collected via injections of synthetic human luteinizing hormone-releasing hormone (LHRH) in a method similar to those described for *Xenopus* and chorionic gonadotropin (¶ *a* above). These techniques do not require destroying animals but are not frequently used.[12–14]

c. Collection and maintenance of embryos: If necessary for certain experiments, begin dejellying anuran embryos immediately after egg laying. Gently swirl embryos for 1 to 3 min in a 2% w/v L-cysteine solution prepared in FETAX solution (¶ *a* above). Adjust pH of cysteine solution to 8.1 with 1*N* NaOH, and continue dejellying until all jelly is removed. Do not treat embryos too long because survival will be reduced. Place embryos in 60-mm glass or plastic Petri dishes (25 embryos and 10 mL FETAX solution) and keep these in a constant-temperature room or a suitable incubator. Renew solution every 24 to 48 h.[1–4] Periodically identify developmental stage of embryos according to standard guides.[15–17]

d. Rearing larvae and metamorphs: Transfer free-swimming larvae of *Xenopus* sp. or *Rana* sp. to glass aquariums. Loading depends on size of larvae and type of tank (static versus flow-through). Determine developmental stages using standard guides.[15–17] Transfer metamorphs and small adults of *Xenopus* sp. to tanks similar to those of adults. Transfer *Rana* larvae to adult tanks with dry areas when forelimbs emerge and tail reabsorption begins.[18–20]

e. Food and feeding: Feed *Xenopus* sp. larvae a finely ground animal chow (e.g., Salmon Starter® pellets or Sera Micron®). Suggested rations are 30 to 80 mg/animal/d for *X. laevis*[8–9] (6 to 10 mg/animal/d for *X. tropicalis*[21]), depending on developmental stage. *Rana* larvae can be fed a variety of foods, including trout chow, boiled lettuce and spinach, and fish flakes. A convenient diet for *Rana* larvae is a combination of ground rabbit chow (Harlan Teklad®), agar, and gelatin, which is heated and then cooled until congealed.[22–24] Feed *Rana* an equivalent *Xenopus* diet if studies require comparison between the two genera. If larvae will be used for experiments, feed early-stage larvae (actively feeding for <20 d) until the test begins. Withhold food from later-stage larvae for 48 h before test begins.[25,26]

Feed post-metamorphic and adult *Xenopus* sp. raw beef liver three times a week. *X. tropicalis* can be fed Salmon Starter or equivalent at a rate of 40 to 50 mg/female and 25 to 30 mg/male.[2–4] This food should also be adequate for *X. laevis,* but amounts should be increased due to this species' larger size. Supplement food with liquid multiple vitamins and screen it for materials that will be tested.[1–4] Adult *Rana* sp. will not eat non-moving food. Feed them appropriate-sized crickets and other insects or earthworms. Dust food with a vitamin powder. If food animals—particularly crickets—will be maintained for ex-

tended periods, feed diets fortified with calcium and vitamins. Anurans will absorb these supplements. Large *R. catesbeiana* will eat fish, crawfish, and small laboratory mice. Offer food on the tank's dry area, but items that float or swim will be eaten from the water.[5,27–29]

f. Parasite and disease control: Observe animals in quarantine daily for signs of disease. Treat diseased frogs appropriately. Look for dull or dry skin, wounds or infection, poor posture, decreased body condition, and lethargy.[30,31] Withhold sick frogs from breeding for 4 to 6 weeks after treatment. Remove dead individuals and waste, and use separate cleaning and transfer equipment for each tank.

3. Acclimating and Holding Test Organisms

Use embryos immediately for embryo–larval stage tests.[1–4] Acclimate later-stage larvae before use by changing water from 100% holding water to 100% dilution water over 48 h. Hold subjects at 100% test water for 48 h.[25,26]

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8930 C. Toxicity Test Procedures

1. Frog Embryo Teratogenesis Assay–*Xenopus laevis* (FETAX)

FETAX is used to evaluate how toxicants affect animals' developmental processes. It is designed not just for amphibians but also other species (e.g., mammals) and, therefore, useful for screening substances that are potential teratogens in humans.[1] The FETAX assay can be modified to accommodate species other than *X. laevis* (e.g., *X. tropicalis*[2–4]) with different developmental times or maintenance requirements. Details on FETAX assays are presented in other sources.[1–4] The general procedure and modifications to the standard protocol are discussed below.

a. Scope and application: FETAX procedures can be used to test chemicals either individually or in formulations, as well as temperature, DO, pH, physical agents, aqueous surface and ground waters, leachates, aqueous extracts of water-insoluble materials, and solid-phase samples (e.g., soils and sediments, particulate matter, sediment, and whole bulk soils and sediment). The assay uses *X. laevis* embryos, so it requires no feeding, little maintenance, and minimal laboratory space.

b. Range-finding test and selection of concentrations: Conduct range-finding tests to select proper concentrations for definitive tests. Use at least seven concentrations that differ by a factor of ten. Conduct the test and calculate the 96-h lethal concentration (LC) (LC_5, LC_{16}, LC_{50}, LC_{84}, and LC_{95}) and the effective concentration (EC) (EC_5, EC_{16}, EC_{50}, EC_{84}, and EC_{95}). If necessary, use results of the first definitive experiment as another range-finder and readjust test concentrations.

c. Specific test procedures:

1) Facilities and equipment—Conduct tests in an incubator to house the embryos (see 8930B.2*c*). Ensure that all equipment and facilities that contact stock solutions, test solutions, or water in which embryos will be placed do not contain leachable or dissolvable substances at levels that would adversely affect embryo growth or development. For new test facilities, conduct a non-toxicant test in which all test chambers contain FETAX solution with no added test material. The embryos should grow, develop, and survive in numbers consistent with an acceptable test. To count and evaluate abnormal embryos, obtain a binocular dissection microscope (magnifications up to 303×), a darkroom enlarger (to enlarge embryo images), and a map measurer or an ocular micrometer. Alternatively, count embryos using a digitizer interfaced to a microcomputer.

For toxicity tests of fluids, use 60-mm glass or plastic Petri dishes as the exposure containers and modify them as necessary.[1–4] For aquatic sediment testing, use a 250-mL (9-oz) specimen bottle with a glass tube/TFE* mesh insert. Add 35 g test soil to bottle, followed by insert and 140 mL FETAX solution. Place embryos on top of insert.[5] For *in situ* field exposures, particularly with native species, construct exposure chamber from a 2.5-cm-wide, 7.0-cm-diam circlet of plastic polyethylene mesh (1-mm mesh). Use a central nylon bolt/wing nut to hold the chamber together, and line the chamber with TFE mesh to retain embryos. Anchor the chamber in water with a stainless steel stake, and mark the location with a fishing bobber. Equilibrate embryos by placing laboratory shipping container directly into sample location until temperatures are within 2°C. Use four to six exposure cages per onsite sample unit, with 10 to 25 embryos in each cage.[6]

2) Test initiation—Induce breeding in three mating pairs and harvest clutches separately. Conduct three definitive tests using embryos from only one mating pair in each test. Keep clutches separate because embryos from one mating pair that initially appear acceptable might develop poorly. If the embryos were mixed (from different mating pairs), then all embryos would have to be discarded.

For each test concentration, use two dishes each containing 25 embryos and 10 mL test solution. For each control, use four dishes of 25 embryos each. Maintain a temperature of 20 ± 2°C for *X. laevis*[1] (26 ± 0.5°C for *X. tropicalis*[2–4]) throughout test to prevent malformations and allow proper growth of controls. For a positive control or reference toxicant, use 6-aminonicotinamide at 5 mg/L (the 96-h LC_{50}) and 2250 mg/L (the 96-h EC_{50} for malformations)—four dishes of each.

3) Solution renewal—Replace test material every 24 h, and measure pH of control and highest test concentration during renewal. Remove test solution using a Pasteur pipet with an orifice that has been enlarged and fire-polished to accommodate embryos without damage in case embryos are accidentally picked up. Complete renewal as quickly as possible to minimize embryo desiccation.

4) Biological data recording—Measure mortality by counting and removing dead embryos during solution renewal. At 24 h, death is determined by embryo's pale skin pigmentation, decomposition, and lack of response to prodding. At 48, 72, and 96 h, use the lack of heartbeat as an unambiguous sign of death. Record final number of dead embryos at 96 h of exposure and the time of first hind-limb buds in controls. Fix dead embryos and remaining live embryos in 3% formalin. Record developmental stage and malformations at the end of 96 h according to standardized guides.[7–9] Compare exposed embryos to control embryos. Measure head-to-tail length at the end of each test after embryos are fixed in 3% formalin.

5) Exogenous metabolic activation system (MAS)—Use an exogenous MAS when using FETAX to evaluate developmental toxicity for human-health hazard assessment, because early *X. laevis* embryos have limited xenobiotic metabolic capabilities, particularly cytochrome P-450. Make the MAS from rat liver microsomes and nicotinamide adenine dinucleotide (the generator), and Aroclor 1254 (the cytochrome P-450 inducing agent). Use MAS-only dishes as the negative control, and 4 mg/mL cyclophosphamide with and without MAS as the positive control. Make a P-450 control (to demonstrate that the cytochrome P-450 system is responsible for observed bioactivation) by adding dithionite directly to the microsomes and bubbling carbon monoxide through for 3 min to inactivate P-450. To prepare 20 mL test solution, place an appropriate volume of FETAX solution into a 50-mL flask, add MAS components and appropriate volume of test material stock to give desired concentration, then adjust final volume to 20 mL with FETAX solution.

* Teflon®, or equivalent.

Divide this test mixture between replicate Petri dishes to which embryos will be added.

6) Duration of test—For *X. laevis*, run FETAX assay for 96 h, at which point the control embryos should have hind-limb buds (Nieuwkoop and Faber Stage 46[8]). If 90% of controls do not show hind limb buds by 96 h, then extend the test another 3 h. The FETAX assay can be conducted in 48 h for *X. tropicalis*.[2–4] For *Rana* sp. or other species, test duration may need to be modified to accommodate differences in developmental time. Report deviations from standard FETAX conditions.

7) Mixture-interaction studies—Interaction studies can be conducted to evaluate synergistic or antagonistic developmental toxicity among two or more combined substances. Two methods have been used successfully with the FETAX assay.[10–12] In toxic unit analysis, binary mixtures are made in specific ratios (1:3, 1:1, and 3:1), and LC_{50}s are plotted on an isobole diagram to determine additive, synergistic, or antagonistic effects. In the second method, simply test for greater or less than expected responses from a particular concentration of chemicals with known effects. This method requires less time but does not yield the quality of information of toxic unit analysis.

2. Other Short-Term Acute Tests for Embryos and Later-stage Larvae

Procedures for testing toxicants on post-embryonic, free-swimming amphibian larvae (tadpoles) in an acute exposure can be designed similar to tests for fish and macroinvertebrates. Standard methods for acute exposures have been described in detail[13,14] and are discussed in general below.

a. General procedure: Larvae are maintained for 2 to 8 d in each of two or more treatments in one or more test chambers. In each of the one or more control treatments, larvae are maintained in dilution water to which no test solution has been added. This procedure measures the test's acceptability based on the quality of test subjects, dilution water, test conditions, handling procedures, etc. It also provides a basis for interpreting data obtained from the other treatments. In each treatment, larvae are maintained in one of several concentrations of test solution. Data on biological effects in each test chamber usually are obtained periodically during the test and analyzed to determine LC_{50}s or EC_{50}s for various exposure periods.

b. Types of experimental design: Two types of experimental design are appropriate in most cases. For an acute test intended to allow calculation of an LC_{50}, EC_{50}, or inhibitory concentration (IC) (IC_{50}), use one or more control treatments and a geometric series of at least five concentrations of test material. Except for the control(s) and the highest concentration, each concentration should equal at least 60% of the next higher one. Alternatively, if it is only necessary to determine whether a specific concentration is acutely toxic to larvae or whether the LC_{50}, EC_{50}, or IC_{50} is above or below a specific concentration, then only that concentration and the control(s) are necessary. Use two more concentrations at about one-half and twice the specific concentration to increase confidence in the results. For both test designs, at least one concentration of test material should kill or affect a percentage of larvae (other than 0 or 100%) near the percentage for which the LC, EC, or IC is to be calculated.

c. Test setup: In static and renewal tests, load 0.5 to 0.8 g organism/L in each test chamber, adding fewer animals at higher temperatures. In flow-through tests, load no more than 1 g organism/L/d in each test chamber. For consistency, use temperatures similar to those in other assays (e.g., FETAX). For static tests, maintain a DO level between 60 and 100% of saturation in each test chamber during the first 48 h of the test, and between 40 and 100% of saturation afterward. For renewal and flow-through tests, maintain a DO level between 60 and 100% of saturation in each test chamber at all times.

d. Specific test procedures:

1) Test initiation—Distribute test organisms randomly among test chambers. For static and renewal tests, place test organisms in chambers within 30 min after test material is added. For flow-through tests, place test organisms in chambers after test solutions have been flowing through chambers long enough for their concentrations to have reached steady state.

2) Feeding—Do not feed larvae 24 to 48 h before or during an acute toxicity test. Food can add another uptake route, and fecal matter and uneaten food will decrease the DO concentration and biological activity of some test materials.

3) Physical and chemical data recording—For static and renewal tests, measure water quality parameters at the beginning, the end, and at least every 48 h during the test in the control(s) and the high, medium, and low test concentrations as long as live organisms remain. Measure maximum and minimum temperatures daily in at least one test chamber. For flow-through tests, measure water quality parameters (e.g., hardness, alkalinity, conductivity, DO, and pH) at the beginning of the test and measure temperature daily in at least one test chamber. To monitor test-solution concentrations, take water samples from a point midway between the top, bottom, and sides of the test chamber. In static and renewal tests, measure the concentration of test material in at least the control(s) and the high, medium, and low concentrations of test material at the beginning of the test, at the end of renewal periods, and at the end of the test. For flow-through tests, measure concentration of test material in the chambers as often as practical during test. In each treatment, the highest concentration measured during the test should be less than 1.5 times the lowest concentration.

4) Biological data recording—Measure the number of dead and affected organisms (lack of movement or response to gentle prodding, loss of equilibrium) in each test chamber every 24 h after test begins. Remove dead organisms at least once every 24 h if it can be done without stressing live organisms. Measure weights of test organisms after exposure is complete.

5) Test termination—Conduct test for at least 96 h or long enough to ensure that a time-independent toxicity level can be determined or estimated mathematically. If organisms will not be substantially affected by starvation for at least 8 d, conduct test for at least 8 d to determine whether more organisms are affected or killed after 96 h. As an optional test, place remaining test organisms in dilution water that does not contain any added test material for 2 to 8 d and feed them to determine whether delayed effects occur.

3. Amphibian Metamorphosis Assay (AMA)

A variety of environmental chemicals adversely affect endocrine systems. The thyroid system is one of the most vital endocrine systems in vertebrates; it is particularly important in regulating development and metamorphosis in amphibians. The

Amphibian Metamorphosis Assay (AMA) is a simple, standardized screen for substances that affect the hypothalamic–pituitary–thyroid (HPT) axis.[15–18] The assay uses Nieuwkoop–Faber (NF) Stage 51 *X. laevis,*[8] and consists of a 21-d exposure to the suspected chemical. Periodic observations during exposure include mortality, hind-limb length, snout-to-vent length, developmental stage, weight, and thyroid histology. The AMA is a test guideline suggested by the Organisation for Economic Cooperation and Development (OECD)[15] and is also incorporated into Tier 1 of the U.S. Environmental Protection Agency's Endocrine Disruptor Screening Program.[16] This procedure has been adapted for *X. tropicalis*.[19] The general procedures are described below.

a. Obtaining test subjects: Breed 3 to 5 pairs of adult *X. laevis* (see 8930B.2*b*). Retain the best individual spawns and do not mix. Discard all obvious dead or abnormal eggs via pipet. Transfer healthy embryos from each spawn into hatching tanks for 4 d, and transfer the best spawn to a rearing tank (see 8930B.2*c*). Through the first week, remove dead larvae and replace daily. Clean tanks daily by siphoning, and feed larvae twice each day (see 8930B.2*e*). During pre-exposure, use the same culture water, light, and and feeding regime to be used during the exposure phase. Do not exceed rearing densities of 4 larvae/L (static exposure) or 10 larvae/L (flow-through exposure). Larvae should develop from NF Stage 45/46 to 51 in 12 d.

b. Test vessels and solutions: Conduct tests in glass or stainless steel aquaria (22.5 × 14 × 16.5 cm) with 4 to 10 L test solution (10 to 15 cm minimum water). Flow-through test systems are preferred over static renewal systems. Ensure that iodide concentrations in test water are between 0.5 and 10 μg/L because iodide needs to be available for proper thyroid gland function. When determining concentrations of test material, set the high test concentration by either the test substance's solubility limit, the maximum tolerated concentration (MTC), or 100 mg/L, whichever is lowest. If solvent carriers for the test substance are required, then include a solvent control among the test solutions. Use at least three test concentrations and a clean water control. Separate the highest and lowest concentrations by a minimum of one order of magnitude.

c. Test initiation and conduct: Initiate the exposure on Day 0 when enough larvae have reached NF Stage 51[8] and are ≤17 d post-fertilization. Hind-limb morphology is the most prominent and preferred landmark for morphological staging. To select test animals, pool normal-looking larvae into one vessel with dilution water. To determine developmental stage, remove larvae individually and transfer to a transparent chamber (e.g., 100-mm Petri dish) containing dilution water. Although anesthesia should be avoided, if necessary for proper staging, anesthetize larvae with 100 mg/L buffered tricaine methanesulfonate (MS-222). Distribute larvae randomly into treatment tanks, 20 per tank (5 per L), with four replicate tanks per treatment. Replace unhealthy larvae from the pooling tanks. Continue monitoring mortality every day and remove dead larvae. Record any abnormal behavior or excessive visible malformations throughout the test. Sample test solutions from each replicate tank for chemical analysis on Day 0 and once every week thereafter.

On Day 7, remove five randomly chosen larvae from each test tank, euthanize in 150 to 200 mg/L tricaine methanesulfonate, rinse, and blot dry. Measure body weight (nearest mg), hind-limb length, snout-to-vent length, and developmental stage. On Day 21, terminate exposure by removing and euthanizing all remaining larvae. Rinse, dry, and determine body weight, snout-to-vent length, and developmental stage for each larva. For any histological assessments, place all larvae or head tissue samples containing the lower jaw in Davidson's fixative for 48 to 72 h. Segregate larvae in each test tank according to stage. Evaluate the distribution of stages across the test by comparing larvae from all treatments to the median developmental stage of all larvae from the control treatment group. Choose five stage-matched larvae from each replicate tank for histopathological observations. A pathologist should be consulted for determination of histological effects due to the test chemical, because basic statistical analysis is not useful for these endpoints.

d. Data analysis: Tests considered to be negative for thyroid activity are accepted as valid if (*a*) mortality in any treatment does not exceed 10%; (*b*) at least two treatment levels, with all four uncompromised replicates, are available, and (*c*) at least two treatments with no obvious toxicity are available. Tests considered to be positive for thyroid activity are accepted as valid if mortality in the control group does not exceed two tadpoles/replicate. Accelerated development, asynchronous development, or marked histopathology of the thyroid gland relative to the control specimens indicates the test material's potential effects on the thyroid. Advanced development is determined via statistical analyses of

- hind-limb length (normalized by snout-to-vent length) on Study Day 7;
- hind-limb length (normalized by snout-to-vent length) on Day 21;
- developmental stage on Day 7; and
- developmental stage on Day 21.

Consider the test positive for accelerated development if a statistically significant effect is detected in any of the four endpoints above.[20]

4. Chronic Toxicity Tests

Few toxicology protocols have been developed to expose amphibians from embryo through the larval period to adulthood. Investigators have rarely attempted to expose amphibian larvae for the entire metamorphic period because this lasts several months for many species, and because larvae are extremely sensitive and difficult to keep alive at metamorphic climax. Examining toxicant effects during amphibian metamorphosis would be particularly valuable because certain critical stages of development might be sensitive to even low levels of toxicant. The following methods are useful in examining later-stage larvae under longer exposure times than a few days and as a complement to FETAX assays.

a. 10- and 30-d embryo–larval exposures: Transfer 4-d-old embryos (e.g., after FETAX assay) to glass aquariums and continue exposure through 10 d. Monitor same endpoints as in FETAX assay (change exposure water on Day 7).[21] If desired, extend exposure for 30 d.[22–26] Feed subjects as needed, depending on tank size, and renew exposure water every 48 h. After 30 d, measure length and weight and identify hind-limb abnormalities. Fix representative specimens in 3% formalin at pH 7.0.

b. Exposures through metamorphosis: The following procedures have been used successfully to expose *R. pipiens* from embryo through tail reabsorption[27–29] and should be adaptable to

other species (with necessary adjustments for larval size and development duration). Collect embryos as above (8930B.1). Maintain in 150-mL Petri dishes, 30 to 40 embryos per dish, with 100 mL of appropriate water or test solution. Renew solutions daily. When larvae reach Gosner Stage 25,[7] transfer from respective Petri dishes to 18.9-L, low-density polyethylene (LDPE) plastic tanks† containing 15.14 L test solution. Feed larvae ground rabbit chow daily, *ad libitum*. Change the full volume of water every 48 h. Check periodically for dead larvae and record length, weight, and malformations on a subset of larvae; the frequency of data collection will depend on the experiment's objectives. As larvae reach Gosner Stage 42 (forelimb emergence[7]), record time to metamorphosis, size, and mass. Transfer individual Stage 42 larvae into plastic boxes with divided compartments (115 × 51 × 45 mm) (e.g., a plastic tackle box). Add enough test solution to keep each larva hydrated, and change solution daily. Slant the boxes so larvae can emerge from the water. Do not feed larvae; nutrition will come from reabsorbing the tail. Maintain larvae until complete tail reabsorption (Gosner Stage 46).[7] Euthanize larvae in 0.5 g/L tricaine methanesulfonate (MS-222) and freeze for tissue analysis.

5. Performance and Behavioral Tests

Toxicity tests that use changes in performance or behavior as endpoints are more useful in some circumstances than examinations of mortality or gross abnormalities. Performance and behavioral responses change at sublethal concentrations and after chronic exposure. Changes in performance or behavior can compromise an otherwise healthy individual's ability to find food, avoid predators, and locate mates. Avoiding toxicants can cause individuals to relocate to suboptimal habitat or reduce population densities.

There are standard guidelines for examining behavioral alterations in amphibians; they are similar to those designed for aquatic organisms in general.[30] The following assays were developed specifically to measure amphibian performance and behavior, and the utility of some of these tests has been demonstrated with toxicant-exposed subjects. These tests can be conducted during or after toxicant exposure. Although direct observation of some of these activities is possible, usually a remote camera and video recording equipment are necessary.

a. Swimming performance and activity: To evaluate swimming performance, place an amphibian larva in an appropriately sized water-immersed track and prod it at the base of the tail. Measure the larva's responsiveness and speed.[31–33] Alternatively, measure unforced activity and distance traveled within a set time period (e.g., 5 min) to observe hyper- or hypoactivity in exposed subjects.[34] For more elaborate activity observations, add refuges or predators and measure time spent being active, hiding, feeding, or performing other activities.[35]

b. Preference/avoidance of toxicants: To measure preference/avoidance of toxicants, place an amphibian larva in an appropriate chamber where both control and test water are available and the larva can move toward or away from either water. One chamber design consists of a Y-shaped apparatus in which two water sources flow toward the subject. Measure the number of times the larva enters each stream or the amount of time spent in either water.[36]

Alternatively, construct an octagonal fluviarium with eight outputs into a circular swimming arena.[37–42] Each output should maintain an isolated plume, so the test toxicant does not flow into the control streams. Place a larva in the central arena, where it has equal access to all eight output streams. Give the subject 5 min without any test plume, 5 min with the test plume flowing, and then another 5 min without the test plume. Note how much time the larva spends in each stream.

In both exposure chambers, use either unexposed subjects or subjects previously exposed to a toxicant.

c. Respiratory behavior in larvae: Place larvae in individual tanks, or uniquely mark individuals in group tanks.[43,44] Monitor larvae for 5 d. For each individual, count the number of movements longer than one body length (activity), the number of trips to the surface for 15 min, and the number of times the buccal floor is elevated for 1 min. Larvae with compromised respiratory abilities will become less active, head to the surface more frequently to gulp aerial oxygen, and increase their buccal pump rate.[45]

6. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

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PART 9000

MICROBIOLOGICAL EXAMINATION

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Joint Task Group Chairs for The 23rd Edition

9020 Quality Assurance/Quality Control ..... Margo E. Hunt
9030 Laboratory Apparatus ..... Gil Dichter
9040 Washing and Sterilization ..... Margo E. Hunt
9050 Preparation of Culture Media ..... Margo E. Hunt
9060 Samples ..... Gil Dichter
9215 Heterotrophic Plate Count ..... Gil Dichter and Mark W. LeChevallier
9221 Multiple-Tube Fermentation Technique for Members of the Coliform Group ..... Ellen B. Braun-Howland
9222 Membrane Filter Technique for Members of the Coliform Group ..... Nancy H. Hall
9223 Enzyme Substrate Coliform Test ..... Jennifer Best
9230 Fecal Enterococcus/Streptococcus Groups ..... Rachel T. Noble
9250 Detection of Actinomycetes ..... Margo E. Hunt
9610 Detection of Fungi ..... Joseph O. Falkinham, III

Summary of Major Changes since 2012

Quality Assurance/Quality Control (9020) has been expanded to address requirements for regulatory compliance, along with updates on personnel training, biosafety, and temperature-sensing devices.

The language was clarified in Laboratory Apparatus (9030), Washing and Sterilization (9040), and Preparation of Culture Media (9050). Samples (9060) contains new information on sample collection, preservation, and storage.

Heterotrophic Plate Count (9215) contains new information on sample preparation and agar use, as well as summarizing incubation times and temperatures for media.

The Multiple-Tube Fermentation Technique for Members of the Coliform Group (9221) now includes quality control (QC) information and a procedure for the simultaneous determination of thermotolerant coliforms and *E. coli*. The procedure for the confirmed phase of the Presence–Absence test was rewritten.

The Membrane Filter Technique for Members of the Coliform Group (9222) includes new QC information and new laboratory apparatus lists. MF Partition Procedures (formerly 9222G) has been split into Partitioning Thermotolerant Coliforms from MF Total Coliform using EC Broth (now 9222G), Partitioning *E. coli* from MF Total Coliform using EC-MUG Broth (9222H), and Partitioning *E. coli* from MF Total Coliforms using NA-MUG Agar (9222I). Simultaneous Detection of Total Coliform and *E. coli* by Dual-Chromogen Membrane Filter Procedure (formerly 9222H) is now 9222J. Simultaneous Detection of Total Coliforms and *E. coli* by Fluorogen/Chromogen Membrane Filter Procedure (formerly 9222I) is now 9222K.

Enzyme Substrate Coliform Test (9223) now includes information on sample collection and QC. Also, incubation times and temperatures were clarified—including instructions for pre-warming samples—and specific directions for analyzing samples in various MPN formats are given.

Fecal Enterococcus/Streptococcus Groups (9230) includes new QC procedures to test each lot of commercial media. Editorial revisions were made to Detection of Actinomycetes (9250) and Detection of Fungi (9610), and references were updated in 9610.

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9010 Introduction*

The following sections describe procedures for examining a water sample's microbiological content. These methods were developed primarily to permit rapid examination of water samples, so analysts often assume they only apply to routine examinations. However, they are suitable for both compliance monitoring and research studies of ambient, drinking, waste, and marine waters. The methods are used by many laboratories and are the best techniques currently available, but to be effective, their limitations must be thoroughly understood and appropriate quality control practices must be followed. Likewise, all techniques should be investigated to establish their specificity, improve their procedural details, and expand their uses in analyzing various types of water.

Keep in mind that routine bacteriological analyses do not provide a complete water quality picture. Always consider bacteriological results in light of information available about sanitary conditions in and around the sample source. Water quality evaluations based on test results of one sample from a given source are inadequate. When possible, base water quality evaluations on the results of a series of samples collected and analyzed over a known, protracted period.

Also, experience has shown that when un-iced samples are mailed, noticeable changes may occur in the types or numbers of bacteria (even if shipping time is brief). So, samples should be refrigerated during transportation—particularly when ambient air temperature exceeds 13°C—and minimal time should elapse between collection and analysis.

One of the principal indicators of a water's suitability for domestic, industrial, or other uses is the coliform group of bacteria, as herein defined. Experience has established the significance of coliform density—in particular, that of *Escherichia coli* or thermotolerant coliforms (previously called *fecal coliforms*) as a water quality criterion, and the group's cultural reactions and characteristics have been studied extensively.

Tests for detecting and enumerating indicator organisms and pathogens are presented, including two for coliforms: the membrane filter technique and the multiple-tube fermentation test. The membrane filter technique involves direct plating to detect and estimate coliform densities. Procedural modifications—particularly the culture medium—have made its results comparable to those of the multiple-tube fermentation procedure. Although the membrane filter technique has limited applications, it is equivalent when used appropriately. Either procedure can be used to evaluate water quality or treatment process effectiveness.

Coliform density can be reported as a most probable number (MPN) index or membrane filter count per 100 mL. It is customary to report results of the multiple-tube fermentation procedure as MPN, which is not an actual enumeration but rather an index of the number of coliform bacteria most likely to give the results produced during testing. In contrast, the membrane filter procedure and other direct plating methods enable analysts to actually count coliform colonies.

Not all sample types require a quantitative assessment of coliform bacteria, so qualitative, presence–absence tests are included.

Pathogen contamination also can be indicated by fecal streptococci and enterococci. Methods for their detection and enumeration include multiple-tube dilution and membrane filter procedures.

Methods for differentiating coliforms are included. Generally, differentiation is of limited value in assessing drinking water quality because the mere presence of coliform bacteria—particularly thermotolerant coliforms or *E. coli*—renders the water potentially unsatisfactory and unsafe. However, identification may confirm the validity of coliform results or show how a distribution system is colonized. Differentiation also yields valuable information about the possible source of water pollution—especially its remoteness—because nonfecal coliforms may be expected to survive longer than thermotolerant coliforms in water (an unfavorable environment). Procedures for thermotolerant coliforms and *E. coli* include a 24-h, multiple-tube test using A-1 medium; a rapid (7-h) method; and chromogenic substrate coliform tests. Genetic testing also is becoming available.

Unlike other coliforms, those found in the guts and feces of warm-blooded animals generally include organisms capable of producing gas and/or acid from lactose in a suitable culture medium at 44.5 ± 0.2°C. Both the multiple-tube dilution technique and the membrane filter procedure have been modified to incorporate incubation in confirmatory tests at 44.5°C so analysts can estimate the density of thermotolerant organisms.

Heterotrophic plate counts may be done via pour plate, spread plate, membrane filter, or multiwell-enzyme substrate methods. The methods provide an approximation of viable bacteria that may yield useful information about water quality and indicate the significance of coliform test results. They may be used to judge the efficiency of various treatment processes, application as an in-plant control test, and to check water quality in the laboratory's reagent-grade water system or in distribution systems (by indicating microbial colonization and sediment buildup in slow-flow sections and dead ends).

Procedures for isolating certain pathogenic bacteria and protozoa are presented. These procedures are tedious, complicated, and followed by laboratory personnel with related expertise. Likewise, tentative procedures for enteric viruses are included, but their routine use is not advocated. However, these methods are useful in investigating waterborne diseases, characterizing watersheds, or determining groundwater quality.

Pollution in tidal estuaries and other bodies of saline water has raised concerns about whether existing bacteriological techniques need to be modified to analyze such waters effectively. However, the methods used to analyze fresh waters generally are also satisfactory when testing saline waters.

Methods for examining waters in swimming pools and other bathing places are included. The standard plate count procedures for thermotolerant coliforms and streptococci are identical to those used for other waters. Procedures for *Staphylococcus* and *Pseudomonas aeruginosa*—organisms commonly associated with the skin or upper respiratory tract—also are included, as are procedures for aquatic fungi and actinomycetes.

Sections on rapid coliform tests and on recovering stressed organisms are included. The quality control section has been expanded because of increased interest in this topic.

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Joint Task Group: 22nd Edition—Margo E. Hunt.

9020 QUALITY ASSURANCE/QUALITY CONTROL*

9020 A. Introduction

1. General Considerations

Due to the emphasis on microorganisms in water quality standards and enforcement activities and their continuing role in research, process control, and compliance monitoring, laboratories need to implement, document, and effectively operate a quality management system (QS) for microbiological analyses. The QS establishes an environmental testing and management operation describing both

- a quality assurance (QA) policy or program and
- quality control (QC) operational techniques and practices.

These are designed to substantiate the validity of analytical processes and data and ensure compliance with regulatory requirements, customer and project quality objectives and requirements, and applicable standards of accreditation or certification.

The laboratory practices set forth in Section 9020 represent best practices to ensure high-quality data, so use of these procedures is highly recommended for both stand-alone and mobile laboratories. These practices may be required by regulatory agencies (e.g., under the U.S. Safe Drinking Water Act, standard-setting organizations, and laboratory certification or accreditation programs).

Each laboratory develops its own QS suitable for its needs. A laboratory documents its QS's policies and objectives in a quality management plan or quality manual. The document denotes the laboratory's commitment to the QA program for integration of intra- and inter-laboratory QC activities, standardization of laboratory operating procedures, and management practices. It also clearly defines responsibilities and duties to ensure that the data are the type, quality, and quantity required.

The program should be practical. Staff should spend about 15% of overall laboratory time on the various aspects of an established QA program. That said, more time may be needed for crucial analytical data (e.g., data for enforcement actions). When properly administered, a balanced, conscientiously applied QS will optimize data quality, identify problems early, and increase satisfaction with analytical results without affecting laboratory productivity.

Microbiological analyses are inherently variable because they measure dynamic living organisms. Several of the QC tools available to microbiologists are different from those routinely used by chemists because many of the microbiologists' measurements involve discrete variables rather than continuous ones. Discrete variables have only integer values; continuous variables are not limited to particular values but rather the accuracy of the measuring tool used. Therefore, different statistics and probability distributions are also used to evaluate chemical and microbiological data.

Documented QSs will vary among laboratories as a result of differences in organizational mission, responsibilities, and objectives; laboratory size, capabilities, and facilities; and staff skills and training.

In 2012, the U.S. Environmental Protection Agency (EPA) published 12 essential QC elements for chemical pollutants analyzed under the Clean Water Act that need to be incorporated when the analytical method lacks QA/QC procedures.[1] AOAC International has developed a document[2] to address, using the EPA terminology, the 10 essential QC elements for microbiology laboratories:

- demonstration of capability (DOC),
- method blanks/sterility checks,
- laboratory QC samples/fortified blanks,
- matrix spike/matrix spike duplicates,
- calibration,
- control charts,
- corrective action,
- QC acceptance criteria,
- batch/test runs, and
- minimum frequency QC checks.

These are discussed throughout 9020 and in Part 9000 (e.g., laboratory QC samples may be considered positive and negative culture controls and matrix spikes are employed during detection of matrix effects on an analytical method and during protozoan testing).

2. Guidelines for a Quality System

The laboratory must develop, document, and implement its processes to result in controlled experimental conditions that meet its specific needs and the planned use of the data.

a. Management responsibilities: Management must evaluate the risks associated with errors, recognize the need for and actively support the QS, involve staff in QS development and operations, commit monetary and personnel resources, and assume a leadership role. Management should meet with the laboratory supervisor and staff to develop and maintain a comprehensive program; to establish specific responsibilities for management, laboratory supervisors, and analysts; and to maintain awareness of conditions through periodic and systematic review of laboratory functions. Management has overall responsibility to the end-user or customer for the QA/QC program and activities performed by laboratory analysts. While management delegates responsibilities to the QA officer, laboratory supervisor, and laboratory analyst so they may effectively carry out their individual job duties, management is ultimately responsible for the QA/QC program.

b. Quality assurance officer/quality manager responsibilities: In large laboratories, a QA officer is responsible for overseeing the QA program. Ideally, this is a staff position reporting directly to upper management, so this person has the authority and operational independence necessary to succeed. The QA officer

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should have coursework, job experience, or specialized training in microbiological testing; be acquainted with all aspects of laboratory work; be aware of and familiar with the laboratory's QA program and QC practices; and be familiar with statistical techniques for evaluating data. The QA officer is responsible for implementing the QA program and providing necessary technical support and training where needed. Once the QA program is functioning, the QA officer should sign off on all standard operating procedures (SOPs), ensure that documents are updated routinely, and conduct frequent (weekly to monthly) reviews with laboratory management and staff to ensure that the program is being followed correctly and resolve any problems that may arise. The QA officer also reports periodically to management to secure backing for any actions needed to correct problems that threaten data quality. In small laboratories, these responsibilities may be assigned to one or more staff on a part-time basis, or staff may form a QA unit. Unavoidable conflicts of interest (e.g., reviewing and signing off on one's own data) must be clarified in advance and documented.

c. Staff responsibilities: Laboratory and field staff should help management plan the QA program, help prepare SOPs, and—most importantly—incorporate the QA program and QC activities in their daily tasks (e.g., collecting samples, conducting analyses, and calculating and reporting results). Staff members are the first informed and credible sources in identifying potential problems and should work with the QA officer and laboratory supervisor to correct and prevent them. It is critical to QA program success that staff members understand what is expected of them and actively support the QA program.

3. Quality System Objectives

A major QS objective is to implement a system to produce data of known quality and provide a standard mechanism for ensuring and evaluating data quality and project objectives. In addition, other objectives include the assurance of excellent laboratory performance, continuously assessing laboratory operations, identifying weaknesses in laboratory operations, detecting analysts' training needs, improving documentation and recordkeeping, developing adequate and clear reporting systems to ensure traceability, and ensuring compliance with both regulations and the client's requirements.

4. Elements of a Quality System Manual

This written management plan or manual, describing the laboratory's policies and plans for ensuring the quality of their work for their clients, is to be reviewed annually, updated routinely, and signed by both management and the QA officer to indicate their approval and acceptance of their responsibilities. For a small laboratory, the plan should be signed by the owner/operator.

The plan should address the following:

a. Quality policy statement, which describes the QS's specific objectives, includes an ethics statement, and notes laboratory staff's and management's commitment to quality and data integrity.

b. Organization and management structure, which includes an organizational chart and describes the functions of key laboratory staff and management.

c. Personnel policies, which indicates specific qualifications, training requirements, and job responsibilities for all analysts and supervisors.

d. Equipment and instrument requirements, which includes a list of critical equipment and instruments available (including their serial and/or laboratory-assigned identification numbers), as well as the calibration, accuracy-check, and preventive-maintenance procedures and frequency required to ensure acceptable functionality before an item is put into service.

e. Specifications for supplies, which notes procedures to identify, track, and ensure that reagents and supplies are of sufficient quality and acceptable for use.

f. Specifications for subcontracting tests and calibrations, which establishes standards for the laboratory's oversight and acceptance of products.

g. Sampling procedures (if performed by the laboratory) and sample-acceptance criteria, which describes procedures for identifying, collecting, handling (e.g., transport conditions, transport time, and temperature maintenance), accepting, storing, and tracking submitted samples, along with required chain-of-custody procedures if data may be subjected to litigation.

h. Analytical methods, which lists the laboratory's scope for testing, its validation procedures for nonstandard or new methods, the accreditation/certification status for individual methods and analytes, and the requirements for initial and ongoing demonstrations of capability.

i. Analytical quality control measures, which states the laboratory's requirements for measurement assurance (e.g., method verification and documentation; error prevention; analytical checks, such as replicate analyses, positive and negative culture controls, blanks, sterility checks, verification tests, performance evaluations/proficiency tests; and tests for determining analyst variability) and the statistical methods to be used, where necessary.

j. Standard operating procedures (SOPs), listing all generic laboratory processes and specific routine laboratory analyses. These are documented in a manner that reflects actual methodologies in use, signed by management, as well as appropriate staff and the QA officer, include the dates they were last revised, are readily accessible to staff, and are available to clients upon request.

k. Documentation control and recordkeeping requirements, which identifies the recordkeeping format(s) (e.g., hard-copy, e-notebooks, and computer files) and procedures to ensure data review, traceability, and accountability. It describes the procedures required to ensure customer confidentiality, where applicable; to maintain original data when revision is required; to establish levels of data access for revisions; to ensure security for data stored both onsite and offsite; and to handle other issues, such as record retention time and record disposal.

l. Assessments, which describes the laboratory's processes to monitor and report on the effectiveness of its QA program.

1) Routine internal audits of laboratory operations, performed at least annually by the QA officer and supervisor. For a small laboratory, an outside expert may be needed. These audits should be comprehensive, including analyses conducted, analyst technique, data manipulations, and reporting.

2) Onsite evaluations by third-party experts to ensure that the laboratory and its personnel are following an acceptable QA program. This is a required component of laboratory certification or accreditation and of analyst certification.

3) Proficiency test (PT) studies, in which the laboratory generally participates once or twice a year. These collaborative studies should confirm the laboratory's ability to generate acceptable data comparable to both the reference laboratory and other participants. They also should identify any potential issues to address.

m. Corrective and preventive activities, which identify procedures used to determine the causes of identified problems and to record, correct, and prevent their recurrence. They indicate continual improvement. Another name for this process is *root-cause analyses* (the systematic process of identifying the cause of a problem or issue, generally through a multi-step process, and developing corrective action plans to prevent recurrences).

n. Customer service, which denotes the laboratory's commitment to internal and external customers. It describes procedures for responding to customer requests and complaints, as well as ensuring customer confidentiality and proprietary rights.

The QC guidelines discussed in 9020B and C are recommended as useful source material of elements that need to be addressed when developing policies for a QA program and QC activities. More information is available from several standards-setting organizations, such as the American Association for Laboratory Accreditation (A2LA), Association of Official Analytical Chemists (AOAC) International Inc., International Organization for Standardization (ISO), The NELAC Institute (TNI), and the U.S. Environmental Protection Agency (EPA).

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9020 B. Intralaboratory Quality Control Guidelines

Quality control (QC) practices are designed to ensure that the laboratory's processes are under control. All laboratories have some intralaboratory QC practices that have evolved from common sense and controlled-experimentation principles to indicate method efficiency and laboratory performance. A laboratory's QS sets in place the QA policies or program and QC activities necessary to minimize systematic and random errors resulting from variations in personnel, instrumentation, equipment, reagents, supplies, sampling and analytical methods, data handling, and data reporting. It is especially important that laboratories performing only a limited amount of microbiological testing exercise strict QC. A listing of key QC practices is given in Table 9020:I and discussed below. Additional sources of information about laboratory QC practices are available.[1–10] Laboratories should address all of the QC guidelines discussed herein, but the depth and details may differ for each laboratory. Many items mentioned here are also applicable to other laboratories (e.g., chemical and radiological laboratories). However, microbiology laboratories testing under Good Manufacturing Practices (GMP)/Good Laboratory Practices (GLP) regulations should note that certain QC practices may differ from those listed here.

1. Personnel

Microbiological testing should be performed by a professional microbiologist or technician with an appropriate level of education, training, and laboratory bench experience in general microbiological techniques that are employed at the laboratory. If such personnel are unavailable, a professional microbiologist must provide training in specific techniques and be available to review work.

TABLE 9020:I. KEY QUALITY CONTROL PRACTICES

Item	Action	Frequency	Further Information in Section 9020B, ¶
Air in workplace	Monitor bacterial density	Monthly	3*e*
Autoclave	Check temperature with max-registering device	Weekly	4*h*
	Check performance with bioindicator	Monthly	
	Check timing	Quarterly	
Balances	Check zero	Daily before use	4*b*
	Check accuracy with at least 2 weights		
	Service and recalibrate	Monthly, preferably	
Biosafety cabinet	Inspect for airflow	Each use	4*m*
	Have certified	Annually	
Conductivity meter	Calibrate	Monthly	4*q*
Dilution water bottles	Check sterility, pH, and volume	Each batch or lot	5*c* and 9050C.1*a*
Freezer	Check temperature	Daily	4*j*
	Defrost	Annually	
Glassware	Inspect for cleanliness, chips, and etching	Each use	5*a*
	Check pH with bromthymol blue	Each wash batch	
	Conduct inhibitory residue test	Initial use and new washing procedure (also may be annual)	
	Check for autofluorescence if used for testing	Each batch or lot	
Hot-air sterilizing oven	Check temperature	Each use	4*g*
	Check performance with bioindicator	Monthly	
Incubator	Check temperature	Twice daily when in use	4*n* and *o*
Media	Check sterility, pH, and appearance	Each batch or lot	5*j*
	Check performance with + and – culture controls	Each batch or lot	
	Check recovery of new vs. old media	Before first use	
Media-dispensing apparatus	Check volume dispense accuracy	Each volume change	4*f*
Membrane filters	Check sterility and properties	Each new lot	5*i*
Membrane-filtration equipment	Check for leaks and surface scratches	Each use	4*k*
	Check sterility	Pre- and post-test	
	100-mL volume check	Initially	
Micropipetters	Check dispense accuracy and precision	Quarterly or more frequently if heavily used	4*s*
	Calibrate	Annually	
Microscope	Clean optics and stage, check alignment	Each use	4*p*
Multi-well sealer	Check performance	Monthly	5*e*
pH meter	Standardize with at least 2 buffer solutions	Each use	4*c*
	Determine slope	Daily	
Plate counts	Perform duplicate analyses	Monthly	9*a*
	Repeat counts	Monthly	
Reagent water	Monitor quality	See Table 9020:II	
Refrigerator	Check temperature	Daily	4*i*
Sample bottles	Check sterility	Each batch or lot	5*d*
	Check decholorination agent efficiency	Each batch or lot	
	Check 100 mL line	Each lot	
	Check for autofluorescence if also used for testing	Each lot	
Temperature devices:			4*a*
Working units	Check accuracy	Annually, preferably semiannually	
Reference units	Recertify	Every 5 years	
Timer:			4*h*
Autoclave	Check timing with stopwatch	Quarterly	
Stopwatch	Check against National Time Signal	Annually	
UV lamps, short-wave disinfection	Monitor bulb use	Each use	4*l*
	Test with UV meter or perform plate count check	Quarterly	
Weights:			4*b*
Working	Check with reference weights		
Reference	Recertify	Annually	

Education provides the theory and basic science of microbiology. Training should detail proper techniques and demonstrate the negative consequences (e.g., when SOPs are not followed properly). For specialized testing, such as protozoan or molecular analyses, additional training and bench experience is required. For each analytical method performed, analysts must demonstrate capability in performing laboratory operations before generating reportable data (initial DOC) and periodically thereafter (ongoing DOC) using blind samples (preferred) or known positive samples.

The supervisor should routinely evaluate and document the technician's skills. Sample collection (if performed by the laboratory), sample handling, media and glassware preparation, sterilization, clean room gowning and access requirements, aseptic techniques, routine analytical testing, counting, data handling, and QC techniques to identify and eliminate problems should be closely monitored. Management should help laboratory personnel obtain additional training and course work to enhance their technical skills and advance their careers. Employee training records and performance scores obtained by analyzing single-blind samples, especially for enumeration methods, and DOCs should be reviewed/monitored and maintained.

2. Biosafety Criteria

Biosafety is a concern for all microbiological laboratories to prevent exposure. There are three elements to consider: laboratory practices, safety equipment, and facility design. Risk assessments of the work performed for each biological agent will determine the appropriate combination of these elements. Personnel must be trained in aseptic techniques and wear personal protective equipment (PPE) (safety glasses, protective clothing, gloves, etc.). For example, PPE clothing and gloves should not be worn outside the laboratory, nor should equipment be routinely moved in and out of the microbiology laboratory. Also, report all accidents and "near-misses."

The U.S. Centers for Disease Control and Prevention (CDC) Public Health Prevention Service divides laboratories handling potentially hazardous biological agents into four biosafety levels (BSLs). Each BSL denotes a combination of laboratory facilities, practices, techniques, and safety equipment appropriate for the laboratory function or activity, the operations performed, the infectious agents' suspected transmission routes, and risk mitigation. A brief discussion of each BSL follows; however, detailed information on special practices, containment, and facilities for BSLs 3 and 4 are not included here. For further information on all BSLs, review CDC's protocols.[11]

a. Biosafety level 1 (BSL 1): According to CDC, BSL 1 is suitable for work involving well-characterized agents not known to consistently cause disease in healthy adults and of minimal potential hazard to laboratory personnel and the environment. Work is generally conducted on open bench tops using standard microbiological practices. The agents listed in *Standard Methods* that should be handled under BSL 1 practices are total and thermotolerant (fecal) coliform bacteria, *E. coli*, enterococci, iron and sulfur bacteria, actinomycetes, and other nonpathogenic microorganisms. It is up to the laboratory director to determine which biosafety practices to follow based on the samples and agents involved.

The standard practices and safety equipment for BSL 1 are as follows:

1) Access to the laboratory is limited or restricted at the laboratory director's discretion by posting a sign (e.g., "Restricted Area—Biohazard Laboratory Personnel Only") when experiments or work with samples are in progress. Ensure that doorways and windows are closed when aseptic work is in progress.
2) Wash hands thoroughly with soap and water after handling viable materials, after removing gloves, and before leaving the laboratory.
3) Do NOT eat, drink, smoke, handle contact lenses, apply cosmetics, operate personal cell phones or portable music devices, wear open-toed shoes, or store food for human use in work areas.
4) Do NOT pipet by mouth.
5) Establish and follow policies for safely handling sharp items.
6) Decontaminate work surfaces before and after each use and after any spill of viable material.
7) Decontaminate all cultures, stocks, and other regulated wastes before disposal by an approved decontamination method, such as autoclave sterilization. Keep related decontamination records.
8) Establish and maintain an insect- and rodent-control program.
9) Wear laboratory coats, gowns, or uniforms to avoid contaminating or soiling street clothes. Safety glasses are recommended. Wear gloves, especially if there is a rash or open lesion on the hands. Perform all procedures so no aerosols or splashes occur.

b. Biosafety level 2 (BSL 2): BSL 2 involves work with agents of moderate potential hazard to personnel and the environment. The agents listed in *Standard Methods* that require BSL 2 practices are the pathogenic microorganisms described in the various sections of Part 9000. In addition to BSL 1 practices listed above, BSL 2 requires that laboratory personnel have specific training in handling pathogenic agents; access to the laboratory is limited when work is in progress; extreme precautions are taken with contaminated sharp items; and procedures that could create infectious aerosols be conducted in biological safety cabinets (BSC). BSCs are designed to protect microbiologists from microbial contaminants in samples. If available, appropriate immunizations should be given.

The standard practices and equipment for BSL 2 include all those listed for BSL 1 and the following:

1) Always be very careful with any contaminated sharp items, including needles, syringes, slides, pipets, capillary tubes, and scalpels.
2) Decontaminate work surfaces whenever work is started and completed, at the end of the day, and after any spill or splash of viable material, using disinfectants that are effective against the agents of concern.
3) Place cultures or potentially infectious wastes in a container labeled "Biohazardous Waste" with a cover that prevents leakage during collection, handling, processing, storage, transport, or shipping.
4) Use BSCs (preferably Class II) or other appropriate PPE whenever conducting procedures that could create infec-

tious aerosols or splashes and whenever using high concentrations or large volumes of infectious agents.
5) Use face protection to avoid splashes or sprays of infectious materials whenever the microorganism must be manipulated outside the BSC.
6) Wear appropriate laboratory coats, gowns, or uniforms; gloves; and safety glasses while in the laboratory. Leave these in the laboratory before exiting to non-laboratory areas.
7) Wear gloves when hands could contact potentially infectious materials, contaminated surfaces, or equipment.

c. Biosafety levels 3 and 4: BSLs 3 and 4 involve working with indigenous, dangerous, or exotic agents that may cause serious or potentially lethal disease as a result of inhalation and contact. Because *Standard Methods* does not address agents in these categories, the special practices, containment, and facilities for these levels are only outlined here.

1) BSL 3—The standard practices and equipment for BSL 3 include all those listed for BSLs 1 and 2. BSL 3 also requires that personnel be professionally trained in handling infectious materials. The laboratory must be secured and access limited. Work must be conducted within BSCs by personnel wearing appropriate PPE. No one with open lesions should enter the laboratory. There should be passages between the outer hallway and the laboratory entrances where personnel can change into PPE; the doors at each end of these passages should NOT be able to open at the same time or else these safety barriers are compromised. All potentially contaminated material (gloves, laboratory coats, etc.) must be decontaminated before disposal or reuse.

2) BSL 4—BSL 4 is for biological agents, often exotic, that are extremely hazardous both to personnel and/or the environment. The standard practices and equipment for BSL 4 include all those listed for BSLs 1, 2, and 3. BSL 4 also requires that laboratory access be strictly controlled and situated in a clearly marked area removed from normal operations or in a separate building. Personnel must completely disrobe and put on laboratory-specific clothing before entering test areas and be decontaminated before leaving.

3. Facilities

Develop an environmental control policy to ensure that environmental conditions do not invalidate results, impair measurement quality, or harm personnel.[12] Health and safety policies and procedures must be posted or readily available. The factors to be considered and monitoring to be performed are described below. Much of this information applies to any laboratory facility.

a. Ventilation: Design and construct well-ventilated laboratories that can be kept free of dust, drafts, and extreme temperature changes. Install heating, ventilation, and air-conditioning (HVAC) and humidity-control systems to reduce contamination, permit more stable operation of incubators, and decrease moisture problems with media and instrumentation. Adjust HVAC vents so airflow does not blow directly on the working surface areas. Where feasible, ensure that air only flows into (rather than out of) the laboratory to avoid the possibility of contaminating other areas of the building.

b. Space utilization: To ensure test and sample integrity and minimize potential contamination, design and operate the laboratory to minimize through traffic and visitors. Do not obstruct entrances and exits.

Ensure that there is sufficient workspace available for the volume of work to be performed. For example, maintain separate work areas for sample receipt; media, glassware, and equipment preparation and sterilization, and decontamination of media and glassware; testing and culturing; and data handling and storage. Maintain heat-generating equipment, such as autoclaves, in a room separate from incubators. Using a hood or BSC to dispense and prepare sterile media, transfer microbial cultures, or work with pathogenic materials is recommended. In smaller laboratories it may be necessary, although undesirable, to perform these activities in the same room; however, do not perform them near open doorways or windows. Have sufficient storage space available in the laboratory to store materials (e.g., reagents, glassware, and laboratory supplies) appropriately.

c. Laboratory bench areas: It is optimal to provide at least 2 m (6 ft) of linear bench space per analyst and additional areas for preparation and support activities. Bench height should be reasonable and comfortable for the analysts. For stand-up work, typical bench dimensions may range from 90 to 97 cm (35 to 38 in.) high and 70 to 76 cm (27 to 30 in.) deep. For sit-down activities, such as microscopy and plate counting, benches may range from 75 to 80 cm (29 to 32 in.) high. Specify benchtops of stainless steel, epoxy plastic, or other smooth, impervious surfaces that are inert and corrosion-resistant with minimal seams and NO cracks or crevices. Install even, glare-free lighting with about 1000 lux (100 ft-c) intensity at the working surface; test using a photometer.

d. Walls, floors, and ceilings: Ensure that walls are covered with a smooth finish that is easily cleaned and disinfected. Specify floors of smooth concrete, vinyl, asphalt tile, or other impervious, sealed washable surfaces. Specify smooth, nonfibrous ceiling surfaces and recessed lights.

e. Work area: Keep work areas clean. Disinfect surfaces before and after testing. Sterilize contaminated supplies and media promptly after use. Institute a regular preventive-maintenance policy for work areas and equipment, such as incubators, waterbaths, and refrigerators. Avoid buildup of water in refrigerator drip pan, and clean all vent filters.

Monitor air quality routinely—at least monthly or more frequently if area is heavily used or biocontamination risk analysis indicates more frequent monitoring is needed. In aseptic work areas, use air-density settling plates (a passive sampling process wherein particles can settle on the agar surface). If risk assessment indicates the potential for aerosol contamination, use active air samplers.[4] Replicate organism detection and counting (RODAC) contact plates or the swab method[1] can be used weekly or more frequently to monitor bench-surface contamination.

Although uniform limits for bacterial density have not been set, each laboratory can use passive or active air-monitoring systems to establish baselines for specific work areas, evaluate trends, establish alert and action levels, and take appropriate action when necessary. Start by averaging the results obtained from tests over a period of time to determine the typical bacterial density for a given location. In general, airborne bacterial populations should not exceed colonies/plate/15 min exposure, or 1 colony-forming unit (CFU) per minute. Longer exposure times can be used, but water loss may occur and reduce growth potential. In addition to this surveillance system, the laboratory

may wish to identify contaminants recovered with commercially available automated identification systems.

Prevent adverse sound and vibration levels in the laboratory. Install easy-to-clean sun shades on large glass windows to prevent heat buildup.

f. Laboratory cleanliness: Regularly clean laboratory rooms and wash benches, shelves, floors, windows, overhead lights, and exposed pipe surfaces. Wet-mop floors and treat with a disinfectant solution weekly; do not sweep or dry-mop. Wipe benchtops and treat with a disinfectant at least daily, or more frequently depending on the biosafety level required for the work being done (see 9020B.2). Do not permit laboratory to become cluttered. Store supplies and paperwork away from benchtops. Eliminate or cover any overhead pipes that cannot be cleaned routinely. Keep liquid hand soap (preferably in a gravity-fed touchless sensor dispenser) and paper towels (touchless paper roll dispenser can be used) available at laboratory sinks. Do not allow smoking or consumption of food or drink in the laboratory. Dispose of laboratory materials properly (e.g., by autoclave sterilization or incineration).

g. Electricity: Ensure a stable source of electricity, a sufficient number of outlets [including ground fault circuit interrupter (GFCI) outlets where needed], and appropriately placed surge protectors. An emergency power backup and alarm system may be necessary where the work is critical.

4. Laboratory Equipment and Instrumentation

Identify equipment by serial number or unique laboratory reference number. Implement procedures to verify that each identified piece of equipment is installed properly and operating consistently and satisfactorily. Verify by constant monitoring, routine maintenance, and a regular calibration schedule that each item meets the user's needs for precision and minimization of bias. Provide written procedures on the use, operation, calibration, and maintenance of relevant equipment and instruments, along with relevant QC acceptance criteria (see 9020B.6). Keep manufacturers' manuals available for easy retrieval. Perform equipment standardization/calibration using reference standards and maintain equipment regularly, as recommended by the manufacturer, or obtain preventive-maintenance contracts on autoclaves, balances, microscopes, and other critical equipment. Directly record all QC checks in dedicated logbooks, three-ring binders, or electronic records, and maintain documentation so it is accessible for the time period mandated by law. Develop a system for "flagging" problems and related corrective actions.

Ensure that the laboratory has all equipment and supplies required to perform environmental tests and calibrations. For molecular testing, the laboratory's equipment and supplies need to be dedicated to specific rooms.[9] Keep enough equipment and supplies where needed so they are not routinely moved from one laboratory area to another. When certain equipment is only available offsite, document how the laboratory will ensure that all QC factors will be satisfactory. Maintain all documentation showing determination of acceptability for equipment, instruments, and supplies, as well as all analytical analyses. Keep the records in a permanent record format, such as a notebook, e-notebook, or computer file.

Use the following QC procedures for both applied and research laboratories (equipment needed for specialized testing may not be listed here):

a. Temperature-sensing and -recording devices: Historically, microbiology laboratories used mercury-filled thermometers, but many states have discouraged the use of such thermometers due to environmental concerns about mercury's neurotoxicity. The National Institute of Standards and Technology (NIST) stopped calibrating mercury thermometers in 2011 (http://www.nist.gov/pml/mercury.cfm). Instead, microbiology laboratories can use organic fluid-filled analog thermometers or digital sensing devices. Review established thermometer guidance[13] for information on the three types of sensors—platinum resistance sensors, thermistors, and thermocouples—used in digital thermometers. Ensure that the thermometer markings are legible and the liquid column or glass case has no break or change. Discard thermometers with illegible graduation marks.

Use thermometers with temperature increments of 0.5°C or less, as appropriate (e.g., for a 44.5 ± 0.2°C water bath used for incubation of thermotolerant bacteria, use a thermometer with 0.1°C increments). Thermometers used in refrigerators or sample-receipt areas may have temperature increments of 1 or 0.5°C. If using liquid-based thermometers to measure temperatures in air incubators and refrigerators, keep thermometer bulb in water or glycerol. When testing temperatures exceed 50°C (e.g., autoclave spore check functionality—50 to 64°C), place the thermometer bulb in a glass container filled with sand.

Another option is to equip incubators, water baths, etc. with temperature-recording instruments that continuously monitor the operating temperature. These wired or wireless data-logging systems can be downloaded into a computerized or printed record. Data-logging units must meet the same requirements as temperature-sensing devices. Establish a system for recording information from data-logging units so analysts are aware of temperature violations shortly after they occur; can invalidate test samples, as appropriate; and can collect new samples. Also, establish a documentation system of data-logger results so time/temperature readings can be used to trace a sample and its testing conditions during laboratory assessments.

Annually, or preferably semiannually, verify the accuracy of all working temperature-sensing devices (e.g., liquid-in-glass thermometers, thermocouples, and temperature-recording instruments) at the use temperature(s). To do this, compare each device's measurements to those of a certified NIST temperature-sensing device or one traceable to NIST and conforming to NIST specifications. Discard temperature-sensing devices that differ by >1°C from the reference device.

For ambient water-temperature readings, ensure that the water has come to equilibrium by letting it sit for at least 1 h. Use a circulating water bath or beaker of water with stir bar set at the appropriate test temperature. When conducting an ice-point check, use reagent grade water and ice (i.e., the concentrations of minerals, salts, etc. in the water and ice should NOT inhibit reaching the true ice-point determination).

Perform a three-point verification testing (at, below, and above the temperature at which the temperature-sensing device will be used). Record accuracy-check results—along with the date, device identification number, and the technician's signature or initials—in a QC logbook. If a correction calculation is necessary, mark the appropriate correction factor on the device so only

corrected temperature values are recorded. For example, when ice-point temperature determination does not match certificate value, adjust all subsequent temperature readings by the same amount (difference in temperature) or submit the unit for recertification (a new certificate of accuracy). Verify accuracy of the certified reference temperature-sensing device as often as specified on the certificate of accuracy or at least once every 5 years. Some accreditation organizations or federal or state agencies may require more frequent verification/certification.

b. Balances: Locate balances in areas without rapid air movement (e.g., drafts) and level them on firm, even surfaces to prevent vibrations. Re-level balance each time it is moved to a new location. Check balance routinely (preferably daily before use) using at least two working weights that bracket the normal usage range. Before each use, clean balance and tare weight before adding reagents to weigh paper or boats. Clean balance pans after use and wipe spills up immediately with a laboratory tissue. Follow manufacturers' instructions for operation and routine maintenance of analytical and top-loading balances.

Use only plastic-tip forceps to handle weights. Check working weights monthly for accuracy, precision, and linearity against a set of reference weights of known tolerance[14] [e.g., ANSI/ASTM Class 1, 2, or 3 or NIST Class S (redefined as ASTM Class 1 weights and no longer available), accompanied by appropriate calibration certificate]. Record results along with date and technician's initials. If weights are corroded or dropped, have them professionally cleaned and recertified or replace them.

Service balances annually, or more often as conditions change or problems occur, following in-house protocols or through service contracts. Recertify reference weights as often as specified in the calibration certificate, or at least once every 5 years.[15,16] Some regulators or accreditors may require that reference weights be recertified more frequently.

c. pH meter: Use a digital meter, graduated in 0.1 pH units or less, that includes the theoretical slope of temperature compensation because the electrode pH response is temperature-dependent. Use electrodes suitable for a wide temperature range, and use a flat-head electrode to measure solid agar media. Measure test solution's pH at a temperature near that used to calibrate the meter. The most desired temperature range for determining pH is 18 to 25°C (room temperature). Keep the probes clean and store electrode immersed in the manufacturer-recommended solution.

Use only commercial buffer standard solutions for calibrations, and standardize pH meter with at least two certified pH buffers (usually pH 4 and 7 or pH 7 and 10) that bracket the pH of the sample being measured (two-point standardization). Record standardization results, date, and technician's initials. Immediately after use, discard the buffer-solution portions or single-use/ready-to-use solution packets used to standardize meter. Discard all buffer solutions made from packets after 1 d.

Each time a fresh bottle of buffer solution is opened, inscribe the date on bottle and in logbook; thereafter, check the bottled solution monthly against another pH meter, if possible. Replace pH buffer supply containers by the expiration date, preferably 6 months after opening because the solution may absorb carbon dioxide.

To verify that the pH meter is functioning properly, measure and record its slope after standardization daily (or each day it is used). Most meters provide slope values automatically. If the pH meter does not calculate the slope automatically, but can provide the pH in millivolts (mV), use the following formula to calculate the slope:

$$\text{Slope, as } \% = |\text{mV at higher pH} - \text{mV at lower pH}| \times 100/177$$

If the slope is $<95\%$ or $>105\%$, the electrode or meter may need maintenance. If all three buffers are used in sequence to standardize the meter (three-point standardization), analysts may provide either both slopes or an average. For full details of pH meter use and maintenance, see Section 4500-H^+ or follow manufacturer's instructions.

d. Water-purification system: The quality of laboratory-prepared reagent water depends on the quality of the source water and the water-purification equipment used to develop and store it. Commercial systems are available that include some combination of prefiltration, activated-carbon filter, ion-exchange cartridge or cylinder, and reverse osmosis with final filtration to produce reagent-grade water. Such systems tend to produce the same water quality until the ion-exchange resins or activated carbon is near exhaustion; then, the quality abruptly becomes unacceptable. Some deionization components now automatically regenerate the ion-exchange resins.

Do not store laboratory-prepared reagent water unless a commercial ultraviolet (UV) irradiation device is installed and confirmed to maintain sterility. Maintain and monitor the equipment routinely to ensure that the water meets the appropriate standards. Every day laboratory-prepared reagent water is used, monitor it with a standardized conductivity meter (see ¶ *q* below). Each month (or use, as appropriate), determine total chlorine residual and heterotrophic bacteria concentrations, which may provide an early warning of potential problems. Increasing bacteria numbers indicate the possible presence of complex organic compounds, inorganic compounds, and endotoxins that can be nutrient sources for bacteria. At least once a year, analyze reagent water for trace metals. Also, perform the bacteriological water quality test annually, and whenever the water-purification system is modified or repaired. The water quality test described in 9020B.5*f*1) is not required for Type II water or medium-quality water or better, as defined in 20th, 21st, 22nd, and Online Editions of *Standard Methods*, Section 1080C, or in other widely accepted standards.[17] Most systems used today meet or exceed these standards. Perform the use test [see 9020B.5*f*2)] whenever there is a new source of water or new water system employed in the laboratory.

Replace cartridges at manufacturer-recommended intervals based on the estimated usage and source water quality. Do not wait for column failure. If bacteria-free water is desired and a UV irradiation device is unavailable, use a 0.2-μm-pore membrane filter for aseptic final filtration and collect in a sterile container. Monitor treated water for contamination and replace filter as necessary.

e. Water still: Stills produce good water that characteristically deteriorates slowly over time as corrosion, leaching, and fouling occur. These conditions can be controlled with proper maintenance and cleaning. Stills efficiently remove dissolved substances but not dissolved gases or volatile organic chemicals. Freshly distilled water may contain combined chlorine and ammonia (NH_3), and stored distilled water will absorb more NH_3 and carbon dioxide (CO_2) from the air. Drain and clean still and reservoir according to manufacturer's instructions and usage. To

reduce cleaning frequency, use softened water as the source water.

f. Mechanical media-dispensing apparatus: Check apparatus' accuracy by dispensing a sample volume of medium into a graduated cylinder just after filling/refilling it and periodically throughout extended runs; record results. Before dispensing medium for sample analyses, flush apparatus with a small volume of medium to ensure that evaporation has not blocked the tip or changed the concentration of reagent. Between runs, rinse apparatus by pumping hot reagent-grade water through it. Correct leaks, loose connections, or malfunctions immediately. At the end of use, break apparatus down into parts, wash, rinse with reagent-grade water, and dry. Lubricate parts according to manufacturer's instructions or at least once per month.

g. Hot-air sterilizing oven: Test performance monthly with commercially available strips of a spore-forming microorganism (e.g., *Bacillus atrophaeus*) that ideally has a minimum spore density of 1×10^6. Test the strip in glassware similar to the items being sterilized. Measure oven temperature with a thermometer whose bulb is placed in sand, a thermocouple-type probe, or a continuous-read temperature recorder. The temperature-measuring device must be accurate in the 160 to 180°C range. Record results and contents when in use. Use heat-indicating tape, chemical strips, or Diack tubes to identify supplies and materials that have been exposed to sterilization temperatures.

h. Autoclave: For new autoclaves, conduct an initial temperature profile to determine any hot or cold spots throughout the unit, using probes placed in various areas.

When filling the autoclave, avoid overcrowding (e.g., do not place racks on top of each other; leave space between racks and flasks so steam can flow past individual test tube racks and flasks). After each run cycle, record the items sterilized, sterilization temperature, total run time (heat exposure), programmed/preset sterilization period, actual pressure readings, and analyst initials.[18,19] New units may print most of this information on tape automatically (i.e., time, temperature, and pressure at selected time interval). For older units, if possible, use a recording thermometer chart or electronic high-temperature data logger (HTDL).

For general sterilization tasks, the recommended autoclave temperature range is 121 to 124°C (at 200 kPa), although higher temperatures (≥121°C) are acceptable for decontaminating laboratory materials. Ensure that the autoclave maintains 121°C with minimal temperature variation at ≥15 lb/in.^{2} (≥103 kPa) for 15 min during the media sterilization cycle and that media are withdrawn from the autoclave in 45 min or less. Autoclave temperature control tolerances may vary, depending on the nature of the media being sterilized. In these cases, follow the relevant recommended procedures. Typically, however, keep temperature within ±2°C of prescribed temperature for media (and ±10 kPa of recommended P).

Some regulatory programs and new media may require a different temperature/pressure/time sequence. Certain media may be heat sensitive and require narrow temperature tolerances. If media contain lactose, for example, excessive heat exposure (i.e., autoclaving too long or at too high a temperature) will result in lactose hydrolysis, rendering the medium unsuitable for its intended use. Other media may need a shorter autoclave cycle. Review all pertinent information for these cases. See 9020B.5*j* for further discussion.

For routine use, verify autoclave temperature weekly with a maximum registering thermometer (MRT) (generally a mercury-filled Teflon-coated device) or accurate HTDL able to withstand 15 to 20 lb/in.^{2} If neither device is available, use a strip or pie chart recorder with interpretations written on the chart. Maintain verification records. Using a chemical steam indicator for each cycle will show if minimum exposure conditions were met but will not indicate whether sterilization was achieved. Heat-indicating tape can quickly identify supplies and materials that have been sterilized.

Maintaining proper autoclave functions is critical. Test monthly for sterilization efficacy at the media's normal sterilization time and temperature using a biological indicator (e.g., commercially available *Geobacillus stearothermophilus* in spore strips, suspensions, or capsules, preferably at a 1×10^6 concentration). Place the indicator in glassware containing a liquid to simulate actual autoclave sterilization performance on media.[20] Some biological indicators may require more time at the sterilization temperature than is used for most carbohydrate media. If this becomes problematic, use biological indicators for autoclave runs that exceed 20 min (e.g., dilution water and contaminated materials).

Each quarter, use a calibrated timer or national time signal to check the timing of all three cycles for a media run (≤15 min conditioning cycle, 15 min sterilization cycle, and ≥15 min exhaust cycle). Keep autoclave clean and free of debris by checking both trap and seals monthly. Service autoclaves annually either in-house or through service contracts.

i. Refrigerator: An initial temperature profile is suggested to determine any hot or cold spots in the unit. Maintain temperature at 2 to 8°C and monitor it using either thermometers whose bulbs are submerged in distilled water or glycerol solution, or digital temperature-sensing devices placed on the top and bottom shelves of each use area. Every day while in use, check and record temperature (corrected, if necessary), also noting date and observer's initials. Identify and date materials stored in refrigerator, and discard outdated materials monthly. Clean units annually, or more frequently if needed.

Frost-free units may dehydrate stored media more quickly because heating is used to prevent ice buildup. Flammable materials should be stored in explosion-proof refrigerators. Volatile organic chemicals should not be stored in the same refrigerator used for microbiological media, reagents, or cultures.

j. Freezer: The freezer temperature range depends on analytical need (e.g., a standard laboratory freezer may range from −15 to −25°C, while an ultra-low freezer may range from −70 to −90°C). A recording thermometer and alarm system are highly desirable. Every day while in use, check and record corrected temperature(s), also noting date, time, and observer's initials. Avoid opening units repeatedly because frost will build up and make the freezer less efficient. Identify and date (e.g., manufacturer- or lab-expiration) materials stored in freezer. Storing materials in insulated boxes with snug-fitting lids and away from freezer walls may be beneficial. Defrost and clean units annually (or more frequently, as needed); discard outdated materials.

Frost-free units may dehydrate stored media more quickly because heating is used to melt ice buildup. Store flammable materials in explosion-proof freezers.

k. Membrane filtration equipment: Before initial use, assemble filtration units and check for leaks. Discard units if chipped or inside surfaces are scratched. Units that leak should be repaired accordingly or discarded. Replace damaged screens on stainless steel units. Wash and rinse filtration assemblies thoroughly after use, wrap in nontoxic paper or foil, and sterilize via autoclave or dry heat oven. When measuring sample volumes using funnels with volumetric graduation marks, initially check the marks' accuracy using a Class A graduated cylinder or volumetric pipet. Record results. For presterilized single-use funnels, check one per lot or a set percentage (e.g., 1 to 4%) to confirm the accuracy of volumetric graduation mark.

l. Ultraviolet lamps:

1) Short-wave ultraviolet lights (254 nm)— Germicidal short-wave UV lights are commonly used to sanitize, not sterilize, such items as membrane filtration units. When in use, disconnect lamps monthly and clean bulbs with a soft cloth moistened with ethanol (70% ethanol/30% reagent-grade water) or with spectroscopic grade 2-propanol in areas where baked-on material is collecting. Test lamps quarterly with an appropriate (short-wave) UV light meter, and replace bulbs when output drops to <70% of initial output. Alternatively, expose spread plates containing 200 to 300 CFU/mL of a selected bacterial suspension for 2 min. Incubate plates at 35°C for 48 h and then count colonies. Replace bulb if colony count is not reduced 99%. It also is advisable to ask the manufacturer for the bulb's expected lifespan and then track hourly usage.

2) Long-wave ultraviolet lights—Long-wave (365–366-nm) UV lights are used to determine fluorescence. Analysts should use 6-W lamps because faint fluorescence may not be visible when using 4-W lamps. Keep units clean, periodically use a light meter to check that the bulb remains at the proper wattage, and replace the UV light yearly.

CAUTION: Although the short-wave (254-nm) UV light is known to be more dangerous than long-wave (365-nm) UV light, both can damage eyes and skin and are potentially carcinogenic.[21] Protect eyes and skin from exposure to UV light. Consider installing a lockout mechanism so laboratory lights cannot be turned on without turning off overhead UV lights, if used. (See Section 1090B.)

m. Biohazard safety cabinet (BSC): Properly maintained Class I and II BSCs, along with good microbiological techniques, provide an effective containment system for safely manipulating moderate- and high-risk microorganisms (BSL 2 and 3 agents). Both Class I and II BSCs have inward face velocities (80 to 100 linear ft/min) designed to protect laboratory workers and the immediate environment from infectious aerosols generated inside the cabinet. Class II BSCs also protect the material itself through high-efficiency particulate air (HEPA) filtration of the airflow down across the work surface (vertical laminar flow). Standard operating procedures are as follows:

1) Before and after use, purge air for 10 to 15 min and wipe down unit with disinfectant. Use a piece of tissue to confirm inward airflow.
2) Enter straight into cabinet and perform work slowly and methodically. Place material well within cabinet—not on front grill—and do not disrupt or block laminar airflow. Place discard pan within cabinet.
3) Decontaminate interior of BSC after work is completed and before it is removed. Allow cabinet to run for 10 to 15 min and then shut off.[22]

Provide for testing and certification of Class I and II BSCs *in situ* when they are installed, moved, and at least annually thereafter. Maintain cabinets as directed by the manufacturer. Avoid using a Bunsen burner inside BSCs because it will change airflow and may destroy the HEPA filter. Do not allow workspace to become crowded because objects may disturb airflow pattern, allowing contaminant(s) to exit at the face opening. Place working objects at least 6 in. from the face.

n. Water bath incubator: Verify that water bath incubators maintain the set temperature, such as 35 ± 0.5°C or 44.5 ± 0.2°C; use an appropriately marked total immersion thermometer if available (¶ *a* above). When incubator is in use (i.e., samples are being incubated), monitor and record corrected temperature twice daily separated by 4 h.

Electronic temperature-sensing devices (i.e., data loggers) may be used so long as the laboratory's system for recording information from the devices also promptly notifies analysts of temperature violations so they can invalidate test samples, as appropriate, and request that new samples be collected. This system also must document the data such that time/temperature readings can be used to trace a sample and its testing conditions during laboratory assessments. Each data logger should be marked with any correction factor needed.

Fill unit only with reagent-quality water. Maintain water level so it is above the upper level of the medium in either tubes or flasks. Equip water bath with a gable cover to prevent evaporation and with a circulating pump to maintain even temperature distribution. Use only stainless steel, plastic-coated, or other corrosion-proof racks. Use screens or weights to keep materials from floating. Empty and clean bath as needed to prevent buildup of salts and microbial growth, and disinfect before refilling.

o. Incubator (gravity convection or mechanical forced hot-air, water-jacketed, or aluminum block): Place incubators in an area where room temperature is maintained between 16 and 27°C (60 and 80°F), or else in a separate, well-insulated room with forced air circulation. Clean and then sanitize incubators routinely. Determine that incubators maintain appropriate, uniform spatial test temperatures. It may take longer for media to reach the set incubation temperature in gravity convection hot-air incubators.

While in use, check and record corrected temperature twice daily (morning and afternoon, separated by at least 4 h) on the shelves in use, or at least on the top and bottom shelves to ensure consistency throughout unit. If using a glass thermometer, submerge bulb and stem in water or glycerin to the immersion mark. For best results, use a recording thermometer and an alarm system that promptly notifies analysts of temperature violations so they can invalidate test samples, as appropriate, and request that new samples be collected. Maintain a logbook or digital records of temperature readings and alarms. Electronic temperature-sensing devices (i.e., data loggers) may be used so long as the laboratory's system for recording information from the devices also promptly notifies analysts of temperature violations and documents results such that time/temperature readings can be used to trace a sample and its testing conditions during laboratory assessment. Each data logger should be marked with

a correction factor, as needed. Allow sufficient space between items to permit unobstructed airflow; do not overload nor stack Petri dishes more than four plates high.

Incubator humidity may be a concern if Petri agar media are dehydrated because less water is available for metabolic growth and cells may lyse. Incubated agar plates should be evaluated for the percent moisture weight loss for the method's incubation period. Moisture weight loss should be performed annually. If agar weight loss is >15%, moisture needs to be added to the environment by either using humidified incubators or enclosing the media in tight-sealing containers or bags. If additional moisture is needed, wet paper towels can be added to container or a large shallow pan filled with water can be added to the incubator, refilling as necessary. If there is no weight loss and smearing of colonies on media is evident, humidity needs to be reduced accordingly.

p. Microscopes: Before each use, check Kohler illumination to confirm that alignment is correct. When available on binocular microscopes, adjust ocular lenses for diopter (difference of visual acuity between an analyst's eyes) to reduce or eliminate headaches, motion-sickness symptoms, and the potential for personal errors. After each use, clean optics and stage with lens paper. Cover microscope when not in use.

Permit only trained technicians to use fluorescence microscope and light source. Monitor fluorescence lamp and replace when a significant loss in fluorescence is observed, according to manufacturer recommendations, or when maximum hour usage specified by a rule or laboratory guidance document has been reached, whichever occurs first. Record lamp operation time/usage, efficiency, and alignment. Always realign lamp after bulb has been replaced. Use known positive fluorescence slides as controls.

Establish an annual service contract. Review the microscope-manufacturer's manual; for further information, visit the manufacturer Web site or refer to Section 9030.20[20] and elsewhere.[23]

q. Conductivity meter: A conductivity meter measures the presence of dissolved ions, such as aluminum, calcium, chloride, iron, magnesium, nitrate, phosphate, sodium, and sulfate. Conductivity measurements are temperature-dependent, and the temperature's effect is solution-dependent. Follow manufacturer's instructions for meter check and calibration procedures. Check daily before use and calibrate, if needed. Every month, standardize meter or determine cell constant using certified low-level standards that bracket the expected sample conductivity (e.g., 10 μS/cm) at 25°C. For on-line conductivity meters that cannot be calibrated, remove a portion of reagent water and measure its conductivity with another meter. When solutions must be measured at another temperature, use a meter with automatic temperature compensation or take solution's temperature, record reading, and then correct reading to 25°C using the formulae in Section 2510B.5*b* (usually 2%/°C). Open sample bottle as rarely as possible because conductivity measurements will change when the sample is exposed to ambient air.

r. Microwave ovens: Microwave ovens vary in power and acceptable placement of material, but they have been used successfully to melt presterilized agar media. Set microwave power and time to minimum settings. Check unit's performance and undertake comparison studies to confirm that microwaving is comparable to standardized melting procedures. Take care to avoid media bubbling over.

s. Micropipetters: Micropipetters are high-precision laboratory instruments for dispensing extremely small volumes. Use with sterile precision tips supplied by manufacturer or equivalent that securely fix to the nose cone to ensure a tight seal. Maintain technique consistency in pipetting action, such as pre-wetting, plunger release, and tip immersion depth (between 1 and 3 mm). Operate only in a vertical position and have both sample and equipment at equivalent temperature. Avoid over-dialing the micropipetter's recommended range, which can cause mechanical damage. Follow manufacturer's instructions to perform routine maintenance, such as cleaning, seal replacement, and re-lubrication. Check accuracy and precision of volume dispensed by each pipetter before first use after purchase, maintenance, or repair, and at a frequency related to its usage (e.g., quarterly or sooner if pipetter is showing overt signs that it is inaccurate or if tip manufacturer changes). Calibrate at least annually either in-house or by the manufacturer. Record results of calibration. If water is used to calibrate or check accuracy of pipetter, remember that changes in liquid viscosity can affect the volume dispensed.

5. Laboratory Supplies

Retain records and manufacturer certificates of analysis, purity, or tolerance level (if supplied) for all laboratory supplies.

a. Glassware: Here, the term *glassware* refers to both borosilicate glass and heat-resistant plastic materials. Markings must be legible. Volumetric glassware, pipets, graduated cylinders, and beakers with calibration marks should be accurate to the specified volumetric tolerances. See established standards[24] for calibration of laboratory volumetric apparatus. Volumetric glassware is generally either Class A or Class B (undesignated); Class A is more precise. Determine tolerance once per lot or at a set percentage (e.g., 1 to 2.5%). Graduated cylinders should be accurate to within ≤2.5%.

Before each use, examine glassware and discard items with chipped edges or etched inner surfaces—especially screw-capped dilution bottles and flasks with chipped edges that could leak and contaminate the sample, analyst, and area. After washing, inspect glassware for excessive water beading, stains, and cloudiness, and rewash or discard if necessary. Replace glassware with excessive writing if markings cannot be removed. Store glassware either covered or bottom up to prevent dust from settling inside it. If glassware is being used for fluorescence detection [i.e., with EC + 4-methylumbelliferyl-β-D-glucuronide (ECMUG) medium], check it for autofluorescence before use.

Perform the following tests for clean glassware:

1) pH—Because some cleaning solutions are difficult to remove completely, spot check batches of clean glassware for pH reaction, especially if soaked in alkali or acid. (A *batch* is all glassware washed in the same load.) To test clean glassware for an alkaline or acid residue, add a few drops of 0.04% bromothymol blue (BTB) or other pH indicator to dry glassware and observe the color reaction. If there is no residual, the reaction should be neutral (blue-green for bromothymol blue). However, if the indicator turns yellow (acid residual) or deep blue (alkaline residual), then the glassware must be rewashed and tested again. If the re-test indicates a problem, review the washing equipment, procedures, and detergent used.

Use commercially or laboratory prepared reagents for this pH check. To prepare 0.04% BTB solution, add 16 mL 0.01*N* NaOH to 0.1 g BTB and dilute to 250 mL with reagent water.

2) Inhibitory residues—The main objective of this test is to determine whether the laboratory's washing procedure leaves an inhibitory substance on the glassware. Certain wetting agents or detergents may contain bacteriostatic, inhibitory, or stimulatory substances that may take 6 to 12 rinses to remove. If each batch of glassware is pH tested, then this test is only needed when changing washing compounds or procedures. However, if glassware is not consistently pH-tested or the detergent is not laboratory-grade, then conduct the inhibitory residue test just before first use and annually thereafter. Record results. The following procedure is suitable for both Petri dishes and other glassware.

a) Procedure—Wash and rinse six Petri dishes (Group A) according to usual laboratory practice. Wash six more Petri dishes (Group B) as above, and then rinse 12 times with successive portions of reagent water. Rinse six more Petri dishes (Group C) with water containing the detergent (in use concentration), and air-dry without further rinsing.

Sterilize dishes in Groups A, B, and C by the usual procedure. For presterilized plasticware, set up six plastic Petri dishes (Group D). Prepare and sterilize 200 mL plate count agar and temper in a 44–46°C water bath. Prepare a culture of *Enterobacter aerogenes* ATCC® 13048 known to contain 50 to 150 CFU/mL. Preliminary testing may be necessary to achieve this count range. Inoculate three dishes from each test group with 0.1 mL culture and the other three with 1 mL culture.

Follow the heterotrophic plate count method (Section 9215B) for all inoculated plates and incubate at 35°C for 48 h. Count plates with 30 to 300 colonies and record results as CFU/mL.

b) Interpretation of results—The averaged counts on plates in Groups A through D should differ by <15% if there are no toxic or inhibitory effects. If averaged counts differ by <15% between Groups A and B and >15% between Groups A and C, then the cleaning detergent has inhibitory properties that are eliminated during routine washing. If averaged counts differ by >15% between Groups A and B, then inhibition is occurring because more colonies grew when there was additional rinsing. If the difference between B and D is ≥15%, then an inhibitory residue is present after the normal washing process and plasticware must not be used for microbiological analyses. A new washing procedure, equipment, or detergent supply may be needed.

b. Utensils and containers for media preparation: Use utensils and containers of borosilicate glass, stainless steel, aluminum, or other corrosion-resistant material (see Section 9030B.8). Do not use copper utensils.

c. Dilution water bottles: Use bottles scribed at 99 mL and made of nonreactive and autoclavable borosilicate glass or plastic with screw caps that are either linerless or have inert liners. Clean before use. Commercially available bottles prefilled with dilution water are acceptable. Before using each batch or lot, conduct sterility test (9020B.9*d*); check one per lot or a set percentage (e.g., 1 to 4%) for pH and volume (99 ± 2 mL). Examine dilution water bottles for a precipitate; discard if present. Reclean bottles with acid if necessary, and remake the dilution water. If precipitate repeats, procure bottles from a different source. Recheck volume at regular intervals to determine volume loss rate under holding conditions. Discard by expiration date.

d. Sample bottles: Use wide-mouth reusable, nonreactive, autoclavable borosilicate glass or plastic bottles with screw caps that are either linerless or have inert liners, or else commercially prepared sterilized plastic bottles or bags with ties of sufficient size. The bottles or bags must be large enough to collect the needed sample and still have an adequate headspace (1 in.) to allow sample to be shaken in the container.

Clean and sterilize bottles before use and, depending on use, add sufficient dechlorination agent to neutralize residual chlorine (Section 9060A.2). Sample container may be purchased with added dechlorination agent. Test for sterility at least one or a set percentage (e.g., 1 to 4%) of each batch sterilized in the laboratory or of each presterilized lot purchased from a vendor. Document results. If growth occurs, discard entire batch or lot. Also, check one per batch or lot for efficacy of dechlorination agent, accuracy of 100-mL mark (if present), and auto-fluorescence properties (if used for fluorescence testing). Record results.

e. Multi-well trays and sealers:* When using multi-well trays for growth studies, check one per lot for sterility beforehand by aseptically adding 100 mL of sterile tryptic soy broth or other non-selective medium, sealing, and incubating at 35 ± 0.5°C for 24 and up to 48 h. No growth indicates sterility. If the wells become very turbid (indicating nonsterile condition), there could be gas production and concomitant blowout between wells. See 9020B.9*d*.

Every month, evaluate the heat sealer's performance by adding one to two drops of a food-color dye to 100 mL deionized water sample, run the multi-well tray through the sealer, and visually check each well for leakage. Clean and conduct preventive maintenance on sealer annually, or more frequently if needed.

Microtiter plates are used in a variety of analytical procedures (e.g., DNA hybridization and immunoassay studies) and may contain >96 wells. Examine the tray wells for consistency and perform appropriate QC controls, as indicated by the manufacturer. Use controls from an approved certified vendor; these may be labeled for the system being tested. The laboratory may need to detoxify or sterilize the plates if their use requires this.

f. Reagent-grade water: Use reagent-grade water to prepare solutions and media, and for final glassware rinses. The water must be proven to be free from inhibitory and bactericidal substances. The quality of water obtainable from a water-purification system depends on the system and how it is maintained (see 9020B.4*d* and *e*). See Table 9020:II for recommended reagent water-quality limits for the microbiology laboratory. If these limits are not met, investigate and correct or else change water source. NOTE: Reagent water's pH tends to drift, but extreme readings indicate chemical contamination.

1) Test for bacteriological quality[25]—This test, also called the *water suitability test*, is based on the growth of *Enterobacter aerogenes* ATCC® 13048 in a chemically defined minimal-growth medium. The presence of a toxic agent or a growth-promoting substance will increase or decrease the 24-h population by 20% or more, compared to a control. Perform the test at least annually, whenever the reagent-water source is

* Quanti-Tray® or Quanti-Tray®/2000, available from IDEXX Laboratories, Inc., Westbrook, ME, 04092, or equivalent.

TABLE 9020:II. QUALITY OF REAGENT WATER USED IN MICROBIOLOGY TESTING

Test	Monitoring Frequency	Maximum Acceptable Limit
Chemical tests:		
Conductivity	Continuously or usage day	<2 μmhos/cm (μmsiemens/cm) at 25°C
Total organic carbon	Monthly	<1.0 mg/L
Heavy metals, single (Cd, Cr, Cu, Ni, Pb, and Zn)	Annually*	<0.05 mg/L
Heavy metals, total	Annually*	<0.10 mg/L
Total chlorine residual	Monthly or with each use	<0.1 mg/L
Bacteriological tests:		
Heterotrophic plate count†	Monthly	<500 CFU/mL or MPN <500/mL
Use test [see 9020B.5*f*2)]	For a new source	Student's $t \leq 2.78$
Water quality test [see 9020B.5*f*1)]‡	Annually	0.8–3.0 ratio

* Or more frequently if there is a problem.
† See Section 9215.
‡ This water-quality test is not needed for Type II water or better, as defined in *Standard Methods* (18th and 19th Editions), Section 1080C, or medium quality water or better, as defined in *Standard Methods* (20th, 21st, 22nd, and Online Editions), Section 1080C.

changed, and whenever an analytical problem occurs. This bacteriological quality test is not needed for Type II water or better [as defined in *Standard Methods* Section 1080C (18th and 19th Editions)] or medium-quality water or better [as defined in *Standard Methods* Section 1080C (21st, 22nd, and Online Editions)]. Test to ensure continued quality of this water to meet the above ATCC growth standards. Because of its complexity and because most laboratories use Type II water or better, this test is seldom performed but may be used when an analytical problem occurs.

The test is complex, requires skill and experience, and is not easily done on an infrequent basis. It requires work over 4 d, ultrapure water from an independent source as a control, high-purity reagents, and extremely clean culture flasks, Petri dishes, test tubes, pipets, and other equipment. A contract laboratory familiar with the test can be used.

a) Apparatus and material—Use borosilicate glassware; presterilized plastic Petri dishes may be used in plating steps. Rinse glassware in water freshly redistilled from a glass still and then sterilize it with dry heat (steam sterilization will recontaminate these specially cleaned items). Test sensitivity and reproducibility depend in part on the cleanliness of sample containers, flask, tubes, and pipets. It often is convenient to set aside new glassware for exclusive use in this test. Use any strain of coliform with IMViC type – – + + (*E. aerogenes*) obtained from an ambient water or wastewater sample or reference culture.†

b) Reagents—Use only ACS-grade reagents and chemicals. Test sensitivity is partly controlled by reagent purity. Use medical-grade water or water freshly redistilled from a glass still; the water can be purchased (see Table 9020:III). Prepare reagents as follows:

- *Sodium citrate solution:* Dissolve 0.29 g sodium citrate ($Na_3C_6H_6O_7 \cdot 2H_2O$) in 500 mL water.
- *Ammonium sulfate solution:* Dissolve 0.26 g $(NH_4)_2SO_4$ in 500 mL water.
- *Salt-mixture solution:* Dissolve 0.26 g magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 0.17 g calcium chloride ($CaCl_2 \cdot 2H_2O$), 0.23 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$), and 2.50 g sodium chloride (NaCl) in 500 mL water.
- *Phosphate buffer solution/dilution water:* Dilute stock phosphate buffer solution (Section 9050C.1*a*) 1:25 in water.

Filter sterilize or boil all reagent solutions 1 to 2 min to kill vegetative cells. Store solutions in sterilized glass-stoppered bottles in the dark at 5°C for up to several months, provided that they are tested for sterility before each use. Because the salt-mixture solution will develop a slight turbidity within 3 to 5 d as the ferrous salt converts to the ferric state, prepare the salt-mixture solution without $FeSO_4$ for long-term storage. To use the mixture, add an appropriate amount of freshly prepared and freshly boiled iron salt. When solutions become turbid, discard them and prepare new ones.

c) Samples—To prepare test samples, collect 150 to 200 mL laboratory reagent water and control (redistilled) water in sterile borosilicate glass flasks and boil for 1 to 2 min. Avoid longer boiling to prevent chemical changes.

d) Procedure—Label two flasks or tubes A and B. Add water samples, media reagents, and redistilled water to each flask as indicated in Table 9020:III. Add a suspension of *E. aerogenes* ATCC® 13048 (IMViC type – – + +) of such density that each flask will contain 30 to 80 cells/mL, prepared as directed below. Cell densities below this range result in inconsistent ratios while densities above 100 cells/mL are less sensitive to nutrients in the test water.

e) Preparation of bacterial suspension—On the day before the distilled-water suitability test, inoculate a strain of *E. aerogenes* ATCC® 13048 onto an approximately 6.3-cm-long nutrient agar slant in a 125- × 16-mm screw-cap tube. Streak entire agar surface to develop a continuous-growth film, and incubate 18 to 24 h at 35°C.

f) Harvesting of viable cells—After incubation, pipet 1 to 2 mL sterile dilution water from a 99-mL water blank onto the culture. Emulsify growth on slant by vortexing, gentle sonication or swirling; then pipet suspension back into original 99-mL water blank.

g) Dilution of bacterial suspension—Make a 1:100 dilution of original bottle into a second water blank, a further 1:100 dilution of second bottle into a third water blank, and a 1:10 dilution of third bottle into a fourth water blank, shaking vigorously 22 times after each transfer. Pipet 1.0 mL of the fourth dilution (1:105) into Flasks A and B. This procedure should produce a

†ATCC 13048, or equivalent.

Table 9020:III. Reagent Additions for Water Quality Test

	Control Test mL		Optional Tests mL		
Media Reagents	Control A	Test Water B	Carbon/Nitrogen Available C	Nitrogen Source D	Carbon Source E
Sodium citrate solution	2.5	2.5	—	2.5	—
Ammonium sulfate solution	2.5	2.5	—	—	2.5
Salt-mixture solution	2.5	2.5	2.5	2.5	2.5
Phosphate buffer (7.3 ± 0.1)	1.5	1.5	1.5	1.5	1.5
Unknown water	—	21.0	21.0	21.0	21.0
Redistilled water	21.0	—	5.0	2.5	2.5
Total volume	30.0	30.0	30.0	30.0	30.0

final dilution in the range of 30 to 80 viable cells per milliliter of test solution.

h) Verification of bacterial density—Variations among strains of a given organism, different organisms, media, and surface area of agar slopes may require that the dilution procedure be adjusted to achieve an appropriate cell density. To determine the bacterial density for a specific organism and medium, make a series of plate counts from the third dilution. Then, choose the proper volume from this dilution, which when diluted by the 30 mL in Flasks A and B, will contain 30 to 80 viable cells/mL. If the procedures are standardized as to slant surface area and laboratory technique, it is possible to reproduce results on repeated experiments with the same strain of microorganism. Run tests in triplicate.

i) Procedural difficulties—Problems in this method may be due to:

- test water sample stored in soft-glass containers or glass containers with linerless metal caps;
- reagents prepared with chemicals that are not analytical-reagent grade or of recent manufacture;
- reagent contaminated by distilled water containing background levels of bacteria (to avoid this, make a heterotrophic plate count on all media and reagents before starting the suitability test, as a check on stock solution contamination);
- bacterial density outside 30- to 80-viable cells/mL range (e.g., incorrect dilution chosen for 24-h plate count);
- inconsistent mixing;
- delay in pouring plates; or
- samples incubated for longer than 26 h, thereby desensitizing growth response.

j) Calculation—For growth-inhibiting substances:

$$\text{Ratio} = \frac{\text{colony count/mL, Flask B}}{\text{colony count/mL, Flask A}}$$

If the ratio is 0.8 to 1.2 (inclusive), no toxic substances are present; if the ratio is <0.8, there are growth-inhibiting substances in the water sample.

A value >1.2 indicates an available nutrient source for bacterial growth; however, the test is very sensitive and the ratio could go as high as 3.0 without any undesirable consequences. Do not calculate if the first ratio indicates a toxic reaction.

k) Interpretation of results—The colony count from the control, Flask A, after incubation will depend on the strain of *E. aerogenes* used and the number of organisms initially inoculated in the flask. Therefore, run Flask A for each individual series of tests. If the strain of *E. aerogenes* ATCC® 13048, initial inoculum, and environmental conditions are the same, the terminal count should be reasonably constant. On the other hand, a difference in initial inoculation of 30 to 80 will result in a final count about threefold larger for the 80 organisms if the growth rate remains constant. Thus, it is essential that initial colony counts on Flasks A and B be approximately equal.

Specific corrective measures cannot be recommended for every instance of defective distillation apparatus. Carefully inspect the distillation equipment and review the distilled-water production and handling processes to help locate and correct the cause of difficulty. Feed water to a still often is passed through a deionizing column and a carbon filter. If these columns are well maintained, most inorganic and organic contaminants will be removed. If maintenance is poor, then feed water quality may be lower than that of raw tap water.

The best distillation system is made of quartz or high-silica-content borosilicate glass with special thermal endurance. Tin-lined stills are not recommended. For connecting plumbing, use stainless steel, borosilicate glass, or special plastic pipes made of polyvinyl chloride (PVC). Protect storage reservoirs from dust.

2) Use test for evaluating reagent water—Before using a new reagent-water source, analysts should compare it for equivalence with the current lot in use (reference lot). Note: It may not be possible to compare reagent-water sources because the previous system may no longer be available.

a) Procedure—Use a single batch of control water (redistilled or distilled water polished by deionization), glassware, membrane filters, or other needed materials to control all variables except the one factor under study. Perform replicate pour, spread, or membrane-filter plate tests on both reference and test lots (see Sections 9215 and 9222). At a minimum, analyze five different water samples known to be positive for the target organism or culture controls of known density. Replicate analyses and additional samples can be tested to better detect any differences between reference and test lots.

When analyzing reagent water, perform the quantitative bacterial tests in parallel using a known high-quality water as the

control water. Prepare dilution/rinse water and media with new source of reagent and control water. Test water for all uses (dilution, rinse, media preparation, etc.).

b) Counting and calculations—After incubation, compare bacterial colonies from both lots for size and appearance. If colonies on the test lot plates are atypical or noticeably smaller than those on the reference lot plates, record the evidence of inhibition or other problem, regardless of count differences. Count plates and calculate the individual count per 1 or 100 mL. Transform the count to decimal logarithms and enter the log-transformed results for both lots in parallel columns. Calculate the difference, *d*, between the two transformed results for each sample, including the + or − sign; the mean, $\bar{d}$; and the standard deviation, *sd*, of these differences (see Section 1010B).

Calculate Student's *t* statistic:

$$t = \frac{\bar{d}}{s_d/\sqrt{n}}$$

where n = the number of samples.

These calculations may be made using various statistical software packages available for personal computers.

c) Interpretation—Compare the calculated *t* value to the critical *t* value from a Student's *t* table. At the 0.05 significance level, Student's *t* is 2.78 for five samples (four degrees of freedom). If the calculated *t* value is ≤2.78, the test lot is acceptable (i.e., the two lots' results are not significantly different). If the calculated *t* value is >2.78, the test lot is unacceptable.

If the colonies are atypical or noticeably smaller on the test lot or Student's *t* exceeds 2.78, then review test conditions and repeat the test or else reject the test lot and obtain another one.

g. Reagents:[26] Because reagents are an integral part of microbiological analyses, their quality must be assured. Use only ACS-grade chemicals or equivalent because impurities can inhibit bacterial growth, provide nutrients, or fail to produce the desired reaction. Maintain any safety data sheets (SDS) provided with reagents or standards and have them available to all personnel.

Date chemicals and reagents both when received and when first opened for use. Maintain records for receipt, expiration, and subsequent preparation. During preparation, bring all reagents to room temperature, make reagents to volume, preferably in volumetric flasks, and store them in good-quality inert plastic or borosilicate glass bottles with borosilicate, polyethylene, or other plastic stoppers or caps. Label prepared reagents with name, concentration, date prepared, preparer's name, and expiration date (if known). Store under proper conditions and discard by expiration date. Include positive and negative control cultures with each series of cultural or biochemical tests.

h. Dyes and stains: In microbiological analyses, organic chemicals are used as selective agents (e.g., brilliant green), indicators (e.g., phenol red), and stains (e.g., Gram stain). Dyes from commercial suppliers vary from lot to lot in percent dye, dye complex, insolubles, and inert materials. Because microbiological dyes must be strong and stable enough to produce correct reactions, only use those certified by the Biological Stain Commission. Prepare minimal quantities and before use, test dyes using at least one positive and one negative control culture. Record results. For fluorescent stains, test for positive and negative reactivity each day of use. Do not freeze dyes or stains. Read and follow manufacturer's information for storage time and temperature.

i. Membrane filters and pads: The quality and performance of membrane filters vary with the manufacturer, type, brand, and lot due to differences in manufacturing methods, materials, QC, storage conditions, and application.[27]

1) Specifications—Manufacturers of membrane filters and pads for water analyses must meet standard specifications for flow rate, retention, percent recovery, and inorganic and organic chemical extractables.[28,29] Some manufacturers also report pore size, sterility, and pH, and certify that their membranes are satisfactory for water analysis. Although the standard membrane filter evaluation tests were developed for manufacturers, a laboratory can conduct its own tests, if desired.

2) Use test—Each new lot of membrane filters should perform satisfactorily in the use test to ensure that it does not yield low recoveries, poor differentiation, or malformation of colonies due to toxicity, chemical composition, or structural defects. For procedure, see ¶ *f*2) above.

3) Standardized use tests—When each lot of membranes arrives at the laboratory, record lot number and date received. Inspect each lot before use and during testing to ensure that membranes are round and pliable. If lot is held for one or more years, carefully check for brittleness and discard lots that appear brittle. Confirm sterility prior to first use of the lot by placing a membrane filter on a pad saturated with tryptone glucose extract broth (or equivalent non-selective broth or agar) and incubating it at 35 ± 0.5°C for 24 h; the filter is sterile if no growth occurs. Alternatively, run a sterility control with each analytical test run.

After sample incubation, colonies should be well-developed with appropriate color and shape, as defined by the test procedure. The gridline ink should not channel growth along the ink line nor restrict colony development. Colonies should be distributed evenly across the membrane surface. Reject membrane lot if these criteria are not met, and inform manufacturer.

j. Culture media: Because culture methods depend on properly prepared bulk media, use the best available materials and consistent techniques to prepare, store, and use media, and prepare the correct medium for the intended application. For QC, use commercially prepared bulk media whenever available, but note that the quality and ingredient composition of such media may vary both from lot to lot and from manufacturer to manufacturer. Before first use, compare the growth recovery of newly purchased lots of bulk media to those of proven lots, using positive and negative reference cultures (preferably), recent pure-culture isolates, or natural samples [see ¶ *f*2) above]. This is known as a *use test* as applied to media. Test using cultures whose estimated density is similar to samples normally tested in the laboratory. Observe media for growth promotion, inhibitory properties, physical appearance, and pH.

File any SDS accompanying media.

Order media in quantities expected to be used within 1 year (preferably within 6 months) after opening. Order commercially prepared media in quantities expected to be used by the manufacturer's expiration date. Use media on a first-in, first-out basis. When practical, order media in smaller containers (e.g., 0.25 lb or 125 g) rather than 1-lb or 500-g bottles so most of the supply remains sealed as long as possible. Keep written or digital records of the type, amount, and appearance of media received,

lot number, expiration date, and dates received and opened; also, mark containers with the expiration date and date opened. Check inventory quarterly for reordering.

Each lot of media for detecting fluorescence should be checked for autofluorescence before use. This can be done by dissolving the medium in reagent water and examining with UV light.

1) Preparation of media—Prepare media in clean containers that are at least twice the volume of the medium being prepared. Use reagent-grade water. Measure both water and media with graduates or pipets that conform to NIST and APHA standards, respectively. Use TD (to deliver) pipets, NOT blowout ones. Stir media, particularly agars, while heating. Avoid scorching or boil-over by using a boiling water bath for small batches of media and by continually attending to larger volumes heated on a hot plate or gas burner. Preferably use hot plate-magnetic stirrer combinations. Label and date prepared media.

After sterilization, check and record pH of a portion of each medium because the specified pH of the medium is the actual pH required for adequate growth. If pH adjustment is needed, use filter-sterilized 1*N* NaOH or 1*N* HCl solutions to make minor adjustments so medium's pH meets that specified in the formulation. (Commercially available media will seldom need pH adjustment.) If medium is known to require pH adjustment, adjust it appropriately before sterilization and record final pH. If the pH difference is >0.5 units, discard the batch and check both preparation instructions and reagent water's pH to resolve the problem. Incorrect pH values may be due to reagent water quality, deterioration of medium, or improper preparation. If reagent water's pH is unsatisfactory, prepare a new batch of medium using water from another source (see 9020B.4*d* and *e*). If water is satisfactory, remake medium and check pH; if pH is still incorrect, prepare medium using a different lot or source of media. NOTE: Certain specific isolation media prepared with organic or fatty acids will have marked changes in pH following sterilization. Discard media if crystal formation or color variations are found. NOTE: A precipitate is normal in Endo-type media.

Document preparation activities, such as name of medium, volume produced, format, final pH, date prepared, and name of preparer. Record pH problems in the media record book and inform the manufacturer if the medium is indicated as the source of error. Examine prepared media for unusual color, darkening, or precipitation, and record observations. Consider whether variations in sterilization time and temperature could be the cause of problems. If any of the above occurs, discard the medium.

2) Sterilization—Sterilize media at ≤121°C with minimal temperature variation for minimum time specified. Follow manufacturer's directions for sterilizing specific media. The required exposure time varies with form and type of material, type of medium, presence of carbohydrates, and volume. Table 9020:IV gives guidelines for typical items in small units (e.g., test tubes and small flasks). Do not expose media containing carbohydrates to elevated temperatures for >45 min; some media cannot be exposed to heat for that long. For example, presence–absence media cannot be exposed to heat for >30 min. *Exposure time* is the period from initial heat exposure to removal from the autoclave. Overheating media can result in nutrient degradation. Maintain autoclave printout records.

TABLE 9020:IV. TIME AND TEMPERATURE FOR AUTOCLAVE STERILIZATION*

Material	Time at 121°C *min*
Membrane filters and pads	10
Carbohydrate-containing media (lauryl tryptose, BGB broth, etc.)	12–15†
Contaminated materials and discarded cultures	30
Membrane filter assemblies (wrapped), sample collection bottles (empty)	15
Buffered dilution water, 99 mL in screw-cap bottle	15
Rinse water, volume >100 mL	Adjust for volume

* Except for media, times are guidelines.
† Certain media may require different sterilization conditions.

NOTE: Where possible, avoid sterilizing large amounts of media in containers because it will take longer for the media to reach sterilization temperature. Use a temperature probe in a media flask to determine the length of time needed to reach sterilization temperature.

Remove sterilized media from autoclave as soon as chamber pressure reaches zero or, if using a fully automatic model, as soon as the door opens. Use extreme care to avoid boiling over due to superheated liquids. Do not re-autoclave media.

Sterilize heat-sensitive solutions or media by filtration through a 0.2-μm-pore-diameter filter in a sterile filtration and receiving apparatus. Filter and dispense medium in a laminar-flow hood or safety cabinet, if available. Sterilize glassware (e.g., pipets, Petri dishes, sample bottles) in an autoclave or hot-air sterilizing oven (170 ± 10°C for ≥2 h). Sterilize equipment, supplies, and other heat-sensitive solid or dry materials by exposing to ethylene oxide in a gas sterilizer. Use commercially available spore strips or suspensions to check dry heat and ethylene oxide sterilization.

3) Use of agars and broths—Temper melted agars in a water bath at <50°C (preferably 44 to 46°C) until used, but for ≤3 h. To monitor agar temperature, expose a bottle of water or medium to the same heating and cooling conditions as the agar. Insert a thermometer in the monitoring bottle to determine when the temperature is suitable for use in pour plates. Add heat-sensitive solutions (e.g., antibiotics) to tempered agar. Ideally, prepare media ≥2 d before tests to allow sufficient time for sterility and positive- and negative-control culture testing to be completed. If agar medium is solidified for later use, then melt in boiling water bath or beaker or a unit with a flowing stream of steam (e.g., an autoclave set at 100°C for 5 to 10 min, or low-wattage microwave[30]), use, and then discard any remainder. Because microwaves vary, run comparison tests to ensure that medium integrity has not been compromised. Some media are not suitable for melting in the microwave (i.e., M-Endo/Endo LES). Do not re-autoclave media. Agar may be melted only once, and some media cannot be melted in the microwave without destroying their selective nature.

The volume dispensed depends on the size of the Petri dish and its intended use. Invert plates as soon as poured medium has solidified. Handle tubes of sterile fermentation media carefully to avoid entrapping air in Durham (inner) tubes, thereby producing false positive reactions. (*Durham tubes* are very small test tubes

TABLE 9020:V. HOLDING TIMES FOR PREPARED MEDIA

Medium	Holding Time
Broth in screw-cap flasks*	96 h
Poured agar in plates with tight-fitting covers*	2 weeks
Agar or broth in loose-cap tubes*	2 weeks
Agar or broth in tightly closed screw-cap tubes†	3 months
Poured agar plates with loose-fitting covers in sealed plastic bags*	2 weeks
Large volume of agar in tightly closed screw-cap flask or bottle*	3 months

* Hold under refrigerated conditions (2–8°C).
† Hold at <30°C.

inverted in larger test tubes to entrap any gas produced.) Examine freshly prepared tubes to determine that there are no gas bubbles in the Durham tubes.

4) Storage of media—Store all media under controlled conditions to maintain quality until expiration date. Dehydrated media are hygroscopic; avoid excessive humidity. Store dehydrated media in a tightly closed container in a cool (15 to 25°C), dry, controlled-temperature room or desiccator away from direct sunlight. Discard media that cake, discolor, or show other signs of deterioration. Discard unused media by manufacturer's expiration date. A conservative time limit for unopened bottles is 2 years at room temperature.

Use opened bottles of media preferably within 6 months. Immediately after use, close bottles as tightly as possible. Store opened bottles in desiccator, if available.

Prepare media in amounts that will be used within holding time limits given in Table 9020:V. Fresh medium is required to ensure that target microorganisms are isolated properly, especially bacteria stressed or injured during treatment. Protect laboratory- and commercially-prepared media containing dyes from light; if color changes, discard the media.

If prepared ready-to-use commercial medium has an expiration date later than that noted in Table 9020:V, have the manufacturer supply evidence of medium quality for that entire period. Verify usability weekly by testing recoveries with known densities of culture controls that will also meet QC check requirements.

Controlling moisture content is important because prolonged storage and subsequent dehydration may alter recovery and selectivity. When media are used for research purposes, establish appropriate media expiration dates and document results. Protect laboratory-prepared and purchased-prepared media containing dyes from light; if color changes occur, discard the media. Refrigerate any poured agar plates not used on preparation day. To prevent dehydration, seal agar plates in plastic bags or other sealed container if they will be held >2 d. Store plates inverted to prevent condensation from falling on medium. If condensate has formed, consider placing plates briefly in a 35 to 37°C incubator. For media in test tubes, tighten caps before storage. Weigh plates or mark liquid level in several tubes (10% of each batch) after sterilization and monitor for liquid loss by weight or volume when stored for >2 weeks. If loss is 10% or more, discard batch. Discard all Petri dishes with solid media that have been stored for >2 weeks; discard earlier if they are dried out (e.g., wrinkled, cracked, or pitted).

If media are refrigerated, bring to room temperature before use and reject batch if growth or false-positive responses occur. Commercially prepared sterile broths and agars may offer advantages when analyses are done intermittently, staff is unavailable for preparation work, or cost can be balanced against other laboratory-operation factors. Check performance of these media as described in ¶s 5)–7) below.

5) Use test—Subject laboratory-prepared media to the use test. For procedure, see ¶ *f*2) above.

6) Quality control of laboratory-prepared media—Compare new lots and previously acceptable ones [¶ 5) above] for their quantitative recoveries of the microorganism of concern. Include media sterility checks and positive- and negative-control culture checks to determine specificity on all media, as described below. Culture controls can be used to detect growth promotion and medium selectivity, as well as monitor analyst technique. Maintain information in a bound book.

A good laboratory practice is to periodically challenge prepared media with low numbers of an appropriate microorganism. Growth would be affected by media quality, preparation, sterilization, storage time, and storage conditions.

7) Quality control of commercially prepared media—Shipping this media should not invalidate any of the media holding times or conditions described above. The manufacturer should supply validation information if shipment conditions are otherwise. However, the laboratory should perform its own enumerative test by challenging media with low numbers of an appropriate microorganism. Record receipt and expiration dates, lot number, and then measure and record medium used. Store as directed by manufacturer and discard by expiration date. Comparing quantitative recoveries with laboratory-prepared media, as directed in ¶ 5) above, is recommended. Test each new lot for sterility and with positive- and negative-control culture checks (suggested control organisms can be found in Table 9020:VI). If commercially prepared medium has a longer shelf life than the laboratory-prepared version, perform these tests more frequently.

6. Standard Operating Procedures (SOPs)[31–33]

The operational backbone of an analytical laboratory, generic and specific SOPs are designed to prevent deviations due to a misinterpreted process or method. Each specific SOP describes, step by step, the details of a routine task or procedure tailored to the laboratory's own equipment, instrumentation, and sample types. Such tasks include preparing reagents, reagent water, standards, and culture media; using balances properly; sterilizing media; washing items; disposing of contaminated material; collecting and analyzing samples; maintaining a chain of custody, keeping records, performing appropriate QC, and confirming that QC acceptance criteria are met. Simply citing a published analytical method is not an SOP, although that information can be included in the laboratory's own SOP.

SOPs are laboratory-specific, written by the person who does the work, and approved in writing by the supervisor (with the effective date indicated). Follow SOPs as written, keep them current via routine reviews, and have them accessible to all necessary personnel. When changes are needed, document them and have the supervisor approve the updated SOP. Keep a file of outdated SOPs for future reference, as needed. If maintained in electronic form (eFiles), SOPs may need to be password pro-

Table 9020:VI. Suggested Control Cultures for Microbiological Tests*

Group	Control Cultures	
	Positive	Negative
Total coliforms	*Escherichia coli*	*Staphylococcus aureus*†
	Enterobacter aerogenes‡	Proteus vulgaris§
	Klebsiella pneumoniae (ATTC 4352)	*Pseudomonas aeruginosa*†
Thermotolerant coliforms	*Escherichia coli*	*Enterobacter aerogenes*
	Klebsiella pneumoniae (thermotolerant)‖	
Escherichia coli	*Escherichia coli (MUG-positive s strain)*	*Enterobacter aerogenes*
		Klebsiella pneumoniae (thermotolerant)
Enterococci#	*Enterococcus faecalis*	*Staphylococcus aureus***
	Enterococcus faecium	*Escherichia coli*††

* Use appropriate ATCC strains. Note: Other cultures may be used.
- *Enterococcus faecalis* ATCC 11700
- *Enterococcus faecium* ATCC 6057
- *Enterobacter aerogenes* ATCC 13048
- *Escherichia coli* ATCC 11775 or 25922
- *Klebsiella pneumoniae* (thermotolerant) ATCC 13883 or 4352
- *Proteus vulgaris* ATCC 13315
- *Pseudomonas aeruginosa* ATCC 27853
- *Serratia marcesens* ATCC 14756
- *Staphylococcus aureus* ATCC 6538

† *S. aureus*, *P. aeruginosa*—not lactose fermenter.
‡ *E. aerogenes*—ferments lactose, but is not typically thermotolerant.
§ *P. vulgaris*—not lactose fermenter, uses hydrolyzed lactose, indicating "overcooked" medium.
‖ *K. pneumoniae*—ferments lactose, but does not hydrolyze MUG.
Do not use closely related strains from genus Streptococcus as a positive control.
** *S. aureus*—sensitive to sodium nalidixic acid medium.
†† *E. coli*—sensitive to sodium azide in medium.

tected to prevent unauthorized changes. Also, the electronic system used to develop and store such files must be retained when no longer in use (e.g., when replaced by a new system).

Consistent use of SOPs helps ensure uniform operations. They also are an effective training tool and a means for determining competency when conducting an assessment.

7. Sampling

Although microbiology-laboratory personnel generally do not collect samples themselves, they need to be familiar with the sample-collection process.

a. Planning: Microbiologists should participate in the planning of monitoring programs that will include microbial analyses. They can provide valuable expertise on the selection of sampling sites, depths, and points; the number of samples and analyses needed; workload; and supplies. For natural waters, their knowledge of probable microbial densities and the effects of season, weather, tide and wind patterns, known sources of pollution, and other variables is needed to formulate the most effective sampling plan. Microbiologists also can indicate when replicate samples will be needed (e.g., when a new water source is being tested or a sample is being collected from a different area of the same locale). For compliance monitoring, the sampling plan must be approved by the state.

b. Methods: Sample-collection guidance generally addresses the factors that must be considered for each site. Sample-collection SOPs describe sampling equipment and its cleaning, techniques, frequency, handling, chain of custody, holding times and conditions, safety rules, etc., that will be used under various conditions at different sites to ensure sample integrity, the avoidance of cross-contamination, and representativeness. Use the information in these SOPs to draw up sample-collection plans, which must be site-specific and based on appropriate statistical sampling designs. Sample-collection techniques for detecting and recovering microorganisms should be validated.[34]

c. Sample acceptance: The laboratory must determine whether sample integrity, holding conditions and time, and accompanying documentation are acceptable for the intended use of the resulting data. Sample-receipt information should include names or identifiers of both sampling site and sampler, turbidity, and date and time of sample collection. Sample-receipt records also must include date and time received, name or initials of individual accepting the sample, temperature of sample upon receipt, and any deficiencies noted (e.g., frozen, heated, or leaking samples). Note: The number of recoverable microorganisms can increase or decrease over time after sample collection.

d. Sample analysis: The laboratory is responsible for ensuring that analyses are initiated within an acceptable holding time.

8. Analytical Methods

The essential QC elements for microbiology laboratories are described in 9020A. Calibration and maintenance of equipment and supplies, and sterility tests are critical to the successful operation of an analytical method. Conduct appropriate QC checks with each batch or test run of samples. When a matrix changes and analysts anticipate that isolating a particular microorganism may be difficult, analyze matrix spike and matrix spike duplicate samples. This is particularly important for recreational

water programs. Ensure that documentation can successfully follow a sample from receipt in the laboratory to the final data report.

a. Method selection: Microbiological methods are used for a variety of matrices, including drinking water, municipal wastewaters, recreational waters, ground waters, marine waters, storm waters, and direct discharges. Some regulatory monitoring programs specify which analytical methods are approved for monitoring and they may differ for drinking water programs. Factors such as media compatibility with sample matrix, temperature, time at incubation temperature, and minor variations in techniques must be applied consistently to ensure appropriate microbial recovery for qualitative and quantitative determinations. Also, microbiological methods must be standardized so multiple laboratories produce uniform data. Select analytical methods appropriate for the sample type from *Standard Methods* or other sources of standardized methods and ensure that methods have been properly validated in a multi-laboratory study and approved by regulatory authority if used for compliance monitoring with the sample types of interest. Validate any new method or nonstandard method that the laboratory intends to use, as well as any method being used for a matrix it was not specified for. See the discussion in 9020B.11.

b. Data objectives: Review available methods and determine which best produce data meeting the program's needs for precision, bias, specificity, selectivity, detection limit, and recovery efficiency under actual test conditions. Methods that are rapid, inexpensive, and less labor-intensive are desirable, but not if there is a high potential for false-positive or -negative results that could affect water-quality decisions.

c. Internal QC: Published analytical methods must contain the required QC checks to ensure data quality, such as the use of positive and negative control cultures, sterility method blanks, replicate analyses (precision), and bacterial cultures having a known density level for quantitative methods. These must be part of a laboratory's internal QC program with any additional internal requirements, such as the frequency of QC analyses and verification requirements for new sample types.

d. Method SOPs: As part of the series of SOPs, provide each analyst with a copy of the analytical procedures written exactly as they are to be performed step by step, with QC requirements identified, and specific to the sample type, equipment, and instrumentation used in the laboratory.

9. Analytical Quality Control Procedures for Established Methods[6–8,19,35]

In order to estimate uncertainty in analytical measurements, analysts must determine a method's repeatability, reproducibility, and false positive and negative rates. Therefore, replicate analyses, reference cultures, blanks (sterility tests), intra- and inter-laboratory tests and spiked samples become necessary.

General quality control procedures:

a. Colony-counting variability: For routine performance evaluations, analysts should repeat counts on one or more positive samples at least monthly and record results. Only one count is made during official sample testing. When comparing two analysts, each should count the same plate once. When comparing three or more analysts, use a statistical evaluation method. (See 9020B.13*b* for a statistical calculation of data precision.) Replicate counts by one analyst should agree within 5% (repeatability); counts made by two or more analysts should agree within 10% (reproducibility). If counts do not agree within the acceptable margin, determine why and correct as needed. Chart these results in a QC chart.

b. Positive and negative control cultures: Use certified reference cultures obtained from nationally or internationally recognized sources. The reference cultures must come from established commercial sources and be impregnated onto discs or strips or in liquid culture. Subculture the reference culture to develop one or more primary working stocks,[36] but make no more than five transfers (i.e., to a fresh medium to promote growth) from the original culture. Minimize subsequent transfers to ensure that working stocks retain phenotypic and genotypic identity and to reduce potential contamination. Test strains periodically to ensure their viability and that performance remains unchanged. If a laboratory lacks the facilities to maintain a pure culture, its personnel should use single-use culture strips or submit the relevant samples to another laboratory for testing. NOTE: Proficiency test (PT) samples [also called *performance evaluation (PE) samples*] are unknowns and should not be considered replacements for positive and negative culture controls.

For each lot of medium received, each laboratory-prepared batch of medium, and each lot of commercially prepared medium, verify appropriate response by testing with known positive and negative control cultures for the organism(s) under test. See Table 9020:VI for examples of test cultures. Record results.

c. Duplicate analyses[37,38]: Precision (repeatability) of quantitative analytical results when counting plate colonies is evaluated through replicate analyses. Note that the three dilutions are not to be considered replicates for the purpose of determining precision. Replicate analyses are particularly important when a laboratory or analyst is new to a method, or a method or matrix is expected to generate considerably variable results. The results can be charted in a control chart.

Perform duplicate analyses at least monthly, or more often as needed (e.g., 10% of samples when required by the analytical method or regulations, one sample per batch or test run, or one sample per week for a laboratory that conducts <10 tests/week). A *batch* or *test run* is defined as an uninterrupted series of analyses, generally 20 samples including appropriate QC controls. Evaluate and record results. An adequate sample volume is essential. Balance frequency of replicate analyses against the time, effort, and expense incurred. Replicate analyses of environmental samples can result in widely different counts and can be considered estimates only.

d. Sterility checks: Test media sterility before first use to ensure that there is no potential interference, and record results. Incubate at least one aliquot per lot or a set percentage (e.g., 1 to 4%) of laboratory- and commercially-prepared medium, broth, or agar at an appropriate temperature for the same time period as the actual test (e.g., 24 to 48 h for coliforms) and observe for growth. For enzyme-defined substrate tests, check for sterility by adding a media packet to 100 mL sterile deionized water and incubating at 35°C for the time specified in the method. Certain granulated ready-to-use enzyme-substrate media may be free of coliforms but not sterile; using nonselective broth could result in growth and turbidity but should not produce a positive reaction when compared to the positive sample tube supplied by the vendor.

TABLE 9020:VII. CALCULATION OF PRECISION CRITERION

Sample No.	Duplicate Analyses		Decimal Logarithms of Counts		Range of Logarithms (R_{log})
	D_1	D_2	L_1	L_2	$(L_1 - L_2)$
1	89	71	1.9494	1.8513	0.0981
2	38	34	1.5798	1.5315	0.0483
3	58	67	1.7634	1.8261	0.0627
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
14	7	6	0.8451	0.7782	0.0669
15	110	121	2.0414	2.0828	0.0414

Calculations:

$$\Sigma \text{ of } R_{log} = 0.0981 + 0.0483 + 0.0627 + \ldots + 0.0669 + 0.0414 = 0.718\ 89$$

$$\bar{R} = \Sigma\frac{R_{log}}{n} = \frac{0.71889}{15} = 0.0479$$

Precision criterion $= 3.27\ \bar{R} = 3.27\ (0.0479) = 0.1566$.

Check each new batch (or lot, if commercially prepared) of buffered water for sterility before first use by adding 50 mL of it to 50 mL of a double-strength non-selective broth (e.g., tryptic soy, trypticase soy, or tryptose broth). Alternatively, aseptically pass 100 mL or more dilution water through a membrane filter and place filter on nonselective medium. Incubate at 35 ± 0.5°C for 48 h and observe for growth. Record results.

If any contamination is indicated, discard dilution water, invalidate any data associated with that batch, and check for contamination source. Request immediate resampling.

Check sterility of process methodology as follows:

1) For each manifold used in membrane filter tests, check sterility of the entire process by using sterile dilution water as the sample at the beginning and end of each filtration series of samples and test for growth. If a processing interruption lasts >30 min, use new sterilized funnels and repeat sterility test. Record results.
2) For multiple-tube and presence–absence procedures, check sterility of prepared media and dilution water as outlined above.
3) For pour plate procedures, check sterility by pouring at least one uninoculated plate per batch or lot of media and record results.
4) If any contamination is indicated, determine the root cause. Invalidate analytical data from sample(s) tested. Document both cause/problem and corrective action taken. Request resampling. Laboratories interested in contaminant identification can use either standardized phenotypic testing systems or genotypic procedures.

e. Precision of quantitative methods[37,38]: For plate-based analyses [e.g., membrane filtration and some heterotrophic plate counts (HPC)], calculate precision of duplicate counts using the best dilution for reading each type of sample examined (e.g., drinking water, ambient water, or wastewater) according to the following procedure, and record results. NOTE: SimPlate for HPC does not require duplicate counts in the EPA-approved method.

1) Perform duplicate analyses on first 15 positive samples of each matrix type, with each set of duplicates analyzed by one analyst. Record duplicate analyses as *D*1 and *D*2. Calculate the logarithm of each result. If either of a set of duplicate results is <1, add 1 to both values before calculating the logarithms. Calculate the range (R) for each pair of transformed duplicates and the mean ($\bar{R}$) of these ranges (see sample calculation in Table 9020:VII). If more than one analyst regularly runs the tests, include them all, with each analyst performing an approximately equal number of tests.

2) Thereafter, analyze 10% of routine samples in duplicate, or one per test run. Transform the duplicates and calculate their range as above. If the range is >3.27 R, there is >99% probability that the laboratory variability is excessive, so discard all analytical results since the last precision check (see Table 9020:VIII). Identify and resolve the analytical problem before making further analyses. If sample test results have already been reported, it may be impractical to discard all test results. Resampling may have already been performed.

3) Update by periodically repeating the procedures using the most recent sets of 15 duplicate results. Using software can make these calculations easier to handle.

10. Verification

Verification is a general process used to determine whether the method and the analyst are both performing as expected to provide reliable data (i.e., determining false-positive and false-negative rates). If the verification percentage for a certain water supply or matrix is low, either another test method or more training is needed. For the most part, the confirmation/verification procedures for drinking water differ from those for other waters because of specific regulatory requirements. Microorganisms often are defined via method or operation, not taxonomy. A false positive occurs when a positive well, fermentation tube, or colony counted as the target bacterium is transferred to a confirmation medium and has a negative result. A false negative is determined when atypical colonies or media from a negative well or fermentation tube gives a positive confirmation result. The following is a brief summary; further information may be

Table 9020:VIII. Daily Checks on Precision of Duplicate Counts*

	Duplicate Analyses		Logarithms of Counts			
Analyses	D_1	D_2	L_1	L_2	Range of Logarithms	Acceptance of Range†
8/29	71	65	1.8513	1.8129	0.0384	A
8/30	110	121	2.0414	2.0828	0.0414	A
8/31	73	50	1.8633	1.6990	0.1643	U

* Precision criterion = (3.27 $\bar{R}$) = 0.1566.
† A = acceptable; U = unacceptable.

found in the appropriate discussions of the specific microorganism or microbial group.

a. Multiple-tube fermentation (MTF) methods:

1) Total coliform procedure (Section 9221B)

a) Drinking water—Carry tests through confirmed phase only. The completed test is not required.

If positive results have not normally occurred within a quarter, analyze at least one positive source-water sample to confirm that the media, laboratory procedures, and equipment produce appropriate responses (for both QC purposes and maintenance of analyst proficiency). For samples with a history of heavy growth without gas in presumptive-phase tubes, carry the tubes through the confirmed phase to check for false-negative responses for coliform bacteria. Verify any positives for thermotolerant (fecal) coliforms or *E. coli.*

b) Other water types—Verification can be achieved by performing the completed phase at a frequency established by the laboratory (e.g., 10% of positive samples, one sample per test run, or a certain percentage of normal laboratory workload). For large laboratories analyzing a significant number of samples daily, 10% of positive samples may be an unnecessary burden; choose an appropriate lower percentage.

2) Fecal streptococci and enterococci procedures—Verification can be performed as outlined in Section 9230C.5 at a frequency established by the laboratory.

b. Membrane filter methods:

1) Total coliform procedures

a) Drinking water—Swab entire membrane or pick up five typical and five atypical (nonsheen) colonies from positive samples on m-Endo or Endo LES agar medium and verify as directed in Section 9222B. Also verify any positives for thermotolerant (fecal) coliforms as described in ¶ *b*2) below. Adjust counts based on percent verification. If there are no positive samples, test at least one known positive source-water sample quarterly or, if the laboratory is running positive and negative culture controls, consider that this confirms the analysts are competent to determine a positive sample result.

b) Other water types—Verify positives monthly by picking at least 10 typical and atypical colonies from a positive water sample, as directed in Section 9222B. Adjust counts based on percent verification.

c) To determine false negatives, pick representative atypical colonies of different morphological types and verify as directed in Section 9222B.

2) Thermotolerant (fecal) coliform procedure—Verify positives monthly by picking at least 10 blue colonies from one positive sample using lauryl tryptose broth and EC broth as directed in Section 9221E.1. Adjust counts based on percent verification. To determine false negatives, pick representative atypical colonies of different morphological types and verify as directed in Section 9221B.3.

3) *Escherichia coli* procedure

a) Drinking water—Verification is not required.

b) Other water types—Verify one positive sample monthly by picking from well-isolated colonies while taking care not to pick up medium, which can cause a false-positive response. Perform the indole test and the citrate test as described in Sections 9225D.4 and 7, or other equivalent identification procedures or systems. Incubate the indole test at 44.5°C. *E. coli* are indole-positive and yield no growth on citrate. Adjust counts according to verification percentage.

c) To determine false negatives, pick representative atypical colonies of different morphological types and verify as in ¶ b) above.

4) Fecal streptococci procedure—Monthly, pick at least 10 isolated red colonies from m-Enterococcus agar to brain–heart infusion (BHI) media and verify as described in Section 9230C.5. Adjust counts based on percent verification.

5) *Enterococcus* procedures—Monthly, pick at least 10 well-isolated pink to red colonies with black or reddish-brown precipitate from EIA agar. Transfer to BHI media and verify as described in Section 9230C.5. Adjust counts based on percent verification.

c. Enzyme defined substrate tests:

1) Total coliform test (Section 9223)

a) Drinking water—Verification is not required.

b) Other water types—No confirmation/verification step is required. A positive result is based on the presence and reaction of a specific enzyme, and these tests use a defined substrate with inhibitors for noncoliform bacterial growth. The following is a brief description for those who want to conduct verification testing.

For total coliform analyses, aseptically transfer material from a certain percentage (e.g., 5%) of enzyme-substrate-positive wells and enzyme-substrate-negative wells to M-Endo or Levine EMB or other suitable media. Streak for isolation. For confirmation, test for lactose fermentation (note that a number of coliforms can be either slow lactose fermenters or may not ferment lactose at all) or for β-D-galactosidase by the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) test and indophenol cytochrome oxidase (CO) test or organism identification. See Section 9225D for test descriptions or use other equivalent identification procedures or systems.

2) *E. coli*—For *E. coli* analyses, no confirmation/verification is normally required; a positive result is based on the presence of a specific enzyme. Use of a comparator and negative culture control assists in the determination of a weak fluorescence. If verification is desired, aseptically transfer material from a certain percentage (e.g., 5%) of MUG-positive and MUG-negative wells to MacConkey or Levine EMB or other suitable media. Streak for isolation. Verify by confirming MUG reaction using EC+MUG or NA+MUG media or *E. coli* biochemical identification (as described in Section 9225D) or other equivalent identification procedure or system. Adjust counts according to verification percentage.

3) Simultaneous detection of total coliform bacteria and *E. coli*—Review the information in Section 9222J for the dual-chromogen MF procedure and Section 9222K for the flurogen/chromogen MF procedure. As noted above for *E. coli* analyses, verification typically is not required for drinking water samples; a positive result is based on the presence of a specific enzyme. For other water types, verify at the laboratory-established frequency based on need and sample type.

11. Validation of New or Nonstandard Methods[39–46]

The laboratory must validate all nonstandard methods, laboratory-developed methods, and standard methods applied to new test conditions (e.g., matrix) before using them to gather data. *Validation* is the process of demonstrating that a method, when properly performed, provides data that are accurate and reliable for their intended use. Although historically limited to the field of chemistry, validation now also applies to microbiology, using the same terms. The main difference is that when discrete variables (e.g., plate counts) are involved, analysts use different statistics and probability distributions.

For culture-based analyses, validation focuses on whether and how well a test method can detect and/or quantify a specific microorganism or group of microorganisms with set characteristics in the matrix of concern. For culture-independent methods (e.g., immunoassays and molecular genetic techniques), the same need exists to demonstrate process control and confidence in the information's reliability. This is essentially a proof of concept.

For compliance methods, obtain validation data from the manufacturer and/or regulator. Before adopting a new method, conduct parallel tests with the standard or reference procedure to determine the new method's suitability and to compare its performance to the standard's stated performance criteria. Obtain at least 30 positive data points over a period of time (e.g., 4 to 8 months) so analysts can statistically determine equivalence before replacing an established method with the new one for routine use. This can be called a *secondary* or *cross-validation*.

For methods in development (e.g., research methods), establish confidence in the analytical method by conducting full intralaboratory validation studies on a statistically significant number of samples in the applicable matrix or matrices to ensure reliability before making a final determination of usability. Conduct interlaboratory studies (also called *collaborative studies* or *round robin tests*) to validate the method for wider use. The following is a brief discussion of microbial method validation and the desired quality of performance criteria. Review the cited references for further information and for programs involved with microbial method validation.[39–46]

To determine the effect of matrix on recoveries, add a known concentration (set at an anticipated ambient level) to a field sample collected from the same site as the original. Use commercial laboratory-prepared cell suspensions of the target microorganism from a reputable source. The supplier should provide third-party evidence of competence and compliance with global standards. Microorganisms should be traceable to a culture collection, which can be verified through a license agreement.

a. Qualitative test methods: Validation of presence or absence (growth versus no-growth) methods involve establishing method performance characteristics in the matrix of choice, such as:

1) Accuracy and precision (repeatability and reproducibility)—For qualitative tests, analysts would need an extremely large number of replicates to statistically evaluate comparability, so these data-quality indicators generally are not determined.
2) Specificity/selectivity—These indicators show how well a test method can preferentially select or distinguish target organisms from nontarget ones in the matrix of choice under normal laboratory sample-analysis conditions (i.e., a method's fitness for use). For qualitative methods, the indicator is growth of the target organism and is determined by verifying all responses (e.g., by microbial identification testing).
3) Detection limit—This indicator reveals the lowest microbial density that can be determined under the stated conditions. Analysts do this by using dilutions of reference cultures and measuring recoveries among replicates of each dilution.
4) Robustness—This indicator measures how well a test method can perform under changing conditions. This test is conducted by the method's initial developer; it is determined by changing variables (e.g., sample holding time or conditions, incubation temperature, medium pH, and incubation time) and determining how much the resulting data vary.
5) Repeatability—This indicator shows the degree of agreement between replicate analyses or measurements conducted under the same conditions (e.g., laboratory, technician, and equipment). Use a target microorganism or microbial group density such that at least 75% will be positive (i.e., growth) so enough responses can be detected[47] for either a quantitative or qualitative test. This can serve as one measure of uncertainty.

b. Quantitative test methods: Validation of a method concerned with numerical determinations (e.g., count per unit volume) involves ascertaining the method's performance characteristics as noted above, in addition to the following:

1) Accuracy—This indicator notes the degree of agreement, or lack of uncertainty, between the observed and true values. Accuracy is estimated by using known reference cultures at the anticipated range of environmental densities and then comparing the new method's results to those of the reference or standard method. It is usually expressed as the percentage of recovery.
2) Precision/repeatability—This indicator reveals the degree of agreement between replicate analyses or measurements conducted under the same conditions (e.g., laboratory,

technician, and equipment). Use a target microorganism or microbial group density such that at least 75% will be positive, so enough responses can be detected.[46] This can serve as one measure of uncertainty.

3) Precision/reproducibility—This indicator shows the degree of variability when the same method or process is conducted under changed conditions (e.g., more than one analyst following the method in another area or room in the laboratory and/or using different equipment). This serves as another measure of uncertainty.
4) Recovery/sensitivity—This indicator notes a test method's ability to recognize or detect the target microorganism or a component thereof in the matrix of choice. Determine by analyzing enough samples using at least two added suspension levels of the target microorganism or by increasing or decreasing the sample volume or dilution analyzed, followed by determination of statistical confidence.
5) Detection limit—This indicator shows the lowest microbial density that can be determined. Determine by using dilutions of reference cultures and measuring recovery among replicates of each dilution.
6) Upper counting limit—This indicator reveals the level at which quantitative measurements become unreliable (e.g., due to overcrowding of typical and atypical colonies, which may mask target organisms on an agar plate). Determine as above.
7) Range—This indicator notes the interval between the upper and lower detection limits, determined as above.

12. Documentation and Recordkeeping

a. QA Plan: The laboratory's QA Plan or Quality Manual documents management's commitment to a QA policy and sets forth the requirements needed to support program objectives. The plan describes overall policies, organization, objectives, and functional responsibilities for achieving the quality goals and specifies the QC activities required to achieve the data representativeness, completeness, comparability, and compatibility. In addition, the QA plan includes the laboratory's implementation plan to ensure maximum coordination and integration of QC activities within the overall program (sampling, analyses, and data handling) and indicates compliance with federal, state, and local regulations and accreditation requirements where applicable. See 9020B.1.

b. Sampling records: A written SOP describing sample-handling records composed of the laboratory's procedures for sample collection, acceptance, transfer, storage, analyses, and disposal is necessary. Records associated with sample handling (i.e., chain-of-custody forms) should be completed for each sample entering the laboratory. Such records should be maintained long term, because it is critical that this record be exact and complete if there is any chance litigation may occur. Some federal or state programs may require chain-of-custody forms in order to ensure sample integrity. Details on chain-of-custody are available in Section 1060B.2 and elsewhere.[1] A laboratory system that uniquely identifies samples in the laboratory and that is tied to the field sample number will ensure that samples cannot be confused.

c. Recordkeeping: An acceptable recordkeeping system provides needed information on sample collection and preservation, analytical methods, medium and temperature used to conduct the test, date and time analyses were initiated and completed, QC results, raw data, calculations through reported results, and a record of persons responsible for sampling, sample acceptance, and analyses. Choose a format agreeable to both the laboratory and the customer (the data user). Use preprinted forms if available. Ensure that all data sheets are signed and dated by the appropriate analyst(s) and supervisor(s). The preferable record form is a bound and page-numbered notebook, with entries in ink, or a computer file (e.g., an e-notebook). Any change will be indicated by a single line drawn through the original text, the corrected text inserted adjacently, with the date of change and the recorder's initials next to the correction. Keep records of microbiological analyses for at least 5 years in a secure location. Offsite storage is recommended as backup for all records. Data expected to become part of a legal action must be maintained for a longer period of time. Actual laboratory reports may be kept, or data may be transferred to tabular summaries so long as the following information is included:

- date, place, and time of sampling;
- name of sample collector;
- sample identification;
- date and time of sample receipt;
- condition and temperature of received sample;
- dates of sample analysis start and completion;
- person(s) responsible for performing analysis;
- analytical method used;
- the raw data; and
- the calculated results of analysis.

Verify that each result was entered correctly from the bench sheet and initialed by the analyst.

When a laboratory information management system (LIMS) is used, verify the software input and output and arithmetic computations. Also, verify that no errors occurred when copying the data to the LIMS. Back up all laboratory data on disk or hard-copy system to meet the customer and laboratory needs for both data management and reporting. Verify data on the printouts. Always back up electronic data by protected tape or disk or hard copy.[45] If the system (hardware or software) is changed, transfer old data to the new system so it remains retrievable within the specified period of time. Data expected to become part of a legal action must be maintained for a longer period of time; check with the laboratory's legal counsel. Further guidance is available.[48–50]

13. Data Handling

a. Distribution of bacterial populations: Microbiological data can have wide uncertainty ranges due to non-homogeneous samples and bacteria's variable growth characteristics. In most chemical analyses, the distribution of analytical results follows a normal (Gaussian) curve, which has a symmetrical distribution of values about the mean (see Section 1010B). Microbial distributions, on the other hand, are not necessarily symmetrical and rarely fit a normal distribution curve. Bacterial counts often have a skewed distribution due to many low values and a few high ones, leading to an arithmetic mean that is considerably higher than the median. The frequency curve of this distribution has a long right tail (see Figure 9020:1), which is referred to as *positive skewness*.



Figure 9020:1. Frequency curve (positively skewed distribution).

The microorganism distribution in a sample may be natural and unique to the sample and matrix, rather than a function of laboratory performance.[51] Also, microbial counts represent colony-forming units (CFUs), which may have resulted from one or more bacterial or fungal cells or filaments,[52] leading to variations in colony counts in replicate plates or multiple dilutions. In addition, the number of CFUs on the agar surface depends on the type of medium used, its growth potential, and incubation conditions. Simply using the same medium produced by different manufacturers may result in different colony counts.

The more common statistical techniques assume data symmetry (e.g., the normal distribution), so skewed data usually must be converted to a more symmetrical distribution before such techniques can be applied. An approximately normal distribution may be obtained from positively skewed data by converting numbers to their decimal logarithms, as shown in Table 9020:IX. A comparison of the frequency tables for the original data (Table 9020:X) and their logarithms (Table 9020:XI) shows that the logarithms approximate a symmetrical distribution.

b. Central tendency measures of skewed distribution: Analysts use two calculations to determine the central tendency (if any) of microbiological data: Poisson distributions and geometric means. A Poisson distribution indicates the likelihood of observing the organism(s) of interest, and the geometric mean indicates the most likely number of such organism(s) to be found in a given sample.

A multiple Poisson distribution indicates the probability of observing organisms via multiple dilutions.[53,54] The resulting curve appears skewed to the right, much like a log-normal distribution curve, because individual Poisson distribution curves indicate colony counts for different organisms—including those not of interest, which further skew the overall distribution curve. When the maximum likelihood approach[55,56] is used, the maxima of these organisms are spread out under the overall distribution curve because different organisms respond differently to the same nutrients, media, temperature, pH, and incubation time. Analysts should study the maximum-frequency data to ensure that they select the correct organism for colony counting.

When analysts examine the most probable number (MPN) curves for 1, 2, 3, and 4 positive tubes out of 5 total tubes incubated, the log-normal probability graph is close to being linear (thus indicating approximate normality) but bows upward. The bowing could indicate kurtosis (a sharpness) brought about by measuring the cumulative probability on the low and high ends of the distribution curve, which is difficult to do and, therefore, more error-prone. The log-normal probability assumption is confirmed when analysts plot the log of values against colony-count MPN on log-normal–cumulative probability graph paper.

Table 9020:IX. Coliform Counts and Their Logarithms

MPN Coliform Count *No./100 mL*	log MPN
11	1.041
27	1.431
36	1.556
48	1.681
80	1.903
85	1.929
120	2.079
130	2.114
136	2.134
161	2.207
317	2.501
601	2.779
760	2.881
1020	3.009
3100	3.491
$\bar{x}$ = 442	$\bar{x}_g$ = antilog 2.1825 = 152

The geometric mean best estimates the central tendency of log-normal data; it is used when a probability distribution is anticipated. The term *mean* in geometric mean is misleading; what a geometric mean determines is the maximum likelihood estimate, which is based on the mode (maximum frequency) of the distribution curve (i.e., both frequency of *n* observations and the count of a random sample on *n* observations). It is calculated as the *n*th root of the product of all the data values.[57]

The geometric mean of the maximum likelihood estimates is a better estimate than the arithmetic average for living organisms because the geometric mean considers both frequency and variability in colony counts. When deriving the maximum likelihood[58] for a Poisson probability distribution, the log of the products of MPN can be shown to be a function of the log of frequency, thereby justifying the use of geometric mean. The geometric mean is the log of the inverse of the average log of likelihoods of the measured parameter. This value is generally lower than the arithmetic average of MPNs.[58]

When the likelihood ratio is observed before and after the log transformation of the variable *x*, it can be shown that the ratios are the same.[57] By means of the log-likelihood ratio, product properties are converted into summation properties, which are easy to understand and deal with.

c. "Less than" (<) values: There has always been uncertainty as to the proper way to include "less than" values when calculating and evaluating microbiological data because such values cannot be treated statistically without modification. Proposed modifications involve changing such numbers to zero, choosing values halfway between zero and the "less than" value, or

Table 9020:X. Comparison of Frequency of MPN Data

Class Interval	Frequency (MPN)
0–400	11
400–800	2
800–1200	1
1200–1600	0
1600–2000	0
2000–2400	0
2400–2800	0
2800–3200	0

Table 9020:XI. Comparison of Frequency of Log MPN Data

Class Interval	Frequency (log MPN)
1.000–1.300	1
1.300–1.600	2
1.600–1.900	1
1.900–2.200	5
2.200–2.500	1
2.500–2.800	2
2.800–3.100	2
3.100–3.400	0
3.400–3.700	1

assigning the "less than" value the value itself (i.e., changing <1 values to 0, 1/2, or 1, respectively).[59–61]

There are valid reasons for not including "less than" values, whether modified or not. If the database is fairly large and contains few such values, then their influence would be minimal and of no benefit. If the database is small or contains a relatively large number of "less than" values, then they would exert an undue influence and could artificially bias results either negatively or positively. Including "less than" values is particularly inappropriate if the values are <100, <1000, or higher because the unknown true values could be anywhere from 0 to 99, 0 to 999, etc. When such values are first noted, adjust or expand test volumes. The only exception to this caution would be regulatory testing with defined compliance limits (e.g., the <1/100 mL values reported for drinking water systems where the 100-mL volume is required).

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9020 C. Interlaboratory Quality Control

1. Background

Interlaboratory QC programs are a means of establishing an agreed-upon, common performance criteria system that will ensure an acceptable level of data quality and comparability among laboratories with similar interests and/or needs. A number of publications[1–6] and organizations* address interlaboratory programs.

A *certification program* is one in which an independent authority issues a written assurance or certificate that a laboratory is managed in compliance with that authority's standards. An *accreditation program* is one in which a specialized accredita-

* American Association for Laboratory Accreditation, www.a2la.net, and National Cooperation for Laboratory Accreditation, www.nacla.net.

tion body sets standards and then a certification body determines whether the laboratory exhibits competence in following the standards. If so, the laboratory receives formal recognition. Often the term *accreditation* is used interchangeably with *certification*.

Usually, interlaboratory QA programs have three elements: uniform criteria for laboratory operations, external review of the program, and external proficiency testing. These programs help laboratories address continual-improvement efforts.

2. Uniform Criteria

Interlaboratory QC programs begin as a voluntary or mandatory means of establishing uniform laboratory standards for a specific purpose. The participants may be from one organization or a group of organizations with either common interests or common regulatory requirements. Often one group or person may agree to draft the criteria. If the participants are regulated, the regulator may set the criteria for compliance-monitoring analyses.

Uniform sampling and analytical methods and QC criteria for personnel, facilities, equipment, instrumentation, supplies, and data handling and reporting are proposed, discussed, reviewed, modified if necessary, and approved by the group for common use. Criteria identified as necessary for acceptable data quality should be mandatory. A formal document is prepared and provided to all participants.

The QA/QC responsibilities of managers, supervisors, and technical staff are described in 9020A.2. In large laboratories, the QA officer is a staff position, but a supervisor or other senior person may assume the role in smaller laboratories.

Once the QA program has been incorporated into laboratory operations and confirmed to be in routine use, the laboratory supervisor and QA officer jointly conduct an internal program review of all operations and records for acceptability, to identify possible problems and help resolve them. If this is done properly, there should be little concern that subsequent external reviews will find major problems.

3. External Program Review

Once a laboratory has a QA program in place, managers inform the certifying or accrediting organization and request an external quality assessment. The choice of assessor and type of assessment will depend on a number of variables, such as accreditation request(s) and whether the sample analyses will be for compliance purposes. An experienced external QA professional or team then arranges an onsite visit to evaluate the QA program for acceptability and to work with the laboratory to solve any problems. Laboratories applying for review will have their laboratory documentation and procedures reviewed. An acceptable rating confirms that the laboratory's QA program is operating properly and that the laboratory can generate valid, defensible data. Such onsite evaluations are periodic and may be announced or unannounced.

4. External Proficiency Testing

Laboratories applying for certification or accreditation must participate in routine proficiency testing for the analytical, technological, or matrix-specific procedures that they intend to use. On a set schedule, the accrediting authorities send challenge samples (unknowns) to the laboratories for analyses. Each unknown must be processed as a routine sample by the analyst who typically runs the related method, and the results are reported back for evaluation. The certifier/accreditor codes the results for confidentiality, evaluates them according to an agreed-upon scheme, and summarizes them for all laboratories. Each participant then receives an individual report that indicates how well its personnel conduct routine analyses compared to the rest of the group. Also, the overall group's results characterize the performance that can be expected for each analytical method tested. Failure to evaluate unknowns successfully can result in loss of certification/accreditation.

Laboratories not applying for certification/accreditation can purchase unknowns for their own use.

5. Maintenance

After passing an external evaluation and analyzing a set number of unknowns successfully, the laboratory will be formally notified that it has been certified/accredited. To maintain this status, the laboratory must continue to analyze proficiency-testing samples successfully on an annual or semi-annual basis (established by the certifier/accreditor) and pass an onsite assessment about once every 3 years.

6. Example Programs

In the U.S. Drinking Water Laboratory Certification Program, public water supply laboratories must be certified according to minimal criteria, procedures, and QA described in the EPA manual on certification:

- criteria are established for laboratory operations and methodology;
- the certifying state agency or its surrogate must conduct onsite inspections to verify that such criteria are met;
- laboratories must perform acceptably on annual proficiency tests; and
- if problems are identified during inspections or proficiency testing, the certifying state agency must follow up and require corrections within a set timeframe.

Individual state programs may exceed federal criteria.

In addition, there are several Clean Water Act (CWA) programs that monitor recreational water quality, assess impaired waters, and develop total maximum daily loads (TMDLs) for discharges through the National Pollutant Discharge Elimination System (NPDES). The CWA program also requires laboratory certification through either state programs or the National Laboratory Accreditation Institute (TNI). To maintain accreditation by TNI, laboratories must have performed acceptably during two of the last three proficiency tests and successfully pass routine onsite assessments.

Previous onsite inspections of drinking water laboratories indicate that the primary causes of discrepancies have been inadequate equipment, improperly prepared media, incorrect analytical procedures, and insufficiently trained personnel.

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9030 LABORATORY APPARATUS*

9030 A. Introduction

This section contains specifications for basic microbiological laboratory equipment. Additional equipment needed to analyze specific microorganisms is discussed in the sections pertaining to those organisms. Ensure that all laboratory equipment is calibrated and its performance verified before initial use and on a routine basis thereafter. Conduct and document maintenance at least annually, either in-house or via service contract, following manufacturer's and analytical requirements. For testing and maintenance procedures related to quality control (QC), see Section 9020.

* Approved by Standard Methods Committee, 2015.
Joint Task Group: Gil Dichter (Chair), Ellen B. Braun-Howland, Nancy H. Hall, Margo E. Hunt, Kimberly Phillips.

9030 B. Equipment Specifications

1. Incubators

Use incubators that are large enough to prevent internal crowding and can maintain a constant, uniform temperature at all times in all areas. To obtain such accuracy and sensitivity, use a water-jacketed, forced-air, or convection incubator with thermostatically controlled, low-temperature electric heating units. The units must be properly insulated, located in or next to the chamber walls or floor, and preferably equipped with a mechanical means of circulating air.

Incubators with high-temperature heating units are unsatisfactory because, when improperly placed, the units frequently cause localized overheating and excessive media dehydration, thereby inhibiting bacterial growth. Such incubators can become satisfactory if mechanical air-circulation devices are installed and the high-temperature heating units are replaced with suitable wiring arranged to operate at a lower temperature.

If ordinary room temperatures vary excessively, equip the incubators with compressors to remove heat or else keep them in special rooms maintained at a few degrees below the recommended incubator temperature. Alternatively, use special well-insulated incubating rooms equipped with properly distributed heating units, forced-air circulation, and air-exchange ports (provided that they conform to desired temperature limits). When using such rooms, record the daily temperature range in areas where Petri plates or tubes are incubated. For all incubators, use open metal-wire or perforated shelves that are spaced to ensure temperature uniformity throughout the chamber. Leave a 2.5-cm space between walls and stacks of dishes, baskets of tubes, or multi-well trays. See Section 9020B.4*o*.

Use a water-bath incubator, sized for the laboratory's workloads, with a gabled cover to reduce water and heat loss and a circulating pump to maintain set temperature. Keep water depth in the incubator sufficient to immerse tubes to upper level of sample. Use weights (ensure that the weights are totally immersed in water and at incubation temperature before adding samples) or metal or plastic tube racks, ensuring that plastic bags containing plated media and multi-well trays are completely immersed. Water baths also may be used to temper agar and for other purposes. Never place samples directly on the heating element.

Calibrate heat-block incubators and determine their stability before initial use; calibrate the built-in thermostat annually thereafter with a National Institute of Standards and Technology (NIST)-traceable thermometer or an external temperature-sensing device. (See Section 9020B.4*a*.)

2. Hot-Air Sterilizing Ovens

Use hot-air sterilizing ovens large enough to prevent internal crowding. They are constructed to provide uniform and adequate sterilizing temperatures of 170 ± 10°C, and equipped with suitable glass thermometers or equivalent (inserted in sand). Use of a calibrated temperature-recording instrument is optional. The ovens also may be used at lower temperatures to dry glassware.

3. Autoclaves/Steam Sterilizers

Use autoclaves large enough to prevent internal crowding; constructed to provide uniform temperatures within the chambers (up to and including the sterilizing temperature of 121°C); and capable of reaching the desired temperature within 15 min. It is important that all trapped air is removed from the autoclave because it is a very poor medium for achieving sterility. Autoclaves should be equipped with an accurate thermometer whose bulb is properly located in the exhaust line to register temperature in the sterilizing chambers (temperature-recording instrument is optional). They also should be equipped with a pressure gauge and properly adjusted safety valves directly connected either to a suitable special steam generator or to saturated-steam supply lines equipped with appropriate filters to remove particulates and oil droplets. Do not use steam from a boiler treated with amines for corrosion control.

Some currently available autoclave models are automatic and include such features as vertical sliding, self-sealing and -opening doors; programmable sterilization cycles; and continuous multipoint monitoring of chamber temperature and pressure. They also may include solution-cooling and vapor-removal

features. If a medium is to be autoclaved for no more than 10 min, the maximum heat-exposure time must not exceed 30 min.

Vertical autoclaves and pressure cookers are not recommended because of difficulty in adjusting and maintaining sterilization temperature. However, a vertical autoclave could be used if it can adjust and maintain sterilization temperature. If a pressure cooker is used in an emergency or under special circumstances, equip it with an efficient pressure gauge and a thermometer with the bulb 2.5 cm above the water level.

4. Gas Sterilizers

Use a sterilizer with automatic controls that can carry out a complete sterilization cycle and a sterilizing gas (e.g., ethylene oxide diluted to 10 to 12% with an inert gas). Provide an automatic control cycle to

- evacuate sterilizing chamber to at least 0.06 kPa,
- hold the vacuum for 30 min,
- adjust humidity and temperature,
- charge with the ethylene oxide mixture to a mixture-dependent pressure,
- hold such pressure for at least 4 h,
- vent gas,
- evacuate to 0.06 kPa, and finally,
- bring to atmospheric pressure with sterile air.

The sterilizing cycle's humidity, temperature, pressure, and time depend on the gas mixture used. A wet gauze or paper towel may be needed to achieve the correct humidity for proper sterilization. **CAUTION: Ethylene oxide is toxic—avoid inhalation, ingestion, and skin contact. Also, ethylene oxide forms an explosive mixture with air at 3 to 80% proportion and provides a much narrower margin of safety than steam sterilization. Malfunctions can occur that are not easily detected. CAUTION: Take care with other gases used for sterilization, such as chlorine dioxide and hydrogen peroxide.**

Store gas-sterilized sample bottles (with loosened caps) overnight to allow last traces of gas mixture to dissipate. Incubate gas-sterilized media overnight to ensure that gas dissipates. An aerator may also be used to remove traces of gas.

In general, mixtures of ethylene oxide containing chlorinated hydrocarbons (e.g., trichloro-trifluoroethane) are harmful to plastics, although the effect is minimal at temperatures $<55°C$, gas pressure <35 kPa, and sterilization time <6 h. If carbon dioxide is used to dilute ethylene oxide, increase exposure time and pressure (depending on the temperature and humidity that can be used).

Determine proper cycle and gas mixture for objects to be sterilized and confirm via sterility tests.

5. Optical Counting Equipment

a. Pour and spread plates: Use a dark-field model colony counter with a magnification of 1.5×, satisfactory visibility, and adequate lighting.

b. Membrane filters: Use a binocular or zoom microscope with a magnification of 10 to 15×. Provide daylight fluorescent light source at 60 to 80° angle above the colonies; use low-angle lighting for non-pigmented colonies.

c. Tally equipment: Do not use automatic colony counters when determining count for compliance purposes or when enumerating pour plates, which typically have submerged pinpoint colonies in clear-colored medium. When automatic colony counters are used, have units calibrated when installed and check performance via conventional counting methods.

When counting colonies, use either a mechanical hand tally or an electronic tally unit that touches the agar surface (the latter after any colony culture transfer, if needed, has occurred). Test unit for accuracy before initial use and annually thereafter.

6. pH Equipment

Use electrometric pH meters accurate to at least 0.1 pH units to measure the pH values of media; preferably, the meters should also have automatic temperature compensation. Use appropriate probes to determine pH of liquid and solid agar media. Refer to pH manual for information on slope and corrective action. Record slope monthly or as required. The pH meter must be calibrated with at least two buffers that bracket the measurement range. The buffers should be obtained from an approved vendor. See Section 9020B.4*c* and Table 9020:I.

7. Balances

Use balances whose sensitivity is at least 0.1 g at a load of 150 g, with weights traceable to appropriate national standards. Use an analytical balance whose sensitivity is 1 mg under a load of 10 g to weigh small quantities (<2 g) of materials. Single-pan rapid-weigh balances are most convenient. Place balances on solid surfaces to avoid vibrations and in locations where drafts and humidity levels are reduced.

8. Media-Preparation Utensils

Use equipment made of borosilicate glass, stainless steel, or other suitable noncorrosive materials. Use glassware that is clean (washed as noted in Section 9040) and free of residues, dried agar, or other foreign materials that may contaminate media.

9. Pipets, Micropipets, and Graduated Cylinders

Use pipets of any convenient size that can deliver the required volume accurately and quickly. The calibration error for a given manufacturer's lot must not exceed 2.5%. Use pipets with distinctly marked graduations and unbroken tips. Bacteriological-transfer pipets or pipets conforming to American Public Health Association (APHA) standards may be used. **CAUTION: Do not pipet by mouth; use a pipet aid.**

Calibrate and maintain adjustable micropipettors according to manufacturer's instructions because differences will exist between different units. Use sterile tips specific to the micropipettor and their intended use. For example, units used for PCR analyses may contain aerosol-resistant tips and should be certified as RNase-free, DNase-free, and pyrogen-free.

Use graduated cylinders meeting ASTM International standards (D-86 and D-216) and with accuracy limits established by NIST, where appropriate.

10. Pipet Containers

Use cylindrical or rectangular boxes made of aluminum or stainless steel that are 5 to 7.5 × ~40 cm. When these are not available, use paper wrappings or equivalent for individual pipets. To avoid excessive charring during sterilization, use best-quality sulfate pulp (kraft) paper. **CAUTION: Do not use copper or copper alloy cans or boxes as pipet containers.**

11. Refrigerator and Freezers

Use a refrigerator that can maintain 2 to 10°C to store samples, media, reagents, etc. Do not store volatile solvents, food, or beverages in the same refrigerator with media or cultures. Frost-free refrigerators may cause excessive dehydration of media stored for longer than 1 week; do not use them if this is a concern.

Freezer temperature range depends on analytical need (e.g., culture storage). Standard laboratory freezers may range from –10 to –20°C down to −70 to −90°C. Monitor freezer temperature.

12. Temperature-Monitoring Devices

Use glass or metal thermometers, probes, or sensors linked to temperature-recording devices or digital-display computers to monitor required analytical temperature range for incubators and refrigerators; non-mercury thermometers enclosed in bottles of water or glycerol can be placed on each shelf. Thermometers must be graduated in appropriate increments; for example, use thermometers graduated to 0.1°C for incubators operated >35°C. Alternatively, analysts can use continuous recording devices or digital temperature-measuring devices instead of thermometers. Calibrate and record accuracy of all temperature-monitoring devices at least annually, via comparison with a NIST-certified thermometer or equivalent. See Section 9020B.4*a*.

13. Dilution Bottles or Tubes

Use resistant-glass bottles or tubes (preferably borosilicate glass) closed with glass stoppers, lined screw caps, or equivalent that do not produce toxic or bacteriostatic compounds when sterilized. Do not use cotton plugs as closures. Mark graduation levels indelibly on side of dilution bottle or tube, and confirm that marks are within required level of accuracy. Plastic bottles of nontoxic material and acceptable size may be substituted for glass if they can be sterilized properly. Discard any items with chips, cracks, or scratches. See Section 9020B.5*a*.

14. Petri Dishes

For plate count and environmental monitoring, use glass or plastic Petri dishes about 100 × 15 mm or 150 × 20 mm. Dish bottoms must be bubble- and scratch-free and flat so the medium thickness will be uniform throughout the plate. For the membrane filter technique, use loose-lid glass or plastic dishes (60 × 15 mm) or tight-lid dishes (50 × 12 mm). Sterilize glass Petri dishes and store in metal cans (aluminum or stainless steel, but not copper), or wrap them in paper—preferably best-quality sulfate pulp (kraft)—before sterilizing.

15. Multi-Well Trays and Sealer Units

A variety of trays for most-probable-number (MPN) determination is available. Use trays and seal them using the unit supplied by the manufacturer. QC information is discussed in Section 9020B.5*e*. Sealer units should be cleaned monthly (or at frequency established by laboratory) and maintained following manufacturer recommendations.

16. Membrane Filtration Equipment

Use filter funnel and membrane holders that are made of seamless stainless steel, glass, or autoclavable plastic; do not leak; and are not scratched or corroded. If metal grids break, replace them. Field laboratory kits are acceptable for in-field use only; in the laboratory, use standard laboratory filtration equipment and procedures. See also Section 9020B.4*k*.

17. Fermentation Tubes, Vials, and Bottles

Use any type of fermentation tubes whose design conforms to the medium and volume requirements for concentrating nutritive ingredients. If used for a gas-production test, enclose an inverted Durham tube or vial sized so it will be filled completely with medium, be at least partly submerged in the tube, and let gas bubbles be easily visible.

18. Inoculating Equipment

Use ≥3-mm-dia. wire loops made of 22- or 24-gauge nickel alloy* or platinum-iridium that can be sterilized via flame or electric micro incinerator. Another option is single-use hardwood or plastic applicators that are 0.2 to 0.3 cm in diameter and at least 2.5 cm longer than the fermentation tube. Sterilize wooden applicators via dry heat and plastic ones via autoclave, and store in glass or other nontoxic containers. A third option is prepackaged sterile, disposable, plastic loops and needles.

19. Sample Bottles

Use suitably sized and shaped bottles that can hold enough sample for all required tests with enough air space left over (at least 1 in.) to permit proper mixing, and that can be capped to keep samples uncontaminated until examinations are completed. Wide-mouthed bottles are recommended. If preparing sample bottles in-house, use nontoxic glass or plastic ones (e.g., polypropylene). Commercial sampling bottles are single-use; for example, presterilized disposable plastic bags or bottles (with or without dechlorinating agent) are available commercially. Most plastic containers reduce shipping weight and eliminate the possibility of breakage during shipment.

Sample bottles may have metal or plastic screw-cap closures with liners so long as no toxic compounds are produced when they are sterilized.

* Chromel, nichrome, or equivalent.

Before sterilizing sample bottles with glass closures, cover their tops and necks with aluminum foil or heavy kraft paper. See Section 9020B.5*d*.

20. Microscopes

Laboratory needs will dictate the microscope properties, such as magnification (ocular and objective lenses), and lighting components required. Most basic microbiology laboratories will have a compound brightfield light microscope.

The manufacturer or qualified service representatives should check stage micrometer calibration and lens quality and focus. Follow manufacturer's recommendations to adjust lighting and focus eyepieces and check alignment routinely. Follow Kohler illumination procedures for each objective used. After each use, clean the microscope body and lenses. Use manufacturer-recommended immersion oil for oil-immersion lenses only, and ensure that oil is removed from lens after each use. Use pressurized air or rubber bulb to blow off dust; do not blow onto lenses. Use only optically safe tissues and cleaning solutions designed for microscopes.

When not in use, cover microscope and store it in areas where temperature and humidity levels are consistently low. For some microscopes (e.g., fluorescence microscopes), record light usage times and limit technician time at the microscope. See Section 9020B.4*p*.

21. Centrifuges

Centrifuge speed(s) should meet the analytical method criteria. Follow manufacturer's instructions for usage, maintenance, and calibration. Use only with lab equipment designed for that centrifuge and capable of withstanding the relative centrifugal forces to be used. Ensure that centrifuge tube loads are balanced. Inspect rotor and carriers according to manufacturer recommendations. Clean units whenever contamination is suspected and as often as the lab's standard operating procedure (SOP) requires, using appropriate disinfectant solutions (e.g., a freshly prepared 0.5% sodium hypochlorite solution) followed by an ethanol (70%) rinse to remove residual bleach and avoid metal pitting.

22. Laminar-Flow Hoods/Biological Safety Cabinets

Purchase units designed for analytical needs. Laminar-flow hoods (also known as *laminar-flow clean benches*) may duct to the outside, and unidirectional airflow blows sterile air towards the operator. These units do not protect the environment unless filtered exhausts are installed.

Biological safety cabinets (BSCs) are classified according to the degree of protection each affords to the technician, the product, and the environment. Class I cabinets have high-efficiency particulate arrestance (HEPA) filtered exhaust air systems, but do not supply air. They provide worker protection only. Class II cabinets provide HEPA filtered supply and exhaust air, as well as worker and product protection. Class III BSCs provide HEPA filtered supply and exhaust air, provide worker and product protection, and are sealed (gas tight) to provide an additional level of worker protection.

Sanitize all units after each use (and, in some situations, before use), visually check airflow before use, and have units serviced annually. Maintain certificates.

NOTE: Bunsen burners may disrupt airflow patterns within any hood and may harm HEPA filters used within units.

23. Ultraviolet Lights

Short-wave ultraviolet (UV) lights (254-nm) can be used for sanitation purposes and to decrease nucleic acid contamination. Long-wave UV lights (365- to 366-nm) are used to detect fluorescence in enzymatic methods. All laboratory lights need to be turned off and incident light blocked (e.g., from windows) for effectiveness. QC and safety considerations are discussed in Section 9020B.4*l*.

24. Spectrophotometer

A spectrophotometer measures light absorption and can be used, for example, to determine turbidity/growth of microbial cultures. Have unit calibrated at installation and check performance each day of use with a method-specific blank and an appropriate calibration standard obtained from an outside source.

25. Homogenization Apparatus

Blenders and stomachers are used to extract bacteria from materials in which they may be found or attached to (e.g., filters). Select variable-speed units with appropriate capacity for intended use. Vortex mixers and rotary shakers are designed to resuspend bacteria in a solution. Rotary shakers also are used in incubator rooms to shake cultures. Follow manufacturer's instructions to determine required stroke measurement and revolutions per minute on initial use and quarterly thereafter.

26. Media and Solution Dispensers

Follow manufacturer's instructions to verify the performance (i.e., delivery-volume accuracy and precision) of automatic diluters, micropipettors, and media-dispensing apparatus at initial use and whenever volume amounts dispensed are changed. If delivery-volume amounts do not change, verify continued capability at least quarterly. This can be determined by taking replicate mass/volume measurements.

27. Bibliography

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9040 WASHING AND STERILIZATION*

Clean glassware† is essential in microbiology laboratories to ensure reliability in analytical tests. Clean before first use. All contaminated laboratory ware must be sterilized before cleaning. Wash labware after use. If material has dried on glassware, a pre-soaking may be needed. Consistently follow the same washing procedures, whether using an automated system or washing by hand. Where possible, use mechanical glassware washers. Do not use units designed for home use or with copper plumbing. Instead, use stainless steel or other nontoxic material for influent, distribution, and rinse-water systems. Ensure that the water jets' spray reaches all parts of the vessels, including deep vessels.

Cleanse all glassware thoroughly with warm to hot water and a suitable laboratory-grade detergent containing no phosphates. To remove all traces of residual washing compound, rinse five to ten times with cold water after bubbles/foam are gone. Then, rinse two to three times with reagent-grade water. Alternatively, any washing method in a commercial glass washer can be used.

Perform the bromothymol blue pH check on each batch (all items washed at the same time) and type of glassware to demonstrate that no alkali or acid residues are present. If residues are found, rewash glassware. If this test is done on each batch of washed glassware, run the glassware inhibitory residue test before initial use of a washing compound and whenever a new formulation or washing procedure is used. If the bromothymol blue test is not done consistently, also run the toxicity test annually. NOTE: Glass items washed and sterilized in the laboratory may contain toxic detergent residues not detected by the bromothymol blue pH test. These items must be checked periodically for toxic detergent residues. Review the information in Section 9020B.5*a*2). Perform the bromothymol blue pH check [Section 9020B.5*a*1)] on each batch and type of glassware to demonstrate that no alkali or acid residues are present. If residues are found, rewash glassware. NOTE: Glassware used for some analyses [e.g., biochemical oxygen demand (BOD) and assimilable organic carbon (AOC)] must be organic-free. Use a furnace set at 550°C for ≥4 h.

Protect clean glassware and materials against dust during storage, in conditions that will maintain its cleanliness.

To sterilize glassware via dry heat, use a hot-air oven set at ≥170°C for 2 h or longer. Alternatively, sterilize glassware by autoclaving at 121°C for at least 30 min. For all bottles, loosen caps before autoclaving. If desired after autoclaving, remove moisture present in empty sterile containers by placing items in a drying oven. For glass pipets in metal containers, sterilize using a hot-air oven set at ≥170°C for at least 2 h.

As long as the bromothymol blue pH test is being done on *each* batch of washed glassware, only run the glassware inhibitory residue test [Section 9020B.5*a*2)] before initial use of a washing compound and whenever a new formulation or washing procedure is used. If the bromothymol blue test is not done consistently, run the glassware inhibitory residue test on a per-lot or annual basis, whichever is more frequent. Review Sections 9020B.4*h* and 5*d* for quality-control checks on autoclaves and sterilized bottles.

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Joint Task Group: Margo E. Hunt (chair), Gil Dichter, Nancy H. Hall, Robin K. Oshiro.

† The term *glassware* refers to both borosilicate glass and heat-resistant plastic materials.

9050 PREPARATION OF CULTURE MEDIA*

9050 A. General Procedures

1. Storage of Culture Media

Store dehydrated media (powders) in tightly closed bottles in the dark at <30°C in a low-humidity atmosphere (e.g., a desiccator). Do not use them if they are discolored, caked, or no longer a free-flowing powder. Use stocks of dehydrated media before their expiration date or within a year of purchase if they contain selective agents (sodium azide, bile salts, antibiotics, sulfur-containing amino acids, etc.) in order to maintain optimum selectivity.

Use commercially prepared media wherever available. Avoid preparing media from essential ingredients unless necessary. See also Section 9020B.5*j*.

For media prepared onsite, review manufacturer's instructions, product material safety data sheets (MSDSs), and analytical methods. Prepare culture media in batches that will be used within 2 weeks unless the method specifies otherwise. If media are contained in screw-capped tubes, however, they may be stored for up to 3 months at <30°C. (See Table 9020:V for specific details on storage time and temperature.) Store media out of direct sunlight and avoid excessive evaporation. Place prepared Petri dishes in air-tight containers or plastic bags, close with twist-ties, and store under refrigerated conditions. Invert Petri dishes to prevent moisture condensation on agar. Do not use plates with condensation drops because this will cause colonies to spread. If necessary, dry plates by placing them with lids slightly ajar in a laminar-flow hood.

If refrigerated, liquid media in fermentation tubes may dissolve enough air to produce an air bubble in the inner Durham tube when later incubated at 35°C. Bring all media (especially fermentation or carbohydrate broth) to room temperature before use and discard tubes containing any air bubbles.

After prolonged incubation or storage, selective agents may break down and evaporation may change media ingredients' concentrations. Discard tubes with growth due to contamination or an evaporation loss of more than 1 mL. A loss of 10% or more (e.g., 1 mL or more from an initial 10 mL) can affect most-probable-number (MPN) calculations.

2. pH Adjustment

After sterilization, determine and record medium's pH. The directions for preparing each medium typically specify the final pH; if so, adjust pH as needed. If a specific pH is not prescribed, adjustment is unnecessary. During sterilization, the pH usually will drop 0.1 to 0.2 but occasionally as much as 0.3 in double-strength media. If the media contain buffers, the decrease in pH will be negligible. The initial pH required to obtain the correct final reaction will have to be determined.

To determine final pH, cool medium to 44–46°C, aseptically remove a small quantity, set meter for the higher temperature (if not done automatically), and measure pH. Alternatively, completely cool a sample of the medium to room temperature before determining final pH. If pH adjustment is necessary, use a sterile stir bar and pipet a sufficient quantity of filter-sterilized 0.1*N* sodium hydroxide (NaOH) or 0.1*N* hydrochloric acid (HCl) into the bulk medium to reach the proper pH.

If dehydrated media are reconstituted according to directions, their pHs seldom require adjustment. However, the final pH may be unacceptable if dehydrated medium was weighed improperly, reconstituted medium was overheated, or there was a problem with the medium itself. If the prepared media's pH values are consistently outside the allowed range, determine the cause. The pH may need to be adjusted before sterilization.

3. Sterilization

After rehydrating a medium, dispense promptly into clean culture vessels without cracks or chips and sterilize within 2 h. Do not store nonsterile media.

Sterilize media in an autoclave at 121°C. Review method and manufacturer's requirements. The required exposure time will vary with form and type of material, medium, presence of carbohydrates, and volume. Sterilize most carbohydrate broths at 121°C for 12 to 15 min; however, there are exceptions. For example, A-1 media must be autoclaved for 10 min at 121°C. When the pressure reaches zero, remove medium from autoclave and cool quickly to avoid decomposition of sugars due to prolonged heat exposure. To permit uniform heating and rapid cooling, loosely pack materials in small containers. The maximum heat exposure for most carbohydrate broths (from closing loaded autoclave to unloading) is <45 min. The maximum heat exposure for A-1 medium is <30 min. Preferably use a double-walled autoclave to permit preheating before loading to keep total heating time within the limit. Adjust autoclave times as volumes/loads increase. Presterilized media may be available commercially. Do not re-autoclave media.

After sterilization, examine media to determine whether any unanticipated color or clarity variations occurred or media components precipitated. Mark media containers with preparation date and record all pertinent information in appropriate logbooks.

4. Quality Control

See Section 9020B.5*j*.

5. Bibliography

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9050 B. Water

1. Specifications

To prepare culture media and reagents, use only distilled or demineralized reagent-grade water that has been tested and found free from traces of dissolved metals and bactericidal or inhibitory compounds. Toxicity in distilled water may be derived from fluoridated water high in silica. Other sources of toxicity are silver, lead, and various unidentified organic complexes. Where condensate return is used as feed for a still, toxic amines or other boiler compounds may be present in distilled water. Residual chlorine or chloramines also may be found in distilled water prepared from chlorinated water supplies. If chlorine compounds are found in distilled water, neutralize them by adding an equivalent amount of sodium thiosulfate or sodium sulfite.

Distilled water also should be free of contaminating nutrients. Such contamination may be derived from flashover of organics during distillation, continued use of exhausted carbon filter beds, deionizing columns in need of recharging, solder flux residues in new piping, dust and chemical fumes, and storage of water in unclean bottles.

Store distilled water out of direct sunlight to prevent algae growth. Aged distilled water may contain toxic volatile organic compounds absorbed from the atmosphere if stored for prolonged periods in unsealed containers. Good housekeeping practices that minimize the presence of airborne particulates usually will eliminate nutrient contamination.

See Section 9020B.5*f* and Table 9020:II.

2. Bibliography

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9050 C. Media Specifications

The need for uniformity dictates the use of dehydrated media. Never prepare media from basic ingredients when suitable commercially prepared dehydrated media are available. Follow manufacturer's directions for rehydration and sterilization. Commercially prepared media in liquid form (sterile ampule or other) also may be used if known to give equivalent results. See Section 9020B.5*j* for quality-control specifications.

The terms used for protein source in most media (e.g., peptone, tryptone, and tryptose) were coined by the media developers and may reflect commercial products rather than clearly defined entities. It is not intended to preclude the use of alternative materials, provided that they produce equivalent results.

NOTE: In the following directions, the term *percent solution* means "grams of solute per 100 mL solution."

1. Dilution Water[1]

Various dilution water solutions can be prepared in the laboratory or purchased commercially. Below are two of the most commonly used solutions in the basic water microbiology laboratory.

a. Buffered water:

1) Stock phosphate buffer solution—Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL reagent-grade water, adjust to pH 7.2 ± 0.5 with 1*N* NaOH, and dilute to 1 L with reagent-grade water. Sterilize via filtration or autoclave. Store stock solution under refrigerated conditions and discard if turbidity develops.

2) Magnesium chloride stock solution—Add magnesium chloride (38 g/L $MgCl_2$ or 81.1 g $MgCl_2 \cdot 6H_2O$) to 1 L reagent-grade water. Sterilize and store stock solution under refrigerated conditions, discarding if solution becomes turbid.

3) Working solution—Add 1.25 mL stock phosphate buffer solution and 5.0 mL magnesium chloride stock solution to 1 L reagent-grade water. Dispense in amounts that will provide 99 ± 2.0 mL or 9 ± 0.2 mL after autoclaving for 15 min. Final pH should be 7.2 ± 0.1. NOTE: pH values will change with time. Store under refrigerated conditions after opening and discard if turbidity develops. Use within 6 months.

b. Peptone water, 0.1%: Prepare by adding 1 g peptone to 1 L reagent water. Final pH should be 7.0 ± 0.2 after sterilization.

Dispense in amounts to provide 99 ± 2.0 mL or 9 ± 0.2 mL after autoclaving for 15 min. Store as above.

Do not suspend a sample in any dilution water for >30 min at room temperature because injury, death, or growth (in peptone water) may occur.

2. Culture Media

Specifications for individual media are included in subsequent sections. Details are provided where use of a medium is first described.

3. Reference

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1978. Microbiological Methods for Monitoring the Environment, Water and Wastes; EPA-600/8-78-017. Cincinnati, Ohio.

9060 SAMPLES*

9060 A. Collection

1. Containers

Collect samples for microbiological examination in clean, sterile, wide-mouth, nonreactive borosilicate glass or plastic bottles, or in presterilized plastic bags appropriate for microbiological use. The bottles should have non-leaking ground glass stoppers or caps with nontoxic liners that should withstand repeated sterilization. For sludge analysis, disposable wide-mouth sterile cups may be convenient. If legal action may be involved, consider using tamper-evident closures.

2. Dechlorination

Add a reducing agent to containers intended for the collection of water containing residual chlorine or other halogens, unless they contain broth for direct incubation of sample. Sodium thiosulfate ($Na_2S_2O_3$) is a satisfactory dechlorination agent that neutralizes any residual halogen and prevents bactericidal action from continuing during transit.

When sampling chlorinated wastewater effluents, add enough $Na_2S_2O_3$ to a clean sample bottle so the final concentration in the sample is 100 mg/L. For example in a 120-mL bottle, 0.1 mL of a 10% solution of $Na_2S_2O_3$ will neutralize a sample containing up to 15 mg/L residual chlorine. The dechlorination agent may be less concentrated in drinking water samples: 0.1 mL of a 3% solution of $Na_2S_2O_3$ in a 120-mL bottle will neutralize up to 5 mg/L residual chlorine. See Table 9060:I for preparation of $Na_2S_2O_3$ solutions. Where possible, determine typical residual chlorine before sampling at a new site (e.g., pool water may contain a higher chlorine level than tap water) so the laboratory can prepare an adequate amount of dechlorination agent per sample bottle. Discard turbid (due to bacterial growth) 10% $Na_2S_2O_3$ stock solutions.

Loosely cap bottle and sterilize by either dry or moist heat, as directed (Section 9040) and perform sterility checks as noted in Section 9020B.5*d*. Presterilized plastic bags or bottles containing $Na_2S_2O_3$ are available commercially; they will neutralize up to 15 mg/L residual chlorine. Check and record efficacy of dechlorination agent, one per batch or lot (see Section 9020B.5*d*). The dechlorinating agent's efficacy can be checked by the use of a sodium hypochlorite (NaOCl) quality-control (QC) control at 5 or 15 mg/L (or equivalent), for example.

3. Sampling Procedures

Proper sample-collection technique is important in order to maintain the sample's integrity. Improper sample handling can invalidate the results of any laboratory analysis.

Table 9060:I. Sodium Thiosulfate Equivalents

Solution Strength and $Na_2S_2O_3$ Form	Weight of Compound Required
3%, anhydrous	3 g/100 mL
3%, pentahydrate	4.6 g/100 mL
10%, anhydrous	10 g/100 mL
10%, pentahydrate	15.21 g/100 mL

Systematically plan to collect samples that are representative of the water being tested. When planning sample-collection activities, consider temporal, spatial (horizontal and vertical), and hydrodynamic conditions (e.g., wet versus dry weather and seasonal lake turnover effects). Sampling frequency and number of samples collected will depend on ultimate data usage.

Bottles used for sample collection should be large enough to collect desired sample volume and still maintain adequate headspace (2.5 cm) to ensure proper sample mixing (via shaking) before analyses. If a sample bottle arrives at the laboratory without adequate headspace for proper mixing, either reject it and request resampling or else (to maintain sample integrity) pour entire sample volume into a sterile container large enough to ensure adequate mixing and then withdraw 100 mL (or required sample volume) aseptically into another suitably sized sterile container.

Keep sample bottle closed until just before collecting sample. Remove cap or stopper (if used) as a unit and do not set on any surface. Avoid external contamination during sample collection and do not contaminate inner surface of stopper/cap and bottle neck. Fill container without rinsing, replace stopper/cap immediately, and secure hood (if used) around neck of bottle. Take precautions to avoid contaminating sample (e.g., wear clean disposable gloves when collecting sample and avoid touching bottle mouth with either hands or the faucet tap).

a. Potable water: Carefully choose sample locations that are conveniently located and readily accessible to collectors so routine sample collection may occur. If testing distribution-system water, for example, avoid taps connected to private water-treatment equipment, such as water softeners or filters. Also, avoid taps subject to exterior contamination if they are too close to a sink bottom or the ground. If taking samples from a distribution-system tap without attachments, select a tap that is supplying water from a service pipe directly connected with the main (e.g., NOT one served from a cistern or storage tank). Remove any attachments (e.g., filters, aerators, flow directors, or screens) from the tap because these may harbor bacteria that do not reflect the source's water quality. Open cold-water tap fully and let water run to waste just long enough to clear the service line (~2 or 3 min). Reduce water flow so bottle can be filled without splashing. If tap cleanliness is questionable, choose another tap. If a questionable tap is required for special sampling purposes, disinfect faucet (inside and outside) by applying a sodium hy-

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pochlorite solution (100 mg NaOCl/L) to faucet and let water run at a steady consistent flow for another 2 to 3 min after treatment. Do not sample from leaking taps that allow water to flow over the outside of the tap. If sampling from a mixing faucet cannot be avoided, run hot water for 2 min, then cold water for 2 to 3 min, and then collect sample as indicated above. Collect a grab sample (i.e., collect all of sample at once and conduct no further manipulations, such as pouring off or adding to sample, because such activities could contaminate sample). Collect sufficient volume to perform analyses but do not try to manipulate collected sample further to adjust sample volume. It is the laboratory's responsibility, not the sample collector's, to measure sample volume for analyses.

If collecting sample from a well fitted with a hand pump, pump water to waste until water temperature has stabilized (~5 to 10 min) before collecting sample. If an outdoor sampling location must be used, avoid frost-proof hydrants. Also, outdoor taps may need additional disinfection (bleach or equivalent) due to the tap's condition. If there is no pumping machinery, collect a sample directly from the well via a sterilized bottle fitted with a weight at the base; take care to avoid contaminating samples with any surface scum. Other sterile sampling devices, such as a trip bailer, also may be used.

In drinking-water evaluation studies, collect samples of finished water from distribution sites selected to ensure systematic coverage during each month. Carefully choose distribution-system sample locations to include dead-end sections to demonstrate bacteriological quality throughout the network and to ensure that localized contamination does not occur via cross-connections, breaks in distribution lines, or reduction in positive pressure. Sample locations may be

- public sites (e.g., police and fire stations, government office buildings, schools, bus and train stations, airports, and community parks);
- commercial establishments (e.g., restaurants, gas stations, office buildings, and industrial plants);
- private residences (e.g., single residences, apartment buildings, and townhouse complexes); and
- special sampling stations built into the distribution network.

Preferably avoid outdoor taps, fire hydrants, water-treatment units, and backflow-prevention devices. Establish sampling program in consultation with state and local health authorities.

b. Raw water supply: When collecting samples directly from a river, stream, lake, reservoir, spring, or well, obtain samples representative of consumers' source water. It is undesirable to take samples too near the bank or too far from the drawoff point, or at a depth above or below the drawoff point.

c. Surface waters: Stream studies may be short-term, high-intensity efforts. Select bacteriological sampling locations to include a baseline location upstream from the study area, industrial and municipal waste outfalls into the main stream study area, tributaries (except those with a flow less than 10% of the main stream), intake points for municipal or industrial water facilities, downstream samples based on stream flow time, and downstream recreational areas. Wastewater dispersion into the receiving stream may necessitate preliminary cross-section studies to determine completeness of mixing. For tributaries, select a sampling point near the confluence with the main stream. Samples may be collected from a boat or from bridges near critical study points. Sampling frequency should reflect changing stream or waterbody conditions.

To monitor stream and lake water quality, establish sampling locations at critical sites. Sampling frequency may be seasonal for recreational waters, daily for water-supply intakes, hourly where waste-treatment control is erratic and effluents are discharged into shellfish harvesting areas, or even continuous.

d. Bathing beaches: Sampling locations for recreational areas should reflect water quality within the entire recreational zone. Include sites from upstream peripheral areas and locations next to drains or natural contours that would discharge accrued storm-water or septic wastes. Collect samples in the swimming area from a uniform depth representative of physical contact through swimming and splashing (approximately 30 cm or 1 ft below the surface). For specific sampling procedure, follow the guidelines listed in Section 9213B.2*b*. Consider sediment sampling of the water–beach (soil) interface to account for young children's exposure at the water's edge.

To obtain baseline data on marine and estuarine bathing-water quality, include sampling at low, high, and ebb tides and at different times of the day (a.m. & p.m.) for all waters.

Relate sampling frequency directly to the peak bathing period, which generally occurs in the afternoon. Preferably collect daily samples during the recognized bathing season; at a minimum include Friday, Saturday, Sunday, and holidays.[1] When limiting sampling to days of peak recreational use, preferably collect a sample in both morning and afternoon. Correlate bacteriological data with turbidity levels and rainfall over the watershed to make rapid assessment of water-quality changes. Heterotroph levels can also vary in response to sunlight conditions.[2]

e. Sediments and biosolids: The bacteriology of bottom sediments is important in water-supply reservoirs; in lakes, rivers, and coastal waters used for recreational purposes; and in shellfish-growing waters. Sediments may provide a stable index of the overlying water's general quality, particularly if its bacteriological quality varies greatly.

Sampling frequency in reservoirs and lakes may be determined by seasonal changes in water temperatures and storm-water runoff. Bottom sediment changes in river and estuarine waters may be more erratic, being influenced by storm-water runoff, increased flow velocities, and sudden changes in effluent-discharge quality.

Microbiological examination of biosolids from water and wastewater treatment processes is desirable to determine how their use or disposal affects receiving waters, land application, or landfilling.

Collect and handle biosolids with less than 7% total solids using the procedures discussed for other water samples. Biosolids with more than 7% solids and a "plastic" consistency or "semisolid" state typical of thickened sludges require a finite shear stress to cause them to flow. This resistance to flow results in heterogeneous distribution of biosolids in tanks and lagoons. Use cross-section sampling of accumulated biosolids to determine distribution of organisms within these impoundments. Establish a length–width grid across the top of the impoundment, and sample at intercepts. A thief sampler (e.g., a VanDorn or Kemmerer sampler) that samples only the solids layer may be useful. Alternatively, use weighted bottle samplers that can be opened up at a desired depth to collect samples at specific

locations. Use gloves when sampling. Rinse exterior of sample bottles onsite and place them in plastic bags.

Processed biosolids without free liquids are best sampled when they are being transferred. Collect grab samples across the entire width of the conveyor and combine into a composite sample. Exteriors of uncovered piles are subject to various environmental stresses (e.g., precipitation, wind, fugitive dusts, and fecal contamination from scavengers), so surface samples may not reflect the microbiological quality of the entire pile. Therefore, use cross-section sampling of these piles to determine the degree of heterogeneity within the pile. Establish a length–width grid across the top of the pile, and sample intercepts. If sample augers and corers are ineffective in sampling piles of variable composition, use hand shovels to remove overburden.

f. Nonpotable samples (manual sampling): Take samples from a river, stream, lake, reservoir, or pool by holding the bottle (with gloves) near its base and plunging it, neck downward, below the surface. Turn bottle until neck points slightly upward and mouth is directed toward the current. If there is no current (e.g., in a reservoir), create one artificially by pushing bottle forward horizontally away from the hand. When sampling from a boat, obtain samples from upstream side of boat. If impossible to collect samples by hand, attach a weight to base of bottle and lower it into the water. In any case, avoid contact with bank or stream bed; that might cause water fouling to occur.

g. Sampling apparatus: When collecting water samples from the depths of a lake, reservoir, or deep well without a pump, use a special apparatus that enables users to mechanically remove bottle stopper below water surface. Various types of deep sampling devices are available. The most common is the ZoBell J-Z sampler.[3] Commercial adaptations of this sampler and others are available.

Bottom sediment sampling may require a special apparatus. Petit Ponar®† samplers are effective for a variety of bottom materials for remote (deep water) or hand (shallow water) sampling. Following manufacturer's instructions, drop the closed sterile sampler through the water column and open it when it reaches the sediment bed. Close after the sample is taken, bring sampler to the surface, and drain excess water. Use a sterile spatula or similar device to transfer sample into a sterile container. Clean and decontaminate sampler between sampling sites; a suggested procedure is to brush with dilute soap, rinse with tap water, soak in 0.005% bleach solution for 10 to 20 min, and then, if chlorination is of concern, soak in 0.005% sodium thiosulfate solution for 5 min or rinse with tap water (and check for chlorine residual).

When sampling wastewaters or effluents, the techniques described above generally are adequate; in addition, see Section 1060.

4. Sample Volume

Sample volumes should be sufficient to carry out all tests required. For potable, surface, recreational, and waste water samples, collect a minimum of 100 mL. For *Cryptosporidium* and *Giardia* analyses in raw water, collect a minimum of 10 L. For finished water, collect a minimum of 50 to 100 L, depending on type of collection filter. Larger volumes may be needed for bacterial pathogen, protozoan, and viral analyses.

NOTE: For tolerances of glassware and vessels, refer to Section 9020B.5*a* for Class A tolerance of graduated cyclinders (<2.5%) and Section 9020B.5*d* for verification of 100 mL mark.

5. Identifying Data

Samples should be accompanied by complete, accurate sample-information forms that include the following information, as applicable: name of system or site; sample type; collection location; sampling depth, date, and time; sampler's name; analyses to be performed; chlorine residual; and reducing agent (e.g., sodium thiosulfate) and EDTA (a chelating agent if sample contains metals), if used. Record abnormalities or departures from specified sample-collection, -handling, or -receipt procedures. If sample results are likely to be used in legal proceedings, the collector should stipulate that chain-of-custody procedures be followed, including chain-of-custody form with appropriate signatures and dates/times. If samples are submitted to the laboratory either refrigerated or stored in a foam chest with gel packs, document the handling and storage temperatures [cold but unfrozen (<10°C)]. Refer to 9060B for preservation and storage. Also, make sure that sample containers are labeled with enough information to adequately identify samples. Do not accept inadequately identified samples.

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9060 B. Preservation and Storage

1. Holding Time and Temperature

a. General: Start microbiological analysis of water samples as soon as possible after collection to avoid unpredictable changes in the microbial population. Do not analyze compromised samples (leakage, chlorine residual, etc.) submitted to the laboratory; instead, request resampling.

For most accurate results, ice samples during transport to the laboratory if they cannot be processed within 1 h after collection. Maintain samples in the dark and keep cool but unfrozen (<10°C) with ice or blue ice. Samples should not be placed in direct contact with frozen ice packs: insulate sample with bubble wrap, crumpled paper, or equivalent. Samples arriving quickly at the laboratory (within 1 h of collection) may not have reached this temperature. Upon receipt, verify and record sample temperature via a control water-sample bottle, infrared thermometer, or other equivalent devices (e.g., i-buttons). Observe regulatory holding-time limits, which vary for different types of samples and in different countries. Hold nonregulatory samples for no more than 24 h. If the results may be used in legal action, use special means (rapid transport, express mail, courier service, etc.) to deliver samples to the laboratory within the specified time limits and maintain chain of custody. If samples cannot be analyzed at a laboratory within the required holding time, consider setting up a mobile field laboratory or pre-incubating sample. Below are examples of U.S. EPA guidelines and requirements.

b. Drinking water for compliance purposes: For total coliform and *E. coli* analyses, the holding time from collection to analysis is 30 h. Although regulations do not specify a preservation temperature, try to keep samples cold but unfrozen (<10°C) during transport to the laboratory. Similarly, keep samples for heterotrophic plate count analysis cold but unfrozen (<10°C) and do not exceed 8-h holding time (collection to analyses). Do not accept any water sample for microbiological analyses that shows evidence of freezing. Record sample-receipt time (and temperature if submitted on ice) in sample-receipt file. Analyze samples on day of receipt whenever possible. If samples arrive too late for processing on same day, refrigerate them overnight as long as holding time limits can still be met.

For analysis of protozoa (*Cryptosporidium* sp. and *Giardia* sp.), the 96-h holding time applies to the period between sample collection and elution for samples shipped to the laboratory in bulk, and to the period between sample filtration and elution for samples filtered in the field. Samples should arrive at <20°C.

c. Nonpotable water for compliance purposes: Keep source water, stream pollution, recreational water, and wastewater samples cold but unfrozen (<10°C) during transport (≤8 h between collection and lab arrival). When samples arrive at the laboratory, refrigerate them, record receipt time and temperature in sample-receipt files, and process samples within 2 h. When transport conditions will prevent samples from being delivered within 6 h, consider using either field laboratory facilities at the collection site or delayed incubation procedures.

For bacterial samples in wastewater sludge (fecal coliforms and *Salmonella* sp.), the regulatory holding time is 24 h.

For analyses of protozoa, see ¶ *b* above.

d. Other water types for noncompliance purposes: Keep samples cold but unfrozen (<10°C) between collection and analysis (≤24 h). Record sample-receipt time and temperature in sample-receipt files.

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9211 RAPID DETECTION METHODS*

9211 A. Introduction

There is a generally recognized need for methods that permit rapid estimation of the bacteriological quality of water. Applications of rapid methods may range from analysis of wastewater to potable water quality assessment. In the latter case, during emergencies involving water treatment plant failure, line breaks in a distribution network, or other disruptions to water supply caused by disasters, there is urgent need for rapid assessment of the sanitary quality of water.

Ideally, rapid procedures would be reliable and have sensitivity levels equal to those of the standard tests routinely used. However, sensitivity of a rapid test may be compromised because the bacterial limit sought may be below the minimum bacterial concentration essential to rapid detection. Rapid tests fall into two categories, those involving modified conventional procedures and those requiring special instrumentation and materials.

* Approved by Standard Methods Committee, 2000.

9211 B. Seven-Hour Fecal Coliform Test

This method[1,2] is similar to the fecal coliform membrane filter procedure (see Section 9222D) but uses a different medium and incubation temperature to yield results in 7 h that generally are comparable to those obtained by the standard fecal coliform method.

1. Medium

M-7 h FC agar: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Proteose peptone No. 3 or polypeptone	5.0	g
Yeast extract	3.0	g
Lactose	10.0	g
d-Mannitol	5.0	g
Sodium chloride (NaCl)	7.5	g
Sodium lauryl sulfate	0.2	g
Sodium desoxycholate	0.1	g
Bromcresol purple	0.35	g
Phenol red	0.3	g
Agar	15.0	g
Reagent-grade water	1	L

Heat in boiling water bath. After ingredients are dissolved, heat additional 5 min. Cool to 55 to 60°C and adjust pH to 7.3 ± 0.1 with 0.1*N* NaOH (0.35 mL/L usually required). Cool to about 45°C and dispense in 4- to 5-mL quantities to Petri plates with tight-fitting covers. Store at 2 to 10°C. Discard after 30 d.

2. Procedure

Filter an appropriate sample volume through a membrane filter, place filter on the surface of a plate containing M-7 h FC agar medium, and incubate at 41.5°C for 7 h. Fecal coliform colonies are yellow (indicative of lactose fermentation).

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9211 C. Special Techniques

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Special rapid techniques are summarized in Table 9211:I. Most are not sensitive enough for potable water quality measurement or are not specific. They may be useful in monitoring wastewater effluents and natural waters but require reagents not generally available, are tedious, or require special handling or incubation schemes incompatible with most water laboratory schedules. Except for the colorimetric test, none are suitable for routine use but they may be used as research tools. The user should refer to the literature citations for the technique listed in the table for procedural details, conditions for use, and method limitations. Only the adenosine triphosphate (ATP) procedure (the firefly bioluminescence system), the colorimetric test to estimate total microbial density, and a radiometric fecal coliform procedure that uses a ^{14}C-labeled substrate can be recommended.

Correlate initial concentration of bacteria with ATP concentration by extracting ATP from serial dilutions of a bacterial

TABLE 9211:I. SPECIAL RAPID TECHNIQUES

Microbial Group	Rapid Method	Test Time *h*	Sensitivity *cells/mL*	Reference
Nonspecific microflora	Bioluminescence	1	100 000	1–3
	Chemiluminescence	1	500 000	3–5
	Impedance	3–12	100 000	6–9
	Colorimetric	0.02	10 000	10
	Epifluorescence/fluorometric	<1–several	—	11–13
Fecal coliforms	Radiometric	4–5	2–20	14
	Glutamate decarboxylase	10–13	0.01–500 000	15–17
	Electrochemical	1–7	1 000 000	18–20
	Impedance	6–12	200–100 000	6–9
	Gas chromatographic assay	9–12	>50	21
	Colorimetric	8–20	5–130 000	22
	Potentiometric	3.5–15	0.1–>10 000 000	23
Gram-negative bacteria	Limulus assay	2	500–3000	24–27
	Fluorescent antibody	2–3	—	28–30

suspension, or for the ^{14}C radiometric method, standardize by determining the $^{14}CO_2$ released by known concentrations of fecal coliform organisms in natural samples, not pure cultures. In using any rapid procedure, determine the initial bacterial density by using an appropriate procedure, such as heterotrophic plate count (9215) or total (9221) or fecal (9222) coliforms, and correlate with results from the special rapid technique.

1. Bioluminescence Test (Total Viable Microbial Measurement)

The firefly luciferase test for ATP in living cells is based on the reaction between the luciferase enzyme, luciferin (enzyme substrate), magnesium ions, and ATP. Light is emitted during the reaction and can be measured quantitatively and correlated with the quantity of ATP extracted from known numbers of bacteria. When all reactants except ATP are in excess, ATP is the limiting factor. Addition of ATP drives the reactions, producing a pulse of light that is proportional to the ATP concentration.

The assay is completed in less than 1 h.[1–3] For monitoring microbial populations in water, the ATP assay is limited primarily by the need to concentrate bacteria from the sample to achieve the minimum ATP sensitivity level, which is 10^5 cells/mL. When combined with membrane filtration of a 1-L sample, ATP assay can provide the sensitivity level needed.

2. Radiometric Detection (Fecal Coliforms)

In this test, $^{14}CO_2$ is released from a ^{14}C-labeled substrate.[14] The technique permits presumptive detection of as few as 2 to 20 fecal coliform bacteria in 4.5 h. The test uses M-FC broth, uniformly labeled ^{14}C-mannitol, and two-temperature incubation; 2 h at 35°C followed by 2.5 h at 44.5°C for fecal coliform specificity. Add labeled substrate at start of 44.5°C incubation. Use membrane filtration to concentrate organisms from sample and place membrane filter in M-FC broth in a sealable container. The $^{14}CO_2$ released is trapped by exposure to $Ba(OH)_2$-saturated filter paper disk. ^{14}C activity is assayed by liquid scintillation spectrometry. Except for the use of the ^{14}C-mannitol substrate and liquid scintillation spectrometry to count the activity of the $^{14}CO_2$ released by the fecal coliforms, this procedure is similar to that given in Section 9222.

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9212 STRESSED MICROORGANISMS*

9212 A. Introduction

1. General Discussion

Indicator bacteria, including total coliforms, thermotolerant coliforms, and enterococci, may become stressed or injured in waters and wastewaters. These injured bacteria are incapable of growth or colony formation under standard conditions because of structural or metabolic damage. As a result, a substantial portion of the indicator bacteria present (i.e., 10 to greater than 90%) may not be detected.[1,2] These false negative bacteriological findings could result in an inaccurate definition of water quality and lead to the acceptance of a potentially hazardous condition resulting from contamination by resistant pathogens[3] or the penetration of undetected indicator bacteria through treatment barriers.[4]

Stressed microorganisms are present under ordinary circumstances in treated drinking water and wastewater effluents, saline waters, polluted natural waters, and relatively clean surface waters. High numbers of injured indicator bacteria may be associated with partial or inadequate disinfection and the presence of metal ions or other toxic substances. These and other factors, including extremes of temperature and pH and solar radiation, may lead collectively to significant underestimations of the number of viable indicator bacteria.

Injured indicator bacteria still have public health significance because they indicate a potential public health risk.[2,5–7] Reports show that enteropathogenic bacteria are less susceptible than coliforms to injury under conditions similar to those in treated drinking water and wastewater, that injured pathogens retain the potential for virulence, and that they recover after being ingested. Hence, methods allowing for the enumeration of injured coliform bacteria yield more sensitive determinations of potential health risks.

Traditionally in microbiology the terms "viability" and "culturability" have been considered synonymous. However, there exist various physiological states of non-growing organisms that have been classified into several categories relating to metabolic activity, cellular integrity, and the ability to regain culturability.[8] The "viable but nonculturable" (VBNC) response is a temporary state during which microorganisms are not culturable under conventional nonselective culture conditions. The VBNC state has been reported for a wide variety of bacteria including, among others, species of *Aeromonas, Campylobacter, Enterobacter, Enterococcus, Escherichia, Klebsiella, Legionella, Micrococcus, Mycobacterium, Pseudomonas, Salmonella, Shigella*, and *Vibrio*.[9] Whereas entrance into the VBNC state is easily demonstrated, proving resuscitation has been difficult for most bacterial species. Many of these studies have failed to exclude the growth of cells that may have been present but had never lost their culturability. In the case of *Vibrio vulnificus*, which becomes nonculturable in response to low temperature, a simple temperature upshift allows renewed catalase synthesis, and is sufficient for cellular resuscitation and growth in the presence of H_2O_2 found in most plating media.[10–12] *Legionella pneumophila*, which is induced into the VBNC state by nutrient depletion, was shown to resuscitate only when co-incubated with its natural environmental host, *Acanthamoeba castellani.*[13] Other mechanisms for the loss of culturability are likely to exist for other bacteria but are frequently unknown or not rigorously examined.[14] Methods that are routinely used to demonstrate bacterial viability in the nonculturable state include substrate responsiveness,[15] redox dye hydrolysis,[16,17] tetrazolium reduction[18] to detect active electron transport, fluorescent probes to determine membrane condition,[19,20] and reverse transcription polymerase chain reaction (RT-PCR) to monitor gene expression.[11,21]

2. Sample Handling and Collection

Certain laboratory manipulations following sample collection also may produce injury or act as a secondary stress to the organisms.[2,22] These include excessive sample storage time, prolonged holding time (more than 30 min) of diluted samples before inoculation into growth media and of inoculated samples before incubation at the proper temperature, incorrect media formulations, incomplete mixing of sample with concentrated medium, and exposure to untempered liquefied agar media. Excessive numbers of nonindicator bacteria also interfere with detection of indicators by causing injury.[23]

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* Approved by Standard Methods Committee, 2014.
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9212 B. Recovery Enhancement

This section describes some general procedures and considerations regarding recovery of stressed indicator organisms.

For chlorinated samples, ensure that sufficient dechlorinating agent is present in the sample bottle (see Section 9060A.2).[1] Collect water samples with elevated concentrations of heavy-metal ions in a sample bottle containing a chelating agent[2] (see Section 9060A.5) and minimize sample storage time (see Section 9060B). Use buffered peptone dilution water rather than buffered water (see Section 9050C.6) when preparing dilutions of samples containing heavy-metal ions. After making dilutions, inoculate test media within 30 min.

Resuscitation of stressed or injured organisms is enhanced by inoculating samples and initially culturing organisms in an enriched, noninhibitory medium at a moderate temperature.

Although no simple test is available to establish the presence of injured bacteria in a given sample, bacteria in water known to contain stressors, such as disinfectants or heavy metals, frequently will be injured.[1,3] When multiple-tube fermentation or enzymatic substrate test results consistently are higher than those obtained from parallel membrane filter tests, or there is other indication of suboptimal recovery, consider injury probable and use one or more of the following procedures.

1. Recovery of Injured Total Coliform Bacteria Using Membrane Filtration

a. m-T7 agar: Use m-T7 agar[4] in the procedure described for the membrane filter test (see Section 9222B).

Proteose peptone No. 3	5.0 g
Yeast extract	3.0 g
Lactose	20.0 g
Tergitol 7	0.4 mL
Polyoxyethylene ether W1	5.0 g
Bromthymol blue	0.1 g
Bromcresol purple	0.1 g
Agar	15.0 g
Reagent-grade water	1 L

Adjust to pH 7.4 with 0.1*N* NaOH after sterilization at 121°C for 15 min. Aseptically add 1.0 μg penicillin G/mL when medium has cooled to about 45°C.

After filtering sample place filter on m-T7 agar and incubate at 35 ± 0.5°C for 22 to 24 h. Coliform colonies are yellow. Verify not less than 10% of coliform colonies by the procedure in Section 9222B.4*g*. With some drinking water samples containing many noncoliform bacteria, confluent growth occurs. To obtain reliable results, carefully distinguish target yellow colonies from background growth.

b. Addition of sodium sulfite: The addition of sodium sulfite to some media (0.05 to 0.1%) can improve the detection of coliform bacteria following exposure to chloramines but not chlorine.[5] Such modified medium is applicable to clean-water systems using chloramination or to chlorinated wastewater discharges containing ammonia or organic amines.

2. Recovery of Injured Thermotolerant Bacteria Using Membrane Filtration

a. Enrichment-temperature acclimation: Use two-layer agar [M-FC agar with a nonselective overlay medium that does not contain glucose (i.e., tryptic soy agar)] with a 2-h incubation at 35 ± 0.5°C followed by 22 h at 44.5 ± 0.2°C.[6] Prepare the M-FC agar plate in advance but do not add the overlay agar more than 1 h before use.

Alternatively, use a pre-enrichment in phenol red lactose broth incubated at 35 ± 0.5°C for 4 h followed by M-FC agar at 44.5 ± 0.2°C for 22 h.[7]

As a third option, prepare enrichment two-layer medium containing specific additives and incubate for 1.5 h at room temperature (22 to 26°C) followed by 35 ± 0.5°C for 4.5 h and 44.5 ± 2°C for 18 h.[8]

b. Temperature acclimation:[9] Modify elevated temperature procedure by preincubation of M-FC cultures for 5 h at 35 ± 0.5°C, followed by 18 ± 1 h at 44.5 ± 0.2°C. Use a commercially available temperature-programmed incubator to make the change from 35 to 44.5 ± 0.2°C after the 5 h preincubation period to eliminate inconvenience and provide a practical method of analysis.

The m-TEC medium[10] for detection and enumeration of *E. coli* from water can utilize a preincubation step at 35 ± 0.5°C for 2 h before incubation at 44.5 ± 0.2°C for 18 to 22 h to enhance recoveries in fresh and marine waters.

c. Deletion of suppressive agent:[11] Eliminate rosolic acid from M-FC medium and incubate cultures at 44.5 ± 0.2°C for 24 h. Thermotolerant coliform colonies are intense blue on the modified medium and are distinguished from the cream, gray, and pale-green colonies typically produced by non-target bacteria.

d. Alternative medium-temperature acclimation: Use m-T7 medium with an 8 h incubation at 37°C followed by 12 h at 44.5 ± 0.2°C.[12]

e. Verification of stressed thermotolerant bacteria: Modifications of media and procedures may decrease selectivity and differentiation of thermotolerant coliform colonies. Therefore, if any procedural modifications are used, verify not less than 10% of the blue colonies from a variety of samples. Use lauryl tryptose broth (Section 9221B.3*a*) (35°C for 48 h) with transfer of gas-producing cultures to EC broth (Section 9221E.1*a*) (44.5 ± 0.2°C for 24 h). Gas production at 44.5 ± 0.2°C confirms the presence of thermotolerant coliforms.

3. Recovery of Stressed Enterococci Using Membrane Filtration

Using bile broth medium yields enterococci recoveries comparable with multiple-tube fermentation tests.[13] Preincubate membrane filters on an enrichment medium for 2 h at 35 ± 0.5°C and follow by plating on m-Enterococcus agar (Section 9230C.2*a*) for 48 ± 2 h at 35 ± 0.5°C.

Verification of stressed enterococci—Verify not less than 10% of the colonies from a variety of samples using the confirmed test procedure given in Section 9230B.3.

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9213 RECREATIONAL WATERS*

9213 A. Introduction

1. Definition of Recreational Waters and Use of Appropriate Microbiological Indicators

Recreational waters are those in which primary contact occurs. *Primary contact recreation activities* are those that could be expected to result in the ingestion of, or immersion in, water (e.g., swimming, water skiing, and kayaking). There are two general types of recreational waters. The first type includes natural sources of water, such as lakes, streams, and coastal waters, where the water source cannot be disinfected and where these sources are at risk for contamination via point sources, such as sewage and industrial waste, and non-point sources, such as streams, storm drains, and animals (e.g., birds and bathers). This source of water is used primarily for swimming, wading, and surfing. The second type of recreational water is found in facilities using municipally treated water; it should be disinfected continuously. The designated uses of this type of water may be recreation, such as swimming pools, or therapy, such as whirlpools. The quality of recreational water should be suitable for its designated use and characteristic conditions. Historically, concentrations of selected microorganisms have been used to determine the suitability of recreational water for its designated use. Ideally, recreational water quality indicators are microorganisms for which densities in the water can be related quantitatively to the degree of potential contamination and thus health hazards resulting from recreational use, particularly where upper body orifices are exposed to water. They are used to determine how well the water will protect human health. The ideal indicator is the one with the best correlation between density and the health hazards associated with a given type of pollution. It also is consistently and exclusively present when the contaminant is present, occurs in higher densities than the contaminant, cannot proliferate more than the contaminant, is more resistant to environmental stresses and more persistent than the contaminant, and has characteristic, simple reactions for easy, simple identification.[1]

Correlations of microbial density in water and predictable disease have been established for exposure to natural sources of recreational water but not for recreational waters that are routinely disinfected. Epidemiological studies have been conducted[1] to show a correlation between concentrations of *E. coli* and enterococci for natural fresh water sources used for swimming and for concentrations of enterococci for natural marine waters used for swimming. These studies used beaches contaminated by known point sources. A positive correlation was found between *E. coli* or enterococci and gastroenteritis in fresh waters and between enterococci and gastroenteritis in both fresh and marine waters. Water quality standards have been established using these indicators of fecal contamination. National Pollutant Discharge Elimination System (NPDES) permits and state permits authorized by the NPDES program require dischargers to monitor such waters for the parameters included in their permits. For dischargers into coastal recreational waters, any permits including fecal indicators likely require monitoring for enterococci or *E. coli.* For continuously disinfected water, such as water in swimming pools and whirlpools, frequent monitoring is for concentrations of disinfectant (e.g., free and total chlorine, bromine). In many U.S. communities, suitability of such waters is based primarily on hourly (pH, temperature, and disinfectant) or daily (hardness and alkalinity) water-chemistry measurements rather than frequent monitoring for microorganisms or correlation with predictable health effects. Which parameters are measured and how often depends on the individual state, county, or municipal swimming pool codes. While most swimming pools use chlorine, they may also use bromine or a non-halogen-based system. If chlorine is used, free chlorine typically is monitored hourly (occasionally total chlorine also is monitored).

2. Transmission of Enteric and Non-enteric Pathogens by Recreational Use of Waters

In general, infections and disease associated with recreational water contact fall into two categories: those due to enteric pathogens and those due to other (non-enteric) pathogens. Infections in the first category typically cause gastroenteritis symptoms and result from unintentional ingestion of water contaminated with fecal wastes. Enteric microorganisms that have been shown to cause gastroenteritis associated with recreational water contact include *Giardia*, *Cryptosporidium*, *Shigella*, *Salmonella*, *E. coli* O157:H7, *Vibrio*, Hepatitis A, Coxsackie A and B, and Norovirus. One exception is Adenovirus, which has been reported to cause conjunctivitis in those using swimming pools.[2]

Infections in the second category are due to non-enteric pathogens that contaminate water and are transmitted by contact with water. These pathogens generally cause non-enteric diseases and may be transmitted via inhalation, contact with mucosal membranes or abraded skin. The sources of the pathogens differ. For example, the bacteria that cause Leptospirosis have been found in the urine of infected animals.[3] The source may be from human bodies (e.g., *Staphylococcus aureus*) or bacteria indigenous to that water (e.g., *Pseudomonas aeruginosa, Legionella* spp., *Naegleria fowleri, Mycobacterium* spp., and *Vibrio* spp). The illnesses or diseases caused by these non-enteric organisms include dermatitis or folliculitis, otitis externa (inflammation of the external ear), leptospirosis, Pontiac fever, granulomas (nodules of chronically inflamed tissue with granulations), primary amebic meningoencephalitis (PAM), conjunctivitis, and potentially fatal wound infections (*Vibrio vulnificus*).

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3. Microbiological Monitoring Approaches and Limitations

The following sections describe recommended methods for measuring pathogens or microbial indicators of recreational water quality. Several key monitoring factors should be considered (e.g., disinfectant residual, indicator organisms, and various physical and chemical characteristics). When selecting the microbiological method or indicator to be used, consider the type of water examined. No single procedure is adequate to isolate all microorganisms from contaminated water. While bacterial indicators may not adequately reflect risk of viral, fungal, or parasitic infection from recreational waters, lack of feasible methods limits monitoring for such organisms in routine laboratory operations.

Routine examination for pathogenic microorganisms is not recommended except for investigations of water-related illness and special studies. Certain pathogenic organisms, such as *Giardia, Cryptosporidium, Mycobacterium, Naegleria,* Hepatitis A, and Norovirus are more resistant to changes in environmental conditions than indicator bacteria; therefore, routine monitoring may not always reflect the risk of infection from these organisms. In the case of water-related illness, focus microbiological analyses on the known or suspected pathogen and on the venue. For instance, if the venue is a disinfected swimming pool and symptoms include gastrointestinal distress, the initial focus should be on such organisms as *Cryptosporidium, Giardia,* and Norovirus. If the venue is heated whirlpools or spas where the issue is folliculitis or respiratory illness, the focus should be on *P. aeruginosa* and *Legionella*, which are more likely to be found. Methods for detecting several of these pathogens are given in Section 9260, Detection of Pathogenic Bacteria, Section 9510, Detection of Enteric Viruses, and Section 9711, Pathogenic Protozoa.

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9213 B. Swimming Pools

1. General Discussion

a. Characteristics: A *swimming pool* is a body of water of limited size contained within a holding structure.[1] Most public swimming pools use potable fresh water and are designed to be filtered and disinfected continually to maintain high water quality. However, some public swimming pools use natural sources of water, including thermal springs and coastal marine waters, and rely on continuous circulation to maintain water quality. These natural water sources generally are not disinfected.

b. Monitoring requirements:

1) General—Monitor water quality in pools for changes in chemical and physical characteristics that may irritate a bather's skin, eyes, and mucosal barriers or adversely affect disinfection. Microorganisms of concern typically are those from the bather's body and its orifices and include those causing infections of the eye, ear, upper respiratory tract, skin, and intestinal or genitourinary tracts. Accidental release of feces into swimming pools occurs and is a serious source of contamination because the volume of water is restricted and exposure of other swimmers is highly likely. This kind of contamination in a swimming pool has been reported to be responsible for transmission of such microorganisms as *Cryptosporidium*, *Giardia*, Norovirus, and *E. coli* O157:H7.[2] Under these conditions, all fecal-borne pathogens may be transmitted to other swimmers. Water quality depends on the disinfection efficacy, sanitary conditions, filtration efficacy, the number of bathers in the pool at any one time, and the total number of bathers per day. While the following describes various monitoring and sampling protocols, individual localities and states have their own pool codes that must be complied with.

2) Disinfected indoor pools—Swimming pools should be disinfected continuously when in use. Test swimming pool water for total and free chlorine (to calculate combined) and pH when the pool is initially opened and at least 3 times/d. Collect samples from at least two locations for these determinations. Evaluate clarity of the swimming pool water before opening for the day and during periods of heavy use.[3] The heterotrophic plate count is the primary indicator of disinfection efficacy. Indicators of health risk include normal skin flora that are shed, such as *Pseudomonas* and *Staphylococcus*.[4–7] These organisms account for a large percentage of swimming-pool-associated illnesses. Take samples for microbiological examination while the pool is in use. The American Public Health Association (APHA) recommends that not more than 15% of samples collected for public swimming pools during any 30-d period shall have a heterotrophic plate count of 200/mL or show a positive confirmed total coliform test in any of five 10-mL portions of sample examined via the multiple-tube fermentation test or more than 1 total coliform/50 mL when the membrane filter test is used. Whenever swimming pool samples are examined for total staphylococci or *Staphylococcus aureus*, not more than 50 organisms/100 mL should be present.[3]

3) Disinfected outdoor pools—As well as the disinfectant levels (if chlorine, total and free) and pool chemistry, the level of

cyanuric acid or stabilizer (if used and required by appropriate pool code) may be monitored. Thermotolerant coliforms (formerly known as fecal coliforms) and *Pseudomonas* species are the primary indicators of contamination from pets, rodents, stormwater runoff, and humans. Supporting indicators include coliform bacteria, heterotrophic plate count, and staphylococci.

4) Untreated pools—The primary indicator may be thermotolerant coliforms. Supporting indicators are those described for disinfected pools. Untreated pools are not recommended for recreational use because of increased health risks.

2. Samples

a. Containers: Collect samples for bacteriological examination of swimming pool water as directed in Section 9060A. Use containers with capacities of 120 to 480 mL, depending on analyses to be made. Add sufficient sodium thiosulfate ($Na_2S_2O_3$) to the sample to provide a concentration of approximately 100 mg/L in the sample. Do this by adding 0.1 mL of 10% solution of $Na_2S_2O_3$ to a 120-mL bottle or 0.4 mL to a 480-mL bottle. After adding $Na_2S_2O_3$, stopper or cap and sterilize container.

b. Sampling procedure: Collect samples during periods of maximum bather load. Information on number of bathers may be helpful in subsequent interpretation of laboratory results. Use sampling frequency consistent with state and local health regulations.

Collect samples by carefully removing cap of a sterile sample bottle and holding bottle near the base at an angle of 45°. Do not rinse bottle (i.e., retain sodium thiosulfate). Fill in one slow sweep down through the water, with the mouth of the bottle always ahead of the hand. Avoid contamination of the sample by floating debris. Replace cap. For pools equipped with a filter, samples may be collected from sampling ports provided in the filter's return and discharge lines.

Most bacteria shed by bathers are in body oils, saliva, and mucus discharges that occur near the surface; collect additional samples of the surface microlayer from the area in 1-m-deep water. Collect microlayer samples by plunging a sterile glass plate (approximately 20 × 20 cm) vertically through the water surface and withdrawing it upward at a rate of approximately 6 cm/s. Remove surface film and water layer adhering to both sides of plate with a sterile silicone rubber scraper and collect in a sterile glass bottle. Repeat until desired volume is obtained. To minimize microbial contamination, wrap glass plate and scraper in metal foil and sterilize via autoclaving before use. Wear sterile rubber or plastic gloves during sampling or hold glass plate with forceps, clips, or tongs.

Determine pH, temperature, total and free chlorine or other disinfectant levels at pool side at the time of sample collection (see Section 4500-Cl.G). Disinfectant levels and chemical and physical quality of pool water should be consistent with local, state, or APHA standards. The permissible bathing load should adhere to local or state pool codes, or APHA-recommendations.

c. Sample storage (holding time): Analyze microbiological samples as soon as possible after collection (see Section 9060B).

d. Sample storage temperature: Less than 10°C, but not frozen.

e. Sample volume: See Section 9222B.5.

f. Sample dilution: If sample dilutions are required, use 0.1% peptone water or buffered dilution water as diluent to optimize recovery of stressed organisms (see Section 9222B.5*a* for suggested sample volume). Because peptone water has a tendency to foam, avoid air bubbles when pipetting to ensure accurate measure.

3. Heterotrophic Plate Count

Determine the heterotrophic plate count as directed in Section 9215. Use at least two plates per dilution.

4. Tests for Total Coliforms

Determine total coliform bacteria as directed in Sections 9221, 9222, or 9223.

5. Tests for Thermotolerant Coliforms

Test for thermotolerant coliforms according to the multiple-tube fermentation technique (Section 9221), the membrane filter technique (Section 9222), or rapid methods (Section 9211).

6. Test for Staphylococci or *Staphylococcus aureus*

a. Single-plate procedure:

1) *Baird–Parker agar base:*

Tryptone	10.0 g
Beef extract	5.0 g
Yeast extract	1.0 g
Glycine	12.0 g
Sodium pyruvate	10.0 g
Lithium chloride	5.0 g
Agar	20.0 g
Reagent-grade water	1 L

Sterilize via autoclaving. Cool to 50°C and aseptically add 50 mL commercial egg yolk tellurite enrichment/L. Mix well. Final pH should be 7.0 ± 0.2.

2) Procedure—Use membrane filter technique to prepare samples. Be certain to use 0.22-μm filters, because it has been reported that chlorine-stressed *S. aureus* will contract and can slip through 0.45-μm membranes.[8] Place membrane filter on Baird–Parker agar and incubate at 35 ± 0.5°C for 48 ± 4 h. Staphylococci typically form slate-gray to jet-black, smooth, entire colonies. If *S. aureus* is present, egg yolk clearing may be observed if the membrane filter is raised from the medium. Verify some differentiated colonies with a commercial multi-test system or on the basis of such key characteristics as catalase reaction, coagulase production, aerobic and anaerobic acid production from certain carbohydrates, and typical microscopic morphology.

b. Two-plate procedure: Alternatively, a two-plate method may be used, which will also help recover chlorine-stressed *S. aureus*. As above, use membrane filter technique to prepare samples. Place membrane filters on R2A agar (Section 9215A.6*c*) and incubate at 37 ± 0.5°C for 24 h. Aseptically transfer the membranes to Baird–Parker agar and incubate at 35 ± 0.5°C for another 24 h. Random and atypical colonies can be verified as described in ¶ 2) above.

c. Modified multiple-tube procedure:

1) *M-staphylococcus broth:*

Tryptone	10.0	g
Yeast extract	2.0	g
Lactose	2.0	g
Mannitol	10.0	g
Dipotassium hydrogen phosphate (K_2HPO_4)	5.0	g
Sodium chloride (NaCl)	75.0	g
Sodium azide (NaN_3)	0.049	g
Reagent-grade water	1	L

Sterilize by boiling for 4 min; pH should be 7.0 ± 0.2. For 10-mL inocula, prepare and use double-strength medium.

2) *Lipovitellenin-salt-mannitol agar*—This medium may not be available in dehydrated form and may require preparation from the basic ingredients or by adding egg yolk to a dehydrated base.

Beef extract	1.0	g
Polypeptone	10.0	g
Sodium chloride (NaCl)	75.0	g
D-Mannitol	10.0	g
Agar	15.0	g
Phenol red	0.025	g
Egg yolk	20.0	g
Reagent-grade water	1	L

Sterilize via autoclaving; pH should be 7.4 ± 0.2.

3) Procedure—Inoculate tubes of M-staphylococcus broth as directed in Section 9221. Incubate at 35 ± 1°C for 24 h. Hold original enrichment sample but streak from positive (turbid) tubes on plates of lipovitellenin-salt-mannitol agar and incubate at 35 ± 1°C for 24 h. Opaque (24 h), yellow (48 h) zones around the colonies are positive evidence of lipovitellenin-lipase activity (opaque) and mannitol fermentation (yellow).

If the plate is negative, streak another plate from the original enrichment tube before discarding. Lipovitellenin-lipase activity has a 95% positive correlation with coagulase production. If necessary, confirm positive isolates as catalase-positive, coagulase-positive, fermenting mannitol, fermenting glucose anaerobically, yielding typical microscopic morphology, and Gram-positive.

7. Tests for *Pseudomonas aeruginosa*

Tests for *P. aeruginosa* are presented in 9213E and F and include a membrane filter procedure and a multiple-tube technique.

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9213 C. Whirlpools

1. General Discussion

a. Characteristics: A *whirlpool* is a shallow pool with a maximum water depth of 1.2 m; it has a closed-cycle water system, a heated water supply, and usually a hydrojet recirculation system. It may be constructed of plastic, fiberglass, redwood, or epoxy-lined surfaces. Whirlpools are designed for recreational as well as therapeutic use and may accommodate one or more bathers. These pools usually are not cleaned, drained, and refilled after each use. They are located in homes, apartments, hotels, athletic facilities, rehabilitation centers, and hospitals.

b. Monitoring requirements: Whirlpool-associated infections are common because of the whirlpool's inherent design and characteristics, which include high temperature, reduced disinfection efficacy, and increased organic material. All these factors contribute to favorable conditions for the growth of microorganisms, especially *Pseudomonas aeruginosa*. Thus, frequent test-

ing for disinfectant levels and pH, along with scheduled maintenance, is necessary for safe whirlpool water quality.[1–5] Studies have also shown that whirlpools can serve as a reservoir of *Legionella pneumophila*. This organism is often detected in water and filter samples during outbreaks; spray nozzles also should be checked because they can aerosolize the organism. Methods for *P. aeruginosa* are presented in 9213E and F. They include a membrane filter procedure and a multiple-tube technique. Methods for *Legionella* can be found in Section 9260J.

c. Microbiological indicators: The primary indicator of disinfection efficacy is *P. aeruginosa*, with total coliforms, heterotrophic plate count, and staphylococci as supporting indicators of water quality. The standard index of water quality (i.e., total coliforms) may be insufficient to judge the microbiological quality of whirlpool water. *Pseudomonas aeruginosa* is frequently isolated from whirlpool water that is coliform-negative.[6] In the event of a whirlpool-associated outbreak, collect samples as soon as possible. Analyze for the suspected pathogen and *P. aeruginosa*.

d. Sample preservation: Examine samples as soon as possible after collection. See Section 9060B.

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9213 D. Natural Bathing Beaches

1. General Discussion

a. Characteristics: A *natural bathing beach* is any area of a stream, lake, ocean, impoundment, natural pool, or hot spring that is used for recreation. A wide variety of pathogens can be transmitted to humans through use of natural fresh and marine recreational waters contaminated by point sources, such as sewage and industrial wastes, and non-point sources, such as streams, storm drains, and animals (e.g., birds and bathers).[1,2] Contaminating microorganisms may include enteric pathogens, such as *Salmonella, Shigella,* enteroviruses, protozoa, and "opportunists," such as *P. aeruginosa*, *Klebsiella* spp., and *Aeromonas hydrophila,* which can multiply in recreational waters with sufficient nutrients. Other organisms of concern are those associated with the skin, mouth, or nose of bathers, such as *Staphylococcus aureus* and other naturally occurring organisms (e.g., nontuberculous mycobacteria, *Vibrio* spp., and *Naegleria*).[3–9]

b. Monitoring requirements: Historically, thermotolerant coliforms have been recommended as the indicator of choice for evaluating the microbiological quality of recreational waters. Many states use this indicator in their water quality standards. Studies have demonstrated that *E. coli* and enterococci showed a stronger correlation with swimming-associated gastroenteritis than do thermotolerant coliforms, and that both indicators were equally acceptable for monitoring fresh-water quality. For marine waters, *E. coli* and other enteric bacteria have now been documented in numerous studies to enter the viable but nonculturable state in response to such stresses as elevated osmotic levels, so the inability to isolate such indicator organisms may not prove a lack of fecal pollution in such waters. In marine waters, enterococci concentration had the strongest relationship to the gastroenteritis incident rate. The recommended densities of these indicator organisms were calculated to approximate the degree of protection previously accepted for thermotolerant coliforms. EPA-recommended water quality criteria are based on these findings.[10] While the primary indicators of water quality are *E. coli* and enterococci, the enumeration of *P. aeruginosa*, *Aeromonas hydrophila*, *Klebsiella* spp., and *Staphylococcus aureus* in recreational waters may be useful in cases that involve discharge of pulp and paper wastes and effluents from textile finishing plants into receiving waters or in waters with higher bather densities.

2. Samples

a. Containers: Collect samples as directed in Section 9060A. Select container size according to the number and variety of tests to be performed. Adding $Na_2S_2O_3$ to the bottle is unnecessary.

b. Sampling procedure: Collect samples 0.3 m below the water surface in the areas of greatest bather load. Samples may be taken ankle deep (at ~0.075 m below water surface). In deeper waters, if desired, take another sample approximately 0.075 m below the water surface. This area may be somewhere between the knees and the chest, depending on how deep the water is where the sample is taken. Take samples over the range of environmental and climatic conditions, especially during times when maximal pollution can be expected (i.e., periods of tidal, current, and wind influences; stormwater runoff; and wastewater treatment bypasses). See 9213B.2*b* for methods of sample collection and Section 9222 for suggested sample volumes.

c. Sample storage: See Section 9060B.

3. Tests for *Escherichia coli*

a. Media: Media described in this section are available commercially. Manufacturer's instructions should be followed for storage and disposal after preparation. The formulation may be available commercially for use in the multiple-tube procedure, the multi-well procedure, or the presence–absence procedure. The need for good quality assurance and uniformity requires the use of a commercial substrate media.

1) *mTEC agar:** [2]

Proteose peptone No. 3	5.0	g
Yeast extract	3.0	g
Lactose	10.0	g
Sodium chloride (NaCl)	7.5	g
Dipotassium phosphate (K_2HPO_4)	3.3	g
Monopotassium phosphate (KH_2PO_4)	1.0	g
Sodium lauryl sulfate	0.2	g
Sodium desoxycholate	0.1	g
Bromcresol purple	0.08	g
Bromphenol red	0.08	g
Agar	15.0	g
Reagent-grade water	1	L

Sterilize via autoclaving; pH should be 7.3 ± 0.2. Pour 4 to 5 mL liquefied agar into culture dishes (50 × 100 mm). Store in refrigerator.

2) *Urea substrate:**[2]

Urea	2.0	g
Phenol red	10	mg
Reagent-grade water	100	mL

Adjust pH to 5.0 ± 0.2. Store at 2 to 8°C. Use within 1 week.

3) Procedure—Filter sample through a membrane filter (see Section 9222), place membrane on mTEC agar, incubate at 35 ± 0.5°C for 2 h to rejuvenate injured or stressed bacteria, and then incubate at 44.5 ± 0.2°C for 22 h. Transfer filter to a filter pad saturated with urea substrate. After 15 min, count yellow or yellow-brown colonies using a fluorescent lamp and a magnifying lens. Verify a portion of these differentiated colonies via a commercial multi-test system [see Section 9222B.4*g*2)b)].

b. Modified mTEC method:

1) *Medium—Modified mTEC agar*—Prepare in same manner and with same ingredients as for mTEC agar, but omit bromcresol purple and bromphenol red and add 0.05 g 5-bromo-6-chloro-3-indoxyl-β-D-glucuronide chromogen. Sterilize via autoclaving; final pH should be 7.3 ± 0.2. Pour 4 to 5 mL liquefied agar into culture dishes (50 × 40 mm). Store in refrigerator.

2) Procedure—Filter sample through a membrane filter (see Section 9222), place membrane on modified mTEC agar, incubate at 35 ± 0.5°C for 2 h to rejuvenate injured or stressed bacteria, and then incubate at 44.5 ± 0.2°C for 22 h. After incubation, count red or magenta colonies (*E. coli*) using a fluorescent lamp and a magnifying lens. Verify a portion of these differentiated colonies via a commercial multi-test system [see Section 9222B.4*g*2)b)].

c. Enzyme substrate test:

See Section 9223B.

* Difco, or equivalent.

4. Tests for Enterococci

Perform tests for enterococci using the multiple-tube technique (Section 9230B), membrane filter technique (Section 9230C), or fluorogenic substrate technique (Section 9230D).

5. Tests for *Pseudomonas aeruginosa*

Perform tests for *P. aeruginosa* as directed in 9213E and F. Use the multiple-tube test with samples but note that the procedures may not be applicable to marine samples.

6. Tests for *Aeromonas hydrophila*

See Section 9260L.

7. Tests for *Klebsiella* spp.

See Section 9222F.

8. Tests for *Staphylococcus aureus*

Another method for enumerating total staphylococci and *S. aureus* in marine waters and swimming pool water (but not natural freshwater) is a modification of the membrane filtration method using Baird–Parker medium [9213B.6*a*1)].[11] This method adds 0.005% sodium azide to Tellurite Glycine Agar (TGA) or Vogel–Johnson Agar (VJA) as an additional inhibitor to increase the selectivity of these media to enumerate concentrations of total staphylococci and *S. aureus* from natural coastal marine waters. TGA and VJA use the same basic ingredients (lithium chloride, glycine, and tellurite) as selective agents against non-staphylococci bacteria, which form black target colonies on these media. However, supplementing TGA or VJA with 0.005% sodium azide was required so they could be used to enumerate total staphylococci and *S. aureus* from marine waters but not from natural freshwater streams. Limited data show that this method also works in swimming pool water. Sodium azide inhibited the low percentages of non-staphylococci bacteria in marine waters from forming black colonies, but not the much higher concentrations of non-staphylococci bacteria found in freshwater streams. All black colonies are presumptive counts of total staphylococci colonies and must be verified as total staphylococci or *S. aureus* by further testing Gram staining (Gram-positive cocci), catalase (positive), or lysostaphin (positive) to confirm as total staphylococci and via coagulase (positive) to confirm as *S. aureus*.

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9213 E. Membrane Filter Technique for *Pseudomonas aeruginosa*

1. Laboratory Apparatus

See Section 9222B.1.

2. Culture Media

a. M-PA agar: This agar may not be available in dehydrated form and may require preparation from the basic ingredients.

L-Lysine HCl	5.0 g
Sodium chloride (NaCl)	5.0 g
Yeast extract	2.0 g
Xylose	2.5 g
Sucrose	1.25 g
Lactose	1.25 g
Phenol red	0.08 g
Ferric ammonium citrate	0.8 g
Sodium thiosulfate ($Na_2S_2O_3$)	6.8 g
Agar	15.0 g
Reagent-grade water	1 L

Adjust pH to 6.5 ± 0.1 and sterilize via autoclaving. Cool to 55 to 60°C; readjust pH to 7.1 ± 0.2 and add the following dry antibiotics per liter of agar base: sulfapyridine,* 176 mg; kanamycin,* 8.5 mg; nalidixic acid,* 37.0 mg; and cycloheximide,* 150 mg. After mixing, dispense in 3-mL quantities in 50 × 12 mm Petri plates. Store poured plates at 2 to 8°C. Discard unused medium after 1 month.

b. Modified M-PA agar†

c. Milk agar (Brown and Scott Foster Modification):

1) *Mixture A:*

Instant nonfat milk‡	100 g
Reagent-grade water	500 mL

2) *Mixture B:*

Nutrient broth	12.5 g
Sodium chloride (NaCl)	2.5 g
Agar	15.0 g
Reagent-grade water	500 mL

Separately prepare and sterilize Mixtures A and B; cool rapidly to 55°C; aseptically combine mixtures and dispense 20 to 25 mL per Petri dish.

3. Procedure

a. Presumptive tests: Filter 200 mL or less of natural waters or up to 500 mL of swimming pool waters through sterile membrane filters. Place each membrane on a poured plate of modified M-PA agar so there is no air space between the membrane and the agar surface.

Invert plates and incubate at 41.5 ± 0.5°C for 72 h.

Typically, *P. aeruginosa* colonies are 0.8 to 2.2 mm in diameter and appear flat with light outer rims and brownish to greenish-black centers. Count typical colonies, preferably from filters containing 20 to 80 colonies. Use a 10- to 15-power magnifier as an aid in colony counting.

A two-plate method also may be used; this method will help recover chlorine-stressed *P. aeruginosa* (e.g., from swimming pools and whirlpools). Use membrane filter technique to prepare samples. Place membrane filters on R2A agar (Section 9215A.6*c*) and incubate at 37 ± 0.5°C for 24 h. Aseptically transfer the membranes to M-PA agar and incubate at 35 ± 0.5°C for another 24 h. Random and atypical colonies can be verified using confirmation tests.

b. Confirmation tests: Use milk agar to confirm a number of typical and atypical colonies. Make a single streak (2 to 4 cm long) from an isolated colony on a milk agar plate and incubate at 35 ± 1.0°C for 24 h. *P. aeruginosa* hydrolyzes casein and produces a yellowish to green diffusible pigment.

4. Interpretation and Calculation of Density

Confirmation is not routinely required. In the absence of confirmation, report results as the number of presumptive *P. aeruginosa*/100 mL.

* Sigma Chemical Co., St. Louis, MO, or equivalent.
† Commercially available as M-PA-C agar. Contains magnesium, sulfate, kanamycin, and nalidixic acid.
‡ Carnation, or equivalent.

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9213 F. Multiple-Tube Technique for *Pseudomonas aeruginosa*

1. Laboratory Apparatus

See Section 9221.

2. Culture Media

a. Asparagine broth: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Asparagine, DL	3.0 g
Anhydrous dipotassium hydrogen phosphate (K_2HPO_4)	1.0 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5 g
Reagent-grade water	1 L

Adjust pH to between 6.9 and 7.2 before sterilization.

b. Acetamide broth: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Acetamide	10.0 g
Sodium chloride (NaCl)	5.0 g
Anhydrous dipotassium hydrogen phosphate (K_2HPO_4)	1.39 g
Anhydrous potassium dihydrogen phosphate (KH_2PO_4)	0.73 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5 g

Dissolve 1.2 g phenol red in 100 mL 0.01*N* NaOH and add 1 mL/L of acetamide broth. Use phenol red stock solution within 1 year. Adjust pH to between 7.1 and 7.3 before sterilization. Final pH should be 7.0 ± 0.2. Prepare acetamide broth as described above. If agar slants are preferred, prepare as described above but add 15 g agar/L, heat to dissolve agar, and dispense 8-mL quantities in 16-mm tubes. After autoclaving, incline tubes while cooling to provide a large slant surface.

3. Procedure

a. Presumptive test: Perform a five-tube test. Use 10 mL single-strength asparagine broth for inocula of 1 mL or less and 10 mL double-strength asparagine broth for 10-mL inocula. Higher dilutions may be necessary for swimming pools. Incubate inoculated tubes at 35 to 37°C. After 24 h and again after 48 h of incubation, examine tubes under long-wave ultraviolet light (black light) in a darkened room. Production of a green fluorescent pigment constitutes a positive presumptive test.

b. Confirmed test: Confirm positive tubes by inoculating 0.1 mL of culture into acetamide broth or onto the surface of acetamide agar slants. Development of purple color (alkaline pH) within 24 to 36 h of incubation at 35 to 37°C is a positive confirmed test for *Pseudomonas aeruginosa.*

c. Computing and reporting results: Refer to Table 9221:V and Section 9221C.

9215 HETEROTROPHIC PLATE COUNT*

9215 A. Introduction

1. Applications

The *heterotrophic plate count* (HPC), formerly known as the *standard plate count*, is a procedure for estimating the number of live, culturable heterotrophic bacteria in water and for measuring changes in swimming pools or during water treatment and distribution. Colonies may arise from pairs, chains, clusters, or single cells—all of which are included in the term *colony-forming units* (CFU). The final count also depends on interaction among developing colonies. Choose the procedure and medium that best suit how the resulting information will be used. Only compare data generated using the same procedure and medium. Four methods and five media are described.

2. Selection of Method

a. Pour plate method: The pour plate method (9215B) is simple to perform and can accommodate sample or diluted-sample volumes ranging from 0.1 to 2.0 mL. It produces subsurface colonies that are relatively small, compact, and less apt to encroach on each other than surface colonies do. However, submerged colonies often are slower growing and difficult to transfer. A thermostatically controlled water bath is essential for tempering the agar.

b. Spread plate method: The spread plate method (9215C) causes no heat shock. All colonies are on the agar surface, where analysts typically can easily distinguish them from particles and bubbles, and discern different colony morphologies. If not, they can transfer colonies quickly and easily discern colony morphology via comparisons to published descriptions. However, the agar in this method can only absorb a small volume of sample or diluted sample (0.1 to 0.5 mL, depending on the degree to which prepoured plates have been dried). Also, spreading colonies could be interferences. To use this procedure, maintain a supply of suitable pre-dried, absorbent agar plates (see 9215C.4).

c. Membrane filter method: The membrane filter method (9215D) can be used to analyze large volumes of low-turbidity water and is the method of choice for waters with low numbers of heterotrophic organisms (<1 to 10 CFU/mL). It produces no heat shock. However, there is the expense of the membrane filter, the smaller area for colony development, possible damage to cells by excessive filtration pressures, possible variations in membrane-filter quality (see Section 9020B.5*i*), and the need to detect colonies via reflected light against a white background if colored filters or contrast stains are not used.

d. Enzyme substrate method: The enzyme substrate method, SimPlate® (9215E), can be used to analyze drinking- and source-water samples with a wide range of bacterial concentrations. It produces no heat shock. Comparable to the pour plate method, this method† uses a medium in which substrates are hydrolyzed by multiple microbial enzymes, causing the release of 4-methylumbelliferone after 48 h of incubation at 35 ± 5°C. The 4-methylumbelliferone fluoresces when exposed to long-wavelength (365–366 nm) ultraviolet (UV) light, and the number of blue fluorescing wells corresponds to a most probable number (MPN) of bacteria in the sample. Unfortunately, individual colonies cannot be directly recovered for subsequent analysis

3. Work Area

Provide a level table or bench top with ample area in a clean, draft-free, well-lighted room or within a horizontal-flow laminar hood. Use tables or bench tops with nonporous surfaces, and disinfect before any analysis is made (Sections 9020B.3*c* and *e*).

4. Samples

Collect water as directed in Section 9060A. Initiate analysis as soon as possible after collection to minimize changes in bacterial populations, and do not exceed a holding time of 24 h (or, if the sample was collected for regulatory purposes, the maximum holding time specified by the regulation). If the sample cannot be tested within 30 min after collection, maintain it at <10°C but do not allow it to freeze during transit.

5. Sample Preparation

Mark each plate with sample number, dilution, date, and any other necessary information before examination. For pour- or spread-plate methods, use sterile glass (65 cm^2) or presterilized disposable plastic (57 cm^2) Petri dishes. For pour-plate, spread-plate, or membrane-filtration methods, prepare at least two replicate plates for each volume of sample or dilution examined for compliance testing. Duplicates also are recommended for non-compliance testing.

Thoroughly mix all samples or dilutions by shaking vigorously for 7 s by hand (approximately 25 times with a 1-ft movement) or via a mechanical shaker for 15 s at low speed. Analytical results depend on adequate sample mixing; if the sample is not adequately shaken before aliquots are removed, the actual bacterial density could be underestimated.

6. Media

Users should ensure that the formulations of purchased media match those described below. Compare new lots of media with current lot in use according to Sections 9020B.5*j* and 9*b*.

* Approved by Standard Methods Committee, 2016.
Joint Task Group: Gil Dichter (co-chair), Mark W. LeChevallier (co-chair), Jennifer Best, Eric Duderstadt, Dennis Hill, Kimberly Phillips.

† SimPlate® for HPC, Idexx Laboratories, Westbrook, ME.

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a. Plate count agar (also called tryptone glucose yeast agar*):* This medium can be used to examine heterotrophic bacteria in a wide variety of matrices. It can be used for both pour- and spread-plate methods. This high-nutrient agar, widely used in the past, may give lower counts than R2A or NWRI agar. Commerically available dehydrated medium should be used when available.

Tryptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar	15.0 g
Reagent-grade water	1 L

The pH should be 7.0 ± 0.2 after autoclaving at 121°C for 15 min.

b. m-HPC agar (formerly called SPC agar*):* This can be used to count heterotrophic bacteria in waters with low levels of heterotrophs, such as treated potable water. This high-nutrient medium is used only with the membrane-filter method. Commerically available dehydrated medium should be used when available.

Peptone	20.0 g
Gelatin	25.0 g
Glycerol	10.0 mL
Agar	15.0 g
Reagent-grade water	1 L

For medium produced from individual ingredients, adjust to pH 7.2 with 1 *N* NaOH. Heat slowly to dissolve thoroughly, add glycerol, and sterilize at 121°C for 5 min. Commercially prepared medium should not require post-sterilization pH adjustment; see Section 9020B.5*j*1). Final pH is 7.1 ± 0.2.

c. R2A agar: Use for pour-plate, spread-plate, and membrane-filter methods. This low-nutrient agar was developed for use with potable treated water to monitor trends in potable-water production; this agar and a longer incubation period can improve the recovery of stressed and chlorine-tolerant bacteria. This medium may yield higher counts than high-nutrient formulations.

Yeast extract	0.5 g
Proteose peptone No. 3 or polypeptone	0.5 g
Casamino acids	0.5 g
Glucose	0.5 g
Soluble starch	0.5 g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.3 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0.05 g
Sodium pyruvate	0.3 g
Agar	15.0 g
Reagent-grade water	1 L

For medium produced from individual ingredients, adjust to pH 7.2 with solid K_2HPO_4 or KH_2HPO_4 before adding agar. Heat to dissolve agar and sterilize at 121°C for 15 min. Commercially-prepared medium should not require post-sterilization pH adjustment; see Section 9020B.5*j*1). Final pH is 7.2 ± 0.2.

d. NWRI agar (HPCA): Use for pour-plate, spread-plate, and membrane-filter methods. This low-nutrient medium is likely to produce higher colony counts than high-nutrient media. This agar typically is not available in dehydrated form; it must be prepared from individual ingredients, making its use less desirable.

Peptone	3.0 g
Soluble casein	0.5 g
K_2HPO_4	0.2 g
$MgSO_4$	0.05 g
$FeCl_3$	0.001 g
Agar	15.0 g
Reagent-grade water	1 L

Final pH is 7.2 ± 0.2 after autoclaving at 121°C for 15 min.

e. Enzyme substrate medium: SimPlate® medium is available commercially‡ in sterile vessels for 100-mL multi-dose procedures or in test tubes for 10-mL unit-dose procedures. Store medium between 2 and 25°C and use before expiration date.

7. Incubation

The media described in 9215A.6 should be incubated as follows:

a. Plate count agar (PCA): Incubate at 35°C for 48 ± 3 h.

b. m-HPC agar: Incubate at 35°C for 48 ± 3 h.

c. R2A agar: Incubate at 20–28°C for 5 to 7 d.

d. NWRI agar: Incubate at 20°C for 7 d.

e. SimPlate® medium: Incubate at 35 ± 0.5°C for 48 h (range of 45 to 72 h).

During incubation, maintain humidity so agar plates will not lose >15% moisture weight; this is especially important during prolonged incubation. A pan of water placed in the bottom of the incubator may be sufficient so long as the incubator's interior walls and shelving are made of high-grade stainless steel or anodized aluminum, which will not rust or oxidize. If the incubator is not humidified, seal plates in plastic bags, removing as much air as possible before sealing bag.

8. Counting and Recording

a. Pour and spread plates: Count all colonies on selected plates promptly after incubation. If counting must be delayed temporarily, store plates refrigerated for ≤24 h, but avoid this as routine practice. Record results of sterility controls on the report for each lot of samples.

Count colonies manually using a dark-field colony counter, such as a Quebec colony counter or equivalent. If such equipment is unavailable, then other counters can be used on non-compliance samples so long as they provide equivalent magnification. Automatic plate-counting instruments are available; they generally use a computer program and scanner and give computed results. Their use is acceptable if, when evaluated by being run in parallel with a manual method, counting results are comparable.

When preparing plates, pipet sample volumes that will yield between 30 and 300 colonies/plate. The aim is to have at least one dilution yield colony counts within these limits, except as provided below.

Ordinarily, do not pipet >2.0 mL of sample; however, if this sample volume yields <30 colonies, record the result observed.

‡ Idexx Laboratories, Westbrook, ME.

Otherwise, use only plates containing 30 to 300 colonies when determining plate count. Compute bacterial count per milliliter as follows:

$$\text{CFU/mL} = \frac{\text{colonies counted}}{\text{actual volume of sample plated, mL}}$$

If no plate contains 30 to 300 colonies, and one or more plates have >300 colonies, use the plate(s) whose count is nearest 300 colonies. Compute the count as above and report as estimated CFU/mL.

If plates from all dilutions of a sample have no colonies, report the count as <1 divided by the corresponding largest sample volume used. For example, if no colonies develop from the 0.01-mL sample volume, report the count as <100 CFU/mL.

If the number of colonies per plate exceeds 300, do not report results as "too numerous to count" (TNTC). If there are <10 colonies/cm^2, count colonies in 13 squares (of the colony counter) with representative colony distribution. If possible, select seven consecutive squares horizontally across the plate and six consecutive squares vertically, being careful not to count a square more than once. Compute estimated colonies per plate as follows: When the plate is 65 cm^2 (the typical area of a glass plate), multiply the sum of the number of colonies in 13 representative square centimeters by 5; when the plate is 57 cm^2 (the typical area of a plastic plate), multiply the sum of the number of colonies in 19 representative square centimeters by 3. When there are >10 colonies/cm^2, count four representative squares, take the average count per cm^2, and multiply by the appropriate factor (57 for disposable plastic plates and 65 for glass plates) to estimate colonies/plate. (NOTE: The nominal diameter of both disposable plastic and nondisposable glass plates is 100 mm. However, the internal diameter of disposable plates is closer to 85 mm and that of nondisposable plates is closer to 90 mm.) When bacterial counts on crowded plates are >100 colonies/cm^2, report results as >6500 divided by the smallest sample volume plated for glass plates or >5700 divided by the smallest sample volume plated for plastic plates. Report as estimated CFU/mL.

If spreading colonies (spreaders) are encountered on the plate(s) selected, count colonies on representative portions only when colonies are well distributed in spreader-free areas and the area covered by the spreader(s) does not exceed one-half the plate area.

When spreading colonies must be counted, count each of the following types as one: a chain of colonies that appears to be caused by disintegration of a bacterial clump as agar and sample were mixed; a spreader that develops as a film or growth between the agar and bottom of the Petri dish; and a colony that forms in a film of water at the edge or over the agar surface. The last two types largely develop due to moisture accumulation at the point from which the spreader originates. They frequently cover more than half the plate and interfere with obtaining a reliable plate count.

Count similar, adjacent colonies as individual colonies so long as they do not touch and the distance between them is at least equal to the diameter of the smallest colony. Count impinging colonies that differ in appearance (e.g., morphology or color) as individual colonies.

If plates have excessive spreader growth, report as "spreaders" (Spr.). When plates are uncountable due to missed dilution, accidental dropping, or contamination, or else the control plates indicate that the medium or other material was contaminated, report as "laboratory accident" (LA).

b. Membrane filter method: Count colonies on membrane filters using a stereoscopic microscope at 10 to 15× magnification. Preferably slant Petri dish at a 45° angle on a microscope stage and adjust light source vertical to the colonies. Optimal colony density per filter is 20 to 200. If colonies are small and uncrowded, a higher limit is acceptable.

Count all colonies on the membrane when there are ≤2 colonies per square. For 3 to 10 colonies per square, count 10 squares and determine an average count per square. For 10 to 20 colonies per square, count 5 squares and determine an average count per square. Multiply average count per square by 100 and divide by the sample volume to give colonies per mL. If there are >20 colonies per square, record the count as >2000 divided by the sample volume. Report averaged counts as estimated CFU. Make estimated counts only when there are discrete, separated colonies without spreaders.

c. Enzyme substrate method: Count the number of blue fluorescent wells by holding a 6-W, 365–366 nm UV light about 5 in. above plate, preferably with UV-filtering safety glasses or in a UV cabinet. Alternatively, count fluorescent wells through the back of the inverted plate following the procedure indicated above.

Use the appropriate MPN Table (multi-dose or unit dose) provided by the manufacturer to calculate MPN/mL. Adjust MPN to reflect sample volume and/or dilution made to yield a corrected MPN value. Record as MPN/mL.

9. Computing and Reporting Counts

The term *colony-forming unit(s)* (CFU) is descriptive of the methods used; therefore, report all counts as CFUs. Also report the method used, the incubation temperature and time, and the medium. For example: CFU/mL, pour plate method, 35°C/48 h, plate count agar.

To compute the heterotrophic plate count for pour plate, spread plate, and membrane filter methods (CFU/mL), divide either the total or average number of colonies per plate by the sample volume. (Use the average number of colonies if duplicate plates of the same dilution.) Record sample volumes used and number of colonies on each plate counted or estimated.

When colonies on duplicate plates and/or consecutive dilutions are counted and results are averaged before being recorded, round off counts to two significant figures only when converting to CFUs.

Avoid creating fictitious precision and accuracy when computing CFUs by recording only the first two digits. Round up the second digit when the third digit is 5 through 9 and round down when the third digit is 1 through 4 (e.g., report a count of 142 as 140, 155 as 160, and 35 as 35).

For the enzyme substrate method, record the MPN/mL obtained from the MPN tables provided by the manufacturer, adjusted for sample dilution if required.

10. Analytical Bias

Avoid counting inaccuracies due to carelessness, damaged or dirty optics that impair vision, or failure to recognize colonies. Laboratory workers who cannot duplicate their own counts on the same plate within 5% and other analysts' counts within 10% should discover the cause and correct such disagreements.

9215 B. Pour Plate Method

1. Samples and Sample Preparation

See 9215A.4 and 5.

2. Sample Dilution

Prepare water used for dilution blanks as directed in Section 9050C.1.

a. Selecting dilutions: If possible (e.g., based on historical information), select dilutions so at least one plate in a series will contain 30 to 300 CFU (Figure 9215:1). For example, if experience indicates that heterotrophic plate counts can be as high as 3000 CFU, prepare plates at 10^{-1} and 10^{-2} dilutions.

For most potable water samples, plating 1 mL and 0.1 mL undiluted sample and 1 mL of the 10^{-2} dilution will produce plates suitable for counting.

b. Measuring sample portions: Do not prepare dilutions and pour plates in direct sunlight. Use a sterile pipet for all transfers from each container; one pipet per dilution. If any pipet becomes contaminated before transfers are completed, replace it with a sterile one. Use caution when removing sterile pipets from the container; to avoid contamination, do not drag pipet tip across exposed ends of pipets in the pipet container or across lips and necks of dilution bottles. When removing sample, do not insert pipet >2 to 3 cm below the surface of sample or dilution.

c. Measuring dilutions: Add sample to a sterile Petri dish before adding melted, tempered culture medium. When discharging sample portions, hold pipet or micropipet tip at an angle of about 45°, with tip touching bottom of Petri dish or inside neck of dilution bottle. Lift Petri-dish cover just high enough to insert pipet or micropipet tip. Allow 2 to 4 s for liquid to drain from 1-mL graduation mark to pipet tip. When measuring 0.1-mL quantities, let diluted sample drain from chosen reference graduation until 0.1 mL has been delivered. Use decimal dilutions when preparing sample volumes <0.1 mL. When examining sewage or turbid water, do not measure a 0.1-mL inoculum of original sample; instead, prepare an appropriate dilution (9215A.5).

If pipet is not a blow-out type, touch tip of pipet *once* against a dry spot on Petri dish bottom. If using a cotton-plugged blow-out type pipet and a pipet bulb (less preferable), gently blow out remaining volume of sample dilution. Remove pipet without further touching it to dish.

After depositing test portions for each series of plates, pour culture medium and mix carefully. Do not let >20 min elapse between starting pipeting and pouring plates.

3. Media for Plating

a. Melting medium: Melt sterile solid agar medium in boiling water, via exposure to flowing steam in a partially closed container, or via microwave [Section 9020B.5*j*3)]. Avoid prolonged exposure to unnecessarily high temperatures during and after melting. Do not re-sterilize plating medium. If medium is melted in two or more

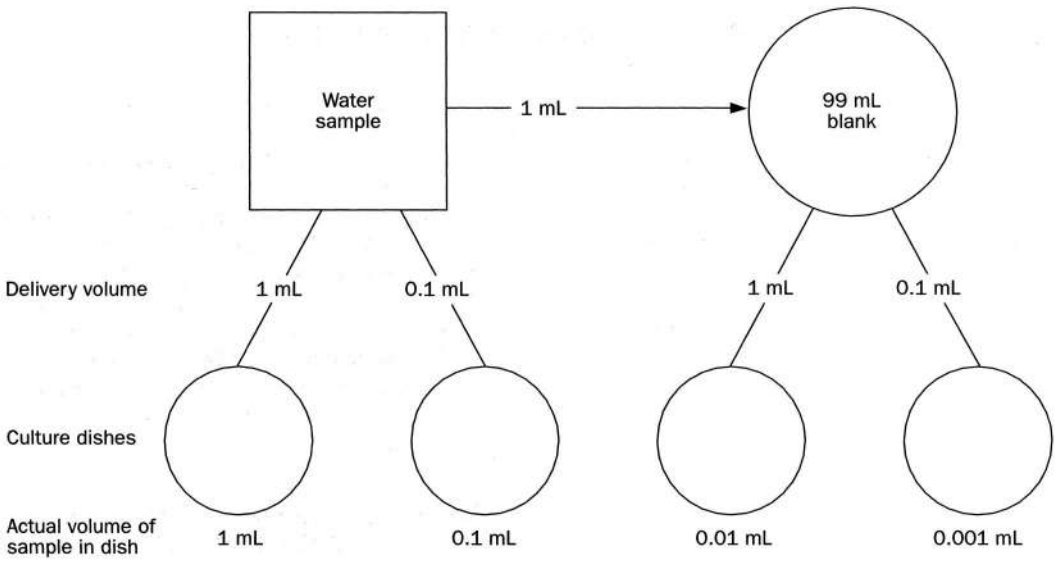


Figure 9215:1. Preparation of dilutions.

batches, use all of each batch in order of melting, so long as contents remain fully melted. Discard melted agar that contains precipitate.

Maintain melted medium in a water bath between 44 and 46°C until used, preferably no longer than 3 h. To track the temperature, place a thermometer in a separate container of medium that has been exposed to the same heating and cooling as the plating medium; do not depend on the sense of touch to indicate proper medium temperature when pouring agar.

Prepare media [plate count agar (PCA), R2A agar, or NWRI agar] as described in 9215A.6.

b. Pouring plates: Limit number of samples to be plated in any one series so no more than 20 min (preferably 10 min) elapse between dilution of first sample and pouring of the last plate in the series. After adding sample to Petri dish, gently lift cover just high enough to pour at least 10 to 12 mL liquefied medium (maintained at 44 to 46°C) in each dish. Carefully avoid spilling medium on outside of dish lid when pouring. When pouring agar from flasks or tubes that have been held in a water bath, wipe outside of bottle with a clean towel and avoid dripping water from water bath onto work surface. As each plate is poured, mix melted medium thoroughly with test portions in Petri dish—taking care not to splash mixture over the edge—by rotating the dish clockwise and counterclockwise, or by rotating and tilting. Let plates solidify (within 10 min) on a level surface. After medium solidifies, invert plates and place in incubator. For plates incubated at 35°C, stack plates no more than four high.

c. Sterility controls: Check sterility of medium and dilution water blanks by pouring control plates for each series of samples. Prepare additional controls to determine contamination of plates, pipets, and room air.

4. Incubation

See 9215A.7 for incubation temperatures and times.

5. Counting, Recording, Computing, and Reporting

See 9215A.8 and 9.

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9215 C. Spread Plate Method

1. Samples and Sample Preparations

See 9215A.4 and 5.

2. Laboratory Apparatus

a. Glass rods: Bend 4-mm-diam, 200-mm-long, fire-polished glass rods, 45° about 40 mm from one end. Sterilize before use. Traditionally, the glass rod is dipped into ethanol and flamed. It should not be overheated, as it is not left in the flame. Sterile disposable plastic spreaders may also be used.

b. Pipet: 1.0-mL sterile glass or plastic disposable pipets or micropipets with sterile disposable tips.

c. Turntable (optional).*

d. Incubator or drying oven, set at 42°C, or laminar-flow hood.

3. Media

See 9215A.6*a*, *c*, and *d*. If R2A agar is used, best results are obtained when incubated at 28°C for 5 to 7 d; if NWRI agar is used, incubate at 20°C for 7 d.

* Fisher Scientific, No. 08-758 (hand-operated), No. 1580 (Lab-Line, motor-driven), or equivalent.

4. Preparation of Plates

Pour 15 mL of desired medium into sterile 100- × 15-mm or 90- × 15-mm Petri dishes; let agar solidify. Use pre-dried plates immediately after drying or store for up to 2 weeks refrigerated at 2–8°C in sealed plastic bags. If pre-drying and using plates the same day, pour 25 mL agar into Petri dish and dry in a laminar-flow hood at room temperature (24–26°C) with the lid off to obtain the desired 2- to 3-g weight loss.

5. Procedure

Prepare sample dilutions as directed in 9215B.2.

a. Glass rod: Pipet 0.1 or 0.5 mL sample onto surface of a pre-dried agar plate. Using a sterile bent glass or disposable plastic rod, distribute inoculum over surface of medium by rotating plate manually or on a turntable. Let inoculum absorb completely into medium before inverting and incubating.

b. Pipet: Pipet desired sample volume (0.1, 0.5 mL) onto surface of pre-dried agar plate, preferably while plate is being rotated on a turntable. Slowly release sample from pipet while making one to-and-fro motion, starting at center of plate and stopping 0.5 cm from plate edge before returning to center. Lightly touch pipet to plate surface. Let inoculum absorb completely into medium before inverting and incubating.

6. Incubation

See 9215A.7.

7. Counting, Recording, Computing, and Reporting

See 9215A.8 and 9.

8. Bibliography

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9215 D. Membrane Filter Method

1. Samples and Sample Preparation

See 9215A.4 and 5.

2. Laboratory Apparatus

See Section 9222B.1.

3. Media

See 9215A.6. Use m-HPC agar or else R2A or NWRI agar.

4. Preparation of Plates

Dispense 5-mL portions of sterile medium into 50- × 9-mm Petri dishes. Let solidify at room temperature. Prepared plates may be stored inverted in a plastic box or tight container in a refrigerator at 2–8°C for ≤2 weeks.

5. Sample Size

The volume to be filtered will vary with the sample. Select a maximum sample size to give 20 to 200 CFU per filter.

6. Procedure

Filter appropriate volume through a sterile 47-mm, 0.45-μm-pore-diam, gridded membrane filter under partial vacuum. Rinse funnel with three 20- to 30-mL portions of sterile dilution water. Using flame-sterilized forceps, aseptically place filter on agar in Petri dish. Make sure to position the filter carefully, rolling from one side to the other so air bubbles are not trapped under it.

7. Incubation

Place dishes in close-fitting box or plastic bag containing moistened paper towels. See 9215A.7 for incubation temperatures and times.

8. Counting, Recording, Computing, and Reporting

See 9215A.8 and 9. Report as CFU/mL, membrane filter method, time, and medium.

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9215 E. Enzyme Substrate Method

1. Samples and Sample Preparation

See 9215A.4 and 5.

2. Laboratory Apparatus

a. Pipets, 0.1-, 1.0-, and 10-mL, sterile, graduated, glass or disposable plasticor micropipeters with sterile disposable tips.

b. Incubator set at 35 ± 0.5°C.

c. Ultraviolet light source, long wavelength, 6-W, 365–366 nm.

*d. Sample plates.**

3. Medium

SimPlate® medium is available commercially† in sterile vessels for 100-mL multi-dose procedures or in test tubes for 10-mL unit-dose procedures. Store medium between 2 and 25°C, and use before expiration date.

Test each lot for sterility by following the inoculation procedure (9215E.5), using 10 mL rehydrated medium without sample. Incubate at 35 ± 0.5°C for 48 h. No wells should fluoresce after incubation when viewed under a 6W 365–366 nm UV lamp.

4. Sample Diluent

Use either sterile deionized water, distilled water, buffered water, or 0.1% peptone (see Section 9050C.1).

5. Procedure

a. Multi-dose: Rehydrate medium by filling medium vessel to 100-mL mark with sterile diluent, re-capping, and shaking until medium has dissolved. Aseptically pipet 1.0 mL sample and then 9 mL rehydrated medium onto center of the sample plate. Alternatively, aseptically pipet 0.1 mL sample and then 9.9 mL rehydrated medium onto center of the sample plate. (NOTE: Final volume of sample plus medium must be 10 ± 0.2 mL.)

Cover plate with lid and gently swirl to distribute mixture into wells. Tip plate 90° to 120° to drain excess mixture into the absorbent pad. Invert plate and incubate at 35 ± 0.5°C for 48 h (range of 45 to 72 h). If a sample is suspected to have a count >738 MPN/mL, dilute by adding 1 mL sample to a sterile vessel containing 99 mL sterile diluent. Make additional dilutions as required to keep counts <738 MPN/mL in final dilution.

b. Unit-dose: Rehydrate medium by either adding 10 mL sample or, if sample is expected to be >73.8 MPN/mL, pipet 9 mL of sterile diluent and 1 mL of sample, re-cap, and shake until medium has dissolved. Add entire contents to center of plate. (NOTE: Final volume of sample plus medium must be 10 ± 0.2 mL.)

Cover plate with lid and gently swirl to distribute mixture into wells. Tip plate 90° to 120° to drain excess mixture into absorbent pad. Invert plate and incubate at 35 ± 0.5°C for 48 h (range of 45 to 72 h).

6. Counting, Recording, Computing, and Reporting

After incubation, count the number of blue fluorescent wells by holding a 6-W, 365–366 nm UV light about 5 in. above plate, preferably with UV-filtering safety glasses or in a UV cabinet. Alternatively, count fluorescent wells through the back of the inverted plate following the procedure as indicated.

Use the appropriate MPN Table (multi-dose or unit dose) provided by the manufacturer to calculate MPN/mL. Adjust MPN to reflect sample volume and/or dilution made to yield a corrected MPN value. Record as MPN/mL.

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* SimPlates®, IDEXX Laboratories, Westbrook, ME, or equivalent.
† Idexx Laboratories, Westbrook, ME.

9216 DIRECT TOTAL MICROBIAL COUNT*

9216 A. Introduction

Staining using deoxyribonucleic acid (DNA)-specific fluorochromes, such as 3,6-bis (dimethylamino) acridinium chloride (acridine orange) or 4',6-diamidino-2-phenylindole (DAPI), followed by epifluorescence microscopy, has been used to count total microbial cells in aquatic samples. Other fluorochromes, such as 5-cyano-2,3-ditolyl tetrazolium (CTC) or SYTO 9/propidium iodide, can be used for viable or total bacteria cell counts.

Direct total cell counts of bacteria in water or wastewater usually exceed counts obtained via heterotrophic plate counts (HPC) and most probable number (MPN) methods because they preclude the errors caused by viability-related phenomena, such as selectivity of growth media, cell clumping, and slow growth rates or errors inherent to the methods. Some water samples might contain large amounts of dead or nonculturable cells.

The method described in this section uses acridine orange as the staining agent.

* Approved by Standard Methods Committee, 2014.
Joint Task Group: 22nd Edition—Christian J. Volk (chair), Jean Claude Block, Ellen B. Braun-Howland, Christian P. Chauret, Archie J. Degnan, Isabel Escobar, Marion G. Freeman, Frederik A. Hammes, Joseph King, Daniel B. Oerther, Michele Prevost, Holger T. Sommer, and Margaret M. Williams.

9216 B. Epifluorescence Microscopic Method Using Acridine Orange

1. General Discussion

The epifluorescence microscopy method produces direct total cell counts with relative speed (20 to 30 min from time of sampling) and sensitivity. It does not permit differentiation of bacterial cells on the basis of taxonomy, metabolic activity, or viability, and it cannot be used to estimate microbial biomass because the volume of individual cells varies considerably. The method requires an experienced technician who can distinguish microbial cells from debris based on morphology.

The method consists of sample fixation for storage, staining with a chemical fluorochrome, vacuum filtration onto a nonfluorescing polycarbonate membrane, and enumeration using an epifluorescence microscope.

2. Apparatus

a. Microscope, vertical ultraviolet (UV) illuminator for epifluorescence with flat field 100× oil immersion objective lens sufficient to achieve total magnification of about 1000× (e.g., 63× objective with a 15× ocular, or a 100× objective with a 10× ocular). The microscope can be equipped with a digital camera and image-analyzing software to perform autocounts and calculations. Use an image-analysis system to improve productivity only when processing debris-free water samples, such as drinking water samples. The laboratory must demonstrate that automated counts produce results as precise and accurate as the manual counts (see Section 9020 for quality assurance).

b. Counting graticule, ocular lens micrometer* calibrated with stage micrometer.

c. Filters,† including excitation filters (KP 490 and LP 455), beam splitter (LP 510), and barrier filter (e.g., LP 520 using mercury lamp, HBO 50).

d. Blender or vortex mixer.

e. Ultrasonic bath or processor, for sonication of samples containing bacterial aggregates. First, determine the sonication time that will facilitate disaggregation of bacterial clumps without causing cell lysis. Sonication times may range from a few seconds to several minutes, depending on the type, frequency, and power of the ultrasonication equipment. To determine the optimum sonication time of a specific ultrasonic apparatus, conduct a series of sonication experiments of biofilm/clumped bacteria samples using different times. Optimum sonication conditions lead to the highest increase in bacteria counts as a result of declumping (no cell disruption). It is difficult (sometimes impossible) to completely disperse bacterial aggregates in some samples (e.g., wastewater samples), so their total cell count might be underestimated.

f. Filtration unit, suitable for use with 25-mm-diam membrane filters.

g. Membrane filters, polycarbonate,‡ 25-mm-diam, 0.2-μm pore size [purchase nonfluorescent or prepare by soaking membrane in Irgalan§ black (2 g/L in 2% acetic acid) for 24 h, then rinse in water and air dry]; cellulosic‖ 25-mm-diam, 5-μm pore size.

h. Syringes, 3-mL, disposable, with disposable syringe filters, 0.2-μm pore size (to filter reagents before use).

i. Test tubes, glass, screw-capped, 13- × 125-mm.

* Bausch & Lomb No. 31-16-13.

† C. Zeiss, or equivalent.
‡ Nuclepore Corp., or equivalent.
§ Chemical alternately named acid black 107 from Ciba Geigy Corp., or equivalent.
‖ Millipore Corp., or equivalent.

3. Reagents

a. Phosphate buffer: Dissolve 13.6 g KH_2PO_4 in water and dilute to 1 L. Adjust to pH 7.2 with a solution of NaOH if necessary; filter through 0.2-μm membrane filter.

b. Fixative, 5.0% (w/v) glutaraldehyde in phosphate buffer. Prepare fresh daily.

c. Fluorochrome, 0.1% (w/v) acridine orange# in phosphate buffer.

d. Immersion oil, low fluorescing**

Because of the potential health effects of exposure to glutaraldehyde and acridine orange, handle chemicals in ventilated areas (fume hood), keep solutions in tightly sealed containers, and wear appropriate personal protective equipment.

4. Procedure

Collect water samples as directed in Section 9060A. Add 9.0 mL sample to a test tube containing 1.0 mL fixative. Fixed samples can be stored in tightly capped containers at 4°C for up to 3 weeks without a significant decrease in cell number.

Disperse and dilute samples from mesotrophic or eutrophic sources to obtain reproducible results. Mix sample using a blender or vortex mixer, then make tenfold dilutions in phosphate buffer as necessary. Clean water samples may not require dilution, but larger sample volumes (>100 mL) may be required to obtain reliable counts (higher volumes of water might be filtered to concentrate samples with low bacteria concentrations). Samples containing clumped bacteria (such as biofilm samples) should be subjected to ultrasonic treatment to disrupt aggregates.

Place 1 mL of sample or dilution on a nonfluorescent polycarbonate filter supported by a cellulosic membrane filter in the filter holder. Using disposable sterile syringe filters, add 1 mL fluorochrome and incubate for 15 min in the dark, then add about 3 mL filtered phosphate buffer to promote a uniform distribution of cells across the membrane. Alternatively, combine fluorochrome and sample in a small clean vial, allow to react, and add the mixture to the filter holder. Filter under vacuum (about 13 kPa). Wash membranes with 2 mL phosphate buffer and apply vacuum. Remove polycarbonate filter with clean forceps and air dry for 1 to 2 min. The filter can be cut into quarter sections and saved, if needed. Place dried filter onto a drop of immersion oil on a clean glass microscope slide. Add a small drop of immersion oil to filter surface. Gently cover filter with a clean glass cover slip. Samples can be stored in the dark for several months without significant loss of fluorescence.

Examine at least 10 randomly selected fields on the filter using the oil immersion lens to establish that distribution of microbial cells is uniform and that individual cells can be enumerated (if not, dilute sample and repeat). Preferably count 10 to 50 cells per field. If the distribution and number of cells is adequate, count number of cells in at least 20 squares of the counting graticule in the ocular lens. When processing samples free of debris, an image analysis system can be used for microscopic counts and calculations of cell concentration.

Include negative and positive controls to confirm sterility and staining efficacy. For the negative control, perform the staining on an aliquot (same volume as sample to be tested) of filtered phosphate buffer. As a positive control, use a suspension of laboratory cultivated cells (e.g., *E. coli*) that has been fixed and diluted in filter-sterilized phosphate buffer to a predetermined cell concentration.

To test for interference of the sample matrix with the procedure, add an aliquot of the positive control cell suspension to 1 mL of sample and perform acridine orange staining. Enumerate total cells, subtract sample cell number from the sample + positive control cell number, and perform the calculation in 9216B.5, accounting for the dilution of the positive control aliquot in the dilution factor to obtain the number of cells per milliliter. Compare the positive control enumeration with the predetermined positive control cell concentration to determine staining efficacy for the sample.

5. Calculations

Calculate the average number of cells per filter. Obtain effective filter area from filtration unit specifications. Extrapolate to determine number of cells per milliliter of sample:

$$\text{Total cells/mL} = (\text{avg cells/square}) \times (\text{squares/filter}) \times (\text{dilution factor})/\text{sample volume, mL}$$

6. Bibliography

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Sigma Chemical Co. biological grade, or equivalent.
** Cargille Laboratories, Inc., Type B, or equivalent.

9217 ASSIMILABLE ORGANIC CARBON*

9217 A. Introduction

1. Significance

Growth of bacteria in drinking water distribution and storage systems can lead to the deterioration of water quality, violation of water quality standards, and increased operating costs. Growth or regrowth results from viable bacteria surviving the disinfection process and utilizing nutrients in the water and biofilm to sustain growth.[1] Factors other than nutrients that influence regrowth include temperature,[2] residence time in mains and storage units,[3] and the efficacy of disinfection.[4] Tests to determine the potential for bacterial regrowth focus on the concentration of nutrients.[5–7]

Not all organic compounds are equally susceptible to microbial decomposition; the fraction that provides energy and carbon for bacterial growth has been called labile dissolved organic carbon,[8,9] biodegradable organic carbon (BDOC),[7] or assimilable organic carbon (AOC).[5] Easily measured chemical surrogates for AOC are not available now.[10,11] As alternatives to chemical methods, bioassays have been proposed.[5–7,12–14]

In a bioassay, the growth of a bacterial inoculum to maximum density can be used to estimate the concentrations of limiting nutrients; the underlying assumptions of the AOC bioassay are that nitrogen and phosphorus are present in excess (i.e., that organic carbon is limiting) and that the bioassay organism(s) represent the physiological capabilities of the distribution system microflora. Various bioassay procedures use an inoculum of one to four species of bacteria[5,12,13,15,16] growing in log phase or present in late stationary phase, or may use undefined bacteria attached to a sand substratum,[7] suspended in the sample,[6] or filtered from the sample and then resuspended.[14] Incubation vessels vary as to material,[17] size,[18,19] closure,[18] and cleaning procedure.[5,18,19] Water to be tested for nutrient concentrations has been variously prepared.[5,7,14] The AOC bioassay is an indirect or surrogate method, wherein nutrient concentrations are not measured directly, but colony-forming units (CFU) of the bioassay organism(s) are the test variable. Nutrient concentrations have been estimated directly from changes in dissolved organic carbon concentrations within the test vessel[7] or indirectly from epifluorescence microscopic counts of the maximum number of bacterial cells grown,[13,14] turbidity,[14] or incorporation of tritiated thymidine into bacterial DNA.[6,20] CFU densities, total cell densities, or bacterial production are converted to nutrient concentration by the growth yield of bacteria, defined as either the ratio between CFU or cells produced and organic carbon used, or biomass produced and organic carbon used.[5,6]

2. Selection of Method

The method described below is a two-species bioassay using *Pseudomonas fluorescens* strain P-17 and *Spirillum* strain NOX (van der Kooij)[10] that has been modified to reduce problems of bacterial and carbon contamination.[18,19] It uses a defined inoculum and miniaturized incubation vessels, requires no specialized equipment, and has been related to the presence of coliforms in a drinking water distribution system.[21] The two-species inoculum probably underestimates the total quantity of AOC, is consistently lower than BDOC estimates, and does not provide an estimate of refractory organic carbon.[22] Critical aspects of the proposed method, including the preparation of the incubation vessel, test water, and inoculum, and enumeration of the test organisms, are transferable to alternate AOC assays that use a different defined inoculum.

With an undefined bacterial inoculum, enumeration by the spread plate technique is not applicable; alternate response variables, such as changes in dissolved organic carbon (DOC) concentration, turbidity, epifluorescence microscopic counts, bacterial mortality, or bacterial growth, have been used.[6,7,14]

3. Sampling and Storage

Follow precautions outlined in Section 9060A and B for collecting and storing samples. Pasteurized and dechlorinated water samples probably can be held for several days without deterioration if properly sealed. Initiate the AOC assay as quickly as possible after pasteurization (see 9217B.4*c*).

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9217 B. *Pseudomonas fluorescens* Strain P-17, *Spirillum* Strain NOX Method

1. General Discussion

a. Principle: The AOC bioassay using *Pseudomonas fluorescens* strain P-17 and *Spirillum* strain NOX involves growth to a maximum density of a small inoculum in a batch culture of pasteurized test water. Pasteurization inactivates native microflora. The test organisms are enumerated by the spread plate method for heterotrophic plate counts (Section 9215C) and the density of viable cells is converted to AOC concentrations by an empirically derived yield factor for the growth of P-17 on acetate-carbon and NOX on oxalate-carbon as standards. The number of organisms at stationary phase is assumed to be the maximum number of organisms that can be supported by the nutrients in the sample and the yields on acetate carbon and

oxalate carbon are assumed to equal the yield on naturally occurring AOC.[1,2]

b. Interferences: Untreated surface waters, especially those with high concentrations of suspended solids or high turbidity, can contain large numbers of spore-forming bacteria that may survive pasteurization, grow, and interfere with the enumeration of P-17 and NOX on spread plates. Such waters generally have high AOC concentrations and can be diluted with organic-free water amended with mineral salts or prefiltered through carbon-free filters. Potable waters that have been disinfected and carry a disinfectant residual will inhibit growth of the test organism unless the disinfectant is neutralized. Surface waters from reservoirs treated with copper sulfate also may be inhibitory unless a chelating agent is added to the sample,[3] and lime-softened waters with elevated pH values may require pH adjustment. Any amendment to a sample requires a control for AOC contamination.

c. Minimum detectable concentration: In theory, concentrations of less than 1 μg C/L can be detected. In practice, organic carbon contamination during glassware preparation and sample handling imposes a limit of detection of approximately 5 to 10 μg AOC/L.

2. Apparatus

a. Incubation vessels: Organic-carbon-free borosilicate glass vials (45 mL capacity) with TFE-lined silicone septa.

b. Incubator, set at 15 ± 0.5°C.

c. Hot water bath capable of achieving and holding 70°C.

*d. Continuously adjustable pipet** capable of delivering between 10 and 100 μL.

e. Erlenmeyer flask, 125-mL, with ground-glass stopper.

f. Apparatus for preparing dilution water and making heterotrophic plate counts: See Sections 9050C.1 and 9215C.

3. Reagents

a. Sodium acetate stock solution, 400 mg acetate-C/L: Dissolve 2.267 g $CH_3COONa \cdot 3H_2O$ in 1 L organic-carbon-free, deionized water. Transfer to 45-mL vials, fill to shoulder, cap tightly, and autoclave. Although standard autoclave practice is to loosen caps, keep vials with septa capped tightly for autoclaving. Store at 5°C in tightly capped vials. Solution may be held for up to 6 months.

b. Sodium thiosulfate solution: Dissolve 30 g $Na_2S_2O_3$ in 1 L deionized water. Transfer to 45-mL vials and autoclave as directed in ¶ *a* above.

c. Buffered water: See Section 9050C.1*a*.

d. R2A agar: See Section 9215A.6*c*.

e. Sodium persulfate solution, 10% (w/v): Dissolve 100 g $Na_2S_2O_8$ in 1 L deionized water.

f. Organic-free water: See Section 5710B.3*e*. Alternatively, use HPLC-grade bottled water.

g. Mineral salts solution: Dissolve 171 mg K_2HPO_4, 767 mg NH_4Cl, and 1.444 g KNO_3 in 1 L carbon-free water. Transfer to 45-mL vials and autoclave as directed in ¶ *a* above.

h. Cultures of strains P-17 (ATCC 49642) and NOX (ATCC 49643).†

4. Procedure

a. Preparation of incubation vessels: Wash 45-mL vials with detergent, rinse with hot water, 0.1*N* HCl two times, and deionized water three times, dry, cap with foil, and heat to 550°C for 6 h. Soak TFE-lined silicone septa in a 10% sodium persulfate solution for 1 h at 60°C; rinse three times with carbon-free deionized water. Alternatively, use pre-cleaned water sampling vials[4] or an equivalent AOC-free vial.‡ Use same cleaning procedure for all glassware.

b. Preparation of stock inoculum: Prepare individual turbid suspensions of P-17 and NOX by transferring growth from a slant culture on R2A agar into 2 to 3 mL filtered (0.2 μm), autoclaved sample. Use slant ≤6 months. The autoclaved sample can be any water that supports growth of P-17 and NOX and is organic-carbon-limited. Neutralize chlorinated samples with sodium thiosulfate (42 μL/50 mL). Transfer 100 μL of suspension to 50 mL filtered, autoclaved sample in a sterile 125-mL ground-glass-stoppered Erlenmeyer flask. Add 125 μL sodium acetate solution (suspension contains 1 mg acetate-C/L). Incubate at room temperature (≤25°C) until the viable cell count reaches the stationary phase. Organic-carbon limitation will ensure complete use of acetate-C so no AOC is transferred with the inoculum. The stationary phase is reached when the viable cell count, as measured by spread plates, reaches maximum value. Store stock cultures for ≤6 months at 5°C. Before inoculating a bioassay vessel, make a viable count of the culture (spread plate) to determine the appropriate volume of inoculum to be added to each bioassay vessel.

c. Preparation of incubation water: Collect samples directly into ten 45-mL vials. Use 9 vials for AOC measurement and 1 for growth control. Fill each vial to the neck (40 mL) within as short a time as possible. Place septa on the vials, TFE side down, and secure with open-topped screw caps. Alternatively, collect 500 mL sample in an organic-carbon-free vessel and pour into each vial. Neutralize samples containing disinfectant residuals with 33 μL sodium thiosulfate solution added to each vial or 0.5 mL per 500-mL sample. Preferably, collect an extra vial to check for residual chlorine after neutralization. In the laboratory, cap vials tightly and pasteurize in 70°C water bath for 30 min.

d. Inoculation and incubation: Cool, inoculate with 500 colony-forming units (CFU)/mL each of P-17 and NOX, either by injecting through the septum or by removing cap and using a carbon-free pipet. Plastic, sterile tips for continuously adjustable pipets are suitable. Use the following equation to calculate volume of inoculum:

$$\text{Volume of inoculum} = \frac{(500\ \text{CFU/mL}) \times (40\ \text{mL/vial})}{\text{CFU/mL stock inoculum}}$$

Hold vials at 15°C in the dark for 1 week. If a 15°C incubator is unavailable, incubate at room temperature not to exceed 25°C. Because incubation temperature influences growth yield, record

* Eppendorf, or equivalent.
† Available from the American Type Culture Collection.
‡ Pierce Vari-Clean.

and report temperature. Determine yields as directed below if an alternative temperature is used.

e. Enumeration of test bacterium: On incubation days 7, 8, and 9, remove three vials from the incubator. Sample an individual vial on only 1 d. Shake vials vigorously for 1 min, remove 1 mL with a sterile pipet, and prepare a dilution series (see Section 9215B). Plate three dilutions (10^{-2}, 10^{-3}, and 10^{-4}) in duplicate. Incubate plates at 25°C for 3 to 5 d and score the number of colonies of each strain. P-17 colonies appear on plates first; they are 3 to 4 mm in diameter with diffuse yellow pigmentation. NOX colonies are small (1- to 2-mm-diam) white dots. It may be necessary to count P-17 and NOX colonies at different dilutions. Sample vials on three separate days to check whether maximum density has been reached. Day-to-day variations of between 11 and 16% of the mean for batch cultures of P-17 in stationary phase are typical.[1] A consistent increase in cell densities of 20% or more over the 3-d period indicates that the cultures are not in stationary phase; repeat assay with longer incubation period. Alternatively, collect more samples (three for each additional sampling day) and prepare as in ¶ *c* above so extended incubation can be used. A sharp population decrease of approximately 0.5 log over the 3 d is unusual, but may occur. If this happens, repeat the assay.

f. Determination of yield of P-17 and NOX: The yields of P-17 and NOX on model carbon compounds should be constant if organic carbon is limiting and the incubation temperature is kept constant. It is acceptable to use the previously derived empirical yield values of 4.1×10^6 CFU P-17/μg acetate-C, 1.2×10^7 CFU-NOX/μg acetate-C, and 2.9×10^6 CFU-NOX/μg oxalate-C at 15°C.[5] However, the determination of a yield control provides an important check on both the bioassay (see also 9217B.6) and carbon limitation in the sample.

5. Calculation

AOC concentration: Average viable count results for the 3 d and calculate concentration of AOC as the product of the mean of the viable counts and the inverse of the yield:

$$\mu\text{g AOC/L} = [(\text{mean P-17 CFU/mL})(1/\text{yield}) + (\text{mean NOX CFU/mL})(1/\text{yield})](1000\ \text{mL/L})$$

When the empirical yield factors[5] are used, the equation becomes:

$$\mu\text{g AOC/L} = [(\text{mean P-17 CFU/mL})(1/\text{yield}) + (\text{mean NOX CFU/mL})(1/\text{yield})](1000\ \text{mL/L})$$

When the empirical yield factors[5] are used, the equation becomes:

$$\mu\text{g AOC/L} = [(\text{mean P-17 CFU/mL})(\mu\text{g acetate-C}/4.1 \times 10^6\ \text{CFU}) + (\text{mean NOX CFU/mL})(\mu\text{g oxalate-C}/2.9 \times 10^6\ \text{CFU})](1000\ \text{mL/L})$$

or

$$\mu\text{g AOC/L} = [(\text{mean P-17 CFU/mL})(2.44 \times 10^{-7}\ \mu\text{g acetate-C/CFU}) + (\text{mean NOX CFU/mL})(3.45 \times 10^{-7}\ \mu\text{g oxalate-C/CFU})](1000\ \text{mL/L})$$

In practice, the densities of organisms vary during the stationary phase. Using average density over 3-d period provides a more accurate estimate of the real maximum density.

Reporting AOC as μg C/L assumes that the yields on acetate and oxalate are equal to the yields on naturally occurring AOC. To permit data comparisons report incubation temperature, contribution of each species to AOC, and yield factors used.

6. Quality Control

See Section 9020B for general quality control procedures. Quality control specific to the AOC bioassay includes testing the inoculum for purity and viability by plating a portion on R2A agar, testing the incubation vessel, inoculum, thiosulfate solution, and any supplemental procedure, such as filtration or dilution for organic carbon contamination, testing the P-17 and NOX inocula for yield, and testing the sample for carbon limitation or inhibition of assay organisms. Test all deviations in procedure (see ¶ *f* below).

To make these tests, use separate controls for blank, yield, and growth. The controls outlined below use a single vial and are meant as a troubleshooting guide. Definitive determination, for example, that the yield is different from a published value or that a sample is inhibitory, requires replication and statistical analysis.

a. Blank control: Dilute mineral salts solution 10:1 with carbon-free water. Follow procedures outlined above: Fill a vial to the shoulder with organic-carbon-free water, add 100 μL mineral salts and 100 μL sodium thiosulfate, pasteurize, inoculate with P-17/NOX, incubate, and enumerate growth.

b. Yield control: Dilute sodium acetate or sodium oxalate solution 10:1 with carbon-free water, preparing 40 mg C/L working concentrations. Follow procedures outlined above: Fill a vial to the shoulder with carbon-free water, add 100 μL mineral salts, 100 μL sodium thiosulfate, and 100 μL sodium acetate or sodium oxalate working solution, pasteurize, inoculate with P-17/NOX, incubate, and enumerate growth. P-17, unlike NOX, will not grow with oxalate as sole carbon source (oxalate is considered a major by-product of ozonation). NOX growth in HPLC-grade water presumed to be organic carbon-free is to be expected. The yield control is a quality control measurement and is not intended to provide a conversion factor for the calculation of AOC.

c. Growth control: Use additional sample of test water collected with the nine AOC vials, (9217B.4*c*) but amend with 100 μL diluted mineral salts and 100 μL of diluted acetate or oxalate solution per vial before pasteurization. As with other controls, inoculate with P-17/NOX, incubate, and enumerate growth.

d. Yield calculations: If previously derived empirical yield values (see 9217B.4*f*) are not used, a conversion factor can be derived empirically by using pure cultures of P-17 and NOX. Mixed cultures of the organisms cannot be used and a separate blank control for each species is required. Convert density units to CFU/L by multiplying CFU/mL by 1000, and divide by

100 μg acetate or oxalate-C/L. Express yield as CFU P-17 or NOX/ μg acetate-C or oxalate-C. For P-17 and acetate-C, the equation is:

$$\text{Yield} = \frac{[(\text{P-17 CFU/mL:yield control}) - (\text{P-17 CFU/mL:blank control})] \times (1000\ \text{mL/L})}{100\ \mu\text{g acetate-C/L}}$$

$$= P\text{-}17\ CFU/\mu g\ acetate\text{-}C$$

e. Interpretation of growth control: Subtract densities of P-17 and NOX that grew in the sample amended with only thiosulfate from the densities of P-17 and NOX that grew in the growth control. Compare difference to the difference between yield and blank controls.

If: (growth control − sample) = (yield control − blank control)

Then: sample is carbon-limited and not inhibitory

If: (growth control − sample) < (yield control − blank control)

Then: sample is inhibitory to bioassay organism

If: (growth control − sample) > (yield control − blank control)

Then: sample is not carbon-limited

f. Supplemental procedure check: When using supplemental procedures, such as filtration, dilution, or chemical amendment, check for carbon contribution to the AOC values. To test a procedure, use carbon-free water and blank control as a base line. Perform the supplemental procedure on additional carbon-free water and compare to densities of P-17 and NOX that grow in the blank control.

7. Precision and Bias

The P-17 bioassay performed in a single laboratory using 45-mL vials had a precision of ±17.5% based on a total of 58 assays with 14 different samples.[6]

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9218 AEROBIC ENDOSPORES*

9218 A. Introduction

1. Description

Endospores are dormant, environmentally resistant structures formed by certain genera of bacteria. The majority of aerobic-endospore-forming bacteria are harmless, saprophytic organisms found in soil and water. Vegetative cells of aerobic-endospore-forming bacteria sporulate in response to adverse environmental conditions. The bacteria can persist in the endospore form for an extended period. Under favorable conditions, such as a readily available nutrient supply, endospores convert back to vegetative bacterial cells in a process known as *germination*. Unlike vegetative cells, endospores resist environmental pressures, such as heat, desiccation, disinfection, and irradiation. Endospores are generally even-sized and refractile.

2. Detection and Applications

Aerobic endospores are ubiquitous in the environment. Indigenously occurring aerobic endospores in water are detected by exposing the sample to a heat treatment to inactivate any vegetative cells; heat treatment does not inactivate the endospores. The sample is then plated onto a nonselective nutrient medium and incubated aerobically at 35°C. The endospores germinate and form bacterial colonies. Most of these organisms will be species of *Bacillus.*

Analysis for aerobic endospores can be used to evaluate a variety of water treatment processes, including physical removal processes (e.g., coagulation, clarification) and disinfection.[1–3] Most surface water sources (except some lakes and reservoirs) contain sufficient levels of endospores to determine order-of-magnitude removal efficiencies. Because endospores are highly resistant structures, they are also used for determining the efficacy of such processes as halogen, ozone, and ultraviolet inactivation. The procedure also has been used to evaluate the efficacy of riverbank infiltration. In addition, the presence of endospores may be used to determine the physical integrity of drinking water distribution systems that may have been compromised via pipeline breaks or maintenance procedures.

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* Approved by Standard Methods Committee, 2007.
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9218 B. Membrane Filter Method

1. Apparatus

a. Bottles or Erlenmeyer flasks with closures, suitable for sample size chosen and capable of withstanding hot and cold temperatures.

b. Culture dishes: See Section 9222B.1*e.*

c. Filtration units: See Section 9222B.1*f.*

d. Water bath, preferably shaking style, capable of heating samples to 80°C.

e. Thermometer.

2. Materials and Culture Media

Nutrient agar plus trypan blue:

Peptone	5.0	g
Beef extract	3.0	g
Trypan blue	0.015	g
Agar	15.0	g
Reagent-grade water	1.0	L

Heat to boiling to dissolve agar. Sterilize by autoclaving for 15 min at 121°C. Final pH should be 6.8 ± 0.2. After autoclaving, cool to 50°C and dispense aseptically into 50- × 9-mm plastic culture plates with loose-fitting lids. The pH should be 6.8 ± 0.2. Refrigerated medium can be held for 20 d at 4 to 8°C.

3. Procedure

a. Sample size: See Section 9222A.2.

b. Heat treatment of sample: Distribute samples into bottles or Erlenmeyer flasks (9218B.1*a*). Loosen caps. Prepare a pilot control flask that contains the same volume as that of the water sample in the test flasks. The control flask must have a closure that includes a thermometer. Place control flask into the water bath along with the samples. Ensure that the level of water in the

bath is higher than that of the samples. When using a water bath with shaker, agitate samples at 60 to 80 rpm. If a shaking bath is not available, manually swirl samples periodically. Monitor temperature in pilot control flask. When pilot flask reaches desired temperature, begin timing and hold sample at that temperature for the specified time. At a temperature of 75°C, heat samples for 15 min; at a temperature of 80°C, heat samples for 10 min.[1,2] Then, cool samples immediately in a bath containing a slurry of wet ice. Let samples cool to approximately room temperature. As a quality control procedure, spike samples and then examine for the presence of spores via staining or phase contrast microscopy.

c. Filtration of samples and counting: Filter samples as described in 9222B.4*c*. Place membrane filter on agar surface and incubate for at least 24 ± 2 h at 35 ± 0.5°C. Some colonies require incubation for 5 to 7 d for intracellular formation of endospores. Count colonies with a low-power (10 to 15×) binocular wide-field dissecting microscope or other optical device. Trypan blue is added to impart a colored background on the membrane. Consider any bacterial colonies growing on the membrane as aerobic spore-forming bacteria.

d. Interpretation: For colony counting procedure, see Section 9215A.8*b*. Colonies should catalase positive.

4. References

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9221 MULTIPLE-TUBE FERMENTATION TECHNIQUE FOR MEMBERS OF THE COLIFORM GROUP*

9221 A. Introduction

Coliform bacteria have long been used as water-quality indicators based on the premise that, because these organisms are present in the intestines of warm-blooded animals, their presence in water could indicate that recent fecal contamination has occurred. Historically, this group of organisms has been defined by their ability to ferment lactose, rather than through the tenets of systematic bacteriology, so the group consists of bacteria from several genera belonging to the family Enterobacteriaceae.

The methods described in this section use a lactose-based broth medium to detect the metabolic end products of lactose fermentation. The presence of coliforms must be confirmed in a lactose- and bile salt-containing medium [brilliant green lactose bile (BGLB) broth]. So when the fermentation techniques in this section are used, *coliforms* are defined as all facultatively anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid production in the presence of bile salts within 48 h at 35°C.

The standard test for the coliform group may be carried out by the multiple-tube fermentation technique or presence–absence procedure (through the presumptive-confirmed phases or completed test) described herein, the membrane filter (MF) technique (Section 9222), or the enzymatic substrate coliform test (Section 9223). Each technique is applicable within the limitations specified and with due consideration of the purpose of the examination. Production of valid results requires strict adherence to quality control (QC) procedures. QC guidelines are outlined in Section 9020.

The fermentation technique can be used to detect coliforms in drinking water or quantitate coliforms in potable and nonpotable water. When multiple tubes are used, coliform density is estimated via a most probable number (MPN) table. This number, generated using specific probability formulas, is an estimate of the mean density of coliforms in the sample. Coliform testing results, together with other information obtained from engineering or sanitary surveys, provide the best assessment of water-treatment effectiveness and the sanitary quality of source water.

The fermentation test's precision in estimating coliform density depends on the number of tubes used. The most satisfactory information will be obtained when the largest sample inoculum examined shows acid and/or gas in some or all of the tubes and the smallest sample inoculum shows no acid or gas in any or a majority of the tubes. Bacterial density can be estimated by the formula given or from the table using the number of positive tubes in the multiple dilutions (9221C.2). The number of sample portions selected will be governed by the desired precision of the result. The MPN tables are based on the assumption of a Poisson distribution (random dispersion). However, if the sample is not adequately shaken before aliquots are removed or if bacterial cells clump, the MPN value will be an underestimate of actual bacterial density.

1. Water of Drinking-Water Quality

When analyzing drinking water to determine if its quality meets U.S. Environmental Protection Agency (EPA) standards, a 100-mL sample must be analyzed; use the fermentation technique with 10 replicate tubes each containing 10 mL, 5 replicate tubes each containing 20 mL, or a single bottle containing a 100-mL sample portion. When examining drinking water via the fermentation technique, process all tubes or bottles demonstrating growth—with or without a positive acid or gas reaction—through the confirmed phase (9221B.4). Drinking water samples that are positive for total coliforms also must be tested for thermotolerant (fecal) coliforms (9221E) or *Escherichia coli* (9221F).

For routine examination of public water supplies, the objective of the total coliform test is to determine the efficiency of treatment plant operations and the integrity of the distribution system. The test is also used to screen for the presence of fecal contamination. Some coliform occurrences in a distribution system may be attributed to coliform growth or survival within bacterial biofilms in the mains rather than treatment failure at the plant or well source, or outside contamination of the distribution system. Because it is difficult to distinguish coliforms entering the distribution system and coliforms already present in the pipe biofilm and sediments, assume that all coliforms originate from a source outside the distribution system.

2. Water of Other than Drinking Water Quality

When analyzing nonpotable waters, inoculate a series of tubes with appropriate decimal dilutions of the water (multiples of 10 mL) based on the probable coliform density. Use the presumptive-confirmed phases of the multiple-tube procedure. Use the more labor-intensive completed test (9221B.5) as a QC measure on 10% (or a set percentage) of coliform-positive nonpotable water samples quarterly. Generally, the objective of analyzing nonpotable water is to estimate bacterial density, determine a pollution source, enforce water quality standards, or trace the survival of microorganisms. The multiple-tube fermentation technique may be used to obtain statistically valid MPN estimates of coliform density. Examine a sufficient number of water samples to yield representative results for the sampling station. Generally, the geometric mean or median value of the results of a number of samples will yield a value in which the effect of sample-to-sample variation is minimized.

3. Other Samples

The multiple-tube fermentation technique is applicable to the analysis of salt or brackish waters, as well as muds, sediments, and sludges. Collect samples as directed in Section 9060A, using sample containers specified in Section 9030B.19. Follow the

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precautions given above on portion sizes and numbers of tubes per dilution.

To prepare solid or semisolid samples, weigh the sample and add diluent to make a 10^{-1} dilution. For example, place 30 g sample in a sterile blender jar, add 270 mL sterile phosphate buffered or 0.1% peptone dilution water, and blend for 1 to 2 min at high speed (8000 rpm). Prepare the appropriate decimal dilutions of the homogenized slurry as quickly as possible to minimize settling.

9221 B. Standard Total Coliform Fermentation Technique

1. Samples

Collect samples as directed in Section 9060A, using sample containers specified in Section 9030B.19. Follow the QC guidelines for sample bottles described in Section 9020B.5*d*. Ensure that samples meet laboratory-acceptance criteria upon receipt.

2. Quality Control

All phases of the fermentation technique (9221B–G) require adherence to the quality assurance/quality control (QA/QC) guidelines presented in Section 9020, including, but not limited to, analytical QC (Section 9020B.9), instrumentation/equipment (Sections 9020B.4 and 9030B), and supplies (Section 9020B.5). Refer to Table 9020:I for key QC procedures. Also, note the sections pertaining to appropriate storage and preparation of dehydrated culture media and water quality (Sections 9050 and 9020B.5*f*).

Use commercial dehydrated media when possible, and ensure that their formulations match those specified here because commercial formulations may vary. Prepared fermentation media can be stored in tightly capped tubes or bottles for up to 3 months in the dark, if temperatures are between 1 and 30°C and evaporation is less than 10% of the original volume. If the tubes were refrigerated after sterilization, they should be incubated overnight at room temperature (20°C) before use and those showing growth or bubbles should be discarded to avoid false-positive results. To demonstrate acceptable medium performance, positive and negative culture controls should be tested before first use and as otherwise specified (see Table 9020:VI). Sterility, volume per tube, and pH should also be verified and recorded. To demonstrate comparability between batches of media, perform a use test [Section 9020B.5*f*2)].

If a laboratory is switching to the multiple-tube fermentation technique, analysts ideally should first conduct parallel tests with the previous method to demonstrate applicability and comparability. The results of many coliform performance studies are available in the literature, and the rates of false-positive and -negative results can differ among various media. Users should carefully select the medium and procedure that best fits their needs.

3. Presumptive Phase

Use lauryl tryptose broth in this phase of the multiple-tube test, following the QC guidelines cited in 9221B.2.

a. Reagents and culture medium:

Lauryl tryptose broth:

Tryptose	20.0	g
Lactose	5.0	g

Table 9221:I. Preparation of Lauryl Tryptose Broth

Inoculum *mL*	Amount of Medium in Tube *mL*	Volume of Medium + Inoculum *mL*	Dehydrated Lauryl Tryptose Broth Required *g/L*
1	10 or more	11 or more	35.6
10	10	20	71.2
10	20	30	53.4
20	10	30	106.8
100	50	150	106.8
100	35	135	137.1
100	20	120	213.6

Dipotassium hydrogen phosphate (K_2HPO_4)	2.75	g
Potassium dihydrogen phosphate (KH_2PO_4)	2.75	g
Sodium chloride (NaCl)	5.0	g
Sodium lauryl sulfate	0.1	g
Reagent-grade water	1	L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. Before sterilization, dispense enough medium into fermentation tubes containing inverted vials (also known as Durham tubes) to cover the inverted vial at least one-half to two-thirds after sterilization. Alternatively, omit the inverted vial and add 0.01 g/L bromocresol purple to lauryl tryptose broth (to determine acid production, an indicator of a positive result in this part of the coliform test). Close tubes with metal or heat-resistant plastic caps.

Prepare in accordance with Table 9221:I, making lauryl tryptose broth concentrated enough that adding 100-, 20-, or 10-mL portions of sample to the medium will not reduce ingredient concentrations below those of the standard medium. Autoclave medium at 121°C for 12 to 15 min. Ensure that inverted vials, if used, are free of air bubbles. Medium pH should be 6.8 ± 0.2 after sterilization.

b. Procedure:

1) Arrange fermentation tubes in rows of five or ten tubes each in a test tube rack. The number of rows and the sample volumes selected depend on the quality and character of the water to be examined. For potable water, 100 mL must be tested. Use five 20-mL portions, ten 10-mL portions, or one 100-mL portion (a single bottle). For nonpotable water, use five tubes per dilution (of 10, 1, 0.1 mL, etc.).

When making dilutions and measuring diluted sample volumes, follow the precautions given in Section 9215B.2. Use Figure 9215:1 as a guide to preparing dilutions. Shake sample and dilutions vigorously 5 s (about 25 times). Inoculate each tube in a set of five with replicate sample volumes in increasing decimal dilutions, if decimal quantities of the sample are used. Mix test portions in the medium by gentle agitation.

2) Promptly incubate inoculated tubes or bottles, any culture controls, and/or sterility blanks at 35 ± 0.5°C. After 24 ± 2 h, swirl each tube or bottle gently and examine it for growth, gas, and/or acidic reaction (shades of yellow color) and, if no gas or acidic reaction is evident, re-incubate and re-examine at the end of 48 ± 3 h. Record presence or absence of growth, gas, and/or acid production. If the inner vial is omitted, growth with acidity (yellow color) signifies a presumptive-positive reaction.

c. Interpretation: Detection of an acidic reaction (yellow color) and/or gas in the tubes or bottles within 48 ± 3 h constitutes a presumptive-positive reaction. Submit tubes or bottles with a presumptive-positive reaction to the confirmed phase (9221B.4).

The absence of acidic reaction and/or gas formation at the end of 48 ± 3 h of incubation constitutes a negative test. Submit drinking water samples demonstrating growth without a positive gas or acidic reaction to the confirmed phase (9221B.4).

4. Confirmed Phase

a. Culture medium: Use BGLB broth fermentation tubes for the confirmed phase, following QC guidelines cited in 9221B.2.

Brilliant green lactose bile broth:

Peptone	10.0	g
Lactose	10.0	g
Oxgall	20.0	g
Brilliant green	0.0133	g
Reagent-grade water	1	L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. Before sterilization, dispense medium into fermentation tubes with an inverted vial, ensuring sufficient volume of medium to cover the inverted vial at least one-half to two-thirds after sterilization. Close tubes with metal or heat-resistant plastic caps. Autoclave medium at 121°C for 12 to 15 min. Ensure that inverted vials are free of air bubbles. Medium pH should be 7.2 ± 0.2 after sterilization.

b. Procedure: Promptly submit all presumptive tubes or bottles showing growth, any amount of gas, or acidic reaction within 24 ± 2 h of incubation to the confirmed phase. If additional presumptive tubes or bottles show active fermentation or acidic reaction at the end of a 48 ± 3 h incubation period, promptly submit these to the confirmed phase. To confirm presumptive coliform colonies growing on a solid medium using fermentation media, see Section 9222B.4*g*.

Gently shake or rotate presumptive tubes or bottles showing gas or acidic growth to resuspend the organisms. With a sterile loop 3.0 to 3.5 mm in diameter, transfer one or more loopfuls of culture to a fermentation tube containing BGLB broth. Alternatively, insert a sterile wooden applicator at least 2.5 cm into the culture, promptly remove, and plunge applicator to the bottom of fermentation tube containing BGLB broth. Remove and discard applicator. Repeat for all other presumptive-positive tubes. Analysts may simultaneously inoculate BGLB broth for total coliforms and EC broth for thermotolerant (fecal) coliforms (see 9221E) or EC-MUG broth for *Escherichia coli* (see 9221F). However, if using the same loop or wooden applicator stick to inoculate a culture into more than one medium, inoculate the most inhibitory medium (BGLB broth) last.

Promptly incubate the inoculated BGLB broth tubes at 35 ± 0.5°C. Any amount of gas formed in the inverted vial of the BGLB broth fermentation tube at any time within 48 ± 3 h constitutes a positive confirmed phase. To estimate the coliform density, calculate the MPN value from the number of positive BGLB tubes as described in 9221C.

c. Alternative procedure: Use this alternative only for polluted water or wastewater known to produce positive results consistently.

If all presumptive tubes are positive in two or more consecutive dilutions within 24 h, then only submit to the confirmed phase the highest-dilution tubes (smallest sample inoculum) in which all tubes are positive, along with any positive tubes in still higher dilutions. Submit to the confirmed phase all tubes in which gas or acidic growth is produced in 24 to 48 h.

5. Completed Phase

The completed test as described here is not required for drinking-water compliance sample analyses. For nonpotable water samples collected under the Clean Water Act, the requirement that 10% of all total-coliform-positive tubes be subjected to the completed test on a seasonal basis no longer exists. The completed test is included here as a QC recommendation and for use when testing results are uncertain. As additional testing for thermotolerant (fecal) coliforms and/or *E. coli* is required of positive coliform tests, further testing using EC and/or EC-MUG broths is considered a completed test. For QC purposes, if no positive drinking-water samples are received within a quarter, then analyze at least one positive source-water sample to confirm that media respond appropriately.

To verify the presence of coliform bacteria and to provide QC data for nonpotable water-sample analysis, use the completed test on at least one positive sample per quarter. If no positive sample occurs within a quarter, perform a QC check using a known positive sample. Analysts may simultaneously inoculate presumptive-positive media into both BGLB broth for confirmation of total coliforms and EC broth for thermotolerant (fecal) coliforms (9221E) or EC MUG broth for *Escherichia coli* (9221F) as long as BGLB broth is inoculated last. Positive results from incubation in EC and/or EC-MUG broths at elevated temperature (44.5 ± 0.2°C) can be considered a completed test. Parallel positive BGLB broth cultures with negative EC or EC-MUG broth cultures indicate the presence of nonfecal coliforms. Parallel positive EC or EC-MUG tubes and negative BGLB broth cultures indicate the presence of thermotolerant (fecal) coliforms or *E. coli*, respectively. Alternatively, the completed test for positive total coliforms may be performed as follows.

a. Culture media and reagents: Follow the QC guidelines cited in 9221B.2.

1) *LES Endo agar*—See Section 9222B.2*a*. Use 100- × 15-mm Petri plates.

2) *MacConkey agar:*

Peptone	17	g
Proteose peptone	3	g
Lactose	10	g

Bile salts	1.5	g
Sodium chloride (NaCl)	5	g
Agar	13.5	g
Neutral red	0.03	g
Crystal violet	0.001	g
Reagent-grade water	1	L

Add ingredients to water, mix thoroughly, and heat to boiling to dissolve. Sterilize by autoclaving for 15 min at 121°C. Temper agar after sterilization and pour into Petri plates (100 × 15 mm). Medium pH should be 7.1 ± 0.2 after sterilization.

3) *Nutrient agar:*

Peptone	5.0	g
Beef extract	3.0	g
Agar	15.0	g
Reagent-grade water	1	L

Add ingredients to water, mix thoroughly, and heat to dissolve. Before sterilization, dispense in screw-capped tubes. Autoclave at 121°C for 15 min. Medium pH should be 6.8 ± 0.2 after sterilization. After sterilization, immediately place tubes in an inclined position so the agar will solidify with a sloped surface. Tighten screw caps after cooling and store in a protected, cool storage area.

4) *Gram-stain reagents*—Reagents are commercially available as prepared solutions.

a) *Ammonium oxalate-crystal violet (Hucker's)*—Dissolve 2 g crystal violet (90% dye content) in 20 mL 95% ethyl alcohol. **CAUTION:** ***Flammable.*** Dissolve 0.8 g $(NH_4)_2C_2O_4 \cdot H_2O$ in 80 mL reagent-grade water. Mix the two solutions and age for 24 h before use. Filter through paper into a staining bottle.

b) *Lugol's solution, Gram's modification*—Grind 1 g iodine crystals and 2 g KI in a mortar. Add reagent-grade water, a few milliliters at a time, and grind thoroughly after each addition until solution is complete. Rinse solution into an amber glass bottle with the remaining water, using a total of 300 mL.

c) *Counterstain*—Dissolve 2.5 g safranin dye in 100 mL 95% ethyl alcohol. Add 10 mL to 100 mL reagent-grade water. **CAUTION:** ***Flammable.***

d) *Acetone alcohol*—Mix equal volumes of ethyl alcohol (95%) with acetone. **CAUTION:** ***Flammable.***

b. Procedure:

1) Using aseptic technique, streak one LES Endo agar (Section 9222B.2*a*) or MacConkey agar plate from each presumptive-positive tube of BGLB broth as soon as possible after gas is observed. Streak plates in a manner to ensure the presence of some discrete colonies separated by at least 0.5 cm. To obtain a high proportion of successful isolations if coliform organisms are present, use the following approach:

a) Use a sterile 3-mm-diam loop or an inoculating needle slightly curved at the tip;
b) tap and incline the fermentation tube to avoid picking up any membrane or scum on the needle;
c) insert the end of the loop or needle into the liquid in the tube to a depth of approximately 0.5 cm; and
d) streak a plate for isolation with the curved section of the needle in contact with the agar to avoid a scratched or torn surface. Flame the loop between the second and third quadrants to improve colony isolation.

Incubate plates, inverted, at 35 ± 0.5°C for 24 ± 2 h.

2) The colonies developing on LES Endo agar are defined as *typical* (pink to dark red with a green metallic surface sheen) or *atypical* (pink, red, white, or colorless colonies without sheen) after 24 h incubation. Typical lactose-fermenting colonies developing on MacConkey agar are red and may be surrounded by an opaque zone of precipitated bile. From each plate, pick one or more typical, well-isolated coliform colonies or, if no typical colonies are present, pick two or more colonies considered most likely to be coliforms. Transfer growth from each isolate to a single-strength lauryl tryptose broth fermentation tube and onto a nutrient agar slant.

If needed, use a colony-magnifying device to provide optimum magnification when colonies are picked from the LES Endo or MacConkey agar plates. When transferring colonies, choose well-isolated ones and barely touch the colony surface with a flame-sterilized, air-cooled transfer needle to minimize the danger of transferring a mixed culture.

Incubate secondary broth tubes (lauryl tryptose broth with inverted fermentation vials) at 35 ± 0.5°C for 24 ± 2 h; if gas is not produced within 24 ± 2 h, reincubate and examine again at 48 ± 3 h. Microscopically examine Gram-stained preparations from those 24-h nutrient agar slant cultures corresponding to the secondary tubes that show gas.

3) Gram-stain technique—The Gram stain may be omitted from the completed test for potable-water samples only because Gram-positive bacteria and spore-forming organisms in drinking water rarely survive this selective screening procedure.

Various modifications of the Gram stain technique exist. Use Hucker's modification (as follows) for staining smears of pure cultures; include a Gram-positive and a Gram-negative culture as controls.

On one slide, prepare separate light emulsions of the test bacterial growth and positive and negative control cultures using drops of distilled water on the slide. Air-dry, fix by passing slide through a flame, and stain for 1 min with ammonium oxalate-crystal violet solution. Rinse slide in tap water and drain off excess; apply Lugol's solution for 1 min.

Rinse stained slide in tap water. Decolorize for approximately 15 to 30 s with acetone alcohol by holding the slide between the fingers and letting acetone alcohol flow across the stained smear until the solvent flows colorlessly from the slide. Do not overdecolorize. Counterstain with safranin for 15 s, rinse with tap water, blot dry with absorbent paper or air dry, and examine microscopically. Gram-positive organisms are blue; Gram-negative organisms are red. Results are acceptable only when controls have given proper reactions.

c. Interpretation: Formation of gas in the secondary tube of lauryl tryptose broth within 48 ± 3 h and demonstration of Gram-negative, nonspore-forming, rod-shaped bacteria from the agar culture constitute a positive result for the completed test, demonstrating that a member of the coliform group is present.

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9221 C. Estimation of Bacterial Density

1. Precision of the Multiple-Tube Fermentation Test

The multiple-tube fermentation test is not very precise unless many sample portions are examined, so use caution when interpreting the sanitary significance of any single coliform result. Precision improves greatly when several samples from a given sampling point are estimated separately and their geometric mean is calculated.

Although most probable number (MPN) tables and calculations are described for use in the coliform test, they also can be used to determine the MPN of any organism so long as suitable test media are available. Online MPN calculators are available, but until a calculator's accuracy has been verified, confirm its results using an MPN table in this section.

2. Use of Tables to Determine MPN

Record coliform concentration as MPN/100 mL. The MPN values for a variety of positive and negative tube combinations are given in Tables 9221:II, III, and IV. The sample volumes indicated in Tables 9221:II and III are chosen especially for drinking-water examinations. Table 9221:IV illustrates MPN values for combinations of positive and negative results when five 10-mL, five 1.0-mL, and five 0.1-mL sample-portion volumes of nonpotable water are tested. If the sample-portion volumes tested are identical to those found in the tables, then report the value corresponding to appropriate combination of positive and negative results as the MPN/100 mL. However, if the series of decimal dilutions is different, then select the MPN value in Table 9221:IV that corresponds to the combination of positive results and calculate the actual MPN using the following formula:

$$\text{MPN/100 mL} = (\text{Table MPN/100 mL}) \times 10/V$$

where:

V = volume of sample portion at the lowest selected dilution.

If the decimal series[1] includes more than three dilutions, use the following guidelines to select the three most appropriate dilutions and then use Table 9221:IV and the equation above to calculate the MPN. See Table 9221:V, which provides several examples (A–G) of combinations of positives. First, remove the highest dilution (smallest sample volume) if it has all negative tubes and at least one remaining dilution has a negative tube. Next, remove the lowest dilution (largest sample volume) if it has all positive tubes and at least one remaining dilution has a positive tube. According to these guidelines, the three dilutions in Example A are selected by removal of the highest (0.001-mL) and the lowest (10-mL) dilutions.

If the lowest dilution does not have all positive tubes, and several of the highest dilutions have all negative tubes, then remove the highest negative dilutions (Example B).

TABLE 9221:II. MPN INDEX AND 95% CONFIDENCE LIMITS FOR ALL COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN FIVE 20-mL PORTIONS ARE USED

No. of Tubes Giving Positive Reaction Out of 5 (20 mL Each)	MPN Index/ 100 mL	95% Confidence Limits (Exact)	
		Lower	Upper
0	<1.1	–	3.5
1	1.1	0.051	5.4
2	2.6	0.40	8.4
3	4.6	1.0	13
4	8.0	2.1	23
5	>8.0	3.4	–

TABLE 9221:III. MPN INDEX AND 95% CONFIDENCE LIMITS FOR ALL COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN TEN 10-mL PORTIONS ARE USED

No. of Tubes Giving Positive Reaction Out of 10 (10 mL Each)	MPN Index/ 100 mL	95% Confidence Limits (Exact)	
		Lower	Upper
0	<1.1	–	3.4
1	1.1	0.051	5.9
2	2.2	0.37	8.2
3	3.6	0.91	9.7
4	5.1	1.6	13
5	6.9	2.5	15
6	9.2	3.3	19
7	12	4.8	24
8	16	5.8	34
9	23	8.1	53
10	>23	13	–

Table 9221:IV. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes Are Used per Dilution (10 mL, 1.0 mL, 0.1 mL)*

Combination of Positives	MPN Index/100 mL	Confidence Limits		Combination of Positives	MPN Index/100 mL	Confidence Limits	
		Low	High			Low	High
0-0-0	<1.8	—	6.8	4-0-3	25	9.8	70
0-0-1	1.8	0.090	6.8	4-1-0	17	6.0	40
0-1-0	1.8	0.090	6.9	4-1-1	21	6.8	42
0-1-1	3.6	0.70	10	4-1-2	26	9.8	70
0-2-0	3.7	0.70	10	4-1-3	31	10	70
0-2-1	5.5	1.8	15	4-2-0	22	6.8	50
0-3-0	5.6	1.8	15	4-2-1	26	9.8	70
1-0-0	2.0	0.10	10	4-2-2	32	10	70
1-0-1	4.0	0.70	10	4-2-3	38	14	100
1-0-2	6.0	1.8	15	4-3-0	27	9.9	70
1-1-0	4.0	0.71	12	4-3-1	33	10	70
1-1-1	6.1	1.8	15	4-3-2	39	14	100
1-1-2	8.1	3.4	22	4-4-0	34	14	100
1-2-0	6.1	1.8	15	4-4-1	40	14	100
1-2-1	8.2	3.4	22	4-4-2	47	15	120
1-3-0	8.3	3.4	22	4-5-0	41	14	100
1-3-1	10	3.5	22	4-5-1	48	15	120
1-4-0	11	3.5	22	5-0-0	23	6.8	70
2-0-0	4.5	0.79	15	5-0-1	31	10	70
2-0-1	6.8	1.8	15	5-0-2	43	14	100
2-0-2	9.1	3.4	22	5-0-3	58	22	150
2-1-0	6.8	1.8	17	5-1-0	33	10	100
2-1-1	9.2	3.4	22	5-1-1	46	14	120
2-1-2	12	4.1	26	5-1-2	63	22	150
2-2-0	9.3	3.4	22	5-1-3	84	34	220
2-2-1	12	4.1	26	5-2-0	49	15	150
2-2-2	14	5.9	36	5-2-1	70	22	170
2-3-0	12	4.1	26	5-2-2	94	34	230
2-3-1	14	5.9	36	5-2-3	120	36	250
2-4-0	15	5.9	36	5-2-4	150	58	400
3-0-0	7.8	2.1	22	5-3-0	79	22	220
3-0-1	11	3.5	23	5-3-1	110	34	250
3-0-2	13	5.6	35	5-3-2	140	52	400
3-1-0	11	3.5	26	5-3-3	170	70	400
3-1-1	14	5.6	36	5-3-4	210	70	400
3-1-2	17	6.0	36	5-4-0	130	36	400
3-2-0	14	5.7	36	5-4-1	170	58	400
3-2-1	17	6.8	40	5-4-2	220	70	440
3-2-2	20	6.8	40	5-4-3	280	100	710
3-3-0	17	6.8	40	5-4-4	350	100	710
3-3-1	21	6.8	40	5-4-5	430	150	1100
3-3-2	24	9.8	70	5-5-0	240	70	710
3-4-0	21	6.8	40	5-5-1	350	100	1100
3-4-1	24	9.8	70	5-5-2	540	150	1700
3-5-0	25	9.8	70	5-5-3	920	220	2600
4-0-0	13	4.1	35	5-5-4	1600	400	4600
4-0-1	17	5.9	36	5-5-5	>1600	700	—
4-0-2	21	6.8	40				

* Results to two significant figures.

More than three dilutions may remain after removal of the lowest dilution with all positive tubes and high dilutions with all negative tubes. In this case, if the highest dilution with *all* positive tubes is within two dilutions of the highest dilution with *any* positive tubes, then use the highest dilution with *any* positive tubes and the two immediately lower dilutions. In Example C, the highest dilution with all positive tubes is 0.1 mL, which is within two dilutions of 0.001 mL, which has one positive tube. In Example D, the highest dilution with all positive tubes is 0.01 mL, which is within two decimal dilutions of 0.001 mL, to yield a combination of 4-5-1.

If, after removal of the lowest dilution with all positive tubes, no dilution with all positive reactions remains, then select the lowest

Table 9221:V. Examples for Choice of Three Combinations of Positives from Five Dilutions

Example	Volume *mL* 10	1	0.1	0.01	0.001	Combination of Positives	MPN Index *No./100 mL*
A	5	**5**	**1**	**0**	0	x-5-1-0-x	330
B	**4**	**5**	**1**	0	0	4-5-1-x-x	48
C	5	2	**5**	**2**	**1**	x-x-5-2-1	7000
D	4	5	**4**	**5**	**1**	x-x-4-5-1	4800
E	5	**4**	**4**	**0**	**1**	x-4-4-1-x	400
F	**4**	**3**	**0**	**1**	**1**	4-3-2-x-x	39
G	4	3	**3**	**2**	**1**	x-x-3-2-1	1700

two dilutions and assign the sum of any remaining dilutions to the third dilution. In Example E, the highest dilution with all positive tubes contains 10 mL; this dilution was removed in the second step. Four dilutions, none of which have all positive tubes, remain. Under these circumstances, select the two lowest remaining dilutions corresponding to 1 and 0.1 mL sample. For the third dilution, add the number of positive tubes in all higher dilutions (0.01 and 0.001 mL sample), to yield a final combination of 4-4-1.

If no dilution has all positive tubes (Example F), select the lowest two dilutions, corresponding to 10 and 1 mL sample. For the third dilution, add the number of positive tubes in the remaining dilutions (0.1, 0.01, and 0.001 mL sample), to yield a final combination of 4-3-2. If the third dilution is assigned more than five positive tubes, then the selected combination will not be in Table 9221:IV.

If the three dilutions selected are not found in Table 9221:IV, then something in the serial dilution was unusual. In this case, the usual methods for calculating the MPN, presented here, may not apply. If a new sample cannot be collected and an MPN value is still desired, use the highest dilution with at least one positive tube and the two dilutions immediately lower as the three selected dilutions. In Example G, the first selection, 4-3-6 (the outcome from the highest three dilutions), is not in Table 9221:IV because 6 is greater than 5. The second selection, according to the above guidelines, would be 3-2-1. If this second set of selected dilutions is not in Table 9221:IV, then use the following formula to calculate the MPN:

$$-\frac{230.3}{z_s}\log_{10}\left(1-\frac{x_s z_s}{\sum_{j=s}^{K} n_j z_j}\right)$$

where:

z_s = the amount of the original sample inoculated into each tube of the sth dilution, and
x_s = the number of positive tubes in the sth dilution,
K = the number of dilutions,
j = a dilution,
s = the highest dilution with at least one positive tube,
n_j = the number of tubes in the jth dilution, and
z_j = the amount of the original sample inoculated into each tube in the jth dilution.

For example, in the series x-x-3-0-0, where the third dilution level (z_s) equals 0.1 mL, $x_s z_s = 0.3$, and $\Sigma\, n_j z_j = 0.555$. Thus, the calculated MPN = 7800/100 mL.

This formula also applies to serial dilutions having all positive tubes in a single dilution, and can serve as an approximation for outcomes like 5-5-5-0-0-0, where five tubes are used per dilution, by using just the last four dilutions.

Table 9221:IV shows all but the improbable positive tube combinations for a three-dilution series. In testing 10 samples, there is a 99% chance of finding all the results among these 95 outcomes. If untabulated combinations occur with a frequency greater than 1%, it indicates that the technique is faulty or that the statistical assumptions underlying the MPN estimate are not being fulfilled (e.g., growth inhibition at low dilutions).

The MPN for combinations not appearing in the table, or for other combinations of tubes or dilutions, may be *estimated* as follows: First, select the lowest dilution that does not have all positive results. Second, select the highest dilution with at least one positive result. Finally, select all the dilutions between them. For example, from (10/10, 10/10, 4/10, 1/10, 0/10) use only (–, –, 4/10, 1/10, –), corresponding to 4/10 @ 0.1 mL sample/tube and 1/10 @ 0.01 mL sample/tube. Likewise, from (10/10, 10/10, 10/10, 0/10, 0/10), select only (–, –, 10/10, 0/10, –), corresponding to 10/10 @ 0.1 mL sample/tube and 0/10 @ 0.01 mL sample/tube. Use only the selected dilutions in the following formula of Thomas:[1]

$$\text{MPN/100 mL (approx.)} = 100 \times P/(N \times T)^{1/2}$$

where:

P = number of positive results,
N = volume of sample in all the negative portions combined, mL, and
T = total volume of sample in the selected dilutions, mL.

That is, $N = \Sigma(n_j\text{-}x_j)z_j$, $P = \Sigma\, x_j$, and $T = \Sigma\, n_j z_j$, where the summations are over the dilutions selected, and x_j = the number of positive tunes in the jth dilution.

In the first example above,

$$\text{MPN/100 mL (approx.)} = 100 \times 5/(0.69 \times 1.1)^{1/2}$$

$$= 500/0.87 = 570/100 \text{ mL}$$

In the second example above,

$$\text{MPN/100 mL (approx.)} = 100 \times 10/(0.1 \times 1.1)^{1/2}$$

$$= 1000/0.332 = 3000/100 \text{ mL}$$

The two examples compare well with the true MPNs, 590/100 mL and 2400/100 mL, respectively. The second example is a special case for which an exact solution can be calculated directly for the two selected dilutions.

When it is desired to summarize the results from several samples with a single MPN value, use the geometric mean or the median. The geometric mean is calculated by averaging the logarithmic values; for example, the geometric mean of A, B, and C is 10^L where:

$$L = (\log_{10} A + \log_{10} B + \log_{10} C)/3$$

Mean values are reported as the antilog of L.

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9221 D. Presence–Absence (P–A) Coliform Test

The presence–absence (P–A) test for the coliform group is a simple modification of the multiple-tube procedure that is intended for use on routine samples collected from distribution systems or water treatment plants. This simplification using one large test portion (100 mL) in a single culture bottle to determine qualitatively whether coliforms are present or absent is justified on the theory that no coliforms should be present in 100 mL of a drinking water sample. Also, it enables analysts to examine more samples in a given time period compared to quantitative methods. Comparative studies with the membrane-filter procedure indicate that the P–A test may maximize coliform detection in samples containing many organisms that could overgrow coliform colonies and cause detection problems.

The P–A broth contains lactose and a pH indicator to detect the presence of acid production. Analysts observe the culture bottles for gas and/or acid production—the metabolic end products of lactose fermentation. Presumptive-positive coliform results obtained from P–A broth must be confirmed using BGLB broth.

1. Samples

Collect samples as directed in Section 9060, using sample containers specified in Section 9030B.19. Follow the QC guidelines for sample bottles described in Section 9020B.5*d*. Ensure that samples meet laboratory acceptance criteria upon receipt.

2. Presumptive Phase

a. Culture medium:

P-A broth: Follow QC guidelines cited in 9221B.2.

Beef extract	3.0	g
Peptone	5.0	g
Lactose	7.46	g
Tryptose	9.83	g
Dipotassium hydrogen phosphate (K_2HPO_4)	1.35	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.35	g
Sodium chloride (NaCl)	2.46	g
Sodium lauryl sulfate	0.05	g
Bromocresol purple	0.0085	g
Reagent-grade water	1	L

Make this formulation triple strength (3×) when examining 100-mL samples. Dissolve P–A medium in water by stirring (do not use heat). Dispense 50 mL prepared medium into screw-capped 250-mL milk dilution bottles or equivalent containers. A fermentation vial insert is unnecessary. Autoclave for 12 min at 121°C; limit total time in the autoclave to 30 min or less. Medium pH should be 6.8 ± 0.2 after sterilization.

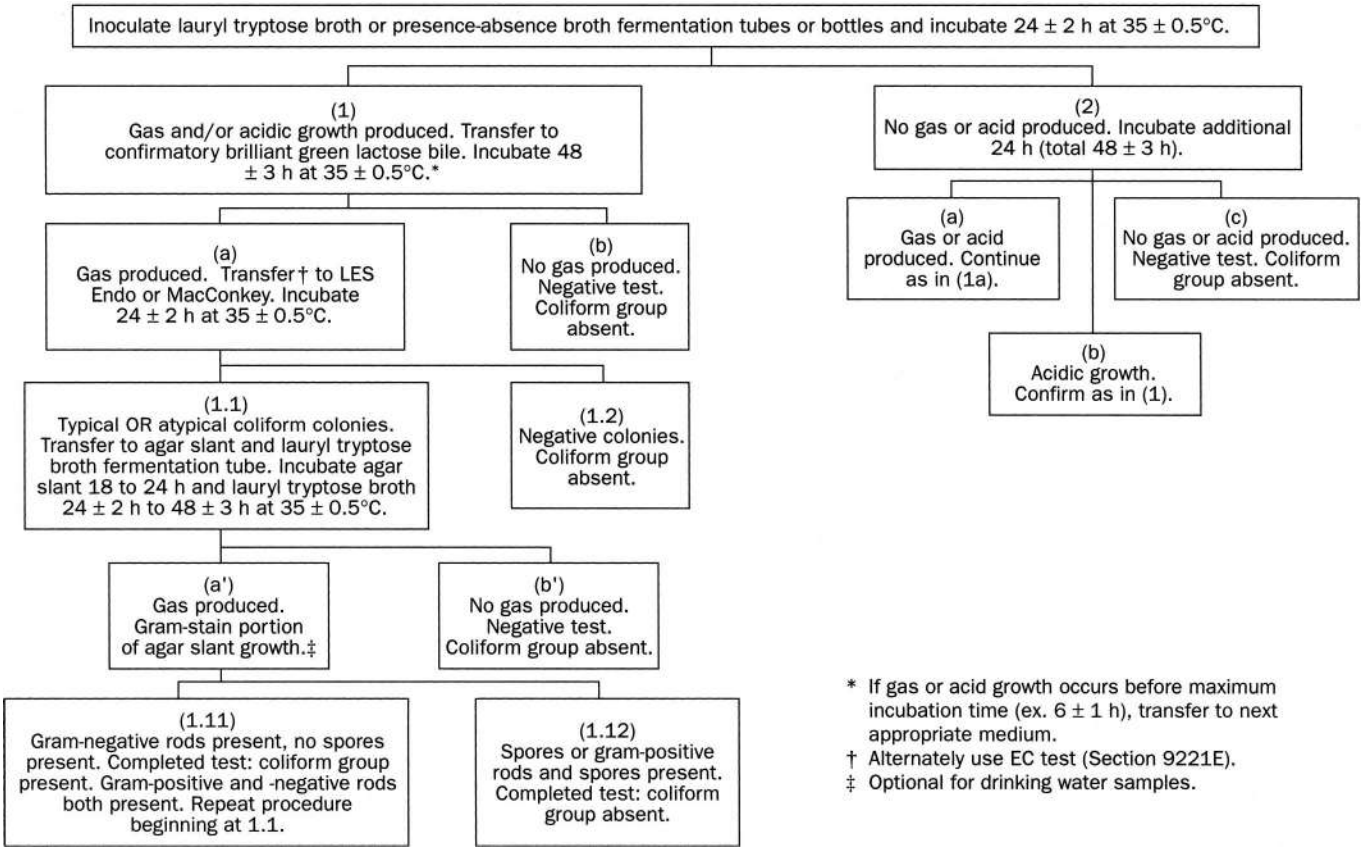


Figure 9221:1. Schematic outline of presumptive, confirmed, and completed phases for total coliform detection.

If sterilized via filtration, a 6× strength P–A medium may be used. Aseptically dispense 20 mL of the 6× medium into a sterile 250-mL dilution bottle or equivalent container.

b. Procedure: Shake sample vigorously for 5 s (approximately 25 times) and inoculate 100 mL into a P–A culture bottle. Mix thoroughly by inverting bottle once or twice to evenly distribute the sample throughout the medium. Incubate at 35 ± 0.5°C and inspect after 24 ± 2 h and 48 ± 3 h for acid reactions.

c. Interpretation: If acidic conditions exist following lactose fermentation, a distinct yellow color will form in the medium. If gas also is being produced, then foaming will occur when the bottle is gently shaken. Any amount of gas and/or acid constitutes a presumptive-positive test that requires confirmation.

3. Confirmed Phase

The confirmed phase is outlined in Figure 9221:1.

a. Culture medium: Use BGLB broth fermentation tubes (see 9221B.4).

b. Procedure: After incubation, promptly use a 3.0- to 3.5-mm-diam sterile loop to transfer one or more loopfuls of culture from a presumptive-positive bottle to a fermentation tube containing BGLB broth. Alternatively, insert a sterile wooden applicator at least 2.5 cm into the culture, promptly remove, and plunge applicator to the bottom of a fermentation tube containing BGLB broth. Remove and discard applicator. Repeat for all other presumptive-positive tubes and inoculate at 35 ± 0.5°C (see 9221B.4).

Loopfuls of culture from presumptive-positive bottles also may be transferred into EC broth [for determination of thermotolerant (fecal) coliforms] and/or EC-MUG broth (for *E. coli* determinations) at the same time, as long as the most inhibitory medium (BGLB broth) is inoculated last.

c. Interpretation: Gas production in the BGLB broth culture within 48 ± 3 h confirms the presence of coliform bacteria. Report result as P–A test positive or negative for total coliforms in 100 mL of sample. Drinking water samples that are positive for total coliforms also must be tested for thermotolerant (fecal) coliforms (9221E) or *E. coli* (9221F).

4. Completed Phase

The completed phase, required for nonpotable water sample analysis, is outlined in 9221B.5 and Figure 9221:1.

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9221 E. Thermotolerant (Fecal) Coliform Procedure

Traditionally called *fecal coliforms,* thermotolerant coliforms (those that ferment lactose to produce gas at 44.5°C) have been documented in organically rich waters or tropical climates in the absence of recent fecal contamination. So when looking for evidence of fecal contamination, testing for *E. coli*—a more specific indicator—is recommended. Nevertheless, regulations may require that thermotolerant (fecal) coliforms be identified and enumerated.

To test for thermotolerant coliforms, use one of the multiple-tube procedures described here or the membrane-filter methods described in Sections 9222D and E. In the multiple-tube fermentation technique, thermotolerant coliforms are identified by their ability to ferment lactose to produce gas at 44.5 ± 0.2°C within 24 ± 2 h.

1. Thermotolerant Coliform Test (EC Medium)

The thermotolerant coliform test using EC medium is applicable to investigations of drinking water, stream pollution, unfiltered raw water sources, wastewater treatment systems, bathing waters, seawaters, and general water-quality monitoring. Do not use EC medium to directly isolate thermotolerant coliforms from water; prior enrichment in a presumptive medium is required for optimum recovery of thermotolerant coliforms. (To test presumptive coliform colonies growing on solid media, refer to Section 9222G.3*c*)

a. EC medium: Prepare EC medium following QC guidelines cited in 9221B.2.

Tryptose or trypticase	20.0 g
Lactose	5.0 g
Bile salts mixture or bile salts No. 3	1.5 g
Dipotassium hydrogen phosphate (K_2HPO_4)	4.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5 g
Sodium chloride (NaCl)	5.0 g
Reagent-grade water	1 L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. Before sterilization, dispense sufficient medium in fermentation tubes with an inverted vial to cover the inverted vial at least one-half to two-thirds after sterilization. Close tubes with metal or heat-resistant plastic caps. Autoclave medium at 121°C for 12 to 15 min. Ensure that inverted vials are free of air bubbles. Medium pH should be 6.9 ± 0.2 after sterilization.

b. Procedure:

1) After incubation, gently shake or rotate fermentation tubes or bottles showing gas, growth, or acidity to resuspend the organisms. Promptly use a sterile 3- to 3.5-mm-diam loop to transfer one or more loopfuls of culture from bottles or tubes showing growth with acid and/or gas production to a fermentation tube containing EC broth. Alternatively, insert a sterile wooden applicator at least 2.5 cm into the culture, promptly remove, and plunge applicator to the bottom of a fermentation tube containing EC broth. Remove and discard applicator. Repeat for all other presumptive-positive tubes and incubate at 44.5 ± 0.2°C.

Simultaneous inoculation into EC broth and/or EC-MUG broth along with BGLB broth is acceptable, if the most inhibitory medium (BGLB broth) is inoculated last.

2) Place all EC tubes into a circulating water bath (preferably with a gabled cover) within 30 min after inoculation. Incubate inoculated EC broth tubes at 44.5 ± 0.2°C for 24 ± 2 h. Maintain a sufficient water depth in the water bath incubator to immerse tubes to the upper level of the medium.

c. Interpretation: Gas production with growth in an EC broth culture within 24 ± 2 h or less is considered a positive thermotolerant (fecal) coliform reaction. Failure to produce gas (with little or no growth) constitutes a negative reaction. If multiple tubes are used, calculate the MPN of thermotolerant coliforms from the number of positive EC broth tubes, as described in 9221C. When using only one tube for subculturing from a single presumptive bottle, report as the presence or absence of thermotolerant coliforms. If heavy growth occurs with no gas production, subject the culture to a thermotolerant coliform or *E. coli* test using a different medium.

2. Thermotolerant (Fecal) Coliform Direct Test (A-1 Medium)

a. A-1 medium: This medium may be used to directly isolate thermotolerant coliforms from unfiltered source water, treated wastewater, and seawater, but not drinking water. Follow guidelines in 9221B.1 for sample collection. Unlike EC medium, A-1 medium does not require prior enrichment in a presumptive medium for optimum recovery of thermotolerant coliforms. Use QC guidelines cited in 9221B.2.

Lactose	5.0 g
Tryptone	20.0 g
Sodium chloride (NaCl)	5.0 g

Salicin	0.5 g
Polyethylene glycol *p*-isooctylphenyl ether*	1.0 mL
Reagent-grade water	1 L

Heat to dissolve solid ingredients, add polyethylene glycol *p*-isooctylphenyl ether, and adjust to pH 6.9 ± 0.1. For 10-mL samples, prepare double-strength medium so the final concentration of ingredients after sample addition is correct. Before sterilization, dispense sufficient medium in fermentation tubes with an inverted vial to cover the inverted vial at least one-half to two-thirds after sterilization. Close with metal or heat-resistant plastic caps. Sterilize by autoclaving at 121°C for 10 min. Ensure that inverted vials are free of air bubbles. Store in the dark at room temperature for not longer than 7 d. Ignore precipitate formed during storage.

b. Procedure: Inoculate tubes of A-1 broth as directed in 9221B.3*b*. Incubate for 3 h at 35 ± 0.5°C. Transfer tubes to a water bath at 44.5 ± 0.2°C and incubate for another 21 ± 2 h.

c. Interpretation: Gas production in any A-1 broth culture within 24 h or less is a positive reaction [i.e., thermotolerant (fecal) coliforms are present]. Calculate the MPN of thermotolerant (fecal) coliforms from the number of positive A-1 broth tubes, as described in 9221C.

* Triton X-100, Sigma-Aldrich, St. Louis, MO, or equivalent.

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9221 F. *Escherichia coli* Procedure Using Fluorogenic Substrate

Escherichia coli is a member of the indigenous fecal flora of warm-blooded animals. The presence of *E. coli* in water is considered a specific indicator of fecal contamination and the possible presence of enteric pathogens. Tests for *E. coli* are applicable to the analysis of drinking, surface, ground, and waste water. Testing for *E. coli* can be performed using the multiple-tube procedure described here, by the membrane filter method described in Section 9222G, or by the chromogenic enzyme substrate tests described in Section 9223. Other *E. coli* procedures are presented in 9221G.

For the *E. coli* test using EC-MUG medium, *E. coli* is defined as the species of coliform bacteria that possesses the enzyme β-glucuronidase, which can cleave the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG), thus releasing the fluorogen within 24 ± 2 h or less when grown in EC-MUG medium at 44.5 ± 0.2°C.

1. *Escherichia coli* Test (EC-MUG Medium)

The use of EC-MUG medium to detect *E. coli* is applicable to investigations of drinking water, stream pollution, unfiltered raw water sources, wastewater treatment systems, bathing waters, seawaters, and general water-quality monitoring. Do not use EC-MUG for the direct isolation of *E. coli*; prior enrichment ina presumptive medium is required for optimum recovery. (To test presumptive coliform colonies growing on solid media, refer to Section 9222G.2.)

Use EC-MUG medium to test for *E. coli* in a total coliform-positive culture, following QC guidelines cited in 9221B.2.

a. EC-MUG medium: Prepare EC-MUG medium following QC guidelines cited in 9221B.2.

Tryptose or trypticase	20.0 g
Lactose	5.0 g
Bile salts mixture or bile salts No. 3.	1.5 g
Dipotassium hydrogen phosphate (K_2HPO_4)	4.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5 g
Sodium chloride (NaCl)	5.0 g
4-Methylumbelliferyl-β-D-glucuronide (MUG)	0.05 g
Reagent-grade water	1 L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. Before sterilization, dispense in tubes that do not fluoresce under long-wavelength (365–366 nm) ultraviolet (UV) light. An inverted tube is not necessary. Close tubes with metal or heat-resistant plastic caps. Medium pH should be 6.9 ± 0.2 after sterilization for 15 min at 121°C.

b. Procedure:

1) Gently shake or rotate fermentation tubes or bottles showing growth, gas, or acidity to resuspend the organisms. Using a sterile 3- or 3.5-mm-diam loop, transfer one or more loopfuls of growth from the fermentation tube or bottle to EC-MUG broth. Alternatively, insert a sterile wooden applicator stick at least 2.5 cm into the culture, promptly remove, and plunge applicator to the bottom of a fermentation tube containing EC-MUG broth.

2) Place all EC-MUG tubes in water bath within 30 min after inoculation. Incubate inoculated EC-MUG tubes and negative controls for 24 ± 2 h in a circulating water bath (preferably with a gable cover) maintained at 44.5 ± 0.2°C. Maintain a sufficient

water depth in the water-bath incubator to immerse tubes to the upper level of medium.

c. Interpretation: Examine all tubes exhibiting growth for fluorescence using a 6W, 365–366 nm long-wavelength UV lamp. The presence of bright blue fluorescence is considered a positive result for *E. coli*. Growth in the absence of bright blue fluorescence is considered a negative result. To help interpret results and avoid misidentifying weak autofluorescence of the medium or glass tubes as a positive response, include in the assay a positive control [a known *E. coli* (MUG-positive) culture], a negative control [a thermotolerant *Klebsiella pneumoniae* (MUG-negative) culture], and an uninoculated medium control. The distance between the UV lamp and the tubes should be such that the *E. coli* positive control shows distinct fluorescence while the MUG-negative and uninoculated controls do not. If using multiple tubes, calculate the MPN for *E. coli* from the number of positive EC-MUG broth tubes, as described in 9221C. When using only one tube, or subculturing from a single presumptive bottle or colony, report as the presence or absence of *E. coli*.

2. Simultaneous Determination of Thermotolerant Coliforms and *E. coli*

The presence of thermotolerant coliforms and *E. coli* can be determined simultaneously by including an inverted vial (Durham tube) in tubes of EC-MUG broth. Prepare EC-MUG broth according to 9221F.1.

a. Setup: Before sterilization dispense, in fermentation tubes with an inverted vial, sufficient medium to cover the inverted vial at least one-half to two-thirds after sterilization. Close with metal or heat-resistant caps. Medium pH should be 6.9 ± 0.2 after sterilization for 15 min at 121°C.

b. Procedure:

1) Gently shake or rotate fermentation tubes or bottles showing growth, gas, or acidity to resuspend the organisms. Using a sterile 3- or 3.5-mm-diam loop, transfer one or more loopfuls of growth from the fermentation tube or bottle to EC-MUG broth. Alternatively, insert a sterile wooden applicator stick at least 2.5 cm into the culture, promptly remove, and plunge applicator to the bottom of a fermentation tube containing EC-MUG broth.

2) Place all EC-MUG tubes in water bath within 30 min after inoculation. Incubate inoculated EC-MUG tubes, along with positive and negative controls, for 24 ± 2 h in a circulating water bath (preferably with a gable cover) maintained at 44.5 ± 0.2°C. Maintain a sufficient water depth in the water-bath incubator to immerse tubes to the upper level of medium.

c. Interpretation: Examine all tubes exhibiting growth and/or gas for fluorescence using a 6W, 365–366 nm long-wavelength UV lamp. Growth with gas production is considered a positive result for thermotolerant coliforms. The presence of bright blue fluorescence is considered a positive result for *E. coli*. Tubes with growth and/or gas *and* fluorescence are considered positive for both thermotolerant coliforms and *E. coli*. Tubes with growth and/or gas but without bright blue fluorescence are considered positive for thermotolerant coliforms and negative for *E. coli.*

Due to indigenous autofluorescence of media or glass tubes/inserts, use caution in interpreting results. To help interpret results, include in each assay a positive control [a known *E. coli* (MUG-positive) culture], a negative control [a thermotolerant *Klebsiella pneumoniae* (MUG-negative) culture], and an uninoculated medium control. The distance between the UV lamp and the tubes should be such that the *E. coli* positive control shows distinct fluorescence while the MUG-negative and uninoculated controls do not. If multiple tubes are used, calculate the MPN for *E. coli* and thermotolerant coliforms from the number of positive EC-MUG broth tubes, as described in 9221C. When using only one tube, or subculturing from a single presumptive bottle or colony, report the presence or absence of *E. coli* and thermotolerant coliforms.

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9221 G. Other *Escherichia coli* Procedures

For the *E. coli* test using the GAD reagent, *E. coli* is defined as the species of coliform bacteria that possesses the enzyme glutamate decarboxylase (GAD), which can produce an alkaline reaction within 4 h in a reagent containing glutamic acid and a lytic agent. This procedure is used to test for *E. coli* after prior enrichment in a medium used to identify coliform bacteria. The procedure is particularly useful for determining the presence of MUG-negative strains of *E. coli,* some of which are pathogenic (see also Section 9260F).

1. *Escherichia coli* Test (GAD Procedure)

Use the GAD procedure to test for *E.* coli in a total coliform-positive culture following the QC guidelines cited in 9221B.2.

a. GAD reagent:

L-Glutamic acid	1.0 g
Sodium chloride (NaCl)	90.0 g
Bromocresol green	0.05 g

Polyethylene glycol octylphenyl ether*	3.0	mL
Reagent-grade water	1	L

Add ingredients to water and mix thoroughly until all ingredients are dissolved. The pH should be 3.4 ± 0.2. The reagent is stable for 2 months when stored at 5°C. It can be filter-sterilized (0.2-μm filter) and treated as a sterile solution.

b. Procedure:

1) Gently shake or rotate presumptive tubes or bottles showing growth, gas, or acidity. Using a graduated pipet, transfer 5 mL broth from the fermentation tube or bottle to 15-mL centrifuge tube.

2) Concentrate the bacterial cells by centrifuging the broth at 2500 to 3000 × *g* for 10 min. Discard supernatant and resuspend cells in 5 mL phosphate buffer. Reconcentrate cells by centrifugation at 2500 to 3000 × *g* for 10 min. Discard supernatant and add 1.0 mL GAD reagent. Vigorously swirl tube to resuspend cells in GAD reagent.

3) Incubate tubes at 35°C and observe after 1 h. Tubes may be incubated for a maximum of 4 h.

c. Interpretation: Examine all tubes for a distinct color change from yellow to blue, which is considered a positive result for *E. coli*. To assist in interpreting results, incorporate in the assay a positive control [a known *E. coli* (GAD-positive) culture], a negative control [a known total coliform organism, such as *Enterobacter cloacae* (GAD-negative)], and an uninoculated GAD reagent control. If multiple tubes are used, calculate the MPN for *E. coli* from the number of positive GAD tubes, as described in 9221C. When using only one tube or presumptive bottle, report as presence or absence of *E. coli*.

2. *Escherichia coli* Test (Indole Production)

For the purposes of this test, *E. coli* is defined as the species of coliform bacteria that can produce indole within 24 ± 2 h when grown in tryptone water at 44.5 ± 0.2°C. There are exceptions: *Klebsiella oxytoca* and some strains of *C. freundii* and *Enterobacter* spp. are also indole positive. Use tryptone water and Kovac's reagent to test for *E. coli* in a total coliform-positive culture.

a. Reagents: Prepare tryptone water and Kovac's reagent following the guidelines cited in 9221B.2.

1) *Tryptone water:*

Tryptone	20	g
Sodium chloride (NaCl)	5	g
Reagent-grade water	1	L

Add ingredients to water and mix thoroughly until dissolved. Adjust pH to 7.5. Dispense 5-mL portions into tubes, cap, and sterilize for 10 min at 121°C.

2) *Kovac's reagent:*

p-Dimethylaminobenzaldehyde	5	g
Amyl alcohol (analytical grade)	75	mL
Hydrochloric acid, conc.	25	mL

Dissolve aldehyde in alcohol. Cautiously add acid to aldehyde-alcohol mixture and swirl to mix. Store in the dark at 4°C. **CAUTION:** ***Reagent is corrosive and flammable.*** This reagent should be pale yellow to light brown in color. Use of low-quality amyl alcohol may produce a dark-colored reagent; do not use such a reagent.

b. Procedure: Gently shake or rotate presumptive tubes or bottles showing growth, gas, or acidity. Using a sterile 3- or 3.5-mm-diam metal loop or sterile wooden applicator stick, transfer growth from presumptive fermentation tube or bottle to a tube containing 5 mL tryptone water. Incubate inoculated tryptone water tubes in a water bath or incubator maintained at 44.5 ± 0.2°C for 24 ± 2 h. After incubation, add 0.2 to 0.3 mL Kovac's reagent to each tube of tryptone water.

c. Interpretation: Examine all tubes for the appearance of a deep red color in the upper layer, which is considered a positive result for *E. coli*. To assist in interpretation of results, incorporate into the assay a positive control [a known *E. coli* (indole-positive) culture], a negative control [a known total coliform organism, such as *Enterobacter cloacae* (indole-negative)], and an uninoculated reagent control. If multiple tubes are used, calculate the MPN for *E. coli* from the number of indole-positive tubes, as described in 9221C. When using only one tube or presumptive bottle, report as presence or absence of *E. coli*.

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* Triton X-100, Sigma-Aldrich, St. Louis, MO, or equivalent.

9222 MEMBRANE FILTER TECHNIQUE FOR MEMBERS OF THE COLIFORM GROUP*

9222 A. Introduction

The membrane filter (MF) technique is reproducible, can be used to test relatively large sample volumes, and usually yields numerical results more rapidly than the multiple-tube fermentation procedure. It is useful in monitoring drinking water and various natural waters. However, the MF technique has limitations, particularly when testing waters with high turbidity or large numbers of noncoliform (background) bacteria. If heterotrophic bacteria interference occurs, for example, sample results may need to be invalidated and new samples collected.

If a laboratory has not used the MF technique before, analysts should conduct parallel tests with the lab's current method to demonstrate applicability and comparability. Many coliform performance studies have been reported in the literature; the rates of false-positive and -negative results can differ among various coliform media, so users should carefully select the medium and procedure that best fit their needs.

1. Terminology

In the MF technique, the *coliform group* is defined as facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that develop colonies with distinctive characteristics on specific media. When purified cultures of coliform bacteria are tested, they produce negative cytochrome oxidase and positive β-galactosidase test reactions. Details of these coliform characteristics are given below for the standard total coliform MF procedure (9222B) (¶ *a* below) and for two procedures for detecting total coliforms and *E. coli* simultaneously (9222J and K) (¶s *b* and *c* below).

a. Endo-type agar medium (Endo agar LES or Endo broth MF): In this procedure, *coliform bacteria* are defined as bacteria that develop red colonies with a metallic (golden-green) sheen within 24 h at 35°C on an Endo-type medium containing lactose. Some members of the total coliform group also produce dark red, mucoid, or nucleated colonies without a metallic sheen; when verified, these are classified as *atypical coliform colonies*. Generally, pink (non-mucoid), blue, white, or colorless colonies lacking sheen on Endo media are considered noncoliforms in this technique.

b. Dual-chromogen m-ColiBlue24 medium: This differential membrane filter medium simultaneously detects and enumerates both total coliforms (TC) and *Escherichia coli* (*E. coli*) in water samples in 24 h based on their specific enzyme activities. Coliform bacteria (other than *E. coli*) are defined as those that produce red colonies within 24 h at 35°C on this medium, which contains lactose and a nonselective dye [2,3,5-triphenyltetrazolium chloride (TTC)]. *E. coli* are distinguished from other coliform bacteria by blue to purple colonies from the action of ß-glucuronidase enzyme on 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (BCIG), also present in the medium.

c. Fluorogen/chromogen MI medium: This differential membrane filter medium simultaneously detects and enumerates both total coliforms (TC) and *Escherichia coli* (*E. coli*) in water samples in 24 h based on their specific enzyme activities. The medium includes two enzyme substrates—the fluorogen 4-methylumbelliferyl-β-D-galactopyranoside (MUGal) and the chromogen indoxyl-β-D-glucuronide (IBDG) to detect the enzymes β-galactosidase and β-glucuronidase produced by TC and *E. coli*, respectively. In this procedure, *coliform bacteria* are defined as bacteria that produce fluorescent colonies within 24 h when exposed to long-wave ultraviolet (UV) light (365–366 nm) at 35°C, while *E. coli* colonies appear blue on this medium.

2. Applications

The MF technique may be used to test drinking, surface, ground, swimming pool, and marine waters. Do not use it to test primary wastewater-treatment effluent unless the sample is diluted, because the high turbidity level may clog the membrane filter before sufficient sample has been filtered. Chlorinated effluents should have low counts and turbidity. Also, do not use the MF technique to test wastewater containing high levels of toxic metals or toxic organic compounds (e.g., phenols) because the filter may concentrate such substances, thereby inhibiting coliform growth. For non-wastewater samples, high turbidity levels may clog the filter and high heterotrophic-bacteria concentrations may interfere with coliform growth on the filter, possibly requiring the use of multiple filters per sample and/or various sample dilutions. To detect stressed total coliforms in treated drinking water and chlorinated secondary or tertiary wastewater-treatment effluents, use a method designed for stressed organism recovery (see Section 9212B.1). A modified MF technique for thermotolerant coliforms in chlorinated wastewater (Section 9212) may be used if 3 months of parallel testing with the multiple-tube fermentation technique shows comparability for each site-specific type of sample.

The standard volume to be filtered is 100 mL for drinking-water samples; this may be distributed among multiple membranes, if necessary. However, for special monitoring purposes (e.g., troubleshooting water-quality problems or identifying coliform breakthrough in low concentrations from treatment barriers), it may be desirable to test 1-L samples. If particulates prevent one filter from processing a 1-L sample, divide sample into four 250-mL portions for analysis. Total the coliform counts on each membrane to report the number of coliforms per liter. Samples other than drinking water should be analyzed using multiple dilution levels. Recommended dilutions for total coliform measurements are presented in Table 9222:I and for thermotolerant coliform and *E. coli* in Table 9222:IV.

Statistical comparisons of results obtained by the multiple-tube method and the MF technique show that MF is more precise

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(compare Tables 9221:III and IV with Table 9222:III). Data from each test yield approximately the same water-quality information, although numerical results are not identical.

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9222 B. Standard Total Coliform Membrane Filter Procedure using Endo Media

This method may be used to measure total coliforms in drinking, nonpotable, and other waters using Endo-type media. Typical colonies grown on Endo-type media can be further partitioned to differentiate thermotolerant (fecal) coliforms and *E.coli* using the partitioning methods in 9222G, H, and I. This method may not be appropriate for samples high in particulates that may plug filters or samples with a high proportion of total coliforms relative to *E.coli*. For simultaneous measurement of total coliforms and *E.coli* using membrane filtration, refer to Methods 9222J and K.

1. Laboratory Apparatus

For MF analyses, use glassware and other apparatus composed of material free from agents that may affect bacterial growth.

a. Sample bottles: See Section 9030B.19.

b. Dilution bottles: See Section 9030B.13.

c. Pipets and graduated cylinders: See Section 9030B.9. Before sterilization, loosely cover opening of graduated cylinders with metal foil or a suitable heavy wrapping-paper substitute. Immediately after sterilization, secure cover to prevent contamination.

d. Containers for culture medium: Use clean borosilicate glass flasks. Any size or shape of flask may be used, but Erlenmeyer flasks with metal caps, metal foil covers, or screw caps provide for adequate mixing of the medium within and are convenient for storage.

e. Culture dishes: Use sterile borosilicate glass or disposable, presterilized plastic Petri dishes, 15- × 60-mm, 9- × 50-mm, or other appropriate size. Wrap convenient numbers of clean, glass culture dishes in metal foil if sterilized via dry heat, or suitable heavy wrapping paper when autoclaved. Incubate loose-lidded glass and disposable plastic culture dishes in tightly closed containers to prevent moisture evaporation with resultant drying of medium and to maintain a humid environment for optimum colony development.

Presterilized disposable plastic dishes with tight-fitting lids that meet the specifications above are available commercially and used widely. Reseal opened packages of disposable dish supplies for storage.

f. Filtration units: The filter-holding assembly (constructed of glass, autoclavable plastic, porcelain, stainless steel, or disposable plastic) consists of a seamless funnel fastened to a base via a locking device or magnetic force. The design should permit the membrane filter to be held securely on the receptacle's porous plate without mechanical damage and allow all fluid to pass through the membrane during filtration. Discard plastic funnels with deep scratches on the inner surface or glass funnels with chipped surfaces. Replace damaged screens on stainless steel units.

Wrap the assembly (as a whole or separate parts) in heavy wrapping paper or aluminum foil, or place in commercial autoclave bags; sterilize via autoclaving; and store until use. Field units may be sanitized by dipping or spraying with alcohol and then igniting or immersing in boiling water for 2 min. Use reagent water to avoid hard-water deposits (see Section 9020B.4*d* for reagent-grade water-quality specifications). After submerging unit in boiling water, cool to room temperature before reuse. Do not ignite plastic parts. Sterile, disposable field units also may be used.

For filtration, mount receptacle of filter-holding assembly on a 1-L filtering flask with a side arm or other suitable device (manifold to hold three to six filter assemblies) such that a pressure differential (34 to 51 kPa) can be exerted on the filter membrane. Connect flask to a vacuum line, an electric vacuum pump, a filter pump operating on water pressure, a hand aspirator, or other means of securing a pressure differential (138 to 207 kPa). Connect a flask of approxi-

mately the same capacity between filtering flask and vacuum source to trap carry-over water.

g. Membrane filters: Use membrane filters with a pore diameter rated to completely retain coliform bacteria (usually 0.45 μm) (for additional specifications, see Section 9020B.5*i*). Only use filter membranes that have been found—through adequate quality control (QC) testing and manufacturer certification—to exhibit the following:

- full retention of the organisms to be cultivated,
- stability in use,
- freedom from chemical extractables that may inhibit bacterial growth and development,
- a satisfactory filtration speed (within 5 min),
- no significant influence on medium pH (beyond ±0.2 units), and
- no increase in the number of confluent colonies or spreaders compared to control membrane filters.

Use membranes grid-marked so bacterial growth is neither inhibited nor stimulated along the grid lines when membranes with entrapped bacteria are incubated on a suitable medium. Preferably use fresh stocks of membrane filters and, if necessary, store them in an environment without temperature and humidity extremes. Obtain no more than a year's supply at any one time.

Preferably use presterilized membrane filters for which the manufacturer has certified that the sterilization technique has neither induced toxicity nor altered the membrane's chemical or physical properties. If membranes are sterilized in the laboratory, autoclave for 10 min at 121–124°C and then let the steam escape rapidly to minimize condensation on filters.

h. Absorbent pads: Use disks of filter paper or other material that the manufacturer has certified, by lot, to be high quality and free of sulfite or other toxic agents at a concentration that could inhibit bacterial growth. Use pads approximately 48 mm in diameter and thick enough to absorb 1.8 to 2.2 mL of medium. Some pads may require 3.0 mL of medium. Sterilize pads in resealable kraft envelopes, or separately in other suitable containers for 15 min in autoclave (dry cycle). Dry pads so they are free of visible moisture before use. See membrane-filter sterilization procedure in ¶ *g* above.

i. Forceps: Use smooth blunt forceps, without corrugations on the inner sides of the tips. Sterilize before use by dipping in 95% ethyl or absolute methyl alcohol and flaming.

j. Incubators: Use incubators to provide a temperature of 35 ± 0.5°C. To avoid excessive drying, maintain a humid environment for the plates during incubation by either using a humidified incubator (between 60 and 90% relative humidity) or placing plates in a sealed container with tight-fitting lid (or sealed bag). The plates should not lose more than 15% of agar weight during incubation.

k. Microscope and light source: To determine colony counts on membrane filters, use 10 to 15× magnification and a cool white fluorescent light source adjusted to give maximum sheen discernment. Optimally, use a binocular wide-field dissecting microscope. Do not use a microscope with an illuminator that concentrates light from an incandescent source when discerning coliform colonies on Endo-type media.

2. Materials and Culture Media

Because test results need to be uniform, use commercial dehydrated media; never prepare media from basic ingredients when suitable dehydrated media are available. Follow manufacturer's directions for rehydration. Store opened supplies of dehydrated media in a desiccator (if necessary). Commercial liquid media (sterile ampule, etc.) may be used if known to give equivalent results. See Section 9020B.5*j*. Test each new medium lot to confirm that performance is satisfactory (see Section 9020B.5*j*). The use of control charts is helpful to identify trends and ensure long-term consistency in media performance. With each new lot of Endo-type medium, verify a minimum 10% of coliform colonies (obtained from natural samples or samples with known additions) to establish the lot's comparative recovery.

Before use, test each batch of laboratory-prepared MF medium for performance with positive and negative culture controls. If commercially prepared medium is unavailable, prepare from individual components as described in ¶s *a* and *b* below.

a. Endo agar LES (Lawrence Experimental Station formulation):

Yeast extract	1.2	g
Casitone or trypticase	3.7	g
Thiopeptone or thiotone	3.7	g
Tryptose	7.5	g
Lactose	9.4	g
Dipotassium hydrogen phosphate (K_2HPO_4)	3.3	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.0	g
Sodium chloride (NaCl)	3.7	g
Sodium desoxycholate	0.1	g
Sodium lauryl sulfate	0.05	g
Sodium sulfite (Na_2SO_3)	1.6	g
Basic fuchsin	0.8	g
Agar	15.0	g
Reagent-grade water	1	L

CAUTION: Basic fuchsin is a suspected carcinogen and mutagen. Avoid skin contact, ingestion, and exposure to mucous membrane. Follow manufacturer's and safety data sheet (SDS) instructions.

Rehydrate product according to manufacturer's directions or individual components in 1 L water containing 20 mL 95% ethanol. Do not use denatured ethanol, which reduces background growth and coliform colony size. Bring to near boiling to dissolve agar, promptly remove from heat, and cool to between 45 and 50°C. Do not sterilize by autoclaving. Final pH should be 7.2 ± 0.2. A precipitate is normal in Endo-type media. Dispense in 5- to 7-mL quantities into lower section of 60-mm glass Petri dishes or 4- to 6-mL quantities into lower section of 50-mm plastic Petri dishes and allow to solidify. If dishes of any other size are used, adjust quantity to give an equivalent depth of 4 to 5 mm. Do not expose poured plates to direct sunlight; refrigerate in the dark, preferably in sealed plastic bags or other containers to reduce moisture loss. Discard unused medium after 2 weeks [or sooner if there is evidence of moisture loss, medium contamination, medium deterioration (darkening of medium), or surface sheen formation].

*b. m-Endo medium:**

Tryptose or polypeptone	10.0	g
Thiopeptone or thiotone	5.0	g
Casitone or trypticase	5.0	g
Yeast extract	1.5	g
Lactose	12.5	g
Sodium chloride (NaCl)	5.0	g
Dipotassium hydrogen phosphate (K_2HPO_4)	4.375	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.375	g
Sodium lauryl sulfate	0.05	g
Sodium desoxycholate	0.10	g
Sodium sulfite (Na_2SO_3)	2.10	g
Basic fuchsin	1.05	g
Agar (optional)	15.0	g
Reagent-grade water	1	L

CAUTION: Basic fuchsin is a suspected carcinogen and mutagen. Avoid skin contact, ingestion, or exposure to mucous membranes. Follow manufacturer's and SDS instructions.

1) Agar preparation—Rehydrate product in 1 L water containing 20 mL 95% ethanol. Do not use denatured ethanol, which reduces background growth and coliform colony size. Do not sterilize by autoclaving. Heat to near boiling to dissolve agar, promptly remove from heat, and cool to between 45 and 50°C. Dispense 5- to 7-mL quantities into 60-mm sterile glass or 4- to 6-mL quantities into 50-mm plastic Petri dishes. If dishes of any other size are used, adjust quantity to give an equivalent depth. Final pH should be 7.2 ± 0.2. A precipitate is normal in Endo-type media.

Refrigerate finished medium in the dark, and discard unused agar after 2 weeks [or sooner if there is evidence of moisture loss, medium contamination, medium deterioration (darkening of medium), or surface sheen formation].

2) Broth preparation—Prepare as above, omitting agar. Dispense liquid medium (at least 2.0 mL per plate) onto sterile absorbent pads (see 9222B.1*h*) and carefully remove excess medium by decanting plate. The broth may have a precipitate but this does not interfere with medium performance if pads are certified free of sulfite or other toxic agents at concentrations that could inhibit bacterial growth. Refrigerated broth in screw-capped bottles or flasks may be stored for up to 96 h.

c. Buffered dilution rinse water: See Section 9050C.1.

3. Samples

Collect samples as directed in Section 9060A.

4. Procedures

a. Selection of sample size: Sample size will be governed by expected bacterial density, degree of turbidity and, if applicable, regulatory requirements. (See Table 9222:I for suggested sample volumes.)

An ideal sample volume will yield 20 to 80 total coliform colonies and ≤200 colonies of all types (typical, atypical, and noncoliform background colonies) on a membrane-filter surface (Table 9222:II). Analyze drinking waters by filtering 100 mL or replicates of smaller sample volumes (e.g., duplicate 50-mL portions or four replicates of 25-mL portions). Analyze other waters by filtering three different volumes (diluted or undiluted), depending on the expected bacterial density. (See Section 9215B.2 for preparation of dilutions.) When filtering <10 mL of sample (diluted or undiluted), add approximately 10 mL sterile buffered dilution water to the funnel and then add sample followed by another 25 to 50 mL dilution water before filtration or pipet the sample volume into sterile dilution water and then filter the entire contents of dilution bottle. This increase in water volume helps disperse the bacterial suspension uniformly over the entire effective filtering surface.

Table 9222:I. Suggested Sample Volumes for Membrane Filter Total Coliform Test

	Volume (X) To Be Filtered *mL*							
Water Source	100	50	10	1	0.1	0.01	0.001	0.0001
Drinking water	X							
Swimming pools	X							
Wells, springs	X	X	X					
Lakes, reservoirs	X	X	X					
Water supply intake			X	X	X			
Bathing beaches			X	X	X			
River water				X	X	X	X	
Chlorinated sewage				X	X	X		
Raw sewage					X	X	X	X

Table 9222:II. Numbers of Colonies in the Ideal Range for Quantitative Determinations

	Colony Counting Range	
Test	Minimum	Maximum
Total coliform	20	80
Fecal coliform	20	60
Fecal streptococci	20	100
Enterococci	20	60
E. coli	20	80

b. Sterile filtration units and quality control: Use sterile filtration units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is interrupted when an interval of 30 min or longer elapses between sample filtrations. After such interruption, treat any further sample filtration as a new filtration series and sterilize all membrane filter holders in use. (See 9222B.1*f* for sterilization procedures and Sections 9020B.4*l* and *m* for UV cleaning and safety guidelines.)

c. Filtration of sample: Using sterile forceps, place a sterile membrane filter (grid side up) over porous plate of the base. Carefully place matched funnel unit over base (lock it in place, if applicable). Thoroughly mix sample or dilution(s) of sample by vigorously shaking (e.g., 25 times up and down in a 1 ft arc in 7 s) to break up clumps of bacteria, which is crucial for a microbial quantitative method. If sample bottle lacks enough headspace for adequate mixing, pour sample into a larger sterile vessel to mix appropriately. Filter sample under partial vacuum (commonly used pressure: 81 kPa, 24 in. Hg, or 79% vacuum).

* Dehydrated Difco m-Endo Broth MF (No. 274920), or equivalent.

With filter still in place, rinse interior surface of funnel by filtering three 20- to 30-mL portions of sterile buffered dilution water from a squeeze bottle (or other appropriate device). This is satisfactory only if squeeze bottle and its contents do not become contaminated during use. Do not reuse partially filled dilution water bottles. Rinsing between samples prevents carryover contamination.

When final rinse and filtration are complete, use aseptic technique to disengage vacuum, unlock and remove funnel, immediately remove membrane filter with sterile forceps, and roll filter onto selected medium to avoid entrapping air. Incorrect filter placement is instantly obvious because patches of unstained membrane indicate entrapped air. If such patches occur, carefully reseat filter on agar surface. Place only one membrane filter per dish. Invert dish, and incubate at 35 ± 0.5°C for 22 to 24 h for m-Endo LES or m-Endo MF. Optionally, to sanitize funnels between samples after filter removal, expose all surfaces of previously cleaned and sterilized assembly to UV radiation for 2 min before reusing units for successive filtrations.

If using broth, aseptically place a sterile pad in the culture dish, saturate it with at least 2.0 to 3.0 mL of medium (depending on pad manufacturer), and carefully remove excess medium by gently decanting it from dish into a disposable terry towel or empty dish. Place sample filter directly on pad, invert dish, and incubate as specified above. If loose-lidded dishes are used, place them in a humid chamber (or humidified incubator).

Differentiation of some colonies may be lost if cultures are incubated >24 h.

For nonpotable water samples, funnels should be rinsed or sanitized with UV after filter removal or after each sample (as described above) because of the high number of coliform bacteria and background flora present in these samples.

d. QC samples: Check for sterility and coliform contamination at the beginning and end of each filtration series, respectively, by filtering 20 to 30 mL of dilution or rinse water through the filter (one funnel per sterilization batch). If controls indicate contamination, reject all data from affected samples and request new samples. Additionally, to check for possible cross-contamination or contaminated rinse water, insert a sterile rinse-water sample (100 mL) after filtration of 10 samples. Incubate these QC samples under the same conditions as the samples being analyzed.

e. Alternative enrichment technique: Use this technique with m-Endo LES media only. Place a sterile absorbent pad in the lid of a sterile culture dish, and pipet at least 2.0 mL lauryl tryptose broth (prepared as directed in Section 9221B.3*a*) to saturate pad. Carefully remove any excess liquid from absorbent pad by decanting plate. Aseptically place filter—through which the sample has been passed—on the pad. Incubate filter, without inverting dish, for 1.5 to 2 h at 35 ± 0.5°C in an atmosphere of at least 60% relative humidity.

Remove enrichment culture from incubator, lift filter from enrichment pad, and roll it onto the m-Endo LES agar surface, which has been allowed to equilibrate to room temperature. Incorrect filter placement is instantly obvious because patches of unstained membrane indicate entrapped air. If such patches occur, carefully reseat filter on agar surface. If broth medium is used, prepare final culture by removing enrichment culture from incubator and separating the dish halves. Place a fresh sterile pad in dish bottom, saturate with at least 2.0 mL of m-Endo medium, and carefully remove excess liquid from absorbent pad by decanting plate. Transfer filter, with same precautions as above, to new pad. Discard used enrichment pad.

With either agar or broth medium and using this two-step procedure, invert dish and incubate for 22 ± 2 h at 35 ± 0.5°C. Proceed to ¶ *f* below.

f. Counting: To count colony-forming units (CFU) on Endo-type membrane filters, use a low-power (10 to 15× magnification) binocular wide-field dissecting microscope or other optical device, with a cool white fluorescent light source directed to provide optimal viewing of sheen. The angle of light on the colony affects sheen detection for coliform colonies growing on m-Endo plates. Rocking and turning the Petri plate reflects light at different angles and helps detect sheen on the colony. The typical coliform colony on Endo-type media has a pink to dark-red color with a metallic surface sheen. Count both typical and atypical coliform colonies promptly after incubation. The sheen area may vary in size from a small pinhead to complete coverage of the colony surface. Atypical coliform colonies can be dark red, mucoid, or nucleated without sheen. Generally pink, blue, white, or colorless colonies lacking sheen are considered noncoliforms. A high count of noncoliform colonies (>200 CFU) may interfere with the maximum development of coliforms. Refrigerating cultures with high densities of noncoliform colonies (after incubation) for 0.5 to 1 h before counting may aid sheen discernment. Samples of disinfected water or wastewater effluent may include stressed organisms that grow relatively slowly and produce maximum sheen in 22 to 24 h.

g. Coliform verification: Occasionally on Endo-type media, noncoliform organisms may produce typical sheen colonies, and atypical colonies (pink, dark red, or nucleated colonies without sheen) may be coliforms; thus verification of all typical and atypical colony types is recommended. Verify all colonies on Endo media by swabbing the entire membrane or picking at least five typical colonies and five atypical colonies from a given membrane filter culture. (See Section 9020B.9.) Based on need and sample type, laboratories may incorporate more stringent QC measures (e.g., for nonpotable samples, verify at least one colony from each typical or atypical colony type from a given membrane filter culture, or verify 10% of positive samples). Adjust counts based on verification results. Verification tests are listed below.

1) Lactose fermentation—Transfer growth from each colony, or swab the entire membrane with a sterile cotton swab (for presence–absence results) and place in single-strength lauryl tryptose broth; incubate broth at 35 ± 0.5°C for up to 48 h. Gas formed in lauryl tryptose broth and confirmed in brilliant green lactose broth within 48 h verifies the colony as a coliform. (See Sections 9221B.3 and 4 for media preparation.) Simultaneous inoculation of both media is acceptable (if using same inoculating sterile loop, needle, or wood stick, inoculate lauryl tryptose broth first). Including EC broth inoculation incubated at 44.5 ± 0.2°C for 24 ± 2 h will provide information on the presence of thermotolerant coliforms. Including EC-MUG inoculation incubated at 44.5 ± 0.2°C for 24 h will provide information on presence of *E. coli*. The inoculation order should always be from least to most inhibitory [1) EC or EC-MUG, 2) lauryl tryptose broth, 3) brilliant green lactose broth]. (See 9222G and H for MF partition procedures.)

2) Alternative coliform verifications—Apply this alternative coliform verification procedure to isolated colonies on the Endo membrane filter media. If a mixed culture is suspected or if colony separation is <2 mm, streak the growth to m-Endo medium or MacConkey agar to ensure culture purity or submit the mixed growth to the fermentation tube method.

a) Rapid test—A rapid verification of colonies uses test reactions for cytochrome oxidase (CO) and β-galactosidase. Coliform reactions are CO negative and β-galactosidase positive within 4 h incubation of tube culture or micro (spot) test procedure.

b) Commercial multi-test systems—Verify and/or identify the coliform bacteria by selecting a well-isolated colony, streaking for isolation, and inoculating a pure colony into a multi-test identification system for *Enterobacteriaceae* that includes lactose fermentation and/or β-galactosidase and CO test reactions.

5. Calculation of Coliform Density

Select the membrane(s) with acceptable number of colonies (Table 9222:II) and ≤200 colony-forming units (CFU) of all types per membrane, by the following equation:

$$\text{(Total) coliforms, No./100 mL} = \frac{\text{coliform colonies counted} \times 100}{\text{mL sample filtered}} = \text{No. CFU/100 mL}$$

For drinking water samples, if no total coliform colonies are observed, then report the total coliform colonies counted as "<1 CFU/100 mL" or report "total coliform bacteria absent per 100 mL sample."

For nonpotable water samples, if 10.0-, 0.1-, and 0.01-mL portions are examined and all counts are 0, then calculate the number of coliforms per 100 mL that would have been reported if there had been 1 CFU on the filter representing the largest filtration volume. For example, report <10 CFU/100 mL for a 10 mL sample volume with no coliform colonies, i.e,

$$1/10 \times 100 = <10 \text{ CFU/100 mL}$$

For verified coliform counts, adjust the initial count based on the positive verification percentage (both typical and atypical) and report as follows:

$$\text{Verified (total) coliforms, No./100 mL} = \frac{\text{number of verified colonies}}{\text{total number of coliform colonies subjected to verification}} \times \text{total number of presumptive colonies}$$

a. Potable water: In its Total Coliform Rule, the U.S. Environmental Protection Agency (EPA) only requires a record of the presence or absence of total coliforms in 100-mL samples of drinking water; however, quantitative information can indicate the magnitude of a contaminating event and/or remediation progress, especially when comparing sample results from different locations (e.g., repeat samples). Quantitative information only provides a gross estimation of the actual coliform population at collection time due to non-uniform distribution within the matrix.

In good drinking water, coliform occurrence generally will be minimal. Therefore, count all coliform colonies (disregarding the lower limit of 20 cited above) and use the formula given above to obtain coliform density.

If confluent growth occurs—covering either the membrane's entire filtration area or a portion thereof with colonies that are not discrete—report results as "confluent growth with (or without) coliforms." If the total number of bacterial colonies—coliforms plus noncoliforms—is >200 per membrane or if the colonies are not distinct enough to count accurately, then report results as "too numerous to count" (TNTC) or "confluent," respectively. For drinking water samples using Endo-type media, the presence of coliforms in such cultures may be confirmed (see 9222B.4*g*). As an alternative, brush entire filter surface with a sterile loop, applicator stick, or cotton swab and inoculate this growth into 1) a tube of single-strength lauryl tryptose and 2) a tube of brilliant green lactose bile broth. If the brilliant green bile broth tube produces gas within 48 h at 35 ± 0.5°C, coliforms are present. To comply with EPA's Total Coliform Rule, report confluent growth or TNTC with at least one detectable coliform colony (verification only required with Endo-type media) as a "total coliform positive sample." Report confluent growth or TNTC without detectable coliforms as "invalid."

For invalid samples, request a new sample from the same location within 24 h. Select more appropriate volumes to filter per membrane (keeping in mind that the standard drinking-water portion is 100 mL, according to the rule). So instead of filtering 100 mL through one membrane, filter 50-mL portions through two membranes, 25-mL portions through four separate membranes, etc. to reduce interference due to overcrowding. If any membrane contains a verified total coliform colony, report the entire sample as "total coliform positive." If a density determination is desired, total the coliform counts observed on all membranes and report as "[number] per 100 mL." (Alternatively, choose another coliform method that is less subject to heterotrophic bacterial interferences.)

b. Other waters: As with potable water samples, if no filter has a coliform count within the ideal range, total the coliform counts on all filters and report as "[number] per 100 mL." For example, if duplicate 50-mL portions were examined and the two membranes had five and three coliform colonies, respectively, then report the total coliform count as 8 CFU per 100 mL:

$$\frac{[(5 + 3) \times 100]}{(50 + 50)} = 8 \text{ CFU/100 mL}$$

Alternatively, if 50-, 25-, and 10-mL portions were examined and the counts were 15, 6, and <1 coliform colonies, respectively, then calculate based on the most nearly acceptable value and report the total coliform count with a qualifying remark as "estimated 30 CFU/100 mL":

$$\frac{[(15) \times 100]}{(50)} = \text{estimated 30 CFU/100 mL}$$

On the other hand, if 10-, 1.0-, and 0.1-mL portions were examined with counts of 40, 9, and <1 coliform colonies,

Table 9222:III. Confidence Limits for Membrane Filter Coliform Results Using 100-mL Sample

Number of Coliform Colonies Counted	95% Confidence Limits	
	Lower	Upper
0	0.0	3.7
1	0.1	5.6
2	0.2	7.2
3	0.6	8.8
4	1.0	10.2
5	1.6	11.7
6	2.2	13.1
7	2.8	14.4
8	3.4	15.8
9	4.0	17.1
10	4.7	18.4
11	5.4	19.7
12	6.2	21.0
13	6.9	22.3
14	7.7	23.5
15	8.4	24.8
16	9.2	26.0
17	9.9	27.2
18	10.7	28.4
19	11.5	29.6
20	12.2	30.8

respectively, then calculate the coliform density based only on the 10-mL portion because this filter had a coliform count within the acceptable range (see Table 9222:II) and report result as 400 CFU/100 mL:

$$\frac{(40 \times 100)}{10} = 400 \text{ coliforms/100 mL}$$

In this last example, if the membrane with 40 coliform colonies also had a total bacterial colony count >200, then report the coliform count as ≥400 CFU/100 mL or with a qualifying remark as "estimated 400 CFU/100 mL."

If 10.0-, 1.0-, and 0.1-mL portions were examined with counts of TNTC, 150, and 92 coliform colonies, respectively, then calculate based on the most nearly acceptable value and report with a qualifying remark as "estimated 92 000 CFU/100 mL":

$$\frac{(92 \times 100)}{0.1} = \text{estimated } 92\,000 \text{ CFU/100 mL}$$

If 1.0-, 0.3-, 0.1-, and 0.03-mL portions were examined with counts of TNTC, TNTC, 78, and 21 coliform colonies, respectively, then sum the total coliform counts on the latter two countable filters and divide by the sum of their volume to obtain the final reported value of 76 000 CFU/100 mL:

$$\frac{(78 + 21) \times 100}{(0.1 + 0.03)} = 76\,000 \text{ CFU/100 mL}$$

If 1.0-, 0.3-, and 0.01-mL portions were examined with counts of TNTC on all portions, then calculate using the maximum number of colonies acceptable for quantitative determination of that indicator with the smallest filtration volume and report result as >800 000 CFU/100 mL (for total coliform):

$$\frac{80 \times 100}{0.01} = >800\,000 \text{ CFU/100 mL}$$

c. Statistical reliability of membrane filter results: Although MF results are considered more precise than multiple-tube most probable number (MPN) results (5- and 10-tube multiple-tube fermentation formats), membrane counts may underestimate the number of viable coliform bacteria and circumstances may affect this precision (background bacteria and dilution levels, sample types, etc.). Table 9222:III illustrates some 95% confidence limits for MF results. These values are based on the assumption that bacteria are distributed randomly and follow a Poisson distribution.

d. Precision of MF results: Calculate precision of replicate analyses for each type of sample examined and method used if there is enough sample available for replicate analyses (drinking water, ambient water, etc.). (See Section 9020B.9*e*.)

6. Bibliography

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9222 C. Delayed-Incubation Total Coliform Procedure

This modification of the standard MF technique permits membrane shipment or transport after filtration to a distant laboratory for transfer to another substrate, incubation, and completion of the test. The delayed-incubation test may be used when

- conventional procedures are impractical;
- desired sample temperature cannot be maintained during transport;
- the elapsed time between sample collection and analysis would exceed the approved time limit; or
- the sampling location is remote from laboratory services (see Section 9060B).

Independent studies using both fresh and marine water samples have shown consistent results between the delayed-incubation and standard MF tests. Determine the delayed-incubation test's applicability for a specific water source by comparing with results of conventional MF methods.

To conduct the delayed-incubation test, filter sample in the field immediately after collection, place filter on transport medium, and ship to laboratory. Complete coliform determination in the laboratory by transferring membrane to standard m-Endo or Endo LES medium, incubating at 35 ± 0.5°C for 20 to 22 h, and counting the typical and atypical coliform colonies that develop. For drinking-water samples collected for compliance with EPA's Total Coliform Rule, report the presence or absence of verified coliforms in 100-mL samples. Verify colonies as outlined previously in 9222B.4*g*.

Transport media are designed to keep coliform organisms viable and generally do not permit visible growth during transit time. Bacteriostatic agents in holding/preservative media suppress growth of microorganisms en route but allow normal coliform growth after transfer to a fresh medium.

The delayed-incubation test follows the methods outlined for the total coliform MF procedure, except as indicated below. Two alternative methods are given: one using the m-Endo preservative medium and the other using the m-ST holding medium. If commercially prepared medium is unavailable, prepare from individual components as described in 9222B.2*a* and 9222C.2*b*.

1. Laboratory Apparatus

a. Culture dishes: Use disposable, sterile, plastic Petri dishes (9 × 50 mm) with tight-fitting lids. Such containers are lightweight and less likely to break in transit. In an emergency or when plastic dishes are unavailable, use sterile glass Petri dishes wrapped in plastic film or similar material. (See 9222B.1*e*.)

b. Field filtration units: See 9222B.1*f*. Ultraviolet light disinfection may be used in the field if an appropriate power source is available (115 V, 60 Hz). Glass or metal filtration units may be sterilized by immersing in boiling water for 2 min. Use reagent water to avoid hard-water deposits. Use a hand aspirator to obtain necessary vacuum.

c. Absorbent pads: See 9222B.1*h*.

d. Forceps: See 9222B.1*i*.

2. Materials and Transport Media

a. m-Endo methods:

1) *m-Endo preservative medium*—Prepare m-Endo medium as described in 9222B.2*b*. After cooling to <45°C, aseptically add 3.84 g sodium benzoate [U.S. Pharmacopeia (USP) grade]/L or 3.2 mL 12% sodium benzoate solution to 100 mL medium. Mix ingredients and refrigerate poured plates. Discard unused medium after 96 h.
2) *Sodium benzoate solution*—Dissolve 12 g $NaC_7H_5O_2$ in sufficient reagent water to make 100 mL. Sterilize by autoclaving or by filtering through a 0.22-μm-pore-size membrane filter. Discard after 6 months.
3) *Cycloheximide**—Optionally, add cycloheximide to m-Endo preservative medium. It may be used for samples that previously have shown overgrowth by fungi, including yeasts. Prepare by aseptically adding 50 mg cycloheximide/100 mL to m-Endo preservative medium. Store cycloheximide solution in refrigerator, and discard after 6 months. **CAUTION: Cycloheximide is a powerful skin irritant. Follow manufacturer's and SDS instructions for proper handling and storage of this chemical.**

b. m-ST method:

m-ST holding medium:

Sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$)	0.1	g
Dipotassium hydrogen phosphate (K_2HPO_4)	3.0	g
Sulfanilamide	1.5	g
Ethanol (95%)	10	mL
Tris (hydroxymethyl) aminomethane	3.0	g
Reagent-grade water	1	L

Dissolve ingredients by rehydrating in water. Sterilize by autoclaving at 121–124°C for 15 min. Final pH should be 8.6 ±

* Actidione®, manufactured by the Upjohn Company, Kalamazoo, MI, or equivalent.

0.2. Dispense at least 2.0 to 3.0 mL (depending on pad manufacturer) to tight-lidded plastic culture dishes containing an absorbent pad, and carefully remove excess liquid from pad by decanting the plate. Store plates in the refrigerator for use within 96 h.

3. Procedure

a. Sample preservation and shipment: Place absorbent pad in bottom of sterile Petri dish and saturate with selected coliform holding medium (see 9222C.2). Remove membrane filter from filtration unit with sterile forceps and roll it, grid side up, onto surface of medium-saturated pad. Protect membrane from moisture loss by tightly closing plastic Petri dish. Seal loose-fitting dishes with an appropriate sealing tape† to prevent membrane dehydration during transit. Place culture dish containing membrane in an appropriate shipping container and send to laboratory for test completion. The sample can be held without visible growth for a maximum of 72 h at ambient temperature on the holding/preservative medium. Visible growth occasionally begins on transport medium when high temperatures are encountered during transit.

† Parafilm, Sigma-Aldrich Co. LLC, or equivalent.

b. Transfer and incubation: At the laboratory, transfer filter from holding medium on which it was shipped to a second sterile Petri dish containing m-Endo or Endo LES medium and incubate at 35 ± 0.5°C for 20 to 22 h.

4. Estimation of Coliform Density

Proceed as described in 9222B.5. Record times of collection, filtration, and laboratory examination, and calculate the elapsed time. Report elapsed time with coliform results.

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9222 D. Thermotolerant (Fecal) Coliform Membrane Filter Procedure

Thermotolerant (formerly *fecal*) coliform bacterial densities may be determined by either multiple-tube procedure or MF technique. (See Section 9225 for differentiation of *Escherichia coli*.) The thermotolerant coliform MF procedure uses an enriched lactose medium and incubation temperature of 44.5 ± 0.2°C for selectivity. Because incubation temperature is critical, submerge waterproofed (plastic bag enclosures) MF cultures in a water bath for incubation at the elevated temperature or use an appropriate solid heat-sink or other incubator that is documented to hold the temperature at 44.5°C ± 0.2°C throughout the chamber over a 24-h period. The best type of incubator is a gable-covered circulating water bath. In general, this method is applicable under the same circumstances as the multiple-tube thermotolerant coliform procedures (see Section 9221E).

There are limitations to the interpretation of a thermotolerant coliform result from thermal waters (e.g., the tropics) and pulp and paper mill effluent samples where thermotolerant *Klebsiella* have predominated and not been indicative of a sewerage source. As with all coliform results, a sanitary survey should be conducted to identify the most plausible source and public health risk interpretation.

1. Laboratory Apparatus

a. Sample bottles: See Section 9030B.19.
b. Dilution bottles: See Section 9030B.13.
c. Pipets and graduated cylinders: See Section 9030B.9.
d. Containers for culture medium: See 9222B.1*d*.
e. Culture dishes: Tight-fitting plastic dishes (see 9222B.1*e*) are preferred when MF culture plates will be submerged in a water bath during incubation. Place thermotolerant coliform culture plates in plastic bags (remove as much air as possible) or seal individual dishes with waterproof (freezer) tape to prevent leakage during submersion.
f. Filtration units: See 9222B.1*f*.
g. Membrane filters: See 9222B.1*g*.
h. Absorbent pads: See 9222B.1*h*.
i. Forceps: See 9222B.1*i*.
j. Water bath or incubator: The specificity of the thermotolerant coliform test is related directly to incubation temperature. To meet the need for greater temperature control, use a gable-covered water bath, a heat-sink incubator, or any properly designed and constructed incubator that can maintain a temperature tolerance of ±0.2°C. Most circulating water baths equipped with a gable top to reduce water and heat loss can maintain a temperature of 44.5 ± 0.2°C. However, static air incubation may be a problem in some types of incubators because of potential heat layering in the chamber, slower heat transfer from air to the medium, and slow temperature recovery each time the incubator is opened during daily operations.

2. Materials and Culture Medium

mFC medium: The need for uniformity dictates the use of dehydrated media. Never prepare media from basic ingredients when suitable dehydrated media are available. Follow the manufacturer's directions for rehydration. Commercial liquid media

(sterile ampule, etc.) may be used if known to give equivalent results. See Section 9020 for QC specifications. If commercially prepared medium is unavailable, prepare from individual components as described below.

mFC medium:

Tryptose or biosate	10.0 g
Proteose peptone No. 3 or polypeptone	5.0 g
Yeast extract	3.0 g
Sodium chloride (NaCl)	5.0 g
Lactose	12.5 g
Bile salts No. 3 or bile salts mixture	1.5 g
Aniline blue	0.1 g
Agar (optional)	15.0 g
Reagent-grade water	1 L

Rehydrate product or individual components in 1 L water containing 10 mL 1% rosolic acid in 0.2*N* NaOH.* Heat to near boiling, promptly remove from heat, and cool to <50°C. Do not sterilize by autoclaving. If agar is used, dispense 4- to 6-mL quantities to 9- × 50-mm Petri plates (approximately 4 to 5 mm deep) and let solidify. Final pH should be 7.4 ± 0.2. Refrigerate finished medium (preferably in sealed plastic bags or other containers to reduce moisture loss) and discard unused broth after 96 h or unused agar after 2 weeks. NOTE: For samples from sources known to have minimal background growth (e.g., drinking water), 1% rosolic acid addition can be omitted from mFC medium, but it should be used for all unknown sources, stormwaters, and ambient water sources.

Before use, test each batch of laboratory-prepared MF medium for performance with positive and negative culture controls (See Table 9020:VI). Check for coliform contamination at the beginning and end of each filtration series by filtering 20 to 30 mL of dilution or rinse water through filter. If controls indicate contamination, reject all data from affected samples and request new samples. Test each new medium lot to confirm that its performance is satisfactory (see Section 9020B.5*j*). The use of control charts is helpful to identify trends and ensure long-term consistency in media performance.

3. Procedure

a. Select sample size: Select volume of water sample to be examined in accordance with the information in Table 9222:IV. Use sample volumes that will yield counts between 20 and 60 thermotolerant coliform colonies per membrane.

When the sample's bacterial density is unknown, filter several volumes or dilutions to achieve a countable plate. Estimate the volume and/or dilution expected to yield a countable membrane, and select two additional quantities representing one-tenth and ten times (or one third and three times) this volume, respectively.

b. Filter sample: Follow the same procedure and precautions given in 9222B.4*c*.

c. Prepare culture dish: Using aseptic technique, place a sterile absorbent pad in each culture dish and pipet at least 2.0 mL mFC medium (prepared as directed above) to saturate pad. Carefully remove any excess liquid from culture dish by decanting plate. After filtration, aseptically place sample filter on medium-impregnated pad [see 9222B.2*b*2)].

As a substrate substitution for the nutrient-saturated absorbent pad, add 1.5% agar to mFC broth [see 9222B.2*b*1)].

d. Incubate: Place prepared dishes in waterproof plastic bags, remove as much air as possible, seal, invert, and submerge Petri dishes in water bath; incubate for 24 ± 2 h at 44.5 ± 0.2°C. Anchor dishes below water surface; if anchor devices (e.g., "O" rings, bricks, or water bottles) will also be submerged, make sure they are prewarmed before sample use, small enough to maintain critical temperature requirements, and do not interfere with sample incubation. Place all prepared cultures in water bath within 30 min after filtration. Alternatively, use an appropriate, accurate solid heat-sink or equivalent incubator. Do not submerge plates in waterproof hard-sided plastic containers; the extra air space does not allow the plates to reach temperature for many hours.

e. Counting: Colonies produced by thermotolerant coliform bacteria on mFC medium are various shades of blue. Non-thermotolerant coliform colonies are gray to cream-colored. Normally, few non-thermotolerant coliform colonies will be observed on mFC medium because the elevated temperature and addition of rosolic acid salt reagent selects against them. Count colonies with a low-power (10 to 15× magnification) binocular wide-field dissecting microscope or other optical device, if needed.

f. Verification: Verify at a frequency established by the laboratory. Verify typical blue colonies and any atypical grey to green colonies (see Section 9020B.10) for thermotolerant coliform analysis. Simultaneous inoculation into single-strength lauryl tryptose and EC broth (or EC-MUG broth) incubated at 35 and 44.5°C, respectively, is acceptable during verification.

TABLE 9222:IV. SUGGESTED SAMPLE VOLUMES FOR MEMBRANE FILTER THERMOTOLERANT COLIFORM OR *E. COLI* TEST

	Volume (X) To Be Filtered, *mL*							
Water Source	100	50	10	1	0.1	0.01	0.001	0.0001
Drinking water	X							
Lakes, reservoirs	X	X						
Wells, springs	X	X						
Water supply intake		X	X	X				
Natural bathing waters		X	X	X				
Sewage treatment plant			X	X	X			
Farm ponds, rivers				X	X	X		
Stormwater runoff				X	X	X		
Raw municipal sewage					X	X	X	
Feedlot runoff					X	X	X	
Sewage sludge						X	X	X

4. Calculation of Thermotolerant Coliform Density

a. General: Compute the density from the sample quantities that produced MF counts within the desired range of 20 to 60 thermotolerant coliform colonies. This colony-density range is more restrictive than the 20 to 80 total coliform range because of the larger colony size on mFC medium. Calculate thermotolerant coliform density as directed in 9222B.5. Record thermotolerant coliform densities as CFU per l00 mL.

* Rosolic acid reagent will decompose if sterilized by autoclaving. Refrigerate stock solution in the dark and discard after 2 weeks, or sooner if its color changes from dark red to muddy brown.

b. Sediment and biosolids samples: For total solids (dry weight basis), see Section 2540G. Calculate thermotolerant coliforms per gram dry weight for biosolids analysis as follows:

$$\text{Thermotolerant coliform, CFU/g dry weight} = \frac{\text{colonies counted}}{(\text{dilution chosen}) \times (\%\ \text{dry solids})}$$

where dilution and % dry solids are expressed in decimal form.

Example 1: Analyst observed 22 colonies on the 1:10 000 dilution plate of a biosolids with 4% dry solids.

$$\frac{22}{(0.0001)\ (0.04)} = 5.5 \times 10^6\ \text{CFU/g dry weight}$$

If no filter has a thermotolerant coliform count falling in the ideal range (20 to 60), total the thermotolerant coliform counts on all countable filters, and report as thermotolerant coliforms per gram dry weight:

Example 2: Analyst observed 18 colonies on the 1:10 000 dilution plate and 2 colonies on the 1:100 000 dilution plate of a biosolids sample with 4% dry solids.

$$\frac{(18 + 2)}{(0.0001 + 0.00001)\ (0.04)} = 4.5 \times 10^6$$

To compute a geometric mean of samples, convert the thermotolerant coliform densities of each sample to $\log_{10}$ values. Determine the geometric mean for the given number of samples† by averaging the $\log_{10}$ values of the thermotolerant coliform densities and taking the antilog of that value.

† Usually seven if collecting for EPA's Pathogen Reduction Rule, 40 CFR Part 503.[1]

5. Reference

1. U.S. Environmental Protection Agency. 1993. Standards for the Use or Disposal of Sewage Sludge: Final Rule. 40 CFR Part 503; *Fed. Reg.* 58:9248.

6. Bibliography

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9222 E. Delayed-Incubation Thermotolerant (Fecal) Coliform Procedure

This delayed-incubation procedure is similar to the delayed-incubation total coliform procedure (9222C). Use this test only when the standard immediate thermotolerant coliform test cannot be performed (e.g., if the appropriate field incubator is unavailable or circumstances indicate that a specialized laboratory service is advisable to examine, confirm, or speciate the suspect colonies).

Results obtained by this delayed method have been consistent with results from the standard thermotolerant (fecal) coliform MF test under various laboratory and field use conditions. However, determine test applicability for a specific water source by comparison with the standard MF test, especially for saline waters, chlorinated wastewaters, and waters containing toxic substances.

To conduct the delayed-incubation test, filter sample in the field immediately after collection, place filter on m-ST holding medium (see 9222C.2*b*), and ship to the laboratory. Complete thermotolerant coliform test by transferring filter to mFC medium, incubating at 44.5°C for 24 ± 2 h, and counting thermotolerant coliform colonies.

The m-ST medium keeps thermotolerant coliform organisms viable but prevents visible growth during transit. Membrane filters can be held for up to 3 d on m-ST holding medium with little effect on thermotolerant coliform counts.

1. Laboratory Apparatus

a. Culture dishes: See 9222B.1*e*.
b. Field filtration units: See 9222B.1*f*.
c. Absorbent pads: See 9222B.1*h*.
d. Forceps: See 9222B.1*i*.

2. Materials and Transport Medium

a. m-ST medium: Prepare as described in 9222C.2*b*.
b. mFC medium: Prepare as described in 9222D.2.

3. Procedure

a. Membrane filter transport: Using aseptic technique, place an absorbent pad in a plastic Petri dish with a tight lid and saturate with m-ST holding medium. After filtering sample, remove membrane filter from the filtration unit and place it on medium-saturated pad. Use only tightly lidded dishes to prevent moisture loss but avoid excess liquid in the dish. Place culture dish containing the filter in an appropriate shipping container and send to laboratory. Membranes can be held on the transport medium at ambient temperature for a maximum of 72 h with little effect on thermotolerant coliform counts.

b. Transfer: At the laboratory, aseptically remove membrane from holding medium and place it in another dish containing mFC medium.

c. Incubation: After transferring filter to mFC medium, place tight-lidded dishes in waterproof plastic bags, invert, and submerge in a water bath at 44.5 ± 0.2°C for 24 ± 2 h, or use a solid heat-sink or equivalent incubator.

d. Counting: Colonies produced by thermotolerant coliform bacteria are various shades of blue. Non-thermotolerant coliform colonies are gray to cream-colored. Count colonies with a binocular wide-field dissecting microscope at 10 to 15× magnification.

e. Verification: Verify colonies at a frequency established by the laboratory. Verify typical blue colonies and any atypical (grey to green) colonies as described in Section 9020B.10 for thermotolerant coliform analysis.

4. Estimation of Thermotolerant Coliform Density

Count as directed in 9222D.3*e* and compute thermotolerant coliform density as described in 9222D.4. Record time of collection, filtration, and laboratory examination, and calculate and report elapsed time.

5. Bibliography

CHEN, M. & P.J. HICKEY. 1983. Modification of delayed-incubation procedure for detection of fecal coliforms in water. *Appl. Environ. Microbiol.* 46:889.

9222 F. *Klebsiella* Membrane Filter Procedure

Klebsiella bacteria belong to the family Enterobacteriaceae and are included in the total coliform group. *Klebsiella* spp. are excreted in the feces of many healthy humans and animals, and are readily detected in sewage-polluted waters. Approximately 60 to 80% of all *Klebsiella* from feces and from clinical specimens are positive in the thermotolerant coliform test and are *Klebsiella pneumoniae*.

Klebsiella bacteria also are widely distributed in nature, occurring in soil, water, grain, vegetation, etc. Wood pulp, paper mills, textile finishing plants, and sugar-cane processing operations contain large numbers of *Klebsiella* spp. in their effluents (10^4 to 10^6 per 100 mL), and *Klebsiella* spp. are often the predominant coliform in such effluents.

Rapid quantitation may be achieved in the MF procedure by modifying mFC agar base through substitution of inositol for lactose and adding carbenicillin or by using mKleb agar. These methods reduce the necessity for biochemical testing of pure strains. Preliminary verification of differentiated colonies is recommended.

1. Laboratory Apparatus

a. Sample bottles: See Section 9030B.19.
b. Dilution bottles: See Section 9030B.13.
c. Pipets and graduated cylinders: See 9222B.1*c*.
d. Containers for culture medium: See 9222B.1*d*.
e. Culture dishes: See 9222B.1*e*.
f. Filtration units: See 9222B.1*f*.
g. Membrane filters: See 9222B.1*g*.
h. Absorbent pads: See 9222B.1*h*.
i. Forceps: See 9222B.1*i*.
j. Incubators: See 9222B.1*j*.

2. Materials and Culture Media

a. mFCIC agar: This medium may not be available in dehydrated form and may require preparation from the basic ingredients:

Tryptose or biosate	10.0 g
Proteose peptone No. 3 or polypeptone	5.0 g
Yeast extract	3.0 g
Sodium chloride (NaCl)	5.0 g
Inositol	10.0 g
Bile salts No. 3 or bile salts mixture	1.5 g
Aniline blue	0.1 g
Agar	15.0 g
Reagent-grade water	1 L

Heat medium to boiling, and add 10 mL 1% rosolic acid* dissolved in 0.2 *N* NaOH. Cool to <45°C, and add 50 mg carbenicillin.† Dispense aseptically in 4- to 6-mL quantities into 9- × 50-mm plastic Petri dishes (approximate depth of 4 to 5 mm). Refrigerate until needed. Discard unused agar medium after 2 weeks. Do not sterilize by autoclaving. Final pH should be 7.4 ± 0.2.

b. mKleb agar:

Phenol red agar	31.0 g
Adonitol	5.0 g
Aniline blue	0.1 g
Sodium lauryl sulfate	0.1 g
Reagent-grade water	1 L

* Rosolic acid reagent will decompose if sterilized by autoclaving. Refrigerate stock solution in the dark and discard after 2 weeks, or sooner if its color changes from dark red to muddy brown.
† Available from Geopen, Roerig-Pfizer, Inc., New York, NY.

Sterilize by autoclaving for 15 min at 121–124°C. After autoclaving, cool to 50°C in a water bath; add 20 mL 95% ethyl alcohol (not denatured) and 0.05 g filter-sterilized carbenicillin/L. Shake thoroughly and dispense aseptically into 9- × 50-mm plastic culture plates. The final pH should be 7.4 ± 0.2. Refrigerated medium can be held for 20 d at 4 to 8°C.

3. Procedure

a. Sample size selection and filtration: See 9222B.4 for selection of sample size and filtration procedure. Select sample volumes that will yield counts between 20 and 60 *Klebsiella* colonies per membrane. Place membrane filter on agar surface; incubate for 24 ± 2 h at 35 ± 0.5°C. *Klebsiella* colonies on mFCIC agar are blue or bluish-gray. Most atypical colonies are brown or brownish. Occasional false-positive occurrences are caused by *Enterobacter* species. *Klebsiella* colonies on mKleb agar are deep blue to blue gray; other colonies most often are pink or occasionally pale yellow. Count colonies with a low-power (10 to 15× magnification) binocular wide-field dissecting microscope or other optical device.

b. Verification: Verify *Klebsiella* colonies from the first set of samples from ambient waters and effluents, and when *Klebsiella* is suspect in water-supply distribution systems. Verify a minimum of five typical colonies by transferring growth from a colony or pure culture to a commercial multi-test system for Gram-negative speciation. Key tests for *Klebsiella* are citrate (positive), motility (negative), lysine decarboxylase (positive), ornithine decarboxylase (negative), and urease (positive). A *Klebsiella* strain that is indole-positive, liquefies pectin, and demonstrates a negative thermotolerant coliform response is most likely of nonfecal origin.

4. Bibliography

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9222 G. Partitioning Thermotolerant Coliforms from MF Total Coliform Using EC Broth

In a drinking water sample, thermotolerant coliform determination can be performed from a total-coliform-positive MF filter within 24 h. This technique may be applicable to other waters if warranted.

1. Laboratory Apparatus

See 9222B.1*a–j*.

2. Materials and Culture Medium

EC broth: See Section 9221E.1*a*.

3. Procedure

a. Selection of sample size and filtration procedure: See 9222B.4.

b. Total coliform verification: Verify total coliforms before using the thermotolerant coliform partition method. Swab surface growth on the total-coliform-positive filter or, if quantification is desired, transfer small portions of each target colony on the filter to the appropriate total coliform verification medium using a sterile needle. See 9222B.4*g* for total coliform verification procedures.

c. Partition method for thermotolerant coliform determination: Using aseptic technique, transfer total-coliform-positive colonies from the membrane filter to a tube containing EC medium by one of the following methods:

- remove membrane containing total coliform colonies from the substrate with sterile forceps and carefully curl and insert membrane into tube of EC medium (do not vortex tube to avoid introducing air bubbles to inverted vial,
- swab the entire membrane filter surface with a sterile cotton swab and transfer the inoculum to EC medium (do not leave cotton swab in the medium), or
- if quantification is desired, inoculate individual total coliform-positive colonies into separate EC tubes. Simultaneous inoculation of both total-coliform verification tests and EC broth is acceptable (order of inoculation should always be EC broth first and then other more inhibitory media).

Incubate tubes in 44.5 ± 0.2°C water-bath incubator within 30 min after inoculation. Maintain a sufficient water depth in incubator to immerse tubes to upper level of medium. Gas production in an EC broth (9221E.1*a*) culture in ≤24 h is considered a positive response for thermotolerant coliform bacteria.

4. Bibliography

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9222 H. Partitioning *E. coli* from MF Total Coliform using EC-MUG Broth

Escherichia coli is a member of the thermotolerant coliform group of bacteria; its presence is indicative of fecal contamination. Rapid quantitation and verification for *E. coli* may be achieved for a total-coliform- or thermotolerant-coliform-positive MF sample by using media containing 4-methylumbelliferyl-β-D-glucuronide (MUG). In this method, *E. coli* is defined as any coliform that produces the enzyme β-D-glucuronidase and hydrolyzes the MUG substrate to produce a blue fluorescence.

When examining drinking-water samples, use one of the two partition methods to determine the presence of *E. coli* from a total-coliform-positive MF sample on Endo-type media: nutrient agar containing MUG (9222I) or EC containing MUG. When examining wastewater and other nonpotable water samples, use one of the partition methods to determine the presence of *E. coli* from thermotolerant (fecal)-coliform-positive MF samples on mFC media.

1. Laboratory Apparatus

a. See 9222B.1*a–k.*

b. Ultraviolet lamp, long-wave (365–366 nm), 6 W. See Section 9030B.23.

2. Materials and Culture Medium

EC broth with MUG (EC-MUG): See Section 9221F.1.

3. Procedure

a. Selection of sample size and filtration procedure: See 9222B.4.

b. Total coliform verification: Verify total coliforms before using the *E. coli* partition method. Swab surface growth on the total-coliform-positive filter or, if quantification is desired, transfer small portions of each target colony on the filter to the appropriate total coliform verification medium using a sterile needle. See 9222B.4*g* for total coliform verification procedures.

c. Partition method for E. coli *determination:* Use aseptic technique to transfer total coliform-positive colonies on the membrane filter to a tube containing EC-MUG medium by one of the following methods:

- remove membrane containing total coliform colonies from the substrate with sterile forceps and carefully curl and insert membrane into tube of EC-MUG medium,
- swab entire membrane filter surface with a sterile cotton swab and transfer the inoculum to EC-MUG medium (do not leave cotton swab in EC-MUG medium), or
- if quantification is desired, inoculate individual total coliform-positive colonies into separate EC-MUG tubes.

Incubate EC-MUG media at 44.5 $\pm$ 0.2°C for 24 $\pm$ 2 h. Place all EC-MUG tubes in water-bath incubator within 30 min after inoculation. Maintain a sufficient water depth in incubator to immerse tubes to upper level of medium.

Observe EC-MUG tubes using a long-wavelength (365–366-nm) UV light source, preferably containing a 6-W bulb. **CAUTION: UV lamp should never be viewed directly.** Preferably view tubes in a viewing box or hold UV light a few inches in front of tubes (e.g., 3 to 4 in.), facing away from the viewer. Also, using a UV lamp equipped with a specific filter to eliminate most of the visible light interference is desirable and will facilitate fluorescence determination. The presence of a bright blue fluorescence in the tube is a positive response for *E. coli*. Record presence or absence of fluorescence. For nonpotable water samples, this partition method can be used to determine *E. coli* from the thermotolerant coliform MF procedure using mFC medium for initial isolation before transfer to EC-MUG medium. The procedure is the same as the above, except for the total coliform verification process.

A positive control consisting of a known *E. coli* (MUG-positive) culture, a negative control consisting of a thermotolerant *Klebsiella pneumoniae* (MUG-negative) culture, and an uninoculated medium control may be necessary to interpret sample results and avoid misidentifying the medium's weak yellow-blue autofluorescence as a positive response. (See Section 9221F.)

4. Bibliography

MATES, A. & M. SHAFFER. 1989. Membrane filtration differentiation of *E. coli* from coliforms in the examination of water. *J. Appl. Bacteriol.* 67:343.

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MATES, A. & M. SHAFFER. 1992. Quantitative determination of *Escherichia coli* from coliforms and fecal coliforms in sea water. *Microbios* 71:27.

SARTORY, D. & L. HOWARD. 1992. A medium detecting beta-glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms from drinking water. *Lett. Appl. Microbiol*. 15:273.

9222 I. Partitioning *E. coli* from MF Total Coliforms using NA-MUG Agar

Escherichia coli is a member of the thermotolerant coliform group of bacteria; its presence is indicative of fecal contamination. Rapid quantitation and verification for *E. coli* may be achieved for a total-coliform- or thermotolerant-coliform-positive MF sample by using media containing 4-methylumbelliferyl-β-D-glucuronide (MUG). In this method, *E. coli* is defined as any coliform that produces the enzyme β-glucuronidase and hydrolyzes the MUG substrate to produce a blue fluorescence.

When examining drinking water samples, use one of the two partition methods to determine the presence of *E. coli* from a total-coliform-positive MF sample on Endo-type media; use nutrient agar containing MUG or EC broth containing MUG (9222H). When examining wastewater and other nonpotable water samples, use one of the partition methods to determine the presence of *E. coli* from thermotolerant (fecal)-coliform-positive MF samples on mFC media.

1. Laboratory Apparatus

a. Culture dishes: See 9222B.1*e*.
b. Filtration units: See 9222B.1*f*.
c. Forceps: See 9222B.1*i*.
d. Incubator: See 9222B.1*j*.
e. Ultraviolet lamp, long-wave (365–366 nm), 6 W: See Section 9030B.23.
f. Microscope and light source: See 9222B.1*k*.

2. Materials and Culture Medium

Nutrient agar with MUG (NA-MUG):

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
4-methylumbelliferyl-β-D-glucuronide	0.1 g
Reagent-grade water	1 L

Add dehydrated ingredients to reagent-grade water, mix thoroughly, and heat to dissolve. Sterilize by autoclaving for 15 min at 121–124°C. Dispense 4- to 6-mL quantities aseptically into 50-mm plastic culture plates (approximate depth of 4 to 5 mm) and allow to solidify. Final pH should be 6.8 ± 0.2. Refrigerated prepared medium may be held for 2 weeks.

3. Procedure

a. Selection of sample size and filtration procedure: See 9222B.4.

b. Total coliform verification: For drinking water samples using Endo-type medium, total coliform verification procedures can be performed before or after the partition method. Swab surface growth on the filter or, if quantification is desired, transfer small portions of each target colony on filter to the appropriate total coliform verification medium using a sterile needle. Alternatively, after transferring filter to NA-MUG media, incubating it, and reading the results on this media, either transfer individual colonies, swab surface growth on filter, or place whole filter into appropriate total coliform verification medium. (See 9222B.4*g* for total coliform verification procedures.)

c. Partition method for E. coli determination: Aseptically transfer membrane filter with at least one coliform-positive colony to NA-MUG plate. If quantification is desired, mark each sheen colony (e.g., use a fine-tip pen to mark sheen colony's location on lid and filter/lid orientation) and transfer lid to NA-MUG plate, or use a sterile needle to make a hole in membrane filter next to sheen colony after transferring membrane to NA-MUG medium. Incubate NA-MUG immediately after transfer at 35 ± 0.5°C for 4 h.

Observe individual colonies (on NA-MUG plates) using a long-wavelength (365–366-nm) UV light source, preferably containing a 6-W bulb. **CAUTION: UV lamp should never be viewed directly.** Preferably view plates in a viewing box or hold the UV light a few inches above the plates (e.g., 3 to 4 in.), facing away from the viewer. The presence of a bright blue fluorescence on the periphery (outer edge) of a colony, or observed from the back of the plate is a positive response for *E. coli*. Record presence or absence of fluorescence, or if quantification is desired, count and record the number of target colonies. For nonpotable water samples, this partition method can be used to determine *E. coli* from the thermotolerant coliform MF procedure using mFC medium for initial isolation before transfer to NA-MUG medium. The procedure is the same as the above, except for the total coliform verification process.

4. Bibliography

MATES, A. & M. SHAFFER. 1989. Membrane filtration differentiation of *E. coli* from coliforms in the examination of water. *J. Appl. Bacteriol*. 67:343.

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9222 J. Simultaneous Detection of Total Coliform and *E. coli* by Dual-Chromogen Membrane Filter Procedure

1. Laboratory Apparatus

For MF analyses, use glassware and other apparatus composed of material free from agents that may affect bacterial growth.

a. Sample bottles: See Section 9030B.19.

b. Dilution bottles: See Section 9030B.13.

c. Pipets, sample containers, and graduated cylinders: See Section 9030B.9.

d. Culture dishes: See 9222B.1*e*.

e. Filtration units: See 9222B.1*f*.

f. Membrane filters: See 9222B.1*g*.

g. Absorbent pads: See 9222B.1*h*.

h. Forceps: See 9222B.1*i*.

i. Incubators: See 9222B.1*j*.

2. Materials and Culture Medium

Purchase this medium from a commercial vendor; it cannot be prepared from basic ingredients. See Section 9020B.5*j* for media QC specifications.

Before use, test each lot with positive and negative culture controls (See Table 9020:VI). Check for coliform contamination at the beginning and end of each filtration series by filtering 20 to 30 mL of dilution or rinse water through the filter. If controls indicate contamination, reject all data from affected samples and request new samples. Test each new medium lot to confirm that its performance is satisfactory (see Section 9020B.5*j*). The use of control charts is helpful to identify trends and ensure long-term consistency in media performance.

*m-ColiBlue24® Broth**

L-Methionine	0.1	g
Methylene blue	0.016	g
Casitone	8.0	g
Yeast extract	0.5	g
Lactose	0.6	g
Sodium chloride (NaCl)	3.0	g
Dipotassium hydrogen phosphate (K_2HPO_4)	1.75	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.25	g
Triphenyl tetrazolium chloride	0.07	g
Sodium pyruvate	1.0	g
Erythromycin	3.0	g
Octyphenol ethoxylate†	0.5	g
Magnesium sulfate ($MgSO_4$)	0.3	g
5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid	(proprietary)	
Sodium azide	0.02	g
Cyclohexylammonium salt	0.2	g
Reagent-grade water	1	L

CAUTION: Sodium azide is highly toxic and mutagenic. Follow manufacturer's and SDS instructions for proper storage and handling of this medium.

Mix broth gently by inverting ampules two or three times before dispensing. Pour liquid medium (approximately 2 mL per plate) evenly onto sterile absorbent pads and place lid on Petri dish. Final pH should be 7.0 ± 0.2.

3. Procedure

a. Selection of sample size: See 9222B.4*a*.

b. Sterile filtration units: See 9222B.4*b*.

c. Filtration of sample: See 9222B.4*c*, with the following exception: incubate m-ColiBlue24® broth plates at 35 ± 0.5°C for 24 h.

d. Counting: To count colonies on membrane filters, use a low-powered (10 to 15× magnification) binocular wide-field dissecting microscope or other optical device with a cool white fluorescent light source directed to provide optimal viewing. Count all red and blue to purple colonies under normal/ambient light and record as the total coliform result. Count only blue to purple colonies and record as *E. coli* result. Clear or white colonies are considered non-coliform colonies. A high non-coliform count may interfere with the development of coliform colonies.

e. Coliform verification: For drinking water, total coliform colony verification is not required for this medium. For waters other than drinking water, verify at a frequency established by the laboratory (see Section 9020B.10). Based on need and sample type, laboratories may incorporate more stringent QC measures (e.g., verify at least one colony from each typical or atypical colony type from a given membrane filter culture, verify 10% of positive samples) (see Section 9020B.10). Adjust counts based on verification results. Verification tests are listed in 9222B.4*g*.

4. Calculation of Coliform Density

See 9222B.5. Calculate the final values using the formula:

$$\textit{E. coli}/100\text{ mL} = \frac{\text{number of blue-purple colonies}}{\text{volume of sample filtered (mL)}} \times 100$$

$$\text{TC}/100\text{ mL} = \frac{\text{number of red } \textit{and} \text{ blue to purple colonies}}{\text{volume of sample filtered (mL)}} \times 100$$

5. Bibliography

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U.S. ENVIRONMENTAL PROTECTION AGENCY. 1999. National Primary and Secondary Drinking Water Regulations: Analytical Methods for Chemical and Microbiological Contaminants and Revisions to Library Certification Requirement Final Rule. 40 CFR Parts 141 and 143; *Fed. Reg.* 64(230):67449.

* m-Coliblue24®, HACH Company, Loveland, CO; EMD Millipore Sigma Corporation, Billerica, MA; or equivalent.
† Triton X-114, or equivalent.

FRANCY, D.S. & R.A. DARNER. 2000. Comparison of methods for determining *Escherichia coli* concentrations in recreational water. *Water Res.* 34:2770.

OLSTADT, J., J.J. SCHAUER, J. STANDRIDGE & S. KLUENDER. 2007. A comparison of the USEPA approved total coliform/*E. coli* tests. *J. Water and Health* 05:267.

9222 K. Simultaneous Detection of Total Coliforms and *E. coli* by Fluorogen/Chromogen Membrane Filter Procedure

1. Laboratory Apparatus

For MF analyses, use glassware and other apparatus composed of material free from agents that may affect bacterial growth.

a. Sample bottles: See Section 9030B.19.

b. Dilution bottles: See Section 9030B.13.

c. Pipets, sample containers, and graduated cylinders: See Section 9030B.9.

d. Culture dishes: See 9222B.1*e*.

e. Filtration units: See 9222B.1*f*.

f. Membrane filters: See 9222B.1*g*.

g. Absorbent pads: See 9222B.1*h*.

h. Forceps: See 9222B.1*i*.

i. Incubators: See 9222B.1*j*.

2. Materials and Culture Medium

Use commercial dehydrated media whenever possible for uniformity between batches; never prepare media from basic ingredients when suitable dehydrated media are available.

Follow manufacturer's directions for rehydration. Store opened supplies of dehydrated media in a desiccator (if necessary). Commercial liquid media (sterile ampule, etc.) may be used if known to give equivalent results. See Section 9020B.5*j* for media QC specifications.

Before use, test each lot with positive and negative culture controls (See Table 9020:VI). Check for coliform contamination at the beginning and end of each filtration series by filtering 20 to 30 mL of dilution or rinse water through the filter. If controls indicate contamination, reject all data from affected samples and request new samples. Test each new medium lot to confirm that its performance is satisfactory (see Section 9020B.5*j*). The use of control charts is helpful to identify trends and ensure long-term consistency in media performance. If commercially prepared medium is not available, prepare as described in ¶s *a*–*c* below.

a. Cefsulodin solution, 1 mg/1 mL: Add 0.02 g of cefsulodin to 20 mL reagent-grade distilled water, sterilize using a 0.22-μm syringe filter, and store in a sterile tube at 4°C until needed. Prepare fresh solution each time MI medium is made. Do not save the unused portion.

*b. MI agar:**

Proteose peptone No. 3	5.0	g
Yeast extract	3.0	g
β-D-Lactose	1.0	g
4-Methylumbelliferyl-β-D-galactopyranoside (MUGal) (final concentration 100 μg/mL)	0.1	g
Indoxyl-β-D-glucuronide (IBDG) (final concentration 320 μg/mL)	0.32	g
NaCl	7.5	g
K_2HPO_4	3.3	g
KH_2PO_4	1.0	g
Sodium lauryl sulfate	0.2	g
Sodium desoxycholate	0.1	g
Agar	15.0	g
Reagent-grade distilled water	1	L

Autoclave medium for 15 min at 121–124°C, and add 5 mL of freshly prepared cefsulodin solution (¶ *a* above)(5 μg/mL final concentration) per liter of tempered agar medium. Final pH should be 6.95 ± 0.2. Pipet medium into 9- × 50-mm Petri dishes (5 mL/plate). Store plates at 4°C for up to 2 weeks.

c. MI broth:† Use same ingredients as MI agar, but omit agar. Prepare and sterilize, and add cefsulodin by the methods described for MI agar. Alternately, the broth can be filter-sterilized. Final pH should be 7.1 ± 0.2. Place absorbent pads in 9- × 50-mm Petri dishes and saturate with 2.0 to 3.0 mL MI broth containing 5 μg/mL final concentration of cefsulodin. Store plates in refrigerator, and discard after 96 h. Pour off excess broth before using plates.

3. Procedure

a. Selection of sample size: See 9222B.4*a*.

b. Sterile filtration units: See 9222B.4*b*.

c. Filtration of sample: See 9222B.4*c*.

d. Counting: To count colonies on membrane filters, use a low-powered (10 to 15× magnification) binocular wide-field dissecting microscope or other optical device with a cool white fluorescent light source directed to provide optimal viewing. Count all blue colonies on each MI plate under normal/ambient light and record as *E. coli* results. Positive results that occur in <24 h are valid, but results cannot be recorded as negative until the 24-h incubation period is complete. Expose each MI plate to long-wave UV light (366 nm), and count all fluorescent colonies [blue/green fluorescent *E. coli*, blue/white fluorescent TC other than *E. coli*, and blue/green with fluorescent edges (also *E. coli*)] to obtain the TC count. Record the data. If any blue, non-fluorescent colonies are found on the same plate, add their total to the TC count.

Calculate the final values using the following formula:

$$E.\ coli/100\ \text{mL} = \frac{\text{number of blue colonies}}{\text{volume of sample filtered (mL)}} \times 100$$

* BBL™ MI prepared plates (No. 214986), or equivalent.

† Dehydrated Difco™ MI Broth (No. 214882), or equivalent.

$$\text{TC/100 mL} = \frac{\text{number of fluorescent colonies} + \text{number of blue, non-fluorescent colonies (if any)}}{\text{volume of sample filtered (mL)}} \times 100$$

e. Coliform verification: For drinking water, total coliform colony verification is not required. For waters other than drinking water, verify at a frequency established by the laboratory (see Section 9020B.10). Laboratories may incorporate more stringent QC measures (e.g., verify at least one colony from each typical or atypical colony type from a given membrane filter culture, verify 10% of positive samples) based on need and sample type (see Section 9020B.10). Adjust counts based on verification results. Verification tests are listed in 9222B.4*g*.

4. Calculation of Coliform Density

See 9222B.5.

5. Bibliography

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9223 ENZYME SUBSTRATE COLIFORM TEST*

9223 A. Introduction

Enzyme substrate tests use hydrolyzable chromogenic and fluorogenic substrates to simultaneously detect enzymes produced by total coliforms and *Escherichia coli* (*E. coli*). In this method, total coliform bacteria produce the enzyme β-D-galactosidase, which cleaves the chromogenic substrate in the medium to release chromogen. Most *E. coli* strains produce the enzyme β-glucuronidase, which cleaves a fluorogenic substrate in the medium to release fluorogen. The release of chromogen indicates that coliform bacteria are present, and the release of fluorogen indicates that *E. coli* are present.

Multiple-tube, multi-well, or presence–absence (single 100-mL sample) formats are available for use with these enzyme substrate tests.

1. Principle

a. Total coliform bacteria: Colilert®, Colilert-18®, and Colisure® media use the chromogenic substrates ortho-nitrophenyl-β-D-galactopyranoside (ONPG) and chlorophenol red-β-D-galactopyranoside (CPRG), respectively, to detect the enzyme β-D-galactosidase, which is produced by total coliform bacteria. The β-D-galactosidase enzyme hydrolyzes the chromogenic substrate that produces a color change, thereby indicating the presence of total coliforms without additional procedures.

Although non-coliform bacteria (e.g., *Aeromonas, Flavobacterium,* and *Pseudomonas* species) may produce small amounts of the enzyme β-D-galactosidase, the growth of these organisms is suppressed so they generally will not produce a false-positive result unless $>10^6$ CFU/100 mL are present.

b. Escherichia coli: The fluorogenic substrate 4-methyl-umbelliferyl-β-D-glucuronide (MUG) is used to detect the enzyme β-D-glucuronidase, which is produced by most strains of *E. coli.* The β-D-glucuronidase enzyme hydrolyzes the fluorogenic substrate that produces bluish fluorescence when viewed under long-wavelength (365–366 nm) ultraviolet (UV) light. Together, the color change (due to β-D-galactosidase) and the fluorescence (due to β-D-glucuronidase) indicate that a sample contains *E. coli.*

Large numbers of some bacteria or strains of bacteria (e.g., some strains of *Shigella* and *Salmonella* spp.) may cause a sample to fluoresce but will not change its color because they lack β-D-galactosidase. Such samples would be considered negative for *E. coli*.

2. Applications

These enzyme substrate coliform tests are recommended for the analysis of drinking water, source water, groundwater, and wastewater samples. If a laboratory has not used this method before, it is desirable to conduct parallel testing (including seasonal variations) with the existing method to assess site-specific effectiveness and to compare results. The results of many method-performance studies are available in the literature and the rates of false-positive and -negative results differ among various media. Users should carefully select the medium and procedure that best fits their needs. See Section 9020B.11 for guidance on validating new methods.

Water samples containing humic or other material may be colored. If there is a natural background color, note what it is. If the water is yellow enough to be misinterpreted as a weak positive after incubation, use a medium that does not turn yellow (e.g., Colisure). Some waters' high calcium-salt content can cause precipitation, but this should not affect the reaction. In samples with excessive chlorine, a blue flash may be seen while adding Colilert or Colilert-18 media. If this occurs, consider sample invalid and discontinue testing.

Do not use the enzyme substrate test to verify presumptive coliform cultures or membrane-filter colonies, because the substrate may be overloaded by the heavy inoculum of weak β-D-galactosidase-producing noncoliforms, causing false-positive results.

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* Approved by Standard Methods Committee, 2016.
Joint Task Group: Jennifer Best (chair), Bennie L. Cockerel, Jr., Gil Dichter, Nancy H. Hall, William W. Northeimer, Viola Reynolds, Helena Solo-Gabriele.

9223 B. Enzyme Substrate Test

1. Samples

Collect samples as directed in Section 9060A, using sample containers specified in Section 9030B.19. When collecting chlorinated water samples, use sodium thiosulfate as described in Section 9060A.2. Follow the quality control (QC) guidelines for sample bottles described in Section 9020B.5*d*. Adhere to sample holding times and conditions as described in Section 9060B or required by regulations. Take care to ensure that samples are held at the appropriate temperature and analyzed as soon as possible after sample collection because failure to do so could compromise results. Ensure that samples meet laboratory-acceptance criteria upon receipt.

2. Quality Control

Method users must adhere to the quality assurance (QA)/QC guidelines in Section 9020, including, but not limited to, analytical QC (Section 9020B.9), instrumentation/equipment (Sections 9020B.4 and 9030B), and supplies (Section 9020B.5). Refer to Table 9020:I for key QC procedures.

Before using each lot of new medium, verify its performance via positive and negative control organisms. To conduct culture controls, inoculate medium with three control bacteria: *E. coli*, a total coliform strain other than *E. coli* (e.g., *Enterobacter cloacae*), and a noncoliform (see Table 9020:VI). An uninoculated negative control should also be analyzed. In addition, test me-

dium and vessels (bottles, multi-well trays, tubes) to confirm sterility and lack of autofluorescence.

3. Substrate Media

Colilert, Colilert-18, and Colisure media are available commercially* in premeasured packets for presence–absence testing or in disposable tubes for use in a multiple-tube format. The Quanti-Tray® and Quanti-Tray/2000* are multi-well formats that may be used with the premeasured packets to quantitate the coliform bacteria present in a sample.

Store media according to directions and use before expiration date. Avoid prolonged exposure of media to direct sunlight. Discard media that have changed color, appearance, and/or texture (media are hygroscopic and will clump and darken if exposed to moisture).

4. Procedure

Begin analysis by mixing the sample properly to promote even distribution of bacteria. For proper mixing to occur, samples should have ≥1-in. headspace and be shaken vigorously for 7 s (back and forth 1 ft approximately 25 times).

Failure to properly mix sample can lead to erroneous results, as bacteria are known to clump together and are therefore not homogeneously distributed throughout sample. For instance, most probable number (MPN) results are based on a Poisson (random) distribution of cells in the sample; failure to properly mix sample before analysis will result in an MPN value that underestimates actual bacterial density. Removing a portion of sample without proper mixing—such as when performing presence–absence analyses with a single bottle (one bottle used to both collect and analyze sample)—may result in false negative results if the target organisms were clumped together and removed from the bottle without being homogenized.

If the bottle lacks enough headspace for adequate mixing, pour sample into a larger sterile vessel so it can be mixed properly. Measure out desired sample volume and proceed with analysis.

For each medium or format used, tests should be placed in the incubator within 30 min after medium is added to sample. No matter which format is used, all media must be incubated at 35 ± 0.5°C. Colilert medium must be incubated for ≥24 h, Colilert-18 medium must be incubated for ≥18 h, and Colisure medium must be incubated for ≥24 h.

The coliform tests described here have been developed to obtain optimal bacterial growth at the indicated incubation temperatures. Failure to maintain this temperature throughout incubation could result in false negative results, especially with the shorter incubation times for Colilert-18. To ensure that samples are at proper temperature for the entire incubation period, laboratories should pre-warm samples after adding medium but before placing them in the incubator.

To pre-warm a test sample, place it in a 35 ± 0.5°C water bath for 20 min or in a 44.5 ± 0.2°C waterbath for 7 to 10 min to bring it to incubation temperature. The laboratory may need to conduct load studies to determine how long samples need to be incubated for effective pre-warming (depends on number of samples being incubated). Pre-warming is unnecessary if the Quanti-Tray format is used.

a. Presence–absence procedure (P/A): Aseptically add contents of packet containing premeasured medium to a 100-mL sample in a sterile, transparent, non-fluorescent borosilicate glass or equivalent bottle or container. Aseptically cap and shake vigorously to dissolve medium. Some medium may remain undissolved, but this will not affect test performance.

b. Multiple-tube procedure:

1) Multiple-tube procedure using a 5- or 10-tube MPN test—A 5-tube series (20 mL sample per tube) or 10-tube series (10 mL sample per tube) can be used when bacteria levels are anticipated to be fairly low or a fixed 100-mL sample volume must be analyzed (e.g., for regulatory compliance).

Add a premeasured packet of medium to a well-mixed 100-mL water sample in a container and shake vigorously to dissolve medium. Arrange tubes in rows of 5 or 10 in a test tube rack, and label each set of tubes. Aseptically dispense 20 mL sample into each of 5 sterile tubes or 10 mL into each of 10 sterile tubes, cap tightly, and mix vigorously to dissolve medium. If using 10 tubes already containing premeasured medium (available from manufacturer), aseptically dispense 10 mL sample into each tube.

Some medium particles may remain undissolved; this will not affect test performance.

After incubation, refer to Tables 9221:II and III to determine the MPN of total coliforms and *E. coli* present.

2) Multiple-tube procedure using 15-tube MPN test—A 15-tube test typically involves three serial dilutions of a sample, with each dilution inoculated into 5 tubes. Typically, 5 tubes contain undiluted sample, 5 contain a 1:10 dilution, and 5 contain a 1:100 dilution.

Use this technique when a water sample may contain higher bacteria levels and there is no requirement to analyze a fixed volume (e.g., when analyzing nonpotable waters). The number of tubes and sample volumes selected depend on the quality and characteristics of the water to be examined. To preclude any unwanted interaction with the medium, use only sterile, non-buffered, oxidant-free water (e.g., deionized or distilled water) to prepare dilutions.

When working with diluted samples, best laboratory practice is to ensure that all tubes are in place and labeled before analysis begins. Additionally, use clean, sterile pipets to pipet each dilution because bacterial carryover from dirty pipets will make test results inaccurate.

a) Using disposable tubes containing premeasured medium (available from manufacturer)

i) Preparing sample for the undiluted series—Aseptically pipet 10 mL of well-mixed sample into each of 5 tubes containing predispensed medium. Cap tubes and mix vigorously to dissolve medium.

ii) Preparing 1:10 dilution—Aseptically pipet 10 mL of well-mixed sample into a sterile vessel containing 90 mL of sterile, non-buffered, oxidant-free water (e.g., deionized or distilled water). Mix well. Aseptically pipet 10 mL of this dilution into each of 5 tubes containing pre-dispensed medium. Cap tubes and mix vigorously to dissolve medium.

iii) Preparing 1:100 dilution—Aseptically pipet 10 mL of well-mixed sample from the 1:10 dilution into a sterile vessel containing 90 mL of sterile, non-buffered, oxidant-free water

* Available from IDEXX Laboratories, Inc., Westbrook, ME.

(e.g., deionized or distilled water). Mix well. Aseptically pipet 10 mL of this dilution into each of 5 tubes containing predispensed medium. Cap tubes and mix vigorously to dissolve medium.

b) Using packets of premeasured medium

i) Preparing sample for the undiluted series—Add one packet of premeasured medium to a sterile vessel containing 100 mL of well-mixed sample, and mix vigorously to dissolve medium. Aseptically pipet 10 mL of sample/medium mixture into each of 5 sterile, non-fluorescing tubes.

ii) Preparing 1:10 and 1:100 dilutions—Add one packet of premeasured medium to 100 mL sterile, non-buffered, oxidant-free water (e.g., deionized or distilled water) in a sterile container, and mix vigorously to dissolve medium. Aseptically pipet 9 mL of prepared medium into 10 sterile, non-fluorescing tubes. This preparation of enzyme substrate medium must be completed ≤1 h of adding sample to prepared medium.

iii) Inoculating tubes for 1:10 dilution—Aseptically pipet 1 mL of well-mixed sample into each of 5 tubes containing 9 mL of prepared medium. Cap and mix well.

iv) Inoculating tubes for 1:100 dilution—Pipet 10 mL of well-mixed sample into a vessel containing 90 mL sterile, non-buffered, oxidant-free water (e.g., deionized or distilled water). Close and mix well to dissolve medium. Aseptically pipet 1.0 mL of this diluted sample into 5 tubes containing 9 mL of prepared medium. Cap and mix well.

For any additional dilutions needed, continue with the dilution process as described above.

After incubation, use Table 9221:IV to determine the MPN for both total coliforms and *E.coli*. If further dilutions were performed, the MPN value must be multiplied by the dilution factor to obtain the proper quantitative results.

c. Multi-well procedure: This procedure is performed with sterilized disposable multi-well trays [either the Quanti-Tray (51 well) or Quanti-Tray/2000]. Aseptically add premeasured medium from packet to a 100-mL water sample in a container and shake vigorously to dissolve medium. To open Quanti-Tray, use one hand to hold unit upright (with the well side facing the palm) and squeeze the upper part of the tray so it bends toward the palm. Gently pull foil tab to separate foil from tray, being careful not to touch the inside of either foil or tray. Add reagent–water sample mixture directly into tray, avoiding contact with foil tab. Gently tap the small wells (Quanti-Tray/2000) 2 to 3 times to release any air bubbles that may be trapped. Allow foam to settle, although some foam is acceptable. Place tray into the appropriate rubber insert with the well (plastic) side facing down, and feed it into the Quanti-Tray sealer. The sealer disperses the sample into the wells and seals the package.

5. Interpretation

a. Total coliform bacteria: The bacterial enzyme β-D-galactosidase hydrolyzes ONPG (Colilert and Colilert-18) to yield a yellow color and hydrolyzes CPRG (Colisure) to yield a red or magenta color. After the minimum incubation period, examine for the appropriate color change (Table 9223:I). If color response is not uniform throughout sample, mix by inversion before reading.

Use an unexpired color comparator (available from manufacturer) to ensure that Colilert and Colilert-18 test results are read accurately. The comparator used must have the same volume in the same type of container as the sample.

TABLE 9223:I. COLOR CHANGES FOR VARIOUS MEDIA

Substrate	Total Coliform Positive	*E. coli* Positive	Negative Result
Colilert® Colilert-18®	Yellow	Blue fluorescence	Colorless or color lighter than the comparator/no fluorescence
Colisure®	Red or magenta	Blue fluorescence	Yellow, pink, or orange/no fluorescence

1) Colilert—If sample color is as yellow as or darker yellow than the comparator, then it is positive for total coliforms. If not, then the sample is negative for total coliforms.

However, if the chromogenic response is ambiguous (color cannot be discerned) after 24 h, incubate sample for up to 4 h longer to allow test color to intensify. If the color does become as yellow as or darker than that of the comparator within this period, then the sample is positive for total coliforms. If not, then the sample is negative for total coliforms.

Colilert can be incubated for ≤28 h. After 28 h, negative test results are still considered valid, but positive results are not.

2) Colilert-18—If sample color is as yellow as or darker yellow than the comparator, then it is positive for total coliforms. If not, then it is negative for total coliforms.

However, if the chromogenic response is ambiguous (color cannot be discerned) after 18 h, incubate sample for up to 4 h longer to allow the test color to intensify. If the color does become as yellow as or darker than that of the comparator within this period, then the sample is positive for total coliforms. If not, then the sample is negative for total coliforms.

Colilert-18 can be incubated for ≤22 h. After 22 h, negative test results are still considered valid, but positive results are not.

3) Colisure—If the sample has a red or magenta color, it is positive for total coliforms. If the chromogenic response is questionable (color may be orange or pink) after 24 h, incubate sample for up to 24 h longer to allow test color to intensify. If color does become red or magenta within this period, then the sample is positive for total coliforms.

Colisure tests turn yellow after medium is added; if color does not change to red or magenta after incubation, then the sample is negative for total coliforms.

Colisure can be incubated for ≤48 h. After 48 h, results are not valid.

Sometimes a sample's high calcium-salt content can cause precipitation, but this will not affect the reaction. However, if the test medium turns an inappropriate color (e.g., green or black) that interferes with test-result reading, another method must be used.

b. Escherichia coli: The fluorogenic substrate MUG is hydrolyzed by the bacterial enzyme ß-D-glucuronidase to yield a bluish fluorescence when viewed under long-wavelength (365–366 nm) UV light. The color change (indicating β-D-galactosidase is active) and fluorescence (indicating β-D-glucuronidase is active) together show that *E. coli* is present.

After the minimum incubation period, examine positive total coliform tests for a bluish fluorescence; use a long-wavelength (365–366 nm) UV lamp with a 6-W bulb and hold it within 5 in. of sample in a dark environment. Use a color comparator (available

from the manufacturer) before its expiration date to ensure that test results are read accurately. The comparator used must have the same volume in the same type of container as the sample.

1) Colilert—If the sample has a bluish fluorescence equal to or greater than that of a total-coliform-positive comparator, then it is positive for *E. coli*. If the fluorescence is ambiguous (cannot be discerned) after 24 h, the sample may be incubated for up to 4 h longer to allow the fluorescence to intensify. If sample fluorescence does intensify to equal to or greater than that of the comparator within this period, then the sample is positive for *E. coli*.

If sample florescence remains less than that of the comparator after 28 h of incubation, then it is negative for *E. coli*. Samples that are negative for total coliform bacteria are also negative for *E. coli*.

2) Colilert-18—If the sample has a bluish fluorescence equal to or greater than that of a total-coliform-positive comparator, then it is positive for *E. coli*. If the fluorescence is ambiguous (cannot be discerned), the sample may be incubated for up to 4 h longer to allow the fluorescence to intensify. If sample fluorescence does intensify to equal to or greater than that of the comparator within this period, then the sample is positive for *E. coli*.

If sample florescence remains less than that of the comparator after 22 h of incubation, then it is negative for *E. coli*. Samples that are negative for total coliform bacteria are also negative for *E. coli*.

3) Colisure—If a total-coliform-positive sample fluoresces, then it is positive for *E. coli*. If the fluorescence is ambiguous (cannot be discerned), the sample should be incubated for up to 24 h longer to allow the fluorescence to intensify. If the sample clearly fluoresces within this period, then it is positive for *E. coli*.

If sample does not fluoresce after 48 h of incubation, then it is negative for *E. coli*. Samples that are negative for total coliform bacteria are also negative for *E. coli*.

6. Reporting

For the presence–absence procedure, report results as total coliforms and *E. coli* present or absent in a 100-mL sample.

For the multiple-tube procedure, calculate the MPN value for total coliforms and *E. coli* from the number of positive tubes, as described in Section 9221C.

For the multi-well procedure, determine the MPN from the appropriate MPN tables obtained from the tray manufacturer.

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9224 DETECTION OF COLIPHAGES*

9224 A. Introduction

1. General Discussion

Coliphages are bacterial viruses that infect and replicate in *Escherichia coli.* They are shed in human and animal feces. Although coliphages are not known to be hazardous to human beings, they are potentially important microorganisms for monitoring the microbial quality of water and wastewaters.[1]

The detection of coliphages has been of increasing interest since it has become clear that bacterial monitoring of waters and wastewaters may not adequately indicate the presence of viruses in those waters.[2] The presence of pathogenic human viruses in waters is a public health concern. Waterborne outbreaks of viral illnesses, such as gastroenteritis and hepatitis A, occur in the United States and elsewhere.[3,4] Detection of human enteric viruses in water and wastewaters, however, is beyond the capabilities of most water laboratories. Such detection traditionally has required the use of cell culture techniques.[5] These techniques are expensive, require skilled personnel, and have been both time- and labor-intensive. Coliphage assays, on the other hand, are relatively inexpensive, are easier to perform with trained personnel, and yield overnight results. Coliphage assays have been proposed as an alternative to human virus assays as an indicator of the viral quality of waters.[6,7]

Recent progress has been made in the development of specific coliphage methods for evaluating waters and wastewaters. Much of this work has focused on the detection of the group of coliphages known as the male-specific RNA coliphages (also referred to as the F-specific RNA coliphages or FRNA coliphages).[8] These coliphages are 20 to 30 nm in size, contain a single-stranded RNA genome, and have an isometric morphology. They exclusively infect bacterial cells that possess an F pilus, an appendage used for bacterial conjugation. Their significance lies in the fact that these coliphages are structurally similar to many human RNA viruses found in fecally contaminated waters. In particular, they resemble viruses of the picornavirus and calicivirus families, which include poliovirus; coxsackievirus; Norwalk and other noroviruses; hepatitis A virus; and hepatitis E virus. The human viruses cannot replicate in the environment. Similarly, the male-specific RNA coliphages have only limited replication in the environment at temperatures below 30°C.[9] Male-specific RNA coliphages also resemble many human enteric viruses in being relatively resistant to disinfection treatment practices. Because of these characteristics, male-specific RNA coliphages are promising candidate indicators of human viruses in environmental waters.

In the procedures presented here, methods have been included for the detection of the male-specific RNA coliphages using host *E. coli* Famp and for the detection of somatic coliphages using *E. coli* C.[10] Somatic coliphages, unlike the male-specific coliphages, are coliphages that do not require the presence of an F pilus to infect host cells. They represent a broad assortment of coliphage types and have often been included in environmental studies. Also presented here is a procedure that uses an alternate host bacterium, *Salmonella typhimurium* WG49. That host has been used by many laboratories to detect male-specific RNA coliphages and it previously has been used in one standard method protocol.[11] Although a double-agar-layer plaque assay has been specified in these procedures, a single-agar-layer method also is presented and can be used as an alternate plaque assay. Such a single-agar-layer assay has been incorporated into a method developed for the examination of ground waters.[12] One additional procedure, a membrane filter method for assaying 100-mL (and larger) sample volumes, is also presented here. Other methods are available elsewhere. One, an enrichment method, has particular usefulness as a presence-absence assay.[13] Unless otherwise indicated in the procedures described here, refer to Sections 9060A and B for guidance about sample collection, preservation, and storage.

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9224 B. Somatic Coliphage Assay

1. General Discussion

This plaque assay procedure for detecting somatic coliphages is a variant of the widely used double-agar-layer method;[1] it is based on the somatic coliphage assay procedure developed for regulatory purposes.[2,3] The procedure can be used, without supplementary methods, to assay small volumes of water and wastewater samples directly. In conjunction with large-scale concentration methods used to detect enteric viruses (Section 9510), the procedure also can be used to assay samples of much larger volume (>100 L). However, the use of electronegative filters at pH 3.5 is not recommended, because substantial loss of coliphage viability may occur. It is advisable to predetermine the suitability of any large-volume method in measured coliphage recovery trials. This plaque assay procedure uses *E. coli* C, one of the most effective host bacteria for detecting somatic coliphages.[4] Plaques produced with this host are readily distinguished, making plaque quantification relatively simple. The major advantage in detecting somatic coliphages is that they frequently are found in waters and wastewaters in greater abundance than the male-specific RNA coliphages. This can be an important consideration, especially for the assay of waters where pollution indices are expected to be low. The somatic coliphage group, however, is composed of different phage types (including both tailed and non-tailed phages) that exhibit widely varied characteristics. Somatic coliphages can replicate in the environment if a suitable host is present. Because of these factors, the somatic coliphage group may be less representative of human enteroviruses than the more uniform RNA phages of the male-specific coliphage group. The somatic coliphage group thus may not be the best group to serve as a specific indicator for human viruses in waters and wastewaters. However, the detection of somatic phages using *E. coli* C may be useful as a general indicator of water quality.[5]

2. Apparatus

a. Cryovials, 2-mL.
b. Erlenmeyer flasks, 125- and 250-mL, and 2-L.
c. Graduated cylinders, 100- and 500-mL.
d. Inoculating loop.
e. Laboratory balance.
f. Pipets, 1-, 5-, and 10-mL.
g. Petri dishes, 100- × 15-mm.
h. Filters, 0.22-μm. When passing material containing phage, always pass about 10 mL 1.5% beef extract through filter just before use to minimize phage adsorption to filter. Use presterilized filters or sterilize filters before use by autoclaving at 121°C for 15 min. At the time of use, use sterile technique to place filters into sterile sample filtration apparatus.
i. Test tubes, 16- × 150-mm, screw-capped and with closures.
j. Water bath set at 44.5 ± 1°C.

3. Media and Reagents

Adjust amount of media prepared proportionally to the number of samples. Use reagent-grade water (see Table 9020:II) in preparing media and reagents.

a. Beef extract, 1.5%: Dissolve 1.5 g beef extract powder and 0.375 g glycine (final glycine concentration = 0.05*M*) in 90 mL water. Adjust pH to 7.0 to 7.5, if necessary, and bring final volume to 100 mL with water. Autoclave at 121°C for 15 min and use at room temperature. Store at 4°C.

b. Glycerol solution, 50%: Add equal volumes of water and undiluted glycerol. Autoclave at 121°C for 15 min. Store at 4°C.

c. Tryptone agar slants:

Tryptone	1.0	g
Yeast extract	0.1	g
Glucose	0.1	g
NaCl	0.8	g
$CaCl_2$	0.022	g
Agar	1.2	g
Reagent-grade water	100	mL

With gentle mixing, add ingredients to water in a 250-mL flask. Dissolve and sterilize by autoclaving at 121°C for 15 min and dispense 8-mL portions into 16- × 150-mm test tubes with tube closures. Prepare slants by letting agar solidify with the tubes held at about a 20° angle. Slants may be stored at 4°C for up to 3 months.

d. Tryptone bottom agar: Prepare before sample analysis, using ingredients and concentrations listed for tryptone agar slants, but use 1.5 g agar. After autoclaving at 121°C for 15 min, pipet 15-mL portions aseptically into sterile 100- × 15-mm Petri dishes and let agar harden. Store dishes in sleeve bags at 4°C for up to 2 weeks and warm to room temperature for 1 h before use.

e. Tryptone broth: Prepare as for tryptone agar slants, excluding agar. Autoclave at 121°C for 15 min, and allow to cool before use. Broth may be stored at 4°C for up to 3 months in sterile, capped containers.

f. Tryptone dilution tubes: Aseptically, dispense 9-mL portions of sterile tryptone broth into 16- × 150-mm screw-capped test tubes presterilized by autoclaving at 121°C for 15 min.

g. Tryptone top agar: Use ingredients and concentrations listed for tryptone agar slants, but use 0.7 g agar. Autoclave at 121°C for 15 min and place in the 44.5 ± 1°C water bath. Agar may be stored at 4°C for up to 3 months in sterile, capped containers. Before using stored agar, melt solidified agar, then place in 44.5 ± 1°C water bath.

4. Procedure

a. Storage of E. coli *C host culture:* For short-term storage inoculate an *Escherichia coli* C* host culture onto tryptone agar slants with a sterile inoculating loop by spreading the inoculum evenly over entire slant surface. Incubate the culture overnight at 36.5 ± 2°C. Store at 4°C for up to 2 weeks. For long-term storage inoculate a 5- to 10-mL tube of tryptone broth with host culture. Incubate overnight at 36.5 ± 2°C. Add 1/5th volume of 50% glycerol solution. Dispense into 1-mL portions in 2-mL cryovials and store at −70°C, or lower.

b. Preparation of host: Inoculate 5 mL tryptone broth with *E. coli* C from a slant with an inoculating loop and incubate overnight (16–20 h) at 36.5 ± 2°C. Transfer 1.5 mL of the overnight culture to 30 mL tryptone broth in a 125-mL flask and incubate for 4 h at 36.5 ± 2°C with gentle shaking. The amount of inoculum and broth used can be altered proportionally according to need.

c. Preparation of coliphage ΦX174 positive control: Rehydrate a stock culture of somatic coliphage ΦX174† according to supplier directions and store at 4°C. Prepare a 30-mL culture of *E. coli* C as described above. Incubate for 2 h at 36.5 ± 2°C with shaking. Add 1 mL rehydrated phage stock and incubate for an additional 4 h at 36.5 ± 2°C. Filter through a beef-extract-treated 0.22-μm filter (see 9224B.2*h*). Prepare 10^{-7}, 10^{-8}, and 10^{-9} dilutions of the filtrate using tryptone dilution tubes. These dilutions should be sufficient for most ΦX174 stocks. Some stocks may require higher or lower dilutions. Add 3 mL melted tryptone top agar held in the 44.5 ± 1°C water bath to fifteen 16- × 150-mm test tubes. Keep these test tubes in heated water bath to avoid premature solidifying of agar. Add 0.1 mL host culture to each test tube. Add 1 mL 10^{-9} dilution to each of five test tubes.

Add 1 mL 10^{-8} dilution to five additional tubes and 1 mL 10^{-7} dilution to the remaining five tubes. Label tubes with appropriate dilution. For each tube, mix and immediately pour contents over bottom agar of a Petri dish labeled with the dilution assayed. Rotate dish to spread suspension evenly and place it on a level surface to let agar solidify. Incubate at 36.5 ± 2°C overnight and examine for plaques the following day. Count number of plaques on each dish. To determine the titer of the stock filtrate, use the dilution with dishes exhibiting 20 to 100 plaques. Average the plaque counts on these five dishes and multiply result by the reciprocal of the dilution to obtain that titer. For use as a positive control in the coliphage assay, dilute stock filtrate to 30 to 80 PFU/mL in tryptone broth. Store original filtrate and diluted positive control preparation at 4°C. Before using the positive control preparation for the first time, assay 10 mL by adding 1-mL volumes of the preparation to ten test tubes containing agar and host culture, pouring their contents into ten Petri dishes and incubating overnight at 36.5 ± 2°C. Count plaques on all dishes and divide by 10. If result is not 30 to 80, adjust dilution of the positive control sample and re-assay.

d. Assay procedure: Add 3 mL melted tryptone top agar held at 44.5 ± 1°C to each of ten 16- × 150-mm test tubes for sample assay and to each of two additional test tubes to serve as negative and positive controls. Hold test tubes in water bath to avoid premature solidifying of agar. Add 0.1 mL of host culture to each of the 12 test tubes. Add 1 mL tryptone broth to test tube serving as negative control. Add 1 mL ΦX174 preparation (30 to 80 PFU/mL) to test tube serving as positive control. To each of the remaining 10 tubes, add 1 mL sample. For each tube, mix and immediately pour contents over bottom agar of a Petri dish that has been suitably labeled. Tilt and rotate dish to spread suspension evenly and place it on a level surface to let agar solidify. Incubate at 36.5 ± 2°C overnight and examine for plaques the following day. Count total number of plaques on the ten dishes receiving the sample. Calculate the somatic coliphage concentration according to the formula:

$$C_a = (P \div 10) \times D$$

where:

C_a = somatic coliphage concentration, PFU/mL,
P = total number of plaques from the 10 dishes, and
D = reciprocal of dilution made on the inoculum before plating (D = 1 for undiluted samples).

If the sample dishes are completely lysed or yield plaques that are too numerous to count, dilute sample and assay again. In the calculation for diluted samples, D will be >1 (e.g., D = 10, for a sample diluted 1:10 or D = 100, for a sample diluted 1:100).

If the sample assayed is the product of a concentration procedure, such as an eluate from a large-volume filter sampling of water or wastewater, calculate coliphage concentration of the sampled water according to the formula:

$$C_b = \frac{C_a \times V_a}{V_b}$$

where:

C_b = somatic coliphage concentration of sampled water, PFU/L,
C_a = coliphage concentration of concentrated material, PFU/mL,
V_a = volume of that material, mL, and
V_b = volume of water processed in sampling procedure, L.

The resultant concentration generally is reported as PFU/L or PFU/100 L. If the sample dishes are completely lysed or yield plaques that are too numerous to count, dilute sample and assay again. Count plaques on the positive control dish. Maintain a record of the plaque count as a check on the virus sensitivity of the *E. coli* C host. Assay any water eluate samples again where the positive control counts are more than one log below their normal average. Should plaques be detected on the negative control dish, discard assay results and repeat assay.

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9224 C. Male-Specific Coliphage Assay Using *Escherichia coli* Famp

1. General Discussion

Like the somatic coliphage assay, this male-specific assay is a variant of the double-agar-layer method[1] and is based on procedures developed for regulatory purposes.[2] Similarly, this male-specific assay can be used, without supplementary methods, to assay small volumes of water and wastewater samples directly. In conjunction with large-scale concentration methods used to detect enteric viruses (Section 9510), the procedure also can be used to assay larger-volume samples (>100 L). However, the use of electronegative filters at pH 3.5 is not recommended because substantial loss of coliphage viability may occur. Determine the suitability of any large-volume method in measured coliphage recovery trials, before using it. This procedure uses *E. coli* Famp as the host bacterium.[3] This strain is resistant to ampicillin and streptomycin antibiotics. It is one of two bacterial strains that have been developed to selectively detect male-specific coliphages. For a procedure using the other bacterial strain, *Salmonella typhimurium* WG49, see 9224D. It has not been universally established that one strain performs better than the other. Individual laboratories may evaluate both strains before selecting one for routine use. Male-specific plaques can be difficult to recognize in comparison to somatic plaques. They are generally less well-defined than somatic plaques. Use diligence when examining dishes for male-specific plaques. As male-specific DNA coliphages and some somatic coliphages also can produce plaques on host *E. coli* Famp, preferably confirm that plaques quantified by the assay are caused by RNA phages. An RNase procedure that permits differential quantification of the RNA coliphages also is presented below. The advantage in detecting male-specific RNA coliphages is that these coliphages more closely resemble human enteroviruses than do the varied phage types of the somatic group. These male-specific RNA coliphages appear to be useful as a model for human viruses.[4,5] As an indicator of the presence of human viruses in waters and wastewaters, their role remains to be fully established.

2. Apparatus

See 9224B.2.

3. Media and Reagents

Adjust amount of media prepared proportionally to the number of samples. Use reagent-grade water (see Table 9020:II) in making media and reagents.

a. Ampicillin solution: Dissolve 0.15 g ampicillin in 100 mL water and filter with the 0.22-μm filter (beef extract pretreatment not necessary). Store at 4°C.

b. Beef extract, 1.5%: See 9224B.3*a*.

c. Glycerol solution, 50%: See 9224B.3*b*.

d. Ribonuclease (RNase) solution: Dissolve 100 mg RNase containing 50 to 100 Kunitz units/mg in 100 mL water by heating to 100°C for 10 min. Store at −20°C in 0.5-mL portions.

e. Streptomycin solution: Dissolve 0.15 g streptomycin sulfate in 100 mL water and filter-sterilize with 0.22-μm filter (beef extract pretreatment not necessary). Store at 4°C.

f. Tryptone agar slants: Mix ingredients, dissolve, and sterilize as directed in 9224B.3*c*. Let autoclaved agar equilibrate in water bath set at 44.5 ± 1°C, then add 1.0 mL filtered ampicillin solution and 1.0 mL filtered streptomycin solution/100 mL volume of warm agar. Dispense portions, prepare, and store as directed in 9224B.3*c*.

g. Tryptone bottom agar: See 9224B.3*d*. After autoclaving at 121°C for 15 min, let agar equilibrate in water bath set at 44.5 ± 1°C, and then add 1.0 mL filtered ampicillin solution and 1.0 mL filtered streptomycin solution/100-mL volume of warm agar. Dispense portions and store as directed in 9224B.3*d*.

h. Tryptone broth: Use ingredients listed in 9224B.3*c*, excluding agar. Sterilize by autoclaving at 121°C for 15 min. Cool and add 1.0 mL filtered ampicillin solution and 1.0 mL filtered streptomycin solution/100 mL broth. Store at 4°C. Use for growth medium. Store as directed in 9224B.3*c*.

i. Tryptone dilution tubes: See 9224B.3*f*.

j. Tryptone top agar: Use ingredients listed in 9224B.3*c*, but use 0.7 g agar. After autoclaving at 121°C for 15 min, let agar equilibrate in a 44.5 ± 1°C water bath, then add 1.0 mL filtered ampicillin solution and 1.0 mL filtered streptomycin solution /100 mL volume of warm agar. Store as directed in 9224B.3*g*.

4. Procedure

a. Storage of E. coli *Famp host culture:* Follow procedures of 9224B.4*a*, using *Escherichia coli* Famp.* For this strain, use media containing ampicillin and streptomycin as described above.

b. Preparation of host: Follow procedures of 9224B.4*b*. Place on ice until used for assay to prevent loss of F pili (use within 3 h).

* American Type Culture Collection Product No. 700891.

c. Preparation of coliphage MS2 positive control: Rehydrate a stock culture of male-specific RNA coliphage MS2† according to supplier directions and store at 4°C. Prepare a 30-mL culture of *E. coli* Famp as described in 9224B.4*b*. Incubate and proceed as directed in 9224B.4*c*, using the MS2 and *E. coli* Famp.

d. Assay procedure: Proceed as in 9224B.4*d*, using MS2 as positive control. Calculate C_a as directed. If the sample dishes are completely lysed or yield plaques that are too numerous to count, dilute sample and assay again.

If the sample assayed is the product of a concentration procedure, calculate C_b as directed in 9224B.4*d*.

e. Assay procedure with additional confirmation: Proceed as in 9224B.4*d*, using MS2 as positive control, but assay an additional 10 mL of sample. For the additional 10 mL of sample, add RNase solution to the melted tryptone top agar to a concentration of 40 μg/mL. Before pouring tube contents to Petri dishes, label dishes so that the 10 dishes containing RNase are readily distinguished from the 10 dishes without RNase. Calculate the male-specific RNA coliphage concentration (C_a) in PFU/mL according to the formula:

$$C_a = [(P \div 10) - (P_{RNase} \div 10)] \times D$$

where:

C_a = male-specific RNA coliphage concentration, PFU/mL,
P = total number of plaques from the 10 dishes without RNase,
P_{RNase} = total number of plaques from the 10 dishes with RNase, and
D = reciprocal of the dilution made on the inoculum before plating.

If the sample assayed is the product of a concentration procedure, calculate coliphage concentration as previously described in ¶ *d* above.

† American Type Culture Collection Product No. 15597-B1.

5. References

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9224 D. Male-Specific Coliphage Assay Using *Salmonella typhimurium* WG49

1. General Discussion

This male-specific assay uses *Salmonella typhimurium* WG49 as host bacterium. It is a variant of the double-agar-layer method[1] and has been adapted from several published procedures.[2,3] The assay can be used with large-scale concentration methods (see 9224C.1). The use of *S. typhimurium* WG49 in this assay effectively eliminates plaque production by somatic coliphages. This is an advantage over the *E. coli* Famp-based assay. However, somatic salmonella phages do form plaques with *S. typhimurium* WG49 and these somatic salmonella phages also may be present in waters under investigation. Because it has not been established that either *S. typhimurium* WG49 or *E. coli* Famp is universally superior as host bacterium for the male-specific phages, individual laboratories may find it useful to evaluate both assays before making a selection. With either assay, take care in examining dishes for male-specific plaques and confirm that the plaques detected are indeed caused by RNA coliphages and not other phages. An RNase procedure for this purpose is presented below. Also presented below is a treatment to neutralize the somatic salmonella phage content of samples before assay. This treatment may be necessary if assay interference by somatic salmonella phages is encountered.[4–6] Such interference may be expected because somatic salmonella phages do outnumber male-specific phages in some waters.[7] The advantage of the *S. typhimurium* WG49-based assay, as with the *E. coli* Famp-based assay, is that the targeted male-specific RNA phages possess a similarity to human enteroviruses not found with the somatic coliphage group. Assay of these male-specific phages can be useful in evaluating water and wastewater treatment processes but as a specific indicator of the presence of human viruses, their role remains to be clearly established.

2. Apparatus

See 9224B.2.

3. Media and Reagents

Adjust amount of media prepared proportionally to the number of samples. Use reagent-grade water (see Table 9020:II) in preparing media and reagents.

a. Beef extract: See 9224B.3*a*.

b. Glycerol solutions, 50%: See 9224B.3*b*.

c. Kanamycin solution: Dissolve 1 g kanamycin monosulfate in 100 mL water and filter with the 0.22-μm filter (beef extract pretreatment not necessary). Store at 4°C.

d. Lipopolysaccharide solution (LPS): Prepare stock solution of phenol-extracted *S. typhimurium* LPS* by dissolving the lyophilized LPS in sterile phosphate-buffered saline to yield an LPS concentration of 2 mg/mL. Store at 4°C.

* Sigma Chemical Co., St. Louis, MO, item No. L6511, or equivalent.

e. Nalidixic acid solution: Dissolve 1 g nalidixic acid in 100 mL water and filter with the 0.22-μm filter (beef extract pretreatment not necessary). Store at 4°C.

f. Phosphate-buffered saline: Dissolve 0.8 g NaCl, 20 mg KCl, 12 mg KH_2PO_4, and 91 mg Na_2HPO_4 in 100 mL water. Sterilize by autoclaving at 121°C for 15 min.

g. Ribonuclease (RNase) solution: See 9224C.3*d*.

h. Tryptone agar slants: Mix ingredients, dissolve and sterilize as directed in 9224B.3*c*. Let autoclaved agar equilibrate in water bath at 44.5 ± 1°C, then add 1 mL filtered nalidixic acid solution and 0.2 mL filtered kanamycin solution/100 mL volume of warm agar. Dispense portions and store as directed in 9224B.3*c*.

i. Tryptone bottom agar: See 9224B.3*d*. After autoclaving at 121°C for 15 min, let agar equilibrate in water bath at 44.5 ± 1°C, then add 1 mL filtered nalidixic acid solution and 0.2 mL filtered kanamycin solution/100 mL volume of warm agar. Dispense portions and store as directed in 9224B.3*d*.

j. Tryptone broth: Use ingredients listed in 9224B.3*c*, excluding agar. Sterilize by autoclaving at 121°C for 15 min. Cool and add 1 mL filtered nalidixic acid solution and 0.2 mL filtered kanamycin solution/100 mL broth. Use for growth medium. Store as directed in 9224B.3*c*.

k. Tryptone dilution tubes: See 9224B.3*f*.

l. Tryptone top agar: Use ingredients listed in 9224B.3*c*, but use 0.7g agar. After autoclaving at 121°C for 15 min, let agar equilibrate in a 44.5 ± 1°C water bath, then add 1 mL filtered nalidixic acid solution and 0.2 mL filtered kanamycin solution/100 mL volume of warm agar. Store as directed in 9224B.3*g*.

4. Procedure

a. Storage of S. typhimurium *WG49 host culture:* Follow procedures of 9224B.4*a*, using *S. typhimurium* WG49† host culture. *S. typhimurium* WG49 is resistant to nalidixic acid and kanamycin. Accordingly, use media containing nalidixic acid and kanamycin as described above.

b. Preparation of host: Follow procedures of 9224B.4*b*. Place on ice until used in assay to prevent loss of F pili (use within 3 h).

c. Preparation of coliphage MS2 positive control: Rehydrate a stock culture of male-specific RNA coliphage MS2‡ according to supplier directions and store at 4°C. Prepare a 30-mL culture of *S. typhimurium* WG49 as in 9224B.4*b*. Incubate and proceed as directed in 9224B.4*c*, using the MS2 and *S. typhimurium* WG49.

d. Assay procedure: Proceed as in 9224B.4*d*, using MS2 as positive control. Calculate C_a as directed. If the sample dishes are completely lysed or yield plaques that are too numerous to count, dilute sample and assay again.

If the sample assayed is the product of a concentration procedure, calculate C_b as directed in 9224B.4*d*.

e. Assay procedure with additional confirmation: See 9224C.4*e*.

f. Sample treatment procedure to reduce somatic salmonella phages: Results of the confirmation procedure may reveal assay interference by somatic salmonella phages present in some water or wastewater samples. To neutralize these somatic salmonella phages before assay, add enough LPS stock solution to the sample to produce a final LPS concentration of 20 μg/mL. Mix thoroughly and hold at ambient temperature for 15 to 30 min. Then assay as described above.

5. References

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† American Type Culture Collection Product No. 700730.
‡ American Type Culture Collection Product No. 15597-B1.

9224 E. Single-Agar-Layer Method

1. General Discussion

This method can be used as an alternative to the double-agar-layer procedures described above. It uses a single-agar-layer format and larger Petri dishes. The method enables more sample material to be assayed per dish and can be used to directly assay 100-mL volumes of water and wastewater.[1] Although originally described for use with *E. coli* C, the procedure is suitable for use with *E. coli* Famp and *S. typhimurium* WG49 host bacteria. The general procedure is described below. For specific details relating to each of the three bacterial hosts, refer to 9224B, C, and D.

2. Apparatus

See 9224B.2. Use 150- × 15-mm Petri dishes for sample assay.

3. Media and Reagents

The amount of media prepared may be increased proportionally to the number of samples to be analyzed. Use reagent-grade water (see Table 9020:II) in preparing media and reagents. For all host cultures:

a. Beef extract: See 9224B.3*a*.

b. Calcium chloride solution: Add 22 g $CaCl_2$ to 50 mL water and sterilize by autoclaving at 121°C for 15 min. Use at room temperature.

c. Glycerol solution, 50%: See 9224B.3*b*.

d. Tryptone agar slants: See 9224B.3*c*.

e. Tryptone agar:

Tryptone	2.0	g
Yeast extract	0.2	g
Glucose	0.2	g
NaCl	1.6	g
$CaCl_2$	0.022	g
Agar	1.2	g
Reagent-grade water	100	mL

Sterilize by autoclaving at 121°C for 15 min. Place in the 44.5 ± 1°C water bath.

f. Tryptone broth: See 9224B.3*d*, excluding the agar.

g. Tryptone dilution tubes: See 9224B.3*f*.

For *E. coli* Famp host only:

h. Ampicillin solution: See 9224C.3*a*.

i. Streptomycin solution: See 9224C.3*e*.

For *S. typhimurium* WG49 host only:

j. Kanamycin solution: See 9224D.3*c*.

k. Lipopolysaccharide solution (LPS): See 9224D.3*d*.

l. Nalidixic acid solution: See 9224D.3*e*.

For *E. coli* Famp and *S. typhimurium* WG49 hosts:

m. Ribonuclease (RNase) solution: See 9224C.3*d*.

4. Procedure

a. Storage of host cultures: Follow procedures of 9224B.4*a*, using appropriate host culture.

b. Preparation of host: Follow procedures of 9224B.4*b*.

c. Preparation of coliphage positive controls: Rehydrate a stock culture of appropriate positive control according to supplier directions and proceed as directed in 9224B.4*c*.

d. Sample filtration (somatic coliphage assay only): The bacteria present in samples for analysis can interfere with the plaque assay. To eliminate such interference, filter sample before assay. Use sterile 0.22-μm filter unit. To minimize phage adsorption to the filter, pass about 10 mL 1.5% beef extract through filter before passing sample through filter. NOTE: As an alternative to sample filtration, use *E. coli* strain CN-13* in place of *E. coli* C in the somatic coliphage assay. *E. coli* CN-13 is a nalidixic-acid-resistant variant of *E. coli* C; using it permits the addition of nalidixic acid to the assay media (see 9224D) to hinder growth of indigenous bacteria. *E. coli* CN-13 appears equivalent to *E. coli* C in somatic coliphage detection.[2]

e. Assay procedure: Place 100 mL sample in the 44.5 ± 1°C water bath for 3 min. Add 5 mL $CaCl_2$ solution and 5 mL appropriate host bacterium preparation to the warmed sample. Mix inoculated sample with 100 mL melted tryptone agar at 44.5 ± 1°C and distribute to eight 150- × 15-mm Petri dishes. For a positive control, mix 1 mL of appropriate positive control preparation (30 to 80 PFU/mL) and 1 mL host bacterium with 12.5 mL warmed agar that has been diluted with an equal volume of warm sterile water. Pour to a single 150- × 15-mm Petri dish. Repeat for a negative control but omit the 1 mL of phage preparation. Incubate the inoculated dishes at 36.5 ± 2°C overnight and examine for plaques the following day. Count total number of plaques on the eight dishes that received the sample; total is coliphage concentration/100 mL sample. However, when assaying for male-specific RNA coliphages using *E. coli* Famp and *S. typhimurium* WG49, this count could include some somatic and male-specific DNA coliphages or some somatic salmonella phages present in the sample. For appropriate procedures to address the presence of undesired phage, see ¶s *f* and *g* below.

f. Assay procedure with additional confirmation (male-specific assays only): Assay an additional 100-mL sample portion as described above, but add RNase solution to the melted tryptone agar. For the additional material, the melted tryptone agar should contain RNase at a concentration of 60 μg/mL (before sample is added). Appropriately label dishes so the eight dishes containing RNase are readily distinguished from the eight dishes without RNase. Calculate the male-specific coliphage concentration according to the formula:

$$C_a = P - P_{RNase}$$

where:

C_a = male-specific coliphage concentration, PFU/100 mL,
P = total number of plaques from dishes without RNase, and
P_{RNase} = total number of plaques from dishes with RNase.

g. Sample treatment procedure to reduce somatic salmonella phages (WG49 host assay only): To neutralize these somatic salmonella phages before assay, add enough LPS stock solution to sample to produce a final LPS concentration of 20 μg/mL. (NOTE: Add LPS after adding $CaCl_2$ to sample and before adding WG49 host.) Mix thoroughly and hold at ambient temperature for 15 to 30 min. Then add WG49 host and assay as described above.

5. References

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* American Type Culture Collection Product No. 700609.

9224 F. Membrane Filter Method

1. General Discussion

This method uses a filter-adsorption technique to recover coliphages from water samples. With this method, 100-mL and larger volume samples can be assayed effectively by a simple procedure in which the sample is first passed through a membrane filter. Coliphages present in the sample are adsorbed onto the filter and the phage-adsorbed filter is then directly plaque assayed.[1,2] The advantage of this method is that, unlike 9224B–E, it does not require multiple assay dishes for each sample. A single assay dish is utilized for each phage-adsorbed filter. This significantly reduces the time and materials required. The disadvantage is that extraneous material, which can interfere with subsequent plaque assay, can accumulate on filters as water is passed through them. The amount of accumulation depends on the quality of the water being sampled and increases in proportion to the sample volume. Keep sample volumes as small as possible to prevent observable accumulation of discoloring material on the filter. However, with poor-quality waters, rapid accumulation may be observed with even small sample volumes. With such waters, use larger-diameter filters to increase sample volume. In the procedure below, the use of 47-mm membrane filters is described, but 90-mm membrane filters may be used if sample volumes prove inadequate with the smaller filters. Although originally described for use with *S. typhimurium* WG49, the procedure is suitable for use with *E. coli* C, and *E. coli* Famp host bacteria. The general procedure is described below. For specific details relating to each of the three bacterial hosts, refer to the preceding sections (Sections 9224B, C, and D). Although a membrane-filter method is presented here, other approaches also may prove effective for the assay of 100-mL and larger volume samples. Methods such as the most probable number method could be adapted to larger volumes,[3] and such non-filter-based methods probably would be less susceptible to poor water quality. Consider their use if water conditions are encountered that make the membrane filter method impractical.

2. Apparatus

See 9224B.2*a–f* and *h–j*, and in addition:

a. Petri dishes, 60 × 15 mm.

b. Sample vacuum filtration apparatus, including a 47-mm membrane filter holder and filtrate receiving vessel. Sterilize apparatus before use by autoclaving at 121°C for 15 min.

c. Sample membrane filters, 0.45-μm pore size with a 47-mm diam and a cellulose nitrate and acetate composition. Sterilize filters before use by autoclaving at 121°C for 15 min (or use pre-sterilized filters). At the time of use, use sterile technique to place sterile filter into sterile sample filtration apparatus.

3. Media and Reagents

The amount of media prepared may be increased proportionally to the number of samples to be analyzed. Use reagent-grade water (see Table 9020:II) in preparing media and reagents.

For all host cultures:

a. Beef extract: See 9224B.3*a*.

b. Magnesium chloride solution, 2*M:* Dissolve 19.04 g $MgCl_2$ in 100 mL water and sterilize by autoclaving at 121°C for 15 min. Use at room temperature.

c. Glycerol solution, 50%: See 9224B.3*b*.

d. Phosphate-buffered saline: Dissolve 0.8 g NaCl, 20 mg KCl, 12 mg KH_2PO_4, and 91 mg Na_2HPO_4 in 100 mL water. Sterilize by autoclaving at 121°C for 15 min.

e. Tetrazolium violet solution: Add 3 g tetrazolium violet to 100 mL water and filter-sterilize with 0.45-μm filter. Use 1 mL tetrazolium violet solution/100 mL agar.

f. Tryptone agar slants: See 9224B.3*c*.

g. Tryptone agar: Use ingredients and concentrations listed for tryptone broth, and add 0.9 g agar and 15 mg $MgSO_4$/100 mL of broth. Sterilize by autoclaving at 121°C for 15 min. Place autoclaved agar in the 44.5 ± 1°C water bath. After agar has cooled, add 1 mL filtered polysorbate solution and 1 mL filtered tetrazolium violet solution/100 mL volume of warm agar. Then add 2 mL of appropriate host bacterium preparation per 100 mL agar. Dispense agar by pipet in 5-mL portions to sterile 60- × 15-mm Petri dishes and let agar harden. If plates are to be used the following day, store at 4°C overnight and warm to room temperature for 1 h before use.

h. Tryptone broth: See 9224B.3*e*.

i. Tryptone dilution tubes: See 9224B.3*f*.

j. Polysorbate solution: Add 30 g polysorbate* to 100 mL water and filter with 0.22-μm filter. Use 1 mL/100 mL agar.

For *E. coli* Famp host only:

k. Ampicillin solution: See 9224C.3*a*.

l. Streptomycin solution: See 9224C.3*e*.

For *S. typhimurium* host only:

m. Kanamycin solution: See 9224D.3*c*.

n. Lipopolysaccharide solution (LPS): See 9224D.3*d*.

o. Nalidixic acid solution: See 9224D.3*e*.

For *E. coli* Famp and *S. typhimurium* WG49 hosts:

p. Ribonuclease (RNase) solution: See 9224C.3*d*.

4. Procedure

a. Storage of host cultures: Follow procedures of 9224B.4*a*, using appropriate host culture.

b. Preparation of host: Follow procedures of 9224B.4*b*.

c. Preparation of coliphage positive controls: Rehydrate a stock culture of appropriate positive control according to supplier directions and proceed as directed in 9224B.4*c*.

d. Assay procedure: For each 100 mL sample, use a single Petri dish assay. After dishes have been prepared, add 2.5 mL $MgCl_2$ solution to sample to yield a final concentration of 0.05*M*. Pass magnesium-chloride-supplemented sample through sterile sample filter at a rate sufficient to clear entire 102.5 mL within 1 to 3 min. Using sterile technique, remove filter from sample filter apparatus. Taking care to prevent formation of bubbles,

* Tween 80, or equivalent.

apply filter, face down, onto surface of one of the pre-poured agar Petri dishes. Incubate at 36.5 ± 2°C overnight and examine for plaques the following day. Count total number of plaques. That total is the coliphage concentration/100 mL sample. However, in assays for the male-specific RNA coliphages using *E. coli* Famp and *S. typhimurium* WG49, this count could include some somatic and male-specific DNA coliphages or some somatic salmonella phages present in the sample. For appropriate procedures to address the presence of undesired phage see ¶s *e* and *f* below.

e. Assay procedure with additional confirmation (male specific coliphage assay only): Assay an additional 100-mL sample portion. Proceed as described above, but add RNase solution to the melted tryptone agar to give a concentration of RNase of 40 μg/mL. Label assay dish containing RNase so that it is readily distinguished from the corresponding assay dish without RNase. After overnight incubation at 36.5 ± 2°C, determine male-specific RNA coliphage concentration/100 mL sample by subtracting total number of plaques counted on the RNase-containing dish from total number counted on the dish without RNase.

f. Sample treatment procedure to reduce somatic salmonella phages (WG49 host assay only): To neutralize somatic salmonella phages before assay, treat sample filters with an LPS solution made in PBS that contains 50 m*M* $CaCl_2$ and has an LPS concentration of 20 μg/mL. After passing 100 mL sample through filter, pass 5 mL of LPS solution through filter. Handle LPS-treated filter as described in the above assay procedure with untreated filters.

5. References

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9225 DIFFERENTIATION OF COLIFORM BACTERIA*

9225 A. Introduction

The significance of various coliform organisms in water has long been a subject of considerable study. Collectively, coliform bacteria (also called *total coliforms*) are considered indicators of possible inadequate sanitation, fecal contamination, and/or pathogenic and opportunistic pathogenic bacteria. However, completely and accurately differentiating coliform bacteria remains a challenge.

Until recently, the definition of *coliform bacteria* did not include the genera and species of *Enterobacteriaceae*. The traditional definition of coliform bacteria, based on lactose fermentation with the production of gas, excluded some strains of *Escherichia coli* and included some strains of enteric pathogens (e.g., *Salmonella* and *Shigella*).[1] However, as a result of DNA hybridization tests and other molecular methods, researchers now classify many species of *Enterobacteriaceae* as coliform bacteria.[1–4] There are 18 *Enterobacteriaceae* genera with species or distinct biogroups that ferment lactose and produce gas during fermentation. Sixty-eight of the 134 named *Enterobacteriaceae* (59%) are coliforms.[2] These must be considered along with traditional coliforms (e.g., *Citrobacter*, *Enterobacter*, *Escherichia*, and *Klebsiella*) during coliform differentiation. Information on nomenclature, classifications, original literature citations, and detailed information for genera and species in the family *Enterobacteriaceae* and newly identified species, with their publication date, is available.†

Users must decide whether a coliform culture requires a precise taxonomical identification due to safety, public health, or a regulatory requirement. This is not a normal practice in the standard water-testing laboratory.

A history of coliform bacteria identification and differentiation can be found in the literature.[3,4] Other recent reviews[1,2,4] detail coliform differentiation and identification, and discuss the many variables involved.

Completely and accurately identifying coliform bacteria is a daunting task; an *Enterobacteriaceae* reference laboratory may be needed. A practical solution for a water laboratory is to consider several coliform-differentiation methods, to word reports precisely, and to include comments qualifying the reported results (see 9225E for examples).

In the following sections, both cited references and a bibliography are available for further information.

1. Terminology

Total coliforms are historically defined as facultative anaerobic, Gram-negative, non-spore-forming rods that ferment lactose with acid and/or gas formation within 48 h at 35°C. However, there are also slow-growing and anaerogenic (non gas-producing) lactose-fermenting strains of *Escherichia coli* and coliforms.

A new definition of coliform bacteria—based on enzyme-substrate tests—includes those that produce the enzyme β-D-galactopyranosidase to hydrolyze chromogenic substrates, such as ortho-nitrophenyl-β-D-galactopyranoside (ONPG) or chlorophenol red-β-D-galactopyranoside (CPRG). This reaction generally occurs within 24 ± 2 h or less at 35°C. When using chromogenic media, *E. coli* are defined as coliform bacteria with the enzyme β-glucuronidase, which hydrolyzes 4-methyl-umbelliferyl-β-D-glucuronide (MUG) to 4-methylum-belliferone, a fluorescent compound.‡

A few coliform bacteria can grow under elevated temperature conditions (e.g., 44.5°C), but up to 10% of *E. coli* cannot grow at an elevated temperature.[5] However, many commercial and traditional microfiltration methods incubate cultures at 35°C with various enzyme/substrates to differentiate *E. coli* from other coliforms. So, a requirement for thermophilic incubation would both be problematic and challenge the specificity of these methods, many of which have been subjected to extensive validation.

2. References

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* Approved by Standard Methods Committee, 2011.
Joint Task Group: 22nd Edition—Margo E. Hunt (chair), Michael H. Brodsky, Archie J. Degnan, Gil Dichter, J.J. Farmer, III, Peter Feng.

† See http://www.bacterio.cict.fr/. Accessed February 2017.

‡ *E. coli* may be confirmed via broth media (e.g., Sections 9221F and 9222G) or media incubated at elevated temperatures.

9225 B. Culture Purification

1. Procedure

A pure culture is essential for accurate identification. "Presumptive positive" tests for coliforms occur either in a broth culture tube (acid and/or gas production) or a lactose-positive colony on a membrane filter. For enzyme substrate tests, use a test broth, which is positive. Both broth and membrane filter cultures are often mixed—not pure—cultures. Streak a loopful of the liquid sample (or a colony) onto a plate containing a suitable growth medium (e.g., MacConkey agar) to obtain 50 to 100 isolated colonies. Alternatively, produce a pour or spread plate with the culture to obtain 50 to 100 isolates (for details, review Sections 9215B and C). MacConkey agar is ideal because it is commercially available and causes colonies that ferment lactose rapidly to turn red-pink, while lactose-negative colonies remain colorless. However, colonies of Gram-positive and even anaerobic organisms may be mixed with or underneath the lactose-fermenting colony. After an appropriate incubation period (generally 35 ± 0.5°C for at least 24 h), use a sterile needle or loop to gently pick up a well-isolated target colony and streak it on another medium (e.g., MacConkey, tryptic soy, or nutrient agar plate) to ensure that the isolated colony is a pure culture.

Test one well-isolated colony via Gram stain to confirm that only Gram-negative, non-spore-forming rods are present (Section 9221B). Also, determine that the culture is oxidase-negative, as noted in Section 9222.

If the colony yields an unusual or "unidentified" result, a contaminating organism may be present and the resulting biochemical profile will not be useful.

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9225 C. Identification

1. Approaches

There are many ways to identify strains of *Enterobacteriaceae* that are coliforms.[1–4] The most practical options for water laboratories seem to be screening tests, biochemical "tube tests," validated commercial identification methods ("identification kits"), and molecular methods. Each has advantages and disadvantages.

First, confirm that the presumptive coliform culture is a member of the *Enterobacteriaceae* family. The dyes, bile salts, and other ingredients in agars and broths are selective when isolating bacteria. Ensure that the culture is Gram-negative, oxidase-negative, and facultative anaerobic. Doing so will eliminate species that could be confused with *Enterobacteriaceae*, such as *Bacillus, Aeromonas,* and *Vibrio* species that can ferment lactose with gas production. The following approaches ensure quality control (QC) of the media and testing conditions (e.g., use known positive and negative culture controls).

2. Traditional Biochemical Tests, Computer Analysis, and Screening Tests

Many of the most commonly used biochemical tests for differentiating coliforms are shown in Table 9225:I. All microbiology laboratories used to perform biochemical testing in tubes, and many reference and public health laboratories still do.[2] Although some laboratories prepare their own media from commercial dehydrated powders, most of the commonly used media are available in commercially prepared, ready-to-inoculate glass tubes. Such tubes are recommended for QC purposes. The test typically involves inoculating each tube with one colony's growth, incubating it at 35°C, and reading the results at 24 and 48 h. In many reference laboratories, analysts often keep the tubes for 7 d to detect delayed reactions. Unfortunately, the media and tests are not completely standardized, and few laboratories use the exact same formulations or procedures. The results for 68 "potential coliform bacteria" of *Enterobacteriaceae* in 47 tests can be found in the literature.[2]

Analysts then compare the culture's biochemical reactions results to charts or tables[1–5]—such as Table 9260:I—to determine which *Enterobacteriaceae* species are compatible with the results.

The next step could be a computer software and database analyses of the coliform culture's biochemical profile. Computer analysis can be a useful supplement to manual analysis because of the "potential coliform species" in the *Enterobacteriaceae* family. *PIBWin** is a free identification program that can be

* See http://www.som.soton.ac.uk/research/sites/pibwin/. Accessed February 2017.

TABLE 9225:I. BIOCHEMICAL REACTIONS OF SEVERAL SPECIES OF THE FAMILY *ENTEROBACTERIACEAE**

Species	Percentage Isolates Positive in 1 to 2 Days†											
	Lactose Fermentation	ONPG Hydrolysis	Indole Production	Methyl Red	Voges–Proskauer	Simmons's Citrate	Ornithine Decarboxylase	Lysine Decarboxylase	Sorbitol Fermentation	Cellobiose Fermentation	Motility, 35–37°C	Yellow Pigment, 25°C
Citrobacter diversus	35	96	99	100	0	99	99	0	99	99	95	0
Citrobacter freundii	50	95	5	100	0	95	20	0	98	55	95	0
Enterobacter aerogenes	95	100	0	5	98	95	98	98	100	100	97	0
Enterobacter agglomerans	40	90	20	50	70	50	0	0	30	55	85	75
Enterobacter cloacae	93	99	0	5	100	100	96	0	95	99	95	0
Escherichia coli	95	95	98	99	0	1	65	90	94	2	95	0
Escherichia coli, variant	25	45	80	95	0	1	20	40	75	2	5	0
Escherichia fergusonii	0	83	98	100	0	17	100	95	0	96	93	0
Escherichia hermannii	45	98	99	100	0	1	100	6	0	97	99	98
Escherichia vulneris	15	100	0	100	0	0	0	85	1	100	100	50
Hafnia alvei	5	90	0	40	85	10	98	100	0	15	85	0
Klebsiella oxytoca	100	100	99	20	95	95	0	99	99	100	0	1
Klebsiella ozaenae	30	80	0	98	0	30	3	40	65	92	0	0
Klebsiella pneumoniae	98	99	0	10	98	98	0	98	99	98	0	0
Klebsiella rhinoscleromatis	0	0	0	100	0	0	0	0	100	100	0	0
Serratia fonticola	97	100	0	100	9	91	97	100	100	6	91	0
Serratia marcescens	2	95	1	20	98	98	99	99	99	5	97	0

* Modified after FARMER, J.J., III, 1985. Clinical identification of new species and biogroups of Enterobacteriaceae. *J. Clin. Microbiol.* 21:46. This list is not all inclusive.
† Reactions that become positive after 2 d are not considered.

downloaded and used with the "Colindale data matrix of *Enterobacteriaceae*"[6] (a current option in the program). Although the matrix is now outdated, it still contains the most common coliform organisms found in water. The Centers for Disease Control and Prevention's (CDC's) *Enterobacteriaceae* data matrix, which includes the more recently described species, will be added to *PIBWin* in the future.

Screening tests can differentiate coliforms or confirm an identification made via a commercial identification system or molecular method. Table 9225:I lists useful screening tests, key reactions, and the properties of some of the most common coliform bacteria. Additional *Enterobacteriaceae* screening tests that may be useful for differentiating coliforms are available and described elsewhere.[2]

3. Commercial Media, Reagents, and Test Kits

There are many commercial products (e.g., dehydrated media, ready-to-use media, and reagents) and commercial identification products (identification kits) for differentiating *Enterobacteriaceae* and similar bacteria.[7,8] Identification kits typically consist of a plastic strip that contains a set of miniaturized biochemical tests, often those traditionally used for identification (indole production, Voges-Proskauer, citrate utilization, lactose fermentation, etc.). The approach is similar to biochemical testing in glass tubes, with the main differences being in miniaturization, simplicity, ease of use, speed of analysis, number of tests available, suspending medium, and the method of reading and interpreting results (sometimes read via machine with simultaneous computer analysis). Many of these miniaturized biochemical tests are AOAC International-validated. Most clinical and food microbiology laboratories now use miniaturized identification kits to identify common *Enterobacteriaceae* cultures, including coliform cultures isolated from water, but these assays may be less accurate for newly described species. Check the instruction manual to determine which organisms are included in the database and the number of strains used to define each organism.

The main problem with identification kits is that the test panels used (usually about 20) are insufficient to differentiate all of the relevant *Enterobacteriaceae* species. (This is also becoming a problem with conventional tube tests, even when 40 to 50 tests are done.) Unusual or "unidentified" results may be tested with another kit, which may have similar limitations. Alternatively, the culture may be sent to a reference laboratory for identification using molecular tests or 16S rRNA sequencing.

When using identification kits to differentiate coliforms, keep in mind that their databases may not include some coliform bacteria likely to occur in water. These kits and databases were originally developed for use in clinical microbiology, hence may not include all the coliform bacteria isolated from water.[8] Note that some semi-automated and automated identification kits are now being developed for environmental samples.†

† Vitek, bioMérieux Inc., Durham, NC; MicroLog Micro station, Biolog, Bremen, Germany; or equivalent.

4. Molecular Methods

Molecular methods can be used for taxonomic identification of a coliform, including family, genus, species, serotype, clone, and strain and even potential virulence. These methods are impractical for routine coliform differentiation, but can be extremely useful in research projects, epidemiological studies, or when a definitive identification is required.

One of the most accurate methods for identifying a coliform isolate is a complete or partial 16S rRNA gene sequence that can be done by fee-for-service laboratories, which will provide strategic sequence data.[9] Such labs often can identify a coliform species based on the sequence of the first 500 base pairs in its 16S rRNA gene. A complete 16S rRNA gene sequence is more accurate, but much more expensive. Typically, the commercial lab will process the culture, sequence its genes, and put together an identification report with technical details and a figure showing the position of the submitted culture in relation to reference strains in the database. The per-test cost may depend on the total number of strains submitted and the "turnaround time" requested.

The advantage of 16S rRNA gene sequencing is that it considers all *Enterobacteriaceae*, not just those found in human clinical species. When a definitive identification is required (e.g., in a judicial proceeding), this may be the best option.

5. References

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9225 D. Media, Reagents, and Procedures

In routine testing of drinking and waste waters, identification down to the species or strain is not a common practice. However, many laboratories are concerned with bacterial identification. There is no "standard method" for identifying *Enterobacteriaceae* (coliform differentiation); all of the existing identification methods simply evolved over 100 years. Likewise, the commercial media and reagents available are also changing or being modified, so keep the following in mind when choosing media, reagents, and procedures.

If identifying coliform cultures via biochemical tests in tubes, remember that there are many variables involved. When possible, use the media and methods that accompany the chosen "biochemical data matrix" or "identification schema." For example, one reference[1] notes the reactions of more than 10 000 strains of *Enterobacteriaceae* tested at CDC in specific media using specific test conditions described in a second reference.[2] On the other hand, the *Enterobacteriaceae* biochemical data matrix generated by England's national reference laboratories at Colindale[3,4] is based on a different set of media and methods (the Colindale methods).

The following are traditionally used media and methods; however, there is no biochemical data matrix or "coliform identification schema" that accompanies them. Use commercial media and reagents to improve media consistency, reduce labor costs, and increase quality. Include negative and positive controls using known stock cultures to ensure continued accuracy and reliability. Follow the QC guidance in Section 9020B for media preparation, handling, and storage conditions.

1. Lactose, Sorbitol, and Cellobiose Fermentation Tests

Suspend 16 g phenol red broth base and 5 g of the selected carbohydrate (lactose, sorbitol, or cellabiose) in 1 L reagent-grade water, and stir to dissolve completely. Dispense in tubes, filling each tube to one-third its length. Then place a small inverted vial (Durham tube) in the tube to determine gas production. Close tubes and sterilize at 121°C for 12 to 15 min. Store tubes in the dark (refrigeration preferred), and discard if medium becomes discolored or evaporation exceeds 10% of volume.

To conduct a test, inoculate a tube with a loopful of growth from a well-isolated colony or slant and incubate for 24 to 48 h at 35 ± 0.5°C. Carbohydrate fermentation (acid production) will lower pH, causing the phenol red pH indicator to change from red-orange to yellow (pH <6.6). Alternatively, for lactose fermentation, lauryl tryptose broth (Section 9221B.3*a*) may be used.

2. Ortho-nitrophenyl-β-D-galactopyranoside (ONPG) Hydrolysis

This test looks for the β-galactopyranosidase enzyme by hydrolyzing the substrate *o*-nitrophenyl-β-D-galactopyranoside. Use one of the numerous commercial test kits and disks available, or an ONPG-containing medium (Section 9223).

To conduct the test, inoculate 10 mL ONPG broth with a heavy loopful of growth from a slant and incubate at 35 ± 0.5°C for up to 24 h. If the medium develops a yellow color—compared to an uninoculated tube or (preferably) a tube inoculated with an ONPG-negative culture—results are positive. Interpret tests of yellow-pigmented organisms with caution.

3. Other Chromogenic Assays for β-galactopyranosidase

The chlorophenol red-β-D-galactopyranoside (CPRG) hydrolysis test is similar to the ONPG test, except the color of a positive result is red to magenta. Similarly, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranodise) is hydrolyzed by β-galactopyranosidase to yield a blue product.

4. Indole Test

Indole is a product of tryptophan metabolism.

a. Reagents:

1) *Medium*—Use tryptophan broth. Dissolve 10.0 g tryptone or trypticase per L reagent-grade water. Dispense medium in 5-mL portions in test tubes, and sterilize.

2) *Kovac's reagent*—Dissolve 5 g *p*-dimethylaminobenzaldehyde in 75 mL isoamyl (or normal amyl) alcohol, ACS grade, and add 25 mL conc HCl. **CAUTION: Reagents are both flammable and corrosive.** The reagent should be yellow.

The amyl alcohol solution's pH should be <6.0. Purchase both amyl alcohol and benzaldehyde in amounts as small as consistent with the volume of work to be done.

b. Procedure: Inoculate 5-mL portions of tryptophan broth medium from a pure culture and incubate at 35 ± 0.5°C for 24 ± 2 h. Add 0.2 to 0.3 mL Kovac's test reagent and gently shake. Let stand for about 10 min, and observe results.

Results are positive if the amyl alcohol surface layer is a dark red color; results are negative if the layer remains yellow. An orange color probably indicates the presence of skatole (a breakdown product of tryptophan), but it is not indole. Record as a variable result.

5. Methyl Red Test

The methyl red test measures organisms' ability to produce stable acid end products via glucose fermentation.

a. Reagents:

1) *Medium*—Use buffered glucose broth. Dissolve 7.0 g proteose peptone or equivalent peptone, 5.0 g glucose, and 5.0 g dipotassium hydrogen phosphate (K_2HPO_4) in 1 L reagent-grade water. Dispense in 5-mL portions in test tubes, and sterilize by autoclaving at 121°C for 12 to 15 min, making sure that total heat-exposure time is no longer than 30 min.

2) *Indicator solution*—Dissolve 0.1 g methyl red in 300 mL 95% ethyl alcohol and dilute to 500 mL with reagent-grade water. **CAUTION: Ethyl alcohol is flammable.**

b. Procedure: Inoculate 10-mL portions of medium from a pure culture. Incubate at 35 ± 0.5°C for 5 d. Then, add 5 drops methyl red indicator solution to 5 mL of the culture and read immediately.

A 48-h incubation period is adequate for most cultures, but do not incubate for less than 48 h. If test results are equivocal at 48 h, repeat with cultures incubated for 4 or 5 d. In such cases, incubate duplicate cultures at 22 to 25°C. Testing culture portions at 2, 3, 4, and 5 d may provide positive results sooner.

Results are positive if the sample is distinctly red, negative if it is distinctly yellow, and questionable if the color is a mixed shade.

6. Voges–Proskauer Test

The Voges–Proskauer test measures organisms' ability to produce a neutral product (acetoin or acetyl methylcarbinol) via glucose fermentation.

a. Reagents:

1) *Medium*—See 9225D.4*a*1).

2) *Naphthol solution*—Dissolve 5 g purified α-naphthol (melting point 92.5°C or higher) in 100 mL absolute ethyl alcohol. When stored at 5 to 10°C, this solution is stable for 2 weeks. **CAUTION: Ethyl alcohol is flammable.**

3) *Potassium hydroxide*, 7*N*—Dissolve 40 g KOH in 100 mL reagent-grade water.

b. Procedure: Inoculate 5 mL medium and incubate for 48 h at 35 ± 0.5°C. To 1 mL of culture, add 0.6 mL naphthol solution and 0.2 mL KOH solution. Shake well after adding each reagent. Results are positive if a pink to crimson color develops at the surface within 5 min. Do not read after 10 min. Disregard tubes developing a copper color as this represents a negative result.

7. Simmons' Citrate Test

The citrate test measures a bacterium's ability to use citrate as the sole carbon source, using bromothymol blue as the pH indicator.

a. Medium: Use Simmons' citrate agar. To make Simmons' citrate agar, add 0.2 g $MgSO_4 \cdot 7H_2O$, 1.0 g ammonium dihydrogen phosphate ($NH_4H_2PO_4$), 1.0 g K_2HPO_4, 2.0 g sodium citrate dihydrate, 5.0 g NaCl, 15.0 g agar, and 0.08 g bromthymol blue to 1 L reagent-grade water. Autoclave at 121°C for 12 to 15 min and cool tubes at an angle to form an agar slant. Final pH after sterilization is 6.8 ± 0.2.

b. Procedure: Inoculate agar medium via the streak technique using a light inoculum.

Incubate 48 h at 35 ± 0.5°C. Uninoculated medium is green in color. Results are positive when the medium develops a blue color; this indicates the metabolism of citrate and production of alkaline byproducts. Results are negative if there is no color change.

8. Motility Test

The motility test measures whether an organism is motile in a semi-solid medium.

a. Medium: Add 3.0 g beef extract, 10.0 g peptone, 5.0 g NaCl, and 4.0 g agar to 1 L reagent-grade water. Adjust pH to 7.4, dispense in 3-mL portions in 13- × 100-mm tubes or 8-mL portions in 16- × 125-mm tubes, and sterilize.

b. Procedure: Inoculate by stabbing an inoculating needle 5 mm deep into the center of the medium. Incubate for 1 to 2 d at 35°C. If results are negative, incubate another 5 d at 22 to 25°C.

Results are positive if there is diffuse growth through the medium from the inoculation point. Results are negative if growth is visible only along the stab line, and the surrounding medium remains clear.

Alternatively, prepare the medium without agar and examine a young culture via the hanging drop slide technique for motile organisms. Examine the wet-mount slide immediately; do not let it dry.

9. Lysine and Ornithine Decarboxylase Tests

This procedure tests bacteria's ability to metabolize (decarboxylate) the amino acids lysine and ornithine. Use of commercially prepared medium is preferred for QC purposes.

a. Reagents:

1) *Media*—Use a basal medium made according to the Moeller or Falkow methods. The Moeller method involves dissolving 5.0 g peptone (Orthana special, thiotone, or equivalent), 5.0 g beef extract, 0.625 mL bromcresol purple (1.6%), 2.5 mL cresol red (0.2%), 0.5 g glucose, and 5.0 mg pyridoxal in 1 L reagent-grade water and adjusting to pH 6.0 to 6.5. The Falkow method involves dissolving 5.0 g peptone, 3.0 g yeast extract, 1.0 g glucose, and 1.0 mL bromcresol purple (1.6%) in 1 L reagent-grade water and adjusting to pH 6.7 to 6.8.

For either decarboxylase test, divide into three portions: make no addition to the first portion (the control), add enough L-lysine dihydrochloride to the second portion to make a 1% solution, and add enough L-ornithine dihydrochloride to the third to make a 1% solution (for the Falkow method, add only 0.5% of the L-amino acid). After adding ornithine, readjust the medium's pH to 6.0 ± 0.2. Dispense in 3- to 4-mL portions in screw-capped test tubes and sterilize via autoclaving at 121°C for 10 min. A floccular precipitate in the ornithine medium does not interfere with its use.

2) *Mineral oil*—Use mineral oil sterilized via autoclaving at 121°C for 30 to 60 min, depending on container size.

b. Procedure: Lightly inoculate each medium, add an approximately 10-mm-thick layer of mineral oil to promote fermentation, tighten caps, and incubate at 35 to 37°C for up to 4 d. Examine tubes daily. Results are positive if the color changes from yellow to violet or reddish-violet, weakly positive if the color changes to bluish gray, and negative if the color remains yellow (unchanged). For a weakly positive culture, a drop of bromocresol purple can be added. If the color does not change, this is considered a negative test.

10. Oxidase Test

The oxidase test determines the presence of oxidase enzymes. Coliform bacteria are oxidase-negative.

a. Reagents:

1) *Media*—Use either nutrient agar or tryptic soy agar plates to streak cultures and produce isolated colonies. From these, obtain the inoculum for oxidase testing on impregnated filter paper. Cultures should be less than 24 h old. Do not use any medium that includes a carbohydrate in its formulation. Use only tryptic soy agar if reagent is dropped on colonies.

Tryptic soy agar:

Tryptone	15.0 g
Soytone	5.0 g
Sodium chloride (NaCl)	5.0 g
Agar	15.0 g
Reagent-grade water	1.0 L

pH should be 7.3 ± 0.2 after sterilization.

2) *Tetramethyl* p-*phenylenediamine dihydrochloride*, 1% aqueous solution—freshly prepared or refrigerated for no longer than 1 week. Impregnate a filter paper strip* with this solution. Alternatively, prepare a 1% solution of dimethyl *p*-phenylenediamine hydrochloride. Commercially available, single-use reagent ampules are convenient and economical, but use them with caution. This reagent is also available in little dropper bottles, with which analysts can add 1 to 2 drops to filter paper. Use tryptic soy agar when the reagent will be dropped directly on colonies, because nutrient agar plates give inconsistent results; when smearing a portion of a picked colony on reagent-impregnated filter paper, do not transfer any medium with the culture material.

b. Procedure: Remove some of a colony from agar plate with a platinum wire, a wooden or plastic applicator stick, or a glass rod, and smear on the test strip. Do not use iron or other reactive wire because it will cause false positive reactions. Results are positive if a dark purple color develops within 10 s. Test positive and negative cultures concurrently. If liquid reagent is used, drop it on colonies on the culture plate. Oxidase-positive colonies develop a pink color that successively becomes maroon, dark red, and finally, black.

11. Yellow Pigment

Observe isolated colonies on tryptic soy agar slants or plates (nutrient agar may be an acceptable substitute) incubated at 25 ± 0.5°C for up to 48 h. Pigmentation often intensifies as incubation time proceeds.

12. References

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* Whatman No. 1, or equivalent.

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9225 E. Reporting Results

Precisely worded reports are essential because of the identification methods' limitations. Specify the method(s) used and provide comments. Note that careful wording of results will not help an unqualified test. Below are some possibilities, with less-precise identifications given first.

1. Identification: *Klebsiella–Raoultella* complex

Klebsiella sp. identification is difficult. The two species of *Raoultella* were formerly identified as *Klebsiella* sp.

Comment: This culture gave a "possible identification" based on its biochemical test profile determined by the XYZ commercial identification system (47 biochemical tests). Further testing would be required for a definitive identification; please contact the laboratory if this is needed.

2. Identification: *Klebsiella* species

Comment: Identification was based on the following screening-test results:

- grew as large, pink, mucoid colonies on MacConkey agar plus 30 μg per mL ampicillin;
- was a Gram-negative rod with large capsules around the cells; and
- was non-motile, lysine decarboxylase-positive, arginine dihydrolase-negative, and ornithine decarboxylase-negative.

These are all typical and defining characteristics of *Klebsiella* species.

3. Identification: *Klebsiella oxytoca*

Comment: "High likelihood identification" based on biochemical test profile determined by the ABC commercial identification system (21 biochemical tests).

Note: This system is not sensitive and specific for differentiating all species in the genus *Klebsiella*.

4. Identification: *Klebsiella varicola*

Comment: This was a "probable identification" based on a biochemical test profile determined via the CDE commercial identification system (95 biochemical tests). It was later confirmed by a partial 16S rRNA gene sequence (451 base) done by a commercial laboratory (report attached).

5. Identification: *Klebsiella terrigena*

Comment: This identification is based on a PCR test our laboratory developed based on a sequence of the *rpoB* gene. This research test is sensitive, specific for *Klebsiella terrigena*, and differentiates other *Klebsiella* species. These methods and results will be published. Technical details are attached to this report.

9230 FECAL ENTEROCOCCUS/STREPTOCOCCUS GROUPS*

9230 A. Introduction

1. Taxonomy and Principles

In 1903, the genus name *Enterococcus* was proposed for Gram-positive, catalase-negative, coccoid-shaped bacteria of intestinal origin.[1] Several years later, it was suggested[2] that the genus name be changed to *Streptococcus* because of their ability to form chains of coccoid-shaped cells. Subsequently, the species name was *faecalis* because of their fecal origin. The genus name *Streptococcus* was adopted for the next 78 years and applied to coccoid-shaped, chain-forming, Gram-positive bacteria of intestinal origin. In 1984, the genus name *Enterococcus* was revived, and it was suggested that *Streptococcus faecalis* and *Streptococcus faecium* be transferred to the genus *Enterococcus* on the basis of genotypic similarity to microbial taxonomy.[3] Shortly after the acceptance of the revived *Enterococcus* genus, *Streptococcus avium* and *Streptococcus gallinarum* also were transferred to the genus.

Although analysts can differentiate the various species of enterococci and streptococci biochemically, it is impractical to carry out the tiered testing required to identify specific species of interest in water. Although enterococci are broadly used as an indicator of fecal pollution in water, and are commonly found in the feces of human and other warm-blooded animals, some species can persist and grow in the natural environment and do not appear to be related to fecal pollution. Liquid and solid culture media eliminate non-enterococci and non-streptococci via sodium azide; cycloheximide; and 2,3,5-triphenyltetrazolium chloride (TTC). Enterococci are extremely hardy and can tolerate a wide variety of growth conditions. Enterococci are differentiated from streptococci by their ability to grow in 6.5% sodium chloride (NaCl), on bile esculin agar (BEA), and at both 10 ± 0.5°C on brain–heart infusion (BHI) agar[4] and 45 ± 0.5°C in BHI broth. See Table 9230:I for other characteristics of enterococci and streptococci. Analysts should note that commercially available media formulations may vary slightly from those described in this chapter. Therefore, if purchasing media from a commercial vendor, make sure that media formulations contain the appropriate components.

Table 9230:I. Selected Characteristics of *Enterococcus* and *Streptococcus* Species Isolated from Feces

Species	Fecal Origin	Bile Esculin Agar	Growth in 6.5% NaCl	Growth at 10 ± 0.5°C	Growth at 45 ± 0.5°C
E. gallinarum	+	+	+	+/+	+
E. hirae	+	+	+	+/+	+
E. columbae	+	+	−	−/+	−/+
E. cecorum	+	+	−	−/+	−/+
E. durans	+	+	+	+/+	+
E. faecium	+	+	+	+/+	+
E. faecalis	+	+	+	+/+	+
E. casseliflavus	+	+	+	+/+	+
E. avium	+	+	+	+/+	+
E. munditii	+	+	+	+/−	−/+
E. dispar	+	+	+	+/+	+
E. saccharolyticus	+	+	+	+/+	−/+
E. asini	+	+	−	+/+	+
S. hyointestinalis	+	−	−	−/+	+
S. bovis	+	+	−	−/+	+
S. intestinalis	+	−	−	−/+	+
S. suis	+	−	−	−/+	+
S. alactolyticus	+	−	−	−/+	+
S. equines	+	+	−	−/+	+

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* Approved by Standard Methods Committee, 2013.
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9230 B. Multiple-Tube Technique

For an introduction and description of the use of the multiple-tube fermentation approach to enumerating bacteria in environmental samples, see Sections 9221A and 9221C. This approach is recommended for analyzing drinking water, source water, and both fresh and marine recreational waters.

1. Materials and Culture Media

Preferably use commercially available medium. Follow manufacturer's instructions for storage and disposal after preparation (see Section 9020B.5*j*). If the dehydrated media must be prepared from basic ingredients, follow directions below. (NOTE: Formulations of all commercially available media must match the specified components.)

a. Azide dextrose broth:[1]

Beef extract	4.5 g
Tryptone or polypeptone	15.0 g
Dextrose (Glucose)	7.5 g
Sodium chloride (NaCl)	7.5 g
Sodium azide (NaN_3)	0.2 g
Reagent-grade water	1 L

CAUTION: Sodium azide is a dangerous chemical requiring special attention and care. It is toxic and mutagenic. Take precautions to avoid contact with this compound. Azide also can form explosive compounds when it contacts metal pipes.

The pH should be 7.2 ± 0.2 after sterilization. If pH is out of range, adjust and retest pH; discard if pH remains out of range.

b. Bile esculin azide agar:

Yeast extract	5.0 g
Proteose peptone No. 3	3.0 g
Tryptone	17.0 g
Oxgall	10.0 g
Esculin	1.0 g
Ferric ammonium citrate	0.5 g
Sodium chloride	5.0 g
Sodium azide	0.15 g
Agar	15.0 g
Reagent-grade water	1 L

CAUTION: Sodium azide is a dangerous chemical requiring special attention and care. It is toxic and mutagenic. Take precautions to avoid contact with this compound. Azide also can form explosive compounds if it contacts metal pipes.

The pH should be 7.1 ± 0.2 after sterilization. If pH is out of range, adjust and retest pH; discard if pH remains out of range. The dehydrated media described in this section are available commercially[2]; follow manufacturer's instructions for storage and disposal after preparation.

2. Presumptive Test Procedure

Inoculate a series of tubes of azide dextrose broth with appropriate graduated quantities of a 100-mL sample. Use sample volumes of 10 mL or less. The strength of the broth used will be proportional to the sample size. Use only decimal multiples of 1 mL (see Section 9221 for suggested sample sizes).

Incubate inoculated tubes at 35 ± 0.5°C. Examine each tube for turbidity at the end of 24 ± 2 h. If no definite turbidity is present, reincubate, and read again at the end of 48 ± 3 h.

3. Confirmed Test Procedure

After 24 or 48 h incubation, subject all azide dextrose broth tubes showing turbidity to the confirmed test for enterococci.

Streak a portion of growth from each positive azide dextrose broth tube on bile esculin azide (BEA) agar.* Invert and incubate the dish at 35 ± 0.5°C for 24 ± 2 h. Brownish-black colonies with brown halos confirm the presence of fecal streptococci and *Enterococcus sp.* NOTE: *S. bovis* and *S. equinus* can also hydrolyze esculin, however, so this step alone cannot be used as final confirmation of *Enterococcus sp*. Further confirmation is required.

Then, transfer brownish-black colonies with brown halos using a sterile inoculating loop to the following media: two tubes of BHI broth: one with 6.5% NaCl (incubate at 35 ± 0.5°C for 48 ± 3 h) and one without NaCl (incubate at 45°C for 48 ± 3 h) and BHI agar (incubate at 10 ± 0.5°C for 48 ± 3 h). (See 9230C.2*e* and *f* for preparation of BHI broth with and without 6.5% NaCl.)

If growth is observed after incubation in BHI broth with 6.5% NaCl at 35°C and either in BHI agar or broth at 10 or 45°C, respectively, the colony is a confirmed member of the *Enterococcus* genus. Note that *S. bovis* and *S. equinus* can grow at 45°C but do not grow in 6.5% NaCl. More accurate results (≥90%) can be achieved by doing all of the following: observing Gram-positive cocci, a catalase-negative reaction, growth on BHI agar at 10 ± 0.5°C, positive pyrrolidonyl arylamidase (PYR) activity, and positive leucine aminopeptidase (LAP) reaction[3,4] using commercially available test kits.† (See Table 9230:I.)

4. Computing and Recording MPN

Calculate the total fecal enterococci density from the number of confirmed positive cultures on BHI agar and corresponding positive tubes of BHI broth with 6.5% NaCl at 35 ± 0.5°C after 48 ± 3 h. Compute the combination of positive and negative tubes, and record as the most probable number (MPN; see Section 9221C).

5. Quality Control

Test each lot of purchased or made medium for performance by inoculating it with three control bacteria. An example positive control is *Enterococcus faecium.*‡ As negative controls, use *Serratia marcescens*§ (a Gram-negative species) and *Aerococcus viridans*‖ (a Gram-positive species) or similar. Avoid using a heavy inoculum. Incubate these controls at the appropriate temperatures for the appropriate time. Read and record results. In addition, include blank samples of sterile dilution water to check the sterility of the process.

6. References

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* Previous editions of *Standard Methods* recommended the use of Pfizer selective enterococcus (PSE) agar as a confirmatory test. Some of the ingredients for this agar are no longer commercially available. BEA agar is a modification of similar medium[2]; it is more selective and provides for rapid growth and efficient recovery of Group D streptococci.[3] See also ISO 7899-2:2000.

† BactiCard® Strep test kit, Remel, Inc., Lenexa, KS, or equivalent. Available from a variety of sources, including Amazon.

‡ American Type Culture Collection Product No. 35667.

§ American Type Culture Collection Product No. 43862.

‖ American Type Culture Collection Product No. 10400.

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9230 C. Membrane Filter Techniques

The membrane filtration approach for enumerating enterococci in environmental waters is recommended for analyzing drinking water, source water, wastewater, and both fresh and marine recreational waters. Membrane filter approaches are not recommended for highly turbid waters.

1. Laboratory Apparatus

See Section 9222B.1.

2. Materials and Culture Media

Preferably use a commercially available dehydrated media. Follow manufacturer's instructions for storage and disposal after preparation (see Section 9020B.5*j*). If media must be prepared from basic ingredients, follow directions below. (Note: Formulations of all commercially available media must match the specified components at the appropriate amounts.)

a. mE agar:[1–4]

Peptone	10.0 g
Sodium chloride (NaCl)	15.0 g
Yeast extract	30.0 g
Esculin	1.0 g
Cycloheximide	0.05 g
Sodium azide (NaN_3)	0.15 g
Agar	15.0 g
Reagent-grade water	1 L

Caution: Cycloheximide is a poison that is extremely toxic if inhaled, swallowed, or absorbed through the skin. Work with this chemical requires special attention and care.

Caution: Sodium azide is a dangerous chemical requiring special attention and care. It is toxic and mutagenic. Take precautions to avoid contact with this compound. Azide also can form explosive compounds if it contacts metal pipes.

Heat to dissolve ingredients, autoclave at 121°C for 15 min to sterilize, and cool in a water bath at 44 to 46°C. Mix 0.24 g nalidixic acid in 5 mL reagent-grade water and add a few drops (approximately 0.2 mL) of 0.1 *N* NaOH to dissolve the antibiotic. Pour the entire antibiotic/NaOH mixture through a sterile filter and add to the basal media. Add 0.15 g 2,3,5-triphenyltetrazolium chloride (TTC) and mix well to dissolve. Pour the agar into 9- × 50-mm Petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL), and let solidify. The final pH should be 7.1 ± 0.2. If pH is out of range, adjust and retest pH; discard if pH remains out of range. Store poured, solidified dishes in the dark at 2 to 10°C. Discard after 14 d.

b. EIA substrate:[1,4]

Esculin	1.0 g
Ferric citrate	0.5 g
Agar	15.0 g
Reagent-grade water	1 L

Heat to dissolve ingredients, autoclave at 121°C for 15 min to sterilize, and cool in a water bath at 44 to 46°C. The pH should be 7.1 ± 0.2 before autoclaving. If pH is out of range, adjust and retest pH; discard if pH remains out of range. Pour media into 50-mm Petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL) and let solidify. Store Petri dishes in the dark at 2 to 10°C. Discard after 14 d.

c. mEI agar:[3,5] Basal media ingredients are the same as mE agar (see ¶ *a* above). Add 0.75 g indoxyl-β-D-glucoside to basal media. Heat to dissolve ingredients, sterilize, and cool in a 44 to 46°C water bath. Mix 0.24 g nalidixic acid in 5 mL reagent-grade sterile water, add a few drops of 0.1*N* NaOH to dissolve, filter-sterilize the solution, and add to the mEI media. Add 0.02 g TTC separately to the mEI media and mix. Or use the following larger-scale solutions in lieu of the previously mentioned smaller-scale media additions.

1) *Nalidixic acid*—Add 0.48 g nalidixic acid and 0.4 mL 10*N* NaOH to 10 mL reagent-grade water and mix. Filter-sterilize the solution and add 5.2 mL per liter of media. Store solution for no more than 14 d at 2 to 10°C.

2) *TTC*—Add 0.1 g TTC to 10 mL reagent-grade water and warm to dissolve. Filter-sterilize the solution and add 2 mL per liter of media. Store solution for no more than 2 weeks at 2 to 10°C. Pour the agar into 9- × 50-mm disposable Petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL) and let solidify. Final pH should be 7.1 ± 0.2. If pH is out of range, adjust and retest pH; discard if pH remains out of range. Store dishes in dark at 2 to 10°C. Discard after 14 d. (Note: This media is recommended for culturing enterococci in fresh and marine recreational waters.)

d. mEnterococcus agar:

Tryptose	20.0 g
Yeast extract	5.0 g
Glucose	2.0 g
Dipotassium phosphate (K_2HPO_4)	4.0 g
Sodium azide (NaN_3)	0.4 g
2,3,5-Triphenyltetrazolium chloride	0.1 g
Agar	10.0 g
Reagent-grade water	1 L

Caution: Sodium azide is a dangerous chemical requiring special attention and care. It is toxic and mutagenic. Take precautions to avoid contact with this compound. Azide also can form explosive compounds if it contacts metal pipes.

Heat to dissolve ingredients. Do not autoclave this solution because doing so will break down selective chemicals. Dispense into 9- × 50-mm Petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL), and let solidify. Final pH should be 7.2 ± 0.2. If pH is out of range, adjust and retest pH; discard if pH remains out of range. Prepare fresh media for each set of samples. (NOTE: This media is recommended for fecal enterococci/streptococci in fresh and marine waters.)

e. Brain–heart infusion (BHI) broth:

Infusion of calf brain	200 g
Infusion of beef heart	250 g
Proteose peptone	10.0 g
Glucose	2.0 g
Sodium chloride (NaCl)	5.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	2.5 g
Reagent-grade water	1 L

This media is used for verification tests of presumptive enterococci colonies. Heat to dissolve ingredients. Dispense 10 mL into tubes, and autoclave at 121°C for 15 min. The final pH should be 7.4 ± 0.2 after sterilization. If pH is out of range, adjust and retest pH; discard if pH remains out of range.

To make BHI broth with 6.5% NaCl, add 60 g NaCl to the BHI broth formulation above.

f. Brain–heart infusion (BHI) agar: Add 15.0 g agar to the ingredients for BHI broth (¶ *e* above). Heat to dissolve agar. Autoclave at 121°C for 15 min. The pH should be 7.4 ± 0.2 after sterilization. If pH is out of range, adjust and retest pH; discard if pH remains out of range.

g. Bile esculin agar:[6]

Beef extract	3.0 g
Peptone	5.0 g
Oxgall	40.0 g
Esculin	1.0 g
Ferric citrate	0.5 g
Agar	15.0 g
Reagent-grade water	1 L

This media is used for verification tests of presumptive enterococci colonies. Heat to dissolve ingredients. Dispense 8 to 10 mL into appropriate tubes for slants, or dispense an appropriate volume into a flask for subsequent pouring into dishes. Autoclave at 121°C for 15 min. Do not overheat because this may darken the media. Cool to 44 to 46°C and slant the tubes or dispense 15 mL into 15- × 100-mm Petri dishes. The final pH should be 6.6 ± 0.2 after sterilization. If pH is out of range, adjust and retest pH; discard if pH remains out of range. Store at 2 to 10°C.

3. Procedures

a. mE method:[1]

1) Selection of sample size and filtration—Filter appropriate sample volumes through a 0.45-μm, gridded, sterile membrane to give 20 to 60 colonies on the membrane surface. Transfer filter to solidified mE agar (9230C.2*a*) media onto a Petri dish, avoiding air bubbles beneath the membrane.

2) Incubation—Invert Petri dishes and incubate at 41 ± 0.5°C for 48 ± 3 h.

3) Substrate test—After 48 ± 3 h incubation, carefully transfer filter to EIA media (9230C.2*b*). Incubate at 41 ± 0.5°C for 20 min.

4) Counting—Count pink to red enterococci colonies that develop a black or reddish-brown precipitate on the underside of the filter.

When counting colonies, use a low-power binocular (10 to 16×), wide-field dissecting microscope or other optical device, along with a cool, white fluorescent light source directed to provide optimal viewing.

b. mEI method:[3,5]

1) Selection of sample size and filtration—See ¶ *a*1) above.

2) Transfer and incubation—Transfer filter to solidified mEI agar (9230C.2*c*) onto a Petri dish, avoiding air bubbles beneath the membrane. Invert culture dishes and incubate at 41 ± 0.5°C for 24 h.

3) Counting—Count as enterococci any colony (regardless of color) with a blue halo. Such colonies also must be ≥0.5 mm in diameter.[5] Use this criterion at your discretion. When counting colonies, use a low-power binocular (10 to 16×), wide-field dissecting microscope or other optical device, along with a cool, white fluorescent light source directed to provide optimal viewing.

c. mEnterococcus method:

1) Selection of sample size and filtration—See ¶ *a*1) above.

2) Transfer and incubation—Transfer filter to solidified mEnterococcus agar (9230C.2*d*) onto a Petri dish, avoiding air bubbles. Let dishes stand for 30 min, then invert and incubate at 35 ± 0.5°C for 48 ± 3 h.

3) Counting—Count all light and dark red colonies as enterococci. When counting colonies, use a low-power binocular (10 to 16×), wide-field dissecting microscope or other optical device, along with a cool, white fluorescent light source directed to provide optimal viewing.

4. Calculation of Enterococci Density

Compute density from sample quantities producing membrane filter counts within the desired 20- to 60-colony range. If the colonies are more dense than this, attempt to provide an estimated number or else note as "too numerous to count" (TNTC) as in Section 9222B.5. Record densities as presumptive enterococci per 100 mL.

5. Verification Tests

Because all of these methods suffer periodically from false positives, verification tests are an important quality-control step (see Section 9020B.10). Include a routine verification procedure with each enterococci method, especially if the results will be used as evidence in a court of law. To verify enterococci colonies, pick selected typical colonies from a membrane and streak for isolation onto the surface of a BHI agar plate (9230C.2*f*). Incubate at 35 ± 0.5°C between 24 ± 2 and 48 ± 3 h.

Transfer a similar looking, well-isolated colony from the BHI agar plate into a BHI broth tube (9230C.2*e*) and to each of two clean glass slides using a sterile inoculating loop. Incubate the BHI broth at 35 ± 0.5°C for 24 ± 2 h. To the smear on one slide, add a few drops of freshly prepared 3% hydrogen peroxide. The appearance of bubbles constitutes a positive catalase test and indicates that the colony is not a member of the *Enterococcus* genus. If the catalase test is negative (i.e., no bubbles are produced), make a Gram stain of the second slide. Fecal strepto-

cocci and enterococci are Gram-positive, ovoid cells, 0.5 to 1.0 μm in diameter, mostly in pairs or short chains.

Transfer, using a sterile inoculating loop, a loopful of the BHI broth to each of the following media: bile esculin agar (incubate at 35 ± 0.5°C for 48 ± 3 h); BHI agar (incubate at 10 ± 0.5°C for 48 ± 3 h); BHI broth (incubate at 45 ± 0.5°C for 48 ± 3 h); and BHI broth with 6.5% NaCl (incubate at 35 ± 0.5°C for 48 ± 3 h).

Growth of catalase-negative, Gram-positive cocci on bile esculin agar (9230C.2*g*) and in 6.5% NaCl broth at 35°C and either in BHI agar at 10 ± 0.5°C or BHI broth at 45 ± 0.5°C confirms that the colony belongs to the *Enterococcus* genus.

When using mEI media, the incidence of non-*Enterococcus* species can be as high as 26% in marine environments.[7] So, additional tests [e.g., growth in BHI broth at 45 ± 0.5°C and assays for pyrrolidonylarylamidase (PYR) and leucine aminopeptidase (LAP) activities[8] using a test kit*] may be required to confirm *Enterococcus* genus with acceptable accuracy (≥90%).

6. Selected Characteristics of *Enterococcus* and *Streptococcus* Species

Selected characteristics[8–14] of *Enterococcus* and *Streptococcus* species isolated from feces are shown in Table 9230:I.

7. Quality Control

Test each lot of purchased or made media for performance by inoculating it with three control bacteria. Use *Enterococcus faecium*† as a positive control. As negative controls, use *Serratia marcesens*‡ (a Gram-negative species) and *Aerococcus viridians*§ (a Gram-positive species). Avoid using a heavy inoculum. Incubate these controls at the appropriate temperatures for the appropriate time. Read and record results. In addition, include blank samples of sterile dilution water to check the sterility of the process.

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* BactiCard® Strep test kit, Remel, Inc., Lenexa, KS, or equivalent. Available from a variety of sources, including Amazon.
† American Type Culture Collection Product No. 35667.
‡ American Type Culture Collection Product No. 43862.
§ American Type Culture Collection Product No. 10400.

9230 D. Fluorogenic Substrate Enterococcus Test

The fluorogenic enzyme test uses a hydrolyzable substrate (4-methylumbelliferyl-ß-D-glucoside) to detect enterococci. The combined components of the media formulation allow enterococci to express the enzyme β-D-glucosidase (esculinase) while suppressing the formulation of detectable fluorescence in non-enterococci. This test can be used in a multiple tube, a multiwell, or a presence–absence format.

1. Principle

See Section 9223. In the method for enterococci, 4-methylumbelliferyl β-D-glucoside (4-MUG) (a fluorogenic substrate) is used rather than esculin. The β-D-glucosidase enzyme hydrolyzes the substrate, yielding a fluorescent signal when excited by long-wavelength (365- to 366-nm) UV light. Non-enterococcus bacteria that produce β-D-glucosidase, such as some species of the genera *Serratia, Klebsiella,* and *Aerococcus,* are suppressed. They should not produce a positive response within the incubation time (as long as starting concentrations of these cells do not exceed 10^5 CFU/100 mL). The fluorogenic substrate *Enterococcus* sp. test is recommended for analysis of drinking water, source water, wastewater, and both fresh and marine recreational waters. Marine waters require a 1:10 dilution with sterile diluent,

such as deionized or distilled water (not sterile seawater), to avoid microbial interference from certain *Bacillus* spp. In some other ambient waters, a 1:10 dilution may be required to reduce interference.

2. Materials and Culture Media

A formulation of the fluorogenic substrate is available commercially* for use with the multiple tube, multi-well, or presence–absence procedures. Use a commercial substrate medium for both uniformity and good quality assurance. Store according to the manufacturer's directions. Avoid prolonged exposure of the substrate to direct sunlight. Use substrate media before manufacturer's expiration date, and discard any that is discolored.

3. Procedure

Proceed according to ¶s *a, b,* or *c* below and/or see the manufacturer's package insert.

a. Multiple-tube procedure: Select the appropriate number of tubes per sample. (NOTE: Verify the tubes do not auto-fluoresce.) When examining freshwater samples, aseptically add a packet of substrate media to a 100-mL sample and mix well to dissolve. For a ten-tube MPN, dispense 10 mL of the sample/media mixture into each of ten tubes. For a five-tube MPN, dispense 20 mL of the sample/media mixture into each of five tubes. Dilute marine water samples 1:10 (add 10 mL of a well-mixed sample to a sterile container containing 90 mL of sterile diluent, as indicated above) and then add substrate media, mix, and dispense as above.

Cap tubes and incubate at 41 ± 0.5°C for 24 h.

b. Multi-well procedure: The multi-well procedure is performed using sterilized, disposable multi-well trays. When examining freshwater samples, aseptically add a packet of substrate media to a 100-mL sample, mix well to dissolve, pour into the multi-well tray (following the manufacturer's directions), and seal using the manufacturer's tray sealer. Marine water samples must be diluted 1:10; then add 10 mL of well-mixed sample to a sterile container containing 90 mL of sterile deionized or distilled water. Next, aseptically add a packet of substrate media to the container, mix well to dissolve the powder, and pour into the multi-well tray (following the manufacturer's directions). To streamline the process, analysts could mix the substrate media with the sterile deionized or distilled water no more than 30 min before adding the sample. Once the sample, substrate media, and diluent are well mixed, transfer the mixture to a multi-well tray, as indicated above.

Incubate at 41 ± 0.5°C for 24 h.

c. Presence–absence procedure: Aseptically, add a packet of media to a 100-mL sample in a transparent, nonfluorescent container. Cap and mix well. Incubate at 41 ± 0.5°C for 24 h.

4. Computing and Reporting MPN

After incubation, examine the tubes, multi-well trays, or containers for fluorescence in a UV viewing cabinet or a dark environment with a long-wavelength (365- to 366-nm) UV lamp (preferably a 6-W bulb). Keep lamp within 13 cm (5 in.) of sample, facing away from the analyst's eyes and toward the sample. If performing the multi-tube procedure, calculate MPN from the number of positive and negative tubes, as described in Section 9221C. For the multi-well procedure, calculate MPN from the number of positive wells, using the tables provided by the manufacturer. For both methods, correct MPN for any dilution used. If using the presence–absence procedure, report the results as *Enterococcus* sp. presence or absence in 100 mL of sample.

5. Quality Control

Test each lot of commercial media for performance by inoculating it with three control bacteria.

Procedure:

a. Streak the culture onto labeled TSA or Blood Agar plates and incubate at 35 ± 0.5°C for 18 to 24 h.

b. For each bacterial strain, touch a sterile 1 μL inoculating loop to a colony and use it to inoculate a labeled test tube containing 5 mL sterile deionized water. Close cap and shake thoroughly.

c. For each bacterial strain, take a 1-μL loop from the test tube and use it to inoculate a labeled vessel containing 100 mL sterile deionized water.

d. Aseptically add a packet of substrate media to a 100-mL sample, mix well to dissolve, and follow 9230D.3*a*, *b*, or *c* for testing.

The following are some commercially available positive and negative controls that might be considered for testing:

- Positive Control
  - *Enterococcus faecium* (*e.g., Enterococcus faecium* ATCC 35667†)
- Negative Controls
  - *Escherichia coli* (Gram −) (*e.g., Escherichia coli* ATCC 25922†)
  - *Streptococcus bovis* (Gram +) (*e.g. Streptocccus bovis* ATCC 33317†)
  - *Serratia marcesens* ATCC 43862 (Gram −)
  - *Aerococcus veridians* ATCC 10400 (Gram +)

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* Enterolert® and Quanti-Tray®, Quanti-Tray®/2000, IDEXX Laboratories, Westbrook, ME.

† Suggested positive and negative controls provided by manufacturer for Enterolert™.

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9240 IRON AND SULFUR BACTERIA*

9240 A. Introduction

Iron and sulfur bacteria are a group of morphologically and physiologically heterogeneous organisms that can transform significant amounts of iron (Fe), manganese (Mn), or sulfur (S), usually depositing it in the form of objectionable slimes. They may cause, or be associated with, fouling and plugging wells. Their growth also may result in consumer complaints of red, black, or brown water in potable-water distribution systems. Sulfate-reducing bacteria may cause rusty water; pipe tuberculation; and odor, taste, frothing, color, and turbidity problems in waters.

The designation "iron and sulfur bacteria" is historical and imprecise; it includes microflora that may be filamentous or single-celled, autotrophic or heterotrophic, aerobic or anaerobic. Some "iron bacteria" deposit manganese oxides. (Chemically, manganese behaves similar to iron in the environment, although it oxidizes under more highly oxidizing conditions than iron.) The taxonomic position of these bacteria is diverse; in some cases, it is uncertain or in dispute.

Because many of these bacteria can use reduced iron or sulfur as their primary energy source, they can grow under highly oligotrophic conditions when attached to a substrate in flowing water that contains iron or sulfur. Temperature, light, pH, and oxygen supply are critical to the growth of iron and sulfur bacteria. Under different environmental conditions, some bacteria may appear either as iron, manganese, or sulfur bacteria. For example, *Acidothiobacillus* (formerly *Thiobacillus*) *ferroxidans,* which contributes to the problem of acid mine drainage, can be identified by the test for transforming ferrous to ferric iron, but it can also grow via oxidation of reduced sulfur compounds at low pH.

So for both practical and historical reasons, they are treated collectively here as iron and sulfur bacteria. These elements often co-occur, and their transformations may be important in the operation and maintenance of water treatment and distribution systems. They may be especially bothersome in industrial waters (e.g., cooling and boiler waters).

* Approved by Standard Methods Committee, 2011.
Joint Task Group: 22nd Edition—Stuart A. Smith (chair), Jennifer L. Clancy, David Emerson, Gary Rogers, Richard F. Unz.

9240 B. Iron Bacteria

1. General Characteristics

Iron bacteria are bacteria that can (directly or through manipulation of the environment) oxidize any reduced iron in their aqueous habitat from the ferrous to the ferric state and deposit it (in the form of amorphous or low-order crystalline hydrated ferric oxide) on or in biofilms.* Some of these bacteria may also precipitate manganese. Most manganese oxidation is microbially mediated at pH <8; however, researchers have not yet shown that bacteria can derive energy from this process. In fact, why bacteria oxidize manganese remains something of a mystery.

Iron oxidation may spontaneously and rapidly oxidize above pH 4 or 5, but some abundantly growing bacteria depend solely on iron oxidation at neutral pH for their energy source. Fe and Mn oxidation produces large amounts of brown slime that result in biofouling, discoloration, and unpleasant odors in drinking water, and may render the supply unsuitable for domestic or industrial purposes unless treatment or remedial steps are taken.

The ferric form is precipitated as ferric hydroxide [$Fe(OH)_3$], typically ferrihydrite.[1] Likewise, Mn-oxidizing and -precipitating bacteria deposit Mn III/Mn IV oxides (MnO_X) primarily as birnessite, todorokite, or amorphous hydrated birnessite (informally, "buserite").[1,2] Iron may be obtained from the pipe itself if it is made of iron (for both Fe and Mn), from the water within it, or directly from matrix minerals.

A number of microbial presumptive "species" have been described as "iron bacteria" based almost solely on morphological characteristics. Morphologically, iron-precipitating microflora can be divided into filamentous, stalked, or unicellular (nonfilamentous) forms. Despite the proliferation of phylogenetic "genus and species" names (phylotypes), the stalked forms can be represented by *Gallionella ferruginea* and the sheathed filamentous forms by the genus *Leprothrix* (either *L. discophora* or *L. ochracea*). Other filamentous forms are described from ground water samples (e.g., *Crenothris polyspora* and *Sphaerotilus natans*) but these do not necessarily play a role in iron precipitation, even though they are present in biofilm samples and sometimes encrusted by Fe oxides.[3] The identification of other bacteria present depends on the observer's training. Those with wastewater backgrounds tend to identify filamentous forms using wastewater sludge terminology, and those with aquatic microbiology backgrounds tend to identify such forms using their familiar terminology.

Gallionella and *Leptothrix* are the two most easily identifiable iron bacteria. *G. ferruginea* forms a twisted helical stalk composed primarily of iron oxihydroxides. The cells are bean-shaped and, when observed, are at the terminal ends of the stalks, where they excrete stalk material. While the stalks are common, the cells themselves are only seen in actively growing cultures.

* Usually polysaccharides.

L. ochracea is one of the most commonly observed iron-oxidizing bacteria in circumneutral, Fe-rich waters. It forms a hollow, tubular sheath that is typically encrusted with Fe oxides. This easily distinguishes it from *L. discophora* (often associated with Mn precipitation) and *G. ferruginea*. Although it is common to see large masses of *L. ochracea* sheaths in iron-rich microbial mats, the bulk of the sheaths are empty. This organism has not been cultured axenically in the laboratory,[3] so little is known about its physiology or relationship to other iron bacteria. However, based on its abundance in Fe(II)-rich waters that contain little organic matter, it is assumed to be a chemolithoautotroph.

In natural samples, it is common to observe both *L. ochracea* sheaths and *G. ferruginea* stalks by light microscopy; one morphotype typically dominates over the other.[4]

Because of the unique morphologies of the iron oxides that they produce, *L. ochracea* and *G. ferruginea* are considered classic indicator organisms for chemolithoautotrophic iron-oxidizing bacteria. There are also unicellular bacteria that can oxidize and grow on iron,[1,3,5] precipitating amorphous iron oxides (ones that do not have any characteristic shape and, therefore, are largely indistinguishable from chemically formed iron oxides). They probably contribute just as much to bacterial iron oxidation and may be numerically dominant to *L. ochracea* and *G. ferruginea* in most habitats where iron-oxidizing bacteria occur.[6] However, they coexist with *L. ochracea* and *G. ferruginea* in environments with standing or flowing water, so the latter two organisms remain excellent indicators of the overall presence of iron-oxidizing bacteria.

Two other bacteria often associated with iron deposits are members of *Siderocapsaceae* and *Pseudomonas*.[1,3] The exact role of these bacteria in precipitation is not clear. Researchers have not yet shown that they can grow directly on ferrous iron.

While some bacteria oxidize dissolved Fe and Mn, others reduce Fe and Mn oxides and mobilize them into the environment under reducing or anaerobic conditions. Generally, these organisms (e.g., *Geobacter* spp.) couple oxidation of organic matter or hydrogen to the reduction of iron oxides (anaerobic respiration). This may result in localized cycles of Fe and Mn oxidation and reduction in aquifers and other mineral-rich environments. This may produce less ferric hydroxide but still cause taste, odor, and fouling problems.

2. Sample Collection and Identification

a. Sampling and examination procedures: Examples of iron bacteria are shown in Figures 9240:1 –6. Nuisance iron bacteria usually have been identified based on microscopic examination of the suspected material. However, culturing is commonly practiced to refine the information provided by microscopy, and biochemical and genetic analysis is available.

Directly examine bulked activated sludge; masses of microbial growth in lakes, rivers, and streams; and slime growths in systems (e.g., wells and cooling-tower water-circulation systems). Special efforts may be required to secure useful samples for identification from water wells or distribution systems. For example, the material trapped by filters in front of back-surge valves often yields excellent specimens of iron bacteria. **CAUTION: Such samples should be handled using appropriate sanitary methods.**

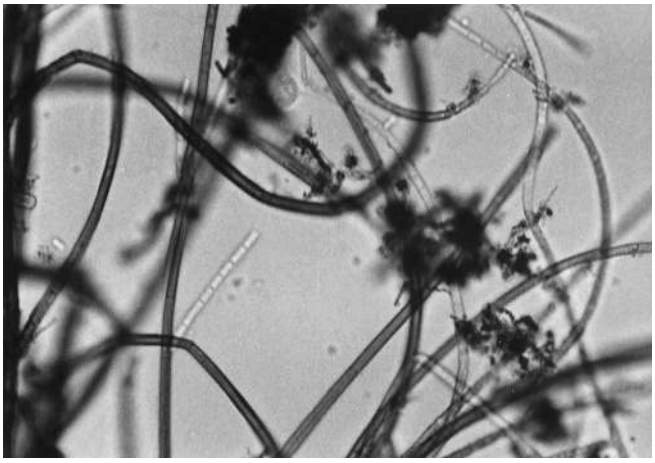


Figure 9240:1. Filaments of *Crenothrix polyspora* showing variation of size and shape of cells in the sheath. Note especially the multiple small round cells (*conidia*) found in one of the filaments. This distinctive feature is the reason for the name *polyspora.* Young growing colonies usually are not encrusted with iron or manganese. Older colonies often have heavily encrusted, empty sheaths. Cells may vary considerably in size: Rod-shaped cells average 1.2 to 2.0 μm wide by 2.4 to 4.5 μm long; conidia's coccoid cells average 0.6 μm in diameter.

Water pumped from wells may be passed through a 0.45-μm membrane filter. Likewise, microscope slides may be immersed in flowing or static (e.g., well column) water to collect biofilm samples. Then, dry the membrane, apply immersion oil directly to it, and examine it microscopically. Alternatively, settle or centrifuge samples drawn directly from wells or collect biofilms on surfaces immersed in water, and examine the sediment microscopically. Place a portion of sediment on a microscope slide, cover with a cover slip, and examine under a low-power microscope for filaments and iron-encrusted filaments. If using conventional light microscopy, stain the culture with india ink or lactophenol blue. Phase-contrast microscopy has improved the examination of unstained culture material. The epifluorescence microscopic method (Section 9216B) also can be used to observe iron bacteria.

Ongoing, heavy iron deposition caused by the oxidation of ferrous iron by air or other environmental changes, as well as activity in the biofilms themselves, often hides the sheaths or stalks of iron bacteria. Cells in the filaments often die and disintegrate, and the filaments tend to be fragmented or crushed by the mass of iron precipitate.

To dissolve iron deposits, place several drops of 1*N* HCl at one edge of the cover slip and draw it under the cover slip by applying filter or blotting paper to the opposite edge. Dilute acetic acid (5% v/v) or reducing compounds (e.g., sodium ascorbate) also may be used to disperse or dissolve deposits and permit observation of cellular structure. To verify that the material is iron, add a solution of potassium ferrocyanide to a sample on a slide, cover, and draw 1*N* HCl under the cover slip. A Prussian blue precipitate will form as iron is dissolved around cells as filaments.

b. Flowcell procedure for collecting iron-related biofilm sample: The flowcell sampling procedure provides a means of col-

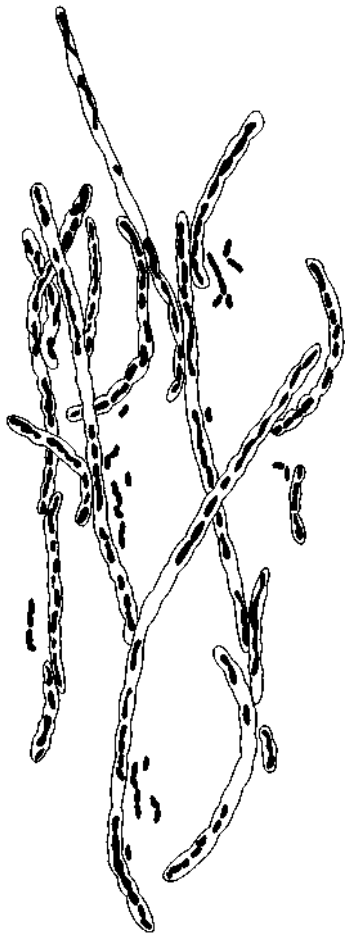


Figure 9240:2. Filaments of *Sphaerotilus natans*, showing cells in the filaments and some free "swarmer" cells. Filaments show false branching and areas devoid of cells. Individual cells in the sheath may vary in size, averaging 0.6 to 2.4 μm wide by 1.0 to 12.0 μm long; most strains are 1.1 to 1.6 μm wide by 2.0 to 4.0 μm long.

lecting biofilm material from pipeline flow (e.g., discharge at a wellhead or flow in a pipeline) for microscopic examination. It permits analysts to collect biofilm samples from systems where grab sampling does not reliably yield useful samples for microscopy.

There are numerous procedures for collecting iron bacteria and other biofouling microflora.[2,7–12] In the following procedure, water is trickled through an enclosed chamber containing collection surfaces[12,13] (a modified flowcell device[11,12]). Because the systems being sampled are not sterile, follow the appropriate QC procedures to ensure that the flowcell itself is not the source of any bacteria in the sample.

1) Example apparatus—The flowcell collection assembly[11,12] consists of a sealable flowcell chamber made of polycarbonate plastic with removable end plates, a ring gasket (fits in end-plate groove), one or more wing-nut bolts, and inlet and outlet taps (Figure 9240:7) . The assembly also includes an inlet stopcock valve and a backflow-prevention check valve. Inlet hoses are adapted to the sampling-point configuration (e.g., a hose connection).

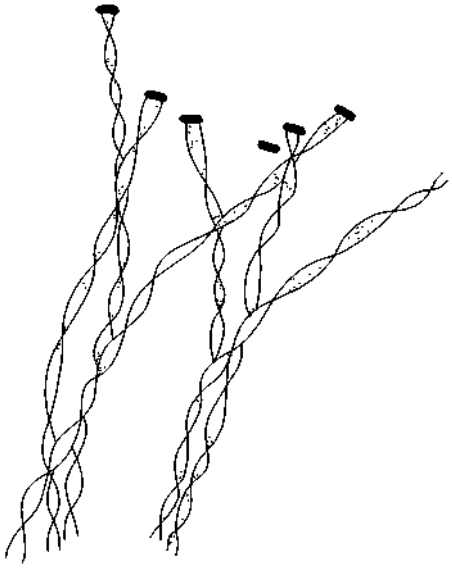


Figure 9240:3. Laboratory culture of *Gallionella ferruginea*, showing cells, stalks excreted by cells, and branching of stalks where cells have divided. A precipitate of inorganic iron on and around the stalks often blurs the outlines. Cells at tip of stalk average 0.4 to 0.6 μm wide by 0.7 to 1.1 μm long.

Insert element in flowcell chamber, with microscope slides or other coupons of interest. Sterilize insert assembly with its collection surfaces via autoclaving or ethylene oxide gas (depending on the selected coupons' heat tolerance). Do not open or handle until ready to use. Similarly, a simplified apparatus may be used (e.g., inserting microscope slides into 25-mm I.D. autoclavable plastic tubing, closing ends with tape, and then autoclaving the assembly).

This method does not limit biofilm collection to the described procedures. It can incorporate any device or procedure derived from relevant work[1–12] that samples the water of interest; prevents or avoids contamination, oxidation, or other environmental interferences; and collects a representative sample.

2) Example sampling procedure—Sample iron bacteria and other biofouling (e.g., sulfur-oxidizing slime) as follows:†

To install apparatus, assemble flowcell unit, attachment hoses, valves, and fittings from clean parts (no encrustation or discoloration). Then, disinfect the unit by immersing it in a chlorine solution of approximately 100 mg/L for 10 min. Rinse with treated water or the water to be sampled. Keep end plate and hose ends from touching nonchlorinated surfaces. Alternatively, if materials permit, sterilize the whole assembly.

Mount assembly so flow enters at the bottom and exits at the top. Attach inlet hose to wellhead tap. Drained water should be directed to a drain that exits the facility. **CAUTION: Follow appropriate safety procedures, and do not compromise operations or safety.**

Open spigot and inlet valves so flowcell fills slowly to rim of opening. Overflow to remove any remaining disinfectant. Un-

† A minimum amount of sediment may collect in the flowcell, but it does not appear to have any effect on biofilm collection, nor does it appear to have an effect due to gradient formation.

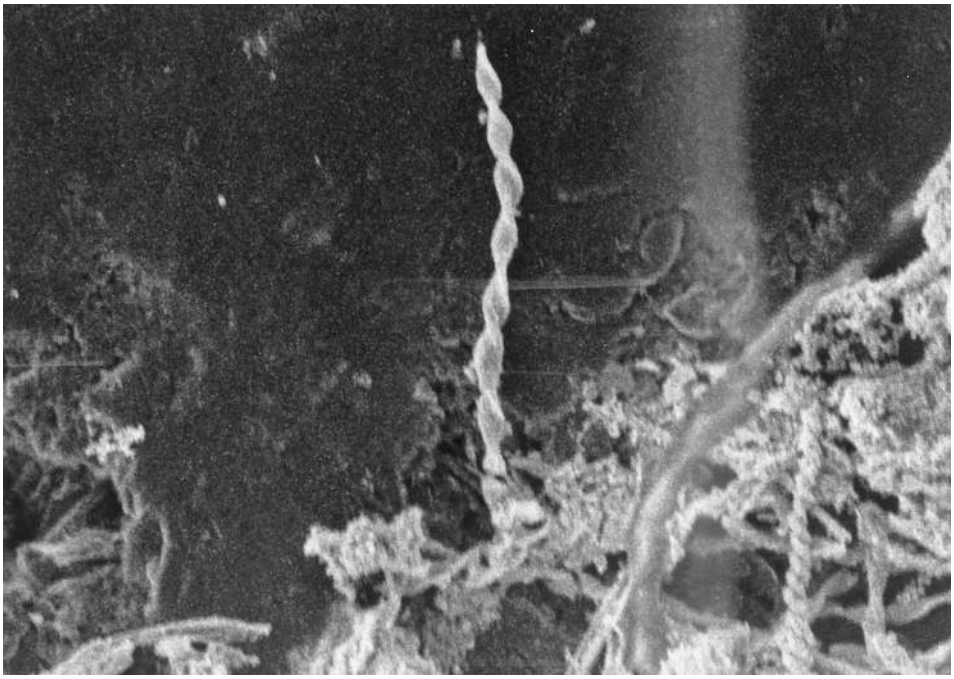


Figure 9240:4. Stalk of *Gallionella ferruginea*. Fragmented stalks appear golden yellow to orange when examined under the microscope. Magnification is 2000×.

wrap sterilized slide-holder insert, handling only by the nut that secures the covering sleeve (preferably with sterile latex gloves). Place insert into filled water column and let it settle to the bottom. Install end plate and secure. Turn on water flow and establish a trickle flow (approximately 0.4 to 1 L/min). Test seals, connections, and joints for leaks, and purge air from flowcell chamber.

Establish a time period for inspecting slides for signs of iron or other discoloration and for removing slides for analysis. For example, raw water typically is sampled from wells every 1 to 4 weeks, but the interval varies. If possible, check daily for flow (which may need to be adjusted) and signs of discoloration.

To remove slides, stop flow through cell and remove end plate, keeping chamber full. Slowly retrieve entire slide-holder insert and immediately transfer to a disinfected or sterile travel container filled with water from the sample source, or sterile water of similar composition. Label container with source, date collected, and days of slide exposure. Record sample-collection circumstances and any relevant observations. Remove slides aseptically from the insert assembly for examination via light or electron microscopy.

3) Examination—For light microscopy, clean one side of the glass microscope slide and fit a clean cover slip on the other side.

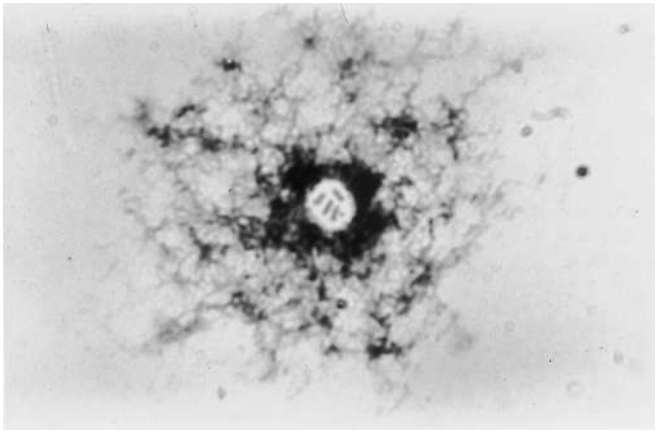


Figure 9240:5. Single-celled iron bacterium *Siderocapsa.* Magnification is 1000×. Cells are surrounded by a deposit of ferric hydrate. Individual cells average 0.4 to 1.5 μm wide by 0.8 to 2.5 μm long.

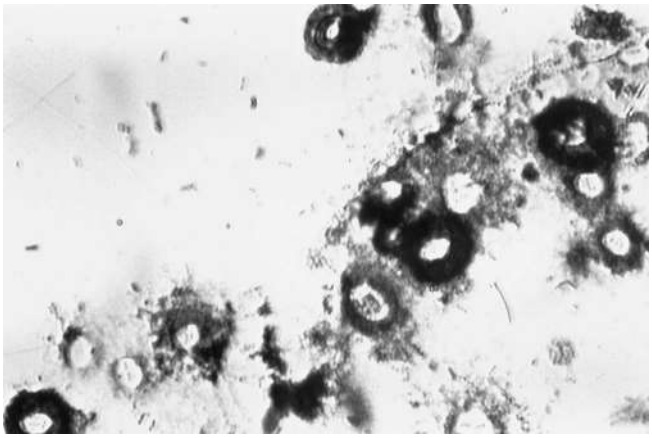


Figure 9240:6. Multiple colonies of *Siderocapsa* spp.

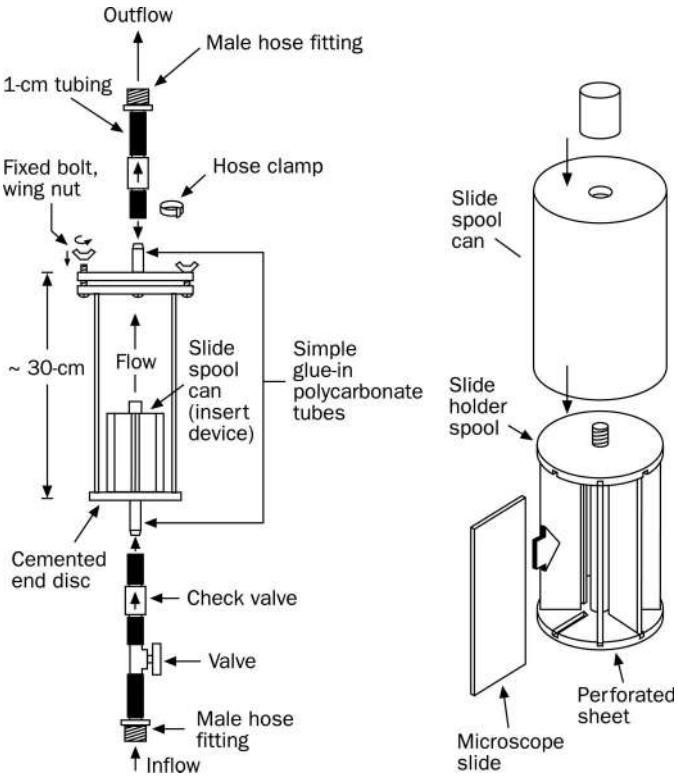


Figure 9240:7. Schematic of flowcell: (left) assembly; (right) slide holder insert.

Alternatively, subsample a biofilm-coated coupon for microflora culturing, electron microscopy, or other elemental or crystallinity analyses relevant to the biofouling or its associated effects on the exposed coupon.

4) Alternative procedures—Simplified apparatus may be immersed directly into flowing water (e.g., soil or geotechnical drains)[2] or enclosed in the specially fabricated flow chamber or any disinfected pipe enclosure. The slide-immersion method can be adapted for wells by suspending slides in the well-water column and retrieving them after a period of time[12] (as is done in the flowcell method). These procedures are also suitable for collecting biofilms consisting predominantly of sulfur-oxidizing bacteria. Modifications are necessary for such factors as security (tamper-free operation), high temperature or pressure, or situations in which the flowcell's outflow must be contained (e.g., contaminated ground or process water).

c. Detection and identification: Researchers have developed and tested cultural methods for isolating and selectively enriching iron bacteria (9240D). When examining samples microscopically, identify organisms by comparing those observed with available drawings or photographs of iron bacteria.[11–24] Also, use information from the environment and about microbial occurrence and growth preferences.

Leptothrix, *Sphaerotilus*, and *Crenothrix* are typically characterized by sheathed filaments, although nonsheathed *Leptothrix* strains have been cultured.[25] *L. discophora* can typically be distinguished from *L. ochracea* by its precipitation of platey MnO_X deposits around filament holdfasts; *L. ochracea* has general filament encrustation by ferrihydrite.[1–3] *S. natans* is sheathed like *Leptothrix*, but it is typically not encrusted and is absent from oligotrophic water. *Crenothrix* filaments and cells are larger than *Leptothrix*, often form spores in filaments under stress (e.g., under microscope lights), and may appear vacuolated (the cells may have a spore-like appearance). *Crenothrix* filaments are typically not encrusted with mineral; recent evidence suggests they actually use methane as an energy source.[26]

3. Phylogenetic Relationships among Iron-Oxidizing Bacteria

Understanding the relationships among iron-oxidizing bacteria is the subject of ongoing research. At present, lithotrophic Fe-oxidizing bacteria [those that use Fe(II) as a sole energy source] seem to share relatedness. *G. ferruginea* is in the same family as *S. lithotrophicus*;[3] they are more closely related to one another than they are to *Leptothrix discophora* or *Sphaerotilus natans*, even though all are members of the same class of Beta-proteobacteria. At present, analysts do not know how closely *L. ochracea* is related to other Fe-oxidizing bacteria. Although it shares the genus name *Leptothrix* with *L. discophora*, this is based purely on its morphology and probably does not reflect its true phylogeny.

Researchers could design molecular probes to both indicate the presence of specific bacteria and approximate their numbers in environmental samples.[25] The most promising technologies for doing this are quantitative polymerase chain reaction (qPCR) and fluorescent *in situ* hybridization (FISH). Both would require designing DNA-based probes that are specific to a group of related organisms. While neither method is ready for routine use in monitoring waters for Fe-oxidizing bacteria, the knowledge that we could design specific probes for important indicator organisms makes these technologies promising for the future.

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9240 C. Sulfur Bacteria

1. General Characteristics

The bacteria that oxidize or reduce significant amounts of inorganic sulfur compounds have widely diverse morphological and biochemical characteristics. For example, sulfate-reducing bacteria consist mainly of single-celled forms that grow anaerobically and reduce sulfate (SO_4^{2-}) to hydrogen sulfide (H_2S). One member of this group, *Desulfonema*, is multicellular with gliding motility. Another group, photosynthetic green and purple sulfur bacteria, grows anaerobically in light and uses H_2S as a hydrogen donor for photosynthesis. Members of a third colorless filamentous group are mixotrophic and use organic sources of carbon but may get their energy via oxidizing reduced sulfur compounds. The sulfide is oxidized to sulfur or sulfate. A fourth group, aerobic sulfur-oxidizers, oxidizes reduced sulfur compounds aerobically to obtain energy for chemoautotrophic growth.

The sulfur bacteria most prominently described as a source of problems in the water and wastewater field are sulfate-reducing bacteria (e.g., *Desulfovibrio* and single-celled aerobic sulfur-oxidizers of the genus *Thiobacillus*). These bacteria contribute greatly to tuberculations, galvanic corrosion of water mains, and taste and odor problems in water. *Thiobacillus*, which produces sulfuric acid, has contributed to the destruction of concrete sewers and the acid corrosion of metals. Filamentous *Thiotrix*, *Beggiotoa*, and similar types of aerobic colorless (white) sulfur bacteria are common components of clogging biofilms in geotechnical drains and wells where aqueous sulfide is present in ground water. They are most common in moving oxygenated

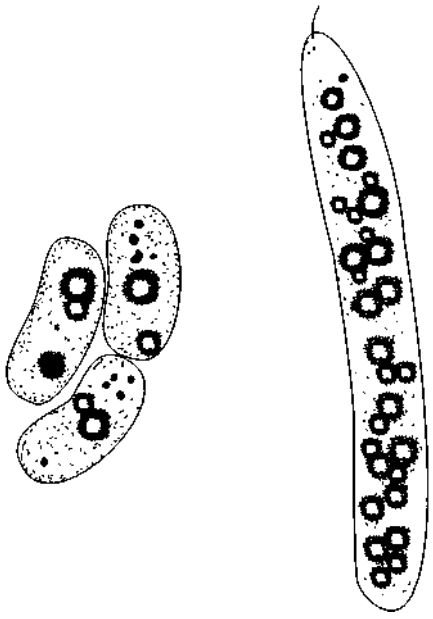


Figure 9240:8. Photosynthetic purple sulfur bacteria. Large masses of cells have brown-orange to purple color and may appear chalky if the cells contain a large amount of sulfur. (Left) Cells of *Chromatium okenii* (5.0 to 6.5 μm wide by 8 to 15 μm long) containing sulfur globules; (right) *Thiospirillum jenense* (3.5 to 4.5 μm wide by 30 to 40 μm long)—cell contains sulfur globules and polar flagellum is visible.

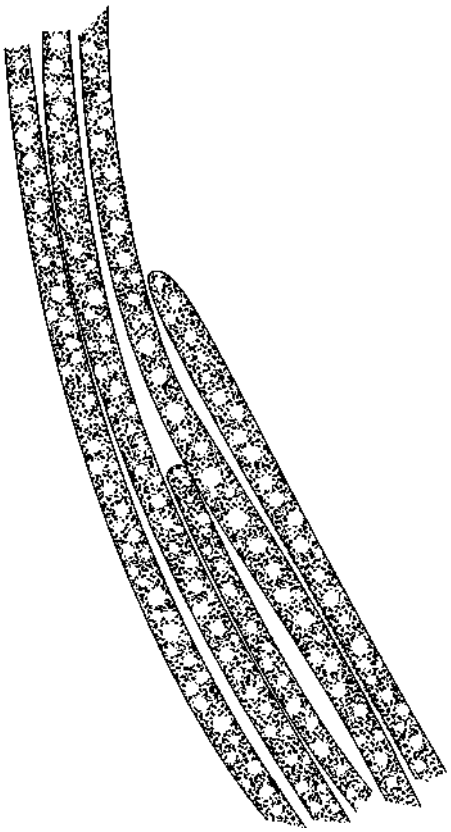


Figure 9240:9. Colorless filamentous sulfur bacteria: *Beggiatoa alba* trichomes, containing globules of sulfur. Filaments are composed of a linear series of individual rod-shaped cells that may be visible when not obscured by light reflecting from sulfur granules. Trichomes are 2 to 15 μm in diameter and may be up to 1500 μm long; individual cells, if visible, are 4.0 to 16.0 μm long.

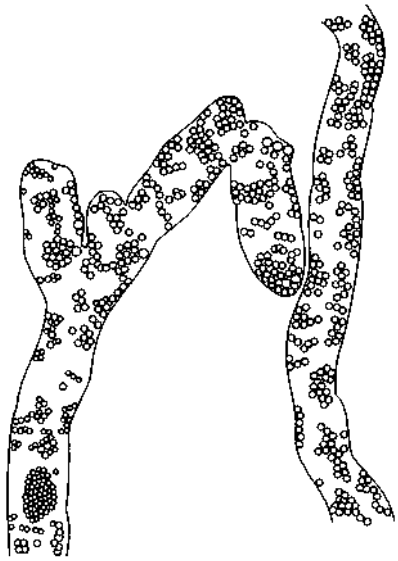


Figure 9240:10. Colorless filamentous sulfur bacteria: portion of a colony, showing branching of the mucoid filament, identified as *Thiodendron mucosum*.[1] Because the name *Thiodendron* previously had been used in bacterial taxonomy, its use here is illegitimate, and this organism remains unnamed. Individual cells (1.0 to 2.5 μm wide by 3 to 9 μm long) have been found in the filaments' jelly-like material. The long axis of the cells runs parallel to the long axis of the filaments.

water (e.g., the water column of an actively pumping well or a regularly flowing drain or spring).

2. Sample Collection and Identification

Nuisance sulfur bacteria usually have been identified based on microscopic examination of suspected material. Examine samples of slimes suspended in waters, scrapings from exposed surfaces, slides exposed in affected water (using the methodology developed for iron and manganese biofilm-forming bacteria), or sediments directly.

Three groups of sulfur bacteria may be recognized microscopically:

- green and purple sulfur bacteria;
- large, colorless filamentous sulfur bacteria; and
- large, colorless, nonfilamentous sulfur bacteria.

A fourth group—sulfate-reducing bacteria and sulfur-oxidizing bacteria of the genus *Acidothiobacillus*—cannot be identified by appearance alone using light microscopy.

a. Green and purple sulfur bacteria:

1) Green sulfur bacteria most frequently occur in waters containing H_2S. They are small, ovoid to rod-shaped, nonmotile organisms, generally less than 1 μm in diameter, and with a yellowish-green color in masses. Sulfur globules are seldom if ever deposited in the cells.

2) Purple sulfur bacteria (Figure 9240:8) occur in waters containing H_2S. They are large, generally stuffed with sulfur globules, and often so intensely pigmented that individual cells appear red. Large, dense, highly colored masses are detected easily by the naked eye. The presence of photosynthetic bacteria

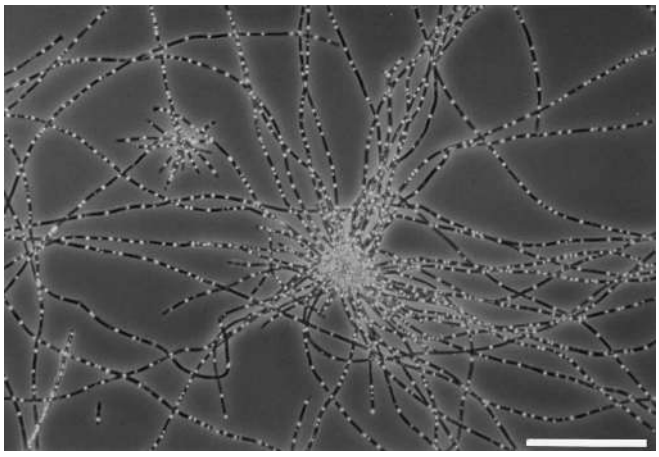


Figure 9240:11. *Thiothrix unzii* after 24 h in lactate-thiosulfate medium. Bright inclusions in filaments are sulfur granules. Bar = 40 μm. Reproduced with permission of the American Society for Microbiology.

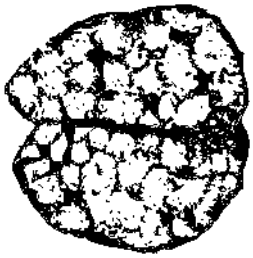


Figure 9240:12. Colorless nonfilamentous sulfur bacteria: dividing cell of *Thiovolum majus,* containing sulfur globules. Cells may measure 9 to 17 μm wide by 11 to 18 μm long and are generally found in nature in a marine littoral zone rich in organic matter and hydrogen sulfide.

in concentrated masses can be confirmed by extracting the mass with ether and scanning the extract absorbance in the infrared region. Bacterial chlorophyll will absorb strongly in the range of 660 to 870 nm.

b. Colorless filamentous sulfur bacteria: Colorless filamentous sulfur bacteria (Figures 9240:9–11) occur in waters where both oxygen and H_2S are present. They may form mats with a slightly yellowish-white appearance due to deposition of internal sulfur globules. They generally are large and may be motile with a characteristic gliding movement. Identify by comparing organisms with available photographs.[1–4]

c. Colorless nonfilamentous sulfur bacteria: Colorless nonfilamentous sulfur bacteria (Figure 9240:12, for example) usually are associated with decaying algae. They are extremely motile, ovoid to rod-shaped organisms with sulfur globules and possible calcium carbonate deposits. They generally are very large.

d. Colorless small sulfur bacteria and sulfate-reducing bacteria: Small single-celled bacteria, *Thiobacillus* spp., and sulfate-reducing bacteria (e.g., *Desulfovibrio*) cannot be identified by direct microscopic examination. *Acidothiobacillus* types are small, colorless, motile, and rod-shaped; they are found in an environment containing H_2S. Sulfur globules are absent. Identify *Thiobacillus* types, *Desulfovibrio,* or other sulfate-reducing bacteria physiologically.

3. References

1. Lackey, J.B. & E.W. Lackey. 1961. The habitat and description of a new genus of sulfur bacterium. *J. Gen. Microbiol.* 26:28.
2. Faust, L. & R.S. Wolfe. 1961. Enrichment and cultivation of *Beggiatoa alba. J. Bacteriol.* 81:99.
3. Morgan, G.B. & J.B. Lackey. 1965. Ecology of a sulfuretum in a semitropical environment. *Z. Allg. Mikrobiol.* 5:237.
4. Vos, P., G. Garrity, D. Jones, N.R. Krieg, W. Ludwig, F.A. Rainey, K.-H. Schleifer & W.B. Whitman, eds. 2005. Bergey's Manual of Systematic Bacteriology: Volume 3: The Firmicutes. Springer-Verlag, New York, N.Y.

9240 D. Enumerating, Enriching, and Isolating Iron and Sulfur Bacteria

Historically, methods for enumerating iron and sulfur bacteria, other than sulfate-reducing bacteria and acidothiobacilli (9240E), have been limited. Cultivating and isolating pure cultures in the laboratory is difficult, and successful isolation is uncertain. This is especially true of attempts to isolate filamentous bacteria from activated sludge or other samples containing many types of bacteria. Recent research suggests that microbial communities living in the oligotrophic conditions typical of water systems operate as interdependent consortia and may not be able to grow in isolation.

Enumeration may depend on MPN (Section 9221C), assuming the microbial community is culturable, or other estimating methods (see discussion following). **Caution: All inoculated microbiological media should be considered potentially biohazardous and handled and disposed of accordingly.**

Note: Refer to Section 9020 for general quality assurance/quality control (QA/QC).

Note on storage time limits and media expiration: Media and media components stored after preparation could degrade due to storage conditions or may become contaminated. As part of general laboratory QA/QC, establish a media-storage and -handling procedure. Specific storage time limits and expiration date guidelines have not been established for individual media formulations cited in the following sections. Table 9020:V provides general guidelines for holding times for prepared agar and broth media. Minimum media (essentially basal salts and mineral amendments) have shelf lives of up to 6 months if maintained at 4°C, with 4 weeks being a conservative guideline. The best recommendation for liquid, broth, and agar media is to prepare what is needed on an as-needed basis. BART media described in the following (dehydrated media that is gas-sterilized and shipped packaged) and liquid commercial MAG media have manufacturer-established expiration dates printed on packaging, and these dates should be used for setting storage and use limits. Where uncertainty exists, use the sterility test in Section 9020B.9*d*.

1. Use of Liquid Media in Industrial and Environmental Analyses of Biofouling Microflora

One practical approach for both presumptively identifying the components of and gauging the degree of water and soil biofouling involves liquid or dehydrated media designed to selectively enrich consortia in water or soil. These methods have been in widespread use since the early 1990s in engineering and industrial water system maintenance and diagnostic practice.[1–3] Two methods in common use are described for iron biofouling, or iron-related bacteria (IRB; not to be confused with iron-reducing bacteria). Variations of these methods exist for sulfate-reducing bacteria (SRB) and other microbial communities of interest in water system, drain operation, and water quality. The utility of these methods has been evaluated for operational and

standard practice use.[1–6] When following these methods, use pre-prepared media and kits where possible to ensure QA/QC standards and vendor support.

*a. Biological activity reaction test (BART™) method:** BART is a method of detecting growth to provide either a presence–absence (P–A) or a quantitative (MPN) result. BART tubes contain dehydrated media formulations and a floating barrier device (a ball that floats on the hydrated sample medium). These devices and their proposed use are described in detail in the literature.[7–8] BART method tubes come with a variety of media mixtures. IRB-BART™, for example, is based on "W–R" medium (an adaptation of Winogradsky's medium)[9] designed to recover micoaerophilic heterotrophic Fe- and Mn-precipitating microorganisms. Upward diffusion of media and downward diffusion of oxygen creates gradient conditions. Meanwhile, SRB-BART uses a dehydrated Postgate medium.

To use BART, collect the sample in the outer sterile tube (as provided) or in any sterile sampling container. Fill the inner tube (containing dried media) to the marked line (about 15 mL). Replace the tube cap, label the tube, and let the sample incubate at ambient (typically room) temperature until a reaction occurs. Alternatively, maintain an incubation temperature analogous to that of the sampled environment. In an SRB-BART test, oil in the dehydrated medium effects a seal around the ball to induce an anaerobic fluid column. Interpret BARTs based on the (*a*) visual appearance of inoculated tubes at initial reaction and as reactions change, and (*b*) days of delay (d.d.) until a noticeable reaction occurs. Changes after the first reaction provide information on microflora likely to be in the sample, and thus information on clogging, corrosive, and biotransformation reactions that can be expected in the system.

Compare observed results to charts designed to aid interpretation; results may be analyzed using vendor-provided software.[7] Based on the vendor's empirical studies,[8] the visual appearance is interpreted to provide information on the types of microorganisms present.[7,8] The d.d. reaction is interpreted in terms of "aggressivity"—a combination of numbers and activity, not further distinguished. The d.d. values can be compared semiquantitatively to CFU/mL values for agar media, also based on the vendor's empirical studies.[7,8] The comparisons are for proportional interpretation only, not precise counts, because d.d. values compare to log CFU/mL. IRB-BART does not rigorously isolate iron-precipitating bacteria, but it does support growth of heterotrophic microflora, which do not precipitate iron under growth conditions.

Independent analyses[1,2] found BART reaction results to be consistent in growth reaction type and d.d. across replicates from split samples, with other comparable media, and with environmental conditions. Results of BART analyses during use in diagnostic practice were consistent with observed conditions (e.g., rapid iron-biofouling results were consistent with observed clogging).[1,6]

b. MAG tests†:[2,3] This is a similar system that has been refined independently. MAG tests for heterotrophic IRB (MAG-CHA), SRB (MAG-BRS), and other microbial types of interest (e.g., acid-producing heterotrophs) consist of a prepared liquid medium in small septum bottles.

Take a 10-mL sample using a syringe and inject it through the septum into the medium. Then, let the sample incubate, as with the BART method. Inoculating one bottle provides a P–A result. Dilution to extinction provides an MPN result.[3] Instructions for use are provided by the vendor.‡ In small-scale tests,[5] MAG methods provided P–A results that seemed comparable to BART results; however, further testing is necessary to make definite conclusions about their comparability.

2. Growth of Iron-Oxidizing Bacteria in Gradient Culture

Fe-oxidizing bacteria that use Fe(II) as a sole energy source at neutral pH are microaerophilic and must be cultured under low O_2 concentrations. Specific methods for growing these bacteria rely on establishing opposing gradients of Fe(II) and O_2. In this way, the chemical oxidation of Fe(II) is minimized by low O_2 levels, and the bacteria grow well at the O_2–Fe(II) interface. In essence, this mimics their natural habitat at the oxic–anoxic surface, where Fe(II)-rich water comes into contact with atmospheric O_2.

A detailed description of methods for growing Fe-oxidizing bacteria under these conditions is available,[10] so only a basic description of a gradient tube method is provided. BART and MAG-CHA tests also establish media–oxygen gradients in their liquid media columns.

a. Preparation of gel-stabilized gradient tubes: Typically, 17 × 60 mm (O.D. × length) borosilicate glass vials are used for gradient tube cultures. Prepare the top layer (the semi-solid mineral media) and the bottom layer (the iron source) separately. The bottom layer contains 1% (w/v) high-melt agarose and equal volumes of modified Wolfe's mineral medium (MWMM) and FeS. In a separate container, prepare the top layer by adding 0.15% (w/v) low-melt agarose to MWMM, 5 m*M* sodium bicarbonate, and 1 μL of trace minerals§ per mL of medium. Autoclave both layers. Shortly after autoclaving, pipet 0.75 mL of the bottom layer into each culture tube. To prevent clogging, use 1-mL pipet tips that have been cut to enlarge the opening. Let the bottom layer cool for at least 30 min to ensure that it is well set. Add 1 mL vitamin solution‖ and adjust the pH to within 6.1 to 6.4 by bubbling with sterile CO_2 gas. The gassing time depends on the medium volume and the CO_2 flow rate; it should be determined empirically.

To make the top layer, pipet 3.75 mL of medium over the bottom layer of each tube. Cap the tubes with either a butyl rubber stopper or a screw cap with a septum to maintain an aerobic headspace. Let the top layer cool to between 30 and 40°C and then allow it to solidify a minimum of 3 h to a maximum of overnight. (Allowing the tubes to sit uninoculated for longer periods will lead to undesirable amounts of abiotic iron oxidation.) To inoculate, draw 10 μL of the desired inoculums into a pipet tip and insert them just above the FeS layer. Draw the pipet tip upward as the inoculums spread into a rust-colored band at the oxic–anoxic interface.

* Droycon Bioconcepts Inc., Regina, Saskatchewan.
† Laboratorio MAG™, La Plata, Argentina.
‡ See http://www.laboratoriomag.com.ar/.
§ See http://www.atcc.org. ATCC MD-TMS.
‖ See http://www.atcc.org. ATCC MD-VS.

Transfer the cultures every 3 to 4 weeks. Refrigerated storage will prolong the culture's useful life. For long-term storage, remove the growth band with a pipet and mix it with an equal volume of sterile 20% (v/v) glycerol. Then, immediately freeze the glycerol stocks in either a −80°C freezer or in liquid nitrogen.

b. Alternative preparation of FeS stock solution (gel-gradient stabilized tubes): Prepare FeS by heating 300 mL d-H_2O to 50°C in a 500-mL beaker with a stir bar present. Separately pre-weigh 46.2 g ferrous sulfate and 39.6 g sodium sulfide. While rapidly stirring the water, add ferrous sulfate followed immediately by sodium sulfide. A thick black precipitate will form instantly. Stir this mixture continuously for 2 to 3 min to ensure that ferrous sulfate and sodium sulfide are completely mixed and dissolved.

Decant the black FeS sludge into a narrow-mouthed glass bottle (500 mL) that can be tightly stoppered. Fill the bottle to the top with d-H_2O and cap it. Allow FeS to settle for several hours, then decant and replace the overlaying water. Repeat this procedure at least five times to wash the FeS. Afterward, the FeS solution's pH should be close to neutral.

The FeS layer is normally quite hydrous and can be pipeted with a standard 10-mL pipet. After removing FeS for use, top the bottle up with d-H_2O and keep it tightly stoppered to limit the influx of oxygen. With limited oxygen exposure, FeS can be maintained at room temperature for up to 3 months. However, FeS ages even under these conditions, slowly losing its ability to release Fe(II). Each batch of FeS is slightly different. If FeS smells strongly of sulfide after washing or has a strongly alkaline pH, discard it. **CAUTION: In the presence of acid, sodium sulfide will immediately release hydrogen sulfide, an extremely toxic gas. Preparing FeS in a chemical fume hood is strongly recommended.**

3. A Selection of Special-Purpose Media for Iron and Manganese-Precipitating Bacteria

There are some media that may be used for enrichment (in some cases, selective). This is not an exhaustive list of media.[11–18]

a. Casitone–glycerol–yeast autolysate broth (CGY), for the Sphaerotilus group: This medium may not be available in dehydrated form and may require preparation from the basic ingredients. It may be solidified by adding 1.5% agar.

Casitone	5.0	g
Glycerol	10.0	mL
Yeast autolysate	1.0	g
Reagent-grade water	1	L

Heat to dissolve and autoclave for 15 min at 121°C. No pH adjustment is necessary.

b. Isolation medium (iron bacteria): This medium may not be available in dehydrated form and may require preparation from the basic ingredients:

Glucose	0.15	g
Ammonium sulfate [$(NH_4)_2SO_4$]	0.5	g
Calcium nitrate [$Ca(NO_3)_2$]	0.01	g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.05	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.05	g
Potassium chloride (KCl)	0.05	g
Calcium carbonate ($CaCO_3$)	0.1	g
Agar	10.0	g
Cyanocobalamin	0.01	mg
Thiamine	0.4	g
Reagent-grade water	1	L

Heat to dissolve and autoclave for 15 min at 121°C. No pH adjustment is necessary.

c. Maintenance (SCY) medium (iron bacteria): This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Sucrose	1.0	g
Casitone	0.75	g
Yeast extract	0.25	g
Trypticase soy broth without dextrose	0.25	g
Agar	10.0	g
Cyanocobalamin	0.01	mg
Thiamine	0.4	mg
Reagent-grade water	1	L

Heat to boiling and sterilize at 121°C for 15 min. Final pH is 7.3 ± 0.2.

d. Mn agar No. 1: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Manganous carbonate ($MnCO_3$)	2.0	g
Beef extract	1.0	g
Ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$]	150	mg
Sodium citrate	150	mg
Yeast extract	75	mg
Cyanocobalamin	0.005	mg
Agar	10.0	g
Reagent-grade water	1	L

Prepare and sterilize the medium at 121°C for 15 min without cyanocobalamin. No pH adjustment is necessary. Separately sterilize cyanocobalamin via filtration and add aseptically just before medium solidifies.

e. Mn agar No. 2:[19] Prepare fresh each time from basic ingredients:

Manganous sulfate ($MnSO_4 \cdot H_2O$)	10	mg
Agar	15.0	g
Natural water	1	L

Heat to dissolve ingredients and sterilize at 121°C for 15 min. No pH adjustment is necessary.

f. Iron oxidizing medium (Acidothiobacillus ferrooxidans): This medium may not be available in dehydrated form and may require preparation from the basic ingredients. Variations on media for this purpose exist.[20–22]

Basal salts:

Ammonium sulfate [$(NH_4)_2SO_4$]	3.0	g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.05	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.05	g
Potassium chloride (KCl)	0.05	g
Calcium carbonate ($CaCO_3$)	0.1	g

Energy source:

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$), 14.74% solution (w/v)	300	mL

Sterilize basal salts and energy source separately at 121°C for 15 min; then, cool and combine. The pH should be 3.3 ± 0.3. Store

in the refrigerator (2 to 8°C) and discard after 2 weeks. A precipitate will form and the medium will be opalescent and green.

g. Ferrous sulfide agar (Gallionella ferruginea): This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Agar layer:		
Ferrous sulfide (FeS) (washed precipitate and liquid)	500.00	mL
Sodium sulfide (Na_2S)	15.6	g
Ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$]	78.4	g
Boiling reagent-grade water	1	L
Agar (liquid) (30 g/L)	500.00	mL
Liquid overlay:		
Ammonium chloride (NH_4Cl)	1.0	g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.5	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.2	g
Calcium chloride ($CaCl_2$)	0.1	g
Reagent-grade water	1	L

Prepare FeS by reacting equal molar quantities of Na_2S and $Fe(NH_4)_2(SO_4)_2$ in boiling reagent-grade water. Let precipitate settle from the hot solution in a completely filled and stoppered bottle, limiting oxygen exposure. Wash precipitate four times by decanting supernatant and replacing with boiling water. Store FeS in a glass stoppered bottle completely filled with more boiling water. Add equal volumes of FeS and 3% agar at 45°C. Prepare slants in screw-capped tubes. Prepare liquid overlay, bubble CO_2 through it for 10 to 15 s, and add several milliliters to agar slant. Modifications to this medium and procedure are available.[11–13,23–27]

h. Modified Wolfe's Mineral Medium (MWMM):

Ammonium chloride (NH_4Cl)	1.0	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.2	g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.1	g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.5	g
Reagent-grade water	1	L

Autoclave for 15 min at 121°C. Adjust pH to 7.2 if necessary.

4. Sulfur Bacteria

"Sulfur" bacteria include both sulfate-reducing and sulfur-oxiding and -precipitating bacteria.

a. Sulfate-reducing bacteria:

1) To detect or enumerate sulfate-reducing bacteria (e.g., *Desulfovibrio*) use a sulfate-reducing medium.[28–32] Inoculate tubes and fill completely with sterile medium to create anaerobic conditions. For comparison, add one or two uninoculated controls to each set of inoculated tubes to be incubated. The SRB-BART and BRS-MAG are acceptable prepared alternatives. If sulfate-reducing bacteria are present, tubes (including SRB-BART and BRS-MAG) will show blackening within 4 to 21 d of incubation at 20 to 30°C. If sample volume is larger than 10 mL, the analyst may wish to filter sample through a 0.45-μm membrane and then put the membrane into a screw-cap test tube with medium. This is not necessary with SRB-BART, which are normally inoculated with a 15-mL sample.

2) An agar medium suitable for growing and enumerating sulfate-reducing bacteria also is available.[29]

Trypticase soy agar	40.0	g
Agar	5.0	g
60% sodium lactate (0.4% v/v)	4	mL
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	2.0	g
Ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$]	2.0	g
Reagent-grade water	1	L

Adjust pH to between 7.2 and 7.4 and sterilize via autoclaving (121°C, 15 min). Medium should be clear and free from precipitate.

Inoculate all plates within 1 or at most 4 h after agar hardens to prevent saturation with oxygen. To prevent moisture condensation on Petri dish covers, replace covers with sterile absorbent tops within 10 to 15 min after agar hardens. Place uninverted plates in desiccator or Brewer jars and replace atmosphere with tank hydrogen or nitrogen via successive evacuation and gas replacement. Alternatively, use a disposable anaerobic generating system.# Incubate at room temperature (21 to 24°C) or at 28 to 30°C, the optimum temperature for these organisms. Sulfate-reducing bacteria typically grow and blacken around the colonies; this may occur at any time between 2 and 21 d, although it usually happens within 2 to 7 d.

3) Media suitable for enumerating or isolating various species of sulfate-reducing bacteria are available.[1–3,30–32]

b. Photosynthetic purple and green sulfur bacteria: Because these organisms are so specialized and rarely cause problems in water and wastewater treatment processes, methods for isolating and enumerating them are not included here. Sometimes they can be beneficial because of their ability to oxidize hydrogen sulfide, thereby reducing odor. An excellent review, which includes media formulations and methods for cultivating specific members of this group of bacteria, is available.[33]

c. Thiobacillus *spp. and physiologically related S-oxidizers:* Researchers have carefully evaluated the growth and physiology of different species of the single-celled sulfur-oxidizing bacteria.[34,35] Media[36] suitable for enumerating *Thiobacillus thioparus* and *Acidothiobacillus thiooxidans* via an MPN technique are listed in 9240E. Inoculate medium and incubate for 4 to 5 d at 25 to 30°C. Thiobacilli growth produces elemental sulfur, which sinks to the bottom with a coincident decrease in the medium's pH and turbidity. Chemical tests for sulfate formation are necessary to confirm the presence of *Thiobacillus*.

d. Filamentous sulfur-oxidizing bacteria:

1) *Beggiatoa—Beggiatoa* exist in most aquatic habitats where both sulfide and oxygen are present,[37] including fresh and marine water, sediments, and wastewater systems. Because *Beggiatoa* is a multicellular bacterium, there is no method for accurately determining the number of viable cells in a sample. To determine the population of *Beggiatoa*, make a direct microscopic count of the number of filaments.

Marine beggiatoas may be quite large, up to 100 μm or more in diameter.[38,39] They have not been grown in the laboratory. However, small marine species (up to 5 μm diam) have been isolated.[39] The media for isolating freshwater and marine beggiatoas differ slightly.

a) Enrichment—Inoculate a tube of extracted hay [9240D.5*d*1)] with enough water and a little mud from the sample site to fill the tube to a depth of at least 8 cm. Incubate for at least 1 week and examine for the presence of "tufts" or "puff-balls" consisting of tangled filaments of *Beggiatoa*. Use phase-contrast microscopy to look for individual filaments.[30] If

GasPak, BBL, or equivalent.

no "puff-balls" are found, incubate for another week and repeat the examination. Continue examining the enrichment for up to 4 weeks before discarding it.

b) Isolation—Using sterilized fine-tipped forceps and a dissecting microscope, transfer tufts of *Beggiatoa* from enrichments to a small Petri dish containing sterile basal medium[40] [9240D.5*d*2)]. Shake tufts with forceps to remove adherent bacteria and transfer tufts to a new Petri dish with sterile basal medium. Continue until tufts have been washed at least five times. Transfer tufts to a "drying plate" containing basal medium and 1.6% agar for about 1 min to remove excess fluid, then transfer tufts to the center of separate plates of either MP or MY medium[40,41] [9240D.5*d*4) and 6)]. Incubate plates at room temperature or cooler and examine with a dissecting microscope every 5 to 10 h for gliding filaments of *Beggiatoa*.

Select filaments that have glided well away from other filaments and appear to be uncontaminated, and transfer them to separate plates of the same medium with a sterile, flattened wire; inoculating needle; or toothpick. Take a little agar with the filament to avoid drying the filament during transfer. Examine the first transfer plates every 5 to 10 h as before and transfer pure filaments to fresh media.

2) *Thiothrix*—Obligately mixotrophic *Thiothrix* have been isolated and characterized from sulfur springs and other bodies of flowing water that contain sulfide.[42,43] Heterotrophic strains have been isolated and characterized from activated sludge wastewater treatment plants.[44–46] While the techniques for isolating the mixotrophic and heterotrophic strains are similar, isolation media differ.

a) Isolating mixotrophic *Thiothrix*—Collect tufts of the bacterium from rocks, water pipes, or other substrates. Using a dissecting microscope and a fine-tipped forceps, pick up filaments, shake to remove contaminating bacteria, and transfer to sterile basal medium [9240D.5*d*2)]. Repeat at least five times to try to obtain filaments with little or no contamination. With a sterile pasteur pipet, transfer separate drops to the edge of Petri dishes containing either MP or MY agar [9240D.5*d*4) and 6)] and tip the dishes so the drops run from one side of the dish to the other. Draw off excess moisture with the pipet and incubate plates at room temperature or cooler for about 48 h. Examine plates under a dissecting microscope for the appearance of typical filamentous colonies. With sterile toothpicks, pick colonies that are widely separated and transfer them individually to fresh plates of the same medium. Streak transferred material with a wire loop. After about 48 ± 4 h incubation, examine plates and restreak colonies that appear to be pure.

b) Isolating heterotrophic *Thiothrix*—One of two different procedures could be used, depending on the concentration and size of *Thiothrix* filaments in the sample.[34] In one procedure, wash individual filaments or rosettes of large strains of *Thiothrix* several times in MSV broth [9240D.5*e*1)] using a Pasteur pipet and a dissecting microscope. After several washings, transfer filaments to a small amount of MSV (1 to 3 mL) and plate on one or more of the solid media [9240D.5*d*4) and 6)]. If the filaments are small or scarce, concentrate the sample via centrifuging. Then dilute 1:5 in MSV, sonicate at 30 W for 10 s, and wash three times via centrifugation at 1900 × *g* for 2 to 5 min. The supernatant contains free filaments that are used to inoculate one or more of the solid media (9240D.5*e*).

5. A Selection of Special Purpose Media for Various "Sulfur" Bacteria

a. Sulfate-reducing medium: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Sodium lactate	3.5	g
Beef extract	1.0	g
Peptone	2.0	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	2.0	g
Sodium sulfate (Na_2SO_4)	1.5	g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.5	g
Ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$]	0.392	g
Calcium chloride ($CaCl_2$)	0.10	g
Sodium ascorbate	0.10	g
Reagent-grade water	1	L

Prepare medium (excluding ferrous ammonium sulfate and sodium ascorbate), dispense in screw-capped test tubes, and sterilize via autoclaving (121°C, 15 min). Final pH should be 7.5 ± 0.3. Use completely filled tubes. Separately sterilize extra medium to be added to tubes for filling. On day of use, prepare separate solutions of ferrous ammonium sulfate (3.92 g/100 mL) and sodium ascorbate (1.00 g/100 mL), filter through a 0.45-μm membrane filter, and aseptically add 0.1 mL each solution/10 mL basal medium.

b. Thiosulfate oxidizing medium (Thiobacillus thioparus): This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)	10.0	g
Dipotassium hydrogen phosphate (K_2HPO_4)	2.0	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.1	g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.1	g
Ammonium sulfate [$(NH_4)_2SO_4$]	0.1	g
Ferric chloride ($FeCl_3 \cdot 6H_2O$)	0.02	g
Reagent-grade water	1	L

Final pH should be 7.8 after sterilization via autoclaving (121°C, 15 min). Sterilize $Na_2S_2O_3$ and $(NH_4)_2SO_4$ separately, and add before use. If this medium is used to isolate *Thiobacillus thioparus*, check isolates to ensure that they are autotrophs by transferring a well-isolated colony to a liquid medium, followed by several serial subcultures. Then plate on medium designed to support sulfur bacteria.

c. Sulfur medium (Thiobacillus thiooxidans): This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Sulfur, elemental	10.0	g
Potassium dihydrogen phosphate (KH_2PO_4)	3.0	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5	g
Ammonium sulfate [$(NH_4)_2SO_4$]	0.3	g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.25	g
Ferric chloride ($FeCl_3 \cdot 6H_2O$)	0.02	g
Reagent-grade water	1	L

Preparation of sulfur: Sterilize by steaming at 100°C for 60 min on 3 consecutive days.

Preparation of medium: Add components (except sulfur) to reagent-grade water and bring the volume to 990.0 mL. Aseptically add 10.0 g sterile sulfur and mix.[36]

d. Media for Beggiatoa *and mixotrophic strains of* Thiothrix:

1) *Extracted hay*—Extract hay or grass at least five times by boiling in water for 30 min, with two rinses in cold water between extractions. Spread out extracted hay to dry, place several dried blades into a large test tube, and then sterilize via autoclaving (121°C, 15 min). Place 0.5 g of sterilized hay blades into a 125-mL Erlenmeyer flask. Add 60 mL stream water with some debris from study site (e.g., a leaf piece, matted material, or soil) and incubate at 28°C for at least one week. Sample over time to isolate species on following media.[37,38]

2) *Basal medium:*

Ammonium chloride (NH_4Cl), 4% (w/v) solution	5	mL
Dipotassium hydrogen phosphate (K_2HPO_4), 1% solution	1	mL
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 1% solution	1	mL
Calcium sulfate ($CaSO_4 \cdot 2H_2O$), saturated solution	20	mL
Trace elements [see ¶ *d*3) below]	5	mL
Reagent-grade water	968	mL

3) *Trace elements:*

Reagent-grade water	920	mL
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$), 0.1% (w/v) solution	10	mL
Manganous sulfate ($MnSO_4 \cdot 4H_2O$), 0.02% (w/v) solution	10	mL
Copper sulfate ($CuSO_4 \cdot 5H_2O$), 0.00005% (w/v) solution	10	mL
Boric acid (H_3BO_3), 0.1% (w/v) solution	10	mL
Cobalt nitrate [$Co(NO_3)_2$], or cobalt chloride ($CoCl_2 \cdot 6H_2O$), (w/v) 0.01% solution	10	mL
Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), (w/v) 0.01% solution	10	mL
EDTA solution [2% EDTA with 7% (w/v) ferrous sulfate ($FeSO_4 \cdot 7H_2O$), with 1 mL conc. HCl/100 mL]	20	mL

4) *MP agar*—This medium may not be available in dehydrated form and should be made fresh before use.

Basal medium	1	L
Sodium acetate	0.1	g
Sodium sulfide solution [see ¶ *d*5) below]	3	mL
Agar	15	g

Adjust pH to between 7.0 and 7.5 and heat to dissolve agar. Sterilize via autoclaving (no more than 15 min) and cool in a 45 to 50°C water bath. Add sodium sulfide solution immediately before pouring plates. If the medium will be used in screw-capped tubes, the sulfide may be added before autoclaving.

5) *Sodium sulfide solution*—Make up and separately autoclave (15 min at 121°C) a 10% (w/v) solution of $Na_2S \cdot 9H_2O$.

6) *MY agar*—This medium may not be available in dehydrated form and should be made fresh before use.

Basal medium	1	L
Sodium acetate	0.1	g
Nutrient broth powder**	0.1	g
Yeast extract**	0.1	g
Sodium sulfide solution [see ¶ *d*5) above]	3	mL
Agar	15	g

Adjust pH to between 7.0 and 7.5 and heat to dissolve agar. Sterilize via autoclaving (no more than 15 min at 121°C) and cool in a 45 to 50°C water bath. Add sodium sulfide solution, noted above, immediately before pouring plates. If the medium will be used in screw-capped tubes, the sulfide may be added before autoclaving.

e. Media for heterotrophic strains of Thiothrix: Use one or more of the media listed in ¶s *e*3)–9) below with the MSV [¶ *e*1) below] and vitamin mix [¶ *e*2) below] solutions, plus indicated additives. After adding all ingredients, adjust pH to between 7.2 and 7.5. For solid media, add 12 g agar/L. Filter-sterilize vitamin mixture; for other ingredients, heat to dissolve and autoclave at 121°C for 15 min.

1) *MSV:*

Ammonium sulfate [$(NH_4)_2SO_4$]	0.5	g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.11	g
Potassium dihydrogen phosphate (KH_2PO_4)	0.085	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.1	g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.05	g
Ferric chloride ($FeCl_3 \cdot H_2O$)	0.002	g
EDTA	0.003	g
Vitamin mix	1	mL
Reagent-grade water	1	L

2) *Vitamin mix:*

Calcium pantothenate	0.01	g
Niacin	0.01	g
Biotin	0.0005	g
Cyanocobalamin	0.0005	g
Folic acid	0.0005	g
Pyridoxine	0.01	g
p-aminobenzoic acid	0.01	g
Cocarboxylase	0.01	g
Inositol	0.01	g
Thiamine	0.01	g
Riboflavin	0.01	g
Reagent-grade water	100	mL

3) *AcS:*

Sodium acetate	0.15	g
Sodium sulfide ($Na_2 \cdot 9H_2O$)	0.187	g
MSV	1	L

4) *SS:*

Sucrose	0.15	g
Sodium sulfide ($Na_2S \cdot 9H_2O$)	0.187	g
MSV	1	L

5) *GS:*

Glucose	0.15	g
Sodium sulfide ($Na_2S \cdot 9H_2O$)	0.187	g
MSV	1	L

6) *SUC:*

Sodium succinate	0.15	g
MSV	1	L

7) *I:*

Glucose	0.15	g
MSV	1	L

8) *S:*

Sodium sulfide ($Na_2S \cdot 9H_2O$)	0.187	g
MSV	1	L

** Difco, or equivalent.

9) *LT:*

Sodium lactate	0.5	g
Sodium thiosulfate ($Na_2S_2O_3$)	0.5	g
MSV	1	L

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9240 E. Bacteria Living in Acidic Environments

1. General Discussion

Acid mine drainages are low-pH waters (typically 2.5 to 3.0) discharged from mines.[1] They occur when sulfide-laden ores and pyrite-laden coal are exposed to water and oxygen and deposit mineral acids and soluble metals in the water.[2] When acid mine water contaminates neutral but poorly buffered watercourses, the environmental and economic consequences include severe retardation of aquatic life throughout the food chain, corrosion of affected structures, elimination of a drinking water source, reduction in property values, and overall aesthetic degradation.

Acidophilic iron and sulfur bacteria [e.g., *Acidothiobacillus ferrooxidans*, *Acidothiobacillus (Thiobacillus) thiooxidans,* and *Leptospirillum ferrooxidans*] are an intrinsic part of acid mine drainage reactions. Strains of acidophilic thiobacilli vary considerably; their genetic differences may be reflected in their natural behavior on mineral substrates. For example, iron- and sulfur-oxidizing bacteria are chemolithoautotrophic, so they sustain synthesis and energy requirements by fixing carbon dioxide and oxidizing reduced inorganic forms of iron or sulfur. Other acidophilic and acid-tolerant bacteria metabolize organic compounds;[3] their ecology is not well understood, but they are ubiquitous in mine water and may be isolated directly from water, decaying vegetation, ferric oxyhydroxides, and gelatinous "streamers" (bacteria embedded in glycocalyx). *A. ferrooxidans* and, possibly, *A. thiooxidans*[4] attack metal sulfides and transform the soluble ferrous iron released when pyrite is oxidized to ferric iron.

Acid mine water also may contain *L. ferrooxidans*, *Leptospirillum*-like bacteria, acidophilic mixotrophs, chemoorganotrophs, and other microorganisms.[5] In addition, certain thermoacidophilic, sulfur-oxidizing archaebacteria are involved in leaching metal ions from ores and desulfurizing fossil fuels.

2. Sample Collection and Identification

Iron- and sulfur-oxidizing thiobacilli in natural samples cannot be identified via direct microscopic examination because

- the bacteria have no peculiar morphology,
- colloidal iron may interfere with detection, and
- large numbers must be seen.

The essential characteristics of bacterial species associated with acid mine water environments and bioleaching are presented below.[6–9] In most cases, there are no special precautions for collecting analytical specimens other than using pre-sterilized containers and transporting them to the laboratory at reduced temperatures.

a. Acidothiobacillus ferrooxidans: Cells are rod-shaped, appearing singly or in pairs; 0.3 to 0.5 × 1.0 to 1.7 μm, gram-negative; motile; monopolar flagellated; aerobic; nonspore-forming; mesophilic (10 to 37°C, optimum 30 to 35°C); acidophilic (pH 1.3 to 4.5, optimum pH 2.5 to 2.8); and chemolithoautotrophic using reduced forms of inorganic sulfur (elemental sulfur, thiosulfate, and tetrathionate), ferrous iron, and sulfitic minerals as energy sources, and carbon dioxide as a carbon source. Ammonium nitrogen is the typical nitrogen source, but nitrate may be used. Sulfate ion is required for growth when ferrous iron is the energy source. NOTE: In general, *A. ferroxidans* cannot be grown on plate media with organics because these are toxic to the organism. In a liquid medium containing ferrous iron (pH <1.8), iron transformation causes the initial pale green opalescence to become rust red, indicating soluble ferric iron without precipitates (observed if initial pH ≥1.9). If the sample's pH is <2, then *Leptospirillum* is the dominant organism.

b. Acidothiobacillus thiooxidans: Cells are rod-shaped, appearing singly or in pairs; 0.5 × 1.0 to 2.0 μm; Gram-negative; motile; monopolar flagellated; aerobic; nonspore-forming; mesophilic (10 to 37°C, optimum 28 to 30°C); acidophilic (pH 0.5 to 5.5, optimum pH 2.0 to 3.5); and chemolithoautotrophic using reduced forms of inorganic sulfur (but not ferrous iron) as energy sources, and carbon dioxide as a carbon source. Ammonium nitrogen is the nitrogen source. On solid culture media containing thiosulfate, colonies are circular, 0.5 to 1.0 mm, and transparent or whitish yellow from sulfur deposition.

c. Acidophilum acidophilum (Thiobacillus acidophilus): Cells are rod-shaped; 0.5 to 0.8 × 1.0 to 1.5 μm; Gram negative; some strains motile; mono- and disubterminal flagella; aerobic; nonspore-forming; mesophilic (25 to 37°C, optimum 27 to 30°); acidophilic (pH 1.5 to 5.5, optimum pH 2.5 to 3.0); and facultatively chemolithotrophic using reduced forms of inorganic sulfur (autotrophic), glucose or some organic acids (heterotrophic), or a mixture of glucose and reduced sulfur (mixotrophic) as energy sources. Ammonium and urea nitrogen serve as nitrogen sources. On solid culture media containing glucose, mature colonies are 1.0 to 2.0 mm, round, convex, and slightly translucent to cream to rose colored, depending on age.

d. Leptospirillum ferrooxidans: Cells are curved (vibroid); 0.2 to 0.4 × 0.9 to 1.1 μm; as spirilla, 8.0 μm; may form chains of 3 to 12 cells giving false *Spirillum*-like morphology; Gram-negative; motile; nonspore-forming; monopolar flagellated; aerobic; optimally mesophilic (30°C) with facultatively thermophilic strains (40 to 45°C); optimally acidophilic (pH 2.5 to 3.0); chemolithotrophic using ferrous iron (but not reduced sulfur) as energy sources. Ammonium nitrogen is the nitrogen source. The colony has the typical size and color of iron-oxidizing thiobacilli.

e. Sulfobacillus thermosulfidooxidans: Cells rod-shaped, 0.6 to 0.8 × 1.0 to 3.0 μm; may appear in aggregates; Gram-positive; may be encapsulated; endospore-forming; nonmotile; aerobic; optimally thermophilic (50 to 55°C), optimally acidophilic (pH 1.7 to 2.4); and facultatively chemolithotrophic using

sulfitic materials, elemental sulfur, and ferrous iron (autotrophic) or yeast extract, glucose, sucrose, and glutamic acid (heterotrophic) as energy sources. Ammonium nitrogen is the nitrogen source.

f. Sulfolobus acidocaldarius: Cells irregularly spheroidal with lobes; 0.8 to 1.0 μm; Gram-negative (archaebacterial cell wall); nonspore-forming; nonmotile; aerobic; thermophilic (55 to 85°C, optimum 70°C); acidophilic (pH 1.0 to 6.0, optimum 2.0 to 3.0); and use elemental sulfur (mixotrophic) or several organic compounds (heterotrophic) as energy sources. Colonies vary from small to large.

g. Acidianus brierleyi: Cells spheroidal with lobes; 1.0 to 1.5 μm; Gram-negative (archaebacterial cell wall); nonspore-forming; nonmotile; facultatively aerobic; thermophilic (45 to 75°C, optimum 70°C); acidophilic (pH 1.0 to 6.0, optimum 1.5 to 2.0); and facultatively chemolithotrophic using sulfitic minerals, elemental sulfur (anaerobic with hydrogen as electron donor), and ferrous iron (autotrophic), yeast extract (heterotrophic), or elemental sulfur and organic compounds (mixotrophic) as energy sources.

h. Acidiphilium cryptum: Cells rod-shaped, 0.3 to 0.5 × 0.6 to 1.5 μm; Gram-negative; motile; monopolar or dilateral flagella; aerobic; weakly catalase-positive, mesophilic (31 to 41°C), acidophilic (pH 2.5 to 5.9); chemoorganotrophic using complex, soluble organic matter (e.g., trypticase and yeast extract with glucose) as energy sources. Acetate inhibits growth. Colonies on organic media are 0.5 to 2.0 mm, circular with irregular margins, and white; colonies on ferrous iron media without exogenous carbon source are small (maximum 0.3 mm) and lobate.

3. Enumeration, Enrichment, and Isolation

Acidophilic bacteria can be grown on appropriate culture media, although sometimes with difficulty. However, there are no good means of enumerating them. Procedures calibrated using axenic strains may not accurately reflect the growth characteristics of naturally occurring organisms.

Analysts have long recognized the problems associated with enumerating this species on membrane filters and solidified culture media.[10] Several culture media have been formulated to isolate and mass culture *Acidothiobacillus ferrooxidans,* but there is no universal culture medium because too many strains exist. Also, obtaining axenic cultures is complicated by the invariable association of acidophilic, heterotrophic bacteria with *A. ferrooxidans*.

Chemolithotrophic and mixotrophic iron- and sulfur-oxidizing thiobacilli are not fastidious and may be grown on several mineral salt formulations with appropriate energy sources. Including a surfactant* in the sample to be plated[11] may enhance the colony size of *A. ferrooxidans.* In isolation media for the iron-oxidizing bacteria, keep the ferrous iron level relatively low (20 m*M*) with a final pH of 2.2 to 2.5 to minimize the precipitation of ferric iron salts.

Sulfolobus species are more difficult to isolate and manipulate. The media described below are only suggestive; more complete discussions on isolating and cultivating the organisms are available in the literature.[9,11]

The mineral salt formulations provided below may be appropriate basal salts for acidophilic heterotrophic bacteria when used with suitable organic carbon and energy sources. Solidifying agents given in connection with specific media may be used with any basal salts solution as desired.

a. Medium 9K[12] *maintenance medium for* Acidothiobacillus ferrooxidans*; acidophilic bacteria:*

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	44.22	g
Ammonium sulfate [$(NH_4)_2SO_4$]	3.0	g
Potassium chloride (KCl)	0.1	g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.5	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5	g
Calcium nitrate ($Ca(NO_3)_2 \cdot 4H_2O$)	0.01	g
Reagent-grade water	1	L

Dissolve ferrous sulfate in 300 mL reagent-grade water, acidify to pH 2 to 3 with H_2SO_4 and filter–sterilize. Dissolve remaining salts in 700 mL reagent-grade water, acidify to pH 2.5 with H_2SO_4, and sterilize via autoclaving (121°C, 15 min). Prepare the liquid medium by combining both solutions. To prepare a solid medium, suspend 5 g agarose in 250 mL reagent-grade water, autoclave, and combine with 450 mL ferrous sulfate solution and 300 mL mixed salts solution (9240D.3*f*) at 55°C.

b. TSM 1 medium[13] *for enumeration of* Acidothiobacillus ferrooxidans *on plates or as nutrient base for membrane filters*:

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	22.0	g
Ammonium sulfate [$(NH_4)_2SO_4$]	3.0	g
Potassium chloride (KCl)	0.1	g
Dipotassium hydrogen phosphate (K_2HPO_4).	0.5	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5	g
Calcium nitrate ($Ca(NO_3)_2 \cdot 4H_2O$)	0.01	g
Agarose†	5.0	g
Reagent-grade water	1	L

Dissolve ferrous sulfate in 150 mL reagent-grade water, acidify to pH 2.5 with H_2SO_4, and filter–sterilize. Dissolve remaining salts in 600 mL reagent-grade water, acidify to pH 2.5 with H_2SO_4, and sterilize via autoclaving (121°C, 15 min). To prepare medium, suspend agarose in 250 mL reagent-grade water, autoclave (121°C, 15 min), and cool to 60°C. Mix sterile ferrous sulfate and mixed salts solutions at 60°C; aseptically add agarose suspension; pour into prewarmed, sterile Petri plates.

c. ISP medium[14] *for isolating* Acidothiobacillus ferrooxidans *and acidophilic heterotrophic bacteria on plates:*

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	33.4	g
Ammonium sulfate [$(NH_4)_2SO_4$]	6.0	g
Potassium chloride (KCl)	0.2	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	1.0	g
Calcium nitrate ($Ca(NO_3)_2 \cdot 4H_2O$)	0.02	g
Purified agar	7.0	g
Reagent-grade water	1	L

Dissolve ferrous sulfate in 300 mL reagent-grade water, acidify to pH 2.5 with H_2SO_4, and filter–sterilize. Dissolve remaining salts in 550 mL reagent-grade water, acidify to pH 3.0 with H_2SO_4, and sterilize via autoclaving (121°C, 15 min). To prepare medium for iron-oxidizing bacteria, suspend purified agar in

* For example, 0.5% (v/v) Tween 80, or equivalent.

† Biorad High MR-1620001, or equivalent.

150 mL reagent-grade water, autoclave (121°C, 15 min), and cool to 60°C. Mix sterile ferrous sulfate and mixed salts solutions at 60°C; asceptically add agar suspension.

d. Enrichment medium for Acidothiobacillus acidophilus:[15]

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	44.22	g
Ammonium sulfate [$(NH_4)_2SO_4$]	3.0	g
Potassium chloride (KCl)	0.1	g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.5	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5	g
Calcium nitrate ($Ca(NO_3)_2 \cdot 4H_2O$)	0.01	g
Glucose	0.1	g
Reagent-grade water	1	L

Use medium 9K, supplemented to contain 0.01% (w/v) glucose, to enrich *Acidophilum acidophilum*; adjust pH to 3.5. Inoculate enrichment medium with acidic mine water and incubate for several days. Transfer inoculum from the enrichment to fresh medium 9K (whose ferrous iron concentration is reduced to 10 mg/L and whose glucose level has been increased from 100 mg/L to 10 g/L in four successive steps). To prepare an isolation medium 9K for *Acidophilum acidophilum*, modify a preparation of 9K solid medium (¶ *a* above) by reducing ferrous iron concentration to 10 mg/L, adding 10 g/L glucose, and adjusting final pH to 4.5.

e. Medium of Norris and Kelly[16] *for growth and maintenance of* Leptospirillum ferrooxidans: *L. ferrooxidans* is difficult to grow on solid media; different strains may require different formulations to produce satisfactory results.

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	13.9	g
Ammonium sulfate [$(NH_4)_2SO_4$]	0.2	g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.2	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.2	g
Reagent-grade water	1	L

To produce liquid medium, dissolve ferrous sulfate in 300 mL reagent-grade water, acidify to pH 1.5 with H_2SO_4, and filter–sterilize. Dissolve remaining salts in 700 mL reagent-grade water, acidify to pH 1.5 with H_2SO_4, and sterilize via autoclaving (121°C, 15 min). Make solid medium for isolating *L. ferrooxidans* by adding silica gel as a hardening agent to avoid using agar for the lithoautotrophic organism. Reference literature for procedures to prepare silica gel media.

f. Medium of Starkey[17] *for isolating and maintaining* Acidothiobacillus thiooxidans:

Ammonium sulfate [$(NH_4)_2SO_4$]	0.2	g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.25	g
Dipotassium hydrogen phosphate (K_2HPO_4)	3.0	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5	g
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	0.01	g
Sulfur energy source: either		
Sodium thiosulfate stock solution	5	mL
or		
Sulfur, elemental	10.0	g

If sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) is used as the energy source, prepare stock solution by dissolving 10.0 g in 100 mL reagent-grade water and then filter–sterilize. Dissolve remaining salts in 950 mL reagent-grade water, adjust to pH 4.0 with H_2SO_4, and sterilize via autoclaving (121°C, 15 min). Add 5 mL stock thiosulfate to mixed salts solution, mix, and bring volume to 1 L.

If elemental sulfur is used, heat the sulfur in an empty 1-L flask under flowing steam for 1 h on each of 3 consecutive days before preparing medium. Dissolve mineral salts and sulfur in 1 L reagent-grade water, adjust pH to 4.0, and sterilize via autoclaving (121°C, 15 min). Prepare solid isolation/culture medium from maintenance medium by using a suitable gelling agent, and adjusting volumes and concentrations appropriately.

g. Modified Allen medium[18] *for enriching, isolating, and maintaining* Sulfolobus *and* Acidianus:

Basal medium:		
Ammonium sulfate [$(NH_4)_2SO_4$]	1.3	g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.28	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.25	g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.25	g
Reagent-grade water	1.0	L

Dissolve ingredients in 1 L reagent-grade water, adjust pH to 2.0 to 3.0 with H_2SO_4, and sterilize via autoclaving (121°C, 15 min).

Trace mineral salts:		
Ferric chloride ($FeCl_3 \cdot 6H_2O$)	20.0	g
Manganese chloride ($MnCl_2 \cdot 4H_2O$)	1.8	g
Sodium borate ($Na_2B_4O_7 \cdot 10H_2O$)	4.5	g
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	0.22	g
Cupric chloride ($CuCl_2 \cdot 2H_2O$)	0.05	g
Sodium molybdate ($NaMoO_4 \cdot 2H_2O$)	0.03	g
Vanadyl sulfate ($VOSO_4 \cdot 2H_2O$)	0.04	g
Cobalt sulfate ($CoSO_4 \cdot 7H_2O$)	0.01	g
Reagent-grade water	1.0	L

Dissolve mineral salts in 950 mL reagent-grade water; adjust pH to <1.5 with H_2SO_4, bring volume to 1 L, and filter–sterilize.

To prepare liquid medium, aseptically add 1 mL mineral salts solution to basal medium. When promoting lithotrophic growth on elemental sulfur (5 to 10 g/L), adjust pH to higher initial value to compensate for sulfuric acid production due to sulfur oxidation, heat elemental sulfur as in preparation of Starkey medium (¶ *f* above). To enhance lithotrophic growth, add 0.05 to 0.2 g/L of yeast extract (with or without glucose) by aseptically transferring from sterile stock to medium. To promote heterotrophic growth, increase final concentrations of yeast extract to 1 g/L and glucose to 2 g/L in sterile medium.

Sulfolobus spp. may be enriched selectively, aerobically at 85°C in modified Allen medium[19] containing only 1 g/L yeast extract. *Acidianus* spp. can be enriched via anaerobic incubation (80:20 H_2:CO_2, 300 kPa) in the same mineral salts solution supplemented with 0.2 g/L yeast extract and 5.0 g/L elemental sulfur. To selectively enhance *A. brierleyi,* incubate at 65°C; to maintain it, transfer to a variation of the medium containing 1 g/L yeast extract without sulfur, followed by aerobic incubation. To favor *A. infernus,* incubate the primary enrichment medium anaerobically at 85°C and then incubate it aerobically, subculturing it to the same medium with a reduced yeast extract concentration of 0.1 g/L.

To prepare a solid isolation medium, make a double-strength salts solution and combine with equal volumes of a gelling agent in conjunction with an overlay technique.

Following is an example preparation of a polysilicate gelling solution:

Sodium silicate solution, 1.38 kg ± 0.01 kg/L	6.0	mL

Dissolve 6 mL silicate solution in 94 mL reagent-grade water and mix with 5 g sodium bentonite. Rapidly adjust pH to 6.0 with H_2SO_4[20] while vigorously stirring mixture. Repeat using nutrient solution in place of water. Make trial preparations to determine proper volumes of sodium silicate solution and acid for best results. (Firmness of silica gel increases as pH decreases.) Poured plates harden in 2 to 5 min; incubate at temperatures up to 80°C in a humidified atmosphere. Before use, equilibrate hardened plates with equal volumes of liquid media by carefully superimposing sterile liquid medium on the hardened plates and withdrawing the liquid in five sequential treatments over 24 h.

For other gelling agents:

Gelling agent‡	24 g
Reagent-grade water	2 L

Prepare two concentrations of gelling agent solution in reagent-grade water (16 and 8 g/L), sterilize via autoclaving, and cool to 70°C. Combine the 16 g/L solution with an equal volume of prewarmed, double-strength modified Allen medium (¶ *g* above) (or medium 9K) to which the following have been added:

Tetrathionate	20.0	m*M*
Sodium chloride (NaCl)	2.0	m*M*
Casamino acids or yeast extract	2.0	g/L
Magnesium chloride ($MgCl_2$)	16.0	m*M*

Pour medium into Petri dishes and let solidify. Combine the 8-g/L gelling agent solution with an equal volume of double-strength medium previously inoculated with a suitable level of cells from an enrichment culture. Pour over solid base layer and let harden. Incubate at 65°C in sealed plastic bags.

h. Acidiphilium *medium*[3] *for isolating and maintaining* Acidiphilium *spp.:*

Ammonium sulfate [$(NH_4)_2SO_4$]	2.0	g
Potassium chloride (KCl)	0.1	g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.5	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5	g
Glucose stock solution	100	mL
Reagent-grade water	1.0	L

Prepare glucose stock solution by dissolving 50.0 g glucose in 500 mL reagent-grade water. Sterilize via autoclaving (121°C, 15 min). Dissolve salts in 900 mL reagent-grade water (pH adjusted to 3.0 with H_2SO_4) and autoclave (121°C, 15 min). Aseptically add 100 mL sterile glucose stock solution [alternatively, use yeast extract or trypticase at 0.01% (w/v) final concentration]. To prepare solid medium, suspend 12 g agar in 150 mL reagent-grade water, dissolve mineral salts in 750 mL reagent-grade water, and autoclave both (121°C, 15 min). Then, cool to 60°C and aseptically combine 100 mL glucose stock, agar, and mineral salts solution.

‡ Consisting of Gelrite, Kelco, Monsanto, or equivalent.

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9245 NITRIFYING BACTERIA*

9245 A. Introduction

1. General Discussion

Nitrifying bacteria and Archaea† convert ammonia to nitrite and then nitrate in a sequential process called *nitrification*.[1] Nitrification is crucial to the biogeochemical nitrogen cycle.

Two groups of nitrifiers are necessary to complete nitrification: ammonia-oxidizing bacteria (AOB), which convert ammonia to nitrite (NH_4^+ to NO_2^-), and nitrite-oxidizing bacteria (NOB), which convert nitrite to nitrate (NO_2^- to NO_3^-). The most commonly isolated or identified nitrifiers from freshwater or wastewater systems are in the *Nitrosospira* and *Nitrosomonas* genera for AOB and in the *Nitrobacter* and *Nitrospira* genera for NOB.[2,3] *Nitrosomonas europaea* and *Nitrobacter winogradskyi* have been the most intensively studied species of AOB and NOB, respectively, and the genomes of these bacteria have been sequenced.[4,5] Nitrifying bacteria can occur in various habitats (e.g., soil and fresh, marine, brackish, waste, and treated drinking waters.) They are generally sensitive to visible and ultraviolet light,[6–9] so they are often found in light-free environments. The bacteria are found in a variety of shapes (e.g., rods, curved rods, spheres, spirals, and lobular forms, many with flagella) and range in size from approximately 0.3 to 11.7 μm. Many have intracellular membranes.[10,11]

These bacteria are aerobic, Gram-negative, and generally chemolithotrophic (obtain energy via the oxidation of inorganic chemical compounds, such as ammonia or nitrite).[10,11] Nitrifiers are generally autotrophic, although mixotrophy has also been observed.[10,11] They use carbon dioxide and carbonates as carbon sources. Bacterial chemolithotrophic aerobic nitrifiers, once classified in the family *Nitrobacteraceae*, are now found in three classes (*Alpha, Beta*, and *Gamma-proteobacteria*) of the *Proteobacteria* phylum[12] and in the deeply branching *Nitrospirae* phylum.[13]

Two recent discoveries have significantly changed our understanding of nitrification.[1] One is anammox, the process of anaerobically oxidizing ammonia to N_2 gas using nitrite as the electron acceptor. The oxidizers are novel bacteria related to the *Planctomycetales*.[14] This process was first characterized in wastewater treatment systems.[15]

The second discovery is that aerobic ammonia oxidation is mediated by organisms in the domain Archaea as well as by those in the domain *Bacteria*.[1,16] While this section focuses on bacterial chemolithotrophic nitrifiers because of their known importance in drinking water[17,18] and wastewater systems,[4] keep in mind that anammox and Archaea also probably affect nitrification in natural and managed systems.[1,19]

To promote nitrification at a water or wastewater treatment utility, personnel allow nitrifying bacteria to grow in filter beds or aeration basins, where they convert ammonia to nitrate.[17] The efficiency of this process depends on retention time, temperature, pH, dissolved oxygen, nitrogen, organic matter, toxic substances, and populations of heterotrophic and nitrifying bacteria and Archaea.

Nitrification can either help or hinder a utility's overall effectiveness, depending on the application and the disinfectant the utility uses. In wastewater treatment, nitrification removes ammonia-nitrogen, which is toxic to some forms of aquatic wildlife.[20] In addition, it is often an indicator of the purification process' overall efficiency. Ammonia also exerts an appreciable chlorine demand, while nitrate does not, so nitrification reduces the costs of chlorine disinfection. In water treatment, nitrification in filter beds can reduce source water ammonia, thereby reducing chloramine or breakpoint chlorination requirements (this occurs in some regions of the United States).

However, nitrification is a nuisance in drinking water distribution systems that use chloramines because it significantly reduces water quality.[7,18,21] Nitrification increases biomass in the distribution system and decreases chloramine residual (via abiotic chemical reactions between nitrite and chloramine).[18,21–23] Its biotic and abiotic reactions may substantially increase levels of heterotrophic plate count (HPC) bacteria, coliforms, and opportunistic pathogens.[24] Also, HPC bacteria use the organic compounds secreted by AOB as sources of energy,[21,25] so nitrification may impair a utility's ability to meet the provisions of the U.S. Environmental Protection Agency's Total Coliform Rule and Surface Water Treatment Rule[21] (which requires utilities to maintain a disinfectant residual or keep the HPC level below 500 CFU/mL).

Although nitrification is an important water and wastewater treatment process, isolating and quantifying the related organisms is not a common practice. Because nitrification yields little energy, these bacteria grow at a much slower rate than most heterotrophic bacteria,[26] making them relatively difficult to grow in the laboratory. They also can be tedious to identify because of both their slow growth rate and their poor isolation and colony development on agar plates.

Molecular methods for quantifying nitrifiers using real-time polymerase chain reaction to target group-specific 16S rRNA genes or functional genes (especially the ammonia monooxygenase gene, *amoA*) are under development and appear promising.[27–30] For example, recent investigations using molecular methods have identified the ubiquitous AOB *Nitrosomonas oligotropha* in full-scale drinking water distribution systems receiving chloraminated water.[22,23] However, these methods are still being refined; routine application in non-research laboratories is not yet feasible.

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* Approved by Standard Methods Committee, 2008.
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† Archaea, one of the three domains of life, is distinct from the Bacteria and Eukaryota domains. Archaea are prokaryotic, lacking a true nucleus and other complex membrane-bound intracellular organelles. They were first distinguished by their small subunit ribosomal sequences. They are distinct biochemically from bacteria in that their cell membranes are based on glycerol ethers and their cell walls lack peptidoglycan (murein).

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9245 B. Multiple-Tube Method

1. Principle

The multiple-tube (or multiwell plate) method is based on selective growth of ammonia-oxidizing AOB and NOB in specialized media, followed by spot tests and statistical computation of most probable numbers (MPN) of nitrifying bacteria. Note that there are biological activity reduction tests available for presence–absence determinations of AOB.*

2. Apparatus

Sterile test tubes or multiwell plates: If using plates, choose those with 6 or 24 wells.

3. Reagents and Media

a. Nitrite spot test reagents:

1) *Sulfanilic acid*—Use 8 g sulfanilic acid in 1 L 5*N* acetic acid (1 part glacial acetic to 2.5 parts water).

2) N, N-*dimethyl-1-naphthylamine*—Use 5 g (or 5 mL) in 1 L 5*N* acetic acid. **CAUTION: This solution may be carcinogenic; avoid all contact with skin**.

b. Nitrite/nitrate spot test reagent—diphenylamine:[1,2] Dissolve 0.2 g diphenylamine in 100 mL conc H_2SO_4. Let solution equilibrate for 12 h.

c. Reagent-grade water, low in total organic carbon. Use to prepare culture media.

d. Ammonia-oxidizing bacteria (AOB) medium:[3,4]

Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.04	g
Ammonium sulfate [$(NH_4)_2SO_4$]	0.5	g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2	g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.04	g
Chelated iron†	0.0005	g
Phenol red‡	0.0005	g
Reagent-grade water	1	L

Dispense ingredients into reagent-grade water and adjust pH to between 7.8 and 8.1 with diluted NaOH, allowing for pH changes during autoclaving. Autoclave medium at 121°C for 15 min. If pH after autoclaving exceeds desired range, readjust pH with sterile NaOH or HCl. [See Section 9020B.5*j*1).] Dispense medium into tubes so the ratio of sample to medium is approximately 1:10.

e. Nitrite-oxidizing bacteria (NOB) medium:[5,6]

Potassium nitrite (KNO_2)	0.30	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.1875	g
Potassium bicarbonate ($KHCO_3$)	1.5	g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.5	g
Potassium dihydrogen phosphate (KH_2PO_4)	0.5	g
Sodium chloride (NaCl)	0.1875	g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.0125	g
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	0.01	g
Reagent-grade water	1	L

Dispense other ingredients into reagent-grade water and adjust pH to between 7.8 and 8.1 with diluted NaOH, allowing for pH changes during autoclaving. If pH after autoclaving exceeds desired range, readjust pH with sterile NaOH or HCl. [See Section 9020B.5*j*1).] Dispense medium into tubes so the ratio of sample to medium is approximately 1:10.

4. Procedure

a. Sample collection: Collect water samples in sterile, dark-colored bottles using aseptic technique. If the sample contains chloramines, dechlorinate with sodium thiosulfate. (See Section 9060A.2.) Immediately after collecting samples, store the bottles on ice in a dark container. (See Section 9060 for more detailed information on sample collection, preservation, and storage.)

b. Multi-tube or multiwell plate procedure: The use of screw-cap tubes may be desirable to prevent desiccation. Pre-sterilized multiwell plates (which are compact and convenient when incubator space is limited) may be used instead of tubes, with modifications to volumes. For example, dispense 1.5 mL sterile medium in each well of the 24-well plates (each well's maximum capacity is 3 mL) and 8 mL medium in each well of the 6-well plates (each well's maximum capacity is 12 mL).

Use spot-test procedures to test water samples qualitatively for nitrite and nitrate before selecting dilutions. If a sample yields positive results for nitrite or nitrate, dilute it with enough medium to ensure that nitrite or nitrate is undetectable before initiating the assay. Before dispensing the sample, gently agitate the sample bottle. Use at least three 10-fold serial dilutions with 5, 10, or 15 tubes or wells at each dilution. The number of tubes or wells and the sample volumes selected depend on the quality and character of the water to be analyzed.

Cover or shield the samples to minimize their exposure to light. Inoculate the media at approximately a 1:10 ratio of sample to medium (e.g., 0.15 mL sample or dilution in each well of the 24-well plates, and 0.8 mL in each well of the 6-well plates.) Cross-contamination can easily occur between wells, so use caution when inoculating media. To avoid splashing, place tip of pipet on side of well when dispensing sample, and keep plates level to prevent spillage from adjacent wells. For tubes and multiwell plates, include blanks and positive control with each batch of samples and incubate at same time and temperature as samples. *Nitrosomonas europaea* (ATCC 19718)§ is recommended as a positive control. Use one well per row of six wells as a blank. To prevent desiccation of the medium during incubation, wrap plates in sealable plastic bags.

Incubate tubes or multiwell plates at 25 to 30°C for 23 to 28 d for ammonia oxidizers and at 23 to 28°C for 23 or more d for nitrite oxidizers.[4,6,7] To recover slower-growing nitrifiers, sam-

* Droycon Bioconcepts Inc., Regina, Saskatchewan, Canada; LaMotte Company, Chestertown, MD

† Sequestrene 138, Fe EDTA, or equivalent.

‡ Not required, but useful for indicating bacterial growth in batch cultures.

§ Available from the American Type Culture Collection, www.atcc.org.

ple incubation may be extended for several more weeks until there are no further positive reactions.

After the prescribed incubation period, identify positive tubes or wells as follows:

1) AOB medium—Look for a pH change in the media (indicated by a color change from red to yellow) as a preliminary indicator of growth. To confirm, take a 1-drop portion from each tube or well and place on a clean spot plate (i.e., unused or freshly cleaned and spot tested to ensure no contamination). Add 1 drop each of sulfanilic acid and *N,N*-dimethyl-1-naphthylamine to each portion to test for nitrite, indicating the presence of active AOB. Positive tubes or wells will develop a deep red color within 1 min. For further verification, add 1 drop of diphenylamine to 1 drop of sample on a clean spot plate to determine nitrite/nitrate production. Positive tubes or wells will develop a blue color; negative ones will not.[1,2] Tubes or wells that test negative via *N,N*-dimethyl-1-naphthylamine but positive via diphenylamine indicate complete nitrification.

2) NOB medium—Take a 1-drop portion from each tube or well and place on a clean spot plate. Add 1 drop each of sulfanilic acid and *N,N*-dimethyl-1-naphthylamine onto each portion to determine nitrite consumption by nitrite-oxidizers. The absence of red color development within 1 min indicates the presence of NOB; the presence of red color indicates their absence or only partial consumption.

If the test results in the absence of red color, then NOB have converted all nitrite to nitrate. Under this circumstance only, for additional verification, add 1 drop of diphenylamine to 1 drop of sample on a clean spot plate to determine whether nitrate production is occurring. Positive tubes or wells are identified by development of a blue color. (The absence of a blue color should not occur under these circumstances.)

5. Calculations

Calculate MPN/mL using a standard MPN table (Section 9221C) or MPN program. Although MPN values have been shown to underestimate the density of nitrifying bacteria,[8] this method is useful for quantifying nitrifiers in water and wastewater.[9]

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9250 DETECTION OF ACTINOMYCETES*

9250 A. Introduction

1. General Discussion

Actinomycetes are a large, diverse group of Gram-positive bacteria in the phylum Actinobacteria. (The taxonomy is still developing.) The order Actinomycetales includes facultative, anaerobic, filamentous, and Gram-positive bacteria that reproduce via fission or production of asexual spores or conidia. Their optimal environment has a pH between 7 and 8, and temperature between 15 and 20°C.

Actinomycetes, such as *Streptomyces* and *Nocardia* spp., are widely distributed in nature. They may constitute a considerable proportion of the microbial population of soil and activated sludge. They may be found in lesser numbers in water environments (lake, river, and marine waters). Actinomycetes are considered important in antibiotic production and often are used in biodegradation studies.

Some actinomycetes are considered nuisance microorganisms in the water management industry because they may emit earthy or musty odors that affect the quality and public acceptance of municipal water supplies in many parts of the world. These bacteria are part of the normal flora of sand filters, where they can decompose organic compounds that are otherwise difficult to biodegrade. Actinomycete spores, especially *Micromonospora* spp., tolerate chlorine disinfection so well that a considerable fraction persists.[1]

Water treatment plant operators find these naturally occurring odors difficult to remove via conventional treatment. As early as 1929, it was assumed that these odors could be attributed to volatile metabolites formed during normal actinomycete development.[2] Two such compounds, geosmin and 2-methylisoborneol, have been isolated[3–9] and identified as the agents responsible for earthy, musty odors in surface water and that impart an unpleasant taste to water.[9–11] However, both compounds are produced also by some filamentous cyanobacteria.[12–18] Geosmin and 2-methylisoborneol have threshold odor concentrations well below the microgram-per-liter level. One study found two actinomycete isolates that also produce 2,4,6-trichloranisole from the disinfection byproduct 2,4,6-trichlorophenol, which creates a disagreeable odor.[18] Traces of these products can impart a disagreeable odor to water or a muddy flavor to fish.

In areas periodically plagued by this problem, it is prudent to enumerate actinomycetes. Identifying their relative abundance in a drinking water source or distribution system (e.g., in biofilms) can provide yet another means to assess water quality.[19] The methods described in this section are well-established techniques that have been used successfully to isolate and enumerate actinomycetes related to public water supplies.[20–21] Actinomycetes, especially *Nocardia* spp. and *Gordonia amarae*, also have been recognized as a cause of disruptions in wastewater treatment. Massive growths can produce thick foam in the activated sludge process.[22–23]

In terms of human health significance, some *Nocardia* spp. may be pathogenic,[24–25] while *Streptomyces* spp. are generally considered non-pathogenic.

2. Samples

a. Collection: Collect samples as directed in Section 9060A.

b. Storage: If possible, analyze samples within 24 h of collection. If samples cannot be processed promptly, store them at temperatures between 2 and 8°C.

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9250 B. Actinomycete Plate Count

1. General Discussion

Generally, actinomycetes are isolated by diluting samples and directly plating on a defined medium. The samples may be heated (e.g., 50°C for 10 min), and selective antibiotics may be added.[1] Samples may also be analyzed by membrane filtration. In one study, analysts first concentrated the sample by filtering it through nylon membrane filters (0.2-μm pore size).[2]

The plating method described in this section uses a double-layer agar technique that has been successfully adapted to determine actinomycete density. The U.S. Geological Survey (USGS) uses a similar method and a commercially available medium to evaluate water quality nationally.[3] In both cases, only the thin, top layer of the medium is inoculated with sample, allowing surface colonies to predominate and facilitating colony identification and counting. [Follow all appropriate quality control (QC) activities noted in Section 9020.]

2. Preparation and Dilution

Prepare and dilute samples as directed in Sections 9215A.5, 9610B.1, or 9610B.3*a*. Treated waters may be examined directly, while raw water usually is diluted up to 1:1000 (10^{-3}).

3. Media

a. Antifungal antibiotic solution:

Cycloheximide*	0.1 g
Reagent water	100.0 mL
Sodium chloride (NaCl)	2.0 g

CAUTION: Cycloheximide has significant toxic side effects. Weigh compound in a fume hood and avoid skin contact. Prepared solutions are available.

Prepare in a glass screw-capped standard-sized dilution bottle or small flask. Autoclave for 15 min at 121°C. Store at 2 to 8°C for up to 6 months.

b. International Streptomyces Project (ISP) medium No. 1: This medium is used to revive freeze-dried *Streptomyces albus* and grow the organism for use in plating positive controls.

ISP No. 1†	8.0 g
Reagent water	1.0 L

Follow manufacturer's directions. Heat to dissolve and autoclave at 121°C for 15 min. Liquid broth medium can be stored in screw-cap flasks or bottles for up to 6 months in the refrigerator at 2 to 8°C.

c. ISP medium No. 2: This medium is used to obtain pure colonies of *Strep. albus* from the broth culture and to maintain a refrigerator slant of *Strep. albus* for use as a positive control.

ISP No. 2†	38.0 g
Reagent water	1.0 L

Follow manufacturer's directions. Heat to dissolve and autoclave for 15 min at 121°C. Agar can be stored in standard-sized dilution bottles for up to 6 months in a 2 to 8°C refrigerator.

d. Starch-casein agar: This medium can be used to make both top agar and bottom agar plates for samples and controls. For QC purposes, a commercially prepared medium is preferred.

Soluble starch	10.0 g
Casein	0.3 g
Potassium nitrate (KNO_3)	2.0 g

* Sigma, St. Louis, MO; Actidone®, Upjohn and Company, Kalamazoo, MI; or equivalent.

† VWR, Pittsburgh, PA, or equivalent.

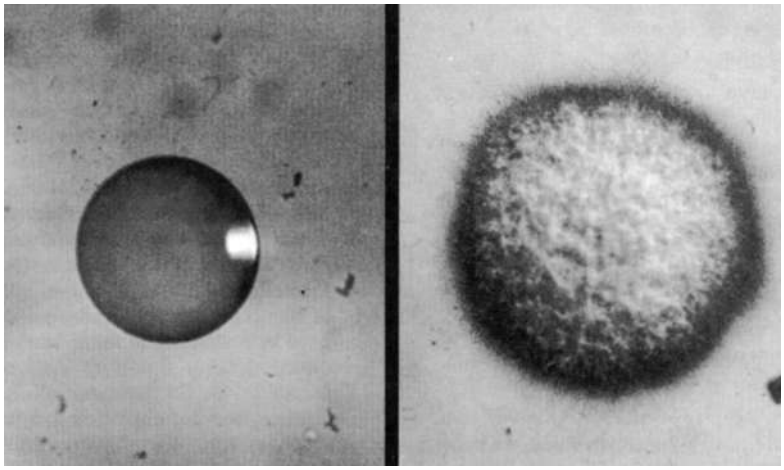


Figure 9250:1. Bacterial colonies—typical colony type vs. actinomycete colony type, 50×. Left: A typical bacterial colony, characterized by a smooth mucoid appearance and a relatively distinct smooth border. Right: An actinomycete colony, characterized by the mass of branching filaments that make its border appear fuzzy and by the dull powdery appearance of the spore-laden, aerial hyphae.

Sodium chloride (NaCl)	2.0	g
Dipotassium hydrogen phosphate (K_2HPO_4)	2.0	g
Magnesium sulfate, hydrate ($MgSO_4 \cdot 7H_2O$)	0.05	g
Calcium carbonate ($CaCO_3$)	0.02	g
Ferrous sulfate, hydrate ($FeSO_4 \cdot 7H_2O$)	0.01	g
Agar	15.0	g
Reagent-grade water	1	L
Glycerol (optional)	5.0	g

Prepare according to manufacturer's instructions. The addition of glycerol is optional; it promotes anaerobic conditions as cultures develop. Heat to dissolve ingredients, mix well, dispense 100 mL into dilution bottles, and then autoclave at 121°C for 15 min. No pH adjustment is required.

Medium poured into tubes or Petri dishes can only be stored for 2 weeks. Medium autoclaved in screw-cap flasks or bottles can be stored for up to 6 months in a 2 to 8°C refrigerator.

Before use, melt agar in flasks or bottles in an autoclave for 2 to 3 min and temper it in a 44 to 46°C waterbath. Warm previously poured Petri dishes in a 35°C incubator.

e. Actinomycetes Isolation Agar (AIA): There are a variety of commercially available AIA preparations, which contain similar ingredients, that may be useful. Glycerol can be added. For example:‡

Sodium caseinate	2.0	g
Asparagine	0.1	g
Sodium propionate	5.0	g
Dipotassium phosphate (K_2HPO_4)	0.5	g
Magnesium sulfate ($MgSO_4 \cdot 7\ H_2O$)	0.1	g
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	1.0	mg
Agar	15.0	g
Reagent-grade water	1	L
Glycerol (optional)	5.0	g

Thoroughly mix 22 g of commercially prepared medium in 1 L water. Heat and boil 1 min to completely dissolve ingredients. Add 5 g glycerol to mixture and swirl. Follow manufacturer's instructions for final pH. Autoclave at 121°C for 15 min.

Medium autoclaved in screw-cap flasks or bottles can be stored for up to 6 months at 2 to 8°C. Medium poured into Petri plates can only be stored for 2 weeks at 2 to 8°C.

‡ Difco, or equivalent

4. Double Agar Layer Procedure

a. Plating: For each sample, prepare two plates for each dilution to be examined, for a minimum of four plates (e.g., undiluted and 10^{-1}). Aseptically transfer 15 to 20 mL of sterile starch-casein agar or AIA to a 100-mm Petri dish and let agar solidify, thus forming the bottom layer. Melt top agar on the day samples are run (as noted above). Add 17.0 mL of liquefied starch-casein agar to a sterile 50-mL tube and then add 2 mL of appropriately diluted sample and 1 mL of sterile antifungal antibiotic solution. Gently mix the inoculated agar. Pipet 5 mL of inoculated agar over the hardened bottom layer and gently swirl the plate to distribute evenly and allow to solidify.

b. Incubation: Invert and incubate at 28 ± 2°C until no new colonies appear. Usually this requires 6 to 7 d, although longer incubation times may be necessary (e.g., 2 to 3 weeks).

5. Single Agar Layer Procedure

a. Plating: Temper agar medium at 44 to 46°C, add the sample or sample dilution, and pour the mixture into a Petri dish. Alternatively, place the sample or sample dilution on the plated agar medium and spread over the medium's surface using a sterile spreader rod.

b. Incubation: Invert plate and incubate at 28 ± 2°C for up to 7 d.

6. Counting

Use all plates when estimating concentration, but try to dilute sample to obtain between 10 and 30 colony-forming units (CFUs) per plate. Counts on plates with more than

Table 9250:I. General Macroscopic Properties of Bacterial Colonies on Solid Medium

Characteristic	Typical Bacterial Colony	Actinomycete Colony*
Appearance	Shiny or opalescent	Mature colonies may have chalky white appearance darker in the center and lighter on the edges, due to fluffy aerial hyphae. Soluble pigments (e.g., melanin), which diffuse into the medium—also are common.
Texture	Soft	Strong and leathery
Degree of adherence to solid medium	Weak	Strong
Edge of colony	Regular, continuous, and not different from colony as a whole	Irregular, intermittent, slightly less dense than colony as a whole, and fuzzy

* Actinomycetes are authentic bacteria, although their hyphal character and mode of spore formation resemble fungi.

30 CFUs may be considered too numerous to count (TNTC). Those plates with more than 30 target colonies often contain considerable non-target microorganisms and are difficult to read. For the double agar layer procedure, the count per plate reflects the number of organisms in a 0.5-mL sample aliquot or dilution thereof. Identify actinomycetes by gross colony appearance. If necessary, verify using a microscope at a magnification of 50 to 100× (see Figure 9250:1). Because of filamentous growth, actinomycete colonies typically have a fuzzy border or may appear compact and leather-like. Table 9250:I lists the distinguishing characteristics commonly used to differentiate actinomycetes from other bacterial colonies. Cycloheximide generally suppresses fungal growth, but any fungal colonies that remain can be recognized by their wooly appearance. Microscopically, fungi have a considerably larger cell diameter than actinomycetes.

7. Calculation

Report actinomycetes per 100 mL of water. Calculate the count by:

$$\text{Count/100 mL} = \frac{\text{Number of colonies counted}}{\text{Volume of sample analyzed (mL)}} \times 100$$

If two plates were used, calculate the average number of colonies per plate and then follow the equation above. If results from multiple dilutions are used, add the total number of colonies for the numerator, add the total volume analyzed for the denominator, and then follow the equation above. For solid or semisolid samples, correct for water content and report actinomycete colonies per Gram of sample (dry weight).

8. Quality Control

A positive control (e.g., *Strep. albus*) should be plated each time samples are analyzed (see 9250B.3*b)*. Transfer a 1-μL loop of *Strep. albus* from the positive-control refrigerated slant to ISP No. 1. Incubate for 7 d at 28 ± 2°C. Dilute the ISP No. 1-grown culture to obtain a count of 10 to 30 colonies per plate. A negative plate control also should be run at the same frequency to ensure that media are not contaminated and test conditions are acceptable. For a negative control, add 2 mL of sterile water to top agar medium before plating.

Maintain positive control cultures at 4 to 8°C on an ISP No. 2 slant, and transfer every 2 months. Inoculate ISP No. 1 medium when positive controls are needed within 2 weeks.

Maintain all documentation of samples and associated QC results.

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9260 DETECTION OF PATHOGENIC BACTERIA*

9260 A. Introduction

1. General Discussion

One purpose of drinking water and wastewater treatment is to reduce the numbers of viable organisms to acceptable levels, and to remove or inactivate all pathogens capable of causing human disease. Despite the remarkable success of water treatment and sanitation programs in improving public health, sporadic cases and point-source outbreaks of waterborne diseases continue to occur. Water and wastewater may contain a wide variety of bacteria that cause intestinal or extra-intestinal infections. Waterborne pathogens enter human hosts through intact or compromised skin, inhalation, ingestion, aspiration, and direct contact with the mucous membranes of the eye, ear, nose, mouth, and genitals. This section provides an introduction to bacterial agents responsible for diseases transmitted by drinking and recreational waters in the United States.

The Centers for Disease Control and Prevention (CDC) and the U. S. Environmental Protection Agency (USEPA) maintain a collaborative system for collecting and reporting waterborne disease outbreak data for both microbial and chemical agents; however, detection and investigation of waterborne outbreaks is primarily the responsibility of the local, state, and territorial public health departments with voluntary reporting to the CDC.[1] From 1971 to 1998, 691 drinking-waterborne disease outbreaks due to a microbiological, chemical, or unknown etiology were reported in the United States (see Figure 9260:1). In the years 1972 through 1994, 291 outbreaks associated with drinking water were attributed to microorganisms as follows: *Giardia lamblia,* 109 (37.5% of total); *Shigella*, 34 (11.7%); nontyphoidal *Salmonella* serotypes, 13 (4.5%); *Salmonella* serotype Typhi, 5 (1.7%); *Vibrio cholerae,* 2 (0.7%), enterotoxigenic *E. coli*, 1 (0.3%); *E. coli* O157, 1 (0.3%). Community waterborne outbreaks have declined since the mid-1980s (see Figure 9260:1), largely because of the promulgation of more stringent drinking water standards, including the Surface Water Treatment Rule,[2] the Total Coliform Rule,[3,4] and other regulations.[5–7] In addition, many water utilities have made voluntary improvements.

The agents responsible for reported outbreaks are predominantly unidentified, microbial (parasitic, bacterial, or viral), or chemical (Figure 9260:2). Large numbers of parasitic outbreaks in the early 1980s were caused mostly by *Giardia*; these outbreaks were reduced by the implementation of the Surface Water Treatment Rule.[2] Relatively few outbreaks due to viruses have been reported, in part because the detection methodologies have difficulty attributing an outbreak to a specific virus. To better address the occurrence of microbial pathogens in drinking water, the USEPA has issued a Contaminant Candidate List that includes 11 microbes for methods development and potential future regulation.[8,9]

Water contamination and disease transmission may result from conditions generated at overloaded and/or malfunctioning sanitary waste disposal and potable water treatment systems. In addition, common outdoor recreational activities, such as swimming (including pools and hot tubs), wind surfing, and waterskiing, all place humans at risk of waterborne diseases from ingestion or direct contact with contaminated water.[10] Outbreaks of gastroenteritis, pharyngoconjunctivitis, folliculitis, otitis, and pneumonia are associated with these recreational activities. Overcrowded parks and recreational areas contribute to the contamination of surface and groundwater.

Laboratory diagnosis of infectious disease depends on detection or isolation of the etiologic agent or demonstration of antibody response in the patient. Environmental microbiological examinations are conducted for compliance monitoring of the environment, to troubleshoot problems in treatment plants and distribution systems, and in support of epidemiological investigations of disease outbreaks. Ideally, the public health microbiologist can contribute expertise in both clinical and environmental microbiology, thereby facilitating epidemiological investigations.

When testing for pathogens in environmental samples, it is advisable to include analyses for indicator organisms. Currently, coliforms (total coliform, thermotolerant coliform, and *E. coli*) are used as water quality indicators. Fecal streptococci, enterococci, *Clostridium perfringens*, *Bacteroides, Bifidobacterium*, and bacteriophages have been proposed as water quality indicators. No single indicator provides assurance that water is pathogen-free. The choice of monitoring indicator(s) presupposes an understanding of the parameters to be measured and the relationship of the indicator(s) to the pathogen(s). Some bacterial pathogens, such as *Pseudomonas, Aeromonas, Plesiomonas, Yersinia, Vibrio, Legionella*, and *Mycobacterium*, may not correlate with coliform indicators. Traditional bacterial indicators also may not correlate with viruses or parasites in pristine waters or groundwaters, and they may be of limited utility in estuarine and marine waters. Nevertheless, tests for total and fecal bacteria and *E. coli* are useful, because it is rare to isolate bacterial enteric pathogens in the absence of fecal contamination.

Other more general indicators may be of value also for assessing the potential for pathogen contamination and interpreting culture results. Heterotrophic plate count provides information about the total numbers of aerobic organotrophic bacteria and an indication of the total organic composition of the aquatic environment. Physicochemical factors, such as turbidity, pH, salinity, temperature, assimilable organic carbon, dissolved oxygen, biochemical oxygen demand, and ammonia, may provide useful information about contamination or the potential of water to support bacterial growth. For treated waters, chlorine residual should be measured at the sample collection point.

* Approved by Standard Methods Committee, 2007.
Joint Task Group: 22nd Edition—Nelson P. Moyer (chair), Edward J. Bottone, Joseph O. Falkinham, III, J.J. Farmer, III, Barry S. Fields, Mark W. LeChevallier.

https://doi.org/10.2105/SMWW.2882.201

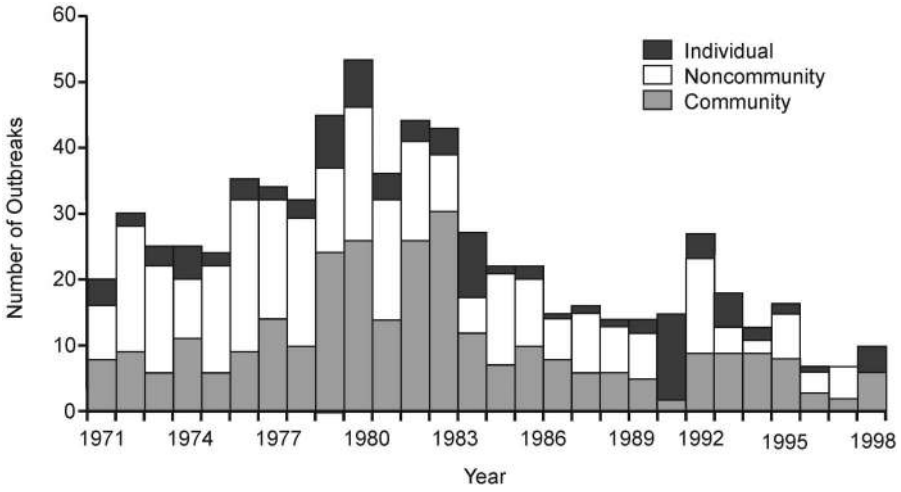


Figure 9260:1. Number of drinking water-related disease outbreaks in the United States, 1971–1998. Individual—private or individual water systems (9% of U.S. population, or 24 million users); community—systems that serve >25 users year-round (91% of U.S. population, or 243 million users); noncommunity—systems that serve <25 users and transient water systems, such as restaurants, highway rest areas, and parks (millions of users yearly). Adapted from BARWICK, R.S., D.A. LEVY, G.F. CRAUN, M.J. BEACH & R.L. CALDERON. 2000. Surveillance for waterborne disease outbreaks—United States 1997–1998. *Morbid. Mortal. Week. Rep.* 49 (SS-4):1.

This section contains methods for *Salmonella, Shigella,* diarrheagenic *E. coli, Campylobacter, Vibrio, Leptospira, Legionella, Yersinia entercolitica, Aeromonas,* and *Mycobacterium*. Methods for isolation and enumeration of *P. aeruginosa* are found in Sections 9213E and F. Methods for other pathogens are found elsewhere.[11]

The methods outlined below may be used to analyze samples associated with disease outbreaks, or in other studies concerned with the occurrence of pathogens in water and wastewater. (The methods presented below are not standardized, and may need modification to fit a particular set of circumstances. No single procedure is available for reliable detection of any pathogen or group of pathogens.)

Because the presence of pathogens is intermittent and the survival times in the environment are variable, routine examination of water and wastewater for pathogenic bacteria is not recommended. Even in outbreak situations, the recovery of pathogens from water and wastewater may be limited by lack of facilities, untrained personnel, inadequate methods, and high costs. Despite these constraints, it is important to recover a substantial number of isolates, especially if molecular fingerprinting methods will be used during outbreak investigations.

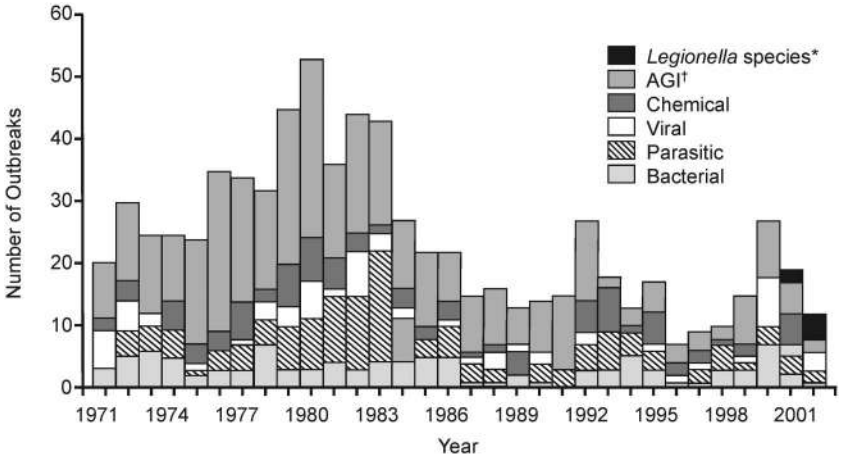


Figure 9260:2. Agents responsible for drinking water-related disease outbreaks. Adapted from BLACKBURN, B.G., G.F. CRAUN, J.S. YODER, V. HILL, R.L. CALDERON, N. CHEN, S.H. LEE, D.A. LEVY & M.J. BEACH. 2004. Surveillance for waterborne-disease outbreaks associated with drinking water—United States, 2001–2002. *Morbid. Mortal. Week. Rep.* 53 (SS-8):23.

A negative result by these methods for pathogenic bacteria does not conclusively prove their absence. The controversial concept of "viable but nonculturable" vs. "injured" organisms should be considered in relation to a negative culture result.[12,13]

All microbes included in this section are human pathogens, and they pose an infectious threat to persons involved in sample collection and laboratory analyses. Biosafety Level 2 precautions are required for all pathogens in this section except the Mycobacteria, which require Biosafety Level 3 precautions.[14]

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9260 B. *Salmonella*

The genus *Salmonella* has been of concern in water analysis for more than 100 years. Much of the history of water bacteriology evolved around methods to isolate and identify the typhoid bacillus *Salmonella* serotype Typhi (also called *Salmonella enterica* serotype Typhi and *Salmonella typhi*), and the corresponding efforts to reduce the incidence of waterborne typhoid fever. Waterborne outbreaks of typhoid fever and salmonellosis are still being reported in both developing and industrialized countries. From 1972 through 1994, 18 *Salmonella* outbreaks (most were of "non-typhoidal" type) associated with drinking water were reported to CDC for the United States. CDC surveillance data on *Salmonella* isolates in the United States since 1995 are available.*

The genus *Salmonella* is comprised of seven phylogenetic groups[1,2] that are often classified into two species: *Salmonella enterica* and *Salmonella bongori*. The genus includes more than 2500 named serotypes. Most water laboratories need to report only the genus name and the O antigen group (i.e., "*Salmonella* group B" or "*Salmonella* group D"). Further study and additional antigenic analysis in public health or reference laboratories can yield a more definitive report.

There is no universally accepted "standard method" for the isolation and identification of *Salmonella* in water, foods, or human clinical specimens. This section is a brief summary of methods for isolating, identifying, and reporting this group of microorganisms. A quantitative procedure (9260B.9) also is included.

Currently available methods used in numerous field investigations demonstrate the presence of *Salmonella* in both fresh and marine water environments; however, the occurrence of *Salmonella* is highly variable. There are limitations and variations in both the sensitivity and selectivity of *Salmonella* isolation procedures, many of which have been adapted from food and clinical microbiology. Thus, a negative result by any of these methods does not prove the absence of *Salmonella* or of other pathogens.

Fluorescent antibody (FA) techniques have been used to detect pathogenic bacteria directly in clinical, food, and water samples. Because equipment and supplies for FA techniques for detecting *Salmonella* are no longer commonly available, this method is not

* See http://www.cdc.gov/biosafety/publications/bmbl5/.

included in this section. Details of the FA method may be found in the 21st Edition of *Standard Methods*.

A newer method for detection after enrichment is polymerase chain reaction (PCR).[3]

1. Safety

Strains of *Salmonella* are almost always enteric pathogens. Use normal safety procedures and standard precautions, such as those available from government agencies.† Biosafety Level 2 (BSL 2) is typically specified because aerosol transmission is not usually involved. If isolation of *Salmonella* serotype Typhi is possible, consider additional personal protection, such as gowns, masks, and gloves, because it is a more hazardous laboratory pathogen than the other serotypes. See 9020B.2 for a discussion of biosafety levels.

Use nonpathogenic and oral vaccine strains of *Salmonella* serotype Typhi‡ in quality control procedures and method-verification studies.

2. Sampling and Concentration

Salmonella and other enteric pathogens isolated from water and other environmental samples usually will be outnumbered by other *Enterobacteriaceae* and other bacteria. Examine a relatively large sample and use enrichment media to maximize the chance of *Salmonella* isolation (see 9260B.4). Consider incubating the sample in noninhibitory media (9260B.3) at 37°C rather than in more toxic enrichment media because enteric pathogens often become "injured" as they survive under less favorable conditions found in drinking and environmental water. These injured cells are more likely to be killed by the toxic components of enrichment media. Small numbers of *Salmonella* cells can often be detected[4,5] with these caveats in mind.

a. Swab technique or "Moore swab": This method has been used to trace typhoid carriers and can be used as a simple method to concentrate other enteric pathogens,[6,7] particularly from rivers, wastewaters, and other flowing waters. Prepare swabs from cheesecloth 23 cm wide, folded five times at 36-cm lengths, and cut lengthwise to within 10 cm from the head into strips approximately 4.5 cm wide. Securely wrap the uncut or folded end of each swab with a wire or coat hanger to secure the swab as it is suspended in water or flowing wastewater. Place the swabs in kraft-type bags and sterilize at 121°C for 15 min. Place swab just below the surface of the sampling location for 1 to 3 d. Longer exposure times apparently do not increase the recovery rate. Gauze pads of similar thickness may be substituted. During sampling, particulate matter and microorganisms attach to the cloth as water passes through and over the swab. After exposure, retrieve the swab, place it in a sterile plastic bag, and send it to the laboratory in an ice chest. Process it as soon as possible, and in all cases, within 6 h. Placing swabs in less inhibitory enrichment media (i.e., media that allow growth of most enteric organisms) before transport may result in overgrowth by competitive organisms that will mask the presence of *Salmonella*. In the laboratory, place the pad or portions of it in pre-enrichment media.

b. Diatomaceous earth technique: Place an absorbent pad (not a membrane filter) on a membrane filter funnel receptacle, assemble funnel, and add 2.5 g sterile diatomaceous earth§ to loosely pack the funnel neck. Apply vacuum and filter 2 L of sample. After filtration, disassemble funnel, divide resulting "plug" of diatomaceous earth and absorbent pad in half aseptically with a sterile spatula (knife edged), and add half of the plug to two enrichment media (see below). Alternatively, place entire plug in a single enrichment medium.

c. Large-volume sampler: Use a filter composed of borosilicate glass microfibers bonded with epoxy resin to examine several liters or more of sample, provided that sample turbidity does not limit filtration.[8] The filter apparatus consists of a 2.5- × 6.4-cm cartridge filter and a filter holder.‖ Sterilize by autoclaving at 121°C for 15 min. Place sterile filter apparatus (connected in series with tubing to a 20-L water bottle reservoir and vacuum pump) in the 20-L sample container appropriately calibrated to measure volume of sample filtered. Apply vacuum and filter an appropriate volume. When filtration is complete, remove filter and place in a selective enrichment medium.

d. Membrane filter technique: To examine low-turbidity water, filter several liters through a sterile 142-mm-diam membrane of 0.45-μm pore size.[9] For turbid waters, precoat the filter: make 1 L of sterile diatomaceous earth suspension (5 g/L reagent-grade water) and filter about 500 mL. Without interrupting filtration, quickly add sample (1 L or more) to remaining suspension and filter. After filtration, place membrane in a sterile blender jar containing 100 mL sterile 0.1% (w/v) peptone water and homogenize at high speed for 1 min. Add entire homogenate to 100 mL double-strength selective enrichment medium. Alternatively, use multiple 47-mm-diam membrane filters to filter the sample. Immerse each membrane aseptically in 50 mL single-strength selective enrichment medium and incubate.

3. Pre-enrichment to Revive Injured Cells

Buffered peptone water was formulated to revive injured *Salmonella* cells found in food and may enhance the yield in water analysis. Pre-enrichment in buffered peptone water,# lactose broth,# universal pre-enrichment broth,# or a similar noninhibitory medium can precede enrichment in one or more inhibitory broths.

4. Enrichment

Although no single enrichment medium is suitable for all *Salmonella* and all conditions, three enrichment media have been widely used: tetrathionate broth, tetrathionate broth with brilliant green, and selenite broth. Direct plating on solid selective media can be done but, because of toxicity to "injured cells," use direct plating only as a supplement to enrichment in broth. Use two or more selective enrichment media in parallel for optimum detection. Elevated incubation temperatures, including 40, 41.5, and

† See, for example, http://www.cdc.gov/od/ohs/? and http://www.osha.gov. Accessed February 2017.
‡ Available from the American Type Culture Collection, http://www.atcc.org.
§ Celite, World Minerals, Inc., Lompoc, CA, or equivalent.
‖ Balston Type AA filter with Type 90 holder, or equivalent.
Difco, or equivalent.

43°C, and the addition of brilliant green dye to media help suppress background growth and may improve *Salmonella* detection. However, these modifications also suppress growth of some serotypes, including *Salmonella* serotype Typhi.

a. Selenite cystine broth: This medium inhibits Gram-positive bacteria and many of the other genera of *Enterobacteriaceae* while allowing for recovery of most *Salmonella* serotypes, including *Salmonella* serotype Typhi. Optimum incubation time for maximum recovery of *Salmonella* is 48 h at 35 to 37°C. Streak from tubes with turbidity several times during first day and then daily up to 5 d to increase potential recovery of *Salmonella*. To enhance recovery of *Salmonella*, transfer 1 mL selenite broth culture to a fresh tube of selenite broth and incubate and subculture as described above.

b. Selenite broth: This medium allows for optimum recovery of most *Salmonella*, including *Salmonella* serotype Typhi, after 24 h at 35 to 37°C. This increased recovery of *Salmonella* is accompanied by a slight decrease in selectivity when compared to selenite cystine. Most significantly, *E. coli* growth is not inhibited. Adding novobiocin (20 μg/mL) to selenite broth may be useful to inhibit swarming strains of *Proteus*. Streak from tubes with turbidity several times during first day and then daily up to 5 d to increase potential recovery of *Salmonella*. Transfer 1 mL selenite broth culture to a fresh tube of selenite broth and incubate and subculture as described above.

Commercial latex agglutination kits, used to test for the presence of *Salmonella* in tubes of selenite enrichment broth, can be useful as screening tests to help predict enrichment cultures that will be *Salmonella*-positive when subcultured.

c. Tetrathionate broth: When incubated at 35°C for 24 to 48 h, tetrathionate broth inhibits coliforms and Gram-positive bacteria, permitting selective enrichment of most *Salmonella*, including serotype Typhi. When incubated for 48 h at 43°C, tetrathionate broth has been reported as more selective for *Salmonella* than selenite-based media. Although this formulation is highly selective, it may not inhibit swarming strains of *Proteus* that can obscure the presence of *Salmonella*. Growth of *Proteus* and *Citrobacter* can be inhibited by the addition of brilliant green. Incubation at 43°C and the addition of brilliant green also will inhibit some serotypes of *Salmonella*, including Typhi. Several commercial products are available with different formulation and modification.

d. Other enrichment broths: Enrichment broths that have proved useful for *Salmonella* isolation include: brilliant green broth, EE broth Mossel, GN broth Hajna, M broth (recommended when immunological assays are being used), Muller Kauffmann tetrathionate broth base, Rappaport-Vassiliadis R10 broth, Rappaport-Vassiliadis soya peptone broth, and TT broth base Hajna (with added iodine and potassium iodide).

5. Immunomagnetic Separation

A recently developed isolation technique, often used in the food industry, is immunomagnetic separation (IMS). Samples that have been incubated for 18 to 24 h in a noninhibitory medium (such as buffered peptone water), and filtered are then reacted with metal beads coated with an antibody for a specific enteric pathogen, such as *Salmonella*. The magnetic beads, with any *Salmonella* cells captured by the antibody, are separated from the enriched sample with a magnet, and then plated on one or more selective media (see 9260B.6) for *Salmonella*. Several manufacturers produce immunomagnetic separation products for *Salmonella* and other pathogens.** An alternative is to purchase uncoated magnetic beads and coat them with antibody that is more specific for the pathogen being sought. In this case, quality control and method validation are necessary to ensure satisfactory performance.

6. Plating Media

Selection of *Salmonella* after enrichment will depend on the plating media chosen and the incubation temperature. All three factors—incubation temperature, enrichment medium, and isolation medium—are interrelated, and no one combination is optimum for recovery of all *Salmonella* serotypes. Method comparisons are encouraged to determine the best combination for a given circumstance.

Solid media commonly used for *Salmonella* and enteric pathogen detection fall into three broad groups: (*a*) differential media of low selectivity, such as MacConkey agar or EMB agar, that inhibit most Gram-positive bacteria but do not inhibit other genera of *Enterobacteriaceae* and many other Gram-negative bacteria; (*b*) more selective media containing bile salts or sodium desoxycholate as inhibitors,[10] such as desoxycholate agar or xylose lysine desoxycholate (XLD) agar; and (*c*) media specifically designed to isolate *Salmonella,* such as brilliant green agar, bismuth sulfite agar, and others. Streaking duplicate plates, one heavily inoculated and one lightly inoculated, often aids in recognition of enteric pathogens in the presence of large numbers of interfering organisms.

a. Brilliant green agar: Typical well-isolated *Salmonella* colonies grown on this medium are pinkish white with a red background. *Salmonella* serotype Typhi and a few other serotypes grow poorly because they are inhibited by the concentration of brilliant green dye used. Bacteria that ferment lactose often grow as greenish colonies, but may produce other colors as well. Occasionally, slow lactose-fermenters (*Proteus, Citrobacter)* or non-fermenters (*Pseudomonas*) will produce colonies resembling *Salmonella*. Increasing the agar concentration to 2% may suppress swarming of undesired bacteria. Swarming of *Proteus* also may be reduced by using agar plates that have dried sufficiently to remove surface moisture. If suspect *Salmonella* colonies are not observed after 24 h incubation, reincubate for another 24 h to permit slow-growing or partially inhibited organisms to develop visible colonies. If typical colonies are not observed or if the streak plate is crowded, isolate in pure culture a few colonies for biochemical characterization. Non-lactose-fermenting colonies in close proximity to lactose-fermenting colonies may be missed.

b. Bismuth sulfite agar (Wilson and Blair medium):[11] Many *Salmonella*, including serotype Typhi, grow well on this medium. Examine bismuth sulfite plates after 24 h incubation for suspect colonies; reincubate for 24 h to detect slow-growing strains. Typical colonies of *Salmonella* usually develop a black color, with or without a metallic sheen, and frequently this blackening extends beyond the colony to give a "halo" effect. A

** For example, Dynal Biotech (http://www.dynalbiotech.com/) and Matrix Microsciences Ltd. (http://www.matrixmsci.com/).

few *Salmonella* species will develop a green coloration; therefore, pick some of these colony types when typical colonies are absent. As with brilliant green agar after 48 h incubation, typical colony coloration may be masked by adjacent heavy growth. *Proteus* and other *Enterobacteriaceae* that are H_2S-positive can also appear as black colonies. One disadvantage of this medium is that it must be used within 24 to 36 h of preparation for *Salmonella* serotype Typhi, and must be refrigerated after preparation for other *Salmonella*.

c. Xylose lysine desoxycholate (XLD) agar: Compared to brilliant green dye, sodium desoxycholate, the selective ingredient in XLD, is only slightly toxic to fastidious *Salmonella,* which grow as black-centered red colonies. *Proteus* and many other *Enterobacteriaceae* grow as yellow colonies. Optimum incubation time is 24 h. If plates are incubated longer, an alkaline reversion and subsequent blackening occur with H_2S-positive genera of *Enterobacteriaceae*, such as *Citrobacter* and *Proteus*.

d. Xylose lysine brilliant green agar: This medium is very good for isolating *Salmonella* from marine samples because the brilliant green inhibits many strains of *Proteus, Enterobacter,* and *Citrobacter*.

e. Other plating media: Several other media that have proved useful in *Salmonella* isolation include: brilliant green agar-modified, brilliant green bile agar-modified, chromogenic substrate plating media,†† desoxycholate citrate agar, Hektoen enteric agar, Rappaport-Vassiliadis Medium with 20 μg/mL novobiocin, SS agar, and XLT-4 agar (with inhibitory surfactant). Check for commercial availability.

7. Screening Tests and Biochemical Identification

There are many approaches to screen for, and identify, colonies as "suspect *Salmonella*"[1,2] and for the other species of *Enterobacteriaceae*, including the other enteric pathogens (Table 9260:I). These approaches include commercial identification kits, commercial *Salmonella*-identification products, screening tests, biochemical and serological identification, fluorescent antibody (FA) screening, genus- and species-specific tests, and molecular methods. These methods are constantly evolving. Complete testing usually will result in a correct identification. Both the sensitivity and specificity of a new method, along with its ease of use, should be considered.

The identification of *Salmonella* only by colony characteristics on selective solid media has obvious limitations. Many other genera and species of *Enterobacteriaceae* can be confused with *Salmonella*;[1] complete biochemical testing will result in the fewest misidentifications.

Table 9260:I lists a series of screening tests that have proved useful for *Salmonella*. Two of the most useful are bacteriophage O1 sensitivity and the reaction with methylumbelliferyl caprylate.‡‡ These are two *Salmonella*-specific tests, and deserve to be evaluated in water analysis because of their high sensitivity and specificity.

Commercially available kits (miniaturized plastic products that contain 20 to 30 biochemical tests and include computer analysis of the results) provide another useful approach[12] to identification and have proved very popular in clinical microbiology laboratories. Their main disadvantage is that many commercial kits include only the genera and species of *Enterobacteriaceae* that occur in human clinical specimens. The omission of organisms that occur in water can lead to misidentifications.

A single-step rapid *Salmonella* test was compared with conventional culture methods for the rapid detection of *Salmonella* in 48 river water samples. The method had a sensitivity of 93% and a specificity of 100%, and was rated as being both rapid and user-friendly.[13]

8. Serological Identification

Serological testing is based on an antigen antibody reaction. The antigen is typically a living or killed *Salmonella* culture that is usually mixed with a commercial rabbit polyclonal antibody that reacts with a surface structure of the bacterium, causing a visible clumping (agglutination). NOTE: It is essential to follow the manufacturer's instructions exactly. Various serological reagents are available and some have been absorbed onto latex or other particles to facilitate recognition of positive reactions. *Salmonella* colonies are first tested in polyvalent antiserum and those that agglutinate strongly are then tested in seven individual sera for O groups A through G. For example, cultures that agglutinate in polyvalent and then only in O group B would be reported "presumptive Group B *Salmonella*." When the culture is confirmed to be *Salmonella* by other methods, a report of "*Salmonella* Group B" can be issued with confidence. Many other *Enterobacteriaceae* (and other bacteria) share antigens with the genus *Salmonella*, so it is essential to do confirmatory testing (Table 9260:I).

Salmonella serotype Typhi cultures are easy to identify because they have characteristic biochemical reactions (Table 9260:I) and typically agglutinate strongly in "Vi" and/or Group D antisera.

Complete serological identification of a *Salmonella* culture is complex and requires determination of O and H antigens and antigen factors; it should be done only by experienced reference laboratories.

9. Quantitative Procedures

The procedure described below is one approach for estimating *Salmonella* density in water samples. Other methods have been described in the literature; a comparative study is strongly recommended to select the best quantitative method for any given application. Modify the following procedure for use with solid or semisolid samples.

Because of the high ratio of coliform bacteria to pathogens, use large samples (1 L or more). Any concentration method in 9260B.2 may be used, but the membrane filter technique (9260B.2*d*) is preferred. After blending the membrane with 100 mL sterile 0.1% (w/v) peptone water, use a quantitative MPN procedure by proportioning homogenate into a five-tube, three-dilution multiple-tube procedure using either selenite cystine, selenite-F, or tetrathionate broth as the selective enrichment medium (see 9260B.4). Incubate for 24 h as required for the enrichment medium used and streak from each tube to brilliant green and xylose lysine desoxycholate agar plates. Incubate for

†† CHROMagar™ *Salmonella, Rambach*™ agar, or equivalent.
‡‡ MUCAP™, or equivalent.

TABLE 9260:I. SCREENING TESTS, KEY REACTIONS, AND PROPERTIES OF *SALMONELLA*, *SHIGELLA*, *ESCHERICHIA COLI*, *YERSINIA* AND OTHER *ENTEROBACTERIACEAE**

Organism (Genus, Species or Serotype)	Test Result or Property†
Salmonella	Lactose$^-$, sucrose$^-$, H_2S^+ (strong, whole tube is black), O1 phage$^+$,‡ MUCAP$^+$,§ agglutinates in polyvalent serum,‡ typical colonies on media selective/differential for *Salmonella* (brilliant green agar, SS agar, CHROMagar™ *Salmonella*, Rambach™ agar, etc.), lysed by the *Salmonella* specific bacteriophage O1,‡ often antibiotic resistant
Salmonella serotype Typhi	Fastidious, H_2S^+ (weak, with characteristic blackening pattern), Citrate$^-$, D-xylose$^-$, agglutinates in group D serum and/or Vi serum
Shigella	Nonmotile, lysine$^-$, gas$^-$, agglutinates in polyvalent serum, biochemically inactive, often antibiotic resistant, molecular test: PhoE+§
Shigella dysenteriae	Agglutinates in group A serum, D-mannitol$^-$
Shigella dysenteriae O1	Catalase$^-$, agglutinates in O1 serum, Shiga toxin$^+$
Shigella flexneri	Agglutinates in group B serum, D-mannitol$^+$
Shigella boydii	Agglutinates in group C serum, D-mannitol$^+$
Shigella sonnei	Agglutinates in group D serum, D-mannitol$^+$, ornithine decarboxylase$^+$, lactose$^+$ (delayed), colony variation: smooth to rough
Escherichia coli	Extremely variable biochemically, indole$^+$, MUG$^+$, grows at 44.5°C, sometimes antibiotic resistant, PhoE$^+$ molecular test§
Escherichia coli O157:H7	Colorless colonies on sorbitol-MacConkey agar, MUG$^-$, D-sorbitol$^-$ (or delayed), agglutinates in O157 serum, and H7 serum
Yersinia	Grow on CIN agar, usually more active biochemically at 25°C than 36°C (motile at 25°C, nonmotile at 36°C), urea$^+$
Yersinia enterocolitica, the pathogenic serotypes	CR-MOX$^+$, pyrazinamidase$^-$, salicin$^-$, esculin$^-$, agglutinate in O typing sera: 3; 4, 32; 5, 27; 8; 9; 13a, 13b; 18; 20; or 21
Yersinia enterocolitica O3 (the most common pathogenic serotype)	D-Xylose$^-$, agglutinates in O3 serum, tiny colonies at 24 h on agar plating media
Yersinia enterocolitica, the non-pathogenic serotypes	CR-MOX$^-$, pyrazinamidase$^+$, salicin$^+$, esculin$^+$, do not agglutinate in typing O sera: 3; 4, 32; 5, 27; 8; 9; 13a, 13b; 18; 20; or 21
Citrobacter	Citrate$^+$, lysine decarboxylase$^-$, often grows on CIN agar, strong characteristic odor
Hafnia	Lysed by *Hafnia*-specific bacteriophage,‡ often more active biochemically at 25°C than 36°C
Klebsiella	Mucoid colonies, encapsulated cells, nonmotile, lysine$^+$, very active biochemically, ferment most sugars, VP$^+$, malonate$^+$, resistant to carbenicillin and ampicillin
Enterobacter	Variable biochemically, citrate$^+$, VP$^+$, resistant to cephalothin
Serratia	DNase$^+$, gelatinase$^+$, lipase$^+$, resistant to colistin and cephalothin
Serratia marcescens	L-arabinose$^-$
Serratia, other species	L-arabinose$^-$
Proteus-Providencia-Morganella	Phenylalanine$^+$, tyrosine hydrolysis$^+$, often urea$^+$, resistant to colistin
Proteus	Swarms on blood agar, pungent odor, H_2S^+, gelatin$^+$, lipase$^+$
Proteus mirabilis	Urea$^+$, indole$^-$, ornithine$^+$, maltose$^-$
Proteus vulgaris	Urea$^+$, indole$^+$, ornithine$^-$, maltose$^+$
Providencia	No swarming, H_2S^-, ornithine$^-$, gelatin$^-$, lipase$^-$, urea$^{+\text{ or }-}$ Very inactive biochemically, no swarming, citrate$^-$, H_2S^-, ornithine$^+$, gelatin$^-$, lipase$^-$
Plesiomonas shigelloides	Oxidase$^+$, lysine$^+$, arginine$^+$, ornithine$^+$, myo-inositol$^+$

* This table gives only the general properties of the genera, species, and serogroups, so there will be exceptions; more details and more precise data, including actual percentages for biochemical test results, have been published.[1] The properties listed for a genus or group of genera generally apply for each of its species, and the properties listed for a species generally apply for each of its serotypes.

† The serological tests refer to slide agglutination in group or individual antisera (O1, O3, etc.) for *Salmonella*, *Shigella*, *Yersinia*, or *Escherichia coli,* respectively.

‡ These are two bacteriophage tests useful for identification.

§ Abbreviations: CIN = cefsulodin-irgasan-novobiocin agar (a plating medium selective for *Yersinia*); CR-MOX = Congo red-magnesium oxalate agar (a differential medium useful for distinguishing pathogenic from nonpathogenic strains of *Yersinia*); MUCAP = 4-methylumbelliferyl caprylate (a genus-specific test for *Salmonella*); MUG = 4-methylumbelliferyl-β-D-glucuronidase; ONPG = *o*-nitrophenyl-β-D-galactopyranoside; PhoE = a test done by PCR that is sensitive and specific for *E. coli*/*Shigella*; VP= Voges–Proskauer.

24 h at 35°C. Select from each plate at least one, and preferably two to three, colonies suspected of being *Salmonella,* confirm their identification as *Salmonella* (see 9260B.7), and then determine serogroup (see 9260B.8). From the combination of *Salmonella* negative and positive tubes, calculate the MPN/1.0 L of original sample (see Section 9221C).

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9260 C. (Reserved)

9260 D. (Reserved)

9260 E. *Shigella*

Shigellosis, an acute intestinal infection of humans, can be caused by any of the different species-serotypes of the genus *Shigella* (family *Enterobacteriaceae*). *Shigella* has been of concern in water analysis for more than 100 years. *Shigella* invades the intestinal mucosa, producing dysentery (shigellosis), which is characterized by abdominal pain; tenesmus (straining to produce feces); and bloody diarrhea. The infectious dose for *Shigella* spp. is low; only a few cells may be sufficient. The low infective dose often results in person-to-person transmission and laboratory-acquired infections. When outbreaks occur, they usually are associated with fecal contamination of foods, but waterborne transmission is also important. *Shigella* accounted for 34 of 291 (11.7%) drinking water-related outbreaks that were reported to CDC for the United States during 1972 through 1994.*

* For subsequent *Shigella* surveillance reports, see http://www.cdc.gov/ncidod/dbmd/phlisdata/shigella.htm. Accessed February 2017.

The genus *Shigella* has four named species, three of which can be further divided into O antigen groups (serotypes): *S. dysenteriae* (serological group A, O antigen groups 1–15), *S. flexneri* (group B, O groups 1–6), *S. boydii* (group C, O groups 1–20), and *S. sonnei* (group D; with a single O group). The latter is biochemically different from the three other *Shigella* species (see Table 9260:I). *Shigella dysenteriae* O1 is an extremely important pathogen in developing countries and frequently causes waterborne outbreaks; *S. sonnei* and *S. flexneri* predominate in most developed countries. Shigellosis is most common among children. Outbreaks with person-to-person transmission have been reported in schools, day-care centers, and institutions providing custodial care. Waterborne outbreaks are associated with the following situations: fecal contamination of private or noncommunity water supplies in which chlorination is inadequate; cross-connections between wastewater and potable water lines; and recreational waters contaminated with feces from cases of shigellosis.

Shigella strains are not unusually resistant to chlorination, and they generally compete poorly with other microorganisms in the aquatic environment. In many types of water their survival time is measured in hours and days, and a survival time of 4 d has been observed in river water. However, if the organic content of the water is very high, survival may be prolonged, depending on extent of fecal pollution, concentration of soluble organic matter, and physical conditions, such as light, temperature, salinity, and pH. Unfortunately, by the time an outbreak is confirmed to be *Shigella* by standard culture-based laboratory methods (2 to 4 d), the organism is unlikely to be found unless there is a continuous source of contamination, such as wastewater seepage or a reservoir of infected individuals who are still shedding the pathogen.

A negative culture result for the presence of *Shigella* in an implicated water supply may be due to nonoptimum sampling location, sample handling, or sample size, or to problems in the sensitivity of isolation and identification methods.

Shigella are normally sought in water samples, either in a search for all *Shigella* species as part of a survey for enteric pathogens in water, or in a search for a particular *Shigella* serotype during the investigation of a specific outbreak. Methods for the quantitative recovery of *Shigella* from the environment are very primitive compared to those for many other organisms. Another difficulty is that *Shigella* and *Escherichia coli* are the "same species" in a phylogenetic sense; this makes selective enrichment for *Shigella*, at the expense of *E. coli*, much more difficult. Classical culture methods that have resulted in isolation of *Shigella* include membrane filtration[1,2] and centrifugation[3,4] with or without subsequent broth enrichment. Recently, the polymerase chain reaction (PCR), immunomagnetic separation, and other methods have shown promise for detection of *Shigella* in environmental samples.[5–7]

1. Safety

All *Shigella* strains are considered enteric pathogens. Use normal safety procedures and standard precautions, such as those available from government agencies.† Biosafety Level 2 (BSL 2) is usually specified because aerosol transmission is not usually involved. However, all *Shigella* strains have high potential for causing laboratory infections. Preferably use additional personal protection, such as gowns, masks, and gloves. In addition, post a sign on each door to the laboratory indicating that *Shigella* is present and listing the necessary precautions for those who enter. Design safety procedures carefully because personnel who perform water analyses may not be accustomed to working with this pathogen. For quality control procedures and method-verification studies, use nonpathogenic strains of *Shigella*, such as those developed for oral vaccines.‡ **CAUTION: Shigella dysenteriae O1 produces a potent toxin and is an extremely hazardous pathogen**. If this organism is being isolated or there is a chance for its isolation, make laboratory managers and staff aware of this danger.

2. Sampling and Concentration

Methods used for sampling and concentration of total coliforms, thermotolerant coliforms, *Escherichia coli*, and *Salmonella* can be used. See membrane filter (9260E.8) and centrifugation (9260E.9) methods below.

3. Enrichment

Choose a selective enrichment medium that has proven successful for isolating *Shigella* from water. Selenite F broth and GN broth have been used most frequently. Enrichment methods that have proved useful in food and clinical microbiology have typically been modified for water analysis, so newer methods in these disciplines also may be considered. Generally, all enrichment broths have been formulated to minimize accumulation of toxic metabolites produced by other *Enterobacteriaceae* and bacteria. Selenite F broth and GN broth have been used successfully to recover shigellae from water and sand.[3,4] Alternatively, use reduced-strength tryptic soy broth adjusted to pH 8.0 (0.15 g tryptic soy broth, added directly to the sample).

During outbreak investigations, the enrichment medium can be made considerably more selective by incorporation of antibiotics. For example, if the outbreak strain is resistant (based on laboratory antibiotic susceptibility studies) to tetracycline and streptomycin at concentrations of 150 μg/mL,[8] these two antibiotics could be incorporated into enrichment broths and plating media. Use careful quality control and method verification to ensure that selective media are not inhibitory to the outbreak strain.

4. Immunomagnetic Separation after Enrichment

See 9260B.5, E.10*b*, and F.4.

5. Plating Media

After incubation, enrichment broths typically are plated on media that are selective and often differential for *Shigella*, such as MacConkey [9221B.5*a*2)], XLD (9260B.6*c*), and SS (*Salmonella Shigella*) agars, with or without antibiotics.

† See, for example, http://www.cdc.gov/od/ohs/? and http://www.osha.gov.

‡ Available, for example, from the American Type Culture Collection, http://www.atcc.org.

TABLE 9260:II. TYPICAL REACTIONS OF COMMON BACTERIA ON TRIPLE SUGAR IRON (TSI) AND LYSINE IRON AGAR (LIA)

Organism	TSI Reactions			LIA Reactions	
	Slant/butt	Gas	H_2S	Slant/butt	H_2S
Shigella	K/A	–	–	K/A	–
Salmonella	K/A	+	+	K/K	+
Salmonella serotype Typhi	K/A	–	weak+	K/K	- or weak+
Escherichia coli	A/A	+	–	K/K	–
Proteus	K/A	+	+	Red/A	+
Providencia	K/A	+	–	K/A	–
Citrobacter	K/A or A/A	+	+	K/A	+
Enterobacter	A/A	+	–	K/A	–
Yersinia	A/A or K/A	–	–	K/A	–
Plesiomonas (oxidase$^+$)	K/A	–	–	K/K or K/A	–
Aeromonas (oxidase$^+$)	A/A	V	–	K/A	–
Pseudomonas and other nonfermentative bacteria	K/K	–	–	V	V

Abbreviations: K = alkaline; A = acid; + = most strains are positive; − = most strains are negative; V = variable reaction reflecting strain-to-strain variation.
TSI determines the fermentation of D-glucose, lactose, and sucrose; gas production during fermentation; and H_2S production from thiosulfate. LIA determines L-lysine decarboxylase and L-lysine deaminase activity; and H_2S production from thiosulfate. The patterns listed are typical for each organism, but there are many exceptions.

6. Screening Tests and Biochemical Identification

There are many approaches to identifying colonies that are suspected of being *Shigella* (see Table 9260:I). Review also 9260B.7 for other approaches for identifying colonies.

a. Screening on TSI and LIA slants: It has been a common practice to screen suspect colonies by inoculating them into triple sugar iron (TSI) agar and lysine iron agar (LIA) slants. *Shigella* strains do not ferment lactose rapidly, are lysine decarboxylase negative, and H_2S negative; their reactions, and those of other common bacteria, on TSI and LIA agar are shown in Table 9260:II. Colonies with typical reactions on these two screening media are then grown on suitable media (as specified by the manufacturer of the serological reagents used), and screened with *Shigella* antisera. However, this is being replaced with more complete testing in clinical and other laboratories.

b. Complete biochemical identification: Biochemical identification can be made with a complete set of 15 to 45 biochemical tests in a wide variety of commercial miniaturized kits or in test tubes of prepared media. These methods are frequently used in state and national reference laboratories. Cultures identified as *Shigella* are then serotyped.

7. Serological Grouping and Complete Serotyping

a. Serological reagents: Cultures that have been identified as *Shigella*, or those with results consistent with *Shigella* on TSI/LIA screenings (Table 9260:II), are tested by slide agglutination with commercial polyvalent and then group-specific antisera. A wide variety of serological reagents have traditionally been available commercially.§ Recently, some manufacturers have formulated tests with antibodies absorbed onto latex particles.‖ Many commercial companies also produce polyvalent grouping antisera and individual O typing sera. Antisera may exhibit problems with both sensitivity and specificity;[9] however, evaluations of commercial products specifically apply only to the individual lot numbers evaluated. Always follow manufacturer's instructions exactly and examine the package insert to determine which *Shigella* serotypes will be detected with each reagent. NOTE: Many other *Enterobacteriaceae* (and other bacteria) share antigens with the genus *Shigella*, so it is essential to do confirmatory biochemical or molecular testing. Refer cultures to a public health reference laboratory for confirmation and additional testing.

b. Testing for a specific Shigella *serotype in water samples:* If a water sample is being analyzed as part of an investigation, in which the species of interest is known (e.g., a recreational lake outbreak due to *S. sonnei)*, test "suspect *Shigella*" colonies with typing sera for that group only. A culture that agglutinates strongly is then confirmed biochemically before being reported.

c. Testing for all Shigella *serotypes in water samples:* If a water sample is being analyzed as part of a general search for all *Shigella* species and serotypes, test "suspect *Shigella*" colonies in each of the *Shigella* polyvalent sera (four to eight, depending on the manufacturer). Test a culture that agglutinates in a polyvalent serum in each dual O sera that is included in the polyvalent. Many state, regional, and national reference laboratories can provide assistance in devising protocols and confirming a water laboratory's identification and serotyping results.

§ Among the sources are BBL, Becton Dickinson Microbiological Systems, Cockeysville, MD; Denka Seiken Co., Ltd., Tokyo, Japan; Difco Laboratories, divisions of Becton Dickinson and Co., Sparks, MD; Murex Diagnostics Ltd., Dartford, U.K.; Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France.

‖ For example, Wellcolex Colour *Shigella*, Murex Diagnostics Ltd., Dartford, U.K.

8. Membrane Filter Procedure

This procedure is suitable for low-turbidity potable and surface waters with low concentrations of coliform bacteria. Filter 100-mL to 1-L samples through 0.45-μm pore size membranes and place filters face up on the surface of XLD or MacConkey agar plates; incubate plates at 35°C overnight. Pre-enrichment can be effected by placing the filter on a noninhibitory medium for several hours and then transferring it to the more selective medium; this treatment may result in the growth of cells that have been injured by exposure to the hostile environment of environmental water. Where growth is confluent, remove some bacterial growth from the plate and inoculate into GN or Selenite F broth enrichment media; incubate for 6 h and then streak onto MacConkey and XLD plates for colony isolation. Isolated colonies then can be identified or inoculated into screening tests. For screening, pick colorless colonies (i.e., those that are lactose negative) from membrane filters or plates and inoculate slants of TSI and LIA; incubate overnight at 35°C. For interpretation, additional biochemical reactions, and serological grouping, see 9260E.6 and 7.

9. Centrifugation Procedure

This procedure is suitable for surface waters, wastewater, and sediments. Centrifuge 200- to 250-mL water samples at 1520 $\times$ *g* for 15 min and pour off all but last 2 mL of supernatant. Resuspend pellet and add 8 mL Selenite F or GN broth. Incubate suspension for 24 h at 35°C. Mix the suspension and inoculate one loopful to each of several MacConkey and XLD plates. Streak plates for isolation and incubate overnight at 35°C. Examine these plates for colorless colonies and pick and then streak suspect colonies onto TSI and LIA slants; incubate at 35°C overnight. For biochemical reactions, and serological grouping, see 9260E.6 and 7.

For solid samples (sediments, soil, sludge, etc.), suspend 10 g sample in 100 mL Selenite F or GN broth and mix thoroughly. Incubate suspension overnight at 35°C. Resuspend sediment and streak one loopful onto each of several MacConkey and XLD agar plates; incubate overnight at 35°C. Pick colorless colonies and streak onto TSI and LIA slants, and proceed as above.

10. Molecular and Research Approaches

a. DNA probe: It is difficult, if not impossible, to devise a plating medium or enrichment broth that selects for *Shigella* at the expense of *E. coli,* which usually outnumber *Shigella* in aquatic specimens. However, all four *Shigella* species contain chromosomal genes, the *ipa* genes (*ipa*B, *ipa*C, and *ipa*D) and plasmid genes (*ipa*H) that are absent on strains of *E. coli* that lack the ability to cause invasive or dysentery-like intestinal infections. This difference between *Shigella* and "generic" *E. coli* offers a unique approach to detect *Shigella*-specific genetic sequences in foods, clinical specimens, or water samples.[7,10–13] Samples that are positive by a molecular method are then cultured to isolate the *Shigella* strain(s) presumably present. Details of a method of enrichment and identification of *Shigella* based on a PCR method are available.[10] An *ipa*C protein-specific monoclonal antibody can be used to detect *Shigella* colonies on nitrocellulose membranes in a colony blot immunoassay.[13] This is an alternative for laboratories without molecular capabilities.

b. Immunomagnetic separations: Another method that shows promise is immunomagnetic separation[14,15] followed by either PCR detection or isolation methods previously described. For example, immunomagnetic particles can be coated with the monoclonal antibody MASFB, which is specific for a common epitope of the O polysaccharides of *S. dysenteriae* type 1 and *S. flexneri*. Cells of these two species attach to the immunomagnetic particles, which are then removed from the sample with a magnet. This or other similar immunomagnetic particles could be added to enrichment cultures to make them much more efficient in detecting *Shigella* strains. Immunomagnetic particles coated with *Shigella* antibodies are not yet commercially available.

c. Other methods: New and novel approaches are constantly being published and evaluated. Methods and procedures described for *E. coli* O157 may provide a useful guide for *Shigella*.

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9260 F. Diarrheagenic *Escherichia coli*

Escherichia coli is a bacterium that has been of concern in water analysis for more than 100 years. *E. coli* is the "type species" of the genus *Escherichia,* which also includes *Escherichia albertii, E. fergusonii*, *E. hermannii*, and *E. vulneris.* A fifth species, *E. blattae,* will probably be removed from the genus. Among microorganisms, *E. coli* is probably the most studied species. Although *E. coli* is a normal inhabitant of the human intestinal tract, some *E. coli* strains cause intestinal infections or attach to intestinal cells and produce enterotoxins.[1–3] The usual result is diarrhea. In this section, these gut pathogens are referred to as "diarrheagenic *E. coli*." This term is more specific,[4] than "pathogenic *E. coli"* because it distinguishes the enteric pathogens from those strains that cause extraintestinal infections because they have evolved in different ways or have acquired different virulence genes. *E. coli* causes a variety of these, such as meningitis, bacteremia, and wound and urinary-tract infections.[4] Specific procedures for the isolation and identification of *E. coli* strains that cause extraintestinal infections can be found in manuals that deal with clinical microbiology.[4]

Diarrheagenic *E. coli* are important causes of intestinal infections worldwide, but particularly in developing countries.[2,3] Many cases of *E. coli* diarrhea are due to foodborne and person-to-person transmission, but waterborne outbreaks also occur.[1,2,5–9] From 1972 through 1994, *E. coli* accounted for only two of 291 (0.7%) drinking water-related outbreaks in the United States that were reported to CDC, one due to an enterotoxin-producing *E. coli* strain and the other to a strain of *E. coli* O157. However, *E. coli* accounted for four of 30 outbreaks associated with recreational water for the years 2001–2002,[9] with three of these attributed to *E. coli* O157:H7.

Four groups of *E. coli* are well established as enteric pathogens:[2,3] Shiga toxin-producing *E. coli* (STEC) [of which enterohemorrhagic *E. coli* (EHEC) is a subgroup], enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enteropathogenic *E. coli* (EPEC). The term *E. coli* O157 is used throughout this section to refer to diarrheagenic members of this group, with or without the H7 antigen. *E. coli* O157 is a member[4] of the STEC group, and is an important pathogen in the United States and many other countries. Because *E. coli* O157 causes life-threatening infections, methods have evolved rapidly for its isolation and identification in human clinical specimens and food, and many commercial reagents are available. These procedures are applicable to water analysis and are covered below.

Other *E. coli* groups have been named[2,3] that are sometimes associated with intestinal infection. They include the enteroaggregative, diffusely adherent, cytotoxic-necrotizing-factor-producing, and cytolethal-distending-toxin-producing *E. coli* groups. These may have a causative role under certain conditions that have not been defined completely. A number of phenotypic properties or specific genes have been found in these different groups and are used in forming operational definitions and as the basis of diagnostic tests.

As described in 9260E.7*b* and *c*, the water laboratory will need to consider two types of situations: a search for all diarrheagenic *E. coli,* or for just one group (e.g., for all enterotoxin-producing *E. coli).* For the typical water laboratory, isolating and identifying diarrheal *E. coli* will be a difficult undertaking because a large number of nonroutine methods are needed to detect one or all of the pathogenicity groups. These methods are best suited for research laboratories and special studies. See 9260F.10 for one molecular solution to this difficult problem. Much easier is the search for a particular *E. coli* strain in the setting of a specific outbreak investigation associated with water. Specific approaches to three outbreak situations are outlined in 9260F.11.

Diarrheagenic *E. coli* often can be differentiated from other *E. coli* on the basis of their O and H antigens but are best defined on the basis of pathogenicity factors or the genes that code for them, such as plasmid-mediated cell invasion, plasmid-mediated colonization and enteroadherence factors, production of several potent cytotoxins, hemolysins, as well as heat-labile and stable enterotoxins.

The general approach for looking for diarrheagenic *E. coli* isolates can be summarized simply: use standard isolation and identification methods for fecal coliforms and *Escherichia coli;* then use special methods to determine if the culture belongs to a diarrheagenic group. Before planning a study to detect diarrheagenic *E. coli* in water, determine the availability of commercial products and reagents.

1. Safety

Diarrheagenic *E. coli* are enteric pathogens; use normal safety procedures and standard precautions, such as those available from government agencies.* Biosafety Level 2 (BSL 2) is usually specified because aerosol transmission usually is not involved. If pathogenic strains of *E. coli* O157 may be isolated, preferably use additional personal protection, such as protective gowns, masks, and gloves. In addition, post a sign on each door

* See, for example, http://www.cdc.gov/od/ohs/? and http://www.osha.gov

to the laboratory indicating that *E. coli* O157 is present and listing the necessary precautions for those who enter. Design safety procedures carefully because personnel who perform water analyses are not accustomed normally to working with this extremely dangerous pathogen. Fatal laboratory infections have occurred with *E. coli* O157, and the infectious dose may be as low as a few cells, as is the case with most *Shigella* strains. This makes it a very hazardous laboratory pathogen. Nonpathogenic and nontoxigenic strains of *E. coli* O157 are available† and should be used in quality control procedures and method-verification studies. **CAUTION: Avoid wild-type and virulent strains whenever possible to protect personnel.**

2. Sampling and Concentration

See sections on coliforms and thermotolerant coliforms (9221 and 9222), and *Salmonella* (9260B); these methods should be generally applicable.

3. Enrichment

Methods described for the enrichment of coliforms, thermotolerant coliforms, and *E. coli* are generally applicable for diarrheagenic strains of *E. coli*.

a. Nonselective enrichment: Enriching a water sample in a noninhibitory growth medium before selective enrichment may aid in reviving "injured cells."[10] Several non-selective broth media can be used: heart infusion broth (HIB), peptone water (PW), buffered peptone water (BPW), tryptic soy broth without D-glucose, tryptone soy broth, and others.

In general, avoid media that contain D-glucose (such as tryptic soy broth) because acidic products from glucose fermentation can reduce the pH to very low levels, resulting in death of the desired pathogen. The exception to this generalization may be *E. coli* O157; acid may actually be selective rather than inhibitory.[4]

"Injured cells" of STEC *E. coli* have been resuscitated in the presence of the trihydroxamate siderophore ferrioxamine, a commercial antioxidant, and by an enterobacterial autoinducer.[10]

b. Selective (and differential) enrichment: After enrichment in a nonselective broth, place a portion in one or more of the more selective or selective/differential enrichment broths, such as EC medium with MUG, GN broth, or vancomycin/cefixime/cefsulodin broth.

c. High-temperature enrichment and incubation time: Most strains of *E. coli* (a notable exception is *E. coli* O157) grow and ferment lactose at 44 to 45°C. Many other species of *Enterobacteriaceae* and other organisms are inhibited at this high temperature. Enrichments for *E. coli* have traditionally been at 37°C, but enrichment at 42 or 44.5°C is more selective. Some enrichment procedures have specified 6 to 8 h; others have specified overnight incubation (16 to 24 h).

d. Broths with antibiotics: E. coli strains that cause outbreaks are often antibiotic-resistant. Test the outbreak strain for its antibiotic susceptibility via the standardized single disk method or a commercial product that determines minimum inhibitory concentration for 10 to 25 antibiotics. Based on the outbreak strain's resistance level, incorporate one or more antibiotics into one of the broths listed above to make it highly selective for the outbreak strain being sought (see 9260F.11).

e. Selective enrichment of E. coli *O157:* Broth media can also be made more selective for *E. coli* O157 by adding cefixime and/or potassium tellurite. Cefixime-tellurite (CT) supplement is commercially available‡ and can be incorporated into liquid and solid media. *E. coli* broth supplemented with 20 μg/mL novobiocin, also known as modified EC medium, is commercially available.§ Vancomycin/cefixime/cefsulodin broth also can be used.[11–13]

4. Immunomagnetic Separation after Enrichment

See 9260B.5. Beads coated with antibodies to the O antigen are commercially available for *E. coli* O157, O26, O103, O111, and O145. To prepare immunomagnetic beads for other *E. coli* O groups, purchase commercial O antiserum for the strain being sought and add it to uncoated immunomagnetic beads. The resulting reagent can then be used for immunomagnetic separation after enrichment.

Immunomagnetic separation is being used in both food and water analysis to improve isolation procedures. Several commercial companies describe specific procedures for separation and subsequent identification with their *E. coli* O157 products and give evaluations and references.

5. Plating Media

See the plating media used for total coliforms, fecal coliforms, and *E. coli.* However, no plating medium will select for, or differentiate, diarrheagenic *E. coli* from the nondiarrheagenic *E. coli*. The exception is *E. coli* O157, for which selective and selective/differential media are available.[4,14,15] The population of diarrheagenic *E. coli* in water usually will be only a fraction of the population of nondiarrheagenic strains.

a. MacConkey agar: On this medium, strains of *E. coli* that ferment lactose rapidly will appear as red colonies, usually with precipitated bile around the colony. Although there are many exceptions, colonies with this appearance can be considered "very suspicious as being *E. coli*." Confirmation of this visual identification is required.

b. Other agar media: Nutrient agar with MUG, mTEC agar, violet red bile agar with MUG, and numerous others are commercially available.

c. Plating media for E. coli *O157:* Since most strains of *E. coli* O157 do not ferment D-sorbitol rapidly (within 48 h of incubation) sorbitol-MacConkey agar (SMAC) was developed[15] and is available commercially. This medium has proved useful for isolating *E. coli* O157 from clinical specimens, foods, and water, and can be made more selective by adding cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L); usually, the resulting medium is referred to as *cefixime-tellurite SMAC*. A few strains of *E. coli* O157 do not grow on this medium.[4] Another plating medium that differentiates *E. coli* O157 from other D-sorbitol-negative organ-

† For example, from the American Type Culture Collection, http://www.atcc.org.

‡ Dynal Biotec, or equivalent.
§ Difco, or equivalent.

isms‖ can be made more selective by adding potassium tellurite (2.5 mg/L) and/or cefixime (0.025 mg/L) to inhibit *Proteus* strains, or 5 mg/L to inhibit strains of *Pseudomonas* and/or *Aeromonas.*

6. Screening Tests and Biochemical Identification

Sections 9260B and E discussed different approaches to identification to the species level and the use of commercially available products, such as kits. These kits are useful for identification, but many of them will not include tests or information for organisms that normally occur in water, which will limit their application and accuracy for water analysis. *E. coli* and *Shigella* are very closely related in a phylogenetic sense, and belong to the "same species" when most phylogenetic definitions are applied. The *phoE* genetic probe is a sensitive and specific single test for the *E. coli-Shigella* Group that can be performed by reference laboratories.

Diarrheagenic and nondiarrheagenic strains of *E. coli* are generally indistinguishable in their biochemical reactions, but there are two important exceptions. *E. coli* O157 has a unique phenotype, and its MUG-negative and D-sorbitol-negative phenotype has been most useful in screening tests. Most invasive strains (EIEC) are nonmotile and negative for lysine decarboxylase and lactose fermentation. This phenotype is in contrast to "gut strains" of *E. coli* but is shared with other "inactive *E. coli*" strains that are not invasive.

Escherichia coli O157:H7 has several unique properties that facilitate its recognition and identification. This microorganism grows as colorless colonies on sorbitol MacConkey agar because it is D-sorbitol negative (or delayed) and is MUG-negative, and will agglutinate in O157 serum and H7 serum.

7. Serological Identification

Although polyclonal antisera have been used for more than 50 years to determine the presence and types of O and H antigens of *E. coli* cultures, monoclonal antibodies and latex agglutination reagents have recently become available for *E. coli* O157 and H7 and a few of the other important serotypes. Check for commercial availability and follow manufacturers' instructions exactly. The procedure below for *E. coli* O157:H7 is a typical example.

a. Serological identification of E. coli *O157 and* E. coli *O157:H7:* Test a pure culture in O157 antiserum or latex beads coated with O157 antiserum. *E. coli* O157 cultures react strongly with the reagent. Complete the manufacturer's confirmatory procedures to ensure that it is O157 and not a cross-reacting O group. Determine that the culture is motile and has the H7 antigen. Determine if the culture produces Shiga toxin (see 9260F.8*a*). The following are examples of interpretation as to whether the strain is a STEC diarrheagenic *E. coli* O157:

Result	Interpretation: Is it an enteric pathogen?
E. coli O157:H7, Shiga toxin-positive	Yes
E. coli O157:NM (non-motile, no H antigen), Shiga toxin-positive	Yes
E. coli O157:NM (non-motile, no H antigen), Shiga toxin-negative	No
E. coli O157, motile**, but not H7, Shiga toxin-negative	No

b. Screening colonies based on H antigen immobilization test: This test was developed[15] as a quick and simple method to screen hundreds of *E. coli* O157:H7 colonies picked directly from Sorbitol-MacConkey agar plates. Prepare tubes of H7 immobilization medium.[15] Touch a sorbitol-negative (colorless) colony on sorbitol-MacConkey agar and stab it a few millimeters in the top of the tube. Incubate overnight. Cultures of *E. coli* O157:H7 are immobilized and are presumptive positives. Other motile microorganisms will grow throughout the medium. Confirm the presumptive positives with commercial *E. coli* O157:H7 latex reagents.

When commercial latex reagents became available for *E. coli* O157:H7, the H antigen immobilization test was relegated to a secondary role. However, it may be the best and quickest option if hundreds of colonies must be tested in outbreak investigations.

8. Commercial Tests for *E. coli* Toxins

Several commercially available immunoassays make it possible to test water isolates identified as *E. coli* and determine if they produce three important toxins.

a. Immunoassays for Shiga toxin: These products†† have been designed to detect Shiga toxin produced by pure cultures, "colony sweeps," stool specimens, foods, and enrichments. The procedures can be modified to detect Shiga toxin-producing strains in water analysis. Check manufacturer's descriptive materials to determine which Shiga toxins are being detected.

b. Immunoassays for heat-labile enterotoxin: Pure cultures of *E. coli* are grown in the specified medium and then tested for toxin production. Use a reverse passive latex agglutination assay.‡‡

c. Immunoassays for heat-stable enterotoxin: Pure cultures of *E. coli* are grown in the specified medium and tested for toxin production. An enzyme immunoassay for heat-stable enterotoxin is available.§§

9. Other Commercial Assays and Reagents

Several useful listings of commercial products are available.[4,11–13] Because availability is always changing, check current catalogs and Internet sites.

‖ CHROMagar O157, Dynal Bioscience, or equivalent.

** The H antigen of this strain could be determined and a reference laboratory's final report might be: "*Escherichia coli* O157:H14, negative for Shiga toxins I and II."

†† Difco, Dynal Bioscience, or equivalent.

‡‡ VET-RPLA, available from Oxoid, Ogdensburg, NY, or equivalent.

§§ ST EIA kit, Denka Seiken Co. or equivalent.

10. Molecular Approaches and DNA-Based Testing

Molecular tests are frequently used to detect diarrheagenic *E. coli*, particularly the two enteropathogenic and enteroinvasive groups, for which there are no simple methods for isolation and identification. Strains of diarrheagenic *E. coli* have genes on the chromosome or on plasmids that code for toxins, colonization factors, bacteriophages, or other factors. Some of these genes are unique to one *E. coli* pathogenic group, but others are shared. Molecular tests have been based on one or more genes but no methodology has as yet emerged as standard.

Several molecular procedures for diarrheagenic *E. coli* in food, with detailed instructions for the assays, have been described.[11–13] These may be useful as a guide for investigations in water.

11. Investigation Methods

Several government and nongovernment organizations have written detailed and rigid procedures for isolating fecal coliforms, *E. coli*, and diarrheagenic *E. coli* from various specimens, particularly food and water. There are advantages and disadvantages to this rigid approach. These rigid procedures can be used as a starting point, with modifications considered for particular situations in water analysis.

In contrast to rigid methods, examples of three flexible procedures are given in ¶s *b–d* below for isolating and identifying a specific outbreak strain of *E. coli* during a waterborne outbreak investigation.

a. First steps: Isolate and study the *E. coli* outbreak strain obtained from human diarrhea cases and determine its phenotypic properties. Knowledge of these properties then can be used to develop more sensitive and specific methods for isolating and identifying the outbreak strain. Next, use the outbreak strain and verify that it grows in the media and conditions being considered. This is often called "method validation." Do this before the provisional methods are used to test water samples.

b. Example: Outbreak caused by a Shiga-toxin-producing (STEC) strain of E. coli *O157:H7 that is lactose-positive,* D*-sorbitol-negative, MUG-negative, and antibiotic-sensitive:* The following are general procedures to consider:

1) Sampling, concentration, pre-enrichment—Use one of the methods previously described.
2) Enrichment—Use one or more of the *E. coli* O157 enrichment broths.
3) Immunomagnetic separation—Test with commercial O157-coated immunomagnetic beads.
4) Plating—Use MacConkey agar, sorbitol MacConkey agar, and CHROMagar O157.
5) Screening—Use *E. coli* O157 latex reagent; test suspicious colonies that grow on the plates.
6) Confirmation—Test colonies that agglutinate the O157 latex for the H7 antigen using H7 antisera or H7 latex reagent.
7) Shiga toxin—Test in one of the commercial kits.
8) Report—*E. coli* O157:H7, positive for Shiga toxins 1 and 2.

c. Example: Outbreak caused by invasive (EIEC) strain of E. coli *O124:NM that is lysine-negative, lactose-negative, nonmotile, and resistant to chloramphenicol (MIC of 1048 μg/mL):* The following are general procedures to consider:

1) Sampling, concentration, pre-enrichment—Use one of the methods previously described.
2) Enrichment—Use one or more of the *E. coli* enrichment broths; include enrichment broth(s) with 100 μg/mL chloramphenicol to select for the outbreak strain.
3) Immunomagnetic separation—Use commercial *E. coli* O124 immunomagnetic beads if available; otherwise prepare *E. coli* O124 immunomagnetic beads using commercial *E. coli* O124 antiserum and uncoated immunomagnetic beads.
4) Plating—Use MacConkey agar and MacConkey agar with 100 μg/mL chloramphenicol.
5) Screening—Test lactose-negative colonies that grow on the agar plates in commercial *E. coli* O124 latex reagent (or in commercial *E. coli* O124 antiserum). If commercial *E. coli* O124 latex reagent is not available, prepare it with commercial *E. coli* O124 antiserum and uncoated latex.
6) Confirmation—Confirm colonies that are positives for *E. coli* O124 as being lysine-negative, lactose-negative, nonmotile, and resistant to chloramphenicol (MIC of 1048 μg/mL).
7) Report—*E. coli* O124:NM, lysine-negative, lactose-negative, nonmotile, and resistant to chloramphenicol (MIC of 1048 μg/mL).
8) Reference laboratory—Refer the culture; it can be tested for specific genes-virulence factors that define EIEC.

d. Example: Outbreak caused by an enterotoxin-producing strain (ETEC) of E. coli *O6:H16 that produces both heat-labile and heat-stable enterotoxins (LT^+, ST^+), is lactose-positive, and resistant to tetracycline (MIC of 1048 μg/mL) and ampicillin (MIC of 512 μg/mL):* The following are general procedures to consider:

1) Sampling, concentration, pre-enrichment—Use one of the many methods previously described.
2) Enrichment—Use one or more of the *E. coli* enrichment broths, but also include an enrichment broth(s) with added tetracycline (100 μg/mL) and ampicillin (100 μg/mL) to select for the outbreak strain.
3) Immunomagnetic separation—Prepare *E. coli* O6 immunomagnetic beads using commercial *E. coli* O6 antiserum and uncoated immunomagnetic beads.
4) Plating—Use MacConkey agar and MacConkey agar with tetracycline (100 μg/mL) and ampicillin (100 μg/mL) to select for the outbreak strain.
5) Colony screening—Test suspicious (lactose positive) colonies in *E. coli* O6 latex reagent (use commercial reagent if available, otherwise prepare one by using commercial *E. coli* O6 antisera and uncoated latex). Test with commercial H16 antisera to confirm, or refer to reference laboratory. As an alternative to screening hundreds of colonies; screen with an H16 immobilization test made with commercial H16 antiserum.[15]
6) Heat-labile enterotoxin and heat-stable enterotoxin—Test with commercial kits.
7) Report—*E. coli* O6:H16, positive for LT and ST, resistant to tetracycline (MIC of 1048 μg/mL) and ampicillin (MIC of 512 μg/mL).

12. Specific Methods

Examples of two specific procedures for *E. coli* O157 are given below.

a. Procedure for E. coli *O157—smaller samples:* The following procedure is a modification of the standard total coliform fermentation technique (9221B) for detecting *E. coli* O157:H7 in water. Inoculate a 100-mL sample into 50 mL 3× lauryl tryptose broth (LTB) and incubate at 35°C for 24 h. Serially dilute the sample, spread plate (0.1 mL) onto sorbitol MacConkey agar and incubate at 35°C for 18 to 24 h. Adding cefixime and tellurite enhances selectivity. EHEC O157:H7 forms colorless colonies because they do not ferment, or are slow fermenters of, sorbitol. Pick ten sorbitol-negative colonies, transfer individually into LTB-MUG (4-methylumbelliferone glucuronide; 0.1 g/L) and incubate at 35°C for 18 to 24 h. *E. coli* O157:H7 ferment lactose, but do not have β-glucuronidase activity to hydrolyze MUG, so cultures will appear gas-positive and will not fluoresce. Assay these for positive glutamate decarboxylase activity and then identify biochemically as *E. coli* [see 9221G.1].

b. Procedure for E. coli *O157—larger samples:* Larger volumes of sample also may be examined by the following procedure, modified from a procedure for detecting O157:H7 in food. This procedure has not been tested for use in water analysis; however, it has been used extensively to detect O157:H7 bacteria in apple juice. Centrifuge 200 mL sample at 10 000 × *g* for 10 min. Suspend pellet in 225 mL EHEC enrichment broth (EEB) and incubate at 35°C for 24 h. Spread plate 0.1 mL from EEB and a 1:10 dilution of EEB onto tellurite-cefixime sorbitol MacConkey agar (TC SMAC). Both EEB and TC SMAC contain antibiotics to reduce growth of normal flora bacteria; therefore, they are best suited for highly contaminated samples. Incubate EEB sample and TC SMAC at 35°C for 18 to 24 h. Observe TC SMAC plates for isolated, colorless colonies. If none are evident, serially dilute the overnight EEB sample and plate onto TC SMAC. Test colorless colonies for positive indole reaction and identify biochemically as *E. coli* before serotyping and analysis for the Shiga toxin or its genes.

See research publication concerning procedures for large-volume concentrations in drinking water.[16]

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9260 G. *Campylobacter*

Campylobacter jejuni is the leading cause of bacterial diarrheal disease, in fact causing more cases than *Salmonella* and *Shigella* combined. The majority of *Campylobacter* infections are sporadic, with few outbreaks.[1] *Campylobacter* infections also have been associated with Guillain-Barre syndrome.[2] Campylobacters are ubiquitous in the environment and throughout the food chain.[1] Poultry,[3] sheep,[4] cattle,[4] and wild birds[5] have all been implicated as reservoirs for *Campylobacter* infection. They have been recovered from water, wastewater, and soil.[6] Peak infections of *Campylobacter* occur in May and September.[1] Outbreaks of *Campylobacter* disease also have been associated with drinking raw milk[1] and private drinking water.[7] In a study of 21 outbreaks of *Campylobacter* infection in England, six were traced to water, five to milk, five to food, and in five the source could not be identified.[8] One difficulty in linking exposure to disease and identifying outbreaks is the long incubation period between exposure and disease: approximately 2 weeks. In addition, it is still difficult to distinguish pathogenic from nonpathogenic *Campylobacter*.[1]

Campylobacter physiology is an important determinant of its presence in water and other environments. These bacteria are microaerophiles and only grow at 3 to 5% oxygen and 2 to 10% CO_2.[9] In the laboratory, campylobacters require a low redox potential for growth and are relatively sensitive to ultraviolet light and desiccation.[6] The presence of campylobacters in streams and rivers is associated with passage through farmland or the introduction of wastewater.[6] Campylobacters have also been recovered from groundwater, an environment consistent with their physiology. The highest numbers of campylobacters in water are found in the winter months[6] and thus do not correlate with the seasonal appearance of disease. Because campylobacters do not grow in surface waters and die off rapidly, their numbers fall rapidly as the distance from the source increases.[6] This fact explains why there was no significant association between *Campylobacter* presence in farmland and water beyond 600 m.[10] Further confounding our understanding of the epidemiology of *Campylobacter* disease is the low infectious dose and the presence of viable but nonculturable campylobacters.[11]

Because of the relatively fastidious requirements for growth, isolation from samples that contain other microorganisms can be increased substantially (e.g., fourfold) by enrichment.

1. Water Collection and Filtration Method

Collect large-volume water samples in sterile 10-L plastic containers. Process samples immediately after collection or store at 4°C and process as soon as possible. Filter one to several liters of the water through a 0.45- or 0.22-μm-pore-size, 47-mm-diam, cellulose nitrate membrane filter. Remove filter and place face down on selective medium plate. Incubate at either 37 or 42°C for up to 5 d.[12] Incubate under microaerobic conditions at either 37 or 42°C for 24 h. Remove filter from the plate and place it face down on another selective medium (see 9260G.2).

For turbid waters, prefilter to remove soil particulates by using a stainless steel filtration device with a 1.5-L reservoir assembled with the following filter sequence. Place a 142-mm, 3.0-μm-pore-size filter on the screen inside reservoir with a 124-mm prefilter on top. In the bottom tubing adapter, place a 47-mm, 1.2-μm filter. Then place filter holders in parallel with a 47-mm, 0.65-μm filter in the upstream filter holder and a 47-mm, 0.45-μm filter in the downstream holder. Add 1 L sample to the reservoir, seal, and apply pressure of about 350 kPa. After filtration, remove the 0.45-μm-pore-size filter, place it on the surface of selective plating medium as described above, and incubate at either 37 or 42°C for 48 h.

2. Isolation

a. Selective media and conditions: Campylobacter isolation requires use of selective media containing antimicrobial agents, microaerophilic atmosphere (5% O_2, 10% CO_2, and 85% N_2), and a 42°C incubation temperature to suppress the growth of most common bacteria.[8] The thermophilic campylobacters (*C. jejuni, C. coli, C. lari*, and *C. upsaliensis*) grow well at 42°C. However, other campylobacters (*C. jejuni* subsp. *doylei* and *C. fetus*) do not grow well at 42°C; incubate plates at both 37 and 42°C for optimal isolation.[12] Microaerobic conditions can be provided by using commercially available systems and equipment.*

Selective media recommended for isolating *Campylobacter* include Skirrow's medium and *Campylobacter* medium, both commercially available. Skirrow's medium contains blood agar base with lysed horse blood, trimethoprim, vancomycin, and polymyxin B. *Campylobacter* medium contains *Brucella* agar base with sheep blood, trimethoprim, vancomycin, polymixin B, amphotericin B, and cephalothin (to which some campylobacters are sensitive). Other media include Butzler's medium, containing thioglycollate agar with sheep blood, bacitracin, novobiocin, cycloheximide, and cefazolin; Preston's medium, containing *Campylobacter* medium base with horse blood, cycloheximide, rifampicin, trimethoprim, and polymyxin B; *Campylobacter* blood-free selective medium; and *Campylobacter* charcoal differential agar.[12]

b. Enrichment media: Several enrichment media, such as *Campylobacter* broth, Campy-thio broth, Gifu anaerobe-modified semisolid medium, and Preston medium, are used to enhance recovery of campylobacters.[13] Add 10 mL water sample to 10 mL *Campylobacter* enrichment broth tubes in duplicate and incubate at 37 and 42°C for 8 h or overnight. Incubation of a water or soil sample in a selective enrichment broth for 4 h at 37°C may be important for recovery of stressed cells of *C. jejuni* that show less tolerance to elevated

* Campy Pak II, BioBag Environmental Chamber or BioBag Type Cfj, Becton Dickenson; Gas Generating Kit System BE56 or Campy Gen, Oxoid; Poly Bag System, Fisher Scientific; or equivalents.

growth temperatures. Following pre-enrichment, transfer cultures to another incubator at either 37 or 42°C for overnight incubation.[13]

C. jejuni may be induced to a nonculturable state in water, and it is not clear whether pre-enrichment or enrichment will facilitate isolation of these bacteria.[11] Use of a decreased substrate concentration enhances metabolic activity in nonculturable campylobacters from water.[14]

3. Identification

a. Culture examination: Examine *Campylobacter* plates at 24 and 48 h for characteristic colonies, which can range from flat, spreading colonies that cover the entire surface of the plate, to very small, convex, translucent colonies with colony colors ranging from gray to yellowish or pinkish.[12]

b. Microscopy identification: Campylobacter spp. do not stain well by the conventional Gram stain. If safranin is used as a counterstain, apply it for 2 to 3 min; 0.3% carbol fuchsin may be substituted for safranin to improve counterstaining. Even in 24-h cultures, campylobacters appear pleomorphic in stained smears, and cells range from small Gram-negative rods and coccoid forms to longer curved, spiral, or S-shaped rods.[12]

c. Motility test: Campylobacters normally are motile by a single polar flagellum at one or both ends. Suspend cells in Mueller-Hinton or nutrient broth and observe darting, tumbling motility using phase contrast microscopy or brightfield microscopy with reduced illumination. Do not use saline or distilled water because they may inhibit motility.[12] Young cells are 0.2 to 0.8 μm wide by 1.5 to 5 μm long, curved or spiral, and motile with darting or corkscrew-like motion.[12]

d. Biochemical tests: Campylobacters are presumptively identified by an absence of growth in air, presence of oxidase and catalase activities, Gram stain, and cell size and morphology.[12]

e. DNA-based identification: Presumptive *Campylobacter* isolates can be identified as *C. jejuni*, *C. coli*, *C. lari, C. hyointestinalis*, or *Campylobacter* by single-reaction PCR with different primers for the 16S rRNA gene.[10] PCR-based identification of *C. jejuni* and *C. coli* based on a gene encoding a lipoprotein of the enterochelin transport pathway (*ceu*) also has been developed.[15]

f. Serological identification tests: Kits† for serotyping campylobacters are commercially available. These kits use latex particles coated with polyvalent antibodies for rapid presumptive identification of the thermophilic, enteropathogenic *Campylobacter* species (*C. jejuni, C. coli*, and *C. lari*).

4. Epidemiological Markers

Both phenotypic and genotypic techniques for typing campylobacters have been developed.[1] Phenotypic methods include biotyping, serotyping, and phage typing.[1] A variety of DNA-based (genotypic) molecular techniques have been used to demonstrate the clonal relatedness of *Campylobacter* isolates from patients and environmental sources.[1] Caution must be taken in interpreting results from fingerprinting because instability of fingerprints or profiles of clones has been reported.[16] Multilocus sequence typing (MLS), based on sequences of conserved housekeeping genes, has been used for characterizing populations of different *C. jejuni* isolates.[17] MLS profiles are available for comparison and expansion.‡ Pulsed field gel electrophoresis (PFGE), relying on comparison of large restriction fragments of whole genomes, has led to identification of clonal groups of *C. jejuni* and *C. coli*.[18]

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† Such as Campyslide, BBL Microbiology Systems; Meritec-Campy, Meridian Diagnostics; and Microscreen, Mercia Diagnostics.

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9260 H. *Vibrio*

Vibrio (family *Vibrionaceae*) is a genus that has been of concern in human diseases and water analysis for many years. It now includes more than 60 named species, but only 12 occur in human clinical specimens (Table 9260:III). Eleven of these apparently cause human infections. *Vibrio* species usually cause either diarrhea or extraintestinal infections,[1–3] but some, such as *V. cholerae*, can cause both. Most human infections are related to water exposure, either water itself or through animals, such as fish and shellfish, that live in water. There are several reviews that consider the whole genus and provide many details for isolation and identification.[1–8] Throughout this section, the general term "vibrio" (plural "vibrios") is defined to be a member of the genera *Vibrio* or *Photobacterium.**

V. cholerae and *V. parahaemolyticus* are well-documented causes of diarrhea, and *V. cholerae* has caused many pandemics of cholera and millions of deaths. It is now divided into three major subgroups: *V. cholerae* O1, *V. cholerae* O139, and *V. cholerae* "non-O1, non-O139" (which comprises hundreds of the remaining numbered serotypes). Any strain of *V. cholerae* may cause diarrhea, but only *V. cholerae* O1 and *V. cholerae* O139 have caused pandemics of cholera.

More recently, *V. fluvialis*, *V. hollisae*, and *V. mimicus* also have been implicated as causative agents of diarrhea, but are less common.[1–3] *V. furnissii*, *V. metschnikovii*, and *V. vulnificus* have been isolated from the feces of patients with diarrhea (particularly after eating raw oysters).[3] Their role as actually causing the diarrhea is unproven, but deserves systematic investigation.[3]

All the *Vibrio* species are primarily aquatic, and the species distribution usually depends on temperature, Na^+ concentration, nutrient content of the water, and the plants and animals present. *Vibrio* species are common in marine and estuarine environments[1–4,6] and on the surfaces and in the intestinal tracts of marine animals. In marine and estuarine environments, vibrios are commonly isolated from sediment, the water column, plankton, and shellfish.[2,4] Seafoods that often harbor *Vibrio* species include bivalve shellfish (oysters, clams, and mussels), crabs, shrimp, and prawns. Vibrios have also been recovered from brackish lakes in the continental United States, and nonhalophilic vibrios have even been isolated from freshwater sources.[2,4,8]

Although all vibrios require Na^+ for growth[1–3] they vary greatly in the minimal amount of Na^+ (almost always expressed as NaCl) they require.[2] They also vary greatly in the amount of NaCl they tolerate (Table 9260:IV). This requirement and tolerance for NaCl has been the basis of many selective broth and agar media.[1–3] *V. cholerae* and *V. mimicus* are defined as "nonhalophilic *Vibrio* species." Although they require small amounts of Na^+ for growth, this requirement is satisfied by the peptones,

* See http://www.bacterio.cict.fr/index.html for a complete species listing.

TABLE 9260:III. GROWTH OF *VIBRIO* CULTURES ON TCBS AGAR

Organism	Colony appearance on TCBS agar, %		Growth-plating Efficiency
	Green	Yellow	
V. cholerae	0*	100*	Good
V. mimicus	100	0	Good
V. parahaemolyticus	99	1	Good
V. alginolyticus	0	100	Good
V. fluvialis	0	100	Good
V. furnissii	0	100	Good
V. hollisae	100	0	Very poor
V. harveyi	0	100	Good
V. damsela	95	5	Reduced at 36°C
V. metschnikovii	0	100	May be reduced
V. cincinnatiensis	0	100	Very poor
V. vulnificus	90†	10†	Good
"Marine vibrios"	Variable	Variable	Variable
Aeromonas and *Enterobacteriaceae*	No growth	No growth	Most strains are totally inhibited

* Percentage of strains that produce green colonies and yellow colonies, respectively.
† The original report describing this species gave the percentage positive for sucrose fermentation as 3%. At the CDC *Vibrio* laboratory, about 15% of the strains have been sucrose positive. The 10% in the table represents a composite value.

TABLE 9260:IV. BIOCHEMICAL TEST RESULTS AND OTHER PROPERTIES OF THE 12 *VIBRIO* SPECIES THAT OCCUR IN HUMAN CLINICAL SPECIMENS

	Percentage Positive for:†											
Test*	*V. cholerae*	*V. mimicus*	*V. metschnikovii*	*V. cincinnatiensis*	*V. hollisae*	*V. damsela*	*V. fluvialis*	*V. furnissii*	*V. alginolyticus*	*V. parahaemolyticus*	*V. vulnificus*-biogroup[1]	*V. harveyi*
Eight key differential tests:												
Growth in nutrient broth with 0% NaCl*	100	100	0	0	0	0	0	0	0	0	0	0
Growth in nutrient broth with 1% NaCl*	100	100	100	100	99	100	99	99	99	100	99	100
Oxidase production*	100	100	0	100	100	95	100	100	100	100	100	100
Nitrate reduced to nitrite*	99	100	0	100	100	100	100	100	100	100	100	100
Inositol (myo-) fermentation*	0	0	40	100	0	0	0	0	0	0	0	0
Arginine, Moeller's, (1% NaCl)*	0	0	60	0	0	95	93	100	0	0	0	0
Lysine, Moeller's, (1% NaCl)*	99	100	35	57	0	50	0	0	99	100	99	100
Ornithine, Moeller's, (1% NaCl)*	99	99	0	0	0	0	0	0	50	95	55	0
Additional differential tests:												
Indole production (HIB, 1% NaCl)	99	98	20	8	97	0	13	11	85	98	97	100
Methyl red (1% NaCl)	99	99	96	93	0	100	96	100	75	80	80	100
Voges-Proskauer (1% NaCl; Barritt*)*	75	9	96	0	0	95	0	0	95	0	0	50
Citrate, Simmons	97	99	75	21	0	0	93	100	1	3	75	0
H_2S on TSI	0	0	0	0	0	0	0	0	0	0	0	0
Urea hydrolysis	0	1	0	0	0	0	0	0	0	15	1	0
Phenylalanine deaminase	0	0	0	0	0	0	0	0	1	1	35	NG
Motility, (36°C)	99	98	74	86	0	25	70	89	99	99	99	0
Gelatin hydrolysis, (1% NaCl, 22°C)	90	65	65	0	0	6	85	86	90	95	75	0
KCN test (percentage that grow)	10	2	0	0	0	5	65	89	15	20	1	0
Malonate utilization	1	0	0	0	0	0	0	11	0	0	0	0
D-Glucose, acid production	100	100	100	100	100	100	100	100	100	100	100	50
D-Glucose, gas production	0	0	0	0	0	10	0	100	0	0	0	0
Acid production from:												
D-Adonito[1]	0	0	0	0	0	0	0	0	1	0	0	0
L-Arabinose*	0	1	0	100	97	0	93	100	1	80	0	0
D-Arabitol*	0	0	0	0	0	0	65	89	0	0	0	0
Cellobiose*	8	0	9	100	0	0	30	11	3	5	99	50
Dulcitol	0	0	0	0	0	0	0	0	0	3	0	0
Erythritol	0	0	0	0	0	0	0	0	0	0	0	0
D-Galactose	90	82	45	100	100	90	96	100	20	92	96	0
Glycero[1]	30	13	100	100	0	0	7	55	80	50	1	0
Lactose*	7	21	50	0	0	0	3	0	0	1	85	0
Maltose*	99	99	100	100	0	100	100	100	100	99	100	100
D-Mannitol*	99	99	96	100	0	0	97	100	100	100	45	50
D-Mannose	78	99	100	100	100	100	100	100	99	100	98	50
Melibiose	1	0	0	7	0	0	3	11	1	1	0	0
α-Methyl-d-glucoside	0	0	25	57	0	5	0	0	1	0	0	0
Raffinose	0	0	0	0	0	0	0	11	0	0	0	0
L-Rhamnose	0	0	0	0	0	0	0	45	0	1	0	0
Salicin*	1	0	9	100	0	0	0	0	4	1	95	0
D-Sorbitol	1	0	45	0	0	0	3	0	1	1	0	0
Sucrose*	100	0	100	100	0	5	100	100	99	1	15	50
Trehalose	99	94	100	100	0	86	100	100	100	99	100	50
D-Xylose	0	0	0	43	0	0	0	0	0	0	0	0

TABLE 9260:IV. CONT.

Test*	Percentage Positive for:†											
	V. cholerae	*V. mimicus*	*V. metschnikovii*	*V. cincinnatiensis*	*V. hollisae*	*V. damsela*	*V. fluvialis*	*V. furnissii*	*V. alginolyticus*	*V. parahaemolyticus*	*V. vulnificus*-biogroup[1]	*V. harveyi*
Mucate-acid production	1	0	0	0	0	0	0	0	0	0	0	0
Tartrate-Jordan	75	12	35	0	65	0	35	22	95	93	84	50
Esculin hydrolysis	0	0	60	0	0	0	8	0	3	1	40	0
Acetate utilization	92	78	25	14	0	0	70	65	0	1	7	0
DNase (25°C)	93	55	50	79	0	75	100	100	95	92	50	100
Lipase*	92	17	100	36	0	0	90	89	85	90	92	0
ONPG Test*	94	90	50	86	0	0	40	35	0	5	75	0
Yellow pigment (25°C)	0	0	0	0	0	0	0	0	0	0	0	0
Tyrosine clearing	13	30	5	0	3	0	65	45	70	77	75	0
Growth in nutrient broth with:												
6% NaCl*	53	49	78	100	83	95	96	100	100	99	65	100
8% NaCl*	1	0	44	62	0	0	71	78	94	80	0	0
10% NaCl*	0	0	4	0	0	0	4	0	69	2	0	0
12% NaCl*	0	0	0	0	0	0	0	0	17	1	0	0
Swarming (marine agar, 25°C)	−	−	−	−	−	−	−	−	+	+	−	100
String test*	100	100	100	80	100	80	100	100	91	64	100	100
O129, zone of inhibition‡	99	95	90	25	40	90	31	0	19	20	98	100
Polymyxin B, % with any zone of inhibition	22	88	100	92	100	85	100	89	63	54	3	100

* Test is recommended as part of the routine set for *Vibrio* identification. 1% NaCl in parentheses indicates 1% NaCl has been added to the standard media to enhance growth; HIB = heart-infusion broth; the Barritt reagent for the Voges–Proskauer test contains α-naphthol for greater sensitivity; TSI = triple sugar iron agar; ONPG = *o*-nitrophenyl-β-D-galactopyranoside; the positive string test indicates cell lysis in the presence of a 0.5% sodium desoxycholate solution.

† The number gives the percentage positive after 48 h of incubation at 36°C (Unless other conditions are specified). Most of the positive reactions occur during the first 24 h. NG (no growth) means that the organism does not grow, probably because the NaCl concentration is too low.

‡ Disk content = 150 μg.

meat extracts, and similar ingredients found in commercial media.[1–3] All of the other *Vibrio* species are *halophilic*—they do not grow at 36°C in nutrient broth that has no added NaCl in its formulation (see Table 9260:IV). *V. fluvialis*, *V. furnissii*, and *V. metschnikovii* are "moderate halophiles," and grow in nutrient broth with only 0.1% added NaCl.[1–3] Most of the other *Vibrio* species require much more than 0.1% NaCl for growth and can be considered to be the "true halophiles" or "marine vibrios." The NaCl content can be adjusted in designing enrichment broths and agars. A low amount, such as 0.1%, will select against the marine vibrios, but will allow the pathogens to grow. Hundreds of methods have been described for the isolation and identification of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and other *Vibrio* species from clinical specimens, foods, seafood, wastewater, and water, although none of these has yet achieved enough acceptance to be considered a standard method. Several commercial companies produce equipment, supplies, media, and reagents useful in *Vibrio* work. An extensive listing of commercial products and their sources is available.[5]

Newer methods based on DNA probes and PCR are extremely promising as research procedures, but will have limited application for water analysis unless they become available as a ready-to-use commercial kit. The following sections describe methods and approaches that have proved useful and can be considered for a particular situation in water analysis.

1. Safety

See the safety discussions in the previous sections. Use normal safety procedures and standard precautions, such as those available from government agencies.† Because aerosol transmission is not normally involved, Biosafety Level 2 (BSL2) is usually specified. "Oral vaccine" strains of *V. cholerae* are available, and are recommended as a replacement for pathogenic "wild" strains in method verification studies.

Vibrio vulnificus causes serious, sometimes fatal wound infections. If this organism is being worked with, or there is a chance that it will be isolated from water samples, use disposable gloves to minimize contact with hands, particularly if the skin is not intact.

2. Sampling and Concentration

Methods used for coliforms, thermotolerant coliforms, *E. coli*, and *Salmonella* are generally applicable. The "Moore Swab" technique also has been particularly useful in water analysis.[9] Swabs can be placed in sewer pipes to detect and then trace cholera cases (*V. cholerae* O1). Vibrios in water[1–4] are often

† See, for example, http://www.cdc.gov/od/ohs/? and http://www.osha.gov.

attached to particulate matter, such as plankton with chitin shells (e.g., copepods), algae, and similar microenvironments. If these particulates are filtered out to permit a greater sample volume for analysis, also culture the filter.

3. Enrichment

a. Nonselective enrichment: Enriching a water sample in a noninhibitory growth medium before selective enrichment may be helpful. It is possible that "injured" or "stressed" cells can be revived by pre-enrichment in special media.[10]

Several nonselective broth media can be used, including alkaline peptone water (see ¶ *c* below), peptone water (PW), buffered peptone water (BPW), heart infusion broth (HIB), and marine broth. In general, it is advisable to avoid media that contain D-glucose (such as tryptic soy broth) because acidic end products from glucose fermentation can reduce the pH to very low levels, resulting in rapid death of the desired *Vibrio* species.

b. Selective enrichment: There are many ways to select for one or more pathogenic *Vibrio* species at the expense of other vibrios and nonvibrios. Some of these include: raising the pH or incubation temperature, incorporating chemicals or antibiotics to reduce undesired vibrios and other organisms, and incorporating a sugar or organic compound used only by the desired *Vibrio* species.[1]

c. Enrichment media:

1) *Alkaline peptone water*—This is perhaps the most used broth to enrich *Vibrio cholerae* and other *Vibrio* species. *Vibrio* species typically grow better than other organisms at the high pH (8.5 to 9.0) of this medium, and also tend to concentrate at the aerobic surface (meniscus) of the liquid, often forming a pellicle. Culturing is done from the surface for this reason. Enrichment in alkaline peptone water usually is followed by plating the liquid's surface onto Thiosulfate Citrate Bile Salts (TCBS) agar and/or other media selective for *Vibrio*.

Alkaline peptone water is not a single medium. Most formulations typically contain 0.5 to 1% NaCl, which allows the growth of both pathogenic and environmental *Vibrio* species. The type of peptone used in the medium also has varied widely depending on several factors, including local availability. The final pH also has varied in different formulations. The formula given below,‡ with 0.5% NaCl, has been used in many laboratory procedures and epidemiological investigations. Others formulations may be equally effective.

Peptone§	10 g
Sodium chloride (NaCl)	5 g
Sodium hydroxide (NaOH), l*N*	~6 mL
Water	~994 mL

Dissolve peptone and sodium chloride in the water. Insert a pH electrode and add 1*N* NaOH dropwise until pH has risen to 8.4; about 6 mL will be required. Final volume will be 1000 mL. Dispense and autoclave at 121°C for 15 min. The final medium will be clear and amber-colored.

2) *Alkaline peptone water, saltless:*[1]‖ —The content of NaCl in alkaline peptone water can be adjusted to be selective for different *Vibrio* groups. If no NaCl is added, the medium will select for the nonhalophilic species *V. cholerae* and *V. mimicus* at the expense of the other species, which are all halophilic.

Prepare as with alkaline peptone water, ¶ 1) above, but omit the 5 g NaCl. While this medium has no added NaCl, the peptone formulation contains enough Na^+ for *V. cholerae* and *V. mimicus* to grow.

3) *Alkaline peptone water, 0.1% NaCl*—This medium is selective for the moderate halophilic *Vibrio* species, because the NaCl content is too low for the true halophilic species to grow.

Prepare as described for alkaline peptone water, ¶ 1) above, but add 1 g, rather than 5 g, of NaCl.

4*) Other alkaline peptone waters*—The NaCl content can be adjusted based on the NaCl tolerance of a particular outbreak strain or *Vibrio* species being sought (Table 9260:IV) to make the medium more selective. For example, alkaline peptone water with 8% NaCl would select for *V. alginolyticus* at the expense of less tolerant species. Similarly, other selective agents could be added based on the phenotypic properties of a specific strain being sought.

d. Procedures for Vibrio *species:*

1) *V. cholerae*

a) Enrichment—Use alkaline peptone water—saltless and a second enrichment medium such as alkaline peptone water or alkaline peptone water—saltless with added colistin or Polymyxin B.[1–3] Incubate 6 to 8 h at 36°C.

b) Tentative identification—After incubation, plate a loopful from the surface onto TCBS agar and sheep blood agar. Test yellow colonies on TCBS agar and typical colonies on sheep blood agar (many will be strongly hemolytic) with commercial latex for *V. cholerae* O1. Confirm the identification for those that agglutinate and test them for the production of cholera toxin.

In successful enrichments of water samples, *V. cholerae* O1 may be present in high enough numbers to allow its immediate detection before subculture. If enough *V. cholerae* O1 antigen is present, it will agglutinate latex coated with antibodies to the O1 antigen. Add a drop of the enrichment culture to a drop of *V. cholerae* O1 latex. Agglutination is a presumptive positive; confirm by culture.

A second method for direct detection in enrichments is the microscopic immobilization test, which shows a rapid loss of motility of *V. cholerae* O1 cells in the presence of commercial *V. cholerae* O1 serum as observed with a microscope.

For tentative identification of *V. cholerae* O139 after enrichment but before subculture, use commercial antisera or latex for *V. cholerae* O139 instead of the O1 reagents in both assays described in the preceding two paragraphs.

2) *V. parahaemolyticus*—Use salt Polymyxin broth# and alkaline peptone water.

3) *V. vulnificus*—Use alkaline peptone water–0.1% NaCl and alkaline peptone water–0.1% NaCl with added colistin or Polymyxin B.[1,3]

‡ CDC Medium 1494 of *Vibrio* Reference Laboratory, Centers for Disease Control and Prevention, Atlanta, GA.
§ Bacto™, or equivalent.

‖ CDC Medium 1495 of *Vibrio* Reference Laboratory, Centers for Disease Control and Prevention, Atlanta, GA.
Nissui Co., or equivalent.

4) *V. fluvialis and V. furnissii*—Use a basal salts medium, such as BM medium of Baumann and Baumann[1], but keep the NaCl concentration at 0.1% by substituting K^+ salts for Na^+ salts. Add D-galacturonate as the sole source of carbon and energy, which will select for these two species.[1]

5) *V. cincinnatiensis*—Prepare enrichment as described in ¶ 4) above, but substitute myo-inositol as the sole source of carbon and energy to select for this species.[1]

4. Immunomagnetic Separation after Enrichment

See immunomagnetic separation of *Salmonella* (9260B.5) and diarrheagenic *E. coli* (9260F.4). Immunomagnetic separation should greatly improve the yield of a particular strain after enrichment.[11,12] Purchase commercial O (or K) antiserum for the strain being sought (such as *V. cholerae* O1 or *V. parahaemolyticus* O3:K6) and add it to uncoated immunomagnetic beads according to manufacturer's directions. Use reagent for immunomagnetic separation after enrichment.

Few sera are commercially available to assist in preparing antibody-coated beads for the other *Vibrio* species. Check for availability from reference and research laboratories and with commercial laboratories that prepare custom antisera with an antigen supplied by the customer.

5. Plating Media

a. General guidance: Marine agar** is a nonselective medium, and essentially all *Vibrio* strains will grow on it. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Table 9260:III) is commercially available†† and is extremely useful for isolating *V. cholerae* and *V. parahaemolyticus* from water as well as from human clinical specimens.[1–4] Cultures of *Vibrio* grow well on sheep blood agar and may be beta hemolytic (*V. cholerae* non-O1 and some *V. cholerae* O1 strains of the El Tor biotype), alpha hemolytic (*V. vulnificus* and many others), or nonhemolytic.[3] *Vibrio* strains usually grow on MacConkey agar (sometimes with a reduced plating efficiency) and will appear as colorless (lactose-negative) colonies. *Vibrio* cultures often do not grow well on more selective "enteric plating media." Oxidase testing[1–3] appears to be a cost-efficient method for detecting *Vibrio* isolates from clinical specimens and water and can be conducted on colonies grown on blood agar and on lactose-negative colonies on selective media. Most vibrios are oxidase-positive; however, lactose-positive colonies tested directly from selective media, such as MacConkey agar, are often oxidase-negative, (i.e., a false negative reaction). Test individual colonies for oxidase production, or add reagent to an area of growth on the plate.

b. TCBS agar minus sucrose, plus a different sugar(s): Sucrose is the sugar in commercial formulations of TCBS agar. Prepare agar from the original ingredients[1] but omit sucrose and add 5 to 10 g/L of one or more other sugars. This modified medium will be differential or selective because vibrio species will have different reactions depending on whether they can ferment the sugar compound added to the medium.[1–3] For example, add D-galacturonate for *V. fluvialis* and *V. furnissii* because most other vibrio species do not ferment this compound (Table 9260:IV), or add myo-inositol for *V. cincinnatiensis.*

c. Other selective-differential agar media: Many media are commercially available, and formulas for some are published elsewhere. Alternative media for *V. parahaemolyticus* are *V. parahaemolyticus* sucrose agar (VPSA)[13] and a chromogenic agar‡‡; media for *V. vulnificus* are modified cellobiose polymyxin colistin agar (mCPC),[13] cellobiose-colistin agar (CC agar),[13] and *Vibrio vulnificus* agar (VVA).[13]

6. Biochemical Identification

Only 11 *Vibrio* species cause human infections, and their identification is not difficult if the key tests (1 through 3) or all the tests listed in Table 9260:IV are done. For *Vibrio* identification with standard tube tests, add NaCl to a final concentration of 1% for several biochemical test media because some commercial media formulations do not include NaCl. If this is not done, halophilic *Vibrio* species will not grow or will grow poorly and give negative reactions in tests that should be positive. Fortunately, commercial media for most of the biochemical tests are formulated to contain 0.5 to 1.0% NaCl.

a. Identification of pathogenic Vibrio *species:* The most common *Vibrio* species that require identification are *V. cholerae* (O1, O139, non-O1, non-O139), *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. fluvialis*, and *V. mimicus*.

In the investigation of cholera outbreaks there is no need to do a large number of biochemical tests to confirm a culture as *V. cholerae*. Agglutination in *V. cholerae* O1 or O139 serum is diagnostic; confirm by biochemical testing for the first few isolates. Phenotypically, *V. cholerae* O139 is almost identical to *V. cholerae* O1 (the El Tor biotype), and is identified by its agglutination in O139 serum. Another possible differential characteristic is susceptibility to the vibriostatic compound O-129. *V. cholerae* O139 strains are usually O-129-resistant, whereas most O1 isolates are sensitive.

Strains identified as *V. cholerae* that do not agglutinate in O1 or O139 sera are identified as *V. cholerae* non-O1, non-O139. Further serotyping would yield a more precise identification, but complete serotyping is done by only a few reference laboratories. The test for Na^+ requirement differentiates *V. cholerae* from the halophilic *Vibrio* species (Table 9260:IV), and sucrose fermentation differentiates it from its close relative *V. mimicus*.

Strains of *V. parahaemolyticus* are usually typical in their biochemical reactions (Table 9260:IV). *V. alginolyticus* is biochemically similar to *V. parahaemolyticus*, but it usually swarms, is Voges-Proskauer-positive, and grows in higher concentrations of NaCl (Table 9260:IV).

V. vulnificus strains grow well on blood agar and TCBS agar. Most strains are sucrose-negative and green on TCBS agar, but occasional strains are sucrose-positive and produce yellow colonies. *V. vulnificus* is unique among *Vibrio* species because it ferments lactose, salicin, and cellobiose and is also ONPG-positive. It has no zone of inhibition or a small zone around colistin, but large zones around ampicillin and carbenicillin. *V. vulnificus* biogroups 2 and 3 have been described[1–3] and are

** BD Biosciences, or equivalent.
†† BD Biosciences, Oxoid, Eiken, or equivalent.
‡‡ CHROMagar Vibrio, CHROMagar, Paris, France, or equivalent.

difficult to identify without doing a complete set of biochemical tests.

V. fluvialis and *V. furnissii* are often confused with *Aeromonas,*[1–3] because all three are usually arginine-dihydrolase-positive and are biochemically similar. In contrast to strains of *Aeromonas*, *V. fluvialis*, and *V. furnissii* are slightly halophilic and will grow in nutrient broth only if NaCl is added. Phenotypically, *Vibrio furnissii* is almost identical to *Vibrio fluvialis*, and gas production in glucose is the key differential test.[1–3]

Key points of identification for other species are as follows: *V. hollisae* strains are fastidious. They grow on blood agar, but not on MacConkey or TCBS agar. Strains are halophilic, triple decarboxylase-negative, poorly motile, and have a characteristic fermentation pattern. Strains also have a unique antibiogram,[1–3] with very large zones around all antibiotics, including penicillin. *V. damsela* has a unique biochemical profile and resistance pattern,[1–3] which make identification easy. *V. metschnikovii* is unique among the pathogenic *Vibrio* species because it is oxidase-negative and does not reduce nitrate to nitrite. *V. cincinnatiensis* ferments myo-inositol. *V. harveyi* is biochemically distinct and is resistant to ampicillin, carbenicillin, and colistin.[1–3]

b. Identification with commercial systems: Consult manufacturer's list of the *Vibrio* species included in product's database, published evaluations of the product, and the formula of the product's suspending medium and tests to determine if the Na^+ content is sufficient. A recent study[14] evaluated six commercial identification products for their accuracy in identifying pathogenic *Vibrio* species, and found some problems with each product. One of the most common errors of commercial systems is that they misidentify cultures of *Aeromonas* as *Vibrio fluvialis*/*V. furnissii* and *vice versa*, because these three organisms are very similar biochemically. A good check on a commercial system's identification of a culture is to test one of the halophilic vibrio species and determine its oxidase reaction, growth in nutrient broth with 1% NaCl and without NaCl, and growth on TCBS agar. Disagreements in any of these key properties warn of a possible misidentification.

c. Identification of water and seafood Vibrio *isolates:* This can be extremely difficult because over 60 species of *Vibrio*, *Photobacterium* and their relatives must be considered.[1–4] Do the key tests (1 through 3) or all of the tests in Table 9260:IV and compare the isolate's profile with each species. If there is a perfect match, the isolate is most likely correctly identified. If several tests are in disagreement, there is the danger that the isolate is not one of the "12 clinical species." Molecular tests and 16S rRNA sequencing[2] may prove good alternatives to phenotypic methods for this complex group of organisms, but currently this is a research rather than a routine test; optimally, send the isolate to a commercial laboratory for definitive identification.

7. Serological Identification

The availability of commercial antisera will determine the procedures that are practical. Several polyclonal antisera and latex agglutination reagents are available for *V. cholerae* O1 and O139.§§ Rapid and complete agglutination is a strong presumptive positive, and can be followed by toxin testing. Biochemical confirmation will reduce the chance of a false positive, and should be done for at least a few positive cultures. Commercial antisera are also available for *V. parahaemolyticus*‖ ‖ but rarely for the other *Vibrio* species. Reference laboratories may be willing to furnish some of their reference sera they have made for *V. vulnificus*, *V. fluvialis*, *V. furnissii*, and other species.

8. Toxin Assays

Commercial kits are now available to test for cholera toxin and the thermostable direct hemolysin (TDH) of *V. parahaemolyticus.*

a. Commercial immunoassay for cholera toxin: Grow pure cultures of *V. cholerae* or *V. mimicus* in specified media and test for cholera toxin production in a reverse passive latex agglutination assay (VET-RPLA).## PCR is another method. Typical reports:

- *V. cholerae*, positive for cholera toxin
- *V. mimicus*, positive for cholera toxin

This assay also detects the heat labile enterotoxin of *E. coli*, which is structurally similar to cholera toxin.

b. Commercial immunoassay for heat-labile enterotoxin and heat-stable enterotoxin of V. cholerae *and* V. mimicus: Grow the culture in special media. Test in one of the commercial products previously described for *E. coli* (see 9260F.8). Typical reports:

- *V. cholerae*, non O1-O139, positive for heat-labile enterotoxin, negative for heat-stable enterotoxin
- *V. mimicus*, negative for heat-labile enterotoxin, positive for heat-stable enterotoxin

c. Commercial immunoassay for the thermostable direct hemolysin (TDH) of V. parahaemolyticus: Strains of *V. parahaemolyticus* that contain the *tdh* gene produce this toxin and are enteric pathogens; strains that lack the *tdh* gene do not produce this toxin. Strains of *V. parahaemolyticus* that contain the *trh* gene and produce the TRH toxin are also potential enteric pathogens. No commercial assays are available to determine the presence of the *tdh* gene or its toxin, and *trh*-positive strains are rarer. *V. parahaemolyticus* is a very common inhabitant of water and the surfaces and intestines of fish and shellfish. However, most strains isolated from these and other environmental samples will be negative for both TDH and TRH, and thus are not considered enteric pathogens.

Grow the strain of *V. parahaemolyticus* in a special medium. Test the supernatant for *tdh* in a commercial kit that uses an immunological assay.##

d. Screening colonies of V. parahaemolyticus *on Wagatsuma agar to detect strains that are* TDH-*positive ("Kanagawa positive"):* This method has an advantage over that of ¶ *a* above if hundreds of *V. parahaemolyticus* colonies must be screened for toxin production. Prepare commercial Wagatsuma agar*** and add washed red blood cells as specified. Spot-inoculate the plates with individual colonies suspected as being *V. parahaemolyticus*. Read for hemolysis around the colonies. Hemolytic colonies are referred to as "Kanagawa-positive." Use method of ¶ *c* above

§§ BD Biosciences, Denka Seiken, Columbia Diagnostics, or equivalent.

‖ ‖ Denka Seiken, Nichimen, or equivalent.
Denka Seiken, distributed by Oxoid Inc., or equivalent.
*** Kyoto Pharmaceutical, or equivalent.

to confirm that Kanagawa-positive colonies really produce the thermostable direct hemolysin.

9. Molecular Approaches and DNA-based Testing

DNA-based testing and molecular approaches have been described for many of the *Vibrio* species[1–5,7,8,11] and include methods for identification of species, such as 16S rRNA sequencing, strain typing such as pulsed-field gel electrophoresis (PFGE), PCR and determining toxins and virulence factors. Detailed instructions for several molecular procedures for *Vibrio* species in food are available.[5,7] Genetic sequences most likely to be of interest in vibrio work include:

- *V. cholerae*—the cholera toxin gene *ctx*, genes that code for the O1 and O139 antigen.
- *V. parahaemolyticus—tdh* and *trh* genes and perhaps the *tlh* gene for pathogenicity, and the urease plasmid.
- *V. vulnificus*—the cytotoxin hemolysis gene *vvhA* for identification of the species. Detection of this gene[7] has proved extremely useful in identifying environmental isolates of this species.

These molecular methods are evolving very rapidly. Consult current literature for technical details such as primer sequences, reaction conditions, and detection methods.

10. Investigation Methods

Many of the methods described in 9260H.1–9 allow for flexibility. However, several detailed and specific procedures have been described to isolate and identify pathogenic *Vibrio* species in food and water. It is helpful to compare general and specific methods before deciding on the best approach for a particular situation in water analysis. Some procedures listed below give all the technical details for their methods describing: enrichment-isolation, media-reagents, immunomagnetic separation, screening, identification, toxin testing, and PCR or other molecular methods. These include procedures for *Vibrio* species,[15] *V. cholerae* O1,[7,16] *V. parahaemolyticus,*[7,11] and *V. vulnificus.*[7]

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9260 I. *Leptospira*

Leptospira spp. are motile, aerobic spirochetes that require fatty acids for growth.[1] Serum or polysorbate enrichments must be incorporated into artificial media, and some pathogenic strains may require CO_2 on initial isolation. Leptospires are divided into two groups based on their pathogenicity and growth characteristics. The pathogenic leptospires make up the Interrogens Complex; they have an optimal growth temperature of 28 to 30°C and grow over a pH range from 5.2 to 7.7. Saprophytic leptospires

are assigned to the Biflexa Complex; they prefer a growth temperature between 5 and 10°C below pathogenic strains, grow in the presence of 8-azaguanine at 225 μg/mL, and fail to form spherical forms in 1*M* NaCl.

Since 1998, the phenotypic serological classification of the genus has been replaced with genotypic classification, resulting in recognition of 10 genomospecies. The new genomospecies for *L. interrogans* and *L. biflexa* do not correspond to the earlier phenotypic and serological characteristics of these species, thus creating difficulty for laboratories unable to perform genotypic identification. However, while reclassification of *Leptospira* spp. based upon genomospecies is taxonomically correct, the older phenospecies and serovars are much more practical in clinical microbiology and epidemiology and probably will be used until genotypic classification systems are readily available and widely used.[2] Leptospires prefer alkaline conditions, and they persist longest in warm, moist environments protected from sunlight. Under favorable temperature and pH conditions, leptospires survive for 3 to 5 d in damp soil and up to 10 d in natural waters. They also survive for 12 to 14 h in undiluted wastewater, up to 3 d in aerated wastewater, and up to 4 weeks in sterile tapwater at pH 7. Nonpathogenic leptospires are ubiquitous and have been isolated from municipal water supplies.[3] Pathogenic leptospires usually require an animal host and do not survive and propagate in the environment.

Leptospirosis is a worldwide zoonotic disease of wild animals.[4] Reservoirs of leptospires in wildlife include deer, foxes, raccoons, skunks, opossums, muskrats, and rodents. Domestic animals harboring leptospires include horses, cattle, goats, pigs, and sheep. Dogs may become infected but not cats. Humans are incidental hosts.

Humans acquire leptospirosis (Weil's disease) directly from animals, and from occupational or recreational exposure to urine-contaminated water or environmental surfaces.[5] Rats and other rodents are the most important reservoir for humans.[6] Occupational and recreational activities placing people in contact with animal urine are the primary risk factors for acquiring leptospirosis. The highest prevalence occurs in tropical and subtropical regions where environmental survival is greatest.[7] Increased human exposure is associated with rainfall resulting in flooding. Triathletes,[8] military personnel,[9] and eco-challenge participants (survivors)[10] are at increased risk of infection. Swimming, kayaking, and other water sports,[10,11] travel to tropical areas with occupational or recreational exposure to surface waters,[12] and natural disasters that affect sewer systems and runoff[13,14] increase risk of the disease. Urine from rats, cows, pigs, and dogs has been implicated in surface water contamination leading to outbreaks. Outbreaks of leptospirosis associated with drinking water are unusual, and are invariably caused by contamination of domestic water reservoirs with urine of infected rodents.[15] Drinking water outbreaks have been reported from urine contamination of water, for example, fountains, holding tanks, and wells.[2]

Leptospirosis ranges from mild nonspecific febrile illnesses to severe or fatal renal, hepatic, or meningeal disease.[16,17] Leptospires enter the blood stream through imperfections in the skin, through mucous membranes, or by ingestion of contaminated water. Urine of infected animals and humans may contain 10^6 to 10^8 microorganisms/mL and leptospires may be shed into the environment up to 3 months after clinical recovery from disease.

Diagnosis of disease in animals and humans usually is based upon serology, darkfield examination of urine sediments, examination of histopathological stains, or culture of the organism from urine or tissues. Recently, polymerase chain reaction (PCR) methods have been introduced for diagnosis and typing of leptospires.[18,19] PCR also has been used to differentiate pathogenic from nonpathogenic leptospires.[20–23] PCR methods are insensitive for detection of leptospires in environmental samples and they suffer from the inability to differentiate between living and dead organisms. Because serovar information has epidemiological and public health significance, culture and serotyping are currently preferred to molecular detection methods.[2]

While leptospirosis remains relatively common in tropical regions of the world, only 40 to 120 cases/year have been reported in the United States over the past 30 years. Leptospirosis was dropped from the list of notifiable diseases in 1995. Only one outbreak has been reported in the continental United States since 1995.[8]

Leptospires are recovered from environmental sources with great difficulty.[24–27] Because both saprophytic and pathogenic strains of leptospires may be recovered from environmental samples, their presence has no public health significance apart from an epidemiological context.

1. Sample Collection

Collect water samples of 100 mL to 1 L in sterile containers. Transport samples to the laboratory at ambient temperature within 72 h of collection. Multiple samples from each sample site usually are required for successful isolation because finding leptospires in 10 to 20% of samples of surface waters receiving farm runoff is considered a high yield. Leptospires find their ecological niche at the interface between sediment and shallow water. Gently agitate the water to bring some of the sediment to the surface of shallow bodies of water to improve the probability of recovering organisms.[28]

2. Sample Processing

Centrifuge a portion of a water sample at 5000 × *g* for 10 min. Examine sediment for leptospires by darkfield microscopy; skill and experience are required to differentiate artifacts from leptospires. Their presence indicates that conditions are favorable for leptospire survival, but does not differentiate saprophytic from pathogenic forms. In the laboratory, thoroughly mix soil samples with three volumes of sterile deionized water and let coarse particulate material settle by gravity. Process remaining suspension as a water sample. *Leptospira* can pass through 0.22-μm membrane filters (¶ *a* below); this ability has been exploited to separate them from other bacteria in environmental samples and in mixed cultures. Similarly, guinea pig inoculation (¶ *b* below) has been used as a biological filter for isolation of leptospires from contaminated samples.

a. Filtration method: Filter surface water samples through filter paper* to remove coarse debris before membrane filtration. Occasionally, samples may have to be passed through a series of prefilters of decreasing pore sizes (8-μm, 4-μm, 1-μm, 0.65-μm, and 0.45-μm) to prevent clogging of the final 0.22-μm filter.

b. Animal inoculation method: Filter water through a 0.45-μm membrane filter and inoculate 1 to 3 mL intraperitoneally into weanling guinea pigs. After 3 to 6 d, inject a small amount of sterile saline and withdraw fluid for darkfield examination. If leptospires are seen, perform a cardiac puncture to obtain blood for inoculation of culture media. If no leptospira are seen by darkfield examination, record rectal temperatures daily until a fever spike indicates infection, then repeat the darkfield examination of peritoneal fluid for leptospires. Exsanguinate guinea pigs at 4 weeks and save serum for serological tests. Culture blood, kidney, and brain of guinea pigs with serological evidence of infection. Details of the method are described elsewhere.[29]

3. Culture

Cultures of environmental samples usually will be contaminated with other bacteria unless the samples are filtered through a 0.22-μm membrane filter before inoculation. Filtration also may be used to isolate leptospires from mixed cultures by direct filtration or another method.[30] Unless sample filtration is used with selective media or animal inoculation, a culture contamination rate of 60 to 80% is not uncommon. The amount of sample cultured will depend on the amount of particulate material in the sample. Generally, culture sample volumes from a few drops to 3.5 mL.

a. Culture media: Pathogenic leptospires have been cultured in liquid, semisolid, and solid media, but not all pathogenic strains will grow on solid media. Solid media have been used to purify mixed cultures and to detect hemolysin production.[31,32] Optimal pH of culture media is 7.2 to 7.4 and optimal incubation temperature is 30°C. Keep glassware free of detergent residues because leptospires are sensitive to detergents [see Section 9020B.5*a*2)]. When using serum enrichments in culture media, use serum free of antibody to leptospires. Bovine serum albumin shows manufacturer and lot variations; test new batches for their ability to support growth of leptospires.

Modifications of the Ellinghausen-McMullough formulation (EMJH) that incorporate bovine serum albumin fraction V and polysorbates are used as serum replacements.[33–36] EMJH base is available commercially. Neomycin is used in culture media at concentrations between 5 and 25 μg/mL to inhibit competing microflora, but it may be toxic to some strains.[37] 5-Fluorouracil is used at 100 or 200 μg/mL in culture media, but it is toxic also for some strains, particularly at concentrations above 100 μg/mL.[38]

b. Culture methods:

1) Direct culture method—To recover leptospires from surface waters, place a few drops of water in EMJH liquid medium and incubate overnight at 30°C. Filter inoculated medium through a 0.22-μm membrane filter into a sterile tube and reincubate at 30°C for up to 6 weeks.

2) Dilution method—When samples may contain reasonable numbers of organisms in the presence of inhibitors or competing microflora, prepare 10-fold dilutions in duplicate and inoculate 0.1 mL undiluted sample and each dilution into EMHJ medium. One tube of each pair may be made selective by addition of a single 30-μg neomycin antimicrobial susceptibility disk to the media before incubation. Incubate cultures at 20 to 30°C for up to 4 months.

3) Animal inoculation method—Add 1 to 2 drops of heart blood from infected guinea pigs to each of three to five tubes of EMJH medium. Incubate cultures at 20°C for up to 4 months.

c. Culture examination: Leptospires usually are detected in cultures of environmental samples within 7 to 14 d; however, incubate and examine cultures weekly for up to 4 months before discarding them as negative. Observe tubes for a lightly turbid ring of growth just below the surface of the medium. This band of maximum turbidity at the zone of optimal oxygen tension is referred to as Dinger's ring. Remove a drop of the culture weekly for darkfield examination and prepare subcultures if motile leptospires are observed. Generally, saprophytic leptospires grow at lower temperatures, and form rings closer to the surface of culture media than pathogenic serovars. Cultures remain viable in semisolid media for at least 8 weeks at room temperature. Cultures may be maintained by repeated subculture, by lyophilization, or by freezing at –70°C.[29]

4. Identification

The biochemical tests previously thought to differentiate between pathogenic and saprophytic serovars do not reliably predict pathogenicity of leptospires, and they are not recommended. *Leptospira* are identified to serogroup by the microscopic agglutination test using reference antisera. Identification to serovar requires use of adsorbed antisera that are available only in reference laboratories. More than 200 serotypes of *Leptospira* are known.

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9260 J. *Legionella*

Legionella were first isolated and identified as part of the investigation of respiratory illness in persons attending an American Legion convention in Philadelphia in 1976.[1,2] This highly publicized investigation documented 239 cases and 34 deaths due to a previously unrecognized cause of pneumonia. It was later shown that this disease occurs when sufficient numbers of legionellae are aerosolized from colonized water sources and subsequently inhaled by a susceptible host.[3] The bacteria are associated with two forms of respiratory illness, collectively referred to as legionellosis.[4,5] Legionnaires' disease is the pneumonic and more severe form of legionellosis. The other form of respiratory illness is named Pontiac fever after the first documented outbreak, which occurred at a health department in Pontiac, Michigan.[4] Pontiac fever is a less severe, self-limited illness. Possible explanations for the manifestation of these two disease syndromes caused by the same bacteria include the inability of some legionellae to multiply in human tissue (for a variety of reasons, including virulence, host range, or viability of the bacteria) and differences in host susceptibility.[6–8] Community-based pneumonia incidence studies have estimated that there are between 8000 and 18 000 cases of legionellosis annually in the United States, approximately 25-fold higher than the number of cases annually reported to the Centers for Disease Control and Prevention.[9] The majority of cases of legionellosis are sporadic, with only about 4% outbreak-related.[9] The sources of community-acquired cases are difficult to identify, partly because of the ubiquitous nature of the bacterium. Although the organisms are relatively common in the environment, they cause disease infrequently.[10] It is generally accepted that in order to cause disease legionellae must, first, be present in an environmental reservoir, then amplify from low to high concentrations, and be disseminated to susceptible hosts.[11] This model explains the epidemiology of legionellosis as it is used to develop prevention strategies.

Bacteria of the genus *Legionella* are Gram-negative, aerobic, rod-shaped bacteria. Cells are 0.3 to 0.9 by 1 to 20 μm and motile, with one or more polar or lateral flagella.[12] Legionellae use amino acids as their carbon and energy sources and do not oxidize or ferment carbohydrates. Currently, there are 49 species comprising 71 distinct serogroups in the genus *Legionella.*[13–15] Species identification and differentiation are performed serologically, although antisera for many species and serogroups are not available commercially.[12] A single species of *Legionella*, *L. pneumophila*, causes approximately 90% of all documented cases of legionellosis.[16] Although there are now 15 serogroups of *L. pneumophila*, 82% of all legionellosis cases are caused by *L. pneumophila* serogroup 1. Approximately half of the species of Legionellae have been associated with human disease. It is likely that most of the legionellae can cause human disease under the appropriate conditions; however, these infections are infrequently reported because they are rare and there is a lack of diagnostic reagents. Some unidentified legionellae cannot be grown on routine *Legionella* media; these organisms have been given the acronym LLAPs (*Legionella*-like amoebal pathogens) because they have been detected through their ability to grow intracellularly in protozoan cells.[17]

Water is the major reservoir for legionellae, and the bacteria are found in freshwater environments worldwide.[18] Legionellae have been detected in as many as 40% of freshwater environments by culture and in up to 80% of freshwater sites tested by polymerase chain reaction (PCR).[14] Several outbreaks of legionellosis have been associated with construction, and it was originally believed that the bacteria could survive and be transmitted to humans via soil. However, legionellae do not survive in dry environments and these outbreaks are more likely the result of massive descalement of plumbing systems due to changes in water pressure during construction.[19,20]

Initially, it was difficult to explain the pervasiveness of legionellae in aquatic environments because these bacteria are fastidious and require an unusual combination of nutrients in bacteriologic medium. These levels of nutrients would rarely be found in aquatic environments and, if present, would serve only to amplify faster growing bacteria that would compete with the legionellae. However, the nutrients required by legionellae represent the need for an intracellular environment, not soluble nutrients commonly found in fresh water. Legionellae survive in aquatic and, possibly, in some soil environments as intracellular parasites of free-living protozoa.[21,22] They infect the protozoa by use of a novel IV secretion system and use the same mechanism to infect and multiply within human macrophages.[23] Protozoa play a crucial role in the ecology of legionellae and this interaction is key to the development of successful prevention strategies. To understand the ecology of legionellae, these bacteria must be considered in the context of their microbial community, not as independent inhabitants of freshwater environments.

Inhalation of legionellae in aerosolized droplets is the primary means of transmission for legionellosis.[3] These aerosolized droplets must be of a respirable size (1 to 5 μm). No person-to-person transmission of Legionnaires' disease has been documented. A number of devices have been implicated as sources of aerosol transmission of legionellae.

These sources are of two general types: those producing aerosols of contaminated potable water, such as showers, faucets, decorative fountains, ultrasonic mist machines, humidifiers, and respiratory therapy equipment, and those producing aerosols of nonpotable water, such as cooling towers, evaporative condensers, hot tubs, and whirlpool spas. Meaningful identification of sources of transmission requires a multidisciplinary approach including epidemiology, molecular epidemiology, and microbiologic techniques including water and, rarely, air sampling.[3]

Detection of legionellae in an environmental source is not necessarily evidence of the potential for disease. As previously stated, legionellae are ubiquitous and could be isolated from 60% of buildings tested in a recent study.[24] The relationship between the presence of the bacterium in the environment and frequency of resulting disease remains poorly defined. Monitoring of building water systems is warranted to identify the source of an outbreak of legionellosis or to evaluate the efficacy of biocides or prevention measures. Monitoring may be warranted in special settings where people are highly susceptible to illness due to *Legionella* infection, such as an organ transplant ward within a hospital.[25]

1. Collection of Samples

Determine on an individual basis the number and types of sites to be tested to detect legionellae. A published sampling protocol[26] can serve as a prototype for identifying sites to be sampled. Generally, consider any water source that may be aerosolized a potential source for the transmission of legionellae. The bacteria are rarely found in municipal water supplies and tend to colonize plumbing systems and point-of-use devices. To colonize a system, the bacteria must multiply, and this requires temperatures above 25°C.[12] Therefore, legionellae are most commonly found in hot-water systems. These bacteria do not survive drying, and so condensate from air-conditioning equipment, which frequently evaporates, is not a likely source.[19]

When sampling for legionellae collect both water samples and swabs of point-of-use devices or system surfaces.[27] Collection of at least 1 L of water allows concentration of the sample if necessary. If the water source has recently been treated with chlorine or bromine, 1 mL of 10% sodium thiosulfate may be added to each 1-L sample to neutralize the disinfectant.

Swabs allow sampling of biofilms, which frequently contain legionellae. These can be taken from various points within plumbing systems or from surfaces of basins of cooling towers or spas. Take swabs of faucet aerators and showerheads in conjunction with water samples from these sites, with the aerator or showerhead removed. The swabs can be streaked directly onto an agar plate or submerged in a small volume of water taken at the same time to prevent drying during transportation to the laboratory.

Transport all samples at ambient temperature to the laboratory in insulated coolers as protection against extreme heat or cold. Refrigerate samples that will not be processed within 24 to 48 h from the time of collection.

2. Pretreatment of Water Samples

The method selected for processing water samples depends on the expected degree of total bacterial contamination in a particular sample. Potable waters generally have low bacterial concentrations and are either cultured directly or concentrated to detect legionellae. Nonpotable waters, such as those from cooling towers, generally do not require concentration because of their high bacterial concentrations.

Samples may be concentrated 10-fold or more by using either filtration or centrifugation. Filtration is used more frequently, although either procedure can be used successfully.[27,28] Filter concentrate water in a biological safety cabinet using 0.2-μm-pore-size polycarbonate filters. Polycarbonate membranes allow suspended particles to collect on the filter surface without being trapped as they are in matrix-type filters. Resuspend the filter membrane into a volume of the sterile water and vortex for 30 s. Concentrate samples by centrifugation at 1000 $\times$ *g* for 10 min, removing all but 10 mL of the supernatant, and vortex.[28]

Use a selective procedure to reduce the number of non-*Legionella* bacteria before culturing some water samples with high total bacterial concentrations. Non-legionellae bacteria can be killed selectively by either acid pretreatment or brief exposure to higher temperatures.[29,30] Legionellae are more resistant to lower pH and brief exposures to higher temperatures than many other freshwater bacteria. For acid pretreatment, mix the sample and incubate with an acid buffer (pH 2.2) for 3 to 30 min.[29] The sample is neutralized by the buffer within buffered charcoal yeast extract (BCYE) agar and therefore must be spread on the agar plate at the end of the period of incubation with the acid buffer. For heat pretreatment, incubate 10 mL sample in a 50°C water bath for 30 min.[30]

If amoebae are present, intracellular legionellae numbers can be increased by "heat enrichment" or incubation of specimens at 35°C. This can improve recovery of legionellae by up to 30%.[31] However, this procedure requires a considerable length of time before results can be obtained and may not be practical in many situations. Heat enrichment relies on autochthonous protozoa to amplify undetectable levels of legionellae. Portions of samples are incubated at 35°C and cultured after 2 to 6 weeks.

3. Culture Media

The medium currently used for the culture of legionellae is buffered charcoal yeast extract (BCYE) agar;[32–34] its most widely used form is supplemented with alpha-ketoglutarate.[33,34] Table 9260:V lists the primary components of BCYE agar and the supplements added for various purposes.[32,35–37]

Culture of environmental samples requires the use of selective and nonselective media in conjunction with the previously described selection procedures. Most laboratories use multiple plates for each sample, including a BCYE agar plate, a BCYE agar plate containing three antimicrobial agents, and a BCYE agar plate containing the three antimicrobial agents plus glycine (Table 9260:V). These media can be prepared with indicator dyes, which impart a color specific for certain species of *Legionella.*[36] Although the majority of *Legionella* spp. grow readily on BCYE agar, some require supplementation with bovine serum albumin to enhance growth. *L. micdadei* and several strains of *Legionella bozemanii* show a preference for BCYE with 1.0% albumin.[35] Inoculate all agar plates with 0.1 mL of sample by the spread plate technique and incubate at 35°C in a humidified 2.5% CO_2 atmosphere or candle extinction jar.

4. Identification of Legionellae Colonies

Colonies of legionellae require approximately 72 h to appear on BCYE agar and may require 7 d or longer. Ideally, examine plates after 4 d incubation and again before discarding them after 7 to 10 d incubation. Examine plates with a dissecting microscope and a light source to detect bacterial colonies resembling legionellae. After approximately 4 d of incubation, these colonies are 2 to 4 mm in diameter, convex, and round with entire edges. The center of the colony is usually a bright white with a textured appearance that has been described as "cut-glass like" or speckled. The white center of the colony is often bordered with blue, purple, green, or red iridescence. Some species of legionellae produce colonies that exhibit blue-white or red autofluorescence.[30] The primary isolation plates can be examined with long-wave UV light to detect these autofluorescent colonies.

Colonies resembling legionellae can be presumptively identified on the basis of their requirement for L-cysteine by subculture on blood agar or BCYE agar without L-cysteine. Subcultured colonies that grow on BCYE agar, but not on blood agar or BCYE without L-cysteine, are presumed to be legionellae. Le-

TABLE 9260:V. COMPONENTS AND SUPPLEMENTS OF BCYE AGAR FOR CULTURING LEGIONELLAE FROM THE ENVIRONMENT

Component	Concentration	Purpose
Charcoal	2.0 g/L	Base component
Yeast extract	10.0 g/L	Base component
ACES* buffer	10.0 g/L	Base component
Ferric pyrophosphate	0.25 g/L	Base component
L-cysteine	0.4 g/L	Base component
Potassium α-ketoglutarate	1.0 g/L	Base component
Agar	17.0 g/L	Base component
Glycine	3.0 g/L	Selective agent
Polymyxin B	50-100 U/mL	Selective agent (Gram negative)
Vancomycin or cefamandole	1-5 mg or 4 mg/L	Selective agent (Gram positive)
Anisomycin or cycloheximide	80 μg/mL (for either)	Selective agent (fungal)
Bromocresol blue	10 mg/L	Indicator dye
Bromocresol purple	10 mg/L	Indicator dye
Bovine serum albumin	10 g/L	Supplement for some fasitidious Legionellae

* N-(2-Acetamido)-2-aminothanesulfonic acid.

Source: FIELDS, B. 2002. Legionellae and Legionnaires' disease. *In* C.L. Hurst, R.L. Crawford, G.R. Knudsen, M.J. McInerney & L.D. Stetzenbach, eds. Manual of Environmental Microbiology, 2nd ed. American Soc. Microbiology, ASM Press, Washington, D.C.

gionellae are relatively inert in many biochemical test media, so these tests are of limited value in their identification. Definitive identification is usually made by using a direct fluorescent antibody (DFA) or slide agglutination test with specific antisera.[27] Fatty acid analysis and DNA hybridization[12] are other identification techniques.

5. Nonculture Methods for Detection of Legionellae

Several nonculture methods have been developed to detect legionellae in environmental samples and offer the potential of greatly increased sensitivity. These nonculture methods include detection of the organisms with specific antisera by DFA staining and procedures to detect nucleic acids of legionellae using polymerase chain reaction (PCR). However, culture remains the method of choice for detecting legionellae, primarily because nonculture methods cannot provide information regarding the viability of the bacteria.

The use of DFA to detect legionellae is limited by the number of specific antisera that can be used. Because no antisera specifically react with all *Legionella* species, a different antiserum must be used for each species or serogroup. Reports on the sensitivity and specificity of DFA testing of environmental specimens vary greatly, with most studies indicating that the test is relatively insensitive and nonspecific.[38]

The use of PCR for detecting nucleic acids of legionellae in the environment has proved to be valuable in some investigations of outbreaks of legionellosis.[7] A number of *Legionella* genes, including 5S rRNA, 16S rRNA, and *mip* genes, have been used as targets for PCR.[39,40] Use of PCR to detect legionellae in the environment has indicated that up to 80% of fresh waters are positive while only 20 to 40% are positive by culture.[41,42] This discrepancy could be due to the presence of nonviable or injured organisms, nonspecific reactions with unrelated organisms, or the presence of related organisms, such as *Legionella*–like amoebal pathogens, that cannot be detected by conventional techniques used for legionellae.

Most investigations of epidemic legionellosis have used culture to detect legionellae in the environment; thus, most epidemiologically relevant information about legionellosis is based on direct culture data. Interpret results from non-culture-based methods cautiously.

6. Subtyping Techniques

Molecular subtyping procedures, as well as epidemiologic evidence, are required to associate an environmental isolate of *Legionella* with a clinical isolate from a patient. *L. pneumophila* serogroup 1 (Lp1), which accounts for most cases of legionellosis, can be divided into a number of subtypes, indicating that this is a fairly heterogeneous serogroup.[43] Identification of the bacterium, even to the serogroup level, is not sufficient to implicate an environmental isolate as the source of disease.

The variety of strains and distribution of Lp1 necessitate more elaborate subtyping procedures to discriminate within these bacteria. Several groups of monoclonal antibodies have been developed for this purpose.[44] An international panel of seven monoclonal antibodies was proposed in 1986;[44,45] use of these monoclonal antibodies has identified 10 type strains within Lp1. Although much information has been gained through the use of this panel, several of the cell lines have been lost and most of these reagents are no longer available.

DNA fingerprinting techniques provide a level of discrimination similar to the use of monoclonal antibodies; these techniques are complementary.[43] Techniques used to discriminate between isolates of legionellae include restriction fragment length polymorphism analysis, plasmid analyses, electrophoretic alloenzyme typing, RNA/DNA probing of DNA digests, pulsed-field gel electrophoresis, and arbitrarily primed PCR.[43,46,47] Amplified fragment length polymorphism (AFLP) has been used since the late 1990s.[48,49] A study using a standardized protocol demonstrated that the AFLP was highly reproducible and epidemiologically concordant with good discrimination. The method has been adopted as the first standardized typing method for the

investigation of travel-associated Legionnaires' disease in Europe.[50] Currently a number of laboratories are investigating the use of multi-locus sequence typing (MLST) as the next generation typing method for legionellae.[51] This method involves comparison of nucleic acid sequence data for a limited number of well-characterized bacterial genes. Given the advances in nucleic acid sequence technology and bioinformatics, it would appear that sequence-based typing systems eventually will replace other molecular typing methods.

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9260 K. *Yersinia enterocolitica*

The genus *Yersinia* comprises Gram-negative coccobacilli, of which three species—*Y. pestis* (the plague bacillus), *Y. pseudotuberculosis*, and *Y. enterocolitica*—are well-known human pathogens. However, not all strains of *Y. enterocolitica* are capable of causing human intestinal infections. Primarily on the basis of biochemical reactions, *Y. enterocolitica* has been classified[1] into six biogroups that have distinct patterns of serogroup designations, human pathogenic potential, and ecologic and geographic distribution (Table 9260:VI). Isolates belonging to biogroup 1A (positive for salicin fermentation, esculin-hydrolysis, and pyrazinamidase production) are thought to be incapable of causing human intestinal infections, while isolates negative for these traits are more likely to cause intestinal infections, which include enterocolitis, mesenteric adenitis, or terminal ileitis.

Yersinia enterocolitica is a Gram-negative bacterium that can cause acute intestinal infection and can be found in cold or temperate U.S. climates.[2,3] It is widespread in nature and occurs in the gastrointestinal tract of numerous animal hosts, including mammals associated with aquatic habitats, avian species, and cold-blooded species.[4] From these reservoirs, terrestrial and aquatic ecosystems may become contaminated and human infections may ensue. Some animal hosts (e.g., domestic dogs and cats and farm animals) carrying pathogenic *Y. enterocolitica* are likely to come in contact with humans either directly or via ingestion (e.g., pork).[5,6]

Y. enterocolitica can grow at temperatures as low as 4°C with a generation time of 3.5 to 4.5 h if at least trace amounts of organic nitrogen are present.[3] Most environmental strains of *Y. enterocolitica* and the closely related species—*Y. kristensenii, Y. frederiksenii,* and *Y. intermedia*—lack the virulence factors to cause intestinal infections, but they cause, or are associated with, extra-intestinal infections, most frequently soft-tissue infections. Disease outbreaks associated with *Y. enterocolitica* have been associated with environmental sources.[7–9] Some strains lacking

TABLE 9260:VI. ASSOCIATION OF *YERSINIA ENTEROCOLITICA* WITH BIOGROUP, SEROGROUP, ECOLOGIC, AND GEOGRAPHIC DISTRIBUTION

Probable Cause of Intestinal Human Infection	Biogroup	Serogroup(s)	Ecologic/Geographic
Yes	1B	O8; O4; O13a,13b; O18; O20; O21	Environment, pig (O8) United States, Japan, Europe, The Netherlands (O8-like)
Yes	2	O9; O5, 27	Pig, Europe (O9), United States (O5, 27), Japan (O5, 27), Sweden, The Netherlands
Yes	3	O1, 2, 3; O5, 27	Chinchilla (O1, 2, 3), Pig (O5, 27)
Yes	4	O3	Pig, Europe, United States, Japan, South Africa, Scandinavia, Canada, The Netherlands
Yes	5	O2, 3	Hare, Europe
No*	1A	O5; O6, 30; O7, 8; O18, O46, nontypable	Environment, pig, food, water, animal and human feces, global

* May cause extra-intestinal infections

SOURCE: WAUTERS, G., K. KANDOLO & M. JANSSENS. 1987. Revised biogrouping schema of *Yersinia enterocolitica. Contrib. Microbiol. Immunol.* 9:14.

classic virulence markers also may be associated with disease.[7–10]

Y. enterocolitica has become recognized worldwide as an important human pathogen. In several countries, it is nearly as common as *Salmonella* and *Campylobacter* as a leading cause of acute or chronic intestinal infection.[11] *Y. enterocolitica* usually is associated with sporadic cases of intestinal infection in the United States. Epidemiologic investigations suggest that the predominant pathogenic serotype isolated in the United States has been changing.[10,12] *Y. enterocolitica* serogroup O3 has replaced O8 as the most common serogroup recovered from intestinal and systemic human infections, reflecting the same pattern seen in other parts of the world.[10,11] The emergence of serogroup O3 *Y. enterocolitica* infection was first noted in New York in 1983[12] and numbers of cases have increased steadily,[6,10] while serogroup O8 has become rare in the United States.

Yersinia strains have been isolated from untreated surface and ground waters in the Pacific Northwest, New York, and other regions of North America, with the highest isolations occurring during the colder months.[13–16] Concentrations have ranged from 3 to 7900 CFU/100 mL; the tests did not discriminate between pathogenic and nonpathogenic strains. Neither enteropathogenic or nonenteropathogenic *Yersinia* strains correlate with levels of total and fecal coliforms or total plate count bacteria.[15] There is also little information on *Yersinia* survival in natural waters and water treatment processes. Two incidents of waterborne gastroenteritis[3,13] and septicemia[2] caused by *Yersinia* have been documented.

In studies of *Y. enterocolitica* in chlorinated–dechlorinated secondary effluent and receiving (river) water, the organism was isolated in 27% of effluent samples, 9% of upstream samples, and 36% of downstream samples.[17] However, determination of biogroup status, and hence virulence potential of the isolates, were not assessed. Mean total and thermotolerant coliform reductions in effluent chlorination were 99.93 and 99.95%, respectively. In a survey of untreated and treated (chlorination or filtration plus chlorination) drinking water supplies, *Y. enterocolitica* was found in 14.0 and 5.7% of the samples, respectively.[15] Further, of all the water samples containing <2.2 coliforms/100 mL, 15.9% were *Yersinia*-positive. *Y. enterocolitica* isolation did not correlate with the presence of total or thermotolerant coliforms in this study. Another study also confirmed that *E. coli* is not a good indicator for the presence of *Yersinia* in water and that *Y. enterocolitica* O3 strains harboring a virulence plasmid have enhanced resistance to chlorine compared to nonvirulent strains.[18]

Yersinia could be an important drinking water pathogen because of its widespread occurrence, its persistence in natural and treated waters (at least in some geographic areas), the existence of animal reservoirs, the evidence for possible waterborne outbreaks, and the lack of definitive data on its reduction via treatment processes.

1. Enrichment and Isolation with Selective Media

Yersinia-selective agar, first proposed in 1979,[19] is the medium of choice for the isolation of *Y. enterocolitica* and other *Yersinia* species. The powdered medium, known as "*Yersinia* selective agar base" or "Cefsulodin Irgasan® Novobiocin (CIN) agar base," contains three agents (sodium desoxycholate, crystal violet, and triclosan) that inhibit Gram-positive bacteria and many Gram-negative bacteria. Because they are heat-sensitive, two other selective agents (cefsulodin and novobiocin) have been formulated separately as a supplement to be added to the prepared agar base. These agents inhibit most other *Enterobacteriaceae* and other enteric bacteria. Most *Yersinia* strains are resistant to the five inhibitory ingredients and will grow on the agar; most enteric bacteria are inhibited, with the exception of some strains of *Aeromonas*, *Citrobacter*, *Serratia*, and *Enterobacter*. These other organisms are easily differentiated by screening tests, biochemical reaction, serological screening, or other methods (see Table 9260:I). The agar also contains D-mannitol as the differential test; most strains of *Yersinia* ferment it rapidly and turn pink-red in the presence of the medium's pH indicator neutral red.

a. Double-strength Yersinia-*selective broth:* Reconstitute powdered *Yersinia* selective agar base* as described by the manufacturer, but use 500 mL rather than 1 L water. Let the insoluble agar settle. Pour off the clear liquid. If desired, filter the liquid through a coarse filter paper to remove any remaining agar particles; however, these should not be detrimental to most enrichment procedures. Autoclave and cool to room temperature. Add one vial *Yersinia* antimicrobic supplement.*

* Difco, or equivalent.

TABLE 9260:VII. DEFINITION OF THE SIX BIOGROUPS OF *YERSINIA ENTEROCOLITICA* BASED ON REACTIONS AT 25°C

Test	Result for Biogroup*					
	1A	1B	2	3	4	5
Pyrazinamidase (48 h)	+	−	−	−	−	−
Salicin fermentation (24 h)	+	−	−	−	−	−
Esculin hydrolysis (24 h)	+	−	−	−	−	−
Tween 80 esterase (lipase)	+	+	−	−	−	−
Indole production (48 h)	+	+	(+)	−	−	−
D-Xylose fermentation	+	+	+	+	−	−†
Ornithine decarboxylase	+	+	+	+	+	−
Voges-Proskauer	+	+	+	+	+	−
Nitrate reduction to nitrite	+	+	+	+	+	−
D-Sorbitol fermentation	+	+	+	+	+	−
D-Sorbose fermentation	+	+	+	+	+	−
Sucrose fermentation	+	+	+	+	+	−
Trehalose fermentation	+	+	+	+	+	−
DNA'ase	−	−	−	−	+	+

Composite reactions[23] from the biotyping schemas proposed by Niléhn,[22] Wauters,[1] and the subsequent revision by Wauters.[1] Variable reactions are excluded in the operational definitions of the biogroups. The length of incubation period is specified if it is critical. The incubation time for a final reaction has varied depending on the particular laboratory and test.

* + = positive; (+) = weak positive; − = negative

† Some positive strains occur.

b. Enrichment procedure: Combine equal volumes of water sample and double-strength broth and incubate at 36 or 25°C. Subculture the enrichment at 24 and 48 h to agar (¶ *c* below). Consider subculturing the enrichment to other plating media, such as MacConkey agar, Congo red-magnesium oxalate (CR-MOX) agar (to determine pathogenic serotypes of *Y. enterocolitica*), and MacConkey agar base with added D-xylose to recognize the xylose-negative *Y. enterocolitica* O3, which is the most common pathogenic serotype in human infection.[20]

c. Growth on Yersinia-*selective agar:* Prepare agar, including supplement, according to manufacturer's directions, or purchase in disposable Petri dishes. This medium can be inoculated with a small volume of a water sample, a membrane filter, an enrichment culture (see above) or a pure culture that is a "suspect" *Yersinia*. Incubation can be at 25 or 36°C; the latter will result in faster growth and larger colonies. At 24 h, cultures of *Y. enterocolitica* typically appear translucent or translucent with dark pink centers. At 48 h, they appear dark pink with a translucent border and may be surrounded by precipitated bile. Other *Yersinia* species grow well and in a similar manner.

2. Membrane Filter Method

A membrane filter method for enumerating and isolating *Yersinia enterocolitica* with mYE medium is available.[21] This method may be used for examining large volumes of low-turbidity water and for presumptively identifying the organism without transferring colonies to multiple confirmatory media.

Filter sample through a 0.45-μm membrane filter and place filter on a cellulose pad saturated with mYE recovery broth. Incubate for 48 h at 25°C. Aseptically transfer the membrane to a lysine-arginine agar substrate and incubate anaerobically at 35°C. After 1 h, puncture a hole in the membrane next to each yellow to yellow-orange colony with a needle, transfer the membrane to a urease-saturated absorbent pad, and incubate at 25°C for 5 to 10 min. Immediately count all distinctly green or deep bluish-purple colonies next to punctures. The green or bluish colonies are sorbitol-positive, lysine- and arginine-negative, and urease-positive. They may be presumptively identified as *Y. enterocolitica* or a closely related *Yersinia* species. Additional biochemical testing will be necessary to determine related species and biogroups (Table 9260:VII). Reasonably simple tests have been described to screen isolates for pathogenicity, and these tests correlate with the most common *Y. enterocolitica* serogroups.[1,22]

3. Identification

Strains of *Yersinia* are distinct from other enteric bacteria (see Table 9260:I), which makes it easy to identify a strain to the genus level (i.e., *Yersinia* species). Identification within the genus is more difficult, and commercial identification products may give incorrect identifications. Similarly, identification of *Y. enterocolitica* can be difficult because the other *Yersinia* species are so similar in their biochemical reactions. *Y. enterocolitica* O3, the most common and important serotype, does not ferment D-xylose, so this sugar is a useful screening test (see Table 9260:I). Cultures of *Yersinia* typically are more active biochemically at 25°C than 36°C; thus a test for definitive identification should be incubated at the lower temperature. Then compare the results to an identification chart that contains the reactions of all the species.[23]

4. Determination of the O antigen

Commercial antisera are available to serotype the most common and important pathogenic serotypes of *Y. enterocolitica;*†

† Denka Seiken, distributed by Oxoid, www.oxoid.com/us; Statens Serum Institut, www.ssi.dk; and others.

check for current availability. Sera for groups 1–6 of *Yersinia pseudotuberculosis* are also available.‡ Subculture colonies from plating media or enrichments, incubate, then test culture for agglutination in each antiserum. See manufacturer's instructions for details. See 9260B.8, E.7, and F.7 and 12 as a guide. Report the O antigen along with the species and biogroup (Table 9260:VI) (i.e., *Y. enterocolitica* O3, Biogroup 4). Refer cultures to a reference laboratory if they do not react in commercial sera but have properties of the enteric pathogens.

These commercial *Y. enterocolitica* antisera could also be used to coat magnetic particles, yielding a specific immunomagnetic separation reagent that would be useful in isolation. See 9260B.5 and F.4 and 11 as a guide.

5. Differentiation of Enteropathogenic Strains

Identify the culture to species, then use screening tests (see Table 9260:I), biotyping results (Table 9260:VII), and the culture's O antigen to determine whether it is likely to have the capacity to cause intestinal infections. In addition, if the culture produced tiny red colonies on CR-MOX agar (see Table 9260:I) it contains the *Yersinia* virulence plasmid and is probably an enteric pathogen. This plasmid is rapidly lost during enrichment and culturing.

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‡ Denka Seiken, or equivalent.

9260 L. *Aeromonas*

1. Introduction

Aeromonas spp. are natural inhabitants of aquatic environments worldwide. These Gram-negative, non-spore-forming facultatively anaerobic, glucose-fermenting organisms have been isolated from groundwater, treated drinking water, surface waters, wastewater, sludge, and sediment. Their populations are seasonal in all natural waters, with the highest numbers present in warmer months. Aeromonads cause serious diseases of aquatic animals and represent an economic threat to the aquaculture industry. Some species among the motile aeromonads have emerged as a potential microbial threat to human populations, especially the immunocompromised.[1]

As a result of recent taxonomic studies, *Aeromonas* bacteria have been removed from the family *Vibrionaceae* and established as a genus of the new family *Aeromonadaceae*. The genus *Aeromonas* comprises 22 phenospecies and 18 genomospecies, three of which are unnamed.[2] Phenotypic characterization of genomospecies has advanced via incorporation of nontraditional substrates into biochemical identification schema. Environmental microbiologists usually combine all motile, mesophilic aeromonads into the *Aeromonas hydrophila* complex, or simply report isolates as *A. hydrophila*. These practices obscure understanding of the medical and public health significance of aeromonads isolated from clinical specimens, environmental samples, and public water supplies; identification of *Aeromonas* isolates according to established taxonomic principles is preferable.[3] *A. hydrophila* (HG-1), *A. caviae* (HG-4), *A. veronii* (HG-8), *A. jandaei* (HG-9), *A. schubertii* (HG-12), and *A. trota* (HG-14) are most frequently associated with clinical specimens.[2]

Although no U.S. outbreaks of aeromonad-related gastroenteritis have been attributed to public drinking water supplies to date, this does not mean that no connection exists. The epidemiologic association between ingestion of untreated well water and subsequent *Aeromonas* gastrointestinal illness has been widely documented. Numerous cases and outbreak investigations of water- and food-transmitted illnesses associated with aeromonads have been reported.[4] Outbreaks of gastroenteritis associated with aeromonads have occurred in custodial care institutions, nursing homes, and day-care centers. *Aeromonas* contamination of drinking water has been associated with travelers' diarrhea.[5]

For many years, *Aeromonas* have been considered nuisance microorganisms by environmental microbiologists because they were reported to interfere with coliform multiple tube fermentation (MTF) methods. While aeromonads comprise 12% of bacteria isolated from drinking water by presence–absence methods, no data have demonstrated inhibition of coliform organisms by aeromonads in drinking water. Slight turbidity of lauryltyptose broth (LTB) tubes, with or without a small bubble of gas in the inverted tube, is suggestive of aeromonads. When the MTF method is used for drinking water samples, cultures producing turbidity at 35°C that remain clear at 44.5°C are suggestive of aeromonads. The presence of aeromonads can be verified by subculturing a loopful of turbid broth to a MacConkey plate and screening colorless colonies for gelatinase and oxidase production. No data are available to support invalidation of coliform MTF tests based on turbidity of tubes in the absence of gas production.

The ecology of mesophilic aeromonads in aquatic environments, including water treatment plants and distribution systems, has been reviewed.[6] The Netherlands and the Province of Quebec have established drinking water standards for *Aeromonas* at 20 CFU/100 mL for water leaving the treatment plant and 200 CFU/100 mL for distribution system water. Canada has established an *Aeromonas* Maximum Contamination Limit (MCL) of 0 (zero) for bottled water. A resuscitation method for recovery of aeromonads in bottled water has been published.[7]

The ability to isolate, enumerate, and identify aeromonads from water and wastewater sources is important because of their role in causing human and animal disease, their ability to colonize treatment plants and distribution systems, and their presence and distribution as alternative indicators of the trophic state of waters. The diversity of aeromonads in drinking water plants and distribution systems was shown by several investigators.[8–10]

Many media and methods have been proposed for the isolation and enumeration of aeromonads.[11,12] The methods presented below represent a compromise, because no single enrichment method, isolation medium, or enumeration method is capable of recovering all aeromonads present in a water sample. The methods were chosen on the basis of reproducibility of results, objectivity of interpretation, availability of materials, and specificity of the method for detecting aeromonads in the presence of other heterotrophic bacteria. Consult the literature for additional methods for use in special circumstances.[13]

2. Sample Collection

Collect water samples in sterile screw-capped glass or plastic bottles or plastic bags.* Sample volumes of 200 mL to 1 L are sufficient for most analyses. For chlorinated waters, add sodium thiosulfate (see Section 9060A.2). The potentially toxic effect of heavy metals is neutralized by adding EDTA (see Section 9060A.2).

Transport samples to the laboratory at 2 to 8°C within 8 h. Samples for presence–absence analyses may be transported at ambient temperatures within 24 h. Grab samples are most common. Moore swabs (see 9260B.2*a*) have been used for wastewater sampling, and Spira bottles have been used for tapwater sampling.[13] Both of these methods are used with enrichment in 1% alkaline peptone water (APW), pH 8.6.[13] Place sediment and sludge samples in bottles or bags and submit in same way as water samples.

3. Enrichment Methods

Do not use enrichment methods for ecological studies because the predominant strain(s) will overgrow other organisms. Reserve enrichments for presence–absence tests for aeromonads in drinking water, foods, stools, or for monitoring aeromonad populations in wastewater or marine environments, where organisms

* WhirlPak™, ZipLoc™, or equivalent.

may be present in low numbers or require resuscitation due to injury from exposure to inimical agents or hostile physical environments. For isolation of aeromonads from clear water samples, filter through 0.45-μm membrane filters, place filters in a bottle with 10 mL APW, incubate overnight at 35°C, and inoculate to plating media for isolation. Optimally, to sample clear water intended for drinking, filter a volume of water through a mini-capsule filter,† decant residual water from inlet, plug ends with sterile rubber stoppers, and fill filter with APW, pH 8.6, through syringe port. Incubate filter at 35°C for 6 h or overnight and streak loopfuls of broth onto selective and differential plating media.[13]

4. Enumeration Methods

a. Spread plates: Enumerate samples expected to contain predominantly aeromonads in high numbers (sludge, sediments, wastewater effluents, polluted surface waters, etc.) directly by spreading 0.1-mL portions of decimal dilutions on ampicillin dextrin agar (ADA) plates.[14–16] Incubate plates at 35°C overnight and count bright yellow colonies 1 to 1.5 mm in diameter. Presumptively identify colonies using the screening methods below.

b. Membrane filtration: Enumerate aeromonads in drinking water samples or other low-turbidity waters by using membrane filtration procedures with ADA medium[15] and incubating aerobically overnight at 35°C. Filter sample volumes equivalent to 1, 10, and 100 mL. To achieve a countable plate (1 to 30 colonies), prepare decimal dilutions when aeromonads are present in high numbers. Count bright yellow colonies, 1 to 1.5 mm in diameter, and pick to screening media.

A recent modification[17] of the membrane filter method incorporates vancomycin into ADA to inhibit *Bacillus* species, which produce yellow colonies that could have been counted as presumptive *Aeromonas* spp.[16–18] This method has been validated and used in surveys to characterize the presence of *Aeromonas* spp. in distribution system water in the United States.

c. Multiple-tube fermentation (MTF) tests: Multiple-tube fermentation tests using APW, pH 8.6, or trypticase soy broth (TSB) containing ampicillin at 30 μg/mL (TSB30) have been applied to foods; however, they have not been used for enumeration of aeromonads in water samples. Some aeromonads are sensitive to ampicillin and will not grow in TSB30 medium. ADA without agar has been used to enumerate aeromonads in drinking water.[8] Use MTF methods only for clean samples, such as groundwater or treated drinking water samples, because the effects of competing microflora present in surface waters on recovery of aeromonads in broth media has not been studied adequately. Similarly, the correlation between MTF population estimates and other enumeration methods has not been examined adequately for matrices other than foods.

5. Screening Tests

Pick 3 to 10 colonies resembling aeromonads on differential and selective plating media or membrane filters and stab-inoculate into deeps of Kaper's multi-test medium[18] or one tube each of triple sugar iron (TSI) agar and lysine iron agar (LIA). Incubate cultures at 30°C for 24 h. Perform a spot oxidase test on growth taken from the LIA slant. Do not test for oxidase on growth from TSI slants, MacConkey agar, or other selective or differential media, because acid production interferes with the oxidase reaction. Reactions of enteric bacteria on TSI and LIA media are shown in Table 9260:VIII. When Kaper's medium is used instead of TSI/LIA slants, colonies may be picked and inoculated onto sheep blood agar plates; incubate at 35°C overnight to provide growth for the oxidase test and to record hemolysin production. Cultures are identified presumptively using Kaper's medium according to the characteristics shown in Table 9260:IX. When using the membrane filter method with vancomycin, test presumptive yellow colonies for oxidase production, trehalose fermentation, and indole production according to the procedures described in the method.[17]

Table 9260:VIII Reactions of Enteric Bacteria on TSI and LIA Media

Organism	TSI Reactions*	LIA Reactions*
Shigella	K/A–	K/A–
Salmonella	K/Ag+	K/K+
Escherichia	A/Ag–	K/A–
Proteus	A/Ag+ or K/Ag+	R/A+
Citrobacter	A/Ag+	K/A+
Enterobacter	A/Ag–	K/A–
Aeromonas	A/A–	K/A–
Yersinia	A/A– or K/A–	K/A–
Klebsiella	A/Ag–	K/A–

* Fermentation reactions = slant/butt; H_2S production = + or −; K = alkaline, A = acid, R = red (deaminase reaction); g = gas produced.

If species identification is desirable, submit presumptively identified *Aeromonas* cultures to a reference laboratory. Cultures with potential public health or regulatory significance may be subtyped using various molecular methods to determine clonality for outbreak investigations and troubleshooting of treatment plant or distribution system problems.[19,20]

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† Gelman 12123, or equivalent.

TABLE 9260:IX. REACTIONS OF *AEROMONAS* AND ENTERIC BACTERIA ON KAPER'S MEDIUM

Organism	Fermentation Pattern*	Motility	H_2S	Indole
Aeromonas hydrophila	K/A	+	−	+
Klebsiella pneumoniae	A/A	−	−	−
Klebsiella oxytoca	A/A	−	−	+
Escherichia coli	K/K or K/A	+ or −	−	+
Salmonella spp.	K/K, K/A, A/K or A/A	+	+	−
Enterobacter spp.	K/K, K/N or N/N	+	−	−
Proteus spp.	R/K or R/A	+	+ or −	+
Yersinia enterocolitica	K/K, K/N or N/N	−	−	+ or −
Citrobacter spp.	K/K or K/A	+	+	−
Serratia spp.	K/K, K/N or N/N	+	−	−

* K = alkaline; A = acid; N = neutral; R = red (deamination reaction).

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9260 M. *Mycobacterium*

The genus *Mycobacterium* comprises more than 70 characterized species that are nonmotile non-spore-forming, aerobic, acid-fast bacilli measuring 0.2 to 0.6 × 1 to 10 μm. They are conveniently separated into slow-growing (>7 d for colony formation) and rapid-growing (<7 d for colony formation) species. Many species are capable of causing disease in humans.[1] The two most important pathogens in this group include *Mycobacterium tuberculosis* and *Mycobacterium leprae,* the causative agents of tuberculosis and Hansen's disease (leprosy), respectively. Recently there has been an increase in the incidence of disease caused by opportunistic mycobacteria (also called nontuberculosis mycobacteria), probably related to the increasing numbers of elderly[2] and immunocompromised patients.[3] In the genus *Mycobacterium*, the most important environmental opportunistic pathogens include *M. avium* and *M. intracellulare, M. kansasii, M. marinum, M. xenopi*, and *M. simiae*. A number of the rapidly growing mycobacterial species (e.g., *M. fortuitum, M. chelonae*, and *M. abscessus*) have been found in cases of nosocomial infections.[4] Some of the common hosts and environmental reservoirs of *Mycobacteria* are shown in Table 9260:X.

Because of the complex nature of the cell wall, which is rich in lipids and therefore has a hydrophobic surface, these microorganisms are impermeable and resistant to many common disinfectants. The ability of the cell walls to retain dyes (i.e., the acid-fast property) is due to their high lipid content. As a result of their hydrophobicity, mycobacteria are present in biofilms on surfaces, such as pipes, and are readily aerosolized from water.[5] Because of disinfectant (e.g., chlorine) resistance, several members of this genus have become important waterborne pathogens in the elderly[2] and immunocompromised population.[3] Their rel-

TABLE 9260:X. MYCOBACTERIA OF WATERBORNE OR UNKNOWN ORIGIN

Mycobacterium species	Environmental Contaminant	Reservoir
M. kansasii	Rarely	Water, swine, cattle
M. marinum	Rarely	Fish, water
M. simiae	No	Primates, possibly water
M. scrofulaceum	Possibly	Soil, water, foodstuffs
M. szulgai	No	Unknown
M. avium-intracellulare	Possibly	Soil, water, swine, cattle, birds
M. xenopi	Possibly	Water
M. ulcerans	No	Unknown
M. fortuitum	Yes	Soil, water, animals, marine life
M. chelonae	Yes	Soil, water, animals, marine life

ative slow growth is due, in part, to their impermeability. Some species, such as *M. avium-intracellulare*, require from 3 to 8 weeks to form colonies on culture media.

Because *Mycobacterium avium* and *Mycobacterium intracellulare* exhibit overlapping properties, speciation is extremely difficult. Because of their close relatedness, these two pathogens are grouped together and called the *M. avium* complex (MAC). Organisms from this group are ubiquitous in the environment and have been isolated from potable water systems,[5] including those in hospitals[4–6] as well as from soil and dairy products.[7] MAC causes a chronic pulmonary disease in immunocompetent hosts, including the elderly, that is clinically and pathologically indistinguishable from tuberculosis.[2,8] MAC also causes disseminated disease in immunocompromised hosts.[3] In children less than 5 years old, *M. avium* causes cervical lymphadenitis.[9] The primary routes of transmission are via ingestion and inhalation of mycobacterial-laden aerosols or dusts.

1. Sample Collection and Concentration

Mycobacteria typically constitute a minority of the microflora in waters and require sample concentration. Collect water samples in sterile 1-L polypropylene containers. For water samples containing disinfectants (e.g., chlorine) add 1 mL 10% (w/v) sodium thiosulfate solution per liter of water collected. Transport samples to laboratory immediately after collection. If samples cannot be analyzed immediately, store at 4°C and begin analysis within 24 h of sampling.

To sample biofilms, scrape material from a defined area of any surface (e.g., pipe or water meter) and suspend it in 1 to 10 mL sterile water. Because of the aggregation of mycobacterial cells due to hydrophobicity, disrupt biofilm suspensions by vortexing with or without 1-mm glass beads or by treatment with a sterilized tissue homogenizer.

2. Screening Water Samples by Direct Fluorescent Assay

Before committing the sample to lengthy culture incubation, survey for acid-fast bacteria by using a combination solution of auramine-rhodamine (A-R) fluorescent dye.* Auramine and rhodamine nonspecifically bind to mycolic acids and resist decolorization by acid alcohol.[10]

Filter a minimum of 500 mL finished water, or 100 mL source water (depending on turbidity), through a sterile 0.45-μm-porosity, 47-mm-diam black filter. Aseptically transfer filter to a sterile polypropylene 50-mL tube and add 5 mL of buffered dilution water. Resuspend organisms from filter by vortexing for 2 min. Aspirate suspension and aseptically transfer to a sterile 15-mL polypropylene centrifuge tube. Centrifuge suspension at 5000 × *g* for 10 min and discard all but about 0.5 mL of supernatant. Vortex to resuspend pellet. Transfer 100 mL of the concentrate to a clean glass slide and air-dry and heat-fix at 60 to 70°C for 2 h or overnight. Stain the smear with A-R (15 min), then decolorize with acid-alcohol for 2 to 3 min, and rinse with deionized water. Next, apply secondary potassium permanganate counterstain (no longer than 2 to 4 min), rinse, and let air-dry. Examine smear at 100 and 400× with a microscope fitted with a BG-12 or 5113 primary filter with an OG-1 barrier filter. Acid-fast organisms will stain yellow-orange on a black background. To confirm for acid-fastness, apply a traditional acid-fast stain (Ziehl-Nielsen with Kenyon modification) directly to the prepared smear following the A-R stain.

For wastewater or highly turbid source waters, collect a 10-mL subsample and transfer to a sterile polypropylene 15-mL tube. Centrifuge at 5000 × *g* for 10 min and discard all but about 0.5 mL of supernatant. Follow slide preparation procedure and staining as above.

3. Sample Decontamination and Culture Methods

Some mycobacteria form colonies on laboratory media only after 7 d incubation at 37°C. Therefore, eliminate from the sample naturally occurring microorganisms that can outcompete and overgrow the mycobacteria. Various isolation and identification methods have been described for the recovery of mycobacteria, especially in the hospital environment.[11–13] Selective decontamination of the sample concentrate is required for the selection of mycobacteria before culturing. In addition, the matrix may affect the success of the recovery of mycobacteria; for example, decontamination is not required for many drinking water samples, while decontamination usually is required for biofilm samples.[5] However, all decontamination methods also reduce the number and, hence, the recovery of mycobacteria.[14] Several methods are detailed below for recovering mycobacteria from water samples [¶s *a*1)–4) below] and from biofilms (¶ *b* below).

a. Methods for water: Determine which of the four methods below performs best with the matrix to be examined.

1) Centrifuge 500-mL water sample (5000 × *g* for 20 min), discard the supernatant, and suspend the pellet in 1 mL sterile water. For decontamination, add 1 mL 1*M* NaOH and immediately centrifuge (5000 × *g* for 20 min), discard supernatant, suspend pellet in 1 mL of phosphate-buffered saline (PBS) at pH 7.4, and spread 0.1-mL samples on Middlebrook 7H10 agar medium.† Use thick plates (30 to 35 mL per plate) and seal

* Catalog No. 40-090, Remel, Lenexa, KS, or equivalent.
† BBL Microbiology Systems, Cockeysville, MD, or equivalent.

plates with thermoplastic self-sealing film‡ to prevent drying of the medium during the long incubation (3 to 8 weeks). Incubate at any temperature between 15 and 45°C and examine plates for the appearance of small (0.2-mm-diam), transparent or yellow-pigmented colonies. The M7H10 agar medium allows detection of the small, transparent mycobacterial colonies. Although other microorganisms are killed, only 5% of mycobacteria survive sample decomtamination.[14] For gentler decontamination, suspend pellet in 0.005% (w/v) cetylpyridinium chloride (CPC) and leave at room temperature for 24 h.[15] Centrifuge (5000 × *g* for 20 min) to pellet the cells, suspend in 1 mL PBS and spread 0.1 mL on M7H10 agar medium.

2) Filter 500-mL water sample through a sterile 0.45-μm-porosity, 47-mm-diam filter. Aseptically transfer filter to a sterile polypropylene 50-mL tube. Add 5 mL sterile distilled water and resuspend organisms off the filter by shaking with two 5-mm glass beads for 1 h on a mechanical shaker.[11] Add a 3% sodium lauryl sulfate and 1% NaOH solution, then incubate on the bench for 10 min.[11,13] Pellet cells by centrifugation (5000 × *g* for 20 min), discard supernatant, suspend cells in 5 mL PBS to wash cells free of detergent and base, and pellet cells again. Suspend cells in 1 mL sterile distilled water and spread portions of this suspension onto M7H10 agar medium.

3) Filter 500-mL water sample through sterile 0.45-μm-porosity, 47-mm-diam filter. Aseptically transfer filter to a sterile polypropylene 50-mL tube. Add 5 mL sterile distilled water and resuspend organisms off the filter by shaking with glass beads for 5 min on a mechanical shaker. Add 10 mL 1*M* NaOH for 20 min followed by centrifugation at 5000 × *g* at 4°C for 20 min. Discard supernatant and add 5 mL 5% aqueous oxalic acid for 20 min. Re-centrifuge, discard supernatant, and add 30 mL sterile distilled water to neutralize. Centrifuge again and resuspend in 0.7 mL distilled water.[11] Spread 0.1 mL on M7H10 agar medium.

4) Add 20 mL 0.04% (w/v) cetylpridinium chloride (CPC) to 500-mL water sample and leave at room temperature for approximately 24 h. Filter sample and wash filter with 500 mL sterile water.[15] A study of decontamination methods for the isolation of mycobacteria from drinking water samples found a CPC concentration of 0.005% (w/v) to yield the highest isolation rate and lowest contamination rate for the water examined.[15]

b. Method for biofilms: Obtain a section of pipe or a water meter. Expose the pipe or meter surface and scrape a defined area (4 cm^2) with a sterile rubber policeman or spatula. Transfer the material to 1 to 10 mL sterile water. For a small-diameter pipe section, measure length and interior diameter of pipe and use a sterilized pipet brush to collect the biofilm. Disperse biofilm suspension by vortexing with 1-mm-diam glass beads or with a tissue homogenizer. Add CPC to a final concentration of 0.005% (w/v) and leave on bench for 24 h. Pellet the cells by centrifugation (5000 × *g* for 20 min), discard supernatant liquid, and suspend pellet in 1 mL water. Spread 0.1 mL on the surface of M7H10 agar medium and incubate at 15 to 45°C.

‡ Parafilm®, or equivalent.

4. Selective Growth

Culture all samples in duplicate. After sample decontamination, either spread 0.1-mL portions of the concentrates or use sterile forceps to place filters on selective media. For environmental samples, use M7H10 agar medium. Pour thick plates (30 to 35 mL/15 × 100 mm Petri dish) to prevent drying during the prolonged incubation. If fungal contamination is a problem, add 100 μg cycloheximide/mL or increase concentration of malachite green to 5 μg/mL. Although the common egg-based media (e.g., Lowenstein-Jensen medium) have been used successfully to isolate mycobacteria from patient samples, it is difficult to detect the small transparent mycobacterial colonies and obtain quantitative results on those media. Seal plates with plastic film§ or place plates in humid chambers or gas-permeable bags to prevent dehydration and incubate at 37°C. Additional plates also can be incubated at any temperature between 15 and 45°C in a humidified chamber to detect mycobacteria that grow optimally at lower or higher temperatures; for example, *M. avium* and *M. xenopi* grow at 45°C. Examine plates or slants periodically during a 3- to 8-week incubation period. Count suspect colonies (acid-fast coccobacilli), streak for isolation on M7H10 agar medium, and subculture to a tube of M7H9 broth.‖ After 5 d, remove subsamples and stain with Ziehl-Nielsen stain with Kenyon modification.

5. Identification

Identification of acid-fast isolates can be conducted by genetic or phenotypic tests.

a. Genetic tests: One genetic test involves PCR amplification of the mycobacterial *hsp-65* heat-shock protein gene followed by restriction endonuclease digestion (with either *Bst*EII or *Hae*III) and identification of species-specific fragments.[16,17] For a few mycobacteria (e.g., *M. avium* and *M. intracellulare*), DNA probe-based tests are available;# these are based on hybridization between 16S rRNA genes and species-specific oligonucleotide probes.

b. Phenotypic tests: These include cultural, biochemical, and enzymatic tests (Table 9260:XI), as well as identification of mycolic acid profiles by HPLC[18] cellular fatty acids by GC.[19] Although phenotypic tests have been the standard for species identification, there are several inherent problems in this approach. First, because initial identification of mycobacteria can take 3 to 8 weeks, observing biochemical changes entails additional time for the isolates (especially those of slowly growing mycobacteria) to metabolize specific substrates or to exhibit certain characteristics. Second, phenotypic traits subject to variation depend on the growth medium and prior growth conditions.

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§ Parafilm®, or equivalent.
‖ BBL Microbiology Systems, Cockeysville, MD, or equivalent.
Gen-Probe, Inc., San Diego, CA, or equivalent.

TABLE 9260:XI. PHENOTYPIC CHARACTERISTICS OF CLINICALLY SIGNIFICANT ENVIRONMENTAL MYCOBACTERIA*

Species	Growth Rate	Pigmentation	Urease	Nitrate Reduction	Hydrolysis of Polyoxyethylene Sorbitan Monooleate†
M. kansasii	S	P	±	+	+
M. marinum	S	P	+	−	+
M. simiae	S	P	±	−	−
M. scrofulaceum	S	S	±	−	−
M. szulgai	S	S/P	+	+	±
M. xenopi	S	S	−	−	−
M. avium-intracellulare	S	N	−	−	−
M. ulcerans	S	N	±	−	−
M. fortuitum	R	N	+	+	±
M. chelonae	R	N	+	−	±

* S = slow (3 to 8 weeks), R = rapid (7 d or longer), P = photochromogenic, S = scotochromogenic, N = nonphotochromogenic, S/P = scotochromogenic at 37°C and photochromogenic at 24°C.
† Tween 80®.

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9510 DETECTION OF ENTERIC VIRUSES*

9510 A. Introduction

1. Occurrence

Viruses in any animal's excreted feces or urine may pollute water, but those in human feces are of particular importance to human health. These include viruses of the genera *Norovirus*, *Rotavirus*, *Enterovirus*, *Hepatovirus*, *Hepevirus*, *Mamastrovirus*, and *Mastadenovirus*. Infections from these viruses may result in a wide range of illnesses (depending on virus type), including acute gastroenteritis, conjunctivitis, myocarditis, and infectious hepatitis. With the possible exception of *Hepatovirus*, each genus consists of several species, which may be further subdivided into distinct genotypic or serological groups; altogether,[1] there are more than 200 human enteric viruses.[2–6] These viruses most frequently transfer from person to person via the fecal–oral route. Because these viruses are excreted with feces in relatively large numbers, they are present in domestic sewage and, therefore, may be present in sewage-contaminated surface and ground waters[7] that are used as sources of drinking water.

In temperate climates, some viruses demonstrate seasonality (e.g., enterovirus concentrations in sewage peak during late summer and early fall). One notable exception may be rotaviruses, whose incidence of disease increases in colder months, but quantitative information on rotaviruses' seasonal patterns of occurrence in water and wastewater is lacking because these viruses cannot be assayed readily with conventional cell culture techniques. That said, several recent studies using modern molecular techniques show that domestic sewage contains many types of viruses year-round.[8,9]

Culture-based methods may be the gold standard for detecting and characterizing viruses, but many remain recalcitrant to culture. For example, human noroviruses have not been cultivated in any cell cultures, although immunochemical assay methods have been developed to detect them as antigens.[10–12] Human rotaviruses and hepatitis A virus have been cultivated recently in cell cultures, but the techniques are difficult and require concomitant use of polymerase chain reaction (PCR) and immunoassays (e.g., immunofluorescence) to detect virus growth or gene probes.[13–17]

Rotaviruses mostly infect infants and young children, while noroviruses infect adults. Infection rates vary considerably from area to area, depending on sanitary and socioeconomic conditions. Viral pathogens are not normal flora in the intestinal tract; they are excreted only by infected individuals. The concentration excreted, however, is usually several orders of magnitude lower than that of coliform bacteria. Enteric viruses multiply only within living, susceptible cells; their numbers cannot increase in sewage. Sewage treatment, dilution, natural inactivation, and water treatment further reduce viral numbers. So, while massive sewage contamination of a water supply may cause large outbreaks of waterborne viral disease,[17] the incidence of waterborne viral infections in technologically advanced nations depends on whether minimal quantities of viruses can produce infections.

Researchers have demonstrated that infection can be produced experimentally by a very few virus units,[15,18] although the risk of infection increases with increasing ingested doses.[19] The risk of infection for an individual living in a community where the water system contains a very few virus units has not been determined.[20] However, risk analysis has suggested that low numbers of enteric viruses in a drinking water system could result in a significant infection risk.[21] The percentage of individuals who develop clinical illness may be as little as 1% for poliovirus and as much as 97% for hepatitis A.[21]

In the United States, most recognized waterborne virus disease outbreaks have been caused by cross-connections and intrusions in distribution systems, and sewage contamination of untreated or inadequately treated private and semi-public water supplies. Virus disease outbreaks associated with community water supply systems usually are caused by contamination via the distribution system.[22–25]

2. Testing for Viruses

Routine examinations of water and wastewater for enteric viruses are not currently recommended, but they may be prudent or essential in special circumstances (e.g., wastewater reclamation, disease outbreaks, or special research studies). Such testing should be done only by competent, specially trained virologists with adequate facilities.[26]

Laboratories planning to concentrate viruses from water and wastewater should do so with the clear understanding that the available methodology has important limitations.[27] Even the most current virus-concentration methods still are being researched and continue to be modified and improved. A method's efficacy may vary widely depending on water quality. Furthermore, none of the available virus-detection methods have been tested adequately with representatives of all virus groups important to public health. However, molecular methods combined with culture-based methods are widely used, and the detection procedures are available in the literature.[28–32] Most virus-concentration methods have achieved adequate virus recoveries for water or wastewater samples that were experimentally seeded with known quantities of a few specific enteric viruses. Although method effectiveness in field trials is difficult to evaluate, some virus-concentration methods have successfully recovered naturally occurring enteric viruses. Some of these methods require large equipment to process samples; virus assay and identification procedures usually require cell culture, molecular, and related virology laboratory facilities.[29]

Detecting viruses in water via recovery of infectious virus requires three general steps:

a) collecting a representative sample,

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b) concentrating the viruses in the sample, and
c) identifying and estimating quantities of the concentrated viruses.

Particular problems associated with detecting waterborne viruses important to public health are:

a) the small size of virus particles (about 20 to 100 nm in diameter),
b) the wide variety and low concentrations of viruses that may be present in water,
c) the inherent instability of viruses as biological entities,
d) the various dissolved and suspended materials in water and wastewater that interfere with virus-detection procedures, and
e) the present limitations of virus-estimation and -identification methods.

3. Selection of Concentration Method

The densities of enteric viruses in water and wastewater usually are so low that virus concentration is necessary, except possibly for raw sewage in certain areas or seasons.[32–35] Numerous methods for concentrating waterborne enteric viruses have been proposed, tested under laboratory conditions with experimentally contaminated samples, and in some cases, used to detect viruses under field conditions.[36,37]

Virus concentration methods often can only process limited volumes of waters of a given quality. When selecting a virus-concentration method, consider the probable virus density, the method's volume limitations for that type of water, and the presence of interfering constituents. A sample volume less than 1 L and possibly as small as a few milliliters may suffice for recovering viruses from raw or primary treated sewage. For drinking water and other relatively nonpolluted waters, virus levels are likely to be so low that hundreds or perhaps thousands of liters must be sampled to increase the probability of virus detection.

Three virus-concentration techniques are described herein: adsorption to and elution from microporous filters (9510B and C); aluminum hydroxide adsorption-precipitation (9510D) ; and polyethylene glycol (PEG) hydroextraction-dialysis (9510E).[33,38] A separate technique (9510F) for recovering viruses from solids in small volumes of water also is described. Virus concentration by adsorption to and elution from microporous filters can be used for both small volumes of wastewater and large volumes of natural and finished waters. Methods 9510D and E are impractical for processing large volumes of sample, but they are suitable for concentrating viruses from wastewater or other waters with relatively high virus densities and for second-step concentration (reconcentration) of viruses in primary eluates obtained by processing large sample volumes through microporous filters.

4. Recovery Efficiencies

When examining a particular water, include a preliminary evaluation of virus-recovery efficiency. To do this, add a known quantity of one or more test virus types to the required volume of sample, process the sample by the concentration method, and assay the concentrate for test viruses to determine virus-recovery efficiency. Ideally, such seeded samples should be used whenever field samples are processed. If seeded samples are used concurrently with field samples, take appropriate steps—including disinfection, sterilization, and the aseptic technique—to prevent accidental contamination of samples.

5. Controls

Depending on the scope of the experiment, two types of quality control samples should be included in the experimental plan: assay control and method control. In each analysis, include a set of positive and negative samples as assay controls. In each assay, cell-viability control should be included to demonstrate that cells remain viable throughout the assay period. In addition, it will confirm the sterility of the cell culture used throughout the assay period and help track down any contamination that occurs during sample processing.

For method control, include a set of blind seeded and unseeded samples with every batch. All controls should be analyzed at the same time as the samples, incubated under the same conditions, and assayed in the same manner.

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9510 B. Virus Concentration from Small Sample Volumes by Adsorption to and Elution from Microporous Filters

1. General Discussion

Viruses can be concentrated from aqueous samples by reversibly adsorbing them to microporous filters and then eluting them from the filters in a small liquid volume.[1] The virus-containing sample is pressure-filtered through microporous filters with large surface areas to which viruses adsorb, presumably by both electrostatic and hydrophobic interactions.[2] Two general types of adsorbent filters are available: electronegative (negative surface charge) and electropositive (positive surface charge). Electronegative filters are composed of either cellulose esters or fiberglass with organic resin binders. They adsorb viruses most efficiently in the presence of multivalent cations (e.g., Al^{3+} and Mg^{2+}) and/or at low pH (usually 3.5). Electropositive filters are composed of either fiberglass or cellulose and a positively charged organic, polymeric resin, or alumina fiber media. They adsorb viruses efficiently over a wide pH range without added polyvalent salts. If the sample is neutral or acidic, these filters can process it without chemical conditioning.

Electropositive filters have given virus recoveries comparable to those of electronegative filters.[3–5] They have been used in field studies[6,7] and were evaluated with a variety of viruses[8–13] and waters.

Adsorbed viruses usually are eluted from the surfaces of microporous filters by pressure-filtering a small volume of eluent fluid through the filters *in situ.* The eluent is either a slightly alkaline proteinaceous fluid (e.g., beef extract) or a more alkaline buffer (e.g., glycine-NaOH, pH 10.5 to 11.5). If glycine-NaOH

is used as eluent, preferably use pH 10.5 because of the greater likelihood of virus inactivation at the higher pH.[14,15] New elution buffers that work at neutral pH are available.*[16]

Microporous filter methods have three main limitations. Suspended matter in the sample tends to clog the adsorbent filter, thereby limiting the volume that can be processed and possibly interfering with the elution process.[17] Dissolved and colloidal organic matter in some waters can interfere with virus adsorption to filters, presumably by competing with viruses for adsorption sites[18–20]; they also can interfere with virus elution. Finally, viruses adsorbed to suspended matter may be removed via any clarification procedure applied before virus adsorption; they are lost from the sample unless special efforts are made to recover solids and process them for viruses.[17] (A method for recovering solids-associated viruses from small volumes of water and wastewater is given in 9510F.) Despite these limitations, virus concentration by adsorption to and elution from microporous filters is a most promising technique for detecting viruses.

2. Equipment and Apparatus

a. Adsorbent filter holder, 47-, 90-, or 142-mm diam, equipped with pressure-relief valve.

b. Pressure vessel, 12- or 20-L capacity.

c. Positive pressure source up to about 400 kPa with regulator: laboratory air line, air pump, or cylinder of compressed air or nitrogen gas.

d. Autoclavable vinyl plastic tubing with plastic or metal connectors (quick-disconnect type), for connecting positive-pressure source, pressure vessel, and filter holder in series.

e. pH meter.

f. Beakers, 50- to 500-mL.

g. Laboratory balance.

h. Graduated cylinders, 25- to 100-mL.

i. Pipets, 1-, 5-, and 10-mL.

3. Materials

a. Electronegative virus-adsorbent filter: Use either:

1) *Cellulose nitrate filter,* 0.45-μm porosity.†

2) *Fiberglass-acrylic resin filter*, 0.45-μm porosity.‡—Filter media available commercially only as flat sheets can be cut to the desired disk diameter with scissors.

b. Electropositive virus-adsorbent filter: Use either:

1) *Surface-modified cellulose and filter aid disk depth-filter*.§

2) *Surface-modified cellulose and filter aid thin-sheet medium, 0.20-μm porosity*.#

Alternatively, argonde filter media also can be used.

c. Prefilter: Use one or more cellulose nitrate or fiberglass-acrylic resin filters or equivalent, with porosities larger than 0.45 μm to prevent suspended matter from clogging the virus-adsorbent filter. Place prefilters on top of the 0.45-μm-porosity virus-adsorbent filter in the same filter holder.

4. Reagents

a. Hydrochloric acid, HCl, 0.1, 1.0, and 10*N*.

b. Sodium hydroxide, NaOH, 0.1, 1.0, and 10*N*.

c. Aluminum chloride, $AlCl_3 \cdot 6H_2O$, 0.15*N*, or magnesium chloride, $MgCl_2 \cdot 6H_2O$, 5*N* (necessary only for electronegative filters).

d. Sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$, 0.5% (w/v).

e. Sodium chloride, 0.14*N*, pH 3.5: Dissolve 8.18 g in 1 L reagent-grade water and adjust to pH 3.5 with HCl (necessary only for electronegative filters).

f. Virus eluent: Use either:

1) *Glycine-NaOH*, pH 10.5 or 11.5—Prepare 0.05*M* glycine solution, autoclave, and adjust to pH 10.5 or 11.5 with 1 to 10*N* NaOH. Add phenol red, 0.0005%, as a pH indicator.

2) *Beef extract*, 3%, pH 9.0—Dissolve 30 g beef extract paste or 24 g beef extract powder in 1000 mL reagent-grade water, adjust to pH 9.0 with 1 to 10*N* NaOH, and sterilize via autoclaving.

g. Glycine-HCl, pH 1.5: Prepare 0.05*M* glycine solution, autoclave, and adjust to pH 1.5 with 1 to 10*N* HCl. Add phenol red, 0.0005%, as a pH indicator.

h. Nutrient broth, 10X, pH 7.5: Dissolve 8.0 g nutrient broth in 90 mL reagent-grade water, adjust to pH 7.5, dilute to 100 mL with reagent-grade water, and sterilize via autoclaving.

i. Antibiotics: Use either:

1) *Penicillin-streptomycin*, 10X—Contains 5000 IU penicillin/mL and 5000 μg streptomycin/mL. Use commercially available form or prepare by dissolving powdered sodium or potassium penicillin-G and streptomycin sulfate in reagent-grade water and sterilizing via filtration. Store frozen.

2) *Gentamycin-kanamycin*, 100X—Contains 5000 μg/mL each of gentamycin (base) and kanamycin (base). Prepare by combining aseptically equal volumes of commercially available sterile gentamycin and kanamycin solutions, 10 000 μg/mL, respectively, or by dissolving powdered gentamycin sulfate and kanamycin sulfate in reagent-grade water and sterilizing via filtration. Store refrigerated or frozen.

j. Hanks balanced salt solution, 10X: Use commercially available form or prepare following a standard protocol.[21]

k. Sodium hypochlorite, 5.25% available chlorine (household bleach).

5. Procedure

a. Sterilization of apparatus, materials, and reagents: Most reagents, virus-adsorbent filters, filter holders, tubing, and labware can be sterilized via autoclaving or made virus-free by streaming steam. To sterilize filters, load into their holders; if several filters will be placed in one holder, place filter with smallest porosity on the bottom and then add progressively larger filters. Do not use an automatic drying cycle when autoclaving virus-adsorbent filters. Sterilize apparatus and material that cannot be autoclaved or treated with streaming steam by treating with 10-mg/L free chlorine solution, pH 7.0, for 30 min and rinse or flush with 50-mg/L sterile $Na_2S_2O_3$ solution. Do not treat adsorbent filters with chlorine. Use aseptic technique during all virus concentration operations to prevent extraneous microbial contamination.

* Optima RE, or equivalent.
† Type HA, Millipore Corp., Bedford, MA, or equivalent.
‡ No. 8025-035, Filterite Corp., Timonium, MD, or equivalent.
§ Zeta-plus 50S or 60S, CUNO, Meriden, CT, or equivalent.
# 1-MDS Virozorb, CUNO, Meriden, CT, or equivalent.

b. Sample size and choice of filter size: Sample size and, hence, filter diameter depend partly on water quality and the probable virus concentration. Single-stage microporous filter adsorption-elution methods have been used to recover viruses from 100 mL raw sewage on 47-mm-diam filters[22] and from 3.8 to 4.6 L secondary and tertiary sewage effluent on 90- or 142-mm-diam filters.[19,22,23] Based on the diameter and solids-holding characteristics of the filters, the scale and volume capacity of the apparatus and materials, and the quality of the samples, the practical limits for sample size are 20, 8, and 2 L for 142-, 90-, and 47-mm-diam filters, respectively.

c. Choice of filter type: Virus adsorption to electropositive filters decreases above pH 8, so pH adjustment may be necessary for optimal virus adsorption.[4] Virus recovery from raw sewage may be less than with electronegative filters.[10]

d. Sample collection and storage: Collect samples aseptically in sterile containers. If they contain residual chlorine, immediately add $Na_2S_2O_3$ solution to give a final concentration of 50 mg/L. Process samples as soon as possible after collection; do not hold samples for more than 2 h at up to 25°C or 48 h at 2 to 10°C. Do not freeze samples unless they cannot be processed within 48 h; then freeze and store at –70°C or colder.

e. Sample processing of electronegative filters: Adjust sample to pH 3.5 and 0.0015*N* $AlCl_3$ or to between pH 6.0 and 3.5 and 0.1*N* $MgCl_2$. Make sample adjustments in either a pressure vessel or another appropriate container. Mix sample vigorously during addition of 1.0 or 0.1*N* HCl and $AlCl_3$ solution (1 part solution to 100 parts sample) or $MgCl_2$ solution (1 part solution to 50 parts sample). Because $AlCl_3$ is an acid salt, it may lower sample pH slightly. Do not let sample pH fall below 3.0.

Place sample in a pressure vessel connected to a source of positive pressure and connect pressure vessel outlet to inlet of virus-adsorbent filter holder. With pressure-relief valve on filter holder opened, apply a slight positive pressure to purge air from filter holder. When sample just begins to flow from pressure-relief valve, quickly close valve and continue filtration at a rate not exceeding 28 mL/min/cm^{2}of filter area (about 130, 250, and 4000 mL/min for 47-, 90-, and 142-mm-diam filters, respectively). After filtering entire sample, let positive-pressure source purge excess fluid from filter holder.

Wash filters with 0.14*N* NaCl to remove excess Al^{3+} or Mg^{2+} from virus-adsorbent filter. Use about 1.5 mL NaCl solution/cm^2 filter area (25, 100, and 240 mL for 47-, 90-, and 142-mm-diam filters, respectively). Place wash solution in a pressure vessel connected to filter holder inlet, use positive pressure to filter solution through virus-adsorbent filter, discard filtrate, and let positive pressure purge virus-adsorbent filter of excess wash solution.

Elute viruses from filters with a recommended eluent. Use about 0.45 mL eluent/cm^2 filter surface area (about 7.5, 28, and 71 mL for 47-, 90-, and 142-mm-diam filters, respectively). With pressure-relief valve on filter holder open, add eluent to filter holder so it completely covers filter surface. When eluent begins to discharge from pressure-relief valve, quickly close valve. If pH 11.5 glycine-NaOH is the eluent, place a sterile beaker under filter outlet and apply positive pressure so filtrate flows slowly from filter-holder outlet. Collect filtrate in sterile beaker and, when filtrate no longer flows, slowly increase pressure to force retained fluid from filters. Quickly check eluate (filtrate) pH. If it is less than 11.0, elute with additional pH 11.5 glycine-NaOH until an eluate with a pH $\geq$11.0 is obtained. Immediately after checking pH, adjust eluate to a pH between 9.5 and 7.5 with pH 1.5 glycine-HCl or 0.1*N* HCl while mixing vigorously. Complete elution and eluate pH adjustment to 7.5 to 9.5 in 5 min or less to avoid appreciable virus inactivation.

If pH 10.5 glycine-NaOH is the eluent, proceed as with pH 11.5 glycine-NaOH, but pass eluate through filters a total of five times. For each elution, collect filtrate, readjust to pH 10.5 with 1.0 or 0.1*N* NaOH, and then pass through filter. After the fifth elution, adjust filtrate to pH 7.4 with glycine-HCl, pH 1.5, or 0.1*N* HCl.

If 3% beef extract, pH 9.0, is the eluent, place a sterile beaker under filter outlet, apply a slight positive pressure to eluent-containing filter holder so filtrate flows slowly from the outlet, and collect filtrate. Slowly increase pressure to force additional retained fluid from filters.

Measure eluate volume and add 1/10 of the measured volume each of penicillin-streptomycin or gentamycin-kanamycin, Hanks balanced salt solution, and 10X nutrient broth (add last item to glycine eluates only). Adjust sample to pH 7.4 with glycine-HCl or 0.1*N* HCl while mixing vigorously. Store at either 4 or –70°C, depending on the time until virus assay. Maximum storage at 4°C is 48 h.

f. Processing of electropositive filters: Processing for electropositive filters is identical to that for electronegative filters except that Al^{3+} and Mg^{2+} additions and sample pH adjustments are unnecessary; because Al^{3+} and Mg^{2+} are not added, it is unnecessary to wash filters with 0.14*N* NaOH before elution. If sample pH is greater than 8.0, adjust to less than pH 8 by adding 1.0 or 0.1*N* HCl.

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9510 C. Virus Concentration from Large Sample Volumes by Adsorption to and Elution from Microporous Filters

1. General Discussion

Viruses in eluate volumes too large to be conveniently and economically assayed directly in cell cultures (e.g., those obtained from processing large volumes of water through cartridge or large disk filters) can be concentrated further (reconcentrated) via several methods. Viruses in proteinaceous or organic-buffer eluates from all types of water can be reconcentrated by either "organic flocculation,"[1,2] aluminum hydroxide adsorption-precipitation (9510D), or polyethylene glycol hydroextraction-dialysis (9510E). Organic flocculation, now used widely, involves precipitating viruses by acidifying eluates to pH 3.5, recovering precipitate via centrifugation, and then resuspending it in a small volume of alkaline buffer.[1]

Additionally, viruses in nonproteinaceous eluates (e.g., glycine-NaOH) can be reconcentrated via adsorption to and elution from small microporous filters. The eluate is adjusted to pH and ionic conditions for optimum virus adsorption, filtered through a secondary adsorbent, and then adsorbed viruses are eluted with a small volume of eluent. This procedure can only be used to reconcentrate primary eluates obtained from processing drinking water and other highly finished waters because of potential interfering substances likely to be present in primary eluates from natural and less finished waters.

Figure 9510:1 shows the alternative microporous filter adsorption-elution and reconcentration methods.

For general information on microporous filter techniques, see 9510B.1.

2. Equipment and Apparatus

a. Apparatus for first-stage concentration (Figure 9510:2):

1) *First-stage virus-adsorbent filter holder.*

2) *Chemical additive system*—Use either:

a) *Fluid proportioner* with four feed pumps (quadraplex) and a mixing chamber.*

b) *Venturi-type proportioning injector*† with plastic or metal connectors (quick-disconnect type) and a length of vinyl tubing for the chemical feed line.[3] To feed two additives, attach a "Y" or "T" connector and two lengths of vinyl tubing to the chemical feed port, or use two proportioning injectors. It may be necessary to use a bypass system with the injector to prevent loss of chemical feed due to back-pressure from the water line.[4] This bypass system consists of "T" pipe fittings on the injector inlet and outlet ports connected by a length of flexible hose with an in-line shut-off/control valve (see Figure 9510:2).

Proportioning injectors available commercially will process water at flow rates of 3 to 33 L/min with water-to-chemical feed ratios between 10 to 1 and 1110 to 1. Select equipment and operating conditions providing a water-to-chemical feed ratio of 100 to 1.

3) *Water flow meter.*

4) *Pressure gauge*, 0 to 400 kPa.

5) *Vinyl plastic tubing*, autoclavable, with plastic or metal connectors (quick-disconnect type).

* Johanson and Son Machine Corp., Clifton, NJ, or equivalent.
† Models 202-P, 203-P, or 204-P, Dema Engineering Co., St. Louis, MO, or equivalent.

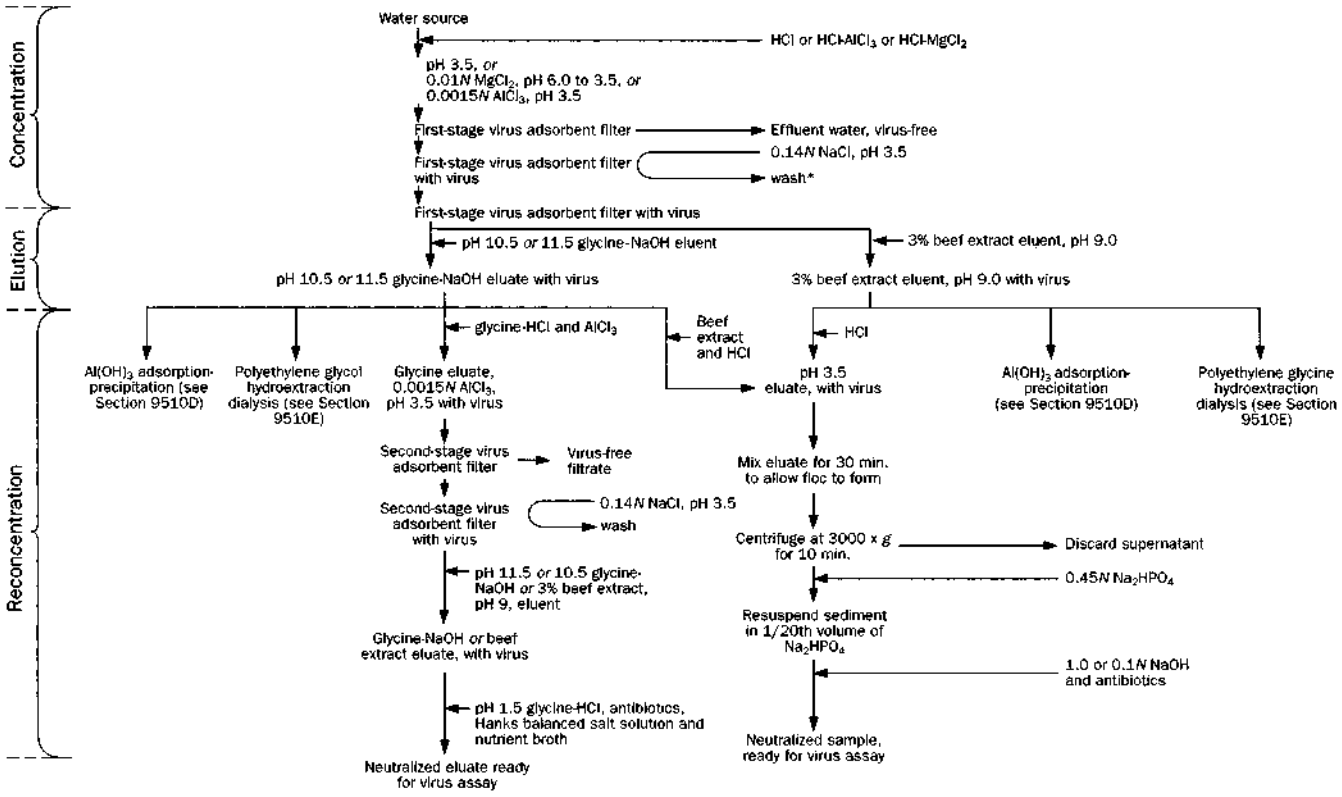


Figure 9510:1. Two-stage microporous filter adsorption-elution method for concentrating viruses from large volumes of water with electronegative filters.

6) *Pressure-relief valve* (optional).

7) *Carboys*, 20- to 50-L, or similar containers.

8) *Positive-pressure source* up to 400 kPa with regulator: laboratory air line, positive-pressure pump, or cylinder of compressed air or nitrogen gas.

9) *Pump* (if source water is not under pressure).

b. pH meter.

c. Laboratory balance.

d. Beakers, 2- or 4-L.

e. Pressure vessel, 4-L.

f. Graduated cylinders, 1- and 2-L.

g. Pipets, 1-, 5-, and 10-mL.

h. Centrifuge with rotor and buckets for 250- to 500-mL-capacity bottles.‡

i. Centrifuge bottles, 250- to 500-mL.

3. Materials

a. First-stage electronegative virus-adsorbent filters: Use one of the following:

1) *Cellulose nitrate filter series*, 293-mm-diam, 8.0- and 1.2-μm-porosity.§

2) *Fiberglass-epoxy filter tube*, 17.8-cm-long, 8.0-μm-porosity.‖

3) *Fiberglass-acrylic resin pleated filter cartridge*, 25.4-cm-long, 0.25- or 0.45-μm-porosity.#

b. Second-stage electronegative virus-adsorbent filters: 47-mm-diam, 3.0-, 0.45-, and 0.25-μm-porosity fiberglass-acrylic resin filter series. Use to reconcentrate highly finished water samples only.

c. First-stage electropositive adsorbent filters: Use one of the following:

1) *Surface-modified cellulose and filter aid filters*, 293-mm-diam.**

2) *Surface-modified thin-sheet media pleated filter cartridge*, 25-cm-long, 0.20-μm-porosity.††

4. Reagents

a. Hydrochloric acid (HCl), 0.06, 1,‡‡ and 6*N*.

b. Sodium hydroxide (NaOH), 10*N*.

c. Aluminum chloride ($AlCl_3 \cdot 6H_2O$), 0.15 and 6*N*.

‡ Required for alternative reconcentration procedure using 3% beef extract.
§ Millipore Corp., Bedford, MA, or equivalent.
‖ Balston, Inc., Lexington, MA., or equivalent.
Filterite Corp., Timonium, MD, or equivalent.
** 50S, 60S, or 1-MDS Virozorb, CUNO, Meriden, CT, or equivalent.
†† 1-MDS Virozorb, CUNO, Meriden, CT, or equivalent.
‡‡ Recommended for first-stage virus adsorption with electronegative filters only.

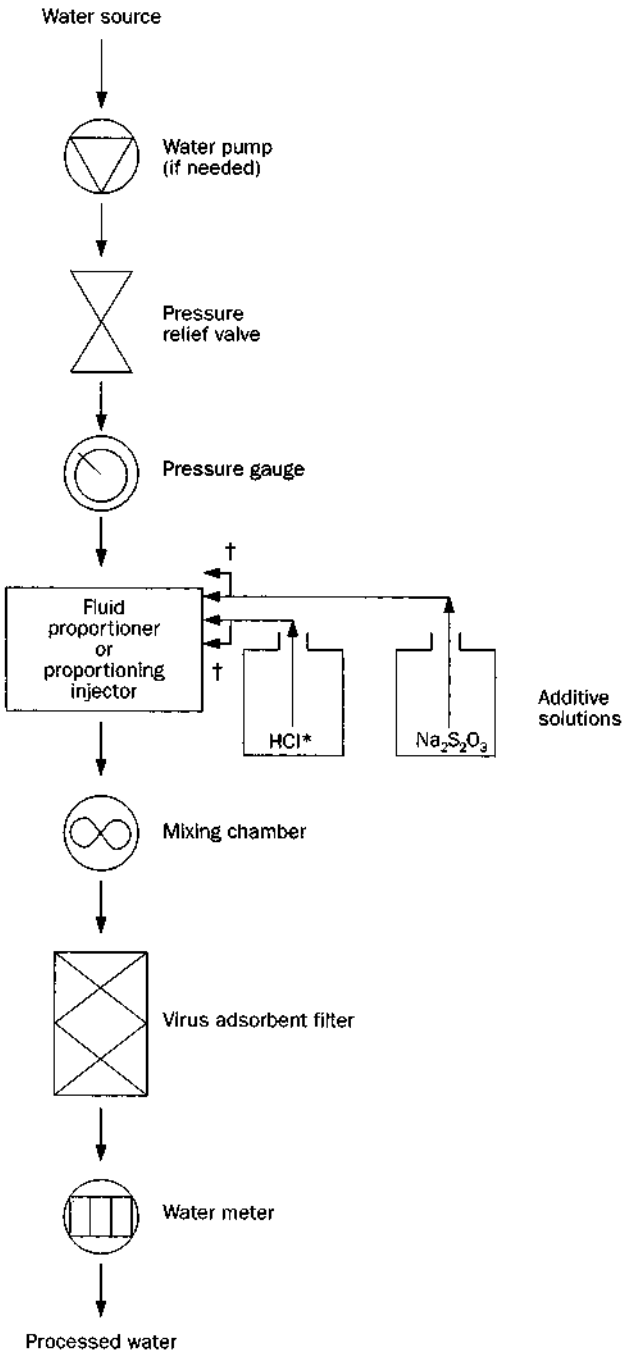


Figure 9510:2. Schematic of apparatus for first-stage concentration with negatively charged filters.

d. Magnesium chloride ($MgCl_2 \cdot 6H_2O$), 10*N*.‡‡

e. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$), 0.5% (w/v).

f. Sodium hypochlorite, 5.25% available chlorine (household bleach).

g. Eluent: Use either:

1) *Glycine-NaOH*, pH 10.5 or 11.5—See 9510B.4*f*1). Use within 2 h of pH adjustment.

2) *Beef extract*, 3%, pH 9.0.§§ See 9510B.4*f*2).

h. Eluate neutralizing solution: Use either:

1) *Glycine-HCl*, pH 1.5—Prepare 0.05*M* glycine solution and adjust to pH 1.5 with 6*N* HCl. Add phenol red, 0.0005%, as a pH indicator. Use within 2 h of pH adjustment.

2) *HCl*, 1.0*N*.

i. Nutrient broth, 10X, pH 7.5: Dissolve 8.0 g nutrient broth in 90 mL distilled water, adjust to pH 7.5 with 10*N* NaOH, dilute to 100 mL with distilled water, and sterilize by autoclaving.

j. Disodium phosphate, 0.45*N:* Dissolve 40.2 g $Na_2HPO_4 \cdot 7H_2O$ in 1 L distilled water and sterilize by autoclaving.

k. Antibiotics: See 9510B.4*i*.

l. Sodium chloride, 0.14*N:* Dissolve 8.18 g NaCl in 1 L distilled water (necessary only with electronegative filters).

m. Hanks balanced salt solution, 10X: See 9510B.4*j*.

5. Procedure

When using electronegative filters, follow ¶s *a–f* below to produce primary eluate. When using electropositive filters, first see ¶ *g* below for procedural modifications.

a. Sterilization of apparatus, materials, and reagents: See 9510B.5*a*.

b. Sample size: For drinking water, use a minimum sample of 400 L—although 2000 L or more may have to be processed to detect viruses at a concentration of 1 to 2 infectious units/400 L.

c. Preparation of feed solutions for electronegative filters: Use an HCl additive solution to adjust sample pH to 3.5 for virus adsorption to filters. If acidification to pH 3.5 is inadequate for obtaining maximum virus adsorption, add either $AlCl_3$ or $MgCl_2$ solution.

When only HCl is used, prepare additive solution as follows: Determine concentration of HCl additive solution by titrating a 1-L sample of dechlorinated water to pH 3.5 with 0.06*N* HCl and noting volume required. The volume, in milliliters, of titrant required is equal to the volume of 6*N* HCl needed/L distilled water for making the additive solution. Make at least 5 L additive solution for 400 L of sample.

When $AlCl_3$ is used to enhance virus adsorption, use pH 3.5 and a final concentration of added $AlCl_3$ of 0.0015*N*. Because $AlCl_3$ is an acid salt, titrate a 1-L sample to about pH 4.0 with 0.06*N* HCl, add $AlCl_3$ to a concentration of 0.0015*N* and continue titration to pH 3.5, noting volume of titrant used. Prepare additive solution by adding titrant volume (mL) of 6.0*N* HCl/L of 0.15*N* $AlCl_3$.

When $MgCl_2$ is used to enhance virus adsorption, use a pH between 3.5 and 6.0, and a 0.1*N* final concentration of added $MgCl_2$. To prepare additive solution, titrate a 1-L sample to desired pH with 0.06*N* HCl (as previously described) and note volume of titrant used. Add the titrant volume of 6.0*N* HCl/L 10*N* $MgCl_2$ to make the additive solution.

d. Preparation of chemical additive system:

1) When using a fluid proportioner, operate at a pressure of 100 to 700 kPa and a water flow rate of 4 to 40 L/min. Adjust each of the proportioner's four chemical additive pumps for a ratio of 1 to 200 (1 part chemical additive to 200 parts water).

§§ For alternative reconcentration procedure: organic flocculation.

Use two pumps (operating reciprocally) for each additive, so the overall dilution for each additive is 1 to 100. One additive is either HCl, HCl-$AlCl_3$, or HCl-$MgCl_2$; the other additive, 0.5% $Na_2S_2O_3$, is needed only when processing samples containing chlorine. For each additive solution, place the two pumps' feed lines into the additive containers and manually operate the pump metering rods to fill feed lines and purge them of air. Connect fluid proportioner to source water and operate briefly without a virus adsorbent in place. Sample conditioned water from proportioner outlet and check pH (it should be 3.5 ± 0.3).

2) When using a Venturi-type proportioning injector, connect injector assembly to water source and to adsorbent-filter inlet, and place additive feed line(s) into additive container(s). Position valve on injector outlet to drain line position (away from adsorbent filter). Begin sample flow. Adjust screw-operated control valve on injector's chemical feed until water collected from drain line is at the desired pH (as measured with a pH meter). If $Na_2S_2O_3$ is used to neutralize chlorine, check to ensure that chlorine is absent. Connect virus concentrator assembly to source water by attaching concentrator inlet hose to valved outlet of a pressurized water source or to outlet of a water pump (the inlet of which has been placed in the source water). Operate for several minutes without a virus adsorbent in place to purge the unit of chlorine solution. Collect a sample from meter outlet to ensure absence of chlorine.

e. First-stage concentration: After preparing concentration apparatus and additive solutions and checking conditioned water for proper pH and absence of chlorine, attach a virus-adsorbent filter to outlet of chemical-additive system. Attach water meter and effluent hose to virus-adsorbent outlet. Record initial meter reading and add to this value the desired volume to be processed plus another 1 or 2% (to account for volume of either one or two additive solutions, respectively). This is the meter reading at which sampling is to be stopped. Turn on water and start a timer (or record starting time). Shortly after filtration begins, collect a sample from filter outlet and check for absence of chlorine and for appropriate pH value. Also check flow rate. Do not use a flow rate above 40 L/min. Recheck pH and chlorine residual several times during sample processing, or monitor continuously. When desired volume has been processed, turn water off. Purge filter holder of excess water with positive pressure from an air or nitrogen gas source.

f. Washing and virus elution: If using $AlCl_3$ or $MgCl_2$, wash excess Al^{3+} or Mg^{2+} from filter with 4 L 0.14*N* NaCl. Omit washing if only using HCl. Place wash solution in a 4-L pressure vessel and pass through filter with positive pressure. Purge filter of excess wash solution with positive pressure and discard entire filtrate.

Using aseptic technique, elute virus from filter as soon as possible in the field or after returning to the laboratory. If filter holders with adsorbed viruses must be returned to the laboratory, seal filter holder openings, place filter holder in a sterile plastic bag, and chill.

Use pH 10.5 or 11.5 glycine-NaOH or 3% beef extract, pH 9.0, to elute viruses from first-stage adsorbent filters. (NOTE: Some viruses are inactivated when pH 11.5 glycine-NaOH is used.[1,3,4])

To elute, place eluent in a pressure vessel. Use minimum eluent volumes of 1 L and 300 mL for cartridge and 293-mm-diam disk filters, respectively. To elute with pH 11.5 glycine-NaOH, connect pressure vessel to inlet of filter holder and, with pressure-relief valve on filter holder open, apply a small positive pressure to the system so eluent fills void volume of filter holder. When eluent begins to discharge from pressure-relief valve, quickly close it. Pass remaining eluent slowly through filter within 1 to 2 min and collect filtrate (eluate) in a sterile 2- or 4-L beaker. When filtrate no longer appears, slowly increase pressure to force more fluid from filter. If using pH 11.5 glycine-NaOH eluent, immediately check filtrate pH; if it is less than 11.0, elute with 1 L more of pH 11.5 glycine-NaOH. Immediately after checking pH, use with pH 1.5 glycine-HCl to adjust filtrate to between 7.5 and 9.5 pH while mixing vigorously. Complete elution and pH adjustment in 5 min or less to avoid appreciable virus inactivation.

To elute with pH 10.5 glycine-NaOH, use either batch or continuous-flow eluent recirculation. For the batch method, begin elution as with pH 11.5 glycine-NaOH. Collect filtrate, measure pH, and readjust to pH 10.5 with 1.0 or 0.1*N* NaOH while mixing vigorously. Then, using this eluate, elute filters four more times, readjusting filtrate to pH 10.5 before each elution. After the fifth elution, adjust filtrate to pH 7.4 with pH 1.5 glycine-HCl or 1.0*N* HCl while mixing vigorously.

Alternatively, elute with pH 10.5 glycine-NaOH by continuous recirculation. Place eluent in a sterile beaker. Attach short lengths of sterile vinyl or rubber tubing to inlet and outlet openings of filter holder and place free ends of tubing in eluent beaker; slip midsection of filter inlet tubing into a peristaltic or roller pump. Open pressure-relief valve on filter holder and operate pump at slow speed so eluent fills void volume of filter holder. When eluent begins to discharge from pressure-relief valve, quickly close it. Increase pump speed so eluent recirculates through filter assembly and beaker at a minimum flow rate of 100 mL/min. After 5 min recirculation, remove filter inlet tube from beaker and pump remaining fluid from filter assembly. Connect filter inlet to positive-pressure source to force more eluent from filter. Adjust eluate to pH 7.4 with pH 1.5 glycine-HCl or 1.0*N* HCl while mixing vigorously.

To elute with 3% beef extract, pH 9.0, follow the procedure described above for pH 11.5 glycine-NaOH. Adjust collected filtrate to pH 7.4 with pH 1.5 glycine-HCl or 1*N* HCl while mixing vigorously. The 5-min time limit to complete elution with pH 11.5 glycine-NaOH is unnecessary when beef extract is used.

g. Sample processing of electropositive filters: Processing for electropositive filters is identical to that for electronegative filters, except that Al^{3+} and Mg^{2+} addition and sample pH adjustments are unnecessary; because Al^{3+} and Mg^{2+} are not added, it is unnecessary to wash filters with 0.14*N* NaOH before elution. If sample pH is greater than 8.0, adjust to less than pH 8 by adding 1.0 or 0.1*N* HCl.

h. Reconcentration of primary eluates: Reconcentrate viruses in primary eluates either by organic flocculation, $Al(OH)_3$ adsorption-precipitation (9510D), polyethylene glycol hydroextraction-dialysis (9510E), or adsorption to and elution from microporous filters. The latter technique can be used only for glycine or other organic buffer eluates.

To reconcentrate viruses in glycine eluates by adsorption to and elution from filters, adjust to pH 3.5 with pH 1.5 glycine-HCl and add $AlCl_3$ to a final concentration of 0.0015*N* while mixing vigorously. Transfer sample to a 4-L pressure vessel.

Filter through a 47-mm-diam 3.0-, 0.45-, and 0.25-μm-porosity fiberglass-acrylic resin filter series at a flow rate of no more than 130 mL/min, and discard filtrate. Rinse filters with 25 mL 0.14*N* NaCl to remove excess Al^{3+}. Pipet NaCl solution directly into filter inlet or place in a small pressure vessel connected to inlet. Use positive pressure to pass NaCl solution through filter and discard filtrate. Elute adsorbed viruses from filter with 7-mL portions of either pH 10.5 or 11.5 glycine-NaOH or 3% beef extract, pH 9.0. Pipet 7 mL eluent directly into filter-holder inlet or into a small pressure vessel connected to filter inlet and connect to a positive-pressure source. Carefully apply positive pressure so eluate flows slowly from filter outlet into a sterile container. When filtrate no longer flows from outlet, increase pressure to force retained fluid from filters. If using pH 11.5 glycine-NaOH, measure eluate pH and immediately adjust pH to between 7.5 and 9.5 with pH 1.5 glycine-HCl. Repeat this elution procedure with another 7-mL portion of pH 11.5 glycine-NaOH. Complete reconcentration within 5 min. If neither eluate portion had a final pH of 11.0 or more, repeat elution procedure with additional 7-mL portions of pH 11.5 glycine-NaOH until an eluate portion has a pH of at least 11.0. Combine all eluates.

If using pH 10.5 glycine-NaOH, elute five successive times with 7-mL volumes of eluent. After each elution, readjust eluate to pH 10.5 with 0.1*N* NaOH while mixing vigorously. After the fifth elution, adjust eluate to pH 7.4 with pH 1.5 glycine-HCl or 0.1*N* HCl while mixing vigorously.

If using 3% beef extract, pH 9.0, elute with two 7-mL volumes, combine filtrates, and adjust to pH 7.4 if necessary.

Measure total eluate volume. For glycine eluates, add the equivalent of 1/10 measured sample volume of 10X Hanks balanced salt solution and 10X nutrient broth. To all eluates, add appropriate volumes of antibiotics (1/10 sample volume of penicillin-streptomycin or 1/100 sample volume of gentamycin-kanamycin, or both). Store at 4 or –70°C, depending on time until virus assay.

Further concentrate viruses in beef extract eluates by precipitation at pH 3.5 (organic flocculation). Viruses in glycine eluates also can be reconcentrated by this technique by first supplementing them with beef extract to a final concentration of 1 to 3%. Use sterile beef extract paste (about 80% beef extract) or sterile 20% beef extract solution made from powder to bring glycine eluates to the desired beef extract concentration. While mixing vigorously, adjust eluate to pH 3.5 by adding 1*N* HCl dropwise. Continue to mix at slow speed for 30 min and centrifuge at 3000 × *g* for 10 min. Decant and discard supernatant. With vigorous mixing, resuspend sediment in 1/20 the initial sample volume of 0.45*N* Na_2HPO_4. Add antibiotics (1/10 final sample volume of penicillin-streptomycin, 1/100 final sample volume of gentamycin-kanamycin, or both) and, while mixing vigorously, adjust to pH 7.4 with 1.0 or 0.1*N* NaOH. Check electrical conductivity of sample. If conductivity is >13 000 μmhos, dialyze sample against Hanks balanced salt solution before assay. Store at 4 or –70°C, depending on time until virus assay.

6. References

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9510 D. Virus Concentration by Aluminum Hydroxide Adsorption-Precipitation

1. General Discussion

Viruses can be concentrated from small volumes of water, wastewater, and adsorbent filter eluates by precipitation with aluminum hydroxide.[1–4] This process probably involves both electrostatic interactions between the negatively charged virus surface and the positively charged aluminum hydroxide [$Al(OH)_3$] surfaces and coordination of the virus surface by hydroxo-aluminum complexes.[5] Viruses are adsorbed to an $Al(OH)_3$ precipitate that is either added to or formed in the sample from a soluble aluminum salt and a base [e.g., sodium carbonate (Na_2CO_3) or sodium hydroxide (NaOH)]. Viruses are allowed to adsorb to the $Al(OH)_3$ precipitate, and the virus-containing precipitate is collected via filtration or centrifugation. The recovered precipitate may be inoculated directly into laboratory hosts for virus assay, or the viruses are eluted from the precipitate with an alkaline buffer or a proteinaceous solution before virus assay.

The major limitations of this method are that sample size is limited to perhaps a few liters, soluble organic matter can interfere with virus adsorption, and virus recovery from the precipitate may be incomplete. Virus adsorption may be improved by forming the $Al(OH)_3$ precipitate in the sample instead of adding it preformed. Although virus adsorption can be maximized by using large amounts of $Al(OH)_3$, the adsorbed viruses become more difficult to elute. Therefore, some intermediate amount of $Al(OH)_3$ is used to achieve maximum virus recovery. Also, $Al(OH)_3$ is a relatively nonspecific adsorbent, so other substances may be concentrated with the viruses. The presence of such impurities may cause the concentrated sample to be toxic for the cell cultures normally used for virus assay.

Several modifications of the $Al(OH)_3$ adsorption–precipitation procedure have been used to concentrate viruses from water, wastewater, and eluates from adsorbent filters. Initially, preformed $Al(OH)_3$ precipitates were made by adding Na_2CO_3 to $AlCl_3$ solutions, and suspending the $Al(OH)_3$ precipitate in 0.15*N* NaCl. This was added to the wastewater,

and the mixture was stirred gently for 1 h or more to allow viruses to adsorb to the precipitate. The precipitate was recovered via filtration, resuspended in cell culture media, and inoculated into cell cultures.[3,4] More recent procedural modifications include:

a) $Al(OH)_3$ precipitate formation in the sample,[1,2,6–8]
b) recovery of $Al(OH)_3$ precipitate via centrifugation followed by virus elution from precipitate with alkaline eluents,[1,2,6,7] and
c) a large-volume method in which precipitate is formed in the sample and collected on a cartridge filter, and viruses are eluted from precipitate on the filter with alkaline eluent.[9]

The method described here is for relatively small sample volumes and uses $Al(OH)_3$ that is either preformed or generated in the sample. The latter modification is preferable because some viruses are not adsorbed efficiently by preformed precipitates.[10]

2. Equipment and Apparatus

a. Centrifuge, with rotor and buckets, capable of operating at about 1900 × *g*.
b. Centrifuge bottles and tubes.
c. Beaker, 100-mL or larger.
d. pH meter.
e. Magnetic stirrer and stirring bars or other mixing device.
f. Graduated cylinders, 100-mL or larger.
g. Pipets, 1-, 5-, and 10-mL.
h. Laboratory balance.
i. Vacuum-type filter holder or Buchner filter funnel,* 47-mm diam or larger.
j. Filter flask.*
k. Spatula,* flat blade, metal or autoclavable plastic.
l. Vacuum source,* vacuum pump or laboratory vacuum line.

3. Materials

*Filter:** Fiberglass-acrylic resin filter† or microporous filter, 0.45-μm porosity,‡ 47-mm diam or larger. To prevent virus adsorption, filter 0.1% polyoxyethylene sorbitan monooleate solution (9510D.4*h*) through filters, using about 1 mL solution/$cm^2$of filter surface area. Rinse filter with distilled water, using about 10 mL/cm^2 of filter surface area. Sterilize treated filters via autoclaving.

4. Reagents

a. Hydrochloric acid, HCl, 0.1 and 1.0*N*.
b. Sodium hydroxide, NaOH, 0.1 and 1.0*N*.
c. Sodium carbonate, Na_2CO_3, 4*N* §.
d. Aluminum chloride, $AlCl_3$, 0.075*N*§ or 0.9*N*.
e. Sodium chloride, NaCl, 0.14*N*.
f. Beef extract, 3%, pH 7.4: Dissolve 3 g beef extract paste or 2.4 g beef extract powder in 90 mL distilled water, adjust to pH 7.4 with 1.0 or 0.1*N* NaOH, dilute to 100 mL with distilled water, and sterilize via autoclaving.
g. Antibiotics: Use either:
1) *Penicillin-streptomycin*, 10X, containing 5000 IU penicillin/mL and 5000 μg streptomycin/mL. Available commercially or prepare by dissolving powdered sodium or potassium penicillin-G and streptomycin sulfate in distilled water and sterilizing via filtration.
2) *Gentamycin-kanamycin*, 100X, containing 5000 μg/mL each of gentamycin base and kanamycin base [see 9510B.4*i*2)].

h. Polyoxyethylene sorbitan monooleate,‖ 0.1% (v/v) in distilled water.

5. Procedure

a. Sterilization of apparatus, materials, and reagents: See 9510B.5*a*.

b. Preparation of preformed Al(OH)$_3$ precipitate: While mixing 100 mL 0.075*N* $AlCl_3$ at room temperature, slowly add 4*N* Na_2CO_3 solution to form precipitate and adjust to pH 7.2. Continue mixing for 15 min and, if necessary, add more Na_2CO_3 to maintain pH 7.2. Centrifuge at 1100 × *g* for 15 min and discard supernatant. Resuspend sediment in 0.14*N* NaCl and recentrifuge. Discard supernatant, resuspend sediment in 0.14*N* NaCl, and sterilize via autoclaving. Cool, centrifuge again, decant supernatant, and resuspend $Al(OH)_3$ sediment in 50 mL sterile 0.14*N* NaCl. Store at 4°C.

c. Sample size, collection, and storage: Process samples of no more than several liters because the method is too cumbersome and time-consuming for larger volumes. See 9510B.5*d* for sample collection and storage procedures.

d. Sample processing: Do not prefilter sample[11,12] because substantial virus losses can occur. Adjust sample to pH 6.0 with 1.0 or 0.1*N* HCl while mixing vigorously. Form $Al(OH)_3$ precipitate in sample by adding 1 part 0.9*N* $AlCl_3$ solution to 100 parts sample to give a final 0.009*N* Al^{3+} concentration. Check sample pH and readjust to 6.0 with 1.0 or 0.1*N* NaOH or HCl, if necessary. Mix slowly for 15 min at room temperature.

Alternatively, use preformed $Al(OH)_3$ precipitate by adding 1 part stock $Al(OH)_3$ suspension/100 parts sample and mix slowly for 2 h at 4 to 10°C to allow for virus adsorption.

Collect virus-containing $Al(OH)_3$ precipitate via centrifugation or filtration. To collect precipitate via centrifugation, centrifuge at 1700 × *g* for 15 to 20 min, discard supernatant, and resuspend sediment in the equivalent of 1/1000 to 1/20 original sample volume of 3% beef extract, pH 7.4.

To collect precipitate via filtration, vacuum filter sample through a treated filter (9510D.3 above) held in a vacuum-type filter holder or Buchner funnel, using additional filters if filter clogs before entire sample is filtered. Carefully scrape precipitate from filter(s) with a sterile spatula and resuspend in the equivalent of 1/1000 to 1/20 original sample volume of 3% beef extract, pH 7.4.

Regardless of collection method, vigorously mix the $Al(OH)_3$ beef extract suspension and, if necessary, adjust pH to 7.4 with

* Required for optional method for collecting $Al(OH)_3$ precipitate from sample.
† Millipore AP20, or equivalent.
‡ Millipore HA, or equivalent.
§ For alternative procedure using preformed $Al(OH)_3$ precipitate.
‖ Tween 80®, ICI United States, Inc., Wilmington, DE, or equivalent. Required for optional method for collecting $Al(OH)_3$ precipitate from sample.

0.1*N* HCl or NaOH. Continue mixing for a total of 10 min. Centrifuge at 1900 × *g* for 30 min. Decant supernatant, add the equivalent of 1/10 concentrate volume of penicillin-streptomycin solution or 1/100 concentrate volume of gentamycin-kanamycin and store at 4 or –70°C.

6. References

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9510 E. Hydroextraction-Dialysis with Polyethylene Glycol

1. General Discussion

Polyethylene glycol (PEG) hydroextraction is an ultrafiltration process in which the sample is placed in a cellulose dialysis bag and exposed to PEG, a hygroscopic material. Water and microsolutes leave the sample by passing across the semipermeable dialysis membrane into the hygroscopic PEG.[1] Viruses and other macrosolutes, including PEG, cannot cross the dialysis membrane. The sample volume in the dialysis bag is reduced by water loss to the PEG, thereby concentrating viruses and other macrosolutes. The viruses retained in the dialysis bag are recovered by opening the bag, collecting the remaining sample, and eluting any viruses possibly adsorbed to the inner walls of the bag with a small volume of slightly alkaline proteinaceous solution (e.g., 3% beef extract, pH 9.0). The collected concentrate and eluate are combined and assayed for viruses.

The main limitations of this method are that only small samples (less than 1 L) can be processed conveniently, virus elution from the bag walls may be incomplete unless the elution is done painstakingly, and other macrosolutes in the sample that are concentrated with viruses may interfere with virus assays by being cytotoxic.

Initial investigations of this method reported low and highly variable virus recoveries from wastewater.[2,3] The type of dialysis tubing and eluent solution, as well as the thoroughness of the elution step, have been found to influence virus-recovery efficiency. More recently, with modified procedures, efficient and consistent virus recoveries have been obtained from wastewater and from adsorbent filter eluates.[4,5]

2. Equipment and Apparatus

a. Beakers, 100-mL or larger.
b. Graduated cylinders, 100-mL or larger
c. Dialysis tubing clamps.*
d. Pan, approximately 30 × 30 × 12 cm, autoclavable.
e. Magnetic stirrer and stirring bars or other mixing device.
f. Centrifuge, with rotor and buckets, capable of operating at about 1900 × *g*.
g. pH meter.
h. Pipets, 1-, 5-, and 10-mL.
i. Tape roller† or similar device to help wash the inside walls of dialysis bags with eluting fluid.
j. Ultrasonic disruptor-emulsifier,‡ probe type, capable of generating 100 W of acoustical output.

3. Materials

a. Dialysis tubing, seamless, regenerated cellulose, 4.8-nm average pore diameter.§
b. Polyethylene glycol (PEG),‖ dry flakes.

4. Reagents

See 9510D.4.

5. Procedure

a. Sterilization of apparatus, materials, and reagents: See 9510B.5*a*. Do not sterilize PEG.

* Fisher Scientific No. 8-670-11A, or equivalent.
† Optional, Fisher Scientific No. 14-245-21, or equivalent.
‡ Optional.
§ Made by Union Carbide Corp. and available from many scientific supply companies.
‖ Carbowax 20 000 or 6000, or equivalent.

b. Sample size, collection, and storage: Process samples of no more than a few hundred milliliters. See 9510B.5*d* for sample-collection and storage procedures.

c. Preparation of dialysis tubing: Cut a length of dialysis tubing long enough to accommodate entire sample. Close one end with a clamp. Do not tie knots to close dialysis tubing. Fill tubing bag with distilled water, sterilize via autoclaving, and let cool.

d. Sample processing: Aseptically remove dialysis bag from distilled water and drain. Fill bag with sample and close open end with a second clamp. Place bag in a pan containing a 5-cm layer of PEG, making sure that bag does not touch pan walls. Cover tubing with another 5 cm PEG and store at 4°C (for about 18 h) until sample volume has been reduced to no more than a few milliliters. (If PEG 6000 is used, the process time shortens to 4 to 6 h.) Although sample may be allowed to dewater completely, do not let it remain in this state.

Remove dialysis bag from PEG and quickly wash PEG from outside of bag with sterile distilled water. Remove clamp from one end of bag and carefully collect sample concentrate. Add the equivalent of about 1/200 to 1/20 original sample volume of 3% beef extract, pH 9.0, and clamp closed. Thoroughly wash inside walls of bag with beef extract by rubbing fluid from one end to the other several times using fingers or a roller device. Remove clamp from one end of bag and collect fluid, kneading or squeezing to recover the last traces. Add recovered fluid to previously collected sample concentrate.

Adjust to pH 7.5 with 1.0 or 0.1*N* HCl while mixing vigorously. To disperse solids-associated viruses in sample, stir overnight (about 18 h) in the cold (about 4°C) or treat with ultrasonics at 100 W for 1 to 2 min. Prevent sample temperature from rising above 37°C during ultrasonic treatment by chilling in an ice bath. Centrifuge at 1900 × *g* for 30 min. Decant supernatant, add the equivalent of 1/10 concentrate volume of penicillin-streptomycin solution or 1/100 concentrate volume of gentamycin-kanamycin, and store at 4 or –70°C.

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9510 F. Recovery of Viruses from Suspended Solids in Water and Wastewater

1. General Discussion

Viruses in the aquatic environment often are associated with solids or particulate matter, either adsorbed to particulate surfaces or embedded in the solid.[1–3] Both freely suspended and solids-associated viruses are concentrated from water by the methods described above. There is evidence that solids-associated viruses are not eluted efficiently from adsorbent filters or from $Al(OH)_3$ precipitates and organic flocs. Nor do microporous filter methods using *in situ* elution recover solids-associated viruses consistently.[2] Solids-associated viruses on adsorbent filters are eluted more efficiently by disrupting filters in elution fluid than by *in situ* elution,[2] but this is cumbersome and time-consuming, especially for large-diameter disk filters and cartridge filters.

For small volumes of water and wastewater, solids-associated viruses can be recovered expediently by separating the solids via centrifuging, decanting supernatant, and eluting viruses from solids by resuspending in a small volume of eluent.[4] Viruses in supernatant can be concentrated via one of the procedures described in 9510B, C, D, or E. Viruses eluted from resuspended solids are separated from the solids via centrifuging and are assayed directly or concentrated further by organic flocculation.[5,6] Major limitations of these methods are incomplete virus elution and poor virus recoveries due to interferences from sample constituents.

2. Equipment and Apparatus

a. Centrifuge, with rotor and buckets for 250- to 1000-mL bottles, capable of operating at about 1250 × *g*.
b. Centrifuge bottles, 250- to 1000-mL.
c. pH meter.
d. Laboratory balance.
e. Graduated cylinder, 250-mL or larger.
f. Beaker, 250-mL or larger.
g. Sample bottles, 250-mL or larger.
h. Magnetic stirrer and stirring bars, or other mixing device.
i. Pipets, 1-, 5-, and 10-mL.
j. Fume hood.

3. Reagents

a. Hydrochloric acid, HCl, 0.1 and 1.0*N*.
b. Sodium hydroxide, NaOH, 0.1 and 1.0*N*.
c. Eluent: Dissolve 10 g beef extract, 1.34 g disodium phosphate heptahydrate, $Na_2HPO_4 \cdot 7H_2O$, and 0.12 g citric acid in 90 mL distilled water, adjust to pH 7.0 with 1*N* HCl or NaOH, dilute to 100 mL with distilled water, and sterilize via autoclaving.

d. Antibiotics: See 9510B.4*i*.
e. Vertrel® XF (1,1,1,2,3,4,4,5,5,5-decafluoropentane).*

4. Procedure

a. Sterilization of apparatus, materials, and reagents: See 9510B.5*a*.

b. Sample size, collection, and storage: Collect and process samples of no more than 10 L, depending on centrifuge capacity. See 9510B.5*d* for sample collection and storage procedures.

c. Sample processing: Aseptically transfer 250- to 1000-mL sample volumes to centrifuge bottles and centrifuge at 1250 × *g* for 20 min. Decant and pool supernatants for subsequent virus processing using one of the water or wastewater methods described previously.

Elute viruses from sedimented solids by resuspending in eluent. Use 40 mL eluent per quantity of sediment from 250 mL original sample. Pool resuspended sediments from multiple centrifuge bottles in a sterile beaker. Alternatively, keep resuspended sediments from small numbers of centrifuge bottles in the bottles and process them individually. While vigorously mixing with a magnetic stirrer, adjust pH to 7.0 by slowly adding 1*N* NaOH or HCl, if necessary. Reduce mixing speed and continue mixing for 30 min while checking sample pH and readjusting to 7.0, as necessary. As an alternative to mixing for 30 min, sonicate samples at 100 W for 15 min in a rosette cooling cell maintained at 4°C. Return sample to centrifuge bottles. Centrifuge at 1250 × *g* and 4°C for 15 min, collect supernatant for subsequent assay or further concentration, and discard sediment.

If desired, further concentrate viruses from this supernatant via organic flocculation (see 9510E). Extract sample concentrates with Vertrel® XF to ensure dispersal of virus particles.[7] If supernatants will be assayed directly for viruses with no further concentration, then adjust pH to 7.4, add the equivalent of 1/10 sample volume of penicillin-streptomycin or 1/100 sample volume of gentamycin-kanamycin, and store at 4 or –70°C.

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9510 G. Assay and Identification of Viruses in Sample Concentrates

1. Storing Sample Concentrates

If sample concentrates cannot be assayed immediately, store them at room temperature (about 25°C) for up to 2 h or at refrigerator temperatures (4 to 10°C) for up to 48 h to minimize virus losses. If samples must be stored for more than 48 h, freeze them at –70°C or colder. Do not freeze samples at –10 to –20°C because some enteric viruses may become extensively inactivated at such temperatures. Store sample concentrates from finished waters in separate freezers or physically separate them from other virus-containing material in common freezers.

2. Decontaminating Sample Concentrates

Sample concentrates, especially those from wastewater, will probably be contaminated with bacteria and fungi, which can overgrow cell cultures and interfere with virus detection and assay. Do not decontaminate via centrifugation or filtration because virus losses are likely. For many samples—especially those from finished waters—contamination is controlled by antibiotics (e.g., penicillin-streptomycin or gentamycin-kanamycin), which are added immediately after the sample is obtained. To provide more protection against fungal contamination, add 2.5 μg/mL amphotericin B or 50 μg/mL nystatin.[1] If penicillin-streptomycin or gentamycin-kanamycin are inadequate, use one or more additional antibiotics (e.g., aureomycin, neomycin, or polymyxin B). To maximize the antibiotic effects, incubate samples for 1 to 3 h at 25 to 37°C after adding antibiotics. Bacterial destruction is further enhanced by freezing at –70°C after incubation with antibiotics. Keep samples frozen until assayed for viruses. To determine if antibiotic treatment has been effective, plate a small subsample on a general-purpose medium (e.g., plate count agar) using the spread plate technique and incubate at 37°C for 24 to 48 h.

If extensive bacterial contamination persists after antibiotic treatment, treat with chloroform ($CHCl_3$) or Vertrel®. Add $CHCl_3$ at 1/10-volume sample and mix vigorously for 30 min at room temperature or homogenize 1 to 2 min at 4 to 10°C. For phase separation, centrifuge at ≥1000 × *g* or store overnight in a refrigerator. Separate sample (upper layer) from $CHCl_3$ (bottom layer) by aspirating with a pipet, and bubble with filter-

sterilized air for about 15 min to remove dissolved $CHCl_3$. If necessary, place sample in a sterile, shallow container and expose it to the atmosphere in a sterile air environment (laminar air flow clean bench or biological safety cabinet) for up to several hours to remove remaining traces of $CHCl_3$. Do not use ether to decontaminate samples because of the hazard of explosion or fire.

3. Laboratory Facilities and Host Systems for Virus Assay

Because viruses are obligate, intracellular parasites, they grow (multiply) only in living host cells. This ability to multiply in, and thereby destroy, their host cells is the basis for virus detection and assay. The two major host cell systems for human enteric viruses are whole animals (usually mice) and mammalian cell cultures of primate origin.

A complete description of the facilities, equipment, materials, and methods for conducting virus assays is beyond the scope of this book; see standard handbooks on virology and cell culture.[1–4] Virus assay is beyond the capability of most water and wastewater microbiology laboratories; it should be done only by a trained virologist working in specially equipped virology laboratory facilities. Take particular care to prevent samples or inoculated hosts from becoming contaminated with viruses from other sources and to prevent virus cross-contamination arising from sample concentrates or inoculated hosts. Process and handle samples in a Class II, Type I biological safety cabinet[5] or in a "sterile" room or cubicle; this is mandatory when testing drinking water or other finished water samples. Receive, store, process, and analyze samples in a segregated area of the laboratory. Overall sample handling and processing flow should be in accordance with established laboratory protocols.

There is no single, universal host system for all enteric viruses. Some—notably hepatitis A virus, human rotaviruses, and norovirus gastroenteritis viruses—cannot be assayed routinely in any convenient laboratory host systems. Most, however, can be detected via two or more cell-culture systems and perhaps suckling mice. (Mice used to be considered essential for detecting group A coxsackieviruses, but recent studies indicate that the RD cell line may be nearly as sensitive in isolating these and other enteroviruses.[6,7]) In general, the more types of host systems used, the greater the enteric virus recovery rate. However, the number of host systems used is limited by practical and economic considerations.

There have been numerous studies of the relative sensitivities of various cell-culture systems in detecting enteric viruses,[6–27] but there has been no systematic, comprehensive study of enteric virus recoveries from water and wastewater. Primary or secondary human embryonic kidney (HEK) cell cultures seem to be the most sensitive host system for isolating enteric viruses, but they are becoming increasingly difficult to obtain regularly. When available from commercial sources, they are expensive. Primary or secondary African green, cynomolgus, or rhesus monkey or baboon kidney cells are sensitive hosts for many enteroviruses and reoviruses, but are not particularly suitable for recovering adenoviruses or group A coxsackieviruses. BGM, a continuous line derived from African green monkey kidney cells, may be comparable in sensitivity to primary monkey kidney cells.[7,18,21,25,26] A number of other continuous cell lines, as well as human fetal diploid cell strains, have been evaluated for enteric virus recoveries. Some human fetal diploid cell strains had virus isolation rates comparable to primary monkey kidney cells, but plentiful supplies of specific strains are not readily available and many are difficult to maintain. Furthermore, each cell strain must be characterized for virus susceptibility. Most continuous cell lines generally are less effective than primary cells, but comparable isolation rates for some enteric virus groups have been obtained with Hep-2[11] and HeLa[17,25] cells.

Assay the entire sample concentrate for enteric viruses using at least two host systems and dividing entire sample equally among hosts. To recover most enteroviruses, adenoviruses, and reoviruses, preferably use primary (or secondary) HEK cells with either primary (or secondary) monkey kidney or BGM cells. Additional use of either suckling mice or RD cells provides for enhanced recovery of group A coxsackieviruses. Different host systems may be substituted for these if they have demonstrated equivalent sensitivity.

4. Virus Quantitation Procedures for Sample Concentrates

a. Advantages and disadvantages of different quantitation procedures: Virus assays in suckling mice or other animals are quantal assays; in cell cultures, they can be done by either quantal (most probable number or 50% endpoint) or enumerative (plaque) methods. The choice of method depends on the sample and whether maximum virus sensitivity or maximum precision and accuracy in estimating virus concentration is preferred. The plaque technique generally is more precise and accurate than the quantal assay because relatively large numbers of individual infectious units can be counted directly as discrete, localized areas of infection (plaques). Quantal assays are more sensitive than monolayer plaque assays, but are less sensitive than an agar cell suspension plaque assay.[26]

Because virus plaques are discrete areas of infection arising from one infectious virus unit, it is relatively easy to recover viruses from individual plaques and then inoculate them into additional cell cultures to obtain a pure virus culture for identification. However, large proportions of so-called "false-positive" plaques, which are not confirmed as virus-positive when further passaged in cell cultures, have been reported.[28,29] Whether this problem is due to nonviral, plaque-like areas of cytotoxicity from the sample or to technical inability to passage viruses successfully from the initial plaques remains uncertain.[28,29]

Using specific plaque assay conditions to optimize the recovery of certain enteric virus groups may preclude efficient recovery of other enteric groups that require different plaque assay conditions. Furthermore, some viruses (e.g., adenoviruses) do not form plaques efficiently under any conditions. Cytotoxicity due to water or wastewater constituents in sample concentrates is difficult to control in plaque assay systems because the agar overlay medium is difficult to remove and replace.

A potential limitation of quantal assays is the possibility that two or more virus types will be inoculated into one cell culture, thus producing a simple positive culture. This not only results in an underestimated virus concentration but also requires that individual virus types be separated via further passage in cell culture. Such mixed cultures may go undetected unless virus isolates are identified serologically. It has been shown that mixed positive cultures rarely occur when samples are divided into

small portions for inoculation into a series of replicate cell cultures.[7,25]

Cytotoxicity due to constituents of sample concentrates usually can be controlled in quantal assay cell cultures by replacing the culture medium before the cells die.

b. Cell culture procedures for virus isolation and assay: To assay sample concentrates in cell cultures by quantal or plaque methods, drain the medium from newly confluent cultures. Rinsing with buffered saline solution may reduce toxicity. Inoculate with unit volumes of sample; use no more than 0.06 mL sample/cm^2 of cell layer surface (e.g., maximum volumes of 1.0, 3.0, and 6.0 mL in cell culture flasks with areas of 25, 75, and 150 cm^2, respectively).[30] If samples are expected to contain such large quantities of viruses that it would be difficult to estimate concentration reliably, then inoculate cell cultures with small sample volumes or dilutions of concentrates. Allow viruses to adsorb to cells for 2 h at 37 ± 0.5°C. Redistribute inoculum over cell layer manually every 15 min or keep cultures on a mechanical rocker during the adsorption period. Add liquid maintenance medium to cultures for quantal assays or agar-containing medium for plaque assays. Invert plaque assay cultures so cell (agar) side of culture faces up and incubate at 37°C.

Microscopically examine quantal assay cultures for cytopathic effects (CPE) daily during the first 3 d and then periodically for a total of at least 14 d. Do not change cell culture medium unless cytotoxicity or cell deterioration occurs. Freeze cultures developing CPE at –70°C when more than 75% of cells become involved. After 14 or more days, freeze at –70°C all remaining cultures, including controls and those still negative for CPE. Thaw cultures and clarify culture fluid-cell lysate by slow-speed centrifugation or filtration through sterile 0.22- or 0.45-μm porosity filters. Inoculate clarified material from each initial (first-passage) culture into a second (second-passage) culture by transferring 20% of total initial culture into newly confluent cell cultures of the same type. Periodically examine second-passage cultures microscopically for CPE development for 14 or more days. Consider second-passage cultures developing CPE as confirmed virus-positive. Freeze and store at –70°C for virus identification. Discard as negative any virus cultures negative for CPE after this second incubation period of 14 or more days.

Periodically examine plaque assay cultures for plaques for 14-d period; mark and tally plaques as they appear. Transfer viruses from each plaque directly to at least two newly confluent, liquid-medium cell cultures of the same type[28] before plaques become too large and grow together or before the entire cell layer deteriorates. Do not store material obtained from plaques before transfer to new cell cultures because this may result in loss of virus titer and unsuccessful transfers. Microscopically examine these second-passage cultures periodically over 14 d for development of CPE. Freeze cultures developing CPE at –70°C for virus identification.

c. Virus isolation and assay in mice: To detect group A and B coxsackieviruses in mice, inoculate samples into animals no older than 24 h using standard procedures.[2,3,8] Use either the intracerebral or intraperitoneal route, inoculating 0.02 and 0.05 mL, respectively. Observe mice daily over a 14-d period for development of weakness, tremors, and either flaccid (due to group A coxsackieviruses) or spastic (due to group B coxsackieviruses) paralysis. Sacrifice animals developing symptoms, and using sterile technique, prepare 20% tissue suspensions in Hanks balanced salt solution of the entire skinned, eviscerated torso or just the brain and legs. Store suspensions at –70°C until used for further passage and identification. For second passage in mice, follow general procedures used for the initial inoculations. However, making a second passage in cell cultures is preferable to making a second passage in mice because it is easier to do subsequent virus identification via neutralization tests.

d. Estimating virus concentration: Determining the amount of virus in a sample concentrate depends on the assay used.[30] If a sample concentrate is assayed in cell cultures by the plaque technique, count all plaques and calculate the virus concentration, expressed as plaque-forming units (PFU).

If a sample concentrate is assayed by the quantal method, estimate the virus concentration by the most probable number (MPN) method and express as MPN of infectious units (MPNIU), or by a 50% endpoint method and express as 50% infectious or lethal dose (ID_{50} or LD_{50}).[2,4,31–33] If the undiluted sample concentrate or a single-sample dilution was inoculated into a series of replicate cell cultures (or mice), calculate MPNIU from the number of confirmed CPE-negative cultures (or mice), q, per total number of cultures (or mice) inoculated, n, according to the formula

$$\text{MPN} = -\ln(q/n)$$

If more than one sample dilution was inoculated into cell cultures (or mice), calculate MPNIU from the formula developed by Thomas:[34]

$$\text{MPN/mL} = P/\sqrt{NQ}$$

where:

P = total number of positive cultures (or mice) from all dilutions,
N = total mL sample inoculated for all dilutions, and
Q = total mL sample in all negative cultures (or mice).

When using this formula, exclude from computation all dilutions containing only positive cultures (or mice).

For MPN values obtained from a single sample dilution, the 95% confidence interval is based on the standard error of the binomial distribution when more than 30 cultures (or mice) are inoculated or from the confidence coefficient table of Crow[33,35] when 30 or fewer cultures (or mice) are inoculated.

Make 50% endpoint estimates arithmetically by either the Reed-Muench or Karber method.[2,4] These methods require results from several equally spaced sample dilutions, preferably with roughly the same number of dilutions above and below the 50% endpoint, and may not be useful for sample concentrates with relatively low virus levels.

e. Identification of virus isolates: Identify enteric viruses isolated from sample concentrates via standard serological techniques, although preliminary identification of genus (enterovirus, reovirus, or adenovirus) sometimes can be made based on information obtained from the isolation procedure. Enteric viruses recovered in suckling mice probably will be either group A or B coxsackieviruses. For enteric viruses isolated in cell cultures, preliminary genus identification often can be made from the characteristic appearance of cytopathic effects (CPE) in infected cell cultures.

Confirm preliminary identification of suspected adenovirus and reovirus isolates by detecting their respective group-specific antigens via complement fixation tests using clarified, second-passage cell-culture lysate as the antigen. Identify specific reovirus serotypes by hemagglutination-inhibition (HI) or neutralization (Nt) tests. Adenovirus serotypes can be separated into four groups based on their ability (or inability) to hemagglutinate rhesus monkey or rat erythrocytes.[2,3,8] Except for Type 18, the first 28 numbered adenoviruses can be identified as to specific serotype by HI. Alternatively, identify all adenovirus serotypes via Nt tests using either individual type-specific antisera or intersecting antisera pools. Also identify specific enterovirus serotypes via neutralization tests in cell cultures using intersecting pools of hyperimmune sera.[2,3,8,36] Use mice for Nt tests for group A and B coxsackieviruses only if the virus isolates fail to propagate in cell cultures.[37] Because polioviruses often are the most prevalent enteroviruses in water and wastewater, test enterovirus isolates for neutralization by an antisera pool against the three types of poliovirus before making neutralization tests with intersecting antisera pools.

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9610 DETECTION OF FUNGI*

9610 A. Introduction

1. Significance

The Kingdom Fungi is composed of diverse eukaryotic and heterotrophic organisms, including filamentous molds and yeasts. Almost all fungi have cell walls composed of rigid, covalently linked polymers, including chitin and glucan, and many fungi produce spores. The majority of fungi are aerobic, although some are obligate anaerobes and a few are facultative anaerobes. Most are also mesophilic (grow at temperatures between 18 and 25°C), but some are thermophilic (grow at temperatures from >20 up to ~50°C) and some are psychrophilic (grow at temperatures between 0 and 5°C, up to a maximum of about 16 to 20°C).

Generally fungi are saprophytic, secreting extracellular enzymes and absorbing nutrients from dead or decaying organic matter. Some fungi are parasitic; however, relatively few species are overt pathogens and some fungi live in a symbiotic relationship with plants or other microorganisms (e.g., lichens and mycorrhizal fungi).

Fungi are ubiquitous in water environments and areas associated with water (e.g., shoreline edges and leaking pipes). Spring water (near the source) usually contains a minimal number of fungal spores, although the species may be diverse. Unpolluted stream water also may contain multiple species, including true aquatic fungi (species with flagellated zoospores and gametes), aquatic fungal-like species, and soil fungi. Moderately polluted water may contain cells or spores of all types, but more of them will be soil fungi. Heavily polluted water contains large numbers of soil fungi. Soil fungi include yeast-like fungi, many species of which have been isolated from polluted waters.

The association between fungal densities and organic loading suggests that fungi may be useful indicators of pollution, but to date no single species or group of fungi has been identified for this role. However, there may be some opportunities for rapid tests. For example, the principal phenotypic distinction between the yeasts *Candida lambica* and *C. krusei* is the ability to use pentose sugars; the former species grows well on pentoses. Theoretically, it might be used to indicate pulp and paper mill wastes, which contain high levels of such sugars. As there are nucleic-acid sequence differences, a PCR-based method is available.[1] Likewise, certain thermophilic species of yeasts and filamentous fungi might be useful indicators of thermal pollution.

Fungi can degrade or deteriorate a wide range of complex natural materials and hazardous compounds.[2] Because they can produce diverse enzymes, they have been used to treat various wastes and wastewaters.

2. Occurrence and Survival

According to a conservative estimate, there are 1.5 million species of fungi.[3] Fungi exist and survive in almost every habitat,[4] although less than 100 000 are formally described. Fungal populations may fluctuate seasonally[5] and usually increase when organic loading in water or soil increases. Large numbers of similar fungi suggest fungal amplification due to excessive organic loading, while a diversified microbiome indicates stable amounts of organics.

a. Fungi in potable water: Fungi have been found in potable water and on the inner surface of distribution-system pipes.[6–11] In Norway, researchers identified 94 fungal species (belonging to 30 genera) in ground- and surface-water-derived drinking water.[10] The dominant fungi were species of *Aspergillus, Penicillium,* and *Trichoderma*, and some occurred throughout the drinking-water system.

These fungi either survived water treatment or entered the system after treatment and remained viable for extended periods. Tuberculate macroconidia of *Histoplasma capsulatum*[12] can pass through a 0.75-m rapid sand filter, but sedimentation or alum flocculation and settling removed 80 to 99% of spores. If these relatively large (8- to 14-μm), globose fungal cells pass through treatment, it is not surprising that species with smaller cells are also found in treated water.

Pathogenic fungi have remained viable in distilled water stored for relatively long periods.[13] Spores of *H. capsulatum*, stored in raw Ohio River water and sterile tap water, remained highly infective for mice after 400 d.[14]

Tastes and odors in potable water are often associated with prokaryotic organisms (e.g., bacteria, actinomycetes, and cyanobacteria), but fungi may also be involved.[10–11,15] Members of the genera *Acremonium, Penicillium,* and *Phialophora* have been shown to be responsible for taste and odor problems in water.[16]

Propagules (e.g., spores or mycelia fragments) from 19 genera of filamentous fungi have been isolated from a chlorinated surface-water system and an unchlorinated groundwater distribution system;[6] researchers found a mean of 18 colony-forming units (CFU)/100 mL in the groundwater system and 34 CFU/100 mL in the surface-water system. Densities of filamentous fungi and yeasts ranged from 4.0 to 25 CFU/cm^2 and 0 to 9 CFU/cm^2, respectively, in the fungal biofilms of a municipal drinking water system.[17] Certain soil-inhabiting genera—*Aspergillus, Penicillium, Mucor, Alternaria,* and *Cladosporium*—seem to be common colonizers of biofilms and potable water.[9,17–19]

In Finland,[6] fungi were isolated from rivers, lakes, and ponds supplying nine communities with sand-filtered water—three with artificially recharged groundwater (two of which used chemical coagulation), and three with chemically coagulated and disinfected water. They found that *Aspergillus fumigatus* was the most common fungus. Mesophilic fungi were common in all raw-water samples; however, thermotolerant fungi were more abundant in river than in lake water. Chemical coagulation and disinfection removed fungi far more effectively than sand filtration and disinfection.[6]

In the United States, one study reported an average of about 5.5 CFU fungi/100 mL per positive sample from five chlorinated

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groundwater systems.[7] In France,[8] yeasts were recovered from 50% and filamentous fungi from 81% of 38 samples.

Except for certain *Aspergillus* spp.,[6–8,20] *Histoplasma capsulatum,*[21] *Candida* spp.,[1] and dermatophytes, the fungi isolated from potable water usually are not considered medically important.

b. Fungi in recreational waters: Fungi may be found in both fresh and marine recreational waters. Marine fungi may have a range of salinity requirements to consider when attempting isolation.[22] Some fungi pathogenic to humans may be expected in recreational waters (e.g., pools and beaches) and in accompanying washing facilities (e.g., shower stalls and changing rooms). They may survive longer than vegetative bacteria due to spore formation.[23,24]

Studies revealed dermatophytes (fungi that grow on skin, nails, and hair) at 42% of beaches surveyed.[25,26] The most common species were *Microsporum nanum* and *Trichophyton mentagrophytes*, the cause of tinea pedis (athlete's foot). There are also reports of finding yeasts (e.g., *Candida albicans* and other *Candida* species) at beaches in the United States, Portugal, and France.[27–31] *Scopulariopsis* and *Candida* are suggested environmental indicators of sand beach water quality for the sand beaches in Portugal.[29] The U.S. Environmental Protection Agency (EPA) has published a document that lists PCR primers and probes for a large number of fungi and yeast with references.[32]

c. Survival after chlorination: Fungi are more resistant to chlorination than most bacteria.[33,34] In one study, more chlorine was required to inactivate *Candida parapsilosis*[35,36] (a commonly isolated yeast known to cause health problems in the tropics) than to inactivate coliform bacteria. In parallel with chlorine inactivation of *C. parapsilosis* cells was a loss of membrane integrity and permeability, but those decreases required higher chlorine concentrations than required for similar changes in *E. coli*.[36] Fungal cells, especially spores and even conidia, can survive higher doses of chlorine than coliform bacteria.[37,38]

3. Growth Patterns and Identification

There are two basic modes of fungal growth in water. True aquatic fungi produce zoospores or gametes that are motile via flagella. Aquatic fungi typically are collected by exposing suitable baits (solid foodstuffs, such as wood, insects, and seeds) in the habitat or in a laboratory sample. This is most effective if the material is kept in a moist chamber (e.g., a large Petri dish with water added). Direct plating involves placing the material or water sample directly onto an agar surface or mixing it with agar and then pouring the mixture into Petri dishes. The material also can be diluted and then directly plated. Cell culture purification may require several transfers. Alternatively, mycelia and spores can be collected by filtration (0.45 μm pore size) from a water sample or soil suspension, their DNA isolated, and then be detected by PCR or enumerated by qPCR. A kit for fungal, yeast, and spore DNA isolation is available.†

† Fungi/Yeast Genomic DNA Isolation Kit, bioWORLD, Dublin, OH, or equivalent.

The second fungal growth mode is nonmotile in all stages of the life cycle. Growth and reproduction usually are asexual (anamorphic). Two growth processes have been recognized:

- filamentous growth with blastic spores or spores produced in special structures, including single-celled growth on each parent cell (called *budding*) (typical of such yeasts as *Candida* and *Cryptococcus*, which include human pathogens); and
- filamentous growth in which the filaments fragment to form separate spores called *arthroconidia* (e.g., *Geotrichum, Trichosporon,* and related genera).

The kingdom Fungi seems to have evolved separately in at least three groups.[39–41] In the past, as with bacteria, identifying fungi depends primarily on colonial morphology on a solid medium, growth and reproduction morphology, and (for yeasts) physiological activity in laboratory cultures.[42] Molecular detection and identification techniques, such as analysis of rRNA sequences,[41,43,44] commercial 18S sequencing services, and real-time polymerase chain reaction (RT-PCR) are increasingly being used because of their speed and accuracy.[45,46] There are commercial identification systems that use molecular methods, especially for yeasts. Methods for extracting DNA from fungal conidia and yeast cells in air and water samples have been evaluated for RT-PCR.[47] Analysts have used RT-PCR with a molecular beacon probe to detect and monitor fungi in hospital water supplies.[48]

Fungal cell measurement is complicated by the fact that a fungal colony may develop from one cell (spore), an aggregate of cells (a cluster of spores or one multi-celled spore), or from a hyphal or pseudohyphal fragment (containing more than one viable cell). Each fungal colony that develops in laboratory culture is assumed to have originated from one colony-forming unit (CFU), which may or may not be a single cell.

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9610 B. Pour Plate Technique

1. Samples

a. Containers: Collect samples as directed in Sections 9060A and 9610A.3. Alternatively, use sterile cylindrical plastic vials with snap-on caps. Transport them in an upright position to minimize the chance of leakage, and discard after use.

b. Storage: Hold samples no more than 24 h. If analysis is not begun promptly after sample collection, refrigerate at 2 to 8°C.

2. Media

Various media (e.g., potato dextrose agar, cornmeal agar, and malt extract agar) are used to isolate, identify, and enumerate yeasts and molds.[1,2] Neopeptone-glucose-rose bengal-aureomycin® agar is the usual medium of choice when estimating viable units of most fungi (molds or yeasts), especially if bacterial contamination may be present. However, experience may indicate that Czapek agar (often used for *Aspergillus*, *Penicillium*, and related fungi), yeast extract-malt extract-glucose agar, or malt extract agar (often used for yeasts) may be preferable.

For consistency in media quality, use commercially prepared versions of the following media whenever available. For inventory or culture maintenance, use neopeptone-glucose agar. Reagent-grade water (as defined in 9020B.4*d*) or laboratory-purified water may be used.

a. Neopeptone-glucose-rose bengal aureomycin agar:

Neopeptone	5.0 g
Glucose	10.0 g
Rose bengal solution (1g/100 mL reagent-grade water)	3.5 mL
Agar	20.0 g
Reagent-grade water	1.0 L

CAUTION: Rose bengal is irritating to eyes, respiratory system, and skin. Combine these ingredients, bring to boil, and then sterilize via autoclaving for 15 min at 121°C. The final pH should be 6.5 ± 0.2.

Because this medium is used to make pour plates, prepare and store basal agar either in bulk or (more conveniently) in tubes in 10-mL amounts.

Separately prepare a solution of aureomycin [1.0 g of chlortetracycline (water-soluble antibiotic) and 150 mL of distilled or deionized water] and refrigerate. Before use, sterilize via filtration through a 0.2-μm pore size sterilizing-grade membrane. To complete the medium, add 0.05 mL sterile aureomycin solution to 10 mL sterilized basal agar tempered at 44 to 46°C.

If this medium is unavailable in dehydrated form, prepare it from the basic ingredients. Dehydrated Cooke's rose bengal agar may be used in place of the agar base, but then store and incubate the resulting medium in the dark to prevent the photosensitized dye from inhibiting fungi.

This medium is useful for isolating a broad spectrum of fungal species.

b. Czapek (or Czapek-Dox) agar:

Sucrose	30.0 g
Sodium nitrate ($NaNO_3$)	2.0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	1.0 g
Magnesium sulfate ($MgSO_4$)	0.5 g
Potassium chloride (KCl)	0.5 g
Ferrous sulfate ($FeSO_4$)	0.01 g
Agar	15.0 g
Reagent-grade water	1.0 L

Combine these ingredients and heat to dissolve. Autoclave for 15 min at 121°C. The pH should be 7.3 ± 0.2 after sterilization.

This medium is useful for isolating species of *Aspergillus*, *Penicillium*, *Paecilomyces*, and some other fungi with similar physiological requirements.

c. Yeast extract-malt extract-glucose agar:

Yeast extract	3.0 g
Malt extract	3.0 g
Neopeptone (or equivalent)	5.0 g
Glucose	10.0 g
Agar	20.0 g
Reagent-grade water	1.0 L

Combine these ingredients and heat to dissolve. Autoclave for 15 min at 121°C. No pH adjustment is required.

This medium is useful for isolating yeasts.

d. Malt extract agar:

Maltose, technical	12.75 g
Dextrin	2.75 g
Glycerol	2.35 g
Peptone	0.78 g
Agar	15.0 g
Reagent-grade water	1.0 L

Combine these ingredients and heat to dissolve. Autoclave for 15 min at 121°C. No pH adjustment is required. The medium will be turbid, but filtration is unnecessary.

This medium is useful in purifying yeast isolates and studying yeast species in various specified tests. It is also useful for maintaining stock cultures. This medium is comparable to neopeptone-glucose-rose bengal aureomycin agar but contains neither rose bengal nor an antibiotic.

e. Neopeptone-glucose agar:

Neopeptone (or equivalent)	5.0 g
Glucose	10.0 g
Agar	20.0 g
Reagent-grade water	1.0 L

Combine these ingredients and autoclave for 15 min at 121°C. The pH should be 6.5 ± 0.2 after sterilization. (This medium is similar to Sabouraud Agar or Sabouraud Dextrose Agar.)

This medium is useful for maintaining stock cultures. It is comparable to neopeptone-glucose-rose bengal aureomycin® agar but contains neither rose bengal nor an antibiotic.

3. Procedure

a. Preparation and dilution: To a sterile 250-mL Erlenmeyer flask, add 135 mL sterile reagent-grade water and 15 mL sample to obtain a 1:10 sample dilution. Use a sterile measuring device for each sample, or (less preferably) rinse the device with sterile reagent-grade water between samples. Mix sample well before withdrawing the 15-mL portion. Shake flask on a rotary shaker at about 120 to 150 oscillations/min for about 30 min, or transfer flask contents to a blender jar, cover, and blend at low speed for 1 min or at high speed for 30 s. Use a sterile blender jar and appurtenances for each sample, or (less preferably) wash jar thoroughly between samples and rinse with sterile water. Further dilutions may be made by adding 45 mL sterile water to 5 mL of a 1:10 diluted suspension.

For stream water samples, a dilution of 1:10 usually is adequate. Dilute samples containing large amounts of organic material (e.g., sediments) to 1:100 or 1:1000. Dilute stream bank or soil samples to 1:1000 or 1:10 000.

b. Plating: Prepare five plates for each dilution to be examined. To use neopeptone-glucose-rose bengal-aureomycin agar, aseptically transfer 10 mL of medium at 44 to 46°C to a 9-cm Petri dish. Add 1 mL of appropriate sample dilution and mix thoroughly by tilting and rotating dish (see plating procedure under heterotrophic plate count, Section 9215B). Alternatively, add 1 mL sample, 0.05 mL antibiotic solution, and 10 mL liquefied agar medium to Petri dish at 44 to 46°C. Solidify agar as rapidly as possible. (In arid areas, use more medium to prevent dehydration during incubation.)

c. Incubation: Plates should be stacked no more than three high, but do not invert. Incubate at room conditions and ambient lighting, or in the dark at 18 to 25°C. Avoid direct sunlight. Examine plates and count colonies on each plate after 3, 5, and 7 d. Continue incubation and observe plates weekly because some fungi grow slowly. Avoid opening these plate cultures because this may increase airborne contamination.

d. Counting and inventory: The fungal plate count will provide the basis for rough quantitative comparisons among samples; the inventory will give relative importance to, at least, the more readily identifiable species or genera.

When preparing plates, use sample portions that will give about 50 to 60 colonies on a plate. Determine this volume by trial and error. When first examining a new habitat, plate at least two sample dilutions. Estimates of up to 300 colonies may be made, but discard more crowded plates. The medium containing rose bengal tends to produce discrete colonies and limits radial growth (and thereby the size of mold colonies), permitting slow-growing organisms to develop and be observed. Counting limits may be raised or lowered at the analyst's discretion.

If five plates are used per sample, the average number of colonies on all plates (total number of colonies counted/5) times the reciprocal of the dilution (10/1, 100/1, 1000/1, etc.) equals the fungus colony count per milliliter of original sample. NOTE: Each colony may have resulted from one or more hyphal fragments. For solid or semisolid samples, use a correction for the water content to report fungus colonies per Gram dry weight. Determine water content by drying paired 15-mL portions of original sample at 100°C overnight; the difference between wet and dry weights is the amount of water lost from the sample.

The inventory includes the direct identification of fungi based on colonial morphology and the counting of colonies assignable to various species or genera. When discrete colonies cannot be identified, and identification is important, use a nichrome wire (20 to 24 gauge, with its tip bent in an L-shape and flattened by hammer) to pick or cut a segment of growth from each selected colony and streak on a slant of neopeptone-glucose agar (9610B.2*e*). Incubate slants at growth temperature until a lawn of growth is observed. Isolation and identification can be attempted again, or the slant can be stored under refrigeration for 3 to 4 weeks.

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9610 C. Spread Plate Technique

The spread plate technique is another procedure for obtaining quantitative data on colony-forming units.

1. Samples

See Sections 9060A, 9610A.3, and 9610B.1.

2. Media

Aureomycin®-rose bengal-glucose-peptone agar (¶ *e* below) and streptomycin-terramycin®-malt extract agar (¶ *f* below) are useful in analyzing sewage and polluted waters.[1] Use commercially prepared media for those listed below whenever available. Reagent-grade water (as defined in 9020B.4*d*) or laboratory purified water (distilled or deionized) may be used.

a. Neopeptone-glucose-rose bengal aureomycin agar: See 9610B.2*a*.

b. Czapek (or Czapek-Dox) agar: See 9610B.2*b*.

c. Yeast extract-malt extract-glucose agar: See 9610B.2*c*.

d. Malt extract agar: See 9610B.2*d*.

e. Cooke's rose bengal medium with Aureomycin:

This medium is similar to ¶ *a* above but is commercially available.

Glucose	10.0	g
Peptone	5.0	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.0	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5	g
Rose bengal	0.035	g
Agar	20.0	g
Reagent-grade water	800	mL

CAUTION: Rose bengal is irritating to eyes, respiratory system, and skin. Heat to dissolve, bring to a boil, and then autoclave for 15 min at 121°C. Dissolve 70.0 mg aureomycin (chlortetracycline) hydrochloride in 200 mL reagent-grade water, filter–sterilize solution using a 0.2-μm pore size sterilizing-grade membrane, and add to the cooled (42 to 45°C) agar base. No pH adjustment is necessary. Pour 25-mL portions into sterile Petri dishes (100 × 15 mm) and let agar harden. Poured plates may be held up to 4 weeks at 2 to 8°C. Check for dehydration before use.

f. Streptomycin-terramycin-malt extract agar:

Malt extract	30.0	g
Peptone	5.0	g
Agar	15.0	g
Reagent-grade water	800	mL

Heat to dissolve and autoclave the 800 mL of agar-based medium for 15 min at 121°C. Dissolve 70.0 mg of streptomycin and 70.0 mg of terramycin (oxytetracycline) in separate 100-mL portions of reagent-grade water, sterilize each via filtration, and add to the cooled (42 to 45°C) agar base. (The total volume is now 1000 mL.) The pH should be 5.4 ± 0.2. Pour about 20-mL portions into sterile Petri dishes (60 × 15 mm) and let agar harden. Poured plates may be held up to 4 weeks at 4°C. Check for dehydration before use.

3. Procedure

a. Preparation and dilution: See Sections 9215A.5 and 9610B.3*a*. Make dilutions with buffered water (Section 9050C.1*a*) and select dilutions that yield 200 to 1500 colonies per 1 mL.

b. Plating: Pre-dry plates separately with lids slightly ajar at room temperature and about 30% relative humidity for 1 to 1.5 h. A sterility control plate is needed to assess possible airborne contamination; it should be carried through the incubation process. Using a sterile pipet, transfer 0.1 mL of sample or dilution onto surface of a pre-dried agar plate. Spread sample over entire agar surface using a sterile L-shaped glass rod or use a mechanical device to rotate plate and ensure proper sample distribution.

c. Incubation: With dish covers on, let plates dry at room temperature, invert plates, and incubate at 18 to 25°C for up to 7 d in an atmosphere of high humidity (90 to 95%). Slow-growing fungi may not produce noticeable colonies until 6 or 7 d.

d. Counting and recording: Using a darkfield colony counter or a binocular microscope, count all colonies on each selected plate. If counting must be delayed temporarily, hold plates at 2 to 8°C for no longer than 24 h to avoid contamination and further spreading of colonies. Depending on colony size, plates with as many as 150 colonies can be counted, but the optimal maximum number is 100 colonies. Counting limits may be raised or lowered at the analyst's discretion.

Multiply counts by dilution factor and then record results as colony-forming units (CFU)/100 mL original sample. For solid or semisolid samples, report CFU/g wet or dry (preferably dry). If three or more plates are used per sample, use average number of colonies times the reciprocal of the dilution (see 9610B.3*d*) to give colony count. If no plates have colonies, record count as <1 for the highest dilution. If the plate colonies are too crowded to count, record as "too numerous to count" (TNTC) but indicate a count of >150 for the appropriate dilution. If colonies are crowded and overlapping with spreaders, record as "obscured" (OBSC) and repeat analysis with higher dilution or earlier observations.

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9610 D. Membrane Filter Technique

For general information on the membrane filter technique and apparatus needed, see Section 9222. However, except for comparisons of different manufacturers' membranes, there are no critical test reports for the fungal isolation efficiency of membrane filters. Media components, pH levels, and antibiotics have been used in routine plating procedures. The reported procedures seem to be satisfactory.

1. Samples

See Sections 9060A and 9610B.1.

2. Media

Use aureomycin-rose bengal-glucose-peptone agar, Cooke's rose bengal agar, or modified streptomycin-terramycin-malt extract agar.[1] These media are prepared identically to the unmodified media described in 9610C.2*e* and *f* except that the concentration of each antibiotic is increased from 70 to 200 mg/L. Dispense media in portions of 5 to 7 mL in glass or plastic Petri dishes (60 × 15 mm); plastic dishes with tight-fitting lids are preferred.

3. Procedure

a. Preparation and dilution: See Sections 9215A.5 and 9610B.3*a*. Select dilutions to yield 20 to 100 colonies per membrane.

b. Filtration: Filter appropriate volumes of well-shaken sample or dilution, in triplicate, through membrane filters with pore diameter of 0.45 or 0.8 μm (see Section 9222).

c. Incubation: Transfer filters to dishes, invert dishes, and incubate at 18 to 25°C for 3 to 5 d in a humid atmosphere to avoid having the plates dry out.

d. Counting and recording: Using a binocular dissecting microscope at a magnification of 10×, count all colonies on each selected plate. If counting must be delayed temporarily, hold plates at 4°C for no longer than 24 h. Ideal plates have 20 to 80 colonies per filter (see 9610C.3*d*.)

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9610 E. Technique for Yeasts

Yeasts are single-cell fungi with typically one nucleus per cell; they do not produce filaments and reproduce via binary fission (e.g., *Schizosacchromyces pombe*) or budding (e.g., *Cryptococcus* spp.). Sparse to extensive hyphal growth may or may not occur. *Candida albicans* was found to form mycelial growth under anaerobic conditions.[1] Solid media, such as those described above, do not permit all yeasts to grow, so an enrichment technique may be useful in addition to the plate count (see also 9610I).

1. Media

For enrichment, use yeast nitrogen base-glucose broth; for isolation, use yeast extract-malt extract-glucose agar or malt extract agar.

a. Yeast nitrogen base-glucose broth: Dissolve 13.4 g yeast nitrogen base in 1 L reagent-grade water; sterilize via filtration. Prepare 500 mL each of 2% and 40% aqueous glucose solutions and sterilize separately via filtration. To make final medium, aseptically add 25 mL yeast nitrogen base solution and 25 mL of either 2% or 40% glucose solutions to a sterile 250-mL Erlenmeyer flask. The final glucose concentration should be 1 or 20%, respectively. Stopper flask with a gauze-wrapped cotton stopper and store until used.

b. Yeast extract-malt extract-glucose agar: See 9610B.2*c*.

c. Malt extract agar: See 9610B.2*d*.

2. Procedure

a. Sample preparation and dilution: Prepare as directed in 9610B.1.

b. Enrichment: In 250-mL Erlenmeyer flasks, prepare one flask of yeast nitrogen base medium containing 1% and one containing 20% glucose. Inoculate with 1 mL of appropriate sample dilution and incubate at room temperature on a rotary shaker operating at 120 to 150 oscillations/min for at least 64 h. Shaken cultures prevent the overgrowth of filamentous fungi.

c. Isolation: Remove flasks from shaker and let settle 4 to 5 h. Yeast cells, if present, will settle to the bottom, bacteria and filamentous fungi will remain in suspension, and filamentous fungi will float on the surface or attach to the glass surface at or above the meniscus. With a nichrome wire loop, remove a loopful of sediment at the sediment–supernatant interface from a tilted flask and smear/streak on yeast extract-malt extract-glucose agar. Use three plates per flask. Incubate at room temperature but out of direct sunlight for 2 to 3 d. It is not necessary to invert dishes. To obtain pure cultures, pick from reasonably isolated colonies and restreak on the same medium or on malt extract agar plates. Obtain pure cultures of as many colonies as can be recognized.

d. Counting: It is impossible to obtain a meaningful plate count after this type of enrichment isolation because this is a qualitative test. An estimate of density could be made if several flasks were used and they were treated as a serial dilution test. An MPN-type calculation could estimate the original density.

3. Reference

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KURTZMAN, C.P. & J.W. FELL. 2004. Yeasts. *In* Biodiversity of Fungi, Inventory and Monitoring Methods, p. 337. Elsevier Academic Press, New York, N.Y.

9610 F. Zoosporic Fungi

1. Occurrence and Significance

The phylum Chytridiomycota is the only fungal group to include representatives with a flagellated stage. *Chytrids* are zoosporic fungi that reproduce asexually via motile, uniflagellate spores. These fungi may be found in lake and river habitats, and many live in parasitic relationships with algae and other fungi.[1] The chytrid *Batrachochytrium dendrobaidis* causes chytridosis in amphibians and has been implicated as contributing to amphibian species decline.[2]

Oomycota (commonly called *water molds*) have a similar morphology, but are not fungi because of their glucan/cellulose cell walls and the diploid nuclei in their non-septate vegetative body.[3] Oomycetes produce both oospores and biflagellate zoospores. (Taxonomists recently placed them with algae, but they will be discussed under fungi in this revision.)

Although polluted river areas have fewer species, they contain more Oomycetes than Chytridiomycetes. Species of the Oomycete genera, *Leptomitus* and *Saprolegnia* (notably *S. ferax*) seem to be more tolerant than other forms. *Saprolegnia* spp. are considered opportunistic facultative parasites that cause infections in fish and fish eggs,[4] important in aquaculture.[5]

2. Sampling and Baiting

These fungi rarely develop in sufficient numbers to be observed or collected directly, so various techniques have been devised for their collection and isolation. Collect samples in sterile 35-mL plastic vials, refrigerate, and (ideally) start analysis within 6 to 8 h. Place each sample in a sterile plate (20 × 100 mm) and dilute with 10 to 15 mL sterile reagent-grade water. As bait, add three to four split hemp seed halves (*Cannabis sativa*)* or whole mustard (*Brassica*) or sesame (*Sesamum*) seeds to each culture. (All bait material should be boiled for 3 to 5 min or autoclaved to reduce or remove extraneous microorganisms.[4]) Incubate at 18 to 25°C (the temperature closest to the environmental condition where found) and examine bait daily for fungal growth. As growth becomes evident (usually within 72 h), remove infected bait, wash it thoroughly using water from a wash bottle, and then transfer the cleaned bait to a fresh plate of water containing two to three halves of hemp or other seed. Alternatively, collect material that may include these fungi (e.g., algae, waterlogged material, or insect bodies[6]) and wash off. The water wash can be placed on a depression slide for 72 h or less for microscopic observation or placed on appropriate culture media and incubated at the same temperature and checked daily for growth and further identification or other studies.

Some chytrids may develop more slowly, and incubation for up to 1 week may be necessary. Other bait material can be used [e.g., purified shrimp exoskeleton (chitin) or corn straw (cellulose)].

Genera may be identified via the spore arrangement in the sporangium and the manner in which spores are released. Specific determination requires microscopic examination of the sexual reproductive structures.

To collect the few naturally occurring parasites or pathogens, place the host organisms in a plate containing sterile water and hemp seed.

3. Isolation

Once grown on baiting material, chytrids can be transferred to a modified nutrient agar medium containing antibiotics.[6] Although most filamentous Oomycetes can be cultivated on plain cornmeal agar, selective media have been developed to isolate *Saprolegnia* from fresh water.[7]

Obtain pure cultures by placing a small portion of material onto a depression slide with one or two drops of distilled water and drawing spores into a micropipet as they emerge from the sporangium. Hyphal tips could be used, but are less preferable because one piece of bait frequently contains several genera and species. Transfer the spore suspension or hyphal tip to a plate of cornmeal agar. Once growth occurs, remove bacteria-free hyphal tips aseptically by cutting out a small block of agar. Transfer to fresh medium or water. If growth is not free from contamination after one transfer, make additional transfers to ensure pure cultures. Contaminants can also be cut out of the agar medium. Other methods have been outlined.[6]

4. Dilution Plating

Make serial dilutions with sterile reagent-grade water (1:1 × 10^5 to 1:7 × 10^5) and spread 1 mL over the surface of a freshly prepared cornmeal agar plate. Remove each developing colony and transfer to water for identification. This method also permits numerical estimation, as well as determination of the Oomycete community's composition; however, at least three to five plates per dilution are needed over several dilutions, or else count 10 plates at one dilution for estimation purposes.

5. References

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9610 G. Aquatic Hyphomycetes

1. Occurrence and Significance

Freshwater Hyphomycetes are a specialized group of conidial fungi that usually occur on the submerged, decaying leaves of angiosperms. Ecological investigations of freshwater hyphomycetes have been limited to substrate, habitat, dispersal, and their role in the enhancement of leaf substrates as food for aquatic invertebrates. The usual habitat of the fungi is well-oxygenated water (e.g., alpine brooks, mountain streams, and fast-flowing rivers), but they also have been found in slow-running, often contaminated rivers, stagnant or temporary pools, melting snow, and soil. The numbers of species and individuals of aquatic hyphomycetes often increase from autumn until spring and decline between late spring and early summer.

The mycelium, which is branched and septate, ramifies through the leaf tissue, especially in petioles and veins. The conidiophores project into the water, and the conidia that usually develop are liberated under water, although they can be aerosoled. Mature conidia also can be found in the surface foam of most rivers, streams, and lakes. Most of these conidia are hyaline, thin-walled, and either tetraradiately branched (four divergent arms) or sigmoid (S-shaped), with the curvature in more than one plane. The conidia do not germinate while suspended in water—even for long periods—but on a solid surface, they will produce germ tubes within a few hours. Their spores' size and morphology make them potentially more prominent in plankton analysis work than the spores of other fungi.

2. Sample Collection and Storage

For most freshwater environments, collect foam or partially decayed, submerged, angiosperm leaves in sterile bottles. Refrigerate sample until analysis.

3. Sample Treatment and Analysis

Wash leaf samples in sterile distilled water and place one to three leaves in a sterile Petri dish (about 1 cm deep) containing sterile pond, river, or lake water. Incubate at room temperature. Within 1 to 2 d, mycelium and conidia develop. Conidiophores and conidia can be observed with a dissecting microscope on any portion of a leaf surface, but they are most frequently seen on petioles and veins. When released, conidia either remain suspended in water or settle to the bottom of the dish.

Using a dissecting microscope, pick up single conidium with a micropipet. Transfer each conidium in a drop of water to a microscope slide for identification. Conidia may be transferred with a sterile needle to a plate of 2% malt extract agar (pH 6.5) for colony production and then maintained on this medium at 20 ± 2°C until re-cultivation or disposal is needed. Taping plates closed will slow dehydration.

Search for conidia in foam samples with a dissecting microscope and isolate single conidia as described above. Submerge mycelial plugs from stock culture isolates of aquatic Hyphomycetes in autoclaved pond water in deep Petri dishes; conidia usually form within 2 to 10 d.

Conidia in all stages of development can be preserved on slides with lactophenol mounting medium in which either acid fuchsin or cotton blue (optional) is dissolved, and sealed with clear fingernail polish. To permit good adherence of the nail polish, avoid excessive amounts of mounting medium.

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9610 H. Fungi Pathogenic to Humans

1. Occurrence and Significance

Opportunistic fungi in hospital water systems are problematic for hospital patients recovering from illnesses and those who are immunocompromised.[1–4]* However, routine isolations of fungi from polluted streams and wastewater treatment plants usually yield relatively few species pathogenic to humans and other higher animals. Most pathogenic fungi are ascomycetes, although there are several human and animal pathogens among the basidiomycetous genera (e.g., *Cryptococcus, Trichosporon*, and *Malassezia*). *Exophiala mansonii*—also called *Phialophora jeanselmei* and *Trichosporium heteromorphum*—is isolated universally and can cause one form of chromomycosis (usually in the tropics). *Aspergillus fumigatus*, which can cause pulmonary aspergillosis, is commonly isolated. *Pseudallescheria* (*Petriellidium, Allescheria*) *boydii* can cause eumycotic mycetomas and other eumycotic conditions grouped under "Pseudallescheriasis";[1] it usually is recovered in its anamorphic state, *Scedosporium* (*Monosporium*) *apiospermum*. (*Scedosporium apiospermum* is the imperfect state of the fungus *Pseudallescheria boydii*, one of 16 species of true fungi that may cause mycetoma in humans.) Infection may be the result of a puncture wound by contaminated materials or of breathing contaminated air or water sprays. For a full discussion of pathogenic fungi, examine the clinical microbiology literature.

The presence of these fungi in stream water probably indicates soil runoff, because soil is the natural habitat of virtually all zoopathogenic fungi. Other zoopathogenic fungi occasionally are recovered from streams (whether polluted or not).

Another fungus, the yeast *Candida albicans*, can be recovered in varying numbers from wastewater treatment plant effluents, streams receiving such effluents, and recreational waters. This is not surprising because *C. albicans* is usually a commensal organism in humans, coexisting in harmony with its host and resistant to several antifungal drugs.[5] Up to 80% of normal, healthy adults have detectable levels of *C. albicans* in their feces, while about 35% harbor it in their oral cavities in the absence of any overt disease. Up to 50% of healthy asymptomatic females may harbor *C. albicans* in their lower genital tract microflora.

C. albicans has been isolated on routine media heavily supplemented with antibacterial antibiotics and cycloheximide, and it also has been isolated from estuarine and marine habitats on a maltose-yeast nitrogen base-chloramphenicol-cycloheximide medium.

2. PCR-based Methods for Human Pathogenic Fungi

Techniques, including primers and PCR-conditions, for rapid detection, identification, and enumeration of yeast (e.g., *Candida* spp. and *Cryptococcus* spp.) and filamentous fungi (e.g., *Aspergillus* spp., *Histoplasma capsulatum*) have been developed for use in the clinical laboratory for human samples. They can be easily adapted for detection, identification, and enumeration of pathogenic yeast and fungi in waters and soils. Refer to 9610I for directions on finding appropriate PCR primers and conditions for specific human fungal and yeast pathogens.

3. Identification of *C. albicans*

C. albicans is a facultative anaerobe and can be detected among the white and pink yeasts growing on a 0.8-μm black membrane filter on maltose-yeast nitrogen base-chloramphenicol-cycloheximide medium. From each colony, inoculate a 0.5-mL portion of calf or human blood serum, incubate at 37°C for 2 to 3 h, transfer a drop or two to a slide, and examine microscopically for the production of germ tubes from most cells. Inoculation must be light, or germination rate can be reduced. Of the white yeasts, only *C. albicans* produces these short hyphae from the parent cell within 2 to 3 h of incubation.[4] Germ tube test interpretation needs to address pseudohyphal formation, which can be mistaken for a germ tube (true hyphal formation).[6]

4. References

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* They are of great concern also in therapeutic pools and recreational waters, both indoors and outdoors.

9610 I. Polymerase Chain Reaction (PCR) Methods

1. Introduction

Polymerase chain reaction (PCR) based methods offer more specificity and speed than culture-based methods. However, because PCR-based methods require quantitative recovery of DNA or RNA from cells, mycelia, or spores, ensure that nucleic acids are completely recovered by running control experiments with cell, mycelial, or spore suspensions. In addition, run control reactions with DNA- or RNA-extracts to ensure that there is no inhibition by extracts, quite commonly encountered with soil extracts.

2. Samples

See Sections 9060A and 9610B.1.

Mycelia, cells, and spores can be collected by filtration (0.45 μm pore size) from a water sample or soil suspension.

3. DNA or RNA Isolation

Using *Candida albicans* as a model yeast and *Aspergillus fumigatus* as a model mycelia-forming fungus, researchers have measured and compared commercial kits' ability to provide PCR-quality DNA. A comparison of six commercial DNA extraction methods using PCR products demonstrated that enzyme-based lysis yielded highest levels of *C. albicans* DNA,* but not *A. fumigatus* DNA, while extraction methods involving mechanical disruption of hyphae† yielded high amounts of *A. fumigatus* DNA.[1] The specific primers for *C. albicans* and *A. funigatus* and the PCR reaction conditions used for assessing the DNA are described.[1]

A variety of kits are available commercially to isolate DNA from fungal and yeast cells, mycelia, and spores collected by either centrifugation or membrane filtration. A kit for fungal, yeast, and spore DNA isolation is available.‡

* Yeast Lysis Genome, Qbiogene, Irvine, CA.
† UltraClean Soil DNA Isolation Kit, MoBio, Inc., Solano Beach, CA, or equivalent.
‡ Fungi/Yeast Genomic DNA Isolation Kit, bioWORLD, Dublin, Ohio.

4. Procedure

The U.S. Environmental Protection Agency has published a document that lists PCR primers for a large number of fungi and yeast.[2] Refer to each reference for the PCR conditions for the fungus or yeast of interest. A qPCR-based method for detecting and enumerating *Batrachochytrium dendrobatidis*, the waterborne chytrid responsible for chytridiomycosis in amphibians, has been described.[3] A PCR assay for detecting *Histoplasma capsulatum* based on the M antigen has been described.[4] PCR primers for the 18S-28S-internal transcribed sequence (ITS) and PCR reaction conditions for identifying *Aspergillus* species have also been described.[5] PCR primers for amplification and sequencing of fungal rRNA genes have been published.[6] Finally, a PCR-based assay was developed for the large number of medically important yeast and fungi, based on amplification of the small subunit rRNA gene sequence.[7]

5. References

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9711 PATHOGENIC PROTOZOA*

9711 A. Introduction

1. Significance

Protozoa are an extremely diverse group of unicellular organisms; while there are many pathogenic species, only a few are considered problems for the water industry. Pathogenic protozoa are widely distributed in water supplies, are relatively resistant to inactivation by chemical disinfectants, and have caused numerous waterborne outbreaks. These organisms generally cause diarrhea or gastroenteritis of varying severity, although more serious consequences (including death) can occur. In the United States, 18% of drinking water-associated outbreaks between 1971 and 2006 were caused by protozoa.[1] Of the 325 water-associated protozoan disease outbreaks reported worldwide, most were caused by *Cryptosporidium* spp. (51%) and *Giardia duodenalis* (41%).[2] The rest were caused by *Entamoeba histolytica* (2.8%), *Cyclospora cayetanensis* (1.8%), *Toxoplasma gondii* (0.9%), *Isospora belli* (0.9%), and *Blastocystis hominis* (0.6%). *Balantidium coli*, microsporidia (*Encephalitozoon* spp. and *Enterocytozoon bieneusi*), *Acanthamoeba* spp., and *Naegleria fowleri* were associated with 0.3% of outbreaks, but a definitive association between drinking water and disease outbreaks has yet to be established for some of these organisms. This group of organisms has a wide variety of morphological and physiological characteristics, and while some are relatively closely related (e.g., *C. parvum*, *C. cayetanensis*, and *T. gondii*), *Giardia* spp. are considered some of the most ancient extant eukaryotes, and microsporidia may be more closely related to fungi than to protozoa.[3] Many parasitic protozoa form cyst, oocyst, or spore stages that allow them to survive in the environment and be resistant to some drinking water treatment practices. Except for the free-living *Acanthamoeba*, they are shed in the feces of infected humans and animals, thus following a fecal/oral route of transmission. Some species of *Cryptosporidium, Giardia,* and *T. gondii* can be transmitted to humans from infected animals. Some are opportunistic pathogens, but most are frank pathogens infecting individuals regardless of their immune status, although the ultimate disease outcomes may be different.

During the 1970s, waterborne outbreaks due to *Giardia duodenalis* (syn. *G. intestinalis, G. lamblia*) were noted with increasing frequency, especially in communities using unfiltered surface water sources. More than 120 outbreaks of disease related to private and municipal drinking water supplies have been reported in the United States.[1] *Giardia* cysts are common in environmental waters, and *G. duodenalis* has historically been the most frequently identified cause of waterborne disease outbreaks worldwide. Between 2003 and 2007, reports of giardiasis cases in the United States ranged from 16 521 to 20 962 per year,[4,5] although the actual incidence was probably much higher. The United States has an estimated 260 000 cases of giardiasis each year, and the overall incidence of *Giardia* carriage may be as high as 2%.[6] It is the most commonly detected protozoan parasite in the world.[7] The relative contributions of waterborne, foodborne, and person-to-person transmission to non-epidemic giardiasis is unknown, but given that cases peak in late summer and fall and that the highest number of cases involve 0- to 5-year-olds, one significant pathway may be communal swimming areas (lakes, rivers, swimming pools, and water parks) heavily used by children in that age group.[7] Cysts are infective immediately after being excreted from the infected host. Symptomatic infection can result in acute, chronic, or recurring bouts of diarrhea of varying severity. Mortality has rarely been reported, and effective drugs (e.g., metronidazole) are available.

In 1993, *Cryptosporidium* caused the largest waterborne outbreak in U.S. history.[8] Both drinking and recreational waterborne outbreaks have been reported. Infection with *Cryptosporidium* (cryptosporidiosis) can cause a severe diarrhea that is usually self-limiting in immune-competent individuals, but may be prolonged and life-threatening for immune-compromised individuals.[9] Nitazoxanide is the only treatment for cryptosporidiosis approved by the U.S. Food and Drug Administration, although its effectiveness is limited primarily to immune-competent individuals.[10] *Cryptosporidium* is identified in 2.2% of all diarrhea cases in developed countries, although it is more common in children (7% of childhood diarrhea cases) and AIDS patients (average of 14%).[11] Between 2003 and 2007, reports of cryptosporidiosis cases in the United States ranged from 3505 to 10 080 per year.[4,5]

Cryptosporidium is particularly problematic for water utilities because it is common in surface waters and because the oocyst stage, which is found in environmental waters, is resistant to chlorine disinfection at the concentrations typically applied during drinking water treatment. Oocysts are infective immediately after being excreted from the infected host. The species of most concern in human infections (accounting for >95% of isolates recovered from humans) are *Cryptosporidium parvum*, which is found in a wide variety of animals, and *Cryptosporidium hominis*, which is primarily a human-specific pathogen.[12,13] *C. meleagridis* accounts for less than 1% of human infections. Other *Cryptosporidium* species that are occasionally isolated from humans include *C. felis*, *C. canis*, *C. andersoni*, *C. suis*, *C. muris*, and various unnamed genotypes.[12–19]

Reports on the occurrence of *Cryptosporidium* spp. oocysts in untreated surface waters vary widely (4 to 100%) but the Information Collection Rule (ICR) survey of 5838 untreated source waters throughout the United States reported an average occurrence of 6.8% with a mean concentration of 0.067 oocysts/L.[20] Except for one recent incident, only *C. parvum* and *C. hominis* have been identified as causes of waterborne and foodborne outbreaks of cryptosporidiosis.[21–23] The only exception is a 2008 outbreak in England caused by a rabbit isolate that is closely

* Approved by Standard Methods Committee, 2011.
Joint Task Group: 22nd Edition—Paul A. Rochelle (chair), George D. Di Giovanni, Rebecca M. Hoffman, Patricia T. Klonicki, Randi M. McCuin, Gregory D. Sturbaum.

related to *C. hominis*.[24] The Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) mandates that U.S. water treatment plants monitor influents for *Cryptosporidium*.[25]

Two waterborne outbreaks due to *Toxoplasma gondii* have been reported: one involving U.S. soldiers exposed to contaminated creek water in Panama,[26] and one involving 100 acute cases associated with municipal drinking water in British Columbia, Canada.[27] In the Canadian outbreak, the implicated water system used unfiltered chloramine-treated surface water. *Toxoplasma* requires intermediate and final (definitive) hosts to complete its life cycle. Felines are the only animals known to act as definitive hosts and to produce oocysts that are excreted in the feces. The oocysts require an external maturation period of 1 to 5 d to sporulate and become infective. Infection (toxoplasmosis) is usually asymptomatic in healthy adults but may result in flu-like symptoms or swollen glands. Pregnant women can pass the infection to their fetus, resulting in birth defects (e.g., mental retardation, loss of vision and hearing) and sometimes death.[28] Disease in immunocompromised individuals can be severe, widely disseminated, and result in brain lesions and death. Oocysts have been detected in both ground and surface waters.[29,30]

Cyclospora cayetanensis is an apicomplexan parasite closely related to *Eimeria* spp. It is increasingly recognized as a human intestinal pathogen worldwide and has been most commonly associated with consumption of contaminated produce (strawberries, raspberries, mesclun, and basil) in North America. The organism has been detected in water used as a source of drinking water,[31] and one waterborne outbreak has been documented in the United States.[32] *C. cayetanensis* is frequently detected in children in the developing world, as well as travelers to these regions. The organism produces oocysts that are shed in feces and require an external maturation period (5 to 14 d at 22 to 32°C) to become infective. Infection is usually self-limiting in immuno-competent individuals but can cause diarrhea, abdominal cramps, nausea, fatigue, and weight loss, and it persists for several months.[33] The disease can be effectively treated with trimethoprim-sulfamethoxazole.

Entamoeba histolytica is an anaerobic parasitic protozoan that causes amoebic dysentery. It is common in developing countries but rare in the United States; it is transmitted in areas with inadequate sanitation, where human feces contaminate drinking water and food. The symptoms of infection may be mild (loose stools, stomach pain, and stomach cramping) but the more severe form of disease is associated with fulminating diarrhea, bloody stools, weight loss, and fever. Rarely, *E. histolytica* trophozoites invade the liver, forming an abscess, and may spread to other parts of the body (e.g., the lungs or brain). It is a significant cause of morbidity and mortality in tropical regions of the world, causing an estimated 40 000 to 100 000 deaths each year.[34] It is morphologically identical to but genetically and biochemically distinct from the non-pathogenic *Entamoeba dispar*, which is more common. Metronidazole treats infections relatively effectively. Two waterborne outbreaks in the United States have been attributed to *E. histolytica.*[1] One was tentatively linked to a community drinking water well, but *E. histolytica* was not detected in the well water even though fecal samples contained *E. histolytica*, *Campylobacter jejuni,* and *Giardia* sp.

Microsporidia are small, spore-forming, obligate intracellular parasites that infect every major animal group and are recognized as the cause of gastroenteritis, renal disease, sinusitis, and keratitis in humans. Traditionally, they were considered protozoa but have been reclassified as fungi.[3] There are more than 1200 species; the human pathogenic species are *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *E. hellem*, *E. cuniculi*, *Trachipleistophora* spp., *Vittaforma corneae*, *Nosema* spp., *Microsporidium* spp., and *Brachiola vesicularum*.[35,36] A microsporidia intestinal infection is primarily characterized by malabsorption, caused by injury to the small intestine epithelium. If untreated, it can lead to death due to chronic malnutrition and dehydration. Many animals may serve as microsporidia reservoirs, but the transmission route to humans is not clearly understood. Because the spores are shed in feces, urine, respiratory secretions, and other body fluids, waterborne transmission is possible. However, only one water-associated outbreak has been reported.[37] Two species of human-pathogenic microsporidia were detected in waters used to irrigate food crops that are traditionally eaten raw; however, there was no association with disease in this instance.[38]

Acanthamoeba are free-living amoebae (FLA) that can cause chronic encephalitis and keratitis.[39,40] They are found worldwide in animal intestines, vegetative material, soil, dust, air, fresh water, marine water, sediments, sewage, compost, tap water, and bottled water.[41,42] High numbers of amoebae are found in sediments and surface layers of fresh water lakes, corresponding to high-density bacterial populations. *Acanthamoeba* colonize hot tubs, chemical showers, eyewash fountains, drinking water fountains, dental units, dialysis units, swimming pools, air conditioning systems, humidifiers, and hot water systems, and their cysts are highly resistant to chlorine.[43] While *Acanthamoeba* spp. are the most prevalent of FLA, not all species or strains are pathogenic for humans. *A. castellanii, A. culbertsoni, A. hatchetti, A. healyi, A. polyphaga, A. rhysodes, A. astronyxis*, and *A. divionensis* have all been implicated in human infections.[44] Portals of entry leading to human infection include the respiratory tract, skin ulceration, contact with corneal tissue, and wounds. Amoebae disseminate to various organs and the brain via the bloodstream. Contact lenses and contaminated contact lens solutions are highly associated with *Acanthamoeba* keratitis.[45] Monitoring drinking water for amoebae is impractical because of the technical difficulty associated with recovering and identifying amoebae, and because no practical method is available to differentiate pathogenic from non-pathogenic strains. In addition, because non-water exposures (e.g., household dust) far exceed drinking water exposure and because ingestion is not associated with encephalitis, enhanced water treatment rules are unlikely to reduce the incidence of disease. The organism was included on the U.S. Environmental Protection Agency's (EPA's) first Contaminant Candidate List (CCL) but was removed after it was decided that no regulatory action was appropriate or necessary.[46]

Naegleria fowleri is a free-living amoebic protozoan that is common in warm bodies of fresh water, hot springs, discharge from industrial plants, and poorly maintained swimming pools. The organism enters nasal passages during water activities and causes a rare but usually fatal infection of the central nervous system: primary amebic encephalitis (PAM). Between 1960 and 1996, there were 175 cases worldwide; in 2007, there were six cases in the United States. Person-to-person transmission has not been documented and there is no effective therapy. One survey

of U.S. groundwaters reported that 16% of samples (n=185) were positive for *N. fowleri*.[47] In 2002, the deaths of two children were linked to *N. fowleri* in non-disinfected well water used for drinking water.[48]

A greater waterborne health risk than direct infection by FLA (e.g., *Acanthamoeba* spp. and *Naegleria* spp.) may be bacterial pathogens that can live and multiply inside FLA cells. *Legionella pneumophila* replicates inside the cysts and trophozoites of *Acanthamoeba* spp.,[49] and *Mycobacterium avium* cells become more virulent after passing through environmental amoebae.[50] Pathogenic *Chlamydiales* have been detected as endosymbionts of environmental and clinical isolates of *Acanthamoeba* spp.[51] At least 30 groups or species of bacteria—many of them pathogens—can infect amoebae. The amoebae may protect such bacteria from disinfection during drinking water treatment and provide a safe haven for proliferation in the distribution system.

2. Overview of Detection and Identification Methods

Methods for detecting and identifying pathogenic protozoa in environmental samples generally consist of three primary steps: sample collection and concentration; purification or separation of target organisms from other particulates in the sample; and a detection assay. Samples are usually filtered or centrifuged to concentrate the organisms. The concentrate may also contain non-target organisms and organic and inorganic debris, so purification procedures [e.g., density gradient centrifugation (flotation) and immunomagnetic separation (IMS)] may be used to minimize the quantity of interfering material in the concentrate.

Assay procedures may involve colorimetric determination, microscopic examination using dyes or fluorescent antibodies, molecular techniques [e.g., the polymerase chain reaction (PCR)], or infectivity in animals or tissue cultures. Primers and probes for molecular detection of a wide diversity of pathogenic protozoa have been published.[52] Selecting appropriate concentration, purification and assay procedures will depend on the matrix being sampled and sampling-program objectives. The method's precision and sensitivity will dictate the optimal procedure for a given application. Considerations include presence/absence detection versus quantification, identification to the genus or species level, and whether the detected microbes are viable and infectious. For example, a method that can produce rapid results might be more important when investigating a suspected waterborne disease outbreak than when determining the occurrence and distribution of organisms in a watershed. Whichever methods are selected, determining their recovery efficiency via spiked samples is critical before inferring pathogen occurrence.

Except for *Cryptosporidium* spp. and *Giardia* spp., there are no standardized or approved methods for detecting pathogenic protozoa in water. However, various approaches have been used to detect some of these protozoa. Procedures typically use 1- to 5-μm-porosity filters or hollow-fiber ultrafilters, centrifugation for primary or secondary concentration, and then microscopy (with or without special staining procedures) and/or PCR.

Many types of filter can be used to collect and concentrate environmental water samples. The most frequently used filters include nominal porosity polypropylene yarn-wound filters† for large volumes (>100 L), 1-μm-absolute-porosity capsule filters‡ for volumes up to 10 L, 1μm-porosity high-volume capsules§ for samples up to 1000 L, and compressed foam filter cartridges‖ for large volumes. Ultrafiltration (through 50 to 80 kDa molecular weight cut-off hollow-fiber filters) also efficiently recovers *Cryptosporidium* oocysts from large volumes of environmental and finished water samples.[53,54]

When examining wastewater, experts recommend direct centrifugation of a small volume (<250 mL) of raw wastewater or primary effluent (with potentially higher concentrations of cysts and oocysts), and filtration of a larger volume (10 to 20 L) of secondary or tertiary effluent.

While these filtration methods were developed primarily to recover *C. parvum* oocysts and *G. duodenalis* cysts, they have been used with varying levels of success for microsporidia, *C. cayetanensis*, and *T. gondii*.[29,55,56] Most of the protozoa of concern should be recovered using 1-μm-porosity filters. However, for smaller microbes (e.g., microsporidia, which have spores <1 μm in diameter), smaller porosity filters may be necessary.

Enterocytozoon bieneusi was detected in river water via filtration and PCR,[57] and *E. intestinalis* and *E. bieneusi* were detected via filtration through yarn-wound filters, density gradient centrifugation, and PCR.[55] A method based on continuous-flow centrifugation, indirect IMS, and PCR has also been proposed.[58] In addition, hollow-fiber ultrafiltration and modified Envirochek® capsules (0.6 μm porosity) have been used to recover microsporidia spores from water.[59] A procedure involving filtration through Envirochek capsules, elution in phosphate buffer containing 1% (v/v) Tween 80 and 1% (w/v) sodium dodecyl sulfate (SDS), centrifugation, and detection by epifluorescence microscopy and PCR was used to detect *C. cayetanensis* in wastewater.[56] *C. cayetanensis* was also found in a drinking water source via PCR on deoxyribonucleic acid (DNA) extracted directly from concentrated water samples.[32] *Naegleria fowleri* was detected in 90% of domestic water sources via filtration (1μm-porosity wound filters), centrifugation, inoculation onto lawns of heat-killed *Escherichia coli* on non-nutrient agar, and PCR.[48] An early method for detecting *T. gondii* oocysts in raw water involved filtering large volumes (>700 L) through 1 μm-nominal-porosity yarn-wound filters, followed by elution, centrifugation through Percoll# sucrose, and a mouse infectivity assay.[29] More recently, Envirochek capsules were used to recover *T. gondii* oocysts from environmental water samples, which were detected by PCR following sucrose gradient centrifugation.[60] Although not yet applied to water samples, an indirect IMS procedure with a recovery efficiency of approximately 70% has been developed for *T. gondii*, and could be incorporated into an overall detection procedure for water.[61]

As an alternative to direct centrifugation or filtration, procedures based on concentrating water samples via continuous-flow centrifugation have also been developed for *Cryptosporidium*, *Giardia*, and microsporidia.[58,62]

† Filterite Corporation, Timonium, MD.
‡ Envirochek capsules, Pall Corp., Ann Arbor, MI.
§ Envirochek HV, Pall Corp, Ann Arbor, MI.
‖ IDEXX Laboratories, Inc., Westbrook, ME.
Sigma, or equivalent.

In 1999, EPA approved Method 1622 to detect *Cryptosporidium* in untreated surface water; since then, the method has been revised to include additional sample collection and concentration methods.[63] EPA Method 1623 is similar to Method 1622 but detects both *Giardia* and *Cryptosporidium*.[64] Method 1623 was incorporated into the most recent round of regulatory *Cryptosporidium* monitoring in the United States under the LT2ESWTR.[25] Laboratories that want to analyze samples under this regulation need to be granted "approved" status through the EPA Laboratory Approval Program. The following sections describe the most commonly used version of Method 1623 for untreated water, along with the different filtration options. In addition, adaptations of the method for wastewater and treated drinking water are provided.

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9711 B. Detection of *Giardia* and *Cryptosporidium* in Water

1. Overview

Cryptosporidium and *Giardia* can be recovered from water samples using a variety of methods. When waters are highly contaminated or field filtration is impractical, collect grab samples (up to 20 L) in carboys and ship them to a laboratory for analysis. Volumes larger than 20 L are impractical for routine shipping. Depending on water quality characteristics and anticipated organism concentration, samples can be concentrated via direct centrifugation (1500 to 2000 × *g* for 15 min), continuous centrifugation, or filtration (e.g., flat membranes, pleated membrane capsules, compressed foam filters, and hollow-fiber ultrafilters). Pleated 1-μm-porosity membrane capsules can process large volumes of water, and have a relatively simple elution process (a wrist-action shaker followed by centrifugation). Compressed foam filters consist of multiple layers of foam disks and can also process large volumes. During elution, the compressed disks are expanded and washed; the eluant is then concentrated via a membrane or centrifugation. Hollow-fiber ultrafilters can simultaneously capture viruses, bacteria, and protozoa.[1–4]

EPA Method 1623, which has been widely adopted in the United States, is recognized as the standard method for detecting *Cryptosporidium* and *Giardia* in surface waters. It involves sample concentration, purification via IMS, and immunofluorescence microscopy.[5] Detected organisms are verified via staining with 4′6-diamidino-2-phenylindole (DAPI) and differential interference contrast (DIC) microscopy. The method was validated using surface waters before it was incorporated into the LT2ESWTR. Although this method is in widespread use, researchers continue to optimize and evaluate various components of it.[6–10] The method does not include viability or infectivity assessment or genetic analyses of detected cysts and oocysts, but these procedures can be incorporated through relatively minor modifications. Although validated for surface waters, the entire method or various components of it can be adapted for use with finished drinking water, wastewater, groundwater, and recycled water. In addition to sample-processing procedures, the published method also includes detailed quality assurance and quality control (QA/QC) procedures; equipment and reagent lists; information on sample collection, storage, and holding times; procedures for assessing precision and incorporating method modifications; equipment calibration; matrix spike preparation and analysis; and QC acceptance criteria. It is a performance-based method that allows for modifications so long as sufficient equivalency testing is conducted. Training modules for the method are available.* Acceptable performance criteria for recovery efficiencies in matrix spike samples are 13 to 111% for *Cryptosporidium* and 15 to 118% for *Giardia*. Revised acceptance criteria for ongoing precision and recovery (OPR) samples (spiked reagent water) are 22 to 100% for *Cryptosporidium*[11] and 14 to 100% for *Giardia.*[5]

Method 1623 includes four options for initial sample concentration: Envirochek and Envirochek HV filter capsules,† Filta-Max® compressed foam filters,‡ and a portable continuous-flow centrifuge (PCFC). The Envirochek filter is a pleated polyethersulfone membrane; the Envirochek HV (high volume) capsule is a polyester membrane. Each of the four options requires different post-concentration elution and processing before sample cleanup via IMS. Hollow-fiber ultrafiltration has not yet been validated for use with Method 1623. Method 1623 requires at least 10 L of untreated surface water for analysis, although depending on sample turbidity, more than one filter may be needed. The Envirochek HV and Filta-Max filters can be used for up to 50 L of surface water and up to 1000 L of finished water. No modifications of Method 1623 are necessary for 50 L surface water samples collected via Envirochek HV capsules, although extra QC samples are required.

After concentration, separate cysts and oocysts from organic and inorganic sample debris. Density gradient centrifugation, flocculation, and fluorescence activated cell sorting (flow cytometry) are useful in some circumstances, but IMS has become the method of choice due to its simplicity, high recovery efficiencies, and relatively high degree of specificity. IMS uses specific high-affinity monoclonal antibodies linked to the surface of magnetic particles to attach to cysts or oocysts. A magnet then separates target organisms from debris. Although a variety of IMS kits have been developed and evaluated, only one§ has been validated and approved for use with Method 1623.

As with most microbial detection procedures, method performance is matrix dependent. Despite removing much of the sample matrix during IMS, enough matrix can carry through the entire process and interfere with the adhesion of cysts and oocysts to the microscope slide. Matrices with high concentrations of silica and iron can be particularly problematic and frequently result in low recovery efficiencies. So, method modifications may be necessary to analyze samples containing high concentrations of extraneous iron and to adjust the pH of concentrated pellets from some source waters.[9] However, all method modifications must meet the QC criteria in the method.

While it is desirable to process samples as quickly as possible after they arrive at the laboratory, the holding times in Method 1623 allow for some flexibility in scheduling sample processing and slide examination. Filter elution must begin within 96 h of sample collection (grab samples) or filtration (filtered in the field). Elution, concentration, IMS purification, and microscope slide application must be completed within 1 work day. Staining must be completed within 72 h of applying the purified sample to the slide. However, spiking experiments on grab samples indicate that holding times can be extended (if necessary) with no significant effect on recovery.[8] If extension of holding times/temperatures is unavoidable, then conduct spiking experiments to determine their effects on recovery. However, specified holding times must be met when using the method for regulatory compliance samples.

For more complete details on the procedure, QA/QC requirements, reagent preparation, matrix spikes, safety, equipment calibration, and verification of analyst performance, see the most

* http://water.epa.gov/lawsregs/rulesregs/sdwa/lt2/compliance.cfm. Accessed February 2017

† Pall Life Sciences, Ann Arbor, MI.

‡ IDEXX Laboratories, Westbrook, ME.

§ Dynabeads®, Invitrogen, Carlsbad, CA.

recent version of Method 1623.[5] The basic procedures using Envirochek HV capsules or Filta-Max filters for untreated surface water samples are described below. The equipment and supplies listed are for all filtration and staining options, so some will be unnecessary (depending on the approach selected).

2. Equipment and Apparatus‖

a. Pump.
b. 10- to 50-L disposable or autoclavable carboys.
c. Wrist-action shaker.
d. Filta-Max processing equipment, manual washing station or automatic filter elution and processing instrument.
e. Portable continuous-flow centrifuge.
f. Flow meter with valve, 0.4 to 4 L/min.
g. Flow totalizer.
h. Vortex mixer.
i. Rotating sample mixer.
j. Centrifuge with swinging bucket rotor.
k. Magnetic particle concentrators (MPC), for 10 mL and microcentrifuge tubes.
l. Epifluorescence/DIC microscope with 20, 40, and 100× objectives, and fluorescein isothiocyanate (FITC) and DAPI excitation/emission filter cubes.
m. pH meter.
n. Laboratory balance.
o. Pipetting devices, pipets, and pipet tips.
p. Stir plates and stir bars.
q. Graduated cylinders.

3. Materials

a. Envirochek HV capsules.
b. Filta-Max compressed foam filters.
c. Dynabeads GC-Combo IMS kit.
d. Microscope well slides and coverslips.
e. Flat sided Leighton-type (L-10) tubes.
f. Centrifuge tubes, 1.5, 50, and 250 mL.
g. Autoclavable tygon tubing.

4. Reagents

a. Envirochek filter eluting solution: Laureth-12, 1 *M* Tris, pH 7.4, 0.5 *M* ethylenediaminetetraacetic acid (EDTA), pH 8, Antifoam A.
b. Filta-Max eluting solution: 0.1% Tween 20 in phosphate-buffered saline.
c. Sodium hydroxide,1 *N*.
d. Hydrochloric acid, 0.1 *N*.
e. Methanol.
f. Phosphate-buffered saline, pH 7.4.
g. Sodium dodecyl sulfate.
h. Tween 20.
i. Tween 80.
j. Fluorescent antibody stain.#
k. 4′6-diamidino-2-phenylindole (DAPI).
l. Non-fluorescing immersion oil.
m. Slide mounting medium: 1,4-diazabicyclo(2,2,2)octane (DABCO)/glycerol, Elvanol, or mounting medium supplied with fluorescent stains.
n. Clear fingernail polish or coverslip fixative.
o. Giardia cysts and Cryptosporidium oocysts for staining positive controls.

5. Procedure for Detecting *Giardia* and *Cryptosporidium* in Raw Water

Consult the most recent version of Method 1623 for complete method details.[3] Document each step of the procedure (time, date, analyst's name, reagent, and material lot numbers) using appropriate sample-processing bench sheets (available from the EPA Microbiology Web site).

Filter 10- to 50-L raw water samples in the field using a pressurized sample tap or portable pump (see Figure 9711:1) . F1 Alternatively, collect 10- to 20-L grab samples in sterile carboys and transport them to the laboratory for analysis. Sample temperature should be 1 to 10°C during storage and between 1 and 20°C during transport. Measure sample temperature during transport and storage via calibrated devices (e.g., a thermometer vial, adhesive temperature strip, infrared thermometer, or temperature-recording Thermochron iButton®).** The flow rate through the filter must not exceed 2 L/min for Envirochek capsules or 4 L/min for Filta-Max filters. The filtered volume should be measured via a flow totalizer or graduated carboy with 0.25-L graduations. All tubing should be new or sterilized by soaking it in a soap/chlorine solution, rinsing it thoroughly, and autoclaving it.

After filtration, remove the Envirochek HV capsule from sample-collection rig and allow excess water to drain through filter. With the outlet cap in place, fill capsule with 125 mL of elution buffer (0.1% Laureth-12, 1 mM EDTA, 10 mM Tris, 0.015% Antifoam A) sufficient to cover the top of the pleated membrane assembly. Install the inlet cap and agitate on wrist-action shaker at ~900 rpm for 5 min with the air bleed valve on the filter capsule in the 12 o'clock position. Remove the inlet cap and pour elution buffer into a 250-mL conical centrifuge tube. Fill capsule with a second volume of 100 to 125 mL elution buffer and agitate for 5 min on a wrist-action shaker with the air bleed valve in the 4 o'clock position. After 5 min, rotate the capsule so the bleed valve is in the 8 o'clock position and shake for another 5 min. Remove inlet cap and drain eluant into the centrifuge tube for a combined volume of approximately 250 mL. To ensure that as much sample as possible is recovered, invert capsule over centrifuge tube, remove outlet cap, and allow capsule to drain into centrifuge tube for approximately 15 min.

Elution from Filta-Max compressed foam filters is accomplished via a Filta-Max wash station or stomacher, after removing the filter from its housing. Elute organisms by expanding and compressing the filter 20 times in 600 mL of elution buffer (0.01% Tween 20 in PBS) using the wash station. Perform a second wash by expanding and compressing the filter 10 times in 600 mL elution buffer. Combine the two volumes of eluate and

‖ Specific needs depend on the filtration method used.
Aqua-Glo G/C Direct FL (Waterborne), Crypt-a-Glo (Waterborne), Giardi-a-Glo (Waterborne), EasyStain C&G (BTF Pty Limited), or MeriFluor *Cryptosporidium/Giardia* (Meridian Diagnostics), or equivalent.

** Maxim Integrated Products, 120 San Gabriel Drive, Sunnyvale, CA 94086.

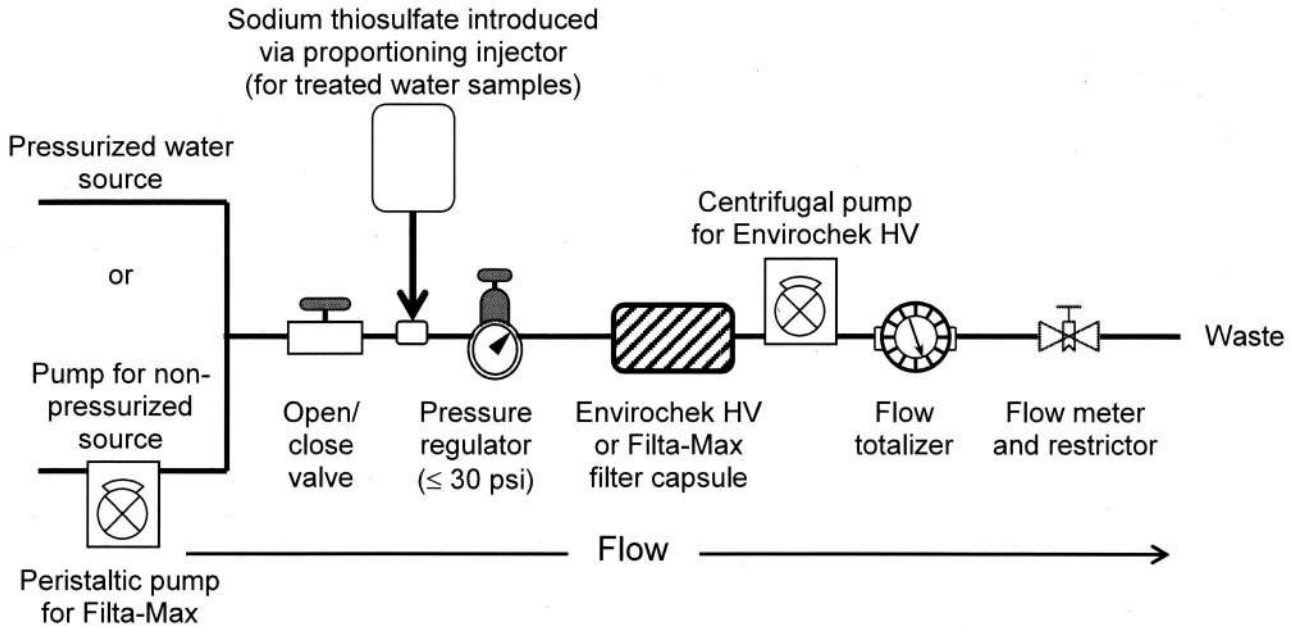


Figure 9711:1. Equipment configuration for sample collection using EPA Method 1623. SOURCE: Paul A. Rochelle, Metropolitan Water District of Southern California.

concentrate via the Filta-Max apparatus, which transfers eluate through a membrane filter. Alternatively, the combined eluate can be concentrated via centrifugation.

Using a swinging bucket rotor and centrifuge, pellet the eluted sample from Envirochek or Filta-Max filters at 1500 to 2000 *g* and ≤20°C for 15 min with no brake. Correct balancing of centrifuge tubes is critical because vibrations caused by imbalanced tubes will reduce recovery efficiencies due to cyst/oocyst loss. Remove supernatant via vacuum or manual aspiration, keeping the pipet tip as close to the liquid surface as possible as supernatant is withdrawn and lowering the center of the centrifuge tube. If using a vacuum aspirator, reduce supernatant volume to approximately 30 mL and then switch to manual aspiration with a 10-mL pipet to reduce volume to 5 mL. Careful aspiration is critical to minimize loss of cysts and oocysts. Refer to EPA Method 1623[5] for guidance on continued processing of samples that yield pellet volumes greater than 0.5 mL.

Vortex sample vigorously for 10 to 15 s to resuspend pellet, and transfer to a flat-side Leighton-type tube (L-10 tube) containing 1 mL of 10× (undiluted) SL Buffer-A and 1 mL of 10× (undiluted) SL Buffer-B. Complete homogenization of the resuspended pellet before transfer is important, particularly for samples with a high clay content. Rinse centrifuge tube twice with enough reagent water to bring the final volume in the L-10 tube to 12 mL. The volume of transferred sample, two reagent water rinses, and IMS buffers totals 12 mL. Add 100 µL each of Dynabeads Crypto-Combo and Giardia-Combo to sample and rotate on a sample mixer at 18 rpm for 1 h at room temperature. Ensure that all IMS reagents are at room temperature before use.

Use a single- or multi-tube MPC to concentrate the magnetic bead-cyst/oocyst complexes on the flat side of the L-10 tube by gently rocking the tube by hand or on a rocking platform for 2 min. Remove the cap and—keeping the tube in the MPC, with the flat side facing up—pour off liquid and discard. Do not allow L-10 tubes to remain stationary before decanting. Remove tube from MPC and gently resuspend all material in the tube in 0.5 mL 1× SL Buffer-A. Transfer the liquid to a 1.5- to 1.7-mL micro-centrifuge tube. Rinse L-10 tube twice with 0.5 mL 1× SL Buffer-A, and combine all liquid in micro-centrifuge tube. Use a smaller-capacity MPC†† to magnetically capture bead-cyst/oocyst complexes on the side of the microfuge tube using a gentle 180° rocking motion for 1 min, with the magnet in the vertical position. Without removing the tube or magnet from the MPC-S, remove supernatant from tube without disturbing the concentrated material attached to the side of the tube. Remove magnetic strip from MPC-S and add 50 µL 0.1*N* HCl to each tube. Vortex for 50 s and then allow tube to stand undisturbed for 10 min before vortexing again for 30 sec. Replace magnetic strip in MPC-S in the slanted position and allow tube to stand for 1 min.

Without removing tube from MPC-S, and with the magnetic strip still in place, transfer liquid sample to a well slide containing 10 µL 1*N* NaOH, being careful not to disturb the pellet of beads at the back of tube. Add another 50 µL 0.1*N* HCl to the tube containing beads and repeat the process. Combine both volumes on a microscope well-slide. Air-dry slide overnight in a dry box at room temperature or on a slide warmer at 35 to 42°C. Stain slides according to manufacturer's recommendations. Several staining reagents have been approved for use with Method 1623.‡‡

†† MPC-S, replaced by DynaMag-2; Invitrogen, Carlsbad, CA.
‡‡ Aqua-Glo™ G/C Direct (A100FLK); Crypt-a-Glo™ (A400FLK) and Giardia-Glo™ (A300FLK), Waterborne, Inc., New Orleans, LA; EasyStain™ C&G,

After 30 min staining, the stain solution and all subsequent rinses and DAPI stain can be removed by: 1) aspirating liquid with a pipet; 2) positioning slide vertically and allowing liquid to drain off; or 3) blotting liquid off the edge of the well with absorbent material. Remove stain and rinse each well with 75 μL PBS, then stain with DAPI at room temperature for 3 min. Rinse wells with 75 μL reagent water, and then cover each well with anti-fade mounting solution and a coverslip. Examine slides via epifluorescence microscopy (450- to 490-nm excitation, 510-nm dichroic mirror, 515- to 520-nm barrier filter) and characterize suspected cysts or oocysts via DAPI fluorescence (340- to 380-nm excitation, 400-nm dichroic mirror, 420-nm barrier filter) and DIC microscopy.

6. Detection of *Giardia* and *Cryptosporidium* in Finished Water

Analysts typically evaluate larger volumes of finished water (up to 1000 L) than of raw water. Collect finished water from pressurized sample taps or via a pump if a pressurized tap is unavailable. Flow rates can range from 0.7 L/min (total run time approximately 24 h for 1000 L sample) to 4 L/min (total run time approximately 4.2 h). Do not exceed 30 lb/in.^{2} or 4 L/min. Neutralize chlorine residual in treated water by adding 2% (w/v) sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) via a proportioning injector.

Although EPA has not validated Method 1623 for finished water, many laboratories have adapted it for this purpose. The only modification required when analyzing large volumes (500 to 1000 L) of finished water is, after sample collection, treat the Envirochek HV filter with the anti-scaling compound sodium hexametaphosphate ($[NaPO_3]_6$) before elution. After filtration, allow residual water to drain from the filter and then fill the capsule with 5% (w/v) sodium hexametaphosphate to a level approximately 1 cm above the pleated filter. Agitate the capsule for 5 min at ~900 rpm on a wrist-action shaker. Remove end caps and allow liquid to drain through the filter. Remove residual sodium hexametaphosphate by filling capsule with deionized water, rotating capsule manually for 30 s, and then allowing water to drain through the filter. Proceed with the rest of the elution, centrifugation, IMS, staining, and microscopy as described in Method 1623.

Until 2009, U.K. regulations required continuous *Cryptosporidium* monitoring of treated drinking water. The method required continuous sampling at a flow rate of at least 40 L/h in which the filter is changed every 24 h (≥960 L in 24 h). As with Method 1623, the U.K.-approved method involves sample collection via filtration through Envirochek HV capsules or Filta-Max compressed foam filters, secondary concentration and purification by IMS,§§ staining with FITC-labeled anti-*Cryptosporidium* antibody, and examination by fluorescence and DIC microscopy.[12] The method uses 0.5% sodium polyphosphate for pre-elution treatment. A slightly modified IMS procedure allows slides to be examined via solid-phase automated scanning cytometry.

BTF, Pty Limited, Sydney, Australia; MeriFluor® *Cryptosporidium/Giardia* (250050), Meridian Diagnostics, Cincinnati, OH; or equivalent.
§§ Dynabeads anti-*Cryptosporidium* and Isolate CryptoIMS are approved.

7. Molecular Detection Methods and Genotyping

Numerous PCR-based methods and primers for detecting *Cryptosporidium* and *Giardia* have been described and compared,[13–15] but PCR has not yet been developed as a routine tool for monitoring or regulatory compliance. The various PCR assays have different nucleic acid targets and include modifications for determining viability by detecting mRNA [reverse transcriptase polymerase chain reaction (RT-PCR)], different specificities (e.g., all *Cryptosporidium* spp. or only *C. parvum*) and sensitivities as low as 1 to 5 cysts or oocysts. DNA and ribonucleic acid (RNA) extraction methods differ greatly among the various published methods. Cyst and oocyst purification methods (e.g., IMS) and advances in nucleic acid purification have reduced, but not eliminated, interference by PCR inhibitors, which are often present in environmental samples. Most PCR-based assays were developed as presence/absence methods, but quantitative-PCR (QPCR) assays have also been developed.[16] Although PCR can be applied directly to DNA extracted from water samples concentrated via filtration and/or centrifugation, maximum sensitivity is achieved when oocysts are purified and concentrated via IMS before DNA extraction.[15] The advantages of PCR-based detection methods are their specificity and—when coupled with restriction fragment length polymorphism analysis or DNA sequencing—their ability to differentiate among species, genotypes, and isolates.

A compilation of genotyping data from 22 waterborne outbreaks demonstrated that 67% were caused by *C. hominis,* while 33% were caused by *C. parvum*.[17–18] Storm water samples were analyzed by PCR–restriction fragment length polymorphism (RFLP) (targeting the 18S rRNA gene), and 93% were positive for *Cryptosporidium* spp.[19] DNA banding profiles indicated that wildlife was the primary source of oocyst contamination in surface water during storms. Using the same method, the predominant genotypes in surface water were *C. parvum* and *C. hominis,* while *C. andersoni* was most commonly detected in wastewater.[20]

G. duodenalis is the only species of *Giardia* known to cause infection in humans, but the species is currently recognized as a heterogeneous complex of organisms. As with *C. parvum*, new genotypes from dogs, cats, rats, and some livestock animals may represent novel and distinct genotypes. The most recent taxonomic review recognizes seven subspecies of *G. duodenalis* (called assemblages A through G).[21] Based on a review of 1458 human fecal samples from different geographical locations, only assemblages A and B infect humans.[22] Assemblages A and B are found in humans and a wide variety of animals, while C through G seem to be host-specific. A new assemblage, H, was recently identified in marine wildlife.[23] Cysts of assemblages A and B have been isolated from water.[24]

Genotyping is also useful for other potentially waterborne pathogenic protozoa. Pathogenic *E. histolytica* is morphologically identical to non-pathogenic *E. dispar,* but they can be differentiated genetically.[25] Similarly, pathogenic and non-pathogenic isolates of *Acanthamoeba* spp. can be differentiated based on sequence differences in the small subunit (SSU) rRNA gene and non-coding genomic regions.[26] *E. cuniculi* has been isolated from many mammals, and there is some evidence for host-adapted genotypes.[27] *T. gondii* isolates also display genetic variation. More than 94% of all *T. gondii* isolates can be clas-

sified into one of three distinct lineages; Type 1 strains are virulent in mice, while Types II and III are not virulent.[28] It is not yet clear whether this characterization also applies to virulence in humans.

PCR-based identification of *Cryptosporidium* in water and molecular speciation or genotyping of oocysts detected by Method 1623 have not been approved by EPA, and it is unlikely that genotyping will be a regulatory requirement soon. However, a method was developed for oocysts isolated during routine water monitoring in the United Kingdom.[29] In addition, a simplified genotyping method for oocysts on regulatory monitoring slides has been developed and is currently undergoing international multi-laboratory evaluation.[30] Various studies and workshops have also been conducted in an effort to standardize genotyping methods and further their application to routine monitoring.[31–33]

One procedure involves sample concentration via filtration, oocyst purification and secondary concentration by IMS, DNA extraction using commercially available kits, PCR amplification of a fragment of the 18S rRNA gene, and restriction digestion or sequencing of the resulting amplicon.[19,34] Alternatively, oocysts can be genotyped following examination by epifluorescence microscopy and removal from the microscope slide.[34,35]

8. Viability and Infectivity

A variety of methods are available to assess the viability or activity of enteric protozoa, with application primarily to laboratory disinfection studies. These include three *in vitro* viability methods (excystation, inclusion/exclusion of fluorogenic dyes, and RT-PCR), infectivity in cell cultures, and animal infectivity. *In vitro* excystation has been used in laboratory disinfection and survival studies, but cannot be used to evaluate ultraviolet (UV) light disinfection or to study viability in environmental samples because of technical limitations. Exclusion of DNA stains or their passage across cell membranes is a measure of membrane integrity and may be interpreted as a marker of cellular viability. For *Giardia* cysts, the hydrolysis of fluorescein diacetate (FDA) by intracellular enzymes cleaving the fluorescein can be visualized by fluorescence microscopy under excitation of 450- to 490-nm wavelength as green fluorescence. Propidium iodide (PI) cannot transverse an intact lipid bilayer in the membrane because it is a large molecule. However, if the membrane is compromised, PI enters the cyst, binds to DNA, and can be visualized microscopically as a red fluorescence using 500-nm wavelength excitation. The inclusion of FDA and exclusion of PI as a marker of viability has been related to animal infectivity, excystation, and cyst morphology,[36,37] and it has been used to assess the potential viability of cysts isolated from contaminated water.[38] For *Cryptosporidium* oocysts, exclusion of PI or inclusion of DAPI was used to indicate a live oocyst.[39] Viewed using epifluorescence microscopy equipped with filters for 350-nm wavelength excitation and 450-nm wavelength emission, the nuclei inside the four sporozoites fluoresce a bright blue. The DAPI/PI test has been correlated with oocyst excystation and used to study oocysts in environmental waters.[38,39] Other nucleic acid stains (SYTO-9, hexidium and SYTO-59) have also been tested for *Cryptosporidium,* and their inclusion correlated with animal infectivity in chemical disinfection studies.[40] However, because these dye-based assays are known to overestimate viability, and the staining can be variable with a portion of cysts and oocysts not staining with either dye, exercise caution when interpreting results obtained with fluorogenic dyes. Although DAPI staining is included in Method 1623, the presence or absence of DAPI staining does not indicate viability or infectivity of the organism detected on the slide.

Methods using RT-PCR to detect messenger RNA (mRNA) from a variety of genes have been developed for assessing viability of *Cryptosporidium* oocysts and *Giardia* cysts.[41–43] Also, incorporating the DNA-intercalating dye propidium monoazide into PCR protocols allowed selective detection of viable *Cryptosporidium* oocysts.[44] However, these PCR-based viability methods have not been widely used.

While mouse infectivity was long considered the gold standard for assessing *Cryptosporidium* infectivity, *in vitro* cell culture has essentially replaced mouse models as the method of choice in the last 10 years. Animal assays are time-consuming and expensive, with significant hidden costs (e.g., maintenance of accredited facilities and license fees). In addition, mouse infectivity models do not support infection with *C. hominis,*[45–47] while both *C. parvum* and *C. hominis* infect cell cultures.[46–48] In extensive evaluations with five isolates of *C. parvum*, a cell culture assay was equivalent to a mouse assay for measuring the infectivity of untreated oocysts.[46] A dual direct detection method using differential immunofluorescent staining that allows detection of total oocysts and cell culture-infectious oocysts in a single analysis has also been developed.[49] Additional information on cell culture infectivity for *Cryptosporidium* is provided in 9711D.

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9711 C. Detection of *Giardia* and *Cryptosporidium* in Wastewater

1. Overview

Due to the widely varying composition of matrices in raw sewage, secondary effluent, and tertiary effluent, different methods are necessary to detect *Cryptosporidium* and *Giardia* in different types of wastewater. For raw sewage and primary wastewater influents, concentrate up to 500-mL samples via centrifugation followed by IMS for sample purification and further concentration, and detect via fluorescence microscopy. Although other methods and reagents are available, those included in Method 1622/1623 were demonstrated to yield relatively high recovery efficiencies (up to 84%) for spiked wastewater samples.[1] Adaptations of the method may be used for raw sewage, primary clarified effluent, and treated wastewater.[1,2] Method 1623 using Envirochek HV filters without modification detected *Cryptosporidium* in 67 to 83% of samples of influent, secondary effluent, and final disinfected effluent from wastewater reclamation facilities (1- to 400-L volumes)[3,4] and some states have approved Method 1623 for analysis of reclaimed wastewater.

2. Equipment, Materials, and Reagents

Most of the equipment and supplies listed in 9711B are required. Additional reagents are:

a. Phosphate-buffered saline containing 0.01% Tween 20 (PBST).

b. Kaolin.

c. Sodium hexametaphosphate, 5%.

3. Procedure

a. Raw sewage and primary wastewater effluent: Collect a 500-mL grab sample and add Tween 80 to a final concentration of 1%. Centrifuge at 1500 × g for 15 min, then aspirate supernatant down to a volume of 5 to 8 mL. Transfer this liquid to a Leighton (L-10) tube, rinse centrifuge tube with 2 to 5 mL PBST, and add rinsate to L-10 tube for a final volume of 10 mL. Add 0.75 g kaolin to each L-10 tube and gently mix. (Note: Do NOT vortex sample once kaolin is added.) Proceed with IMS, staining, and microscopic examination as described in Method 1623.

b. Secondary and tertiary effluent: Filter samples through Envirochek HV capsules. After filtration, allow residual water to drain from filter, then fill capsule with 5% sodium hexametaphosphate to a level approximately 1 cm above pleated filter element. Agitate capsule for 5 min at ~900 rpm on a wrist-action shaker. Remove end caps and allow liquid to drain through outlet port of filter capsule. Remove residual sodium hexametaphosphate by filling capsule with deionized water, rotate capsule manually for 30 s, and then allow water to drain through filter. Proceed with the rest of elution, centrifugation, IMS, staining, and microscopy as described in Method 1623.

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9711 D. Infectivity of *Cryptosporidium* in Cell Culture

1. Overview

Cryptosporidium infectivity assays typically have been used to assess the efficacy of disinfection processes[1–4] but also can be applied to oocysts in environmental water samples. Oocysts recovered via Method 1623 can be used in cell-culture infectivity assays by modifying the oocyst–magnetic bead dissociation step at the end of the IMS procedure and omitting microscopic examination. Although a variety of methods have been developed to detect infection in cell culture, the most widely used techniques are immunofluorescence microscopy, PCR, and RT–PCR.[5–7] Cell-culture assays have been used to detect infectious *Cryptosporidium* oocysts in raw wastewater, disinfected reclaimed effluent, raw source water, treatment plant filter backwash water, and finished drinking water.[3,6,8–10] Optimized cell cultures are sensitive to infections with low levels of oocysts and have been used to assess the infectivity of oocysts recovered from 1000-L samples of finished water.[11]

Although cell culture-based methods for assessing *Cryptosporidium* infectivity have been standardized in individual laboratories, and detailed protocols have been developed,[12] they have not yet been validated in multi-laboratory trials and none have been approved for regulatory use. There are a variety of approaches that include different cell lines and media formulations, oocyst treatments before inoculation, incubation periods, and infection detection assays. The various procedures for assessing *Cryptosporidium* infectivity in cell culture have been reviewed in detail.[13,14] Methods may vary depending on the intended application (e.g., assessing the efficacy of disinfection; detecting infectious oocysts in raw water, finished water, wastewater, or reclaimed water), so the following procedure is a consensus of published methods using the HCT-8 cell line.*

2. Facilities and Equipment

A detailed description of facilities, equipment, materials, and methods for maintaining cell cultures and of their use in conducting *Cryptosporidium* infectivity assays is beyond the scope of this volume; see standard handbooks on cell culture.[15,16] Cell culture assays are beyond the capability of most water and wastewater laboratories; they should only be conducted by trained microbiologists working in specially equipped, dedicated laboratory facilities. Most of the equipment and supplies will be available in a well-equipped microbiology laboratory. Additional specialized equipment and supplies include

a. Liquid nitrogen storage vessels.
b. Biological safety cabinet, Class II Type A/B3.
c. CO_2 incubators, 37°C.
d. Microscope, inverted.
e. Cell-culture flasks and chamber slides.

3. Procedure

Although antibiotics are used in the cell-culture medium to prevent bacterial and fungal growth, cell cultures are prone to contamination, so rigorous aseptic technique and laboratory QA/QC are essential. HCT-8 cells should be passaged at 3- to 4-d intervals (twice per week) using standard cell culture procedures. Culture medium for HCT-8 cells is RPMI-1640 with GlutaMAX† containing penicillin (100 U/mL), streptomycin (100 μg/mL), kanamycin (100 μg/mL), amphotericin B (0.25 μg/mL), and HEPES buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (20 mM). The medium is supplemented with 5% heat-inactivated fetal bovine serum (FBS) for cell maintenance and 10% FBS for cell growth during the infection assay. Adding nystatin and gentamicin to cell culture growth medium is useful for preventing microbial contamination when analyzing sewage samples.[17]

After recovering oocysts via Methods 1622 or 1623, follow a modified IMS oocyst–bead dissociation process. Instead of adding 0.1*N* HCl, resuspend the magnetically separated oocyst–bead complex in 200 μL of freshly prepared acidified Hanks balanced salt solution [pH 2.0 containing 1% w/v porcine pancreas type II-S trypsin (AHBSS/trypsin)]. Vortex for 10 s and then incubate for 1 h at 37°C, vortexing every 15 min. Immediately after the last vortexing, place tubes into the MPC for 10 s. Transfer supernatant containing the dissociated oocysts to a microfuge tube. Resuspend the oocyst–bead complex in an additional 100 μL of AHBSS/trypsin, vortex on high speed for 10 s, separate with MPC as above, and pool sample supernatants. Add 300 μL prewarmed (37°C) growth medium without trypsin to samples. Centrifuge all samples (with cap hinges pointing up) at 15 000 rpm in a microfuge for 2 min at room temperature with no brake. Immediately and carefully aspirate down to 50 μL; aspirate using a 200-μL micropipettor, with the tip just below the meniscus and on the front side of the tube wall away from the pellet. When aspirating, ensure that there are no droplets on the tube wall. Perform a second wash of all samples by adding 500 μL prewarmed (37°C) growth medium without trypsin. Centrifuge as above and aspirate down to 20 μL. Resuspend samples in 480 μL prewarmed growth medium by gentle up and down pipetting and scraping the tube wall with the pipet tip. There should be no clumps. Avoid making bubbles and over-pipetting.

Prepare cell monolayers by seeding 8-well chamber slides with 500 μL of growth medium containing 4×10^5 cells/mL and incubating at 37°C. Incubate cells until monolayers are 80 to 90% confluent, which typically takes 2 d. Inoculate the entire IMS-purified oocyst preparation onto a single monolayer and incubate for 65 to 72 h at 37°C in an incubator providing a 5% CO_2 atmosphere. After incubation, remove medium from well chambers with careful aspiration so monolayers are not disturbed. Do not wash monolayers because this may wash off some *Cryptosporidium* life stages. Add 500 μL of room-temperature absolute methanol to each chamber and incubate at room temperature for 10 min. Aspirate methanol and then allow monolayers to air dry.

Remove chambers from slides according to manufacturer's instructions. Place slides in staining tray with blocking buffer (2% goat serum and 0.002% Tween 20 in PBS) and place on

* ATCC CCL-244, http://atcc.org/.

† Invitrogen, Carlsbad, CA 92008.

rocking platform for 30 min. Slides may be stored overnight at 4°C at this point. Remove slides from blocking buffer and carefully dry around the edges of the slide, being careful not to disturb the cell monolayer. Add 1 mL primary antibody (rat anti-*Cryptosporidium* sporozoite antibody, diluted in PBS) to each slide and place slide in a humidified chamber at room temperature for 1 h. Determine antibody dilution for each lot (for reference, a 1:500 dilution is typically suitable). Remove primary antibody solution and wash cell monolayers four times with PBS, without disturbing monolayers. Add 1 mL labeled secondary antibody (FITC-labeled goat anti-rat IgG, 1:150 dilution in PBS) to each slide, and place slide in a humidified chamber at room temperature for 1 h. Remove staining solution and wash cell monolayers four times in PBS, without disturbing monolayers. Mount slides and examine by epifluorescence microscopy using an FITC filter cube. *Cryptosporidium* infection is identified as bright-green-stained clusters of life stages.

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PART 10000

BIOLOGICAL EXAMINATION

Part Coordinator
Michael K. Hein

Joint Task Group Chairs

10010 Introduction .....Michael K. Hein
10200 Plankton .....Harold G. Marshall
10300 Periphyton .....Robert G. Wetzel
10400 Macrophytes .....Miles M. Smart
10500 Benthic Macroinvertebrates .....Donald J. Klemm
10600 Fishes .....Donald M. Baltz
10700 Benthic Meiofauna .....Byron J. Adams
10750 Nematological Examination .....Byron J. Adams
10900 Identification of Aquatic Organisms .....Donald J. Reish

Summary of Major Changes Since 1998

An introductory section entitled Benthic Meiofauna (10700) is new.
The section on Nematodes (formerly 10550) was renumbered 10750 and moved accordingly.

10010 INTRODUCTION*

The physical and chemical characteristics of waterbodies affect the abundance, species composition, stability, productivity, and physiological condition of aquatic organism populations. Biological methods used to assess water quality include the collection, counting, and identification of aquatic organisms; biomass measurements; measurements of metabolic activity rates; measurements of pollutant toxicity, bioconcentration, and bioaccumulation; and processing and interpretation of biological data.

Information from these methods may serve one or more of the following purposes:

- To explain the cause of color, turbidity, odor, taste, or visible particulates in water;
- To help interpret chemical analyses (e.g., relating the presence or absence of certain biological forms to oxygen deficiency or supersaturation in natural waters);
- To identify the source of one water that is mixing with another;
- To explain the clogging of pipes, screens, or filters, and to help design and operate water and wastewater treatment plants;
- To determine optimum times for treating surface water with algicides and to monitor treatment effectiveness;
- To determine the effectiveness of drinking water treatment stages, to help determine the effective chlorine dose in a water treatment plant, and to indicate treatment problems or deficiencies;
- To identify the nature, extent, and biological effects of pollution;
- To indicate the progress of self-purification in waterbodies;
- To help determine the condition and effectiveness of unit processes and biological wastewater treatment methods in a wastewater treatment plant;
- To document short- and long-term variability in water quality caused by natural phenomena and/or human activities;
- To provide data on the status and trends of an aquatic system;
- To correlate the biological mass or components with water chemistry or conditions. (NOTE: A statistical correlation may not always signify a cause-and-effect relationship because of the presence of confounding variables or unknown covariates.)

The specific nature of a problem and the reasons for collecting samples will dictate which communities of aquatic organisms will be examined and which sampling and analytical techniques will be used.

The following communities of aquatic organisms are considered in the sections that follow:

- PLANKTON (Section 10200): A community of autotrophic (phytoplankton) and heterotrophic (zooplankton, bacteria, fungi) organisms, usually drifting or suspended in water, nonmotile or insufficiently motile to overcome transport by currents. In fresh water, they generally are small or microscopic; in marine or estuarine environments, large plankters are often observed.
- PERIPHYTON (Section 10300): A community of autotrophic (algae) and heterotrophic (bacteria, fungi, protozoa) organisms associated with the surfaces of submersed objects. Some are attached, some move about. Many of the protozoa and other minute invertebrates and algae found in plankton also occur in periphyton.
- MACROPHYTES (Section 10400): Large plants of all types. They are sometimes attached at the bottom (benthic), sometimes free-floating, sometimes totally submersed, and sometimes partly emergent. Complex vascular plants usually have true roots, stems, and leaves. Macroalgae are simpler but may have stem- and leaf-like thalli.
- MACROINVERTEBRATES (Section 10500): The invertebrates defined here are those retained by a U.S. Standard No. 30 sieve (0.6-mm openings). They are generally bottom-dwelling organisms (benthos) that live at least part of their life cycles within or upon available substrates in lentic (standing) and lotic (flowing) waterbodies.
- FISHES (Section 10600): Vertebrates of diverse morphology, ecology, and behavior, inhabiting (and generally limited to) aquatic systems. They have fins and gills.
- BENTHIC MEIOFAUNA (Section 10700): The invertebrates defined here are those that pass through a U.S. Standard No. 35 sieve (0.5-mm openings) and are retained by a No. 230 sieve (0.063-mm openings) or No. 325 sieve (0.044-mm openings). Benthic meiofauna include nematodes (Section 10750), express an extreme range of morphological and life history diversity, and have free-living, parasitic, or symbiotic trophic habits.

Large numbers of bacteria and fungi are present in plankton and periphyton, and constitute an essential element of the total aquatic ecosystem. Although their interactions with living and dead organic matter profoundly affect larger aquatic organisms, techniques for their investigation are not included herein (see Part 9000).

Amphibians, aquatic reptiles, birds, and mammals are useful in monitoring long-term changes in water quality and the presence of toxic substances (see Section 8930). These organisms may be affected directly or indirectly by spills or other discharges of pollutants.

Field observations are indispensable for meaningful biological interpretations, but many biological factors cannot be evaluated directly in the field. These must be analyzed as field data or field samples in the laboratory. Because the significance of the analytical result depends on the representativeness of the sample, attention is given to both field methods and associated laboratory procedures.

Before sampling begins, clearly define study objectives. For example, the frequency of a repetitive sampling program may vary from hourly, for a detailed study of diel variability, to every third month (quarterly) for a general assessment of seasonal conditions. The scope of the study must be adjusted based on limits in personnel, time, and budget. Before developing a study plan, examine historic data for the study area and conduct a literature search to identify related work elsewhere.

Whenever practicable, biologists should collect their own samples. Much of an experienced biologist's value lies in per-

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* Approved by Standard Methods Committee, 2006.
Joint Task Group: 22nd Edition—Michael K. Hein (chair), Byron J. Adams, Steven N. Francoeur, Donald J. Klemm, Ernst B. Peebles, Donald J. Reish, Miles M. Smart, Ann L. St. Amand, Paul V. Zimba.

sonal observations of field conditions and in the ability to recognize signs of environmental changes as reflected in various aquatic communities. Detecting environmental changes also depends on the accurate and consistent identification of the organisms present. Sections 10600 (Fishes), 10750 (Nematological Examination), and 10900 (Identification of Aquatic Organisms) include basic keys, drawings of organisms, and selected references to help biologists identify the plants and animals collected in field surveys. However, these cannot fully replace examination by taxonomic experts for key groups.

The primary orientation of Part 10000 is toward field collection and associated laboratory analyses to help determine the status of aquatic communities under field conditions and interpret the influence of past and present environmental conditions. Principal emphasis is on methods and equipment, rather than on interpretation or application of results. The complex interrelationships existing in an aquatic environment often require many field and laboratory procedures, so frequent cross-references between sections have been made.

Many other types of studies may be, and are being, conducted that are oriented more toward laboratory research. Such laboratory studies will develop further basic knowledge of community and/or organism responses under controlled conditions and will help predict the effects of future environmental changes on aquatic communities. However, such studies are not within the scope of Part 10000.

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10200 PLANKTON*

10200 A. Introduction

Plankton are microscopic aquatic life forms with little or no ability to resist current movement and, thus, live free-floating (suspended) in natural waters. This section covers both phytoplankton and zooplankton. *Phytoplankton* are microscopic algae that occur in unicellular, colonial, or filamentous forms; most are photosynthetic and eaten by zooplankton or other filter-feeding aquatic organisms. Freshwater *zooplankton* principally consist of protozoans, rotifers, cladocerans, and copepods; marine water zooplankton are more diverse. Other planktonic microscopic aquatic organisms are dealt with elsewhere: zoosporic fungi in Section 9610F; aquatic hyphomycetes in Section 9610G; and bacteria in Part 9000.

1. Significance

Plankton, particularly phytoplankton, has long been used as a water quality indicator, both in terms of standing crop and species composition.[1–4] They strongly influence certain nonbiological aspects of water quality (e.g., pH, color, taste, oxygen concentration, and odor). Some species flourish in highly eutrophic or acidic waters, while others are sensitive to (i.e., negatively affected by) organic and/or chemical wastes. Due to narrow environmental tolerances, certain species are extremely useful in determining historical water quality and thus future management direction.[5] In addition, the composition of a phytoplankton community indicates food quality for zooplankton, with implications for fisheries.

So, the compositions of phytoplankton and zooplankton communities are critical components of water-quality assessments. However, because of their transient nature and often patchy distribution, the utility of plankters as water quality indicators is limited.[6–12] Information on plankton as water quality indicators is best interpreted in the context of concurrently collected physicochemical and other biological data. Plankton also may be used to indicate the relative efficiencies of water treatment plants and the probability that groundwater sources are directly influenced by surface water.[13–18]

Some species of plankton develop noxious blooms that can decrease clarity, hurt recreational and aquacultural industries, and create offensive tastes and odors in drinking water.[19] Algal blooms may even create anoxic conditions or produce toxins that poison both aquatic and terrestrial organisms, resulting in animal or human illness or death.[20–29] So, algal blooms in both marine and freshwater environments raise ecologic, economic, and public health concerns. Both marine and freshwater species of cyanobacteria (commonly referred to as *blue-green algae*), dinoflagellates, and diatoms produce toxins. Most marine incidents are associated with dinoflagellates and diatoms, while most freshwater incidents are caused by cyanobacteria.[26,30] Several of these toxins have been found in seafood and drinking water, and have produced illness and fatalities in humans.[31,32] Sampling and analytical guidance for algal blooms and associated toxins currently are not included in *Standard Methods* (taste-and-odor compounds are discussed in Section 6040); however, general guidance is available.[33–37]

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10200 B. Sample Collection

1. General Considerations

The sampling approach and site selection will depend on the study's objectives. Sampling frequency, site location, the time of day samples are collected, the type of samples collected, and how they are collected need to be based on study objectives.[1,2] Put sampling stations as close to chemical and bacteriological sampling stations as possible to ensure maximum correlation of findings. Establish enough stations in as many locations as needed to adequately define the types and quantities of plankton present in the waters studied. The water's physical nature (standing, flowing, or tidal) will greatly influence the choice of sampling stations. Using sampling sites selected by previous investigators usually ensures that historical data are available, which will lead to a better understanding of current results and provide continuity in the study of an area.

In stream and river work, put sampling stations upstream and downstream of suspected pollution sources and major tributaries, as well as at appropriate intervals throughout the reach under investigation. If possible, put stations on both sides of the river because river water may not mix laterally for long distances downstream. Similarly, investigate tributaries suspected of being polluted but interpret data from a small stream carefully because much of the plankton may be periphytic, resulting from the flowing water's scouring of natural substrates. Plankton contributions from adjacent lakes, reservoirs, and backwater areas, as well as soil organisms carried into the stream by runoff, also can influence data interpretation. In addition, the depth at which water is discharged from upstream, stratified reservoirs can affect plankton.

Because river and stream water usually is well mixed vertically, subsurface sampling (i.e., the upper meter or a composite of two or more strata) often is adequate when collecting a representative sample. There may be problems caused by stratification due to thermal discharges, mixing of warmer or colder waters from tributaries and reservoirs, salt intrusion due to tidal influences, or other circumstances that encourage well-defined pycnoclines.[3] Sample in the main channel of a river and avoid sloughs, inlets, or backwater areas unless one of the investigation's goals is to characterize such areas. Samples collected in a river's main channel are most representative of general conditions, while sloughs, inlets, and backwater areas are more representative of localized habitats. In rivers that are mixed vertically and horizontally, measure plankton populations by examining periodic samples collected at midstream 0.5 to 1 m below the surface. When setting up a sampling program, remember that data from separate samples can always be composited,

but data from composites cannot be extrapolated into discrete samples.

If plankton distribution is uniform, use a random sampling scheme to accommodate statistical testing. Include a random selection of sampling sites and transects, as well as a random collection of samples from each site. On the other hand, if plankton distribution is variable or patchy, include more sampling sites, collect composite samples, and increase sample replication. Use appropriate statistical tests to determine population variability.

When sampling a lake or reservoir, use a grid network or transect lines in combination with random procedures. Take enough samples to make the data meaningful. While there is no unequivocal standard procedure, consider sampling a circular lake basin at strategic points along at least two perpendicular transects extending from shore to shore, and include the deepest point in the basin. Consider sampling a long, narrow basin at several points along at least three regularly spaced parallel transects that are perpendicular to the long axis of the basin, with the first near the inlet and the last near the outlet. Similarly, consider sampling a large bay along several parallel transects that are perpendicular to the long axis of the bay. Because many samples are required to appraise the plankton assemblage completely, it may be necessary to restrict sampling to strategic points (e.g., near water intakes and discharges, constrictions in the waterbody, and major bays that may influence the main basin). If only one sampling site can be established, an open water pelagic location near the deepest point in a lake or reservoir is generally considered the most representative.

In lakes, reservoirs, and estuaries where plankton populations can vary with depth, collect samples from all major depth zones or water masses, concentrating on the euphotic zone for phytoplankton and the complete water column for zooplankton. Sampling depths will be determined based on water depth at the station, the depth of a thermocline or pycnocline, or other factors. In shallow areas (2 to 3 m deep), subsurface samples collected at 0.5 to 1 m may be adequate. In deeper areas, collect samples at regular depth intervals. In estuaries, sample above and below the pycnocline. Sampling depth intervals vary for estuaries of different sizes and depths, but use depths representative of the vertical range. Composite sampling above and below the pycnocline is often used. In marine sampling, the extent of sample collection will depend on the study's intent and scope.

Special circumstances may arise over the continental shelf, where it is important to sample the entire vertical range. Take samples at stations approximately equidistant from the shore seaward. At each station, take a vertical series of samples from the water surface to nearly the bottom, gradually adding more stations across the shelf. Benthic grab samples may be taken to collect dormant resting cells or cysts. Beyond the shelf (in pelagic waters), sample in the photic zone from the surface to the thermocline (for phytoplankton) or deeper (for zooplankton). Sampling depths vary, but often are at 10- to 25-m intervals above the thermocline, at 100- to 200-m intervals from the thermocline to 1000 m deep, and then at 500- to 1000-m intervals at deeper levels.

Samples usually are referred to as "surface" or "depth" (subsurface) samples. Depth samples are taken at some stated depth, while surface samples are collected as near the water surface as possible. A "skimmed" sample of surface film plankton (neuston) can be revealing; however, ordinarily do not include a disproportionate quantity of surface film in a surface sample because neustonic plankton[4] often are trapped in the surface film with pollen, dust, and other detritus. Special methods may be needed to sample surface organisms.[5]

Sampling frequency depends on the study's intent, the range of seasonal fluctuations, meteorological conditions, the equipment's adequacy, and personnel's availability. Select a sampling frequency at some interval shorter than the plankton community's turnover rate. This requires consideration of life-cycle length, competition, predation, flushing, and current displacement. Frequent plankton sampling is desirable because of the plankton community's normal temporal variability and migratory character, but is not always practical. Daily vertical migrations occur in response to sunlight, nutrient concentrations, or predators. Random horizontal migrations or drifts are produced by winds, shifting currents, and tides. Both types of migrations will affect plankton data. Ideal characterization may require daily or more frequent sampling at multiple depths. When this is impossible, weekly, biweekly, monthly, or even quarterly sampling may still be useful for determining major population changes.

In river, stream, and estuarine regions subject to tidal influence, expect fluctuations in plankton composition over a tidal cycle. A typical sampling pattern at an estuary station includes a vertical series of samples taken from the surface, across the pycnocline, to near bottom, collected at 3-h intervals over at least two complete tidal cycles. Once a characteristic pattern is recognized, the sampling routine may be modified. If the sampling's focus does not require complete characterization but does require limiting influences, some standardization to match tidal cycles with each sample set may be adequate.

A useful series of references on freshwater and oceanographic methodology has been published.[6–12] Also, numerous taxonomic references for freshwater, estuarine, and marine phytoplankton are available in print.[13–35] In addition, several excellent Internet resources are available for verifying current taxonomy and taxonomic authority.[36–37]

2. Sampling and Storage Procedures

Once sampling locations, depths, and frequency have been determined, prepare for field sampling. Use opaque sample containers because even brief light exposure during storage will alter chlorophyll values. Sample-storage bottles should be made of polyethylene or glass to avoid metallic ion contamination, which can lead to significant errors when making algal assays or productivity measurements. Similarly, in multi-analyte sampling programs, store algal pigments in bottles without acid residues. For example, do not use bottles containing acidic preservatives for nutrients or Lugol's solution when microscopically enumerating phytoplankton: acidic preservatives preclude analyses for chlorophyll and other pigments.

To avoid confusion or error, label each container with the sampling date, cruise number, sampling station, study area (e.g., river, lake, reservoir), type of sample, and depth. Use waterproof labels and waterproof ink. When possible, enclose collection vessels in a protective container to avoid breakage. Do not add preservative to containers before sampling to avoid potentially

overfilled sample bottles, inaccurate preservative additions, and contamination with potentially hazardous preservatives. Sample size depends on the type and number of determinations to be made; the number of replicates depends on the statistical design of the study and statistical analyses selected for data interpretation. Always design a study around an objective with a predefined statistical approach rather than fit statistical analyses to data already collected.

In a field record book, note sample location, depth, type, time, meteorological conditions, turbidity, water temperature, salinity, other significant observations, and if possible, photodocument and record sample coordinates using a hand-held global positioning system (GPS). Field notebooks with waterproof paper are very suitable. Field data are invaluable when analytical results are interpreted and often help explain unusual changes due to the variable character of the aquatic environment. Collect coincident samples for chemical analyses to help define environmental variations that could affect plankton.

a. Phytoplankton:

1) Sampling procedures—If phytoplankton densities are expected to be low (e.g., in oligotrophic waters), collect a sample larger than 1.0 L. In richer, eutrophic waters, collect a 0.1- to 1.0-L sample. Sample size may affect results: be consistent and apply experience with the waterbody or analysis techniques to obtain the correct amount of sample for meaningful analysis. Collect additional samples (0.5 to 1.0 L) if samples need to be acid cleaned for diatoms/Chrysophyte scales or need to be shipped for verification.

Nets are unsuitable for most quantitative phytoplankton sampling. Theoretically, nets capture algae larger than the mesh size, while smaller forms pass through, so nanoplankton (>2.0 to 20 μm) and picoplankton (0.2 to 2.0 um) may be completely missed. In practice, however, nets often capture larger colonies and filaments, while smaller taxa flow through. Also, the passage of larger forms is well-known, though rarely quantified. Net losses are influenced by community composition, mesh quality and size, sampling speed, volume sampled, and net clogging.[22–25]

Even when organisms are captured, differential capture, cell or colony damage, and inefficient net cleaning introduce errors that result in an unreliable, non-quantitative characterization of most phytoplankton communities. However, nets remain a powerful qualitative collection tool, especially in teaching applications.

For qualitative and quantitative evaluations, collect whole (unfiltered and unstrained) water samples with a collection bottle consisting of a cylindrical tube with stoppers at each end and a closing device. Lower the open sampler to the desired depth and trip the closing mechanism (this may involve a messenger or tugging the line). If possible, obtain composite samples from several depths or pool repetitive samples from one depth. The most commonly used samplers that operate on this principle are the Alpha, Kemmerer,[26] Niskin/Nansen, and Van Dorn[27] (Figure 10200:1) samplers.

These samplers collect all sizes of phytoplankton, which can be subsequently segregated by filtering these whole water samples through netting with various mesh sizes. (NOTE: Larger particulates may pass through smaller mesh sizes than their long axis would indicate. Select appropriate mesh sizes to carefully concentrate the various sizes of phytoplankton typical of the aquatic system being studied, and be prepared for overlap.[28,38])

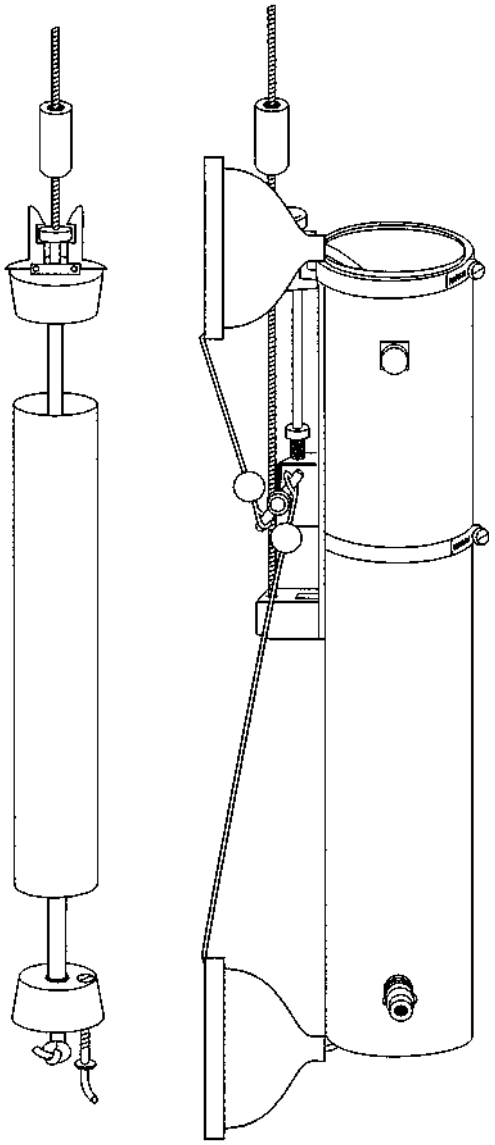


Figure 10200:1. Structural features of common water samplers, Kemmerer (left) and Van Dorn (right).

Van Dorn usually is the preferred sampler for standing crop, primary productivity, and other quantitative determinations because it does not inhibit the free flow of water through the cylinder. In deep-water and marine situations, the Niskin/Nansen bottle is preferred. The Niskin/Nansen sampler has the same design as the Van Dorn sampler except it can be cast in a series on one line to sample multiple depths simultaneously with the use of auxiliary messengers. The triggering devices of these samplers are sensitive, so avoid rough handling. Always lower the sampler into the water; do not drop. Kemmerer and Van Dorn samplers have capacities of 0.5 L or more. Polyethylene or polyvinyl chloride sampling devices are preferred to metal samplers because the latter liberate metallic ions that may contaminate the sample.

In shallow waters, use a Jenkins surface mud sampler,[39] a bottle sampler modified so it is held horizontally,[40] or an appropriate bacteriological sampler.[41]

For greater collection speed, and to obtain large, accurately measured quantities of organisms, use a pump. Diaphragm and peristaltic pumps are less damaging to organisms than centrifugal pumps.[42] Centrifugal pump impellers can damage organisms, as can passage through the hose.[43] Lower a weighted hose, attached to a suction pump, to the desired depth and pump water to the surface. Pumps can supply a homogeneous sample from a given depth or an integrated sample from the surface to a particular depth. If a centrifugal pump is used, draw samples from the line before they reach the impeller. For samples to be analyzed for organochlorine compounds, use tetrafluoroethylene (TFE) tubing.

2) Storage procedures—To examine live samples, partially fill containers and store them in a refrigerator or ice chest in the dark (if not using opaque bottles); examine specimens promptly after collection.

If living material cannot be examined or if phytoplankton will be counted later, preserve the sample. There are multiple phytoplankton preservatives. Lugol's solution and glutaraldehyde are the most commonly used; others include formalin, merthiolate, and "M3" fixative. Also, adding a few crystals of copper sulfate to any preservative stock solution helps maintain the algae's color. **CAUTION: All preservatives are a hazardous chemical risk; consult the appropriate safety data sheets (SDSs) before working with any preservative. Glutaraldehyde and formalin, in particular, must be used in a well-ventilated area or positive flow hood.**

a) Lugol's solution—Lugol's solution, which can be used for most forms (e.g., naked flagellates), stains organisms that store starch (especially chlorophytes and cryptophytes) and tends to cause most cyanobacteria to settle. Unfortunately, acidic Lugol's solution dissolves the coccoliths of Coccolithophores (which are common in estuarine and marine waters), tends to cause freshwater chrysophytes and some cyanobacterial colonies (especially *Microcystis* and *Aphanizomenon*) to disintegrate, and must be spiked every 6 to 12 months because of its volatility. Lugol's solution (and, to a lesser extent, formalin) also squelches autofluorescence.

To preserve samples with Lugol's solution, add 0.3 mL Lugol's solution to 100 mL sample and store in the dark. For long-term storage, add 0.7 mL Lugol's solution per 100 mL sample. The sample should look like weak tea. If Lugol's solution cannot be re-added every 6 to 12 months, add buffered formalin to a minimum of 2.5% final concentration after 1 h (however, formalin tends to distort many cells). Alternatively, glutaraldeyhyde can be added to a final concentration of 0.25 to 0.5%, which results in less cell distortion.

Prepare Lugol's solution by dissolving 20 g potassium iodide (KI) and 10 g iodine crystals in 200 mL distilled water containing 20 mL glacial acetic acid.[44] Utermohl's[45] modification of Lugol's solution results in a neutral or slightly alkaline solution. Prepare modified Lugol's solution by dissolving 10 g KI and 5 g iodine crystals in 20 mL distilled water, then adding 50 mL distilled water in which 5 g anhydrous sodium acetate has been dissolved. This preserves Coccolithophores (which are most common in marine waters) but would be less effective for other flagellates.

b) Glutaraldehyde—Glutaraldehyde is in the same chemical class as formalin but used at a much lower concentration (0.25 to 0.5% versus 3 to 4% final concentration). Glutaraldehyde also causes minimal distortion, tends to preserve colony structure in most algal groups, lasts in samples for years without degradation (formalin can form crystals after 15+ years) and preserves autofluorescence. As long as the preservative percentage does not exceed 2%, no compensation calculation is needed. As a result, glutaraldehyde has gained prominence in freshwater phytoplankton and periphyton work.

Preserve samples by adding neutralized glutaraldehyde to yield a final concentration of 0.25 to 0.5%. If the sample is exceptionally dense, use a maximum concentration of 1% glutaraldehyde. Nalgene or glass bottles are suitable, and amber or opaque bottles are preferred so autofluorescence can be used in analysis. When a preserved sample has been shaken after an appropriate reaction time (about 1 h), the sample should develop temporary foam on the surface. Once preserved with glutaraldehyde, refrigeration is unnecessary and light sensitivity is much reduced; however, keep samples out of direct sunlight. **CAUTION: Keep 25% gluteraldehyde in the fume hood and work with it in a well-ventilated area.**

c) Formalin—To preserve samples with formalin, add 40 mL buffered formalin [20 g sodium borate ($Na_2B_2O_4$) + 1 L 37% formaldehyde] to 1 L of sample immediately after collection. **CAUTION: As with glutaraldehyde, keep concentrated fomaldehyde in the chemical fume hood and work in a well-ventilated area.** In estuarine and marine collections, adjust pH to at least 7.5 with sodium borate for samples containing Coccolithophores.

d) Merthiolate—To preserve samples with merthiolate, add 36 mL merthiolate solution to 1 L of sample and store in the dark. Prepare merthiolate solution by dissolving 1.0 g merthiolate, 1.5 g sodium borate, and 1.0 mL Lugol's solution in 1 L distilled water. Merthiolate-preserved samples are not sterile, but can be kept effectively for 1 year, after which time formalin or glutaraldehyde must be added.[46]

e) "M3" fixative—Prepare by dissolving 5 g KI, 10 g iodine, 50 mL glacial acetic acid, and 250 mL formalin in 1 L distilled water (dissolve the iodide in a small quantity of water to aid in solution of iodine). Add 20 mL fixative to 1 L sample and store in the dark.

Most preservatives distort and disrupt certain cells,[47,48] especially those with delicate forms (e.g., *Euglena, Cryptomonas, Synura, Chromulina,* and *Mallamonas*). Glutaraldehyde solution usually is least damaging for such phytoflagellates, although all preservatives create some level of preservation artifact. To become familiar with live specimens and preservation-caused distortions, use reference collection material from biological supply houses, work extensively with live material, and consult experienced co-workers. Taxonomic consistency over long-term projects is critical. Document the basis for identification carefully, making sure that morphological variation is described clearly and identification references are permanently associated with the data.

b. Zooplankton:

1) Sampling procedures—The choice of sampler depends on the type and size distribution of zooplankton, the kind of study (distribution, productivity, etc.), and the body of water being investigated. Zooplankton populations invariably are distributed in a patchy way, making both sampling and data interpretation difficult.

To collect microzooplankton (20 to 200 μm), such as protozoa, rotifers, and immature microcrustacea, use the bottle sam-

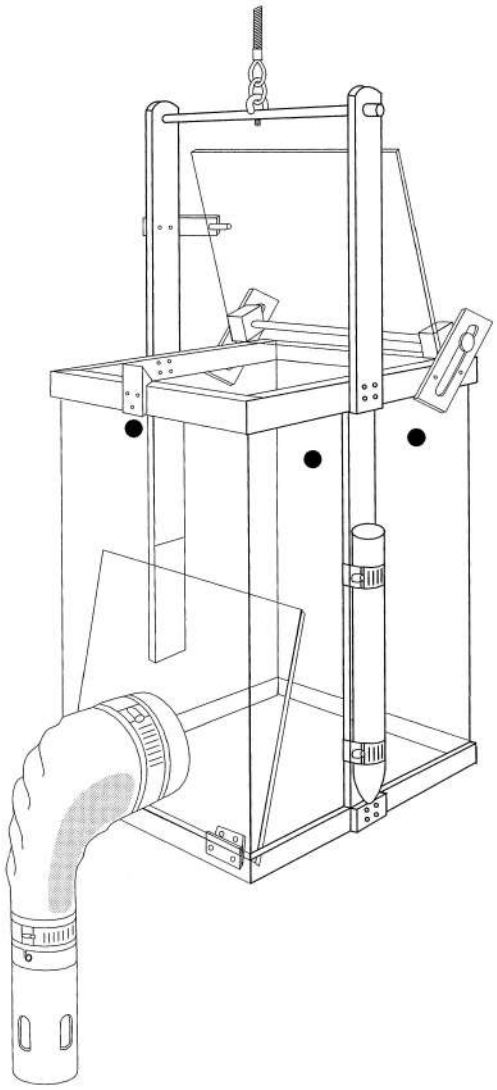


Figure 10200:2. The Schindler–Patalas plankton trap.

plers described for phytoplankton. Small zooplankters usually are sufficiently abundant to yield adequate samples in 5- to 10-L bottles; however, composite samples over depth and time are recommended. Water bottle samplers are suitable especially for discrete-depth samples. If depth-integrated samples are desired, use pumps or nets. Larger and more robust microzooplankters (e.g., loricate forms and crustacea) may be concentrated by passing the whole water through a 20-μm mesh net. If quantitative estimates of other nonloricate, delicate forms are required, do not screen. Fix 0.5 to 5 L of whole water to enumerate these forms.

Bottle samplers usually are unsuitable for collecting larger zooplankton (e.g., mature microcrustacea) that, unlike smaller forms, are much less numerous and are sufficiently agile to avoid capture. Although a pump can be used to sample comparatively large water volumes, and consequently adequate numbers of microcrustacea, avoidance by larger, more agile zooplankters at the pump head can cause sampling error. Consequently, larger trap samplers or nets are the preferred collection methods.

TABLE 10200:I. CHARACTERISTICS OF COMMONLY USED PLANKTON NETS

SILK NO.	SIZE OF APERTURE MM	APPROXIMATE OPEN AREA %	CLASSIFICATION
000	1024	58	Largest zooplankton and ichthyoplankton
00	752	54	Larger zooplankton and ichthyoplankton
0	569	50	Large zooplankton and ichthyoplankton
2	366	46	Large microcrustacea
6	239	44	Microcrustacea
10	158	45	Microcrustacea and most rotifers
20	76	45	Net phyto- and zooplankton
25	64	33	Nanoplankton

The Juday trap[49] operates on the same principle as water bottle samplers but is generally larger (10 L) and so more suitable for collecting zooplankters, especially larger copepods. However, it is awkward to use and its 10-L capacity is inadequate for oligotrophic lakes or other waterbodies with few zooplankters. Also, it is constructed of metal and so is unsuitable if heavy metals analyses are required.

The Schindler–Patalas trap[50] (Figure 10200:2) usually is preferred to the Juday trap because it is constructed of clear acrylic plastic (i.e., is transparent). It can be lowered into the water with minimal disturbance and is suitable for collecting larger zooplankters. Models of 10- to 12-L capacity are available, but the 30-L size is preferred. It has no mechanical closing mechanism, so it is convenient for cold-weather sampling, when mechanical devices tend to malfunction. Like the Juday trap, it can be fitted with nets of various mesh sizes. [NOTE: Mesh sizes less than 125 μm (No. 120 and larger) may rapidly clog when large colonial or filamentous phytoplankton are abundant or zooplankters with sheaths (e.g., *Holopedium*) are present.]

Plankton nets are preferred to sampling bottles and traps in areas where plankters are few, are vertically distributed, or only qualitative data or a large biomass is needed for analysis. Because they originally were designed for qualitative sampling, modifications are required for quantitative work, and nets remain a poor choice for quantitative phytoplankton work.

The mesh size, type of material, orifice size, length, hauling method, type of tow, and volume sampled will depend on the study.[51,52] The type of netting and mesh size determine filtration efficiency, clogging tendencies, velocity, drag, and sample condition after collection. Silk, which used to be the common mesh material in plankton nets, is not recommended because its mesh openings shrink and rot with age. Nylon monofilament mesh is preferred because of its mesh size accuracy and durability. Nylon-net mesh sizes still are labeled by the silk rating system. The characteristics of commonly used nylon plankton nets are listed in Table 10200:I. Finer mesh sizes clog more readily than coarser mesh; when sizing mesh, a compromise must be made between mesh small enough to retain desired organisms effectively and large enough to preclude a serious clogging problem. If clogging occurs, there are several options, depending on whether it is

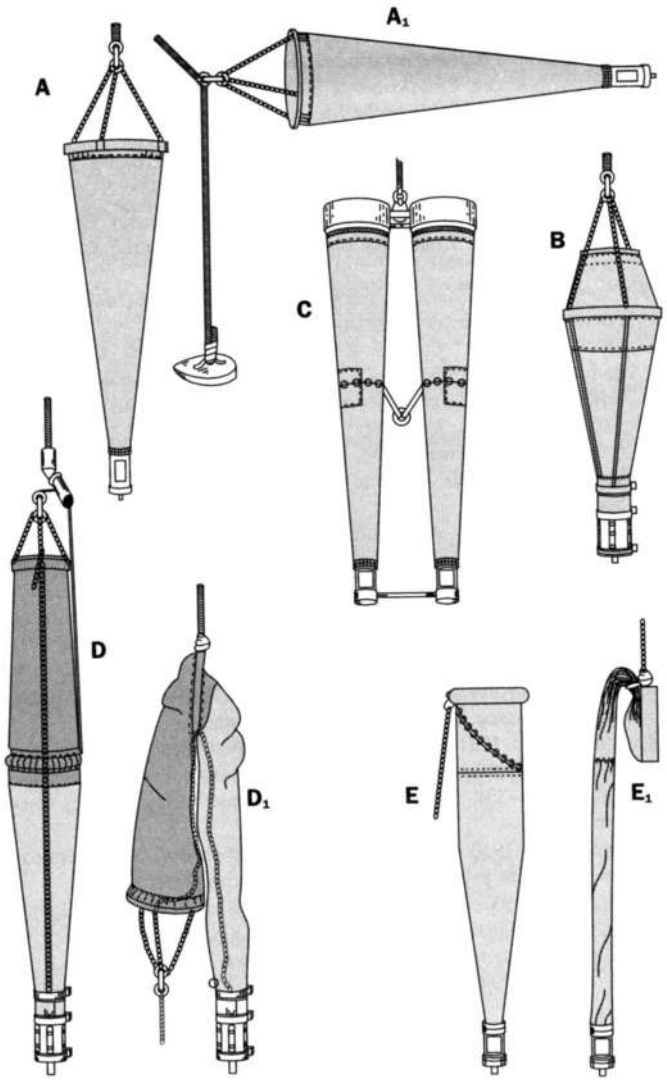


Figure 10200:3. Examples of commonly used plankton sampling nets. (A) Simple conical tow-net: A—rigged for vertical tows; A_1—for oblique or horizontal tows; (B) Wisconsin (Birge) tow-net with truncated cone to improve filtration efficiency; (C) Bongo net—can be fitted with flow meters and opening/closing mechanisms; (D) Wisconsin net fitted with messenger-activated closing mechanism: D—open, D_1—closed; (E) Free-fall net: E—open, E_1—closed.

phytoplankton- or zooplankton-related: decrease tow length, increase mesh size, preserve a larger volume of water, or collect whole water samples.

The maximum volume (V_M) of water that can be filtered through a net during a vertical tow can be estimated as follows:

$$V_M = \pi r^2 d$$

where:

r = radius of net orifice and
d = depth to which net is lowered.

Nets have maximum filtration volumes because mesh clogging by phytoplankton and other particles and, for fine netting, even the netting itself can cause some water to be diverted from the net's path.[53,54] Keep net towing distance as short as practical to alleviate clogging. If the net has a pronounced green or brown color after towing, it is probably clogged.

To estimate sampling volume (V_A), mount a calibrated flow meter midway between the net rims and mouth center (the meter is mounted off-center to avoid the flow reduction associated with the towing bridle).[55] Equip meter with lock mechanisms to keep it from turning in reverse or while in air. Record flow-meter readings before and after collecting sample. Calculate filtration efficiency (E) as follows:

$$E = V_A/V_M$$

If E is less than about 0.8, substantial clogging has occurred. Take steps to increase efficiency. Clogging not only decreases the volume filtered, but also leads to biased samples due to non-uniform filtration efficiency during the tow.[52] Net efficiency on a per-species basis can be determined for each system, tow depth, and sampling interval by using a vertical sampler to composite samples from tow depth to the surface.

Various types of plankton nets are shown in Figure 10200:3. Simple conical nets have been used for many years with little modification in design or improvement in accuracy. Their major source of error is that conical nets' filtration characteristics usually are unknown. Filtration efficiency in No. 20 mesh cone nets ranges from 40 to 77%. To improve efficiency, place a porous cylinder collar or nonporous truncated cone in front of the conical portion of the net. The Juday net is a commonly used net with a truncated cone. For good filtration characteristics, the ratio of the net's filtering area to orifice area should be at least 3:1. Bridles attaching the net to the towing line also adversely influence filtration efficiency and increase turbulence in front of the net, thereby increasing the potential for net avoidance by larger zooplankters. The tandem, Bongo net design (Figure 10200:3C) reduces these influences and permits duplicate samples to be collected simultaneously.

Three types of tows are used: vertical, horizontal, and oblique. Vertical tows are preferred to obtain an integrated water column sample. To make a vertical tow, lower a weighted net to a given depth, then raise vertically at an even speed of 0.5 m/s.

In small waterbodies, haul the net hand over hand with a steady, unhurried motion approximating the speed of 0.5 m/s. In large waterbodies where long net hauls and vessel drifting are expected, use a davit, meter wheel, angle indicator, and winch. Attach a 3- to 5-kg weight to hold the net down. Determine the net's depth by multiplying the length of the extended wire by the cosine of the wire's angle with the vertical direction. Maintain wire angle as close to vertical as possible by controlling the boat's speed null against the wind drift, or wherever feasible, do vertical hauls from an anchored boat.

Vertical and oblique tows collect a composite sample, and horizontal tows collect a sample at a discrete depth. Oblique tows usually are preferred over vertical tows in shallow water or wherever a longer net tow is required. For oblique tows, lower

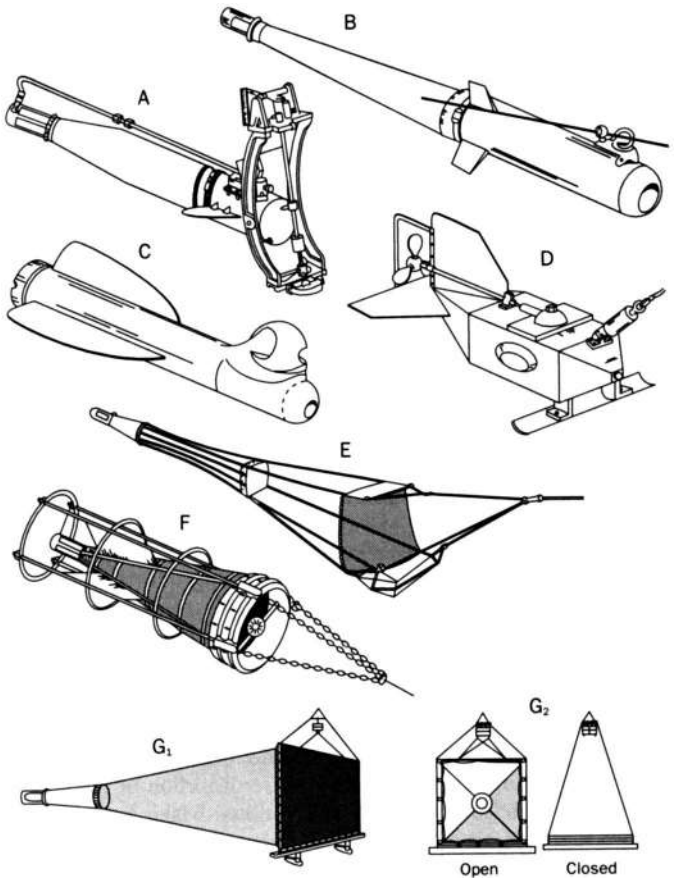


Figure 10200:4. Examples of commonly used high-speed zooplankton samplers. (A) Clarke–Bumpus sampler; (B) Miller sampler; (C) Hardy plankton indicator; (D) Hardy continuous plankton recorder; (E) Issacs–Kidd mid-water trawl; (F) Gulf V sampler; (G) Tucker trawl: G_1—sideview, G_2—front view open and closed.

the net or sampler to some predetermined depth and then raise it at a constant rate as the boat moves forward. Oblique tows do not necessarily sample a true angle from the bottom to the surface. Under best conditions, the pattern is somewhat sigmoid due to boat acceleration and slack in the tow line.

Horizontal tows usually are used to obtain depth distribution information on zooplankton. Although a variety of horizontal samplers is available (see Figure 10200:4), use the Clarke–Bumpus sampler[56] for quantitative collection of zooplankton because of its built-in flowmeter and opening–closing device. For horizontal tows, use a boat equipped as above and determine sampler depth as above. Lower sampler to preselected depth, open, tow at that depth for 5 to 10 min, then close and raise it.

A variety of zooplankton sampling methods can be used in flowing water. The choice depends largely on flow velocity. Properly weighted bottles, traps and pump hoses, and nets can be used in medium- to slow-flowing waters. In turbulent, well-mixed waters, collect surface water via bucket and filter it through the appropriately sized mesh. Select sample size based on zooplankter concentration.

Give plankton nets proper care and maintenance. Do not let particulate dry on the net because it can significantly reduce size of mesh apertures and increase frequency of clogging. Wash net thoroughly with water after each use, and let it dry completely before storage. Periodically clean with a warm soap solution. Because nylon net is susceptible to deterioration from abrasion and sunlight, guard against unnecessary wear and store in the dark.

Traps and nets do not work well in shallow areas with aquatic vegetation, so use a length of lightweight rubber or polyethylene tubing with netting stretched over one end and rope tied to the other.[57] Use tubing that is 5- to 10-cm diameter and long enough to reach from the surface to the bottom. Affix the netting with tape or rubber bands, so it will stay in place in water but can be removed easily after sampling. Lower the open end of the tubing (the end with the rope attached) into the water until it almost touches the bottom and then use the rope to pull it up again, while keeping the covered end above the water surface. When the open end emerges from the water, let the covered end fall in. Pull the tubing into the boat, open end first, and let the water in the tube drain through the netting. When the zooplankton has been concentrated in a small volume, just above the netting, remove the netting over a container and catch the concentrated sample. Wash netting and end of tubing into the container to ensure that all the zooplankton is collected. This method is not limited to areas with aquatic vegetation. It is an excellent method of obtaining an integrated sample from any shallow area. In standing waters, collect tow samples by filtering 1 to 5 m^3 of water.

2) Storage procedures—Zooplankton samples most often are preserved with 70% ethanol[58] or 5% buffered formalin; glutaraldehyde or Lugol's solution will work, but not as well. Ethanol preservative is preferred for materials to be stained in permanent mounts or stored. Formalin may be used for the first 48 h of preservation with subsequent transfer to 70% ethanol. Formalin may distort pleomorphic forms, such as protozoans and rotifers. Make formalin in sucrose-saturated water to minimize carapace distortion and loss of eggs in crustaceans, especially cladocerans.[59] Bouin's fixative—picric acid saturated in calcium carbonate-buffered formaldehyde containing 5% (v/v) acetic acid—produces reasonable results for soft-bodied microzooplankton.[60] Dilute Bouin's fixative 1:19 with the sample. Because rapid fixation is necessary, pour the sample onto the fixative or inject fixative rapidly into the sample.

Use a narcotizing agent (e.g., carbonated water, menthol-saturated water, or neosynephrine) to prevent or reduce the contraction or distortion of organisms, especially rotifers, cladocerans, and many marine invertebrates.[61,62] Adding a few drops of detergent prevents preserved organisms from clumping. Preserve samples as soon as most animal movement has ceased—usually within a half hour of narcotization. To prevent evaporation, add 5% glycerin to the concentrated sample. In turbid samples, differentiate animal and detrital material by adding 0.04% rose bengal stain, which intensely stains the carapace (shell) of zooplankters and is a good general cytoplasmic stain. Taxonomic consistency over long-term projects is critical. Document the basis for identification carefully, making sure that morphological variation is de-

scribed clearly and identification references are permanently associated with the data.

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10200 C. Concentration Techniques

The organisms in water samples often must be concentrated in the laboratory before analysis. Ultimately, when calculating a concentration from an actual count, the multiplication factor should be less than 25 (i.e., every organism or cell counted should not represent more than about 25 organisms or cells in the natural sample). The multiplication factor is a function of the concentrated sample's concentration and volume. Three techniques for concentrating phytoplankton are sedimentation, membrane filtration, and centrifugation. A special technique for zooplankton also is described below.

1. Sedimentation/Settling

Sedimentation is the preferred concentration method because it is nonselective (unlike filtration) and nondestructive (unlike filtration or centrifugation), although many picoplankton, smaller nanoplankton, and actively swimming flagellates (in unpreserved samples) may not settle completely. Also, this approach may be too slow if results are needed quickly. The volume concentrated varies inversely with the abundance of organisms and is related to sample turbidity.

Allow 1 h settling per millimeter of column depth. For a sample preserved with Lugol's solution (2 to 4 ml/L), allow about 0.5 h settling/mm depth.[1] The sample may be concentrated in a series of steps by quantitatively transferring concentrate from the initial container to sequentially smaller ones. Use cylindrical settling chambers with thin, clear glass bottoms. Apply a height-to-diameter ratio no larger than 5:1 to avoid excessive chamber wall influence and currents in the chamber. Fill settling chambers without forming a vortex, keep them vibration-free, and move them carefully to avoid non-random distribution of settled matter. When siphoning supernatant to obtain the desired concentrate (usually 2 to 3 mL; 5 mL for diatom mounts), do it slowly, do not agitate the water, and hold the end of the siphon or pipet directly below the water's surface. Store concentrated sample in a closed, labeled container (remember that samples preserved with Lugol's solution will need to be re-spiked every 6 to 12 months).

2. Membrane Filtration

The filtration method permits the use of high magnification to enumerate small plankters (e.g., flagellates and cyanobacteria); it essentially concentrates the sample while providing a countable preparation. This section emphasizes preparation for microscopy, although glass-fiber filters (GF/F) (and membrane filters) are also used to isolate phytoplankton for pigment analysis (see 10200H). However, delicate forms (e.g., "naked" flagellates) can be distorted by even gentle filtration. When populations are dense and the detritus content is high, the filter clogs quickly and silt may crush organisms or obscure them from view. However, settling under high particulate circumstances also yields a difficult sample. Filtration offers the opportunity to make permanent mounts, allows for fast sample preparation when rapid results are needed to support management decisions (as in water treatment), and enhances the use of autofluorescence (thin preparation).

Pour a measured volume of well-mixed sample into a funnel equipped with a membrane filter (25-mm filter diameter;

0.45-μm pore size). Apply a vacuum of <50 kPa (<25 mm Hg) to the filter until about 0.5 cm of sample remains. Break vacuum, then apply low vacuum (about 12 kPa, 2 to 3 mm Hg) to remove remaining water. Do not dry filter.

For samples with a low phytoplankton and silt content, this method increases the probability of observing less abundant forms.[2] Samples also may be concentrated on a filter, inverted onto a microscope slide, and quick-frozen so plankton can be transferred from the filter to the slide. Alternatively, oil can be added to make the filter slightly translucent.[3,4]

Both autotrophic and heterotrophic picoplankton may be collected and counted via filtration and subsequent epifluorescence microscopy.[5–7] Wet a backing 0.45-μm Nuclepore filter with distilled water and place filter on the stem. Place a black 0.2-μm Nuclepore filter over the other filter. Based on cell concentrations, filter generally 1 to 2 mL of water sample through the filter apparatus, using a hand pump exerting a vacuum of 10 mm mercury. Filter sample until the meniscus disappears from the top filter. Remove the 0.2-μm filter and mount in oil on a slide (see 10200D.2*a*).

3. Centrifugation

Plankton can be concentrated via batch or continuous centrifugation. Centrifuge batch samples at 1000 g for 20 min. The Foerst continuous centrifuge is no longer recommended as a quantitative device, but existing programs may continue using it to ensure continuity with previously collected data. Although centrifugation accelerates sedimentation, it often damages fragile organisms, and is not preferable for quantitative analysis.

4. Sand Filter/Backwash

This method uses sand to filter out phytoplankters and then uses a backwash step to remove algae from the filter media. Although this method destroys many species, especially fragile or large forms, and yields differential recovery of species, it is still in use in some water utilities with limited laboratory resources. It is the least preferable concentration method.

5. Zooplankton Concentration

Zooplankton samples often need to be concentrated in the field, especially when large water bottles or pump sampling methods are used. Moreover, samples obtained via nets or other methods sometimes need to be further concentrated for storage or preparation for examination. When only small volume reductions are needed, pour sample back into the bucket of traps or nets. When processing large volumes of water (as in pump sampling), use larger plankton buckets or funnels with more water-volume retention and filtration surface area. Construct a filter funnel similar to that shown in Figure 10200:5 of clear acrylic plastic or other suitable material.[8] The apparatus' volume and mesh size depend on the volume of water to be filtered and the size of organisms to be retained. The filter funnel's mesh size normally is the same as that of the net or other field sampling device.

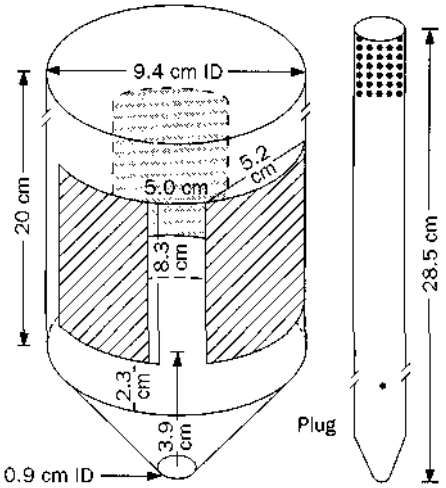


Figure 10200:5. Filter funnel for concentrating zooplankton. This device, originally designed for rotifers, can be modified for other zooplankters by changing the dimensions and mesh size. (After Likens and Gilbert.[8])

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10200 D. Preparing Slide Mounts

Counting methods may use temporary counting cells, semi-permanent slide mounts, or permanent slide mounts. Chamber or mount choice for phytoplankton and zooplankton will depend on available resources, analysis time line, organism size range, and microscope specifications. A method is also included for acid cleaning and mounting diatoms for species identifications and valve counts. Generally, use multiple chambers or mounts (often three per sample), regardless of processing procedure, to help account for subsample variability.[1]

1. Phytoplankton Temporary and Semi-Permanent Wet Slide Mounts

Agitate settled sample concentrate long enough to ensure thorough mixing (50 to 100 times), and withdraw a subsample with an accurately calibrated pipet. Clean pipet thoroughly between samples. To prepare wet slide mounts, transfer 0.1 mL to a glass slide, place a cover slip over the sample, and ring the cover slip with an adhesive (e.g., clear nail polish) to prevent evaporation. For procedures on preparing temporary counting chambers (Sedgwick–Rafter, Palmer–Maloney, nanoplankton and hemacytometer chambers/cells), see 10200F. For semi-permanent mounts, add a few drops of glycerin to the slide. As the sample ages, the water evaporates, leaving the organisms embedded in the glycerin. If the cover slip is ringed with adhesive, the slide can be retained for a few years if stored in the dark.

2. Phytoplankton Permanent Slide Mounts

a. Membrane filter mounts, oil-cleared: Suggested membrane filters include mixed ester/cellulose ester filters.* Method does not work well with non-organic filters (glass fiber filters or polycarbonate filters). Pre-concentration is not required with this method, but a test mount should be completed first to ensure that algal density on the filter is 10 to 20 natural units/field at the counting magnification. If sample volume is <0.5 mL, suspend in 10 mL filtered distilled water before mounting to encourage a random distribution on the filter. Place two drops of immersion oil on a labeled slide. Agitate the sample long enough to ensure thorough mixing (50 to 100 times), and withdraw a subsample with an accurately calibrated pipet. Clean pipet thoroughly between samples.

Immediately after filtering (see 10200C.2), place filter on top of oil with a pair of forceps and add two drops of oil to top of filter. The oil impregnates the filter, making it transparent. Impregnation typically occurs in 24 to 48 h but can be completed in 1 to 2 h by applying heat (70°C). Once the filter has cleared, place a few more drops of oil on it and cover with a cover slip. The mounted filter is now ready for microscopic examination.

Oil-cleared filters will cloud after several months. Alternatively, mount membrane filters in mounting medium.† Immerse filters in 1-propanol to displace residual water and transfer to xylol for several minutes to clear filters. Place a section of filter or entire filter on a microscope slide with the mounting medium, cover with a cover glass, and dry at low temperature.[1]

b. Membrane filter mounts, permanent HPMA: Suggested membrane filters are mixed cellulose ester filters (see ¶ *a* above). Method does not work with non-organic filters (glass fiber filters or polycarbonate filters). The hexamethylphosphoramide (HPMA) method for producing algal sample slides provides an optically clear background while permanently infiltrating and preserving the sample for archival purposes.[2,3] Mounting distortion is minimal, and magnifications of 100 to 1000× can be used on the same specimen. If samples are preserved in glutaraldehyde (final concentration of 0.25 to 0.50%), epifluorescence can be used on the sample while counting.

Agitate sample long enough to ensure thorough mixing (50 to 100 times), and withdraw a subsample with an accurately calibrated pipet. Filter subsample as described in 10200C.2.

Immediately after filtering, carefully place the filter face down on a 25-mm (#1) coverslip with a pair of forceps and add 2 to 3 drops of pre-polymerized HPMA‡ on the back of the filter. The resin clears the filter and impregnates algal cells. Place filters with HPMA in a drying oven at 60°C for 24 h. Once filter has cleared and polymerized, place a few more drops of HPMA on it and adhere it to a 25 mm × 75 mm slide. Place back in the drying oven for 24 to 72 h. The preparation is permanent when the coverslip's outer rim is completely polymerized. The internal part of the preparation may be liquid, but as long as the outer rim is solid, the slide is permanent (HPMA only polymerizes in the presence of oxygen). The mounted filter can be stored indefinitely at room temperature and is now ready for microscopic examination.

c. *Sedimented slide mounts:* There is a technique available for making permanent resin mounts of natural phytoplankton deposited via sedimentation on a microscope slide or cover glass and dehydrated via ethanol vapor substitution.[4,5] This method takes several days to complete; follow settling-duration guidelines and do not exceed a 5:1 height-to-depth ratio for the settling tower (see 10200C.1).

3. Diatom Mounts

Samples concentrated for diatom analysis via settling or centrifugation may contain dissolved materials (e.g., marine salts, preservative, and detergents) that will leave interfering residues. Wash well with distilled water before preparing slide(s). Transfer several drops of washed concentrate via a large-bore disposable pipet or large-bore dropper to a cover glass on a hot plate warmed enough to increase the evaporation rate but not cause boiling (use a large-bore pipet or dropper to prevent accidental exclusion of larger forms or those forming colonies or chains). If the uncleaned material is very concentrated, improve diatom distribution by adding the drops to a cover glass already flooded with distilled water. Redistribute on cover slip, if necessary (using a pipet), to produce a homogenous distribution of frustules. Evaporate to dryness. Repeat addition and evaporation until enough sample has been transferred to the cover

* Millipore HA, Pall GN, or equivalent.
† Permount, Fisher Scientific Co., Millipore HA, Pall GN, or equivalent.
‡ HPMA, SPI Supplies Division of Structure Probe, Inc., P.O. Box 656, West Chester, PA 19381-0656.

glass without producing a residue so dense that organisms cannot be recognized. If in doubt about the density, examine under a compound microscope. After evaporation, incinerate the residue on the cover glass on a 300 to 500°C hot plate; alternatively, use a muffle furnace. This usually requires 20 to 45 min. Mount as described below.

Clean samples chemically for diatom analysis as described elsewhere.[6–8] Mix equal volumes of concentrated nitric acid (HNO_3), sulfuric acid (H_2SO_4), or 50% H_2O_2 and sample. **CAUTION: When working with concentrated acids or caustics, wear safety goggles and an acid-resistant apron and gloves, and work in a chemical fume hood. This reaction will be highly exothermic!** Add a few grains of potassium dichromate ($K_2Cr_2O_7$)[5] to facilitate digestion of the filter and cellular organic matter. Add more dichromate if solution color changes from yellow to green. Place sample on a hot plate and boil down to about one-third the original volume. This process destroys organic matter, leaving only diatom shells (frustules). Alternatively, dichromate may be omitted (or not), and the treated sample may be left to stand overnight. Omitting the boiling and dichromate probably will leave cells intact, which is helpful when identifying heterovalver or heteropolar diatoms. Cool, wash with distilled water, and mount as described below. Transfer cleaned frustules to a cover glass and dry as described below.

Place a drop of mounting medium in the center of a labeled slide. Use 25- × 75-mm slides with frosted ends. Using a suitable high-refractive-index (1.6+) microscopic mounting medium§ ensures permanent, easily handled mounts for examination under oil immersion. Heat slide to near 90°C for 1 to 2 min before applying the heated cover slip with its sample residue to hasten evaporation of solvent in the mounting medium. Remove slide to a cool surface and, during cooling (5 to 10 s), apply firm but gentle pressure to cover glass with a broad, flat instrument. To prevent resin crystallization, ring cover slip with clear fingernail polish.

4. Zooplankton Mounts

For zooplankton analyses, withdraw a 1- to 5-mL subsample from the concentrate and dilute or concentrate further as necessary. Transfer sample to a counting cell or chamber (see 10200G) for analysis as a wet mount. Use polyvinyl lactyl phenol‖ to prepare semi-permanent zooplankton mounts. The mounts are good for about a year, and then the clearing agent causes organisms to deteriorate. For long-term storage, ring cover slip with clear lacquer (fingernail polish) to retard mountant crystallization. For permanent mounting, other mountants are available, and some stains not only highlight features but also partially clear the animals (e.g., Lignin Pink Double stain, Bio-Quip).#

For the protozoan portion of the microzooplankton, a protargol staining procedure[9] not only provides a permanent mount but also reveals the cytological details often necessary for identification.

This procedure is qualitative and especially important in taxonomic studies of ciliated protozoa.

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§ Naphrax, Brunel Microscopes, Unit 2, Vincients Road, Bumpers Farm Industrial Estate, Chippenham, Wiltshire SN14 6NQ U.K., or equivalent.
‖ Biomedical Specialists, Box 1687, Santa Monica, CA.

CMC-10, Master's Chemical Co., P.O. Box 2382, Des Plaines, IL.; Hydramount, Biomedical Specialists, Box 1687, Santa Monica, CA.; or equivalent.

10200 E. Microscopes and Calibrations

1. Compound Microscope

Use either a standard or an inverted compound microscope to identify and enumerate algae. Equip either with a mechanical stage that can move all parts of a counting cell past the objective lens. Standard equipment is a set of 10, 12.5, or 15× oculars and 10, 20, 40, and 100× objectives. Use objectives to provide adequate working distance for the counting chamber. Magnification requirements vary based on the plankton fraction being investigated, the type of microscope, counting chamber used, and optics. With standard objectives, the Sedgwick–Rafter chamber limits magnification to about 200× and the Palmer–Maloney cell limits magnification to

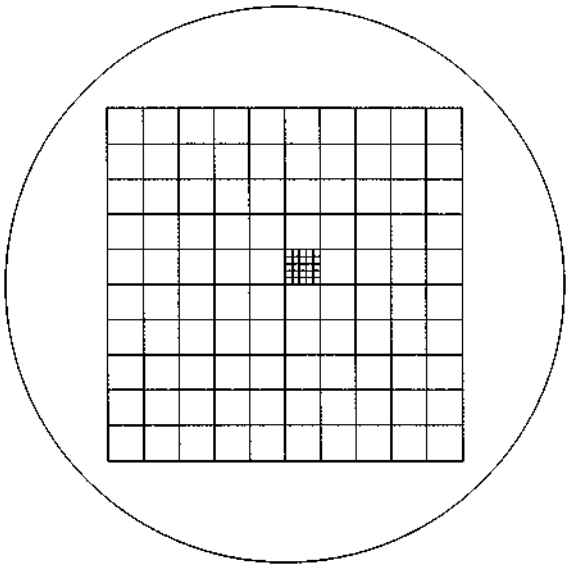


Figure 10200:6. Ocular micrometer ruling. A Whipple micrometer reticule is illustrated.

about 500×. The *useful upper limit of magnification* for any objective is 1000 times the numerical aperture (NA). Above this magnification, no greater detail can be resolved. Use combinations of oculars, intermediate magnifiers, and objectives to obtain the greatest magnification without exceeding the useful limit of magnification. Generally, the upper magnification limit of the standard or inverted compound microscope is 1250×. When the limit is exceeded, empty magnification results. *Empty magnification* is when the image is larger but not any clearer. Optics that enhance contrast [e.g., phase contrast, differential interference contrast (Nomarski), or interference reflection contrast (IRC)[1]] are useful and may be essential to identify phytoplankton accurately.

2. Stereoscopic Microscope

A *stereoscopic microscope* is essentially two complete microscopes assembled into a binocular instrument to give a stereoscopic view and an erect rather than an inverted image. Use this microscope to study and count large plankters (e.g., mature microcrustacea). Combine 10 to 15× paired oculars with 1 to 8× objectives; this bridges the gap between the hand lens and compound microscope, providing magnification from 10 to 120×. Alternatively, use a good zoom-type instrument with comparable magnification.

3. Inverted Compound Microscope

Many laboratories routinely use an inverted compound microscope for plankton counting.[2–5] In this instrument, the objectives are below a movable stage and the illumination comes from above, so analysts can view organisms that have settled to the bottom of a chamber. Place samples in a cylindrical settling chamber with a thin, clear glass bottom. Chambers of various capacities are available; the appropriate size depends on organism density. After a suitable settling period (see 10200C.1), count organisms in the settling chamber.

The major advantage of the inverted microscope is that by simply rotating the nosepiece, a specimen can be examined (or counted) directly in the settling chamber at any desired magnification. When used with an oil that is viscous enough not to run down the objective, oil-immersion objectives are useful and have excellent resolution. No preparation or manipulation other than settling is required. Generally, examine a preserved sample when performing counts. Techniques are available for samples with an abundance of floating organisms.[6]

4. Epifluorescence Microscope

An epifluorescence microscope may be either standard or inverted. It uses incident light to excite electrons in intracellular compounds (e.g., pigments or absorbed stains), and the energy emitted during electron return-to-the-ground state is measured as fluorescent light. The technique has been applied to microscopic identification of chlorophyll *a*, phycoerythrin, phycocyanin-containing cells (autotrophs), and nonpigmented heterotrophic plankton. Fluorescent stains (e.g., primulin or proflavin) also have been used to differentiate nanoplanktonic primary and secondary producers.[7–9] Excitation and emission wavelengths are unique for each pigment and stain, and require distinct light filter combinations and light sources. Select the filter combinations appropriate for the particular application. Concentrate samples via membrane filtration (see 10200C and 10200D, depending on application).

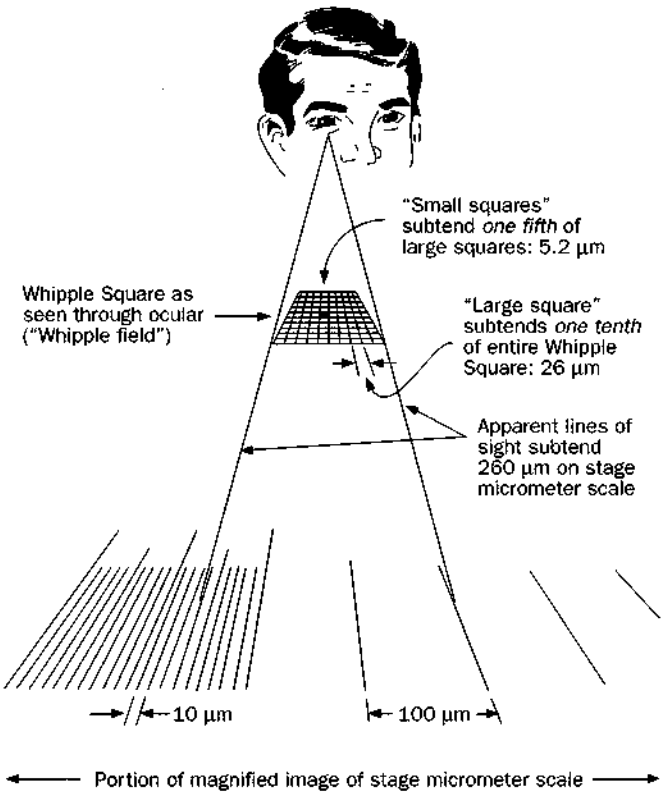


Figure 10200:7. Calibration of Whipple Square, as seen with 10× ocular and 43× objective (approximately 430× total magnification).

Epifluorescence microscopy is particularly useful for enumerating the picoplankton and heterotrophic flagellate populations common to most aquatic systems, or differentiating morphologically similar algal divisions—especially in high particulate samples—because differential pigment composition creates different fluorescent patterns. Use epifluorescence microscopy as a complementary procedure to standard light microscope counting techniques.

5. Microscope Calibration

Microscope calibration is essential. The usual calibration equipment is an ocular micrometer (Whipple grid, reticle, or reticule) placed in the microscope's eyepiece and a stage micrometer with a standardized, accurately ruled scale on a glass slide. There are several designs available for both phytoplankton and zooplankton. The Whipple disk (Figure 10200:6) has an accurately ruled grid subdivided into 100 squares. One square near the center is subdivided further into 25 smaller squares. The grid's outer dimensions are such that, with a 10× objective and a 10× ocular, it delimits an area of about 1 mm^2 on the microscope stage. Because this area may differ from one microscope to another, carefully calibrate the ocular micrometer for each microscope.

With the ocular and stage micrometers parallel and in part superimposed, match the line at the left edge of the Whipple grid with the zero mark on the stage micrometer scale (Figure 10200:7). Determine the width of the Whipple grid image to the nearest 0.01 mm from the stage micrometer scale. If the width is exactly 1 mm (1000 μm), the larger squares will be 1/10 mm (100 μm) on a side and each of the smaller squares will be 1/50 mm (20 μm).

When the microscope is calibrated at higher magnifications, the entire scale on the stage micrometer will not be seen; make measurements to the nearest 0.001 mm. Additional calibration details are available.[10]

6. References

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10200 F. Phytoplankton Counting Techniques

1. Counting Units

Some phytoplankton are unicellular, while others are multicellular (colonial or filamentous). Listed below are suggestions for reporting concentration or density:

Enumeration Method	Counting Unit	Reporting Unit
Total cell count	One cell	Cells/mL
Natural unit count (clump count)	One organism (any unicellular organism, natural colony, or filament)	Natural Units/mL
Areal standard unit count*	400 μm^2	Units/mL

* Areal standard unit = area of four small squares in a Whipple grid at a magnification of 200×, is microscope-specific, and is the least preferred reporting unit.

The variety of configurations poses a problem in enumeration. For example, should a four-celled colony of *Scenedesmus* (see Section 10900, Plate 29, Figure B-40) be reported as one colony or four individual cells? Generally, both cells and natural units should be enumerated. A *natural unit* is the unit that appears in the environment and that aquatic organisms encounter. Making a total cell count can be time-consuming and tedious, especially when colonies consist of thousands of individual cells; however, cells per colony/filament can be estimated closely, if done carefully. The natural unit or clump is the most easily used system; however, it is not necessarily the most accurate quantitatively because handling and preserving samples may dislodge cells from the colony periphery (especially in *Microcystis* and other cyanobacteria with dilute sheaths; this can be a large problem in Lugol's preserved samples). The cell/natural unit method also does not reflect the abundance of biomass or biovolume without additional measurement and calculation (see 10200I).

For most applications, biomass data are preferred, and cell dimensions and abundance are needed to convert from cell counts to biomass, assuming a specific gravity of 1.0. Measure enough cells (generally 10 to 30) to get a reliable average or range (corresponding to size categories applied to each species). If the focus is on biologically meaningful units (e.g., particle sizes), then natural units with an average or range of size dimensions are most appropriate. The most useful counts will provide natural units, average natural unit size [greatest

axial linear dimension (GALD)], average cells per unit, and average size per cell, allowing a series of calculations pertinent to various applications of the data.

Never mix and match units among different taxa within a count. For example, do not report *Merismopedia* as "units" of 4 cells, *Aphanizomenon* as filaments, and *Microcystis* as cells. This will make data interpretation difficult and comparisons of long-term data sets impossible because idiosyncrasies among counters over the years tend to get lost. Whatever method is chosen, identify it clearly in reporting results and understand the implications for data analysis.[1]

If the distribution of organisms is random and the population fits a Poisson distribution, the counting error may be estimated.[2] For example, the approximate 95% confidence limit, as a percentage of the number of natural units counted (N), equals:

$$\frac{2}{\sqrt{N}}(100\%)$$

So if 100 units are counted, the 95% confidence limit approximates ±20%. For a count of 400 units, the limit is about 10%. Natural units, not cells, are used because natural units are statically encountered during counting, not cells. Most counts are conducted to 300 to 400 natural units, spread among multiple slides or counting chambers.

2. Counting Procedures

To enumerate plankton, use a counting cell or chamber that limits the volume and area for ready calculation of population densities. Counting live material with motile taxa is not recommended because they often will avoid heat and light (or be attracted to light, depending on the species), and move in and out of the field of view.

When counting with a Whipple grid, establish a convention for tallying organisms lying on an outer boundary line. For example, when counting a "field" (entire Whipple square), designate the top and left boundaries as "no-count" sides, and the bottom and right boundaries as "count" sides. Thus, tally every plankter touching a "count" side from the inside or outside but ignore any touching a "no-count" side. If significant numbers of filamentous or other large forms cross two or more boundaries of the grid, count them separately at a lower magnification and include their number in the total count.

To identify organisms, use standard bench references (see 10200B.1 and Section 10900) and check current literature. New taxonomic resources are constantly being published.

For aquatic habitats subject to ongoing monitoring, it is often helpful to develop a habitat-specific pictorial key or voucher collection from accumulated data and images. Do not count dead cells or broken diatom frustules. Tally empty centric and pennate diatoms separately as "dead centric diatoms" or "dead pennate diatoms" for use in converting the diatom species proportional count to a count per milliliter, if that methodology applies to your study. Determining which cells were dead or live at collection time is often subjective; results depend on such variables as preservative used, age of collections, and purpose of the study. This becomes especially difficult with many of the tiny diatoms. Generally, if the cell wall and at least one plastid are intact, the natural unit is counted as live.

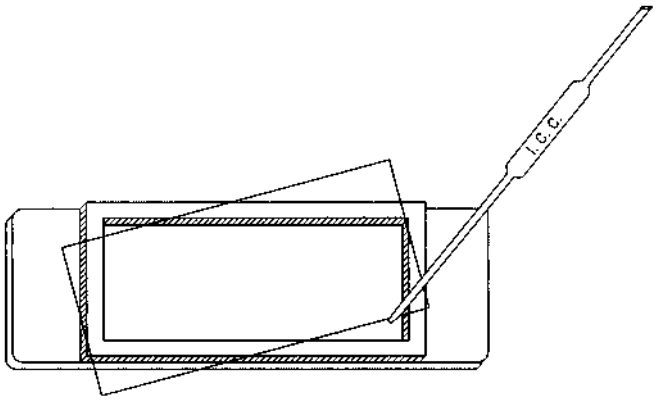


Figure 10200:8. Counting cell (Sedgwick–Rafter), showing method of filling. SOURCE: WHIPPLE, G.C., G.M. FAIR & M.C. WHIPPLE. 1927. The Microscopy of Drinking Water. John Wiley & Sons, New York, N.Y.

Magnification is important in phytoplankton identification and enumeration. Although magnifications of 100 to 200× are useful for counting large organisms or colonies, much higher magnifications often are required. It is useful to categorize phytoplankton-counting techniques according to magnification because this will affect density calculations.

a. Low-magnification (up to 200×) methods: A Sedgwick–Rafter (S–R) cell is commonly used for counting plankton because it is easily manipulated and provides reasonably reproducible data when used with a calibrated microscope equipped with an eyepiece measuring device (e.g., the Whipple grid). The S–R cell is about 50 mm long × 20 mm wide × 1 mm deep. Its total bottom area is about 1000 mm^2 and total volume is about 1000 mm^3 (1 mL). Carefully check the exact length and depth of the cell with a micrometer and calipers before use.

The cell's greatest disadvantage is that high-magnification objectives cannot be used. As a result, the S–R cell is not appropriate for examining nanoplankton.

1) Filling the cell—First, place the cover glass diagonally across the cell and transfer sample with a large-bore pipet (Figure 10200:8). Placing the cover slip diagonally across the cell helps prevent air bubbles from forming in cell corners. It often will rotate slowly and cover the inner portion of the S–R cell during filling. Do not overfill because a sample depth greater than 1 mm would produce an invalid count. During lengthy examinations, do not permit large air spaces (caused by evaporation) to develop in the chamber. To prevent such air spaces from forming, occasionally place a small drop of distilled water on edge of cover glass.

Before counting, let the S–R cell stand for at least 15 min to settle plankton. Count plankton on the bottom of the S–R cell. Some phytoplankton—notably some blue-green algae or motile flagellates in unpreserved samples—may not settle but rise to the underside of the cover slip. When this occurs, count these organisms separately by refocusing, and add to total of those

counted on the cell bottom to derive total number of organisms. Count algae in strips or fields.

2) Strip counting—In this context, a *strip* the length of the S–R cell is a volume about 50 mm long, 1 mm deep, and the width of the total Whipple grid.

The number of strips to be counted is a function of the precision desired and the number of units (cells, colonies, or filaments) per strip. Derive number of plankton in the S–R cell as follows:

$$\text{No./mL} = \frac{C \times 1000\ \text{mm}^3}{L \times D \times W \times S}$$

where:

C = number of organisms counted,
L = length of each strip (S–R cell length), mm,
D = depth of a strip (S–R cell depth), mm,
W = width of a strip (Whipple grid image width), mm, and
S = number of strips counted.

Multiply or divide number of cells per milliliter by a correction factor to adjust for sample dilution or concentration. Ultimately, the factor by which counted cells are multiplied should not be much higher than 25 for reliable results.

3) Field counting—On samples containing many plankton (10 or more plankters per field), make field counts rather than strip counts. Count plankters in random fields, each consisting of one Whipple grid. The number of fields counted will depend on plankton density and statistical accuracy desired (see 10200F.1). Calculate the number of plankton per milliliter as follows:

$$\text{No./mL} = \frac{C \times 1000\ \text{mm}^3}{A \times D \times F}$$

where:

C = number of organisms counted,
A = area of a field (Whipple grid image area), mm^2,
D = depth of a field (S–R cell depth), mm, and
F = number of fields counted.

Multiply or divide the number of cells per milliliter by a correction factor to adjust for sample dilution or concentration. Again, for reliable results, the factor by which counted cells are multiplied should not be much higher than 25.

b. Intermediate magnification (low to 500×) methods: The Palmer–Maloney (P–M) nanoplankton cell[3] is designed specifically for nanoplankton enumeration. It has a circular chamber that is 17.9-mm diam and 0.4-mm deep, and holds 0.1 mL. The shallow depth permits use of 40 to 45× objectives with sufficient working distance. The principal disadvantage of the P–M cell is that these magnifications (400 to 450×) often are insufficient for nanoplankton identification and enumeration.

Because a relatively small sample portion is examined in the P–M cell, do not use it unless the sample contains a dense population (10 or more plankters per field). Such a small sample portion from a less dense population leads to serious underestimation of density. This can be overcome by counting more area over more chambers; again, use the guide of a maximum multiplication factor of 25.

With the cover slip in place, pipet sample into one of the 2- × 5-mm channels on the side of the chamber. After a 10-min settling period, count the plankters in random fields (the number of fields depend on plankton density and variety, and statistical accuracy desired). Strips may be counted in this or any other circular cell by measuring the effective diameter and counting two perpendicular strips that cross at the center.

Calculate the number per milliliter as follows:

$$\text{No./mL} = \frac{C \times 1000\ \text{mm}^3}{A \times D \times F}$$

where:

C = number of organisms counted,
A = area of a field (Whipple grid image), mm^2,
D = depth of a field (P–M cell depth), mm, and
F = number of fields counted.

Multiply or divide the number of cells per milliliter by a correction factor to adjust for sample dilution or concentration.

Other, similar chambers are now available that function much like P–M chambers. For example, the standard medical hemacytometer (used to enumerate blood cells) has a ruled grid machined into a counting plate and is fitted with a ground-glass cover slip. The grid is divided into 1-mm^2 divisions, and the chamber is 0.1 mm deep. Pipet sample into chamber and view under 450× magnification. Count all cells within the grid. The chamber manufacturer provides a detailed instruction sheet on calculations and proper usage. One disadvantage of the hemacytometer is that the sample must have a very high plankton density to yield statistically reliable data, or else analysts must view much more area over multiple chambers.

c. High-magnification methods: Examining phytoplankton at high magnification requires the use of oil-immersion objectives. Suitable procedures include inverted microscope chambers, membrane filter mounts, sedimented slide mounts, the Lackey drop method, and diatom mounts.

1) Inverted microscope counts—Prepare a sample for examination by filling the settling chamber. After the desired settling time (see 10200C.1), transfer the chamber to the microscope stage. Count perpendicular strips across the center of the bottom cover glass. Strip counts may be made via a Whipple grid or special counting oculars with a pair of adjustable parallel hairs and one cross hair. Determine the width of the strip with a stage micrometer, and tally organisms as they pass the cross hair, which functions as a reference point. Hold strip width constant for any series of samples. Alternatively, examine random non-overlapping fields until at least 100 units of the dominant species are counted. To be most accurate, particularly because algae distribution may be non-uniform, count the entire chamber floor. Alternatively, make a random field-minimum count to attain a precision of at least 85%.[4] This can be overcome by counting

more area over more chambers; again, use the guide of a maximum multiplication factor of 25.

$$\text{Strip count (No./mL)} = \frac{C \times A_t}{L \times W \times S \times V}$$

where:

C = number of organisms counted,
A_t = total area of bottom of settling chamber, mm^2,
L = length of a strip, mm,
W = width of a strip (Whipple grid image width), mm,
S = number of strips counted, and
V = volume of sample settled, mL.

$$\text{Field count (No./mL)} = \frac{C \times A_t}{A_f \times F \times V}$$

where:

A_f = area of a field (Whipple grid image area), mm^2,
F = number of fields counted, and
other terms are as defined above.

2) Membrane filter mounts—Concentrate sample as directed in 10200C.2 or prepare membrane filter as directed in 10200D.2*a* and 2*b*.

Examine samples, which are concentrated on unlined membrane filters and mounted in oil or HPMA, as described above. Count enough random fields to ensure desired level of statistical accuracy (see 10200F.1). Select magnification level and size of microscope field (quadrat) so the most abundant species appear in at least 70% but not more than 90% of microscopic fields examined (80% is optimum). Adjust microscope field size by using entire field of view, or part or all of the Whipple grid/ocular micrometer. Examine at least 30 random microscope fields and record number of fields in which each species occurred, depending on density. Report results as organisms per milliliter, calculated as follows:

$$\text{(No./mL)} = \frac{N \times Q}{V \times D}$$

where:

N = density (organisms/field) from Table 10200:II,
Q = number of fields per filter,
V = milliliters filtered, and
D = dilution factor (0.96 for 4% formalin preservative) (Dilution factor is not necessary for preservative at a concentration ≤2%).

3) Sedimented slide mounts—Examine mounts that were prepared as directed in 10200D.2*c*.

4) Lackey drop method—The Lackey drop (microtransect) method[5] is a simple method of obtaining counts of considerable accuracy with samples containing a dense plankton population. It is similar to the S–R strip count.

Prepare slides as directed in 10200D.1. Oil immersion objectives can be used with the semipermanent slides. Count organisms in enough strips to ensure desired level of statistical accuracy (see 10200F.1). Calculate number of organisms per milliliter as follows:

$$\text{(No./mL)} = \frac{C \times A_t}{A_s \times S \times V}$$

where:

C = number of organisms counted,
A_t = area of cover slip, mm^2,
A_s = area of one strip, mm^2,
S = number of strips counted, and
V = volume of sample under the cover slip, mL.

5) Diatom mounts—Prepare samples as directed in 10200D.3.

For diatom species proportional count, examine diatom samples under oil immersion at a magnification of at least 900×. Scan lateral strips the width of the Whipple grid/field until at least 500 valves are counted (2 valves per cell). Available time and required accuracy dictate the number of valves to be counted. Determine percentage abundance of each species from tallied counts, and calculate counts per milliliter of each species by multiplying percent abundance by total live and dead diatom count obtained from the plankton

Table 10200:II. Conversion Table for Membrane Filter Technique (Based on 30 Scored Fields)

Total Occurrence	F* %	N†
1	3.3	0.03
2	6.7	0.07
3	10.0	0.10
4	13.3	0.14
5	16.7	0.18
6	20.0	0.22
7	23.3	0.26
8	26.7	0.31
9	30.0	0.35
10	33.3	0.40
11	36.7	0.45
12	40.0	0.51
13	43.3	0.57
14	46.7	0.63
15	50.0	0.69
16	53.3	0.76
17	56.7	0.83
18	60.0	0.91
19	63.3	1.00
20	66.7	1.10
21	70.0	1.20
22	73.3	1.32
23	76.7	1.47
24	80.0	1.61
25	83.3	1.79
26	86.7	2.02
27	90.0	2.30
28	93.3	2.71
29	96.7	3.42
30	100.0	?

* $F = \dfrac{\text{Total number of species occurrences} \times 100}{\text{Total number of fields examined}}$
† N = number of organisms per field.

counting chamber. For more accuracy (if clearly indicated by material and study objectives), distinguish between living and dead diatoms at the species level.

6) Phytoplankton staining technique—Staining algae enables analysts to differentiate between "live" and "dead" diatoms[6] so total phytoplankton can be enumerated in a single sample without sacrificing detailed diatom taxonomy. It also results in permanent reference slides. This procedure is most useful when diatoms are major components of phytoplankton and distinguishing between living and dead diatoms is important. Other stains can be used to highlight features that facilitate separation of major taxonomic groups. Alternatively, if the sample was preserved in glutaraldehyde, epifluorescence can be used.

For diatom work, preferably preserve samples in Lugol's solution, glutaraldehyde, or formalin (see 10200B.2*a*). For analysis, thoroughly mix the sample and filter a portion through a 47- or 25-mm-diam membrane filter (pore diam 0.45 or 0.65 μm). Use a vacuum of 16 to 20 kPa (<25 mm Hg), and never let sample dry. Add 2 to 5 mL aqueous acid fuchsin solution to the filter and let stand for 20 min. (Create aqueous acid fuchsin solution by dissolving 1 g acid fuchsin in 100 mL distilled water to which 2 mL glacial acetic acid has been added.) After staining, filter sample, wash briefly with distilled water, and filter again. Administer successive rinses of 50, 90, and 100% propanol to the sample while filtering. Soak for 2 min in a second 100% propanol wash, filter, and add xylene. At least two washes are required; let the final one soak 10 min before filtering. Trim the xylene-soaked filter and place on a microscope slide on which there are several drops of mounting medium.* Apply several more drops of medium to top of filter and install a cover glass. Carefully squeeze out excess mounting medium. Make the final mount permanent by lacquering the edges of the cover glass.

Count organisms using the most appropriate magnification. "Live" diatoms typically are red while "dead" ones are unstained. Oil immersion is necessary when identifying diatoms to species and many other algae. Count either strips or random fields, and calculate plankton densities per milliliter:

$$\text{No./mL} = \frac{C \times A_t}{A_c \times V}$$

where:

C = number of organisms counted,
A_t = total area of effective filter before trimming and mounting,
A_c = area counted (strips or fields), and
V = volume of sample filtered, mL.

* Permount, Fisher Scientific Co., or equivalent.

3. References

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10200 G. Zooplankton Counting Techniques

1. Subsampling

Count entire samples with low zooplankton numbers (<200 zooplankters) without subsampling. Most zooplankton samples will contain more organisms than can be enumerated practically, however, so use a subsampling procedure. Before subsampling, remove and enumerate all large uncommon organisms (e.g., fish larvae in fresh water or coelenterates, decapods, fish larvae, etc., in salt water). Subsample by the pipet or splitting method.

In the pipet method, adjust sample to a convenient volume in a graduated cylinder or Imhoff cone. Concentrating plankton via a rubber bulb and clear acrylic plastic tube with fine mesh netting fitted on the end is convenient and accurate (Figure 10200:9). For smaller microzooplankton, use the sedimentation techniques described for concentrating phytoplankton. Transfer sample to a beaker or other wide-mouth vessel for subsampling via a Hensen–Stempel or similar wide-bore pipet. Gently stir sample completely and randomly with the pipet and quickly withdraw 1 to 5 mL. Transfer to a suitable counting chamber.

Alternatively, subsample by splitting via any of a number of devices of which the Folsom plankton splitter[1] is best known (Figure 10200:10). Level splitter before using. Place sample in the splitter and divide into subsplits. Rinse splitter into subsamples. Repeat until a workable number (200 to 500 individuals) is obtained in a subsample. Exercise care to provide unbiased splits. Even when using the Folsom splitter, unbiased subsamples cannot be unquestioningly assumed;[2] therefore, count animals in several subsamples from the same sample to verify that the splitter is unbiased and to determine the sampling error introduced by using it.

Another method permits abundance estimates of more equivalent levels of precision among taxa than obtained with either the Hensen–Stempel pipet or the Folsom splitter.[3] Normal counting procedures tally organisms based on their abundance in a sample. Therefore, in a sample with a dominant organism making up 50% of total numbers, the tally of the dominant taxon will be large and have a small error. However, errors related to subdominants will increase as the tally of each taxon decreases. By accepting one level of precision, the technique[3] has been developed to obtain the same errorabout dominants and subdominants, permitting quantitative comparisons between taxa over successive times or between stations.

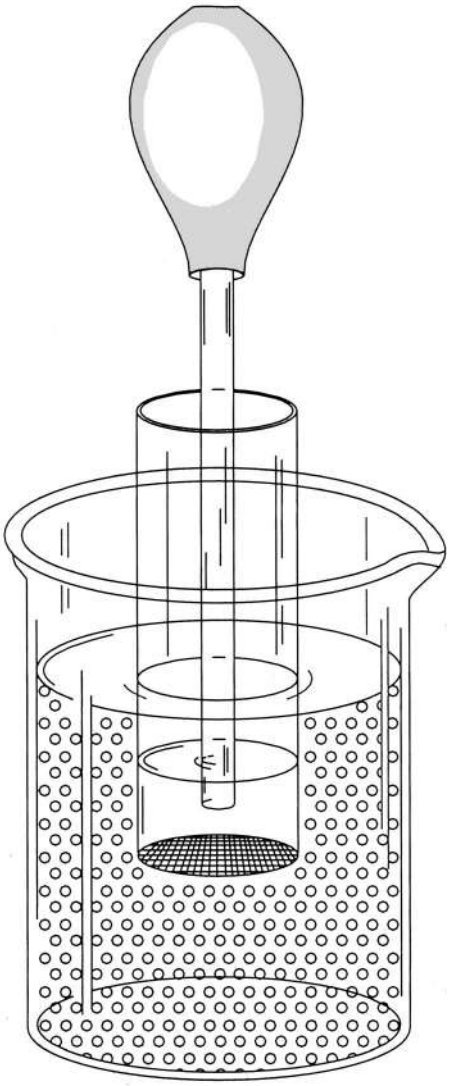


Figure 10200:9. A simple, efficient device for concentrating plankton. The tube is lowered into the beaker containing the sample. Water filtering into the tube is removed via rubber bulb. The filter is nylon monofilament screen cloth that is glued to the bottom of the tube. The mesh size should be small enough to prevent zooplankton from entering filtrate (after Dodson and Thomas[5]).

2. Enumeration

Using a compound microscope and a magnification of 100×, enumerate small zooplankton (protozoa, rotifers, and nauplii) in a 1- to 5-mL clear acrylic plastic counting cell fitted with a glass cover slip. For larger, mature microcrustacea, use a counting chamber holding 5 to 10 mL. A Sedgwick–Rafter cell is unsuitable because of its size. An open counting chamber 80 × 50 × 2 mm deep is desirable; however, an open chamber is difficult to move without jarring and disrupting the count. Place a mild detergent solution in the chamber before counting to reduce organism movements, or use special counting trays with parallel or circular grooves or partitions.[4,5] Count microcrustacea with a binocular dissecting microscope at 20 to 40× magnification. If identification is questionable, remove organisms with a microbiological transfer loop and examine at a higher magnification under a compound microscope.

Report zooplankton as number per liter or number per cubic meter, depending on the system and study objectives:

https://doi.org/10.2105/SMWW.2882.207

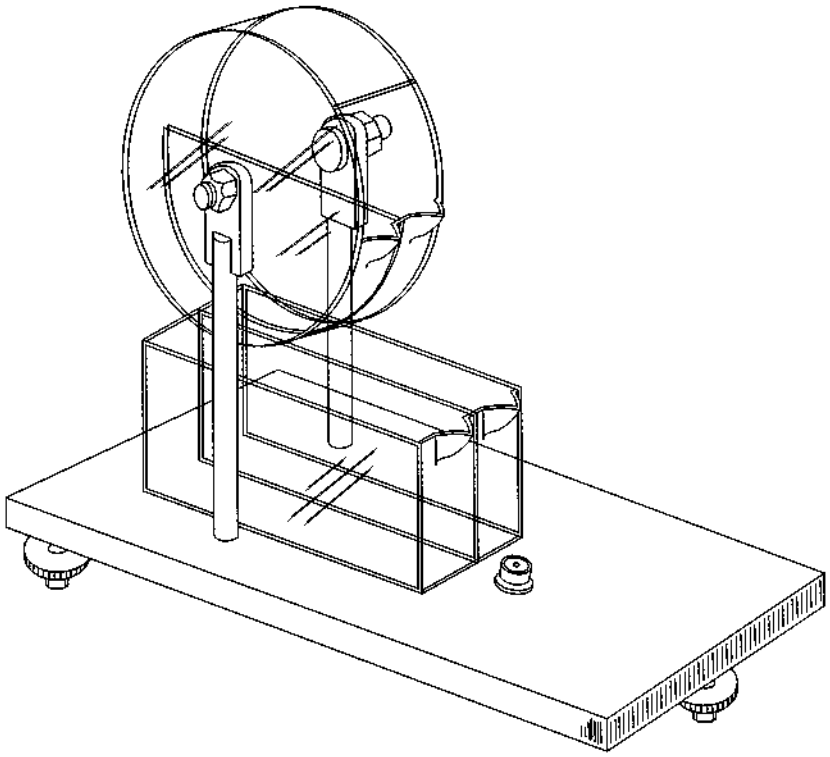


Figure 10200:10. The Folsom plankton splitter.

$$\text{No./m}^3 = \frac{C \times V'}{V'' \times V'''}$$

where:

C = number of organisms counted,
V' = volume of the concentrated sample, mL,
V'' = volume counted, mL, and
V''' = volume of the grab sample, m^3.

To obtain organisms per liter, divide by 1000.

3. References

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10200 H. Chlorophyll

Photosynthetic pigment concentrations are used extensively to estimate phytoplankton biomass.[1,2] All green plants contain chlorophyll *a,* which constitutes about 1 to 2% of the dry weight of planktonic algae. Other pigments in phytoplankton include chlorophylls *b* and c, xanthophylls, phycobilins, and carotenes. Important chlorophyll degradation products found in the aquatic environment are chlorophyllides, pheophorbides, and pheophytins. The presence or absence of various photosynthetic pigments is used, among other features, to identify major algal groups.

Three methods to determine chlorophyll *a* in phytoplankton are the spectrophotometric,[3–5] fluorometric,[6–8] and high-performance liquid chromatographic (HPLC) techniques.[9] Fluorometry is more sensitive than spectrophotometry, requires less sample, and can be used for *in vivo* measurements.[10] These optical methods can significantly under- or overestimate chlorophyll *a* concentrations,[11–18] in part because the absorption and fluorescence bands of co-occurring accessory pigments and chlorophyll degradation products overlap.

Pheophorbide *a* and pheophytin *a,* two common degradation products of chlorophyll *a,* can interfere with the determination of chlorophyll *a* because they absorb light and fluoresce in the same region of the spectrum as chlorophyll *a* does. If these pheopigments are present, significant errors in chlorophyll *a* values will

result. Pheopigments can be measured either via spectrophotometry or fluorometry, but in marine and freshwater environments, the fluorometric method is unreliable when chlorophyll *b* co-occurs, unless a non-acidification method is used (EPA Method 445.0). On acidifying chlorophyll *b*, the resulting fluorescence emission of pheophytin *b* coincides with that of pheophytin *a,* causing underestimation and overestimation of chlorophyll *a* and pheopigments, respectively. The non-acidification method has the following advantages: more accurate chlorophyll *a* data, less labor intensive, fewer resources needed, and very sensitive.

HPLC is a useful method for quantifying photosynthetic pigments,[9,13,15,16,19–21] including chlorophyll *a*, accessory pigments (e.g., chlorophylls *b* and *c*), and chlorophyll degradation products (chlorophyllides, pheophorbides, and pheophytins). Pigment distribution is useful for quantitative assessment of phytoplankton community composition and zooplankton grazing activity.[22]

1. Pigment Extraction

Glass fiber filters are preferred for removing algae from water. The glass fibers help break the cells during grinding, larger volumes of water can be filtered, and no precipitate forms after acidification. Inert membrane filters, such as polyester filters, may be used when these factors are irrelevant. Filters taken from water with pH >6 may be placed in airtight plastic bags and stored frozen for 28 d. Process samples from naturally acidic water with pH <6 promptly after filtration to prevent possible chlorophyll degradation from residual acidic water on the filter. (*Naturally acidic water* has a pH <6 due to humic acid or the contents of senescent cells, not preservatives.)

Chlorophyll can be extracted from cells, typically collected on a filter, with several solvents (e.g., acetone, ethanol, and methanol). The procedure described here uses acetone. Conduct this procedure with chlorophyll extracts in subdued light to avoid degradation. Use opaque containers or wrap with aluminum foil. The pigments are extracted from plankton concentrate with aqueous acetone and the extract's absorbance (optical density) is determined via a spectrophotometer. The ease with which chlorophylls are removed from cells varies considerably with different algae. To completely extract pigments consistently, disrupt the cells mechanically with a tissue grinder.

a. Equipment and reagents:

1) *Tissue grinder**— Successfully macerating glass fiber filters in tissue grinders with grinding tube and pestle of conical design may be difficult. Preferably use round-bottom grinding tubes with a matching pestle that has grooves in the TFE tip. Alternatively, sonication may be used.

2) *Clinical or benchtop centrifuge* (5000 to 7500 rpm) [g = (0.00001118*r*)(*rpm*), where *r* = centrifuge's radius].

3) *Centrifuge tubes*—15-mL graduated, screw-cap.

4) *Filtration equipment*—filters, glass fiber† or membrane (0.45-μm porosity, 47-mm diameter); vacuum pump (or lab vacuum); solvent-resistant filter assembly (e.g., an all-glass filter-support apparatus that can be cleaned with water and organic solvents), 1.0-μm pore size;‡ 10-mL solvent-resistant syringe.

5) *Saturated magnesium carbonate solution*—Add 1.0 g finely powdered $MgCO_3$ to 100 mL distilled water.

6) *Aqueous acetone solution*—Mix 90 parts acetone (reagent-grade BP 56°C) with 10 parts saturated magnesium carbonate solution. For HPLC pigment analysis, mix 90 parts HPLC-grade acetone with 10 parts distilled water.

b. Extraction procedure:

1) Concentrate sample by centrifuging or filtering as soon as possible after collection. If processing must be delayed, hold samples on ice or at 4°C and protect from exposure to light. Use opaque bottles because even brief exposure to light during storage will alter chlorophyll values. Rinse sample storage container with about 20 mL organic-free lab water (which is also passed through the same sample filter to make sure all cells are collected). Use glassware and cuvettes that are clean and acid-free. Add approximately 2 mL of $MgCO_3$ solution to sample just before filtering process is completed. $MgCO_3$ solution acts as a pH buffer to keep chlorophyll from degrading.

2) Place sample in a tissue grinder, cover with 2 to 3 mL 90% aqueous acetone solution, and macerate at 500 rpm for 1 min. Use TFE/glass grinder for a glass-fiber filter and glass/glass grinder for a membrane filter.

3) Transfer sample to a screw-cap centrifuge tube, rinse grinder with a few milliliters 90% aqueous acetone, and add the rinse to the extraction slurry. Adjust total volume to 10 mL with 90% aqueous acetone (additional techniques are used for HPLC analyses; see 10200H.4). Use solvent sparingly and avoid excessive dilution of pigments. Steep samples at least 2 h at 4°C in the dark. Glass fiber filters of 25- and 47-mm diam§ have dry displacement volumes of 0.03 and 0.10 mL, respectively, and introduce errors of about 0.3 and 1.0% if a 10-mL extraction volume is used.

4) Clarify by filtering through a solvent-resistant disposable filter [e.g., a 0.45 μm PTFE 13 mm syringe filter (to minimize retention of extract in filter and filter holder, force 1 to 2 mL air through filter after extract)] or by centrifuging in closed tubes for 20 min at 500 *g* or 3000 rpm [*g* = (0.00001118*r*)(*rpm*), where *r* = centrifuge's radius]. Decant clarified extract into a clean, calibrated, 15-mL, screw-cap centrifuge tube and measure total volume. Proceed as in 10200H.2, 3, 4, or 5.

2. Spectrophotometric Determination of Chlorophyll

a. Equipment and reagents:

1) *Spectrophotometer*—with a narrow band (pass) width (0.5 to 2.0 nm) because the chlorophyll absorption peak is relatively narrow. At a spectral band width of 20 nm, the chlorophyll *a* concentration may be underestimated by as much as 40%.

2) *Cuvettes,* with 1-, 4-, and 10-cm path lengths.

3) *Pipets,* 0.1- and 5.0-mL.

4) *Hydrochloric acid* (HCl), 0.1*N*.

b. Determination of chlorophyll a *in the presence of pheophytin* a: Chlorophyll *a* may be overestimated by including pheopigments that absorb near the same wavelength as chlorophyll *a.* Adding acid to chlorophyll *a* results in loss of the magnesium

* Kontes Glass Co., Vineland, NJ 08360: Glass/glass grinder, Model No. 88855, which requires adaptor 78800 and a stirrer motor 790040 operated at 500 rpm: Glass/TEE grinder, Model 886000; or equivalent.

† Whatman GF/F (0.7 μm), GFB (1.0 μm), Gelman AE (1 μm),[23] or equivalent.

‡ Gelman Acrodisc, or equivalent.

§ Whatman GF/F, or equivalent.

atom, converting it to pheophytin *a.* Acidify carefully to a final molarity of not more than $3 \times 10^{-3}M$ to prevent certain accessory pigments from changing to absorb at the same wavelength as pheophytin *a.*[13] When a solution of pure chlorophyll *a* is converted to pheophytin *a* via acidification, the absorption-peak ratio (absorbance 664/absorbance 665) of 1.70 is used to correct the apparent chlorophyll *a* concentration for pheophytin *a.*

Samples with an absorbance 664 before:absorbance 665 after acidification ratio (664_b:665_a) of 1.70 are considered to contain no pheophytin *a* and to be in excellent physiological condition. Solutions of pure pheophytin show no reduction in absorbance 665 upon acidification and have a 664_b:665_a ratio of 1.0. Thus, mixtures of chlorophyll *a* and pheophytin *a* have absorption peak ratios ranging between 1.0 and 1.7. These ratios are based on the use of 90% acetone as solvent. Using 100% acetone as solvent results in a chlorophyll *a* before-to-after acidification ratio of about 2.0.[3]

Spectrophotometric procedure—Transfer 3 mL clarified extract to a 1-cm cuvette and read absorbance at 750 and 664 nm. Acidify extract in the cuvette with 0.1 mL 0.1*N* HCl. Gently agitate the acidified extract and, 90 s after acidification, read absorbance at 750 and 665 nm. The volumes of extract and acid, and the time after acidification are critical for accurate, consistent results.

The absorbance 664 before acidification should be between 0.1 and 1.0. For very dilute extracts, use cuvettes with a longer path. If a larger cell is used, add a proportionately larger volume of acid. Correct absorbance obtained with larger cuvettes to 1 cm before making calculations.

Subtract the 750-nm absorbance value from the readings before (absorbance 664 nm) and after acidification (absorbance 665 nm).

Using the corrected values, calculate chlorophyll *a* and pheophytin *a* per cubic meter as follows:

$$\text{Chlorophyll } a, \text{ mg/m}^3 = \frac{26.7\,(664_b - 665_a) \times V_1}{V_2 \times L}$$

$$\text{Pheophytin } a, \text{ mg/m}^3 = \frac{26.7\,[1.7(665_a) - 664_b] \times V_1}{V_2 \times L}$$

where:

664_b, 665_a = absorbance of 90% acetone extract before and after acidification, respectively,
V_1 = volume of extract, L,
V_2 = volume of sample, m^3, and
L = light path length or width of cuvette, cm.

The value 26.7 is the absorbance correction and equals

$$A \times K$$

where:

A = absorbance coefficient for chlorophyll *a* at 664 nm = 11.0, and
K = ratio expressing correction for acidification.

$$= \frac{\left(\frac{664_b}{665_a}\right) \textit{pure chlorophyll a}}{\left(\frac{664_b}{665_a}\right) \textit{pure chlorophyll a} - \left(\frac{664_b}{665_a}\right) \textit{pure pheophytin a}}$$

$$= \frac{1.7}{1.7-1.0} = 2.43$$

c. Determination of chlorophyll a, b, *and* c *(trichromatic method):*

Spectrophotometric procedure—Transfer extract to a 1-cm cuvette and measure absorbance at 750, 664, 647, and 630 nm. Choose a cell path length or dilution to give absorbance 664 between 0.1 and 1.0.

Use the absorbance readings at 664, 647, and 630 nm to determine chlorophyll *a, b,* and *c*, respectively. The absorbance reading at 750 nm is a correction for turbidity. Subtract this reading from each of the pigment absorbance values of the other wavelengths before using them in the equations below. Because the extract's absorbance at 750 nm is sensitive to changes in the acetone-to-water proportions, adhere closely to the 90 parts acetone:10 parts water (v/v) formula for pigment extraction. Turbidity can be removed easily via filtration through a disposable, solvent-resistant filter attached to a syringe or by centrifuging for 20 min at 500 *g*.

Calculate the concentrations of chlorophyll *a, b,* and *c* in the extract by inserting the corrected optical densities in following equations:[5]

a) C_a = 11.85(absorbance 664) – 1.54(absorbance 647) – 0.08(absorbance 630)

b) C_b = 21.03(absorbance 647) – 5.43(absorbance 664) – 2.66(absorbance 630)

c) C_c = 24.52(absorbance 630) –7.60(absorbance 647) – 1.67(absorbance 664)

where:

C_a, C_b, and C_c = concentrations of chlorophyll *a, b,* and *c,* respectively, mg/L, and

Absorbance 664, 647, and 630 = corrected optical densities (with a 1-cm light path) at the respective wavelengths.

After determining the concentration of pigment in the extract, calculate the amount of pigment per unit volume as follows:

$$\text{Chlorophyll } a, \text{ mg/m}^3 = \frac{C_a \times \text{ extract volume, L}}{\text{Volume of sample, m}^3}$$

3. Fluorometric Determination of Chlorophyll *a*

The fluorometric method for chlorophyll *a* is more sensitive than the spectrophotometric method, so smaller samples can be used. To achieve acceptable results, calibrate the fluorometer spectrophotometrically with a sample from the same source. Optimum sensitivity for chlorophyll *a* extract measurements is obtained at an excitation wavelength of 430 nm and an emission wavelength of 663 nm. A method for continuously measuring chlorophyll *a in vivo* is available but is reported to be less efficient than the *in vitro* method given here (yielding about one-tenth as much fluorescence per unit weight

as the same amount in solution). Pheophytin *a* also can be determined fluorometrically.[24]

a. Equipment and reagents: In addition to those listed under 10200H.1*a* and 2*a:*

Fluorometer,‖ equipped with a high-intensity F4T.5 blue lamp; photomultiplier tube R-446 (red-sensitive); sliding window orifices 1, 3, 10, and 30×; and filters for light emission (CS-2-64) and excitation (CS-5-60). A high-sensitivity door is preferable.

b. Extraction procedure: Prepare sample as directed in 10200H.1*b*.

1) Calibrate fluorometer with a known concentration of chlorophyll solution as follows. Prepare chlorophyll extract and analyze spectrophotometrically. Prepare serial dilutions of the extract to provide concentrations of approximately 2, 6, 20, and 60 μg chlorophyll *a*/L. Make fluorometric readings for each solution at each sensitivity setting (sliding window orifice): 1, 3, 10, and 30×. Using the values obtained, derive calibration factors to convert fluorometric readings in each sensitivity level to concentrations of chlorophyll *a,* as follows:

$$F_s = \frac{C'_a}{R_s}$$

where:

F_s = calibration factor for sensitivity setting *S,*
C'_a = concentration of chlorophyll *a* determined spectrophotometrically, μg/L, and
R_s = fluorometer reading for sensitivity setting *S*.

2) Measure sample fluorescence at sensitivity settings that will provide a midscale reading. (Avoid using the 1× window because of quenching effects.) Convert fluorescence readings to concentrations of chlorophyll *a* by multiplying the readings by the appropriate calibration factor.

c. Determination of chlorophyll a *in the presence of pheophytin* a: This method normally is not applicable to freshwater samples. See discussions under 10200H and 2*b*.

1) Equipment and reagents—In addition to those listed under 10200H.1*a* and 2*a*, pure chlorophyll *a*# (or a plankton chlorophyll extract with a spectrophotometric before-and-after acidification ratio of 1.70 containing no chlorophyll *b*).

2) Fluorometric procedure—Calibrate fluorometer as directed in ¶ *b*1) above. Determine extract fluorescence at each sensitivity setting before and after acifidication. Calculate calibration factors *(F_s)* and before-and-after acidification fluorescence ratio by dividing the fluorescence reading obtained before acidification by the one obtained after acidification. Avoid readings on the 1× scale and those outside the range of 20 to 80 fluorometric units.

3) Calculations—Determine the "corrected" chlorophyll *a* and pheophytin *a* in sample extracts via the following equations:[8,24]

$$\text{Chlorophyll } a, \text{ mg/m}^3 = F_s \frac{r}{r-l}(R_b - R_a)\frac{V_e}{V_s}$$

$$\text{Pheophytin } a, \text{ mg/m}^3 = F_s \frac{r}{r-l}(rR_b - R_a)\frac{V_e}{V_s}$$

where:

F_s = conversion factor for sensitivity setting *S* (see 10200H.2*b*),
r = R_b/R_a, as determined with pure chlorophyll *a* for the instrument (Redetermine *r* and F_S if filters or light source are changed),
R_b = fluorescence of extract before acidification,
R_a = fluorescence of extract after acidification,
V_e = volume of extract, and
V_s = volume of sample.

d. Extraction of whole water, nonfiltered samples: Alternatively, to prevent cell lysis during filtration, extract whole water sample.

1) Equipment and reagents—Fluorometer equipped with a high-sensitivity R928 phototube** with output impedance of 36 ma/W at 675 nm and a high-sensitivity door. Place neutral density filter (40 to 60N) in the rear light path,†† selected to permit reagent blanking on the highest sensitivity scale.

2) Extraction procedure—Decant 1.5 mL sample into screw-cap test tube and add 8.5 mL 100% acetone. Mix with vortex mixer and hold in the dark for 6 h at room temperature. Filter through glass fiber filter‡‡ or centrifuge. Measure fluorescence as described in 10200H.3 and estimate concentrations as in ¶ *c* above. Humic substances interfere, so if they are present, filter a sample portion (see 10200H.1*b*) and process filtrate with sample. Subtract filtrate (blank) fluorescence from that of sample.

4. High-Performance Liquid Chromatographic Determination of Algal Chlorophylls and Their Degradation Products

a. Equipment and reagents: In addition to those listed for pigment extraction, 10200H.1*a*:

1) *High-pressure liquid chromatograph* capable of a flow rate of 2.0 mL/m.

2) *High-pressure injector valve* equipped with a 100-μL sample loop.

3) *Guard column*—4.0 × 0.5 cm, C_{18} packing material, 3-μm particle size, or equivalent protection system) to extend life of primary column.

4) *Reverse-phase HPLC column.*§§

5) *Fluorescence detector* capable of excitation at 430 ± 30 nm and measuring emissions at wavelengths >600 nm.

6) *Data recorder device*—Strip chart recorder or, preferably, an electronic integrator.

7) *Syringe,* glass, 250-μL.

8) *HPLC eluents*—System A (80:15:5 blend of methanol, reagent water, and ion-pairing solution, respectively) and System B (80:20 blend of methanol and acetone, respectively). Use HPLC-grade solvents; measure volumes before mixing. Filter eluents through a solvent-resistant 0.4-μm filter before use and degas with helium. Prepare the ion-pairing (IP) solution from

‖ Model 10-005, Turner Designs, Sunnyvale, CA, or equivalent.
Purified chlorophyll *a,* Sigma Chemical Company, St. Louis, MO., or equivalent.

** Hammamatsu Corp., Middlesex, NJ, or equivalent.
†† If using Model 10-005, Turner Designs, or equivalent.
‡‡ Whatman GF/F, or equivalent.
§§ Microsorb C_{18} column, 10 cm long, 3-μm particle size, Rainin Co., or equivalent.

15 g tetrabutylammonium acetate‖ ‖ and 77 g ammonium acetate## made up to 1 L with reagent water.[15]

9) *Calibration standards*—Individually dissolve 1 mg each pure chlorophyll *a* and *b*‖ ‖ in 100 mL 90% acetone. Determine the exact concentrations spectrophotometrically (ϵ_{664} for chlorophyll *a* in 90% acetone = 87.67 L/g · cm; ϵ_{647} for chlorophyll *b* in 90% acetone = 51.36 L/g · cm).[5] Prepare pheophytin $a + a'$ and $b + b'$ standards from the primary chlorophyll *a* and *b* standards via acidification with hydrochloric acid; correct respective concentrations for Mg^{2+} loss. Extract chlorophyll *c* with 90% acetone from diatoms, purify by thin-layer chromatography (TLC)[25] and calibrate spectrophotometrically (ϵ_{631} for a mixture containing equal amounts of chlorophylls c_1 and c_2 in 90% acetone containing 1% pyridine = 42.6 L/g · cm; the absence of this small amount of pyridine is presumed to cause only small differences in the absorption properties of chlorophyll *c*.[26] Alternatively, determine the chlorophyll *c* content of a 90% acetone extract made from diatoms spectrophotometrically (chlorophyll $c_1 + c_2$, $\mu g/mL = 24.36E_{630} - 3.73E_{664}$)[5] and use as standard. Prepare chlorophyllide *a* from diatoms,[27] purify by TLC[25] and calibrate spectrophotometrically in 90% acetone (ϵ_{664} for chlorophyllide *a* = 128 L/g · cm).[28] Prepare pheophorbide *a* by acidification of chlorophyllide *a,* purify by TLC,[25] and calibrate spectrophotometrically in 90% acetone (ϵ_{665} for pheophorbide *a* = 69.8 L/g · cm).[28] Standards stored under nitrogen in the dark at –20°C are stable for about 1 month.

b. Procedure:

1) Set up and equilibrate HPLC with solvent System A at a flow rate of 2 mL/min. Adjust fluorometer sensitivity to provide full-scale reading with the most concentrated chlorophyll *a* standard.

2) Calibrate HPLC by preparing working standards from the primary standards (on day of use). Once the standards' retention times are determined for a particular system, simplify standardization by preparing serial dilutions from mixed standards. Prepare separately mixed standards for the chlorophylls and chlorophyllide *a* and for the pheophytins and pheophorbide *a.* Mix 1-mL portions of standards with 300 μL ion-pairing solutions and equilibrate for 5 min before injection (use of ion-pairing agents greatly enhances separation of dephytolated pigments, chlorophyllide *a,* chlorophyll *c,* and pheophorbide *a*).

Prepare blanks by mixing 1 mL 90% acetone with 300 μL IP solution. Rinse syringe twice with 150 μL standard and draw about 250 μL standard into syringe for injection. Place syringe in injector valve and completely fill the 100-μL sample loop. Construct calibration curves by plotting fluorescence peak areas (or heights) against standard pigment concentrations.

3) Prepare samples for injection by mixing a 1-mL portion of the 90% acetone pigment extract with 300 μL IP solution.

4) Use a two-step solvent program to optimize separation of chorophylls from their degradation products.[15] After injection, change from solvent System A to System B over 5 min and follow with System B for 15 min at a flow rate of 2 mL/min. Re-equilibrate the column with System A for 5 min before the next injection, for a total analysis time of about 25 min. Degas the solvent systems with helium during analysis. Increase lifetime of HPLC column by storing it in 100% methanol between runs. Periodically flush the HPLC with reagent water to avoid buildup of ion pairing agents.

‖ ‖ Fluka Chemical Corp., 980 South Second Street, Ronkonkoma, NY, or equivalent.
Sigma Chemical Company, or equivalent.

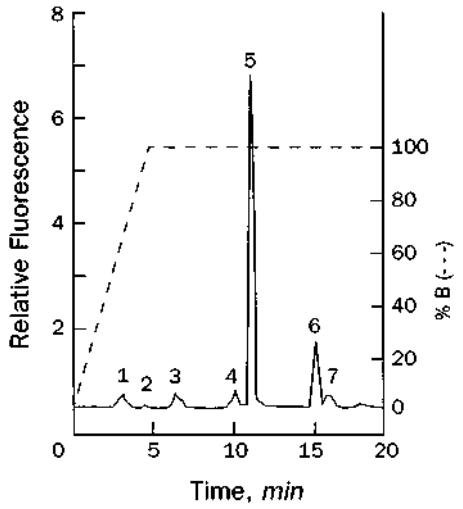


Figure 10200:11. Reverse-phase HPLC chromatogram for a fivefold dilution of EPA sample. Injection volume 100 μL; peaks detected by fluorescence spectroscopy (λ_{ex}: 400–460 nm; λ_{ex}: >600 nm). Peak identities are: 1—chlorophyllide *a,* 2—chlorophyll *c,* 3—pheophorbide *a,* 4—chlorophyll *b,* 5—chlorophyll *a,* 6—pheophytin *a,* and 7—pheophytin *a*′. The chlorophyll *b* degradation products (pheophytin *b* and pheophytin *b*′) were below detection limits. Peak identities confirmed by on-line diode array spectroscopy (350–550 nm).

5) Calculate individual pigment concentrations using the following formula:

$$C_i = \frac{A_s F_i V_E}{V_I V_S}$$

where:

C_i = individual pigment concentration, mg/L,
A_s = area of individual pigment peak from sample injection,
F_i = standard response factor (mg pigment/0.1 mL standard divided by corresponding peak area).
V_E = extraction volume, mL,
V_I = injection volume (0.1 mL), and
V_S = sample volume, L.

6) This method is designed only to quantify chlorophylls and their degradation products. To detect carotenoid pigments, which also are present in 90% acetone extracts but do not fluoresce, use absorbance spectroscopy (at about 440 nm).[21]

7) The elution order and approximate retention times for the major chlorophyll pigments and their degradation products are shown in Figure 10200:11. The detection limits [signal-to-noise (*s/n*) ratio = 2] vary with fluorometer configuration and flow rate; however, they range from 10 to 100 pg per injection for most chlorophylls and their degradation products.[15,21,29] The accuracy of the HPLC method depends primarily on purity of pigment standards. Preferably measure the standards' absorption spectra (350 to 750 nm) and compare with published data. Pigment purity also can be assessed via HPLC analysis if there

TABLE 10200:III. EXTINCTION COEFFICIENTS AND CHROMATOGRAPHIC PROPERTIES OF PIGMENTS SEPARATED BY REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (CF. FIGURE 10200:12)

Pigment Identity	Wavelength (solvent) *nm*	E_{1cm} *L/g · cm*	Ref. No.	Retention Time *min*	% c.v. (n = 3 inj)	Absorption Maxima in Eluent* *nm*		
Chlorophyllide *a*	664 (90% acetone)	128.0	28	7.8	5.7	nd†	nd	nd
Chlorophyll c_{1+2}	631 (90% acetone)	42.6	26	8.9	0.6	444	576	630
Peridinin	466 (acetone)	134.0	33	10.0	1.2		472	
Fucoxanthin	449 (acetone)	160.0	44	11.0	0.9		446	(466)
Neoxanthin	439 (ethanol)	224.3	35	11.5	5.9	416	441	470
Violaxanthin	443 (ethanol)	255.0	35	13.2	2.6	416	440	470
Diadinoxanthin	448 (acetone)	223.0	36	14.6	6.0	422	446	476
Lutein	445 (ethanol)	255.0	35	17.5	0.7	(422)	446	476
Zeaxanthin	450 (ethanol)	254.0	35	18.0	2.2	(428)	454	478
Chlorophyll *b*	647 (90% acetone)	51.36	5	21.1	1.0	456	596	646
Chlorophyll *a*	664 (90% acetone)	87.67	5	22.3	0.8	431	618	665
β,β-carotene	453 (90% acetone)‡	262.0	35	25.4	2.0	427	462	480

* All absorption maxima are from Wright *et al.*,[31] except those for chlorophyll c_{1+2} (R.R. Bidigare and M. Latasa, unpublished data).
† Not determined.
‡ Because of a potential insolubility problem of β,β-carotene in ethanol, prepare this standard in 90% acetone, not ethanol. It is assumed that the extinction coefficient of β,β-carotene in 90% acetone is the same as that in ethanol.

are no co-eluting contaminants with absorption and their fluorescence bands overlap those of the standards. HPLC and spectrophotometrically derived pigment concentrations for available EPA standards agree reasonably well (±20%) if spectrophotometric results are corrected for pheopigments and HPLC results are expressed as pigment equivalents (e.g., chlorophyll *a* equivalents = chlorophyllide *a* + chlorophyll *a* + chlorophyll *a′*, provided that proper molecular weight corrections are applied).[30] Thus, if significant amounts of chlorophyll derivatives are present, pigment concentrations determined spectrophotometrically will be overestimated. The agreement between HPLC and fluorometrically derived results depends on the presence of accessory chlorophylls *b*, *c*, and their derivatives. Triplicate injections of a fivefold dilution of an EPA sample gave coefficients of variation of 7.5% (chlorophyllide *a*), 9.1% (chlorophyll *c*), 13.4% (pheophorbide *a*), 9.6% (chlorophyll *b*), 0.5% (chlorophyll *a*), 6.2% (pheophytin *a*), and 22.9% (pheophytin *a′*), with an average value of 10% for the seven pigments analyzed.

5. High-Performance Liquid Chromatographic Determination of Algal Chlorophyll and Carotenoid Pigments

a. Equipment and reagents: In addition to those listed for pigment extraction, 10200H.1*a*:

1) *High-performance liquid chromatographic pump*—capable of gradient delivery of three solvents at a flow rate of 1 mL/min.

2) *High-pressure injector valve*—equipped with a 200-μL sample loop.

3) *Guard column*—(50 × 4.6 mm, C_{18} packing material,*** 5-μm particle size) to extend life of primary column.

4) *Reverse-phase HPLC column*—with endcapping (250 × 4.6 mm, 5-μm particle size, C_{18} column***).

5) *Variable wavelength or filter absorbance detector*—with low-volume flow-through cell. Detection wavelength is 436 nm.

6) *Data recording device*—Strip chart recorder or, preferably, an electronic integrator or computer equipped with hardware and software for chromatographic data analysis.

7) *Syringe,* glass, 500-μL.

8) *HPLC eluents*—Eluent A [80:20 blend of methanol and 0.5*M* ammonium acetate (v:v), respectively; pH 7.2]; Eluent B [90:10 blend of acetonitrile and water (v:v), respectively], and Eluent C (ethyl acetate). Use HPLC-grade solvents. Measure volumes before mixing. Filter eluents through a solvent-resistant 0.4-μm filter before use and degas with helium.

9) *Calibration standards*—Chlorophylls *a* and *b*, and β,β-carotene can be purchased,††† as can zeaxanthin and lutein.‡‡‡ Other pigment standards can be purified from plant extracts via thin-layer chromatography (TLC)[25] or preparative-scale HPLC. Determine concentration of all standards using a monochromator-based spectrophotometer in the appropriate solvents before calibrating the HPLC system. The recommended extinction coefficients for the most common algal pigments found in freshwater systems are given in Table 10200:III. Measure absorbance in a 1-cm cuvette at the appropriate wavelength (usually at λ_{max}) and 750 nm (to correct for light scattering). Calculate standards concentrations as follows:

$$C_i = \frac{(A_{\lambda max} - A_{750\ nm})}{E_{lcm} \times b} \times 1000$$

where:

C_i = individual pigment concentration, mg/L,
A = absorbance at specific wavelength,
E_{1cm} = weight-specific absorption coefficient, L/g · cm,
b = path length of cuvette, cm, and
1000 = conversion factor, g to mg.

Standards stored under nitrogen in the dark at –20°C are stable for about 1 month.

*** Spherisorb ODS-2, Phase Separations Inc., Norwalk, CT, or equivalent.

††† Sigma Chemical Co., St. Louis, MO, or equivalent.
‡‡‡ Roth Chemical Co., distributed by Atomergic Chemetals Corp., Farmingdale, NY, or equivalent.

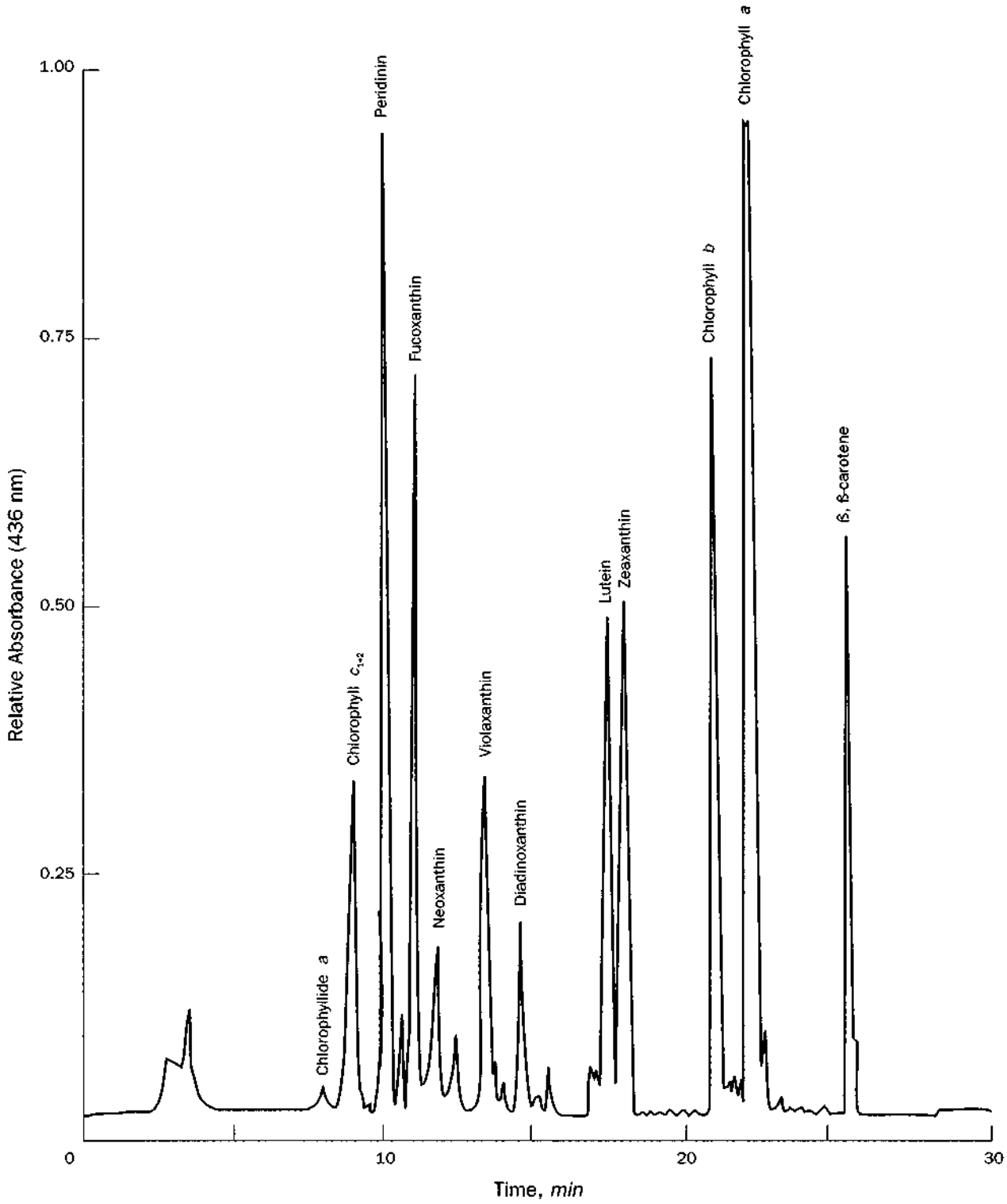


Figure 10200:12. Reverse-phase HPLC pigment chromatogram for a mixture of common algal pigments found in freshwater systems. For further data, see Table 10200:III. Sample contained a natural extract with authentic known additions. The small unlabeled peaks are pigment degradation products.

b. Procedure:

1) Set up and equilibrate the HPLC with Eluent A at a flow rate of 1 mL/min.

2) Calibrate the HPLC using working standards (about 0 to 1000 ng/mL) prepared from primary standards on day of use. Mix 1 mL standard with 300 μL distilled water, shake, and

TABLE 10200:IV. HPLC SOLVENT SYSTEM PROGRAM

Time *min*	Flow Rate *mL/min*	Percentage of Eluent A	B	C	Conditions
Analysis protocol:					
0.0	1.0	100	0	0	Injection
2.0	1.0	0	100	0	Linear gradient
2.6	1.0	0	90	10	Linear gradient
13.6	1.0	0	65	35	Linear gradient
18.0	1.0	0	31	69	Linear gradient
23.0	1.0	0	31	69	Hold
25.0	1.0	0	100	0	Linear gradient
26.0	1.0	100	0	0	Linear gradient
34.0	1.0	100	0	0	Hold
Shutdown protocol:					
0.0	1.0	100	0	0	Analysis complete
3.0	1.0	0	100	0	Linear gradient
6.0	1.0	0	0	100	Linear gradient
16.0	1.0	0	0	100	Washing
17.0	0.0	0	0	100	Shutdown

equilibrate for 5 min before injection (diluting standards and sample extracts with water increases the pigments' affinity for the column in the loading step, resulting in better separation of more polar pigments). Rinse syringe twice with 300 μL standard and draw 500 μL standard into syringe for injection. Place syringe in injector valve, overfilling the 200 μL sample loop 2.5-fold. To check for possible interferences in extraction solvent and/or filter, prepare a blank by extracting a glass fiber filter in 90% acetone; mixing 1 mL 90% acetone filter extract with 300 μL distilled water; and injecting into the HPLC. Plot absorbance peak areas (or heights) against standard pigment concentrations. Calculate response factors as the slope of the regression between the weights of injected standards (ng) and the areas of parent pigment (plus areas of structurally related isomers, if present). These isomers contribute to the standards' absorption signal; disregarding them results in overestimation of pigments in sample extracts.[32]

3) Prepare samples for injection by mixing a 1-mL portion of the 90% acetone pigment extract with 300 μL distilled water, shake, and equilibrate for 5 min before injection.

4) Following sample injection, use a gradient program to optimize separation of chlorophyll and carotenoid pigments. The system described in Table 10200:IV has been developed from the original method[31] to ensure elution of most hydrophobic pigments. Degas solvent system with helium during analysis. Periodically flush HPLC with distilled water to avoid accumulating ion-pairing reagents.

5) Routinely determine peak identities by comparing sample peaks' retention times with those of pure standards. Confirm peak identities spectrophotometrically by collecting eluting peaks from the column outlet (or directly with an on-line diode array spectrophotometer). Table 10200:III lists absorption maxima for the most common pigments found in freshwater systems.

6) Calculate individual pigment concentrations using the formula given in 10200H.4*b*5).

7) This method is designed to separate chlorophyll and carotenoid pigments (Figure 10200:12); however, it also separates major chlorophyll breakdown products.

8) Method precision was assessed by making triplicate injections of a mixture of phytoplankton and plant extracts. Coefficients of variation ranged from 0.6 to 6.0% (Table 10200:III). Using an appropriate internal standard increases precision.

Further information on these pigments and on analysis methods is available.[33–37]

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10200 I. Determination of Biomass (Standing Crop)

Biomass is a quantitative estimate of the total mass of living organisms in a given area or volume. It may include the mass of a population (*species biomass*) or of a community (*community biomass*) but gives no information on community structure or function. The most accurate methods for estimating biomass are dry weight, ash-free dry weight, and volume of living organisms. Indirect methods include estimates of total carbon, caloric content, nitrogen, lipids, carbohydrates, silica (diatoms), and chlorophyll (algae). Adenosine triphosphate[1] (ATP) and deoxyribonucleic acid[2,3] (DNA) also have been used as indirect estimates.

Most biomass estimates can be affected by organic and inorganic detritus; ATP and DNA analyses include contributions from bacterial flora.[4] Biomass calculated from direct cell counts and dimensional measurements will incorporate error but avoid including extraneous matter, including sediments and detritus, that other techniques cannot segregate from their results.

1. Chlorophyll *a*

Chlorophyll *a* is used as an algal biomass indicator.[5] Assuming that chlorophyll *a* constitutes, on average, 1.5% of the ash-free dry weight of algae, estimate the algal biomass by multiplying the chlorophyll *a* content by 67 (see 10200H for chlorophyll *a* methodology). The ratio of chlorophyll to dry weight will vary among major taxonomic groups, however, so the ratio might be adjusted based on knowledge of algal composition.

2. Biovolume (Cell Biovolume)

Plankton data derived on a volume-per-volume basis often are more useful than numbers per milliliter because they allow for analysis of individual taxa or species contributions to community structure and function on a biomass basis (e.g., 1 cell-Chlamydomonas: ~100 μm^3 vs. 1 cell-Ceratium: ~40,000 μm^3).[6] Determine cell biovolume by using the simplest geometric configuration (e.g., sphere, cone, cylinder, wedge) that best fits the shape of the cell being measured.[7–10] Measure only the biovolume of living cells, not sheaths and setae. An organism's cell size can differ substantially in different waters or in the same water at different times of the year, so for each sampling period, average the measurements of 10 to 30 individuals of each species or taxon (use a larger number of individuals for extremely variable genera like *Microcystis*). Calculate the total biovolume

of any species by multiplying the average cell biovolume (in μm^3) by the number of cells per milliliter.

Compute total wet algal biovolume as:

$$V_t = \sum_{i=1}^{n} (N_i \times V_i)$$

where:

V_t = total plankton cell biovolume, mm^3/L,
N_i = number of organisms of the ith species/L, and
V_i = average biovolume of cells of ith species, μm^3.

The carbon biomass in cells of individual phytoplankton components may also be estimated directly from cell biovolume measurements.[8]

3. Volume/Surface Area

An estimation of natural-unit surface area is valuable when analyzing interactions between the natural unit and surrounding waters. Compute average surface area in μm by using similar geometric configurations and multiplying by the number per milliliter of the species being considered.[2] Take care to subtract open areas (lorica openings) or surfaces that abut each other (e.g., valve faces that touch in multiple cell diatom colonies). Natural unit volume and area estimates allow analysis of nutrient dynamics, grazing, and suspension dynamics.

4. Displacement Volume

This method[11] measures the volume of liquid that a sample displaces. Displacement volume may be determined via several methods. For simple, direct measurement, place sample in a sieve whose mesh is equal to or smaller than that of the capture net. Let sample drain and transfer to a measured volume of water in a graduated cylinder. Measure the new volume (sample plus known volume). The displacement volume equals the new volume minus the first measured volume of water. This method works well for macroalgal samples and zooplankton.

5. Gravimetric Methods

The plankton community's biomass can be estimated from gravimetric determinations, although silt and organic detritus interfere. Determine dry weight by placing 100 mg wet concentrated sample in a clean, ignited, and tared porcelain crucible and dry at 105°C for 24 h. Alternatively, filter a known volume of sample through 0.45-μm-porosity membrane or a pre-rinsed, dried, and pre-weighed glass-fiber filter. (NOTE: The small sample used in direct filtration may lead to error if not handled properly.) Cool sample in a desiccator and weigh. Obtain ash-free weight by igniting the dried sample at 500°C for 1 h in a muffle furnace. Cool, rewet ash with distilled water, and bring to constant weight at 105°C. (The ash is rewetted to restore water of hydration to clays and other minerals; this may amount to as much as 10% of weight lost during incineration.[12]) The *ash-free dry weight* is the difference between the dry weight and the weight of the ash residue after ashing. When comparing mixed assemblages, ash-free weight is preferred to dry weight because the ash content of samples varies greatly. Ash may constitute 50% or more of the dry weight of phytoplankton with inorganic structures, such as diatoms. In other samples, the ash content is only about 5% of dry weight.

6. Adenosine Triphosphate (ATP)

The only way to determine the total viable plankton biomass is to measure adenosine triphosphate (ATP) in plankton. ATP occurs in all plants and animals, but only in living cells; it is not associated with nonliving particulate. The ratio of ATP to biomass varies from species to species, but appears to be constant enough to permit reliable estimates of biomass based on ATP measurements.[13] The method is simple and relatively inexpensive, and the instrumentation is stable and reliable. The method also has many potential applications in entrainment and bioassay work, especially plankton mortality studies.

a. Equipment and reagents:

1) *Glassware,* clean, sterile, dry borosilicate glass flasks, beakers, and pipets.

2) *Filters,* 47-mm-diam, 0.45-μm-porosity membrane filters.

3) *Filtration equipment.*

4) *Freezer* (–20°C).

5) *Boiling water bath.*

6) *Detection instruments,* designed specifically for measuring ATP.*

7) *Microsyringes*—10-, 25-, 50-, 100-, and 250-μL.

8) *Reaction cuvettes and vials.*

9) *Tris buffer* (0.02*M*, pH 7.75)—Dissolve 7.5 g trishydroxymethylaminomethane in 3 L distilled water and adjust pH to 7.75 with 20% HCl. Autoclave 150-mL portions at 115°C for 15 min.

10) *Luciferin-luciferase enzyme preparation*†— Rehydrate frozen (–20°C) lyophilized extracts of firefly lanterns with Tris buffer as directed by the supplier; let stand at room temperature 2 to 3 h, then centrifuge at 300 × *g* for 1 min and decant the supernatant into a clean, dry test tube; let stand at room temperature for 1 h.

11) *Purified ATP standard*—Dissolve 12.3 mg disodium ATP in 1 L distilled water and dilute 1.0 mL to 100 mL with Tris buffer; 0.2 mL = 20 ng ATP.

b. Procedure:

1) Calibration—To determine the calibration factor, F, prepare a series of dilutions of purified ATP standard and record the light emission from several portions of each concentration of standard. Correct mean area of standards by subtracting peak reading or mean area of several blanks using 0.2 mL Tris buffer. Calculate calibration factor F_S:

$$F_s = \frac{C}{A_s}$$

where:

F_s = calibration factor at sensitivity S,
C = concentration of ATP in standard solution, ng/mL, and

* Beckman, JRB, Turner Designs, or equivalent.
† Dupont, Sigma Chemical, or equivalent.

A_s = peak reading or mean area under standard ATP curve corrected for blank.

2) Sample analysis—Collect a 1- to 2-L sample in a clean, sterile sampler. Pass through a 250-μm net to remove large zooplankton[13] and filter through a 47-mm, 0.45-μm-porosity filter by applying a vacuum of about 30 kPa. (**IMPORTANT: Break suction before the last film of water is pulled through the filter.**) Quickly place filter in a small beaker. Immediately cover filter with 3 mL boiling Tris buffer, using an automatic pipet. Place beaker in boiling water bath for 5 min and, with a Pasteur pipet, transfer extract to a clean, dry, calibrated test tube. Rinse filter and beaker with 2 mL boiling Tris buffer; combine extracts, record volume, bring volume up to 5 mL with Tris buffer, cover tubes with parafilm and, if samples cannot be analyzed immediately, freeze at –25°C. Extracts may be stored for many months in a freezer. Prepare at least triplicate extracts of each sample.

The analytical procedure depends on detection equipment used. If a scintillation counter is used, pipet 0.2 mL enzyme preparation (blank) into a glass vial. Measure the enzyme preparation's light emissions for 2 to 3 min at sensitivity settings near those anticipated for the sample. Add 0.2 mL sample extract to the vial, record the time, and swirl. Start recording light output 10 s after combining ATP extract and enzyme preparation; record output for 2 to 3 min, using the same time period for all samples. Determine the mean of the areas under the curves obtained and correct by subtracting the mean of the areas under the curves obtained from blanks prepared as directed in Strickland and Parsons.[14]

c. Calculations: Calculate ATP concentration:

$$ATP, \text{ ng/L} = \frac{A_c \times V_e \times F_s}{V_s}$$

where:

A_c = mean corrected area under extract curves,
V_e = extract volume, mL,
F_s = calibration factor, and
V_s = volume of sample, L.

Assuming an ATP content of 2.4 μg ATP/mg dry weight organic matter,[15] total living plankton biomass (B), as dry weight organic matter, is given as:

$$B, \text{ mg/L} = \frac{ATP}{(2.4)(1000)}$$

7. References

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10200 J. Metabolic Rate Measurements

The aquatic community's physiological condition and spectrum of biological interactions must be considered when evaluating the state of natural waters. In earlier studies, numbers, species composition, and biomass were prime considerations. Recognition of this approach's limitations led to the measurement of rates of metabolic processes, such as photosynthesis (productivity), nitrogen fixation, respiration, and electron transport. These provide a better understanding of the aquatic ecosystem's complex nature. An indication of photosynthetic efficiency can be determined via the productivity index (mg C fixed/unit chlorophyll *a*).[1]

1. Nitrogen Fixation

An organism's ability to fix nitrogen is a great competitive advantage and plays a major role in population dynamics. Two reliable methods for estimating nitrogen-fixation rates inthe laboratory are the 15*N* isotope tracer method[2,3] and the acetylene reduction method.[4] Because the rate of nitrogen fixation varies greatly with different organisms and with the concentration of combined nitrogen, nitrogen-fixation rates cannot be used to estimate biomass of nitrogen-fixing organ-

isms. However, the acetylene reduction method is useful in measuring nitrogen budgets and in algal assay work.[5]

2. Productivity, Oxygen Method

In this context, *productivity* is the rate at which inorganic carbon is converted to an organic form. Chlorophyll-bearing organisms (phytoplankton, periphyton, macrophytes) serve as primary producers in the aquatic food chain. Photosynthesis ultimately results in the formation of a wide range of organic compounds, release of oxygen, and reduction of carbon dioxide (CO_2) in surrounding waters. Primary productivity[6] can be determined by measuring the changes in oxygen and CO_2 concentrations.[7]

In poorly buffered waters, pH can be used to detect variations in the system. As CO_2 is removed during photosynthesis, pH rises. This shift can be used to estimate both photosynthesis and respiration.[8] The sea and many fresh waters are too highly buffered to make this useful, but it has been applied successfully to productivity studies in some lake waters.

Two methods for measuring the rate of carbon uptake and net photosynthesis *in situ* are the oxygen method[9] and the carbon-14 method.[10] In both methods, clear (light) and darkened (dark) bottles are filled with water samples and suspended at regular depth intervals for 3 to 4 h (maximum rate) or for a 24-h period (integrate rate), or samples are incubated under controlled conditions in environmental growth chambers in the laboratory.

The basic reactions in algal photosynthesis involve uptake of inorganic carbon and release of oxygen:

$$CO_2 + H_2O \rightarrow (CH_2O)_x + O_2$$

The chief advantages of the oxygen method are that it provides estimates of gross and net productivity and respiration, and that analyses can be performed with inexpensive laboratory equipment and common reagents. The dissolved oxygen (DO) concentration is determined at the beginning and end of the incubation period. Productivity is calculated based on the assumption that one carbon atom is assimilated for each oxygen molecule released.

a. Equipment:

1) *BOD bottles*—numbered, 300-mL, clear and opaque borosilicate glass, with ground glass stopper and flared mouth, for sample incubation. Acid-clean the bottles, rinse thoroughly with distilled water, and just before use, rinse with water being tested. Do not use detergents containing phosphorus.

If suitable opaque bottles are not available, make clear BOD bottles opaque by painting them black and wrapping them with black waterproof tape. As a further precaution, wrap entire bottle in aluminum foil or place in light-excluding container during incubation.

2) *Supporting line or rack* that does not shade suspended bottles.

3) *Nonmetallic, opaque acrylic plastic Van Dorn sampler* or equivalent, 3- to 5-L capacity.

4) *Equipment and reagents for DO determinations*—See Section 4500-O.

5) *Pyrheliometer.*

6) *Submarine photometer.*

7) *Thermometer.*

b. Procedure:

1) Using a pyrheliometer, obtain a profile of the input of solar radiation for the photoperiod.

2) Using a submarine photometer, determine depth of euphotic zone (the region that receives 1% or more of surface illumination). Select depth intervals for bottle placement. To closely approximate the photosynthesis-depth curve, place samples at intervals equal to one-tenth the depth of the euphotic zone. Estimate productivity in relatively shallow water with fewer depth intervals.

3) Measure oxygen concentration via a probe or titration and temperature and salinity to determine whether water is supersaturated with oxygen (see Table 4500-O:I). If water is supersaturated, bubble nitrogen gas through sample to lower initial oxygen concentration to <80% saturation.

4) Keep samples out of direct sunlight during handling. Introduce samples taken from each preselected depth into duplicate clear, darkened, and initial-analysis bottles. Insert sampler's delivery tube to bottom of sample bottle and fill so three volumes of water are allowed to overflow. Remove tube slowly and close bottle. Use water from the same grab sample to fill a "set" (one light, one dark, and one initial bottle).

5) Immediately treat (fix) samples taken to chemically determine initial DO (see Section 4500-O) with manganous sulfate ($MnSO_4$), alkaline iodide, and sulfuric acid (H_2SO_4) or check with an oxygen probe. Analyses may be delayed several hours, if necessary, if samples are fixed or iced and stored in the dark.

6) Suspend duplicate paired clear and darkened bottles at the depth from which the samples were taken and incubate for at least 2 h, but never longer than it takes for oxygen-gas bubbles to form in clear bottles or DO to be depleted in dark bottles.

7) At the end of the exposure period, immediately determine DO as described above.

c. Calculations: The increase in oxygen concentration in the light bottle during incubation is a measure of net production, which (because oxygen is used concurrently in respiration) is somewhat less than total (gross) production. The loss of oxygen in the dark bottle is used as an estimate of total plankton respiration. Thus:

$$\text{Net photosynthesis} = \text{light bottle DO} - \text{initial DO}$$

$$\text{Respiration} = \text{initial DO} - \text{dark bottle DO}$$

$$\text{Gross photosynthesis} = \text{light bottle DO} - \text{dark bottle DO}$$

Average results from duplicates.

1) Calculate the gross or net production for each incubation depth and plot:

$$\text{mg fixed carbon/m}^3 = \text{mg oxygen released/L} \times 12/32 \times 1000\ \text{L/m}^3 \div K$$

where K is the photosynthetic quotient (PQ), which ranges from 1 to 2 depending on nitrogen supply.[11,12]

Use the factor 12/32 to convert oxygen to carbon; under ideal conditions, 1 mole of O_2 (32 g) is released for each mole of fixed carbon (12 g).

2) In this context, *productivity* is the rate of production; it generally is reported in grams carbon fixed per square meter per day. Determine the productivity of a vertical column of water 1 m square by plotting productivity for each exposure depth and graphically integrating the area under the curve.

3) Using the solar radiation profile and photosynthesis rate during incubation, adjust the data to represent phytoplankton productivity for the entire photoperiod. Because photosynthetic rates vary widely during the daily cycle,[13,14] do not attempt to convert data to other test circumstances.

3. Productivity, Carbon-14 Method

Add solution of radioactive carbonate ($^{14}CO_3^{2-}$) to light and dark bottles that have been filled with sample, as described for the oxygen method. After incubation *in situ*, collect the plankton on a membrane filter, treat with hydrochloric acid (HCl) fumes to remove inorganic carbon-14, and assay for radioactivity. The quantity of fixed carbon is proportional to the fraction of assimilated radioactive carbon. This procedure differs from the oxygen method in that it directly measures carbon uptake and measures only net photosynthesis.[15] It is basically more sensitive than the oxygen method but fails to account for organic materials that leach from cells[16,17] during incubation.

a. Equipment and reagents:

1) *Pyrheliometer.*

2) *Submarine photometer.*

3) *BOD bottles and supporting apparatus*—See 10200J.2*a*1) and 2).

4) *Membrane-filtering device and 25-mm filters* with porosities of 0.22, 0.30, 0.45, 0.80, and 1.2 μm.

5) *Counting equipment for measuring radioactivity*—Scaler with end-window tube, gas flow meter, or liquid scintillation counter (see Section 7030B.3). The thin-window tube is the least expensive detector and, when used with a small scaler, provides acceptable data at modest cost.

6) *Fuming chamber*—Use a glass desiccator with conc HCl about 1.4 cm deep in desiccant chamber. The fuming chamber is recommended for filter decontamination.[18,19]

7) *Syringe or pipet,* nonmetallic.

8) *Chemical reagents*—See Sections 4500-CO_2 (Carbon Dioxide) and 2320 (Alkalinity).

9) *Radioactive carbonate solutions*

a) *Sodium chloride dilution solution*, 5% NaCl (w/v)—Add 0.3 g sodium carbonate (Na_2CO_3) and one pellet sodium hydroxide (NaOH) per liter. Use for marine studies only.

b) *Carrier-free radioactive carbonate solution*—commercially available in sealed vials containing about 5 μCi ^{14}C/mL. Confirm absence of suspended and dissolved toxic metals[20] or filter and pass through an ion-exchange column.*

c) *Working solutions* with activities of 1, 5, and 25 μCi ^{14}C/2 mL—For fresh-water studies, use carrier-free radioactive carbonate. For marine-water studies, prepare by diluting carrier-free radioactive carbonate solution with NaCl dilution solution.

d) *Stock ampules*—Prepare ampules containing 2 mL of required working solution. Fill ampules and autoclave sealed ampules at 121°C for 20 min.[21]

b. Procedure:

1) Using a pyrheliometer, obtain a record of incident solar radiation for the photoperiod.

2) Determine depth intervals for sampling and incubation as described above.

3) Use duplicate light and dark bottles at each depth. Also, use dark bottles or bottles harvested at time zero. Fill bottles with sample, add 2 mL radioactive carbonate solution (using a nonmetallic pipet) to the bottom of each bottle, and mix thoroughly by repeated inversion. The concentration of carbon-14 should be about 10 μCi/L in relatively productive waters, to 100 μCi/L or more in oligotrophic (open ocean) waters. To obtain statistical significance, have at least 1000 counts per minute (cpm) in the filtered sample. Take duplicate samples at each depth to determine initial concentration of inorganic carbon (CO_2, HCO_3^-, and CO_3^{2-}) available for photosynthesis (see Section 4500-CO_2). For estuarine and marine samples, estimate total inorganic carbon concentrations with a simple titration procedure[22] and make initial temperature, salinity, and pH measurements.

4) Incubate samples for up to 4 h. If measurements are required for the entire photoperiod, overlap 4-h periods from dawn until dusk. A 4-h incubation period may be sufficient if energy input is used as the basis for extrapolating incubation period to entire photoperiod. For incubation procedure, see 10200J.2*b*6).

5) After incubation, remove sample bottles and immediately place in the dark. Filter unpreserved samples promptly. Avoid sample preservation to avoid lysing cells or inadvertently including extracellular products.

6) Filter two portions of each sample through a membrane filter whose pore size is consistent with quantitative retention of plankton. Although the 0.45-μm pore filter usually is adequate, determine the sample-retention efficiency of a wide range of pore sizes immediately before analysis.[23,24] Apply about 30 kPa of vacuum during filtration. Excess vacuum may cause extensive cell rupture and loss of radioactivity through the membrane.[25] Use maximum sample volume consistent with rapid filtration (1 to 2 min), but do not clog filter.

7) Place membranes in HCl fumes for 20 min. Count filters as soon as possible, although extended storage in a desiccator is acceptable.

8) Determine radioactivity by counting with an end-window tube, windowless gas flow detector, or liquid scintillation counter.

9) Determine counting geometry of thin-window and windowless gas flow detectors.[26] Using three ampules of carbon-14, prepare a series of barium carbonate ($BaCO_3$) precipitates on tared 0.45-μm membrane filters, as directed below. The precipitates will contain the same amount of carbon-14 activity, but their thicknesses will range from 0.5 to 6.0 mg/cm^2. Dilute each ampule to 500 mL with a solution of 1.36 g Na_2CO_3/L CO_2-free distilled water. Pipet 0.5-mL portions into each of seven conical flasks containing 0, 0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 mL, respectively, of a solution of 1.36 g Na_2CO_3/L CO_2-free distilled water. Add, respectively, 0.3, 0.6, 1.2, 1.8, 2.4, 3.0, and 3.6 mL 1.04% barium chloride ($BaCl_2$) solution. Let $BaCO_3$ precipitate stand 2 h with gentle swirling every half hour. Collect each precipitate on a filter (using an apparatus with a filtration area comparable to that of the samples). With suction, dry filters without washing; place in a desiccator for 24 h, weigh, and count. The counting rate increases exponentially as precipitate thickness decreases. Extrapolate graphically (or mathematically) to zero precipitate thickness and multiply the zero-thickness counting rate by 1000 to correct for ampule dilution. This represents the amount of

* Chelex 100, or equivalent.

activity added to each sample bottle used to determine the fraction of carbon-14 taken up in light and dark bottles.

c. Calculations:

1) Subtract the mean dark-bottle or time-zero sample count from the mean light-bottle counts for each replicate pair.

2) Determine the total dissolved inorganic carbon available for photosynthesis (carbonate, bicarbonate, and free CO_2) from pH and alkalinity measurements; make direct measurement of total CO_2 according to Section 4500-CO_2 or methods described in the literature.[27–30]

3) Determine quantity of fixed carbon as follows:

$$\text{mg fixed carbon/L} = \frac{\text{counting rate of filtered sample}}{\text{total activity added to sample}}$$

$$\times \frac{300}{\text{volume filtered}} \times \text{mg/L initial inorganic carbon} \times 1.064\dagger$$

4) Integrate productivity for the entire depth of euphotic zone and express as grams fixed carbon per square meter per day [see 10200J.2*c*2)].

5) Using the solar radiation records and photosynthesis rates during incubation, adjust data to represent phytoplankton productivity for the entire photoperiod. If samples were incubated for less than the full photoperiod, apply a correction factor.

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† Correction for isotope effect.

10300 PERIPHYTON*

10300 A. Introduction

1. Definition and Significance

Microorganisms growing on stones, sticks, aquatic macrophytes, and other submerged surfaces are useful in assessing the effects of pollutants on lakes, streams, and estuaries. Called *periphyton*,[1,2] this group of organisms include zoogleal and filamentous bacteria, attached protozoa, rotifers, and algae, and free-living microorganisms that swim, creep, or lodge among the attached forms.

Unlike plankton, which often do not fully respond to pollution's influence in rivers for a considerable distance downstream, periphyton show marked responses immediately below pollution sources. Examples are the beds of *Sphaerotilus* (see Section 10900, Plate 26:H) and other "slime organisms" commonly observed in streams below organic waste discharges. Because periphyton's abundance and composition at a given location are governed by the water quality there, observations of their condition generally are useful in evaluating the waterbody's conditions.

The use of periphyton in assessing water quality often is hindered by the lack of suitable natural substrata† at the desired sampling station. Furthermore, it often is difficult to collect quantitative samples from natural substrata because of their physical complexity. To circumvent these problems, investigators have used artificial substrata to provide a uniform surface type, area, and orientation.[3]

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† Although the terms *substrate* and *substratum* often have been used interchangeably, technically it is more correct to use substratum in connection with periphyton. In biochemical usage, a *substrate* (plural: substrates) is the substance acted on by an enzyme and the source of energy, while a *substratum* (plural: substrata) is the base or material on which a nonmotile organism lives or grows (i.e., the submerged surfaces used for periphyton colonization).

10300 B. Sample Collection

1. Station Selection

In rivers, locate stations a short distance upstream and at one or more points downstream of the study area or suspected pollution source in areas with central mixing. In large rivers, sample both sides of the stream in main flow areas. Because a pollutant's effects depend on both the stream's assimilative capacity and the nature of the pollutant, progressive changes in water quality downstream from the pollution source may be caused entirely by dilution and cooling—as in the case of nutrients, toxic industrial wastes, and thermal pollution—or by gradual mineralization of degradable organic compounds. A cursory examination of shoreline and bottom periphyton growths on natural substrata downstream of an outfall may indicate conspicuous zones of biological response to water quality that will be useful when determining appropriate sampling-station sites. If an intensive sampling program is infeasible, then using at least three sampling stations—one upstream of the pollution source and the others downstream in areas where the pollutant has completely mixed with the receiving water will provide minimal data on the periphyton community.

In lentic waters (e.g., lakes, reservoirs, ponds) and other standing-water bodies where pollution zones may be arranged concentrically, locate stations in areas next to a waste outfall and in unaffected areas. Use control stations in areas similar to the affected ones (e.g., similar in water depth and distance from shore).

2. Sample Collection

a. Natural substrata: Collect qualitative samples by scraping submerged stones, sticks, pilings, and other available substrata. Many devices have been developed to collect quantitative samples from irregular surfaces. Appropriate techniques for removing periphyton from both living and nonliving surfaces have been described.[1–4]

b. Artificial substrata: The most widely used artificial substratum is the standard, plain, 25- × 75-mm glass microscope slide, but other materials (e.g., clear vinyl plastic) are also suitable. Do not change substratum type during a study because colonization varies with substratum. (NOTE: No community on an artificial substratum is completely representative of the natural community.)

In small, shallow streams and in littoral regions of lakes and reservoirs where light penetrates to the bottom, place slides or other substrata vertically in frames anchored to the bottom. In large, deep streams or standing-water bodies where turbidity varies widely, place slides vertically with the slide face at right

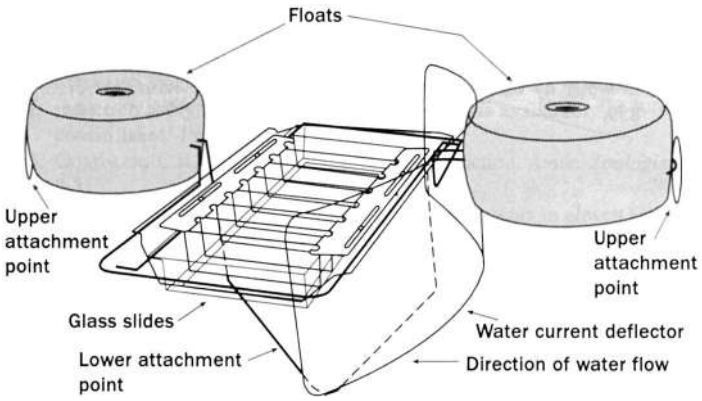


Figure 10300:1. Periphyton sampler. Floating sampler with upstream deflecting baffle and transparent, removable slide rack holding up to eight microscope slides.

angles to the prevailing current. A floating rack is suitable (Figure 10300:1).* Expose several slides (at least five: three for biomass, one for species, and one backup for each time interval) per analysis to ensure that sufficient material is collected and to determine whether variability in results is caused by normal differences in colonization of individual slides. In addition to pollutant effects, length of substratum exposure and seasonal changes in temperature and other natural environmental conditions may profoundly affect sample composition.

Place, expose, and handle all artificial substratum samplers in conditions as nearly identical as possible, whether they are replicates at a particular sampling location or samplers at different locations. Sampler type and/or construction cause changes in surrounding physical conditions, which in turn affect periphyton growth. Variations of 10 to 25% between sample replicates are common. Therefore, to reduce sampling error and increase interpretive power, reduce the magnitude of all possible test variables and use sufficient replication.

c. Exposure period: Colonization on clean slides proceeds exponentially for the first 1 or 2 weeks and then slows. Because exposures shorter than 2 weeks may result in sparse collections, and exposures longer than 2 weeks may result in material loss due to sloughing, sample for 2 weeks during summer. This exposure period precludes collecting sexually mature thalli of larger, slow-growing filamentous algae [e.g., *Cladophora* (see Section 10900, Plate 29, Figure B-8) and *Stigeoclonium* (Plate 29, Figure B-45)]. To obtain optimum growth during winter, use a longer exposure period. For the most exacting work, determine the optimum exposure period by testing colonization rates over approximately 6 weeks.

Secondary problems associated with macroinvertebrate infestation and grazing may occur, often within 7 to 14 d. To reduce the confounding influence of grazing, increase substratum sampling area and expose for 7 to 10 d.

3. Sample Preservation

Preserve counting and identification samples in 5% neutralized formalin, Lugol's iodine, or merthiolate (see Section 10200B.2). Gluteraldehyde (2 to 5%) also is an excellent preservative, and in some ways is superior because it affects cell membranes less severely.

Preserve slides intact in suitably sized bottles or scrape into containers in the field. Air-dry slides for dry and ash-free dry weight in the field and store them in a 3.0- × 7.7-cm glass bottle. Place chlorophyll analysis slides in acetone or methanol in the field or collect and freeze with trichlorotrifluoroethane† (or alternative) or CO_2. Ethanol (95%) is an excellent solvent for chlorophyll extraction; it yields greater extraction, is less toxic, and is less expensive than acetone or methanol.[5,6] The specific absorption coefficient for chlorophyll *a* in 95% ethanol is 83.4 L/g·cm. Alternatively, hold on dry ice until returned to the laboratory. If samples are frozen in the field, remove them from the substratum and concentrate (via filtration or other means) before freezing. Store all samples in the dark. For pollution and eutrophication studies using periphyton biota as indicators, do not preserve samples. Enclose substrata with periphyton in containers filled with water, and transport and analyze them immediately. Also see 10300E.

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* Wildlife Supply Co., 95 Botsford Place, Buffalo, NY 14216, or equivalent.

† Freon, or equivalent.

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10300 C. Sample Analysis

1. Sedgwick–Rafter Counts

Remove periphyton from slides with a razor blade and rubber policeman. Disperse scrapings in 100 mL (or other suitable volume) preservative via vigorous shaking or a blender. Transfer a 1-mL portion to a Sedgwick–Rafter cell, and make a strip count as described in Section 10200F.2*a*. If material in the Sedgwick–Rafter cell is too dense to count directly, discard and replace with a diluted sample.

Sedgwick–Rafter cells do not permit examination at magnifications higher than 200×. A Palmer cell[1] (a thinner version of the Sedgwick–Rafter cell) permits examination at 400 to 500× with a standard compound microscope.

Express counts as cells or units per square millimeter of substratum area, calculated as in 10300C.2.

2. Inverted Microscope Method Counts

Using an inverted microscope to count periphyton permits magnifications higher than 200×. If an inverted microscope is unavailable, use one of the available alternatives for a standard compound microscope.[2,3] Remove periphyton quantitatively from slides with a razor blade and rubber policeman. Transfer a measured portion (after serial dilution, if necessary) into a standardized plankton sedimentation chamber. After a suitable settling period (see Section 10200C.1), count organisms in the settling chamber by counting all organisms within a known number of strips or random fields. Calculate algal density per unit area of substratum as follows:

$$\text{Organisms/mm}^2 = \frac{N \times A_t \times V_t}{A_c \times V_s \times A_s}$$

where:

N = number of organisms (cells or units) counted,
A_t = total area of chamber bottom, mm^2,
V_t = total volume of original sample suspension, mL,
A_c = area counted (strips or fields), mm^2,
V_s = sample volume used in chamber, mL, and
A_s = surface area of slide or substratum, mm^2.

To enhance separation of periphyton from silt and detritus, add a drop or less of a saturated iodine solution to the counting chamber just before counting. This method is especially useful when Chlorophyta are the predominant organisms because iodine stains starch food reserves blue. Iodine can even be added to preserved samples.

3. Diatom Species Counts

Preparing permanent diatom mounts from periphyton samples differs from preparing mounts from plankton samples because extracellular organic matter (e.g., gelatinous materials) must be removed. If this organic matter is not removed, it will produce a

thick brown or black carbonaceous deposit on the cover glass when the sample is incinerated. Very small amounts of organic matter can be cleared via incineration by placing a small, known volume of sample (<1 mL) directly on a cover slip. Let water evaporate, and ash at no more than 525°C for 6 to 10 min. Mount cover slip for direct examination of diatom frustules. Alternatively, decompose organic substances via oxidation with ammonium persulfate, HNO_3, or 30% H_2O_2 and $K_2Cr_2O_7$ (see Section 10200D.3) before mounting sample. To oxidize with persulfate, place a measured sample of approximately 5 mL in a disposable 10-mL vial. Let stand 24 h, withdraw supernatant liquid via aspiration, replace with a 5% solution of $(NH_4)_2S_2O_8$, and mix thoroughly. Do not exceed a total volume of 8 mL. Heat vial to approximately 90°C for 30 min. Let stand 24 h, withdraw supernatant liquid, and replace with reagent-grade water. After three changes of reagent-grade water, use a disposable pipet to transfer a drop of the diatom suspension to a cover glass, evaporate to dryness, and prepare and count a mount (as described for plankton in Section 10200). Count at least 500 frustules and express results as relative numbers or percentage of each species per unit area. Counts of more than 500 frustules may be needed, depending on the questions being addressed.[4]

4. Stained Sample Preparation and Counting

Staining periphyton samples permits analysts to distinguish algae from detritus and "live" from "dead" diatoms. This distinction is especially important because periphyton often contains many dead diatoms of both planktonic and periphytic origins.

In the first method, expose cells to a vital stain and evaluate the percentages of live, senescent, and dead algae—particularly diatoms—by estimating relative metabolic activities. The colorless tetrazolium violet is reduced in the cytochrome system of metabolically active cells to form violet-colored triphenylformazan. When cells are senescent or dead, the reaction fails.

Make tetrazolium violet solution by adding 2.0 g tetrazolium violet to 1.0 L water. The solution may be buffered to a pH between 7.5 and 7.7 with tris-hydroxymethyl amine. Add 1 mL tetrazolium violet solution to 9 mL sample and incubate 2 to 4 h at room temperature. Count diatom frustules and other cells (at least 300/sample) and place into the following categories:

- active: violet precipitate observed within the cell or mitochondria;
- senescent: chlorophyll present, but no violet precipitate; or
- dead: no chlorophyll or violet precipitate present.

In the second method, all algal components of periphyton may be studied in one preparation, without sacrificing detailed diatom taxonomy.[5] This method yields permanent slides for reference collections.

Thoroughly mix preserved samples in the preservative solution. Prepare acid fuchsin stain by dissolving 1 g acid fuchsin in 100 mL reagent-grade water, adding 2 mL glacial acetic acid, and filtering. Place a measured sample in a centrifuge tube with 10 to 15 mL acid fuchsin stain. Mix sample and stain several times during a 20-min staining period; centrifuge at 1000 g for 20 min.

Decant stain, being careful not to disturb sediment or siphon off supernatant. Add 10 to 15 mL 90% propanol, mix, centrifuge for 20 min, and decant supernatant. Repeat using two washes of 100% propanol and one wash of xylene. Centrifuge, decant xylene, and add fresh xylene. At this stage, store sample in well-sealed vials or prepare slides.

Slides for periphyton examinations require random dispersion of a known amount of xylene suspension. Use a microstirrer to break up clumps of algae before removing sample portion from xylene suspension. Count a number of drops of suspended sample into a thin ring of mounting medium* on a slide. Mix the xylene suspension and medium with a spatula until the xylene has evaporated. Warm the slide on a hot plate at 45°C and cover sample with a cover slip.

Count diatoms on the prepared slides using the magnification most appropriate for the desired level of taxonomic identification. Count strips or random fields. Calculate diatom density per unit area of substratum:

$$\text{Organisms/area sampled}^2 = \frac{N \times A_t \times V_t}{A_c \times V_s \times A_s}$$

where the terms are as defined in 10300C.2.

5. Biovolume

Cell volume (biovolume) provides a much more accurate evaluation of cellular biomass because of the large differences in cell dimensions among species (and sometimes seasonally within species under different growth conditions). Cell volumes (based on cell dimensions) are calculated for each species from formulas for solid geometric shapes that most closely match the cell shape. A comprehensive set of geometric shapes and mathematical equations for calculating the biovolume of more than 850 pelagic and benthic freshwater and marine microalgal genera has been compiled.[6]

6. Dry and Ash-Free Weight

Collect at least three replicate slides for weight determinations.[7] Slides air-dried in the field can be stored indefinitely if protected from abrasion, moisture, and dust. Use slides expressly designated for dry and ash-free weight analysis.

a. Equipment:

1) *Analytical balance* with a sensitivity of 0.1 mg.

2) *Drying oven*—double-wall, thermostatically controlled to within ±1°C.

3) *Electric muffle furnace* with automatic temperature control.

4) *Crucibles*, porcelain, 30-mL capacity.

5) *Single-edge razor blades or rubber policeman.*

b. Procedure:

1) Dry slides to constant weight at 105°C, and ignite for 1 h at 500°C. If weights will be obtained from field-dried material, re-wet dried material with reagent-grade water and remove from slides with a razor blade or rubber policeman. Place scrapings from each slide in a separate prewashed, prefired, tared crucible; dry to constant weight at 105°C; cool in a desiccator and weigh; and ignite for 1 h at 500°C.

2) Re-wet ash with reagent-grade water and dry to constant weight at 105°C. This re-introduces water of hydration to clay

* Naphrax®, Brunel Microscopes Ltd., Unit 2 Vincients Road, Bumpers Farm Industrial Estate, Chippenham, Wiltshire, SN14 6QA, U.K., or equivalent.

and other minerals, which is not driven off at 105°C but is lost during ashing. If not corrected for, this water loss will be recorded as volatile organic matter.[8]

c. Calculations: Calculate mean weight from slides and report as dry weight [(crucible + sample weight at 105°C) – (tare weight of crucible)] per square meter of exposed surface. If 25- × 75-mm slides are used and periphyton is removed from only two large faces of the slide, then

$$g/m^2 = \frac{\text{g/slide (average)}}{0.00375}$$

If all six faces (two large faces + four thin edges) of the slide are scraped, then the correct area is 0.00395 m^2. Calculate ash weight for sample [(crucible + sample weight at 500°C) – (tare weight of crucible)]. Subtract ash weight from dry weight to obtain ash-free weight, and report as ash-free weight per square meter of exposed surface.

7. Chlorophyll and Pheophytin

The chlorophyll content of attached algal communities is a useful index of phytoperiphyton biomass. Quantitative chlorophyll determinations require that periphyton be collected from a known surface area. Extract pigments with aqueous acetone, ethanol, or methanol (see Section 10200H.1) and use a spectrophotometer or fluorometer for analysis. If immediate pigment extraction is impossible, samples may be stored frozen for as long as 28 d if kept in the dark.[9] The ease with which chlorophylls are removed from cells varies considerably with different algae; to achieve complete pigment extraction, disrupt cells mechanically via a grinder, blender, or sonic disintegrator, or freeze them. Grinding is the most rigorous and effective of these methods.

The Autotrophic Index (AI) is a means of determining the periphyton community's trophic nature (see Section 10200H). It is calculated as follows:

$$AI = \frac{\text{Biomass (ash-free weight of organic matter), mg/m}^2}{\text{Chlorophyll } a\text{, mg/m}^2}$$

Normal AI values range from 50 to 200; larger values indicate heterotrophic associations or poor water quality. Nonviable organic material affects this index. Depending on the community, its location and growth habit, and the sample-collection method, there may be large amounts of nonliving organic material, which may inflate the numerator to produce disproportionately high AI values. Nonetheless, AI is an approximate means of describing changes in periphyton communities between sampling locations.

a. Equipment and reagents: See Section 10200H.

b. Procedure: In the field, place substrata (individual glass microscope slides) directly into 100 mL of a mixture of 90% acetone (water with 10% saturated $MgCO_3$ solution). Immediately store on dry ice in the dark. (NOTE: Vinyl plastic is soluble in acetone. If vinyl plastic is used as the substratum, scrape periphyton from it before solvent extraction.) If extraction cannot be done immediately, freeze samples in the field and keep frozen until processed.

Rupture cells by grinding them in a tissue homogenizer and steep in acetone for 24 h in the dark at or near 4°C.

To determine pigment concentration, follow the procedures in Section 10200H.

c. Calculation: After determining the extract's pigment concentration, calculate amount of pigment per unit surface area of sample as follows:

$$\text{mg chlorophyll } a/m^2 = \frac{C_a \times \text{volume of extract, L}}{\text{area of substrate, m}^2}$$

where:

C_a is defined in Section 10200H.

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10300 D. Primary Productivity

The productivity of periphyton communities is a function of water quality, substrata, and seasonal patterns in temperature and solar illumination. Measurements of biomass-accrual rates can be useful indicators of pollution and eutrophication, but biomass accrual is not a measure of periphyton productivity. Productivity may be estimated from the rate of oxygen evolution or carbon uptake by the community.[1]

1. Biomass Accumulation

a. Ash-free dry weight: The organic-matter accumulation rate on artificial substrata (via attachment, growth, and reproduction of colonizing organisms) has been widely used to estimate the productivity of streams and reservoirs.[2,3] In this method, expose several replicate clean substrata for a predetermined period, scrape accumulated material from the slides, and ash as described previously.

$$P = \frac{\text{mg ash-free weight/slide}}{tA}$$

where:

P = net productivity, mg ash-free weight/m^2/d,
t = exposure time, d, and
A = area of a slide, m^2.

Obtain estimates of established communities' seasonal biomass changes by placing many replicate substrata at a sampling point and then regularly retrieving a few at a time. Replace removed slides with new clean slides. The recommended collection interval ranges from 2 to 4 weeks for a year or longer.[2] Because biomass losses or multiple growth-and-loss cycles could occur before collection, a gain in ash-free weight per unit area in successive collection periods is often a poor measure of net production, especially when long incubation periods are used.

b. ATP estimates: In recent years, adenosine triphosphate (ATP) measurements have been used to estimate microbial biomass in water, and this technique is applicable to periphyton.[4] It provides another tool for assessing the magnitude and rate of biomass accumulation on substrata in natural waters. At present, the procedure should be limited to communities colonizing artificial substrata.

1) Equipment and reagents—See Section 10200I.6*a*.

2) Procedure—Either scrape periphyton from an exposed artificial substratum or, if standard glass microscope slides are used, place them in polyethylene slide mailers containing preheated (99°C) Tris buffer. Immerse in a boiling water bath for 10 min to extract ATP. If samples are not assayed immediately, freeze at –25°C; they may be stored in a freezer for up to several months. Complete analysis as directed in Section 10200I.6*b*. Slides exposed in highly turbid waters may collect substantial amounts of particulates, including clays. ATP sorbs to these materials; the sorption results in a quenching effect.

3) Calculations—See Section 10200I.6*c*.

2. Standing Water Productivity Measured by Oxygen Method

Analysts can study periphyton's hourly and daily oxygen-evolution and carbon-uptake rates when growing in standing water by confining the community briefly in bottles, bell jars, or other chambers. In contrast, the metabolism of organisms in flowing water highly depends on current velocity and cannot be precisely determined under static conditions. Productivity estimates for flowing waters and standing waters present different problems; therefore, separate procedures are given.

The productivity and respiration of epilithic and epipelic periphyton in littoral regions of lakes and ponds can be determined by inserting transparent and opaque bell jars or open-ended plastic chambers into the substratum along transects perpendicular to the shoreline.[5,6] Leave chambers in place for one-half the daily photoperiod. Determine each chamber's DO concentration at the beginning and end of the exposure period. *Gross productivity* is the sum of the net gain in DO in the transparent chamber and the oxygen used in respiration. The values obtained are doubled to estimate productivity for the entire photoperiod. Alternatively, to more accurately determine how much of the incubation period was subject to total insolation during the photoperiod, measure the incubation period's insolation as a percentage of total daily insolation. Both methods assume that photosynthesis is proportional to irradiance (i.e., not light saturated and no photoinhibition).

Failure to account for DO changes in chambers caused by phytoplankton photosynthesis and respiration may cause serious errors in periphyton metabolism estimates. It is essential that these values be obtained when periphyton is studied via the light- and dark-bottle method (see Section 10200J).

a. Equipment and reagents:

1) *Clear and darkened glass or plastic* chambers*—approximately 20 cm diam and 30 cm high, with a median lateral port, sealed with a serum bottle stopper for removing small water samples for DO analyses or inserting an oxygen probe. Fit the chamber with a small, manually operated, propeller-shaped stirring paddle.

2) *DO probe, or equipment and reagents required for Winkler DO determinations*—See Section 4500-O.

b. Procedure: At each station, place both a transparent and an opaque chamber over the substratum at sunrise or midday and leave in place for one-half the daily photoperiod. In extremely productive environments or to define the hourly primary productivity changes throughout the day, use incubation periods shorter than one-half the photoperiod. The minimum incubation period giving reliable results is 2 h. Determine DO concentration at the beginning of the incubation period.

Include a set of Gaarder–Gran light- and dark-bottle productivity and respiration measurements with each set of chambers to obtain a correction for phytoplankton metabolism. Incubate for the same time period as the chambers (see Section 10200J).

* Users should note that various types of glass and plastic differ in their transparency to UV-A and UV-B radiation; this could influence assay results, as periphyton photosynthesis is sensitive to UV exposure.[7]

At end of exposure period, carefully mix the water in the chambers and determine DO concentration.

c. Calculations: When the exposure period is one-half of the photoperiod, calculate the periphyton community's gross primary productivity as follows:

$$P_G = \frac{2[V_c(C'_{fc} - C'_{ic}) + V_o(C'_{io} - C'_{fo})]}{A}$$

where:

P_G = gross production, mg $O_2/m^2/d_{12h}$;
V_c = volume of clear chamber, L;
C'_{fc}, C'_{ic} = final and initial concentrations, respectively, of DO in the clear chamber, mg/L, corrected for phytoplankton metabolism;
V_o = volume of opaque chamber, L;
C'_{ic}, C'_{fc} = initial and final concentrations, respectively, of DO in the opaque chamber, mg/L, corrected for phytoplankton metabolism; and
A = substratum area, m^2.

Correct for the effects of phytoplankton metabolism in the clear chamber's overall oxygen change via the following equations:

$$C'_{fc} = C_{fc} - C_{flb}$$

$$C'_{ic} = C_{ic} - C_{ilb}$$

$$C'_{fo} = C_{fo} - C_{fdb}$$

$$C'_{io} = C_{io} - C_{idb}$$

where:

C'_{fc} = final DO concentration in clear chamber, mg/L,
C_{flb} = final DO concentration in light bottle, mg/L,
C'_{ic} = initial DO concentration in clear chamber, mg/L,
C_{ilb} = initial DO concentration in light bottle, mg/L,
C'_{fo} = final DO concentration in opaque chamber, mg/L,
C_{fdb} = final DO concentration in dark bottle, mg/L,
C'_{io} = initial DO concentration in opaque chamber, mg/L, and
C_{idb} = initial DO concentration in dark bottle, mg/L.

Calculate periphyton community respiration as follows:

$$R = \frac{24V_o(C'_{io} - C'_{fo})}{tA}$$

where:

R = community respiration, mg $O_2/m^2/d_{24h}$, and
t = length of exposure, h.

Determine the net periphyton community productivity (P_N):

$$P_N = P_G - R$$

If incubation time is different from one-half the photoperiod, modify the daily gross production calculation as follows:

$$P_G = \frac{t_p[V_c(C'_{fc} - C'_{ic}) + V_o(C'_{io} - C'_{fo})]}{tA}$$

where:

t_p = length of the daily photoperiod, h.

Community respiration and net production calculations for different incubation periods are unchanged.

3. Standing Water Productivity Measured by Carbon-14 Method

The approach is similar to that described above for the oxygen method. Transparent and opaque chambers are placed over the substratum, carbon-14-labeled Na_2CO_3 is injected into the chamber by syringe, mixed well, and allowed to incubate with periphyton for one-half the photoperiod. The concentration of dissolved inorganic carbon available for photosynthesis is determined via titration. At the end of the incubation period, periphyton is removed from the substratum and assayed for carbon-14.[5]

a. Equipment and reagents:

1) *Incubation chamber*—See 10300D.2*a*1).

2) *Special equipment and reagents*—See Section 10200J.

3) *Carbon-14-labeled solution of sodium carbonate*—having a known specific activity of approximately 10 μCi/mL.

4) *Other equipment and reagents*—See Section 4500-CO_2.

b. Procedure: At each station, place a transparent and opaque chamber over the substratum and add approximately 10 μCi carbon-14/L of chamber volume. Mix water in the chambers well, taking care to avoid disturbing periphyton. Determine concentration of dissolved inorganic carbon (as described in Section 2320). At end of exposure period, remove surface centimeter of periphyton and sediment enclosed in the chamber, freeze, and store frozen in a vacuum desiccator.

Immediately before analysis, expose sample to HCl fumes for 10 to 15 min to drive off all inorganic carbon-14 retained in the periphyton. Combust sample (or portion) via the Van Slyke method[6] or oxidize by heating in a closed system. Collect all CO_2 for radioassay either by flushing CO_2 into a two-vial train of ethanolamine (2-aminoethanol) or alternative CO_2 absorber, such as methoxyethanol (1:7),[8] or flushing CO_2 produced by combustion into a gas-flow counter or electrometer. Alternatively, extract known amounts of periphyton biomass with a tissue solubilizer† (e.g., 1.0 mL in closed vials at 60°C for 48 h[9]) or sodium hydroxide (e.g., 0.5 *M* NaOH in closed vials at 80°C for 1 h[10]). If necessary, clear color with 30 to 50% H_2O_2, and radioassay subsamples (100-μL) by liquid scintillation.

c. Calculations:

$$P_N = {}^{12}\text{C available} \times \frac{{}^{14}\text{C assimilate} \times \text{conversion factors}}{{}^{14}\text{C available (added)}}$$

$$P_N = \frac{(a)\ (b)\ (d)\ (e)}{(c)}$$

† Beckman BTS-450, or equivalent.

where:

P_N = net primary productivity per unit area of substratum per unit time, mg C/m^2/d,
a = ^{12}C available = dissolved inorganic carbon, mg ^{12}C/L = (total alkalinity – phenolphthalein alkalinity) × 0.240[6] = mg ^{12}C/L,
b = ^{14}C assimilated = [(radioactivity of sample in light chamber × k_1) – (background activity of dark chamber × k_2)] × (isotope effect, 1.06). Express radioactivity as disintegrations per second (dps) (i.e., counts per second corrected to 100% radioassay counter efficiency).
k_1 = correction factor to convert individually different light-chamber volumes to 1 L,
k_2 = correction factor to convert individually different dark-chamber volumes to 1 L,
1.06 = isotope effect to correct for slightly greater mass of ^{14}C than of ^{12}C, which results in a 6% slower assimilation rate,
d = a dimensional factor to convert sampled substratum area to m^2,
e = factor to expand incubation period to the total daylight period. After integration by planimetry or electronic digitizer of the total amount of insolation for the day, determine percentage of total represented by the incubation period, and
c = ^{14}C available = ^{14}C activity added = (μCi ^{14}C added) × (disintegrations of ^{14}C/s/μCi) = 3.7 × 10^4 μCi ^{14}C added, mL.

4. Flowing Water Productivity Measured by Oxygen Method

The primary productivity of a periphyton community in a stream or river ecosystem can be related to changes in DO. These changes are the integrated effects of photosynthesis (affected by light levels and turbidity) by stream phytoplankton, periphyton, and submerged portions of macrophytes that occur during the photoperiod. Water depth, turbulence, and water temperature all influence re-aeration. Oxygen also can enter via groundwater and surface waters.

Daily fluctuations in the photosynthetic production of oxygen are imposed on the relatively steady demand of respiratory activity (due to metabolism of plant communities, aquatic animals, and attached and free-floating microbial heterotrophs). However, respiratory activity may fluctuate greatly in streams receiving a significant load of organic wastes, particularly under intermittent loads (e.g., oxygen demand from urban stormwater runoff). Respiration rates also may vary diurnally under certain conditions, but the factors involved are not well understood.

The rate of change in stream DO (q) in grams per cubic meter per hour is:[11]

$$q = p - r + d + a$$

where:

p = the photosynthetic rate,
r = respiration,
d = re-aeration, and
a = accrual from groundwater inflow and surface runoff.

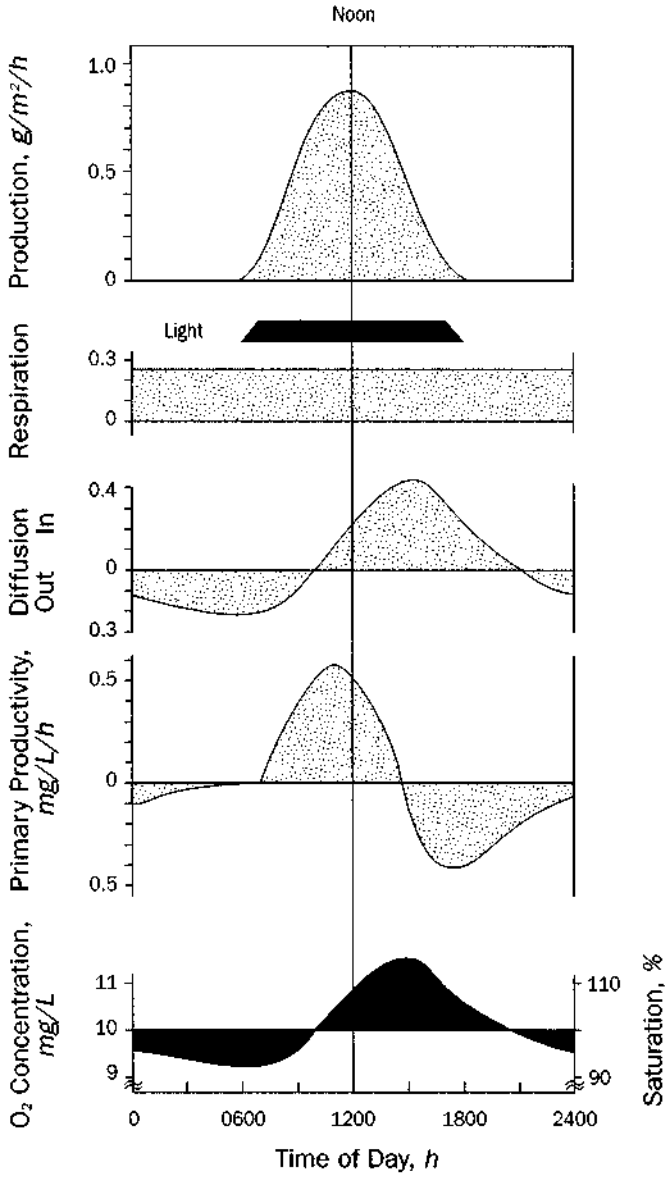


Figure 10300:2. Component processes in the oxygen metabolism of a section of a hypothetical stream during the course of a cloudless day. Production, respiration, and diffusion are given on an areal basis. The combined effect of these rate processes for a 1-m-deep stream is given in mg/L/h (q). The actual oxygen values that would result in a stream with a long homogeneous community are given in the lowermost curve. SOURCE: ODUM, H.T. 1956. Primary production in flowing waters. *Limnol. Oceanogr.* 1:102.

If the equation is multiplied through by depth in meters (z), the resulting values are in terms of grams of oxygen per square meter per hour. Figure 10300:2 illustrates this conceptual relationship between q, primary productivity, and the stream plant community's respiration.

The procedure measures time-variable oxygen concentrations in a stream over a 24-h period. Compensations are made for oxygen changes due to physical factors (accrual and re-aeration)

and for the rate of oxygen change due to biological activity (separated into respiration and primary-production components). The metabolic rates are the sum of the entire stream community's activity. Planktonic productivity and respiration can be separated from overall community activity via the light- and dark-bottle oxygen technique (see Section 10200J). However, in most small streams planktonic production is insignificant. The component of production and respiration due to macrophytes is difficult to separate from periphytic metabolic activity in systems where vascular plants are common.

Because periphyton attach to both plant surfaces and nonliving substrata, radiotracer techniques are required to separate the production component due to macrophytes from that due to attached algae.[12] When vascular plants are present, use techniques discussed in Section 10400 to estimate their contribution to net primary productivity.

Respiration by fish and benthic fauna is also difficult to quantitate directly and usually is not separated from periphyton respiration. If compartmentalized animal metabolism is required, calculate this contribution from laboratory respiration rates extrapolated to the field situation based on animal population sizes.[13,14]

Estimate primary productivity in flowing water by either the free-water demand method or the chamber method.[15,16] The first does not introduce artificiality to the system; however, it is difficult to separate the components of metabolic activity except for the contribution due to plankton. The chamber method measures periphyton activity alone.[17–21]

Depending on the stream system's hydrologic characteristics, accrual and re-aeration may be significant. Accrual can be accounted for by simple mixing equations if estimates of accrued flow and its oxygen concentration are known. In practice, select for study reaches that do not incur significant accrual. Measure re-aeration rates either directly[17–20,22–23] or via estimation from the stream's physical and hydrodynamic features.[19,20]

a. Equipment:

1) *BOD bottles*—for light- and dark-bottle measurements. See Section 10200J.

2) *DO meter(s) and probe(s)* for measuring DO.

3) *Bottom chamber*—60 × 20 × 10 cm, with 32-cm lengthwise dividing baffle, rheostat-controlled submersible pump, temperature thermistor, and DO probe.[16] Use clear and opaque plastic sleeves to cover chamber and Petri dishes, or other means of placing Periphyton within chambers.

4) *Current meter*—capable of detecting water current velocities ranging from 0.03 to 3 m/s in water depths as shallow as 0.3 m.

5) *Tape measure (30-m) and depth staff*—or similar equipment, as required to measure stream cross-sections.

6) *Fluorometer*—capable of detecting fluorescent dye concentration at 0.5 to 100 μg/L, or *conductivity meter* (required only if measuring re-aeration directly).

7) *Liquid scintillation counter*—capable of sensitive detection of ^{85}Kr and ^{3}H, or *gas chromatograph*, capable of quantitatively detecting propane (required only if re-aeration is measured directly).

b. Procedure:

1) Light- and dark-chamber method—Collect natural material or grow samples of typical periphyton communities on artificial substratum. Transfer identical portions to both clear and opaque chambers, taking care to use sufficient periphyton to make the ratio of chamber volume to periphyton area equivalent to the ratio of stream volume to periphyton substratum area. Measure stream current and match the circulation rate in the clear and opaque chambers to that of the stream current. Measure DO concentrations in both clear and opaque chambers at beginning of experiment and after 1 to 3 h to estimate the rate of oxygen increase or decrease. Make concurrent measurements of phytoplankton activity using light- and dark-bottle techniques as described in Section 10200J.2. Incubate light and dark bottles for the same time interval as the chambers.

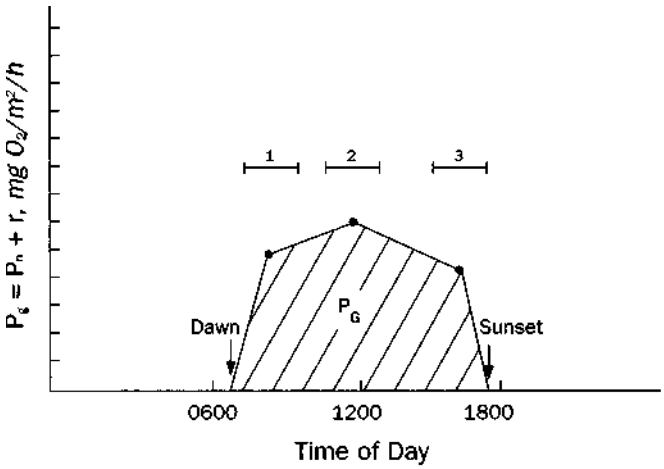


Figure 10300:3. Gross periphytic primary production (P_G) determined by the O'Connell–Thomas Chamber. P_G is the area under the curve obtained by graphical integration planimetry. Each point is the run $P_g = P_n + r$ for incubation periods 1, 2, and 3, which are denoted by the indicated lines.

Make several measurements during the photoperiod to define daily primary productivity. In addition, collect sufficient natural substratum samples of the study reach to estimate periphyton biomass (see 10300B). At end of incubation period, harvest enclosed periphyton and determine ash-free biomass (see 10300C.6).

2) Free-water diurnal curve methods—Measure, hourly or continuously, DO concentration and water temperature for a 24-h period at one or two stations, depending on stream conditions, desired precision, and equipment availability. If similar conditions exist for some distance upstream of the studied reach, diurnal DO measurements at one station are sufficient to estimate productivity. If upstream conditions are significantly different from those in the studied reach, measure DO at the upstream and downstream limits of the reach.

If the single-station method is used, measure depth at several points along the study reach to define average depth. Map and/or make physical surveys to estimate the magnitude of possible sources of accrual via effluents or tributary streams and springs. If the two-station method is used, measure the wetted cross-sectional stream area and the current velocity at several points to define flow (in cubic meters per second) and average cross-sectional area. Correct for phytoplankton activity via light- and dark-bottle measurements (see Section 10200J.2).

3) Direct re-aeration measurement (isotopic technique)[18]—This technique's results may not be more accurate than estima-

tion from the stream's physical and hydrodynamic features. The tracer gas technique is satisfactory but difficult and requires sophisticated equipment not routinely available. Use this method with care and with full recognition of its restrictions. Depending on stream flow, release 10 to 250 μCi ^{85}Kr with 5 to 125 μCi ^{3}H at the upstream end of the reach, along with sufficient fluorescent dye to produce a concentration of 10 μg/L when completely mixed across the river cross-section. Make fluorometric measurements at the downstream end of the reach until the dye peak appears; then, collect water samples to measure the ^{85}Kr:^{3}H ratio via liquid scintillation techniques. Record the dye peak's travel time from the injection point.

4) Direct re-aeration measurement (propane technique)[22,23]—Another method for directly estimating re-aeration involves propane evasion. This method is generally preferable to the isotopic technique because it does not require radioactive substances and seems to be more accurate than re-aeration estimates based on the stream's physical and hydrodynamic features.[22] Inject conservative tracer (NaCl) to raise in-stream conductivity by >100 μS/cm. Calculate reach travel time between upstream and downstream sites from the NaCl pulse's travel time, and streamflow from conservative tracer concentration. Outgas ethane from commercially available propane:ethane (96%:4%) mixture, then bubble propane into stream for at least three times longer than the reach travel time. Collect water samples for gas chromatographic propane analysis at upstream and downstream sites, with downstream samples collected at intervals 1 reach travel-time later than upstream samples.

c. Calculations:

1) Chamber method—Calculation is analogous to that used for the bell jar technique discussed in 10300D.2:

$$P_n = \frac{V_c(C'_{fc} - C'_{ic})B}{tW_c}$$

where:

P_n = hourly rate of net primary production, mg O_2/m^2/h,
V_c = volume of clear chamber, L,
B = average periphyton biomass estimated for the study reach, mg/m^2,
C'_{fc} = final oxygen concentration in clear chamber, corrected for phytoplankton metabolism, mg/L:

$$C'_{fc} = C_{fc} - C_{flb}$$

C_{fc} = final DO in clear chamber,
C_{flb} = final DO in light bottle,
C'_{ic} = initial oxygen concentration in clear chamber corrected for light-bottle measurement, mg/L:

$$C'_{ic} = C_{ic} - C_{ilb}$$

C_{ic} = initial DO in clear chamber,
C_{ilb} = initial DO in light bottle,
t = incubation period, h, and
W_c = total biomass of periphyton contained in clear chamber, mg.

$$r = \frac{V_o(C'_{io} - C'_{fo})B}{tW_o}$$

where:

r = hourly periphyton respiration rate, mg O_2/m^2/h,
V_o = volume of opaque chamber, L,
C'_{io} = initial oxygen concentration in opaque chamber, corrected for phytoplankton respiration, mg/L:

$$C'_{io} = C_{io} - C_{idb}$$

C_{io} = initial DO in opaque chamber, mg/L,
C_{idb} = initial DO in dark bottle, mg/L,
C'_{fo} = final oxygen concentration in opaque chamber, mg/L:

$$C'_{fo} = C_{fo} - C_{fdb}$$

C_{fo} = final DO in opaque chamber, mg/L,
C_{fdb} = final DO in dark bottle, mg/L,
B = average periphyton biomass for the study reach, mg/m^2, and
W_o = total biomass of periphyton contained in opaque chamber, mg.

For each pair of chamber measurements,

$$P_g = P_n + r$$

where:

P_g = hourly gross periphytic primary production, mg O_2/m^2/h.

P_G is the area under the curve of primary production per hour through the photoperiod, mg O_2/m^2/d (Figure 10300:3). Also,

$$R = \left(\frac{\sum_{1}^{n} r_n}{n} \right) \times 24$$

where:

R = total periphyton community respiration, mg O_2/m^2/d, and
n = number of observations.

Thus,

$$P_N = P_G - R$$

where:

P_N = net periphytic production, mg O_2/m^2/d.

2) Free water methods

a) Calculation of re-aeration or diffusion—Calculate k_2 from radio-tracer data as follows:

$$K_{Kr} = \frac{-1}{t} \ln \frac{(C_{Kr}/C_H)_d}{(C_{Kr}/C_H)_u}$$

and

$$k_2 = \frac{K_{Kr}}{0.83}$$

where:

K_{Kr} = base e transfer coefficient for ^{85}Kr, d^{-1},
t = travel time, d,
$(C_{Kr}/C_H)_u$ = ratio of released radioactivities (μCi/mL) ^{85}Kr to ^{3}H at the upstream station,
$(C_{Kr}/C_H)_d$ = ratio of radioactivities (μCi/mL) ^{85}Kr to ^{3}H at the downstream station, and
k_2 = re-aeration coefficient (base e), d^{-1}.

Calculate k_2 from propane data as follows:

$$k_{\text{propane}}(d^{-1}) = T \ln[(G_1 \times CT_2)/(G_2 \times CT_1)]$$

and

$$k_2 = 1.39 \times k_{\text{propane}}$$

where:

k_{propane} = propane evasion coefficient, d^{-1},
T = travel time, d,
G = steady-state propane concentration at upstream (G_1) and downstream (G_2) sites, corrected for background concentrations,
CT = steady-state conservative tracer concentrations at upstream (CT_1) and downstream (CT_2) sites, corrected for background concentrations, and
k_2 = re-aeration coefficient, d^{-1}.

The re-aeration coefficient also can be calculated from an equation relating a stream's energy-dissipation rate to k_2[18,19]:

$$k_2 = K\frac{\Delta h}{t}$$

where:

K = escape coefficient,
Δh = change in water surface elevation in a stream reach, and
T = time of flow through a stream reach.

This can be expressed in terms of hydrodynamic and physical data:

$$k_{2_{20}} = K'\frac{\Delta H}{\Delta X} \times V$$

where:

$k_{2_{20}}$ = re-aeration coefficient, d^{-1}, at 20°C,
K' = 28.3×10^3 s/m·d for stream flows between 0.028 and 0.28 m^3/s; 21.3×10^3 s/m·d for stream flows between 0.28 and 0.56 m^3/s; and 15.3×10^3 s/m·d for stream flows above 0.56 m^3/s,
$\frac{\Delta H}{\Delta V}$ = slope, m/km, and
V = velocity, m/s.

Convert $k_{2_{20}}$ to stream temperature by the following equation:

$$k_{2t} = k_{2_{20}}(1.024)^{(T-20)}$$

where:

k_{2t} = k_2 at ambient water temperature, d^{-1}, and
T = ambient water temperature, °C.

Convert to D in mg/L/h:

$$D = \frac{k_{2t}(C_s - C)}{24}$$

where:

C_s = oxygen concentration at saturation at ambient stream temperatures, mg/L, and
C = measured oxygen concentration, mg/L.

For a two-station energy dissipation method, if oxygen deficits are likely to differ between stations (e.g., because of temperature differences), then calculate D as[23]:

$$D = \frac{(k_2/24) \times [(C_{s1} - C_1) + (C_{s2} - C_2)]}{2}$$

where:

C_s = oxygen concentration at saturation at ambient stream temperatures, mg/L, at upstream (C_{s1}) and downstream (C_{s2}) sites, and
C = measured oxygen concentration, mg/L at upstream (C_1) and downstream (C_2) sites.

b) Calculation of primary productivity and respiration

(1) Single-station method—The calculation of primary productivity and respiration from one station's diurnal oxygen and temperature measurements is summarized in Figure 10300:4 and Table 10300:I.

Tabulate hourly DO measurements and temperatures. Determine C_s (DO of air-saturated H_2O at each temperature) from Table 4500-O:I and compute uncorrected DO consumption, milligrams per liter per hour, for each period:

$$\Delta DO_{\text{hours 1 to 2}} = DO_{\text{hour 2}} - DO_{\text{hour 1}}$$

Plot on the half hour, as shown in Figure 10300:4b.

Calculate the net primary production and respiration of phytoplankton as shown in Section 10200J. Determine the 24-h average hourly plankton respiration, $(\sum_1^n r_p)/n$, in milligrams per liter per hour every half hour. Calculate the hourly net phytoplankton production and tabulate for the approximate hours during the photoperiod. Plot as shown on Figure 10300:4c.

Calculate and tabulate k_{2t} and substitute D for each C_s, as outlined in ¶ a) above. Plot as shown in Figure 10300:4c.

Correct each ΔDO for diffusion and phytoplankton metabolism:

$$\Delta DO_{\text{corrected}}, \text{ mg/L/h} = \Delta DO_{\text{uncorrected}} - D - P_p - R_p$$

Plot each point as shown in Figure 10300:4d.

The gross primary productivity of benthic and attached algal populations is computed as the area under the curve in Figure 10300:4d from sunrise to sunset. This is primary production in

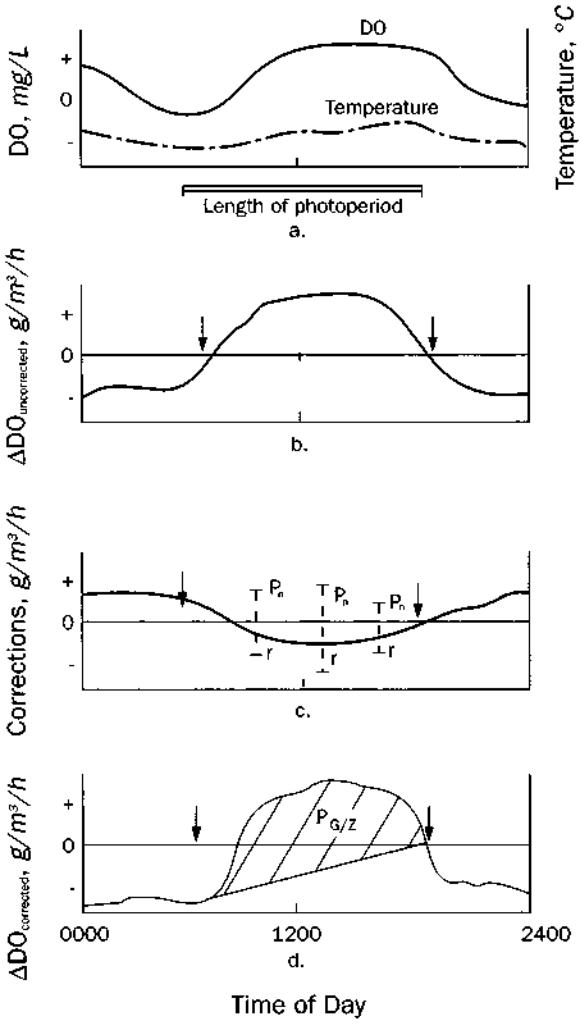


Figure 10300:4. Calculation of gross primary production at a single station. P_g, g $O_2/m^2/h$ = area of corrected rate of change curve integrated for the length of the photoperiod multiplied by average water depth (z) for the reach in meters.

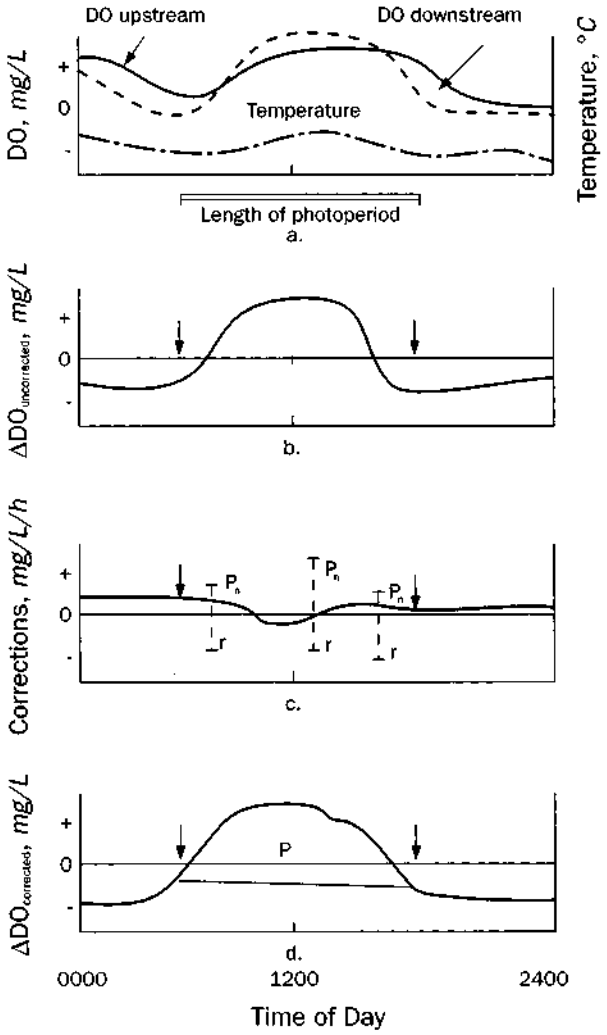


Figure 10300:5. Calculation of gross periphytic primary productivity from upstream–downstream diurnal curves. P is the area under the corrected rate of change graph.

grams per cubic meter per day. Multiply by a reach's average depth (z meters) to obtain P_G in grams per square meter per day. Calculate community respiration:

$$R = 24zF$$

where:

- R = community respiration, g/m^2/d,
- z = depth, m, and
- F = average hourly ΔDO for the dark period (without regard to sign), mg/L/h.

Calculate net primary productivity P_N as:

$$P_N = P_G - R$$

(2) Two-station method—The calculation of a stream reach's primary productivity and respiration from upstream and downstream pairs of diurnal curves of oxygen and water temperature is summarized in Figure 10300:5 and Table 10300:II. Alternatively, calculate as below, with oxygen change expressed as the difference between stations rather than as change per hour. It is also possible to use high-frequency (minutely) DO measurements offset by the reach travel time to calculate DO fluxes on the same parcel of water as it moves from upstream site to downstream site, improving precision.[22] The calculations are analogous. Multiply the area under a curve of oxygen change between two stations, corrected for diffusion and plankton metabolism (expressed in milligrams per liter), by the discharge in cubic meters per hour, and divide by the water surface area between the two stations. This, multiplied by 24, yields gross primary productivity in grams per square meter per day.

To compute gross primary productivity by this method, tabulate upstream and downstream DO and average water temperature for the reach at each hour. Calculate ΔDO between upstream and downstream stations for each hour as

Table 10300:I. Sample Calculation Ledger for Computation of Corrected Rate of Oxygen Change from a Single-Station Diurnal Curve

Time *h*	DO *mg/L*	Water Temp. *°C*	C_s* *mg/L*	Uncorrected ΔDO† *mg/L/h*	P_p‡ *mg/L/h*	R_p§ *mg/L/h*	k^2 d^{-1}	*D* *mg/L/h*	Corrected ΔDO‖ *mg/L/h*
Midnight									
0030									
0100									
0230									
.									
.									
.									
Noon									
1230									
1300									
.									
.									
.									
Midnight									

* DO concentration at 100% saturation for a given water temperature, from Table 4500-O:I.
† Hourly rate of change of DO. For example, for noon to 1300, $DO_{1200-1300} = DO_{1300} - DO_{1200}$; plot at 1230.
‡ Phytoplankton net production.
§ Phytoplankton respiration rate.
‖ $\Delta DO_{corrected} = \Delta DO_{uncorrected} - D - P_p - R_p$

Table 10300:II. Sample Calculation Ledger for Computation of Corrected Rates of Oxygen Change from the Upstream-Downstream Diurnal Curves of Oxygen Concentration and Temperature

Time *h*	DO *mg/L*		Uncorrected ΔDO *mg/L*	Water Temp.° *C*	C_s* *mg/L*	P_p† *mg/L*	R_p‡ *mg/L*	k_2 d^{-1}	Corrected ΔDO§ *mg/L*
	Upstream	Downstream							
Midnight									
0100									
0200									
.									
.									
.									
Noon									
1300									
.									
.									
.									
Midnight									

* DO concentration at 100% saturation for a given water temperature, from Table 4500-O:I.
† Change in oxygen concentration in the light bottle per hour multiplied by travel time between the upstream and downstream station.
‡ Change in oxygen concentration in the dark bottle multiplied by travel time between the upstream and downstream station.
§ $\Delta DO_{corrected} = \Delta DO_{uncorrected} - D - P_p - R_p$

$$\Delta DO = DO_{downstream} - DO_{upstream}$$

Tabulate C_s and determine planktonic activity. Correct for planktonic respiration by relating average hourly dark-bottle DO change to travel time in the stream reach; correct for planktonic production by the hourly change in DO in the light bottle times travel time (see Table 10300:II).

Calculate or tabulate k_2 and convert it into the reach's total oxygen diffusion. Because diffusion (*D*) is expressed as milligrams per liter per hour, multiply it by travel time to obtain the diffusion correction.

Correct each hourly upstream–downstream ΔDO as shown in Table 10300:II. Integrate the area under this ΔDO curve from sunrise to sunset to give *P* as in Figure 10300:5d.

$$P_G, \text{ g/m}^2\text{/d} = \frac{Q}{A} P$$

where:

Q = flow, m^3/h, and
A = reach area, m^2 (average reach width × reach length).

$$\text{Respiration, } R, \text{ g } O_2/m^2/d = \frac{\Delta DO_{dark} \times Q \times 24}{A}$$

and

$$\text{Net production } P_N = P_G - R$$

Metabolism is thus estimated[22,23] using the difference in upstream–downstream data by the graphical technique in Figure 10300:5 as:

$$\text{Net metabolism} = \Delta\ DO_{light} - \text{reaeration}$$

$$\text{Dark metabolism} = \Delta\ DO_{dark} - \text{reaeration}$$

Gross community primary productivity (GPP) then equals net metabolism minus respiration (light), and community respiration (CR_{24}) is average night respiration scaled for 24 h.

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10300 E. Interpreting and Reporting Results

Although several systems have been developed to organize and interpret periphyton data, no single method is universally accepted. The methods may be qualitative or quantitative. Qualitative methods deal with the taxonomic composition of communities in pollution zones, while quantitative methods deal with community structure via diversity indices, similarity indices, and numerical indices of saprobity.

1. Qualitative Methods (Indicator Species and Communities)

The saprobity system developed by Kolkwitz and Marsson is widely used to interpret periphyton data. This scheme divides polluted stream reaches into polysaprobic, α and β mesosaprobic, and oligosaprobic zones, and lists the characteristics of each. The system has been refined[1,2] and enlarged by Fjerdingstad[3,4] and Sládeček.[5–7]

Evaluating the saprobity system requires microscopic evaluation of living indicator biota, particularly for the sensitive sessile protozoans. Glass slides and other transparent substrata are advantageous because they permit direct microscopic examination and identification. Removing periphyton from slides and preserving them for subsequent examination may be acceptable for diatoms and many other algal groups, but observation of preserved material is unacceptable for most flagellated protozoans.

2. Quantitative Methods

These methods use cell counts or biomass estimations per unit area of substratum, as well as numerical indices of pollution or water quality. Considerable data on cell densities and species composition of periphyton in polluted English rivers (collected on glass slides) are available.[8] Other indices include the Shannon Weiner,[9] Simpson's,[10] and Pinkham–Pearson.[11] The saprobity system[12] also may be used when code numbers assigned for the saprobial value and the abundance of individual species are used to calculate a Mean Saprobial Index. Other region-specific indices combining taxon abundance and autecological information are also useful indicators of water quality.[13,14] Results also may be expressed by the truncated-log normal distribution of diatom species,[15,16] as well as the AI.[17] Multivariate techniques provide an excellent way to analyze and present periphyton community composition data with respect to pollution.[18–21] The importance of replication and statistical analysis, particularly in the use of multivariate techniques, has been noted.[22]

3. Water-Quality Applications

Qualitative analyses of periphyton communities can be used to indicate pollution, eutrophication, and hygienic problems when monitoring drinking water quality.[23] Water-quality surveillance can be assisted by bioassays on different types of artificial substrata in which changes and differences in species composition are determined.[24] In addition to the indicator value of individual species, the rates of biomass accrual during periphytic colonization on exposed artificial substrata can serve as another water-quality criterion. Simple periphyton screening assays are useful for classifying the water's biological stability in treatment and distribution systems.[25]

In wastewater treatment, qualitative periphyton analyses coupled with saprobiological evaluations may be used to classify waste treatment plant efficiency and monitor treatment plant effluents.[26] The use of periphyton growing on exposed artificial substrata to reduce nutrients in water supplies also has been proposed for water management practices.[27]

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10400 MACROPHYTES*

10400 A. Introduction

1. General Discussion

Macrophytes consist principally of aquatic vascular flowering plants, but also include the aquatic mosses, liverworts, ferns, and the larger macroalgae. Like other primary producers, macrophytes respond to the quality of the water in which they grow. The use of biota, including macrophytes, is an increasingly important and recognized technique for assessing aquatic habitats.[1–4] Macrophytes often constitute a dominant factor in the habitat of other aquatic organisms.

Freshwater macrophytes range in size from the tiny watermeal (*Wolffia* spp., see Section 10900, Plate 3A:D), about the size of a pinhead, to plants like the cattail (*Typha* spp., see Section 10900, Plate 3C:O), up to 4 m high. Higher aquatic plants often are abundant, occur clustered in high numbers, many in almost pure stands, covering extensive areas of shallow lakes, reservoirs, marshes, and canals. In marine water, intertidal rockweeds (*Fucus* spp., see Section 10900, Plate 2A:D, and *Ascophyllum* spp.) and offshore kelps (*Fucus* spp. and *Macrocystis* spp., see Section 10900, Plate 2A:E) are conspicuous. Vascular marine or estuarine plants, such as eelgrass (*Zostera* spp., see Section 10900, Plate 2B:N) and marshgrass (*Spartina* spp.), are essential to the aquatic ecosystem.

Three growth forms of macrophytes generally are recognized: floating, submersed, and emersed. Floating plants may be rooted or free-floating; their principal foliage or crown floats freely on the water surface. All or most of the foliage of submersed plants grows beneath the water surface: nearly all submersed vascular plants have roots. Growing tips of submersed plants may emerge to flower and some species can produce floating leaves. Emersed plants have their principal foliage in the air at or above the water surface; they are attached by roots to the bottom mud. In some cases, the same species may grow as a floating or emersed type, or as a submersed or emersed type. Submersed and emersed vascular plants typically are rooted to the bottom, but they may be found detached and floating.

The distribution and abundance of higher plants is subject to considerable spatial and temporal variation. Among the many factors that determine their presence, density, and morphology are sediment type, water turbidity, water current, nutrient concentrations, water depth, shoreline disturbance, herbivore grazing, and human activities. Zonation in the littoral zone of lakes and shallow, slow-moving streams is common. Emergent macrophytes generally are found in the most shallow portion of the littoral zone. During periods of low water level, they may occupy the terrestrial as well as the aquatic habitat. The depth of inhabitation seldom exceeds 1 m. Floating-leaved plants commonly are found in the shallower littoral areas (water depths between 1 and 3 m). Submersed plants may occur from the edge of the shore to the interface of the littoral and profundal zones, but rarely extend beyond a depth of 10 m because of limited underwater light.

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10400 B. Preliminary Survey

1. General Considerations

A macrophyte survey includes species identification, location, assessment of health, and quantity. More detailed studies may involve functioning of aquatic plants in nutrient and heavy metal uptake and turnover, use of plants as indicator organisms, and effects of plants on water quality conditions.

Several sampling protocols are required to meet diverse survey needs. The usefulness of a given study and the appropriate types of statistical analyses are determined and fixed initially.

To develop a good sample design, determine what information is desired, prevailing environmental conditions, the life and growth form of the species being sampled, and the methods for obtaining reproducible data that are comparable to other or future studies. In defining reporting requirements, consider such matters as the use of scientific names; the selection of appropriate descriptors (e.g., frequency, density, biomass, cover, diversity, productivity, and outer limit of vegetation growth); and the use of proper statistical techniques.

2. Pre-Field Investigations

During pre-field investigations, assemble maps, charts, aerial photographs, taxonomic keys, and past reports. Maps, charts, and aerial photographs help determine access routes, project size, plant community distribution, habitat characteristics that may influence plant distribution, and sampling obstacles or hazards. These items also provide a base for doing field work and reporting results. They may be available locally from municipal engineering departments, zoning boards, planning commissions, drainage districts, and land conservation commissions. At the state level, information may come from natural resource agencies, natural history survey organizations, universities, and transportation departments. At the federal level, the Geological Survey, Natural Resources Conservation Services, Bureau of Land Management, Forest Service, Park Service, Fish and Wildlife Service, Tennessee Valley Authority, Bureau of Indian Affairs, Army Corps of Engineers, Environmental Protection Agency, and the National Oceanic and Atmospheric Administration have many maps, charts, and aerial photographs available. A final source is private map companies that publish hydrographic maps for fishermen and recreational boaters. Maps, charts, and photographs in digital form from the sources listed above, including private companies, are becoming increasingly common.

Past reports provide historical records useful for planning sampling logistics and interpreting results. Comparable studies taken at different times provide a dynamic look at the vegetation. An often-overlooked resource is a herbarium storing scientific plant specimens. These generally are located in universities, natural history museums, and research botanical gardens.

3. Field Reconnaissance

Sampling efficiency is improved and a sampling scheme can be refined during a field reconnaissance. It provides an opportunity to learn the species of plants present and to sketch their distribution. The species–area curve technique frequently is used to determine the likelihood of finding more species during a preliminary survey. A field reconnaissance allows the investigator to answer logistical questions that are the bane of all sampling efforts.

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Also see 10400A.3.

10400 C. Vegetation Mapping Methods

Mapping vegetation stands may be necessary. Do this during the preliminary survey.[1]

1. Baseline Method

Vegetation maps constructed using the baseline method or the basepoint-stadia rod-alidade method generally are limited to pure stands of floating or emersed littoral macrophytes in all bodies of water. In clear water, the outline of pure stands of submersed vegetation can be determined by using a viewing box (usually a wooden or plastic box with a watertight glass lens) from the surface or by underwater observation by a diver using a snorkel or SCUBA (self-contained underwater breathing apparatus). The baseline method and the basepoint-stadia rod-alidade method provide accurate maps of vegetation in areas up to 1×10^5 m^2 where most of the vegetation outline is visible. The baseline method uses intercepting lines from each end of a predetermined baseline to closely spaced markers (i.e., chaining pins) around the stand. By presetting the map scale, the ratio between the length of the baseline and its reduction on the map (drawn on a plane table) can be determined. The basepoint-stadia rod-alidade method is a modification of the baseline method in that the distance between the vegetational outline and the base point is determined with an alidade, range finder, or portable Loran-C unit. One unit on the stadia rod, as viewed between the cross-hairs of the alidade, is equivalent to a distance of 3.05 m between the stadia rod and the alidade. Chaining pins are not required when this method is used. In practice, more readings closely spaced along the vegetational outline usually are taken using the basepoint-stadia rod-alidade method.

It is not necessary to measure all distances and angles; some can be determined trigonometrically. After all angles and distances are calculated, fill in irregularities via inspection and use of other maps and photographs.

The technology for using global positioning systems (GPS), especially when linked to geographical information systems (GIS) and remote sensing, is evolving rapidly.[2] These systems have wide applicability to mapping aquatic vegetation, thereby rendering older surveying techniques obsolete or at least time-consuming and tedious.

2. Line Intercept Method

The line intercept method is preferable for mapping mixed stands and/or large areas. Select sampling points at equal intervals along a baseline. Choose interval length by the degree of accuracy desired: the closer the sampling points, the more accurate the map. Run transects perpendicularly from the baseline to the boundary of the plant stand. Use an intercept line (transect line) of plastic-coated wire rope to prevent stretching. If line flotation is a problem, use lead weights applied at regular intervals to sink the line and act as interval markers on the rope to designate sampling units. Use 0.5-m (in dense vegetation) to 5-m sampling units (in sparse vegetation) for determining plant species that vertically intercept the line at each segment. During underwater surveys, record data with a wax or soft lead pencil on a writing board constructed of plastic overlays. Construct the vegetation map by placing points where plant species are found within an outline map (or aerial photograph) of the sample area. Determine the area that a single species or total vegetation occupies by planimetry or digitization and computer calculation.

Determine frequency from line intercept or quadrat data, or with a set sampler consisting of a 2-cm steel tube, 2 m long, to which five 0.75-cm by 25-cm steel rods are attached on 40-cm centers. Record vegetation touching each of the five points within 2.5 cm of the distal tip. If more than one plant species is touching, record the plant nearest the tip. If no plant is touched, record bare ground.

3. Remote Sensing

a. General considerations: Remote sensing techniques are used to detect, assess, and monitor aquatic macrophytes. These techniques include analog aircraft and satellite serial photography, digital aircraft and satellite multispectral scanning in the visible, infrared, and thermal bands; microwave techniques, primarily side-looking airborne radar (SLAR); and shuttle imaging radar (SIR). They provide a synoptic view of large areas, and allow quick surveys to further delineate areas of interest and repeated viewing at relatively low cost.

In selecting remote sensing system(s), consider expected results, time for project completion, and available resources. The larger the area, the greater the advantage of remote sensing. Remote sensing also lends itself to studies over time, because each image is a historical record.

For determining general associations of a widespread macrophytic growth, ground resolutions of between 30 and 80 m are recommended. Widespread multitemporal coverage is available at scales ranging from 1:100 000 to 1:1 000 000 at a reasonable cost in the form of paper prints, transparencies, and digital formats. Landsat Multispectral Scanner (MSS) and Thematic Mapper (TM) have been used to identify areas with emergent vegetation or topped-out submergent vegetation. Surface roughness is a requisite for an imaging return with SLAR and SIR. Landsat provides limited capability for species discrimination, but availability, cost, and repeat cycles make it useful in determining the presence of large populations over time. For a detailed vegetation survey, including discrete species identification, use much larger-scale imagery, (1:10 000 or greater). High-altitude aerial coverage available through the National High Altitude Mapping Program (NHAP) and other sources at original scales of approximately 1:60 000 to 1:120 000 provides both good initial areal coverage and the capacity for enlargement up to five times.[3]

After determining scale, select film/filter or sensor combinations. These include black and white imagery, color infrared, and black and white-infrared imagery; color infrared film used with a yellow filter is widely applicable, but other combinations also are useful.[4–7] For detailed flight planning, consider growth stage of plants, water depth and clarity, tidal conditions, cloud cover, and sun angle.[3,8,9] Available resources ultimately determine remote sensing activities.

b. Aerial photography:

1) Equipment—For all-format aerial photography, use a good-quality 35-mm single-lens reflex (SLR) camera with manual or through-the-lens metering or any good 70-mm camera system, preferably with a motor drive. Intervalometers providing for exact exposure intervals for stereo photography are available for both systems. A 28-mm-focal-length lens with a 35-mm SLR camera gives wide coverage at low altitudes; 40-mm and 80-mm lenses are successful with 70-mm camera systems. When photographing with black-and-white or color film, use a skylight or haze filter. When photographing with color infrared film below 1700 m, use a Wratten 12 filter; at altitudes above 1700 m use a Wratten 15 filter. Time of year and condition of vegetation also may influence the choice of yellow/orange filter. In turbid waters, color film* provides better water penetration and is more useful in mapping submersed vegetation than is color infrared film.[4] Color infrared† yields more detail, is more useful in mapping emergent vegetation or wetlands, and may provide more detail in clear, nonturbid waters. Video cameras (camcorders) and digital cameras also are being used to obtain aerial photos of vegetation.

Preferably mount camera in the belly of a high-wing, single-engine aircraft for low-altitude, small-format photography. Belly mounts require special aircraft modification, but provide a stable platform, camera protection, and good access. Alternatively, camera may be mounted on the aircraft door.

2) Procedures—Because sun angle is critical in obtaining high-quality aerial photography, schedule flight for a time when solar altitude is between 40 and 68 degrees.[7]

Set camera at designated ISO reading for the film (assume 100 to 125 for color infrared film) and shoot in the automatic exposure mode. At a typical airspeed of about 190 km/h (approximately 120 mph), a shutter speed of 1/250 or 1/500 is adequate. Determine proper f-stop from an aerial exposure computer; in general, f 5.6 to 11 (f 8 is optimum) gives acceptable color exposure. Proper exposure of color infrared film depends on such factors as time of day and year, altitude, humidity, and type of landscape.

* Kodak Ektachrome Professional film type 5036, or equivalent.
† Kodak Aerochrome Infrared film type 2443, or equivalent.

Process film through the manufacturer, a photo laboratory, or aerial photography service. Development of color infrared film is available solely through the manufacturer. Once an aerial photograph has been developed, it can be scanned into a digital format for computer analysis.

c. Fathometry: Recording fathometers are best applied in water more than 1 m deep where the instruments can determine accurately the height and distribution of subsurface macrophytes.

A recording fathometer can be mounted on most boats and can accurately determine one-dimensional (percent cover) and two-dimensional (percent vertical area) profiles of submersed vegetation. Fathometry is especially useful for determining the outer edge of plant growth. Make linear and planimetric measurements on chart tracings that provide permanent records for ready comparison over time. Calculate percent cover by dividing the linear measurement for a macrophyte species or community by the total chart paper length for any given transect. Dividing the area of the tracing with macrophytes by the total water area gives percent vertical area. Use a fathometer accurate to the nearest 0.1 m.

Mount the transducer for the recording fathometer with brackets on the boat's transom. Keep speed and recorder speed constant to produce tracings of similar length and resolution. Only a few minutes are required to replicate transects several kilometers long. Unless gross morphological differences exist, species discrimination on the chart tracings is difficult or impossible. Mark boundaries of monotypic colonies and community types with a fixed line on the chart tracings. Dense vegetation mats that reach the surface may impede boat movement, prevent the transducer signal from reaching the hydrosoil, and merge tracings of macrophytes with the transducer line. Tracing patterns from water less than 1 m deep may be difficult to interpret.[10] Location of transects or points along a transect also can be determined using GPS techniques.[2]

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10400 D. Population Estimates

1. Sampling Design

The design of a sampling program depends on study aims, collection methods, variation and distribution of vegetation, personnel and funds available, and accuracy expected. Variation in space usually is not random; distribution is determined by water depth, shoreline activity, sediment type, or other factors. The parametric statistic for estimating the true population mean assumes that the population being sampled has a normal distribution and that all sample units have the same probability of being selected. Avoid fixed sampling stations in sampling programs to determine population means, unless they are chosen at random at the beginning of the study. Because normally distributed plant populations may not be a characteristic of contiguous plant communities, use parametric statistics with caution.

The simple random sampling design is best applied to homogeneous, noncontiguous plant communities. The number of stations required to obtain an estimate of the true population mean with a predetermined level of confidence and permissible error can be determined by applying the data from a pilot study to the following equation:

$$N = \left(\frac{t \times S}{d \times \bar{x}}\right)^2$$

where:

N = number of sampling stations,
t = Student's t at a given probability level; because N is unknown, set $t = 2.0$; t is approximately equal to 2.0 for $N > 30$,

S = standard deviation,
d = permissible error of the final mean; $d = 0.1$ is recommended for vegetation studies (±10%); and
$\bar{x}$ = estimator of true population mean usually determined by conducting a pilot study.

An estimate of sampling program cost may be obtained as the sum of initial fixed cost (e.g., cost of equipment purchase) and variable cost (cost per sample multiplied by number of samples).

Apply stratified random sampling to populations having many homogeneous stands. This design is best applied to populations with obvious gradients and, in practice, to gain precision by the minimized variance within strata. Determine placement of strata by a pilot study. To maximize precision, place stratum boundaries around homogeneous areas; generally, the fewer strata, the greater precision. Allocate sampling in stratified random sampling design according to:

$$\frac{N_i}{N} = \frac{W_i S_i}{\Sigma(W_i S_i)}$$

where:

N_i = number of samples in stratum i,
N = total number of samples,
W_i = a weight reflecting the size (e.g., number of quadrats) of stratum i, and
S_i = standard deviation of sampled characteristic within stratum i.

Means for population measurement taken along randomly placed stations on a transect line do not represent large areas of lake populations unless the transect line is placed randomly. Randomization is important; arbitrarily placed transect lines within a sampling area may or may not reflect the true variation of the vegetation within.[1]

2. Collection Methods

a. Field inventory/reconnaissance:

1) Manual collection—If water depth, clarity, temperature, flow, and other circumstances permit, collect specimens by hand. Under ideal conditions, manual collection by wading, snorkeling, or with SCUBA in deeper water habitats permits a detailed and comprehensive evaluation of the macrophyte community.

2) Drag chains—Construct drag chains by welding sharpened U-shaped hooks to a short length (0.6 to 1.0 m) of medium-weight chain. Attach chain to a rope and pull it through the water. Attach a float to the end of the rope to prevent its loss if the chain is snagged and/or dropped. The drag chain can be used readily from a slow-moving or stationary boat and is most efficient in collection of submersed macrophyte species with tall growth forms.

3) Rakes and tongs—Rakes with various handle lengths and oyster tongs may be useful in collecting macrophytes. A rope may be attached to the rake handle for sampling in deep water or to facilitate sampling over a wider radius.

4) Grab samplers—Devices developed for sampling benthic organisms, such as the Ekman, Ponar®,* and similar grab samplers (see Section 10500B.3*a*), may be used to collect macrophytes. The light weight of the Ekman grab makes it preferable for the rapid and numerous samplings often required for survey inventories.

5) Recording fathometers—Use to determine height and distribution of subsurface macrophytes. Species with similar morphology usually cannot be distinguished from chart tracing; use supplemental methods to identify species.

b. Quantitative sampling: Numerical data collected to describe vegetation commonly include such measures of abundance as density, frequency, cover, and biomass/standing crop.[2–4] Collect these data from plots or quadrats or, less frequently, by plotless sampling techniques. The choice of analytical method depends on vegetation density and types, water depth, flow, height of vegetation in the water column, and nature of the sediment.

1) Line intercepts[5]—This plotless sampling technique entails use of a weighted nylon or lead core line laid along the bottom between two known points or oriented by a compass reading or GPS unit. For dense floating mat vegetation, a floating line may be laid on top of the mat. A surveyor measures the linear distance occupied by various species that underlie the transect line. Express these as a percentage of the total line length for individual species as well as for all species combined. If frequency data are desired, mark the line in increments (e.g., 1 m) and treat species presence/absence in a manner similar to data from quadrat sampling. The line intercept has been used to characterize and map aquatic communities[6] and to correlate distribution of macrophytes with selected environmental factors.[7] Line transects also are useful for determining patterns of plant distribution.[8] In aquatic environments, the line-intercept method is time-consuming and may require a diver equipped with SCUBA. Problems arise in determining whether a plant underlies the transect line.[9]

2) Belt transects—This technique is similar to the line transect and is useful for biomass or density determinations. Data are collected along a fixed line, but from a two-dimensional plot or belt. The belt can be treated as a series of contiguous quadrats or quadrat location may be selected based on a fixed or random interval or water depth. Use floating or sinking frames.

3) Quadrats—Quadrats can be used for such population and community estimates as frequency, cover, density, and biomass. Quadrats can be any two-dimensional shape but are typically round or rectangular. The sampling area of quadrat samplers can be of any size, but typically varies from 0.1 to 1 m^2.

With the exception of frames, most sampling devices described have been used to obtain estimates of aboveground biomass (standing crop).[10] Aboveground biomass generally is used because of the difficulty in collecting underground plant parts (e.g., rhizome and roots). Without the underground parts, however, the data are of limited value for estimates of primary production.

a) Manual samplers—These are relatively simple devices for sampling macrophytes, (e.g., cutting shears). Although they can be used in deep water and manipulated by a diver, they work best in shallow water. They are relatively inexpensive and can be constructed easily or purchased from commercial sources.

Frames are suitable for sampling in shallow water. For sampling short, erect plants, use a square sinking frame constructed of metal. For dense or tangled vegetation, a square assembly frame with pins or wing nuts at the corners, or a fixed-corner

* Registered trademark of Morris & Lee, Inc. d/b/a Wildlife Supply Co., Buffalo, NY.

three-sided frame may be useful. Decide whether to include only macrophytes rooted within the frame or also overlapping plants. In deep water, difficulty and bias may occur in sampling tall submersed vegetation. For macrophytes forming a dense floating mat, use a floating frame constructed of wood or PVC pipe.

Box samplers are useful for sampling where water is shallow and the bottom consists of unconsolidated sediments. The sampler consists of an open-ended box with a metal cutting flange at the bottom and lateral handles; a sampler constructed of 7-mm plexiglass with dimensions 0.5 m × 0.5 m × 0.6 m and aluminum cutting flange and corner reinforcements weighs about 12 kg. With modifications, a box sampler can be used in deep water.[11]

Benthic dome (BeD) samplers[12] may be used for sampling in deep flowing waters. The sampler consists of a plastic dome with a stainless steel circular collar that can be pushed into the substrate. It weighs approximately 11 kg and has a sampling area of 0.25 m^2.

Various samplers[13,14] developed for macroinvertebrate sampling also may be used to collect macrophytes. These include the Surber sampler, suitable for shallow rivers with moderate current [see Section 10500B.3*b*1)]; the stovepipe (cylindrical) sampler, suitable for wadable waters with unconsolidated sediment bottoms [see Section 10500B.3*c*4)]; and the Ekman grab sampler, best suited for soft sediment bottoms with short, erect vegetation [see Section 10500B.3*a*6)].

b) Mechanically operated samplers—Mechanical sampling devices are costly and complex, and require a floating platform with winches, cables, and booms. The samplers described below are useful in deep water. They may decrease sample collection time, increase accuracy of aboveground and total biomass estimates, and be subject to less bias than many manual methods.

CAUTION: Use extreme caution for safe operation.

The Louisiana box sampler is an open-ended 35-cm-high box made of sheet metal or similar material that samples a 61- × 61-cm quadrat (sampling area = 0.37 m^2).[15] It can be used from a V-hulled or pontoon boat and is hoisted above the water with a cable and boom. A quick-release mechanism lets the sampler fall free through the water column. Aquatic vegetation is trapped against the bottom and severed by cutting edges along the base of sampler. A nylon net sack over the top retains severed plant fragments. A diver inserts a horizontal cutting blade in a slot at the level of the substrate before the sampler is hoisted to the surface. Manual insertion of the cutting plate by a diver makes use of the Louisiana box sampler comparatively efficient. In soft sediments, the sampler may penetrate too deeply and require lifting before the cutting plate can be inserted. Rocks, stumps, roots, and other debris may prevent complete closure of the cutting door.

The Osborne sampler[16] is a stainless steel box having outside dimensions of 50 cm × 50 cm × 60 cm high and a sampling area of 0.25 m^2. The sampler weighs 110 kg and is operated by winch and cable from a pontoon boat. After hoisting and suspending the sampler alongside the pontoon boat, a quick-release mechanism allows free-fall through the water column. Tempered steel blades along the bottom edge of the sampler cut vegetation during the descent. A wire mesh screen fastened to the top prevents loss of plant fragments. A hinged slotted door is closed with a lift cable and the sampler is winched to the pontoon boat platform for removal of macrophytes and sediments. Because the sampler penetrates and collects sediments, the sample includes roots and rhizomes and can be used to estimate total biomass as well as aboveground biomass. Efficient operation and accurate biomass estimates require an unconsolidated substrate free of rocks and other debris.

The Waterways Experiment Station (WES)[17] sampler is made of perforated stainless steel and operated from a pontoon boat with an overhead beam that allows it to be hydraulically raised and lowered through a circular opening in the pontoon's platform. Two types are available: one is cylindrical, with a sampling area of 0.28 m^2, and the other is square, with a sampling area of 0.39 m^2. Rotating cutting blades at the base of each sampler sever vegetation as the samplers are lowered. The bottom cutting plate of each is closed hydraulically. A major advantage of the WES sampler is its capability to obtain plant samples from any depth. The Louisiana Box and Osborne samplers, once released, free-fall to the substrate; the hydraulic operation of the WES sampler controls its descent. The size and weight of the trailer and pontoon boat for the WES sampler restrict its use in certain waterbodies and require an improved ramp for launching. Although the WES square sampling head is reported to provide a more accurate estimate of aboveground biomass than the circular one, a substantial underestimate of actual aboveground biomass still is reported.[17]

3. Sample Preparation and Analysis

a. Biomass:

1) Fresh weight (wet weight)—Wash samples free of silt and debris, place in a nylon bag (mesh size 0.75 cm) and spin in a garment washer at 560 rpm for 6 to 7 min to remove excess moisture. Weigh sample to nearest 0.1 g.

2) Dry weight—Dry subsample (not less than 10%) in a forced-air oven at 105°C for 48 h or until a constant weight is achieved. The coefficient of variation for a series of subsamples *should not exceed 10%*. Calculate dry weight by dividing dry weight of subsample by fresh weight of subsample times fresh weight of sample.

3) Ash-free dry weight—Transfer dried subsample to a covered and preweighed crucible. Ignite at 550°C for 6 h. The amount of ash is the weight of material remaining after combustion. Calculate ash-free dry weight by determining the ratio between ash and dry weight times dry weight of sample (see Section 10300C.6).

b. Chlorophyll content: Extract fresh plant material with 90% acetone made basic with $MgCO_3$. Grind the plant material and centrifuge at 2500 rpm for 10 to 15 min. Wash residue with acetone and add filtered washings to extract. Dry overnight in a container with anhydrous Na_2SO_4. Dilute with 90% acetone and water. Determine chlorophyll content (see Section 10200H).

c. Carbon content: Most plants (entire) contain 46 to 48% carbon on a dry-weight basis. A factor (46.5%) can be used to calculate carbon content and make comparisons.

d. Caloric content: Determine energy content by bomb calorimetry.

e. Species identification:

1) Sample preparation—Use fresh specimens for identification wherever possible. Avoid immature plants or plants lacking flowers. Because aquatic plants contain 80 to 95% water and have less supportive tissue than terrestrial forms, a different procedure is required for drying, preserving, and mounting them.

Collect plants during peak growth when flowers and/or fruits are present, if practical. Collect the entire plant (stems, rhizomes, leaves, roots, flowers, and fruits).

After collection, either press plants in the field[18–20] or wrap specimen in several layers of paper and submerge in water. Label wrapped specimens with date and location of collection on an index card and place sample and card in a plastic bag. Preferably use an ice chest containing crushed ice for storage in the field. Press plants as soon as practical. They can be kept for several days under refrigeration at 4°C.

Clean plant of all silt and residue. Prepare a mount by centering the plant on 100% rag herbarium paper. Place emergent plants immediately on paper because they take on a natural posture. Place a limp plant in a shallow pan of water and slide the herbarium paper under it; with a slow motion, raise the paper at a 30° angle while keeping the plant centered. Leaves and stems should lie flat on the paper. Drain off excess water, cover with wax paper to prevent plant from sticking to blotters, and place in a plant press between paper and blotters. Place plant press in a dryer. Dry plants at room temperature, but change blotters at least every other day until the plant is sufficiently dry for permanent mounting.

To prepare a wet mount, place specimen in an airtight glass vessel filled with 1 part 10% formalin, 3 parts water, and a trace of powdered copper sulfate. Plants will remain lifelike and retain their color for many years in this condition.

2) Identification—A stereomicroscope is needed to identify many plants, especially aquatic grasses and sedges. Observe vegetative and floral structures by dissecting them (under magnification) with forceps and fine needle probes.

Preferably identify to species. Numerous references are available to help identify aquatic macrophytes (see Section 10900E.4).

3) Plant label—An important part of the species collection is the label that identifies the plant, the collector, the location of collection, and the date of collection.[20] Attach label to the sheet with the mounted plant. The mounted plant is a permanent record that is most useful when placed in an herbarium where it can be used by others.

4. Data Presentation

Express fresh weight (wet weight), dry weight, and ash-free dry weight as grams or kilograms per square meter. Data are best expressed as ash-free dry weight of total biomass. Determine significant digits for dry weight and ash-free dry weight from the accuracy of the scale used to obtain fresh weight: do not use more significant digits than those used for expressing fresh weight. Report pigment as grams chlorophyll per gram dry plant matter and caloric value as gram calories per gram dry plant matter.

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10400 E. Productivity

1. General Discussion

The complexity and heterogeneity of form, function, phenology, and distribution of aquatic macrophytes have resulted in diverse ways of determining their productivity. These methods can be grouped broadly as biomass methods (based on biomass or changes in biomass) and metabolic methods (based on estimates of inorganic carbon or oxygen exchange resulting from photosynthesis). The biomass methods generally are simpler than the metabolic methods and require little specialized equipment or expertise and fewer assumptions. Biomass methods integrate responses to environmental conditions and may provide estimates of aboveground production only. They are best used for long-term comparisons (several months to a year) because they are easily confounded by seasonal changes. Biomass methods are insensitive to losses due to fragmentation, herbivory, and secretion or leaching of dissolved organic compounds. In contrast, metabolic methods provide instantaneous measures of photosynthesis and thus reflect responses of plants to different environmental conditions. Metabolic methods for estimating plant productivity also can provide insight into factors controlling distribution and success. The principal drawbacks of the metabolic methods are that they require specialized equipment and assumptions that may be tenuous and are based on photosynthetic rates (typically measured over a period of minutes to hours) and require extrapolation to net assimilation over longer periods.

In choosing a method, consider why macrophyte production is of interest and the use for the data, the habit (growth form and phenology) and habitat of the population, and the cost and effort required to obtain the desired information. The common methods are listed in Table 10400:I and are described below.

2. Biomass Methods

a. Biomass harvest methods: Biomass measurements vary from a simple, one-time sample of maximum biomass to complicated evaluations of seasonal biomass dynamics by methods originally intended for grassland plants.[1–3] These methods are applicable mainly to emergent and submersed macrophytes. Preferably evaluate productivity of floating plants by using growth rates, permanent quadrats, and random samples[4] or by the turnover and metabolic methods discussed in ¶s 2*b* and 3, below.

1) Aboveground biomass measures—Peak aboveground biomass is measured by the aboveground biomass (usually as ash-free dry weight per unit area) at the time of apparent maximum aboveground biomass (usually time of flowering). This single measurement does not account for biomass carried over from the previous season, losses of material before the peak, or growth after the peak, and therefore generally underestimates net aboveground annual production (NAAP) to an extent, depending on the relationship of annual turnover to maximum biomass.[5] The method provides a reasonable estimate of NAAP for many submersed species.

The seasonal biomass accumulation method[6] is a modification of the peak aboveground biomass method that considers only the positive changes in live material. Live aboveground biomass is determined at the beginning of the growing season and at the time of maximum aboveground biomass; the NAAP is calculated

Table 10400:I. Methods Used to Determine Macrophyte Production*

	Plant Habit					
	Emergent		Floating		Submersed	
Method	Deciduous	Evergreen	Deciduous	Evergreen	Deciduous	Evergreen
Biomass harvest:						
Aboveground biomass	+	−	+	−	+	−
Biomass dynamics	+	−	−	−	−	−
Biomass tagging:						
Turnover, growth increment, and summed shoot maximum	+	+	+	+	+	+
Cohort	+	−	+	−	+	−
Below-ground biomass	+	+	+	+	+	+
Oxygen measurement:						
Light and dark bottle	−	−	−	−	+	+
Open system	−	−	−	−	+	+
Radiocarbon incorporation	−	−	−	−	+	+
Inorganic carbon exchange:						
Continuous CO_2 exchange	+	+	+	+	+	+
Discrete inorganic C measurement	−	−	−	−	+	+
Potentiometric C flux	−	−	−	−	+	+

* + = applicable method; − = method not commonly applied. Evergreen implies retention of substantial aboveground biomass year round; deciduous implies that 10% or less of seasonal maximum biomass is present year round.

as the difference. This method accounts for yearly carryover of living material, but in some cases, this will result in a further underestimate of NAAP relative to the peak aboveground biomass.[2]

When recruitment is continuous during the growing season, the biomass peak is less well-defined and greater losses may occur before the seasonal maximum. Under such conditions, aboveground biomass methods yield poor estimates of NAAP.[3]

2) Biomass dynamics methods—These methods are applicable to emergent plants when dead material remains near the site of decomposition.

The Smalley method[7] estimates net production based on samplings of live and dead material (per unit area) at regular 3- to 6-week intervals. Net production equals the increase in material between samplings: a decrease in live and dead material indicates no net production; an increase in live material and decrease in dead material indicates production equal to the increase in live material; and a decrease in live material and increase in dead biomass with a negative sum indicates no production, while a positive sum indicates production. The method underestimates production if dead material from other areas is present or if new growth is undetected when mortality is high. It is sensitive to sampling frequency[3] and requires a homogeneous area large enough to accommodate replicate sampling. A modification of this method can be used where aboveground biomass varies little from year to year; in this case, net aboveground production is assumed to equal the summed losses of dead material.[8]

More complex procedures based on harvests from a series of paired plots have been proposed.[3,9,10]

b. Biomass tagging methods:

1) Biomass turnover and growth increment methods—Leaf turnover and biomass marking studies involve marking individual plant leaves to follow production, growth, and loss over the year or growing season. For plants with large, long-lived leaves and basal growth (e.g., *Vallisneria, Zostera,* macroalgae) determine short-term growth of individual leaves. Use these methods for studies of populations (species) rather than communities. Marking methods are particularly useful for evergreen plants, where there may be little seasonal change in biomass, and for plants where the ratio of production to biomass is either very much greater than, or very much less than, one. Turnover measures estimate production and biomass loss and are considered better than harvest techniques, both with respect to accuracy and for the additional phenological information. Tagging methods require major efforts in regular censusing and SCUBA diving for sampling deep, submersed populations.

Where leaves or plant parts are about the same size seasonally, the method is relatively simple, requiring only periodic censusing of plant parts. These methods are not appropriate for species with much branching or different rates of leaf production and loss per branch, and are confounded by intense grazing or by sloughing of newly produced parts.

a) Biomass turnover—To determine turnover time of leaves and shoots, where individuals are easy to distinguish, or where vegetative spread is insignificant or easy to account for and leaf size is fairly constant, choose 10 to 50 individual plants and mark each leaf of each plant. If all new leaves are initiated to the inside of older leaves, tag only the newest leaves initially. Mark new leaves or stems with anything that does not interfere with normal growth and is not easily lost (e.g., staples, hole punches, plastic bird rings, fishing line, indelible markers). At regular intervals, revisit plant, tag new leaves, and record the number of new leaves and total number of leaves present. Because new leaves may not be fully expanded at a sampling visit, use a convention concerning the developmental stage of leaves.[11] The sampling interval (weeks to months) depends on research needs and the likelihood of losing the youngest tagged leaf. Compute the annual leaf turnover for each individual plant as the number of new leaves produced annually divided by the maximum number of leaves present at any time in the season. Because the turnover rate is the ratio of production to biomass, calculate NAAP by multiplying the turnover ratio by the maximum aboveground biomass. If vegetative spread is common, modify this method so all stems or leaves of a species within plots of a given area are tagged.[12,13] Revisit plots at regular intervals, tag new leaves, and record total number of leaves present. Calculate production as above. This method will not account for changes in plant size (increase in weight between years), for mortality, or for recruitment into the population, because only plants initially present that survive the season are included.

b) Growth increment measurements—If leaf size changes throughout the growing season or if several types of aboveground parts are present, use methods that account for such differences. Such methods frequently are used for seagrasses.[14–16] Mark each leaf of every plant within a quadrat at a set level above the bottom, relative to a stationary stake or frame. If plants are not buried deeply, use a set distance above the base (i.e., rhizome or root-shoot interface). After a predetermined time, remove all leaves or shoots at the level of the stake or at the set distance. Weigh unmarked leaves produced during the interval. Remove and weigh growth on older leaves (the portion below the marking, but above the ground or base). The combined weights are the net leaf production during the interval. These data also can be used to calculate relative growth rates as [(ln Δ weight)/Δ time]. Make detailed measurements of leaf growth rates by marking plants at the reference level more frequently (e.g., every other day).[17]

A modification of this method[18] permits computation of the growth rate of individual leaves and production of different plant parts.

c) Summed shoot maximum method—Where nondestructive measures are required, determine shoot size and number and estimate production.[3] Choose permanent quadrats of a size allowing easy enumeration of plant parts. Label every stem in each quadrat. On regular sampling visits, label and count new stems. Develop length:weight regressions using plants collected from areas near but outside the quadrats. This procedure is required only once or twice during the year, depending on the characteristics of study species. Estimate net aboveground (shoot) primary productivity using the number of new parts produced and their weights (based on length:weight regressions). Estimate production per unit area as the mean leaf turnover, multiplied by maximum shoot mass.

For certain species, more complex variations of this method have been used.[19,20]

2) Cohort methods—Cohort methods often are used to determine net production of aquatic plants subject to substantial biomass loss before the seasonal maximum biomass is attained. They are useful for species in which groups of individuals, or subunits, initiated at the same time (cohorts) can be identified

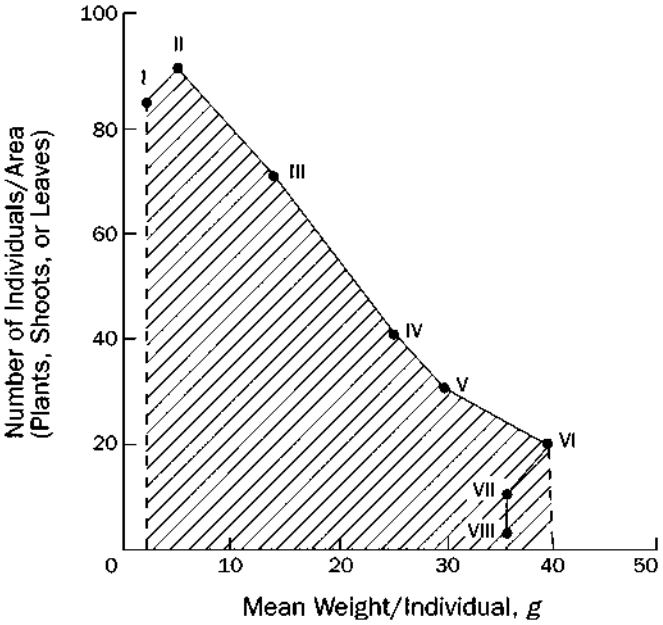


Figure 10400:1. Allen curve for a cohort of a population of aquatic macrophytes. Sample data are indicated by Roman numerals. Net annual aboveground production is proportional to the shaded area.

(i.e., plants where shoots emerge only during one time, or several discrete times, during an annual cycle).

The Allen curve method has been adapted for aquatic macrophytes. It provides an estimate of net production from tables of the numbers and weights of all individuals. It is particularly appropriate for populations for which shoot death and initiation occur throughout the growing season. The method can account for periods of negative production during the year.

Tag all members of a cohort (all leaves or shoots) shortly after initiation or emergence. This sampling is usually the maximum number of individuals present at any time in the cohort, because mortality will decrease the number thereafter.[21] Some new individuals (but still members of the same cohort) may appear by the second sampling visit.[3] Record the number of individuals and their mean weight (dry weight per leaf or shoot). Determine weight from size:weight regressions constructed from data for plants outside the study plot, but using members of the same cohort. Alternatively, harvest adjacent plots of cohort members to estimate the average weight per individual. Repeat for several replicate plots. Revisit plots regularly and record number and mean weight of individuals. Visit frequently enough to minimize potential for loss of young stems or leaves before they have been counted. Plot values (see Figure 10400:1) and determine total area beneath the curve by planimetry, digitally, or gravimetrically, to estimate net annual aboveground production. Repeat if more than one cohort emerges per year; net aboveground annual production then is the sum of the areas beneath several curves.[3,21] If negative production occurs during the year, add this loss back to yield net production. Losses usually can be avoided by initiating studies after winter senescence.[21]

An adaptation of the cohort production method[22] recognizes the hierarchical structure of many aquatic macrophytes. This method may be used when differential turnover rates for plant subunits would confound the simple cohort method.

c. Estimates of below-ground production: The below-ground portions of aquatic macrophytes often comprise a substantial portion of the plant's biomass and net production, and may have important ecosystem implications because of metabolic activity and decay.[5] The amount of below-ground biomass varies dramatically between species, within a species, and seasonally for populations of the same species growing in different habitats.

1) Peak biomass—The maximum below-ground biomass is commonly taken as the net annual below-ground production. This may substantially over- or underestimate actual production, depending on biomass turnover. For many submersed nonevergreens, it provides a reasonable estimate of production, especially where shoot turnover approaches 1 and little below-ground biomass overwinters. For most floating-leaved and emergent macrophytes, it will overestimate biomass.

2) Seasonal biomass accumulation—A more appropriate, but more difficult, method involves repeated sampling and determination of live below-ground biomass at regular intervals during the year. For marsh plants, use sediment cores (25 cm deep, 6- to 10-cm diam) taken at regular intervals (2 to 4 weeks).[23,24] Sieve the cores to remove below-ground biomass and determine weight of live portion. Live material usually can be distinguished by its light color, texture, and turgor. Stains also may be used; chlorazol black stains dead material[25] and tetrazolium stains live material. Use the seasonal change in live biomass as an estimate of production. As for shoots, the loss of plant material before the maximum biomass is attained and any production after the maximum biomass is attained are not accounted for.

3) Root turnover measures—For many applications, the most reasonable way to determine below-ground production is by extrapolation from shoot production or turnover. For many plants, production and turnover of below-ground biomass is directly related to growth and turnover of aboveground plant parts. However, the seasonal translocation of carbohydrate reserves between the below-ground and aboveground portions by most aquatic macrophytes requires that these turnover-based estimates be used only for long-term, annual production estimates, unless morphological observations indicate otherwise. The establishment of a constant relationship between shoot and below-ground turnover[18,26] justifies such extrapolations.

3. Metabolic Methods

Metabolic methods estimate macrophyte productivity on the basis of short-term measures of inorganic carbon and oxygen exchange resulting from macrophyte photosynthesis and respiration. These short-term measures (usually <3 h) must be integrated over time and space and converted to an estimate of organic matter production. Metabolic methods give short-term assessment of processes related to productivity that reflect a metabolic response to existing environmental conditions, and they provide information concerning factors governing plant productivity.

The photosynthetic inorganic carbon budgets of aquatic macrophytes are more complex than those of terrestrial plants. When complex carbon or oxygen exchange patterns occur, a combination of metabolic methods may be used,[27,28] or a biomass method may be preferable.

a. Oxygen measurement: Determining changes in dissolved oxygen (DO) resulting from photosynthesis and respiration is the most common metabolic method for estimating macrophyte productivity. Changes in DO can be determined for plants in chambers (in the field or laboratory) or for the whole system when macrophyte metabolism dominates the oxygen dynamics. Measure DO concentrations with polarographic oxygen electrodes or by titration (Section 4500-O). For the most part, the methodology is similar to that described for phytoplankton and periphyton; however, several additional considerations warrant attention.

Measurements of net oxygen release to water or of changes in the use of inorganic carbon by photosynthesis of submersed macrophytes assume acquisition from, or release to, the surrounding water. Many rooted submersed plants do not release dissolved oxygen at rates proportional to photosynthesis.[29–32] Some oxygen diffuses internally from leaves to sites of high respiratory demand in rooting tissues. Additionally, some oxygen is released from leaves and used by epiphytic bacteria and from roots and used by rhizospheric bacteria.[33]

1) Light and dark bottle (chamber) method—This method is similar to those described for other primary producers, where portions of plants or the aboveground portions of several plants are enclosed in either clear or opaque containers. The change in oxygen per unit plant per unit time is an expression of plant photosynthesis (or respiration in the dark bottle) that is used to calculate productivity during the incubation period. Daily or longer-term production is estimated, using additional information on environmental conditions during the incubation period and during the period over which a production estimate is needed, and the relationship between the response of the plant segment incubated and the response of the entire plant (or population) to those environmental conditions.[34–38] See Sections 10200J.2 and 10300D.2 for procedural details. Mixing to simulate natural water movement is desirable.[39–46] Equipment modifications have been discussed,[47–53] as have specific analytical requirements.[34,54–57]

Determining productivity for periods longer than the test hours requires including respirations during the dark (24-h net production = daytime net production minus nighttime respiration or daytime gross production minus 24-h respiration) and extrapolating from the incubation period to these longer periods. Estimate net photosynthesis during the daylight period from productivity measurements in 3- to 4-h intervals from dawn to dusk daily, or by multiplying the production measured during a 3- to 4-h midday incubation by the fraction of total daily light received during the incubation period. For monthly or yearly periods, there are several methods for extrapolating.[58,59] Most estimates of macrophyte production by these methods rely on a single midday incubation conducted at weekly-to-monthly intervals. There are three ways to estimate production for longer periods. First, measure productivity at 3- to 4-h intervals from dawn to dusk, on a clear day, several times during the growing season, to establish a relationship between midday photosynthesis and total daily photosynthesis. Estimate daily production on the basis of midday incubation at regular intervals (weekly to monthly), using the relationship between light during the incubation period to daily light and photosynthesis during the incubation period to daily photosynthesis, as determined above. Estimate production on intervening days (when photosynthesis is not measured) based on available light on those days. Second, by using experimental evidence (P-I curves from plants incubated at different depths) or literature values, establish a relationship between light and productivity and a midday incubation (weekly to monthly) as a scaling factor. Carefully estimate production based on available light for any day.[60,61] Third, use more involved modeling that accounts for growth characteristics and environmental conditions to provide estimates of macrophyte production.[62,63]

The epiphyton associated with a macrophyte may influence the determination of photosynthesis and respiration,[64–68] but the respective contributions to photosynthesis cannot be resolved by the oxygen method.[66] Alternatively, use the ^{14}C method for estimating macrophyte photosynthesis.

Short-term measures of macrophyte production using the light–dark bottle method do not account for the loss of organic substances that may be as much as 10% of the recently fixed carbon.[69] Other losses of fixed carbon, as well as sloughing, grazing, and fragmentation, also are unaccounted for. Further, the light–dark bottle oxygen method cannot predict the allocation of photosynthate to below-ground growth or reproduction.[5,70] The inability to account for such factors is a shortcoming of all metabolic methods, but oxygen methods provide reasonable estimates of short-term biomass net accumulation for some plants.[38,57]

2) Open-system oxygen method—In flowing-water systems where macrophytes dominate primary production, analysis of diurnal oxygen curves can be used to estimate macrophyte production.[39,71] The change in the oxygen content of a parcel of water is the result of both community metabolism and oxygen diffusion across the water surface (see Section 10300D.4). Entry of groundwater or surface runoff is assumed negligible during measurements. Criteria[72] for determining the suitability of a system for open-system monitoring include extent of uniform area, high biological activity, water depth and residence time (influencing observable changes in water chemistry), turbulence (influencing spatial variation for monitoring and gaseous exchange with the atmosphere), and uniformity of the channel (allowing the calculation of production per unit area and providing uniform residence time). The accuracy and sensitivity of several methods have been compared.[73]

The single-station method (Section 10300D.4) is used most commonly. In addition to the radiotracer method presented, the re-aeration coefficient can be determined by direct methods or calculations based on physical parameters.[73–75] To use a single station, assume stream homogeneity above the region of measurement.

To use an automated two-station system, calculate a continuous function (Fourier series) to determine an exact solution to the oxygen mass-balance equation, rather than the approximate finite difference solution.[76] This system models net productivity so the oxygen concentrations at the downstream station can be predicted accurately from upstream values. Additionally, it provides detailed information, such as hourly variations in net production and seasonal changes in community photosynthetic characteristics.[77]

For analytical details, see Section 10300D.4*a* and *b*.

Determine production from the change in DO between the two stations:

$$dc/dr = K(c_s - c) + \beta(t)$$

where:

dc/dr = change in DO concentration between stations,
K = reaeration coefficient,
c_s = saturation DO concentration, mg/L,
c = DO concentration, mg/L,
$\beta(t)$ = net productivity, and
t = flow time between stations.

For short time intervals, write t as a Fourier cosine series:

$$\beta(t) = A_o/2 + \sum_{n=1}^{\infty} A_n \cos n\omega t$$

where:

A_n = Fourier coefficients and
$\omega = 2\pi/48$.

Determine enough Fourier coefficients to solve for $\beta(t)$.[76] After solving the equation, plot estimated net photosynthetic rates as a function of incoming solar radiation to evaluate the solution for net production. An anomalous relationship between light and net production, or an unreasonable variation in net photosynthesis over time suggests solution errors. The estimate of K is the method's weak point; an iterative approach to estimating K, based on the morning and afternoon relationship between net production and light,[73] has been proposed.

b. Radiocarbon incorporation method: The radiocarbon (^{14}C) tracer method[78] has been used extensively for estimating the productivity of virtually all aquatic primary producers. It is based on the measurement of radiocarbon incorporation when a small but known quantity is made available to a plant. The proportion of tracer added to that incorporated indicates the fraction of stable carbon that is incorporated. This method is more sensitive than oxygen methods, so it can be used where photosynthetic rates are very low or where carbon consumption is excessive.

Although this method directly measures the incorporation of external inorganic carbon, the relationship of this incorporation to net or gross photosynthesis is not without controversy.[79] The consensus is that the ^{14}C method for macrophytes provides a rate of carbon incorporation between net and gross photosynthesis, but closer to net photosynthesis.[35,38,47,52,57,80] The primary drawback of the ^{14}C method is that it provides no measure of dark-period respiration. If the photoassimilation of ^{14}C is assumed to estimate net photosynthesis for macrophytes (gross photosynthesis minus respiration), then respiration during the dark period must be measured separately to account for carbon lost at night in the calculation of 24-h net production (24-h net primary production = gross photosynthesis − 24-h respiration, or here, 24-h NPP = daytime ^{14}C uptake − nighttime respiration).[57] As noted for the oxygen method, respiration usually is assumed to be identical during the day and night for macrophytes.[38,57]

Portions of CO_2 from photorespiration and mitochondrial respiration are stored internally in gas lacunal spaces and are intensively recycled.[81] Some CO_2 from rooting tissues and rhizosphere bacterial metabolism can diffuse internally in these gas channels to photosynthetic tissues.[82] Thus, incorporation rates of $^{14}CO_2$ from the water may not be proportional (underestimate) to true rates of carbon fixation.

For procedural details, see above for the light and dark bottle oxygen method and Section 10300D.3. Analysis of organic fractions into which carbon-14 may be incorporated has been described,[30,64,83] as have procedures for carbon-14.[84–88]

c. Inorganic carbon exchange methods: Changes in the inorganic carbon concentration in air or water surrounding aquatic macrophytes as a result of photosynthesis and respiration can be determined by several methods. These methods provide a highly sensitive, direct measure of photosynthetic carbon uptake, thus requiring few assumptions, and can provide additional information on plant physiology. They require considerable expertise and expensive equipment. Carbon-exchange methods that use closed chambers have the same potential problems as the light–dark chamber oxygen method; however, the formation of bubbles during macrophyte photosynthesis usually does not interfere.

1) Continuous infrared gas analysis (IRGA)—The continuous IRGA method is based on measuring the change in CO_2 (resulting from photosynthesis and respiration) in an air stream that has passed over the leaf or leaves of a plant. The change in CO_2 concentration is determined from the difference in concentration between a reference and a sample air flow. For submersed plants, enclose portions of the plant in a cuvette filled with a bathing solution (e.g., water from the collection site) and bubble a gas stream through the solution. The CO_2 in the air stream equilibrates with the CO_2 (all components of the carbonate system) in the water. The change in CO_2 concentration of the air stream reflects plant metabolism and can be measured as the air stream passes through the IR analyzer. Details of equipment and procedures have been discussed.[80,89–95]

2) Discrete inorganic carbon exchange—Metabolic inorganic carbon exchange with the air or water also can be measured by determining the change in inorganic carbon in sealed containers after a discrete incubation period, using IRGA,[68,92,96–98] gas chromatography,[99] or a total organic carbon analyzer operating in the inorganic carbon mode (see Section 5310). The change in CO_2 in the air (for emergent and floating plants) or the change in dissolved inorganic carbon (DIC) in the water (for submersed plants) is determined for subsamples of the incubation medium. This method is analogous to the light and dark bottle oxygen method, except that changes in the CO_2 or DIC concentrations are determined, rather than the change in DO concentration.[38] This method requires more expensive equipment than the oxygen method does; however, in low-DIC waters it is much easier to measure the change in DIC resulting from plant metabolism than the corresponding change in DO (due to the relative abundance of DO and DOC and the high sensitivity of the DIC method). See references cited above for procedural details.

The change in CO_2 in the light is net photosynthesis. A darkened chamber can be used to determine respiration. Extrapolation to estimate production and additional methodological considerations are covered in the discussion for the light and dark bottle oxygen method. As for other carbon exchange methods, the exchange of CO_2 with the atmosphere, as well as marl formation, can result in errors in the determination of carbon exchange.

3) Potentiometric measurement of inorganic carbon flux—The physical–chemical relationships of the carbonate buffer systems in natural waters dictate a definable relationship between DIC (or alkalinity) and pH. On the basis of such relationships, changes in the water's pH resulting from plant-mediated

changes in DIC concentrations (due to photosynthesis and respiration) can be used to estimate the inorganic carbon exchange for submersed macrophytes. The method presented here is based on the estimation of DIC or total carbon (C_T) determined by a Gran titration.[100] This approach requires only a good pH meter capable of readings to 0.1 pH unit, an electrode, and careful laboratory procedure. However, it is not suitable for waters of very high or low pH, and has limitations similar to those of the oxygen method. While it has been applied primarily to laboratory studies,[101–103] it is adaptable for field use.

To measure inorganic carbon flux,[104] incubate and collect sample as described for the light and dark bottle method. Use 125-mL gastight bottle or polypropylene syringe for sampling. Determine temperature of a 2.0- to 100-mL water sample and add sample to a titration vessel containing a magnetic stir bar, or add stir bar to collection bottle for direct titration. Ensure that titration vessel is partially sealed around the outlet to provide protection from the atmosphere. Record initial pH. Stir sample and use a 0.5- to 5-mL-capacity piston syringe-buret to titrate stepwise with HCl or other appropriate acid of known normality. Use acid of such normality that the total solution volume is not changed by >10% by the end of titration. Record pH and volume of titrant added at three points between pH 7.6 and 6.7 (or lower if necessary) and another three points between pH 4.4 and 3.7.

Calculate net photosynthesis by the change in carbon in the light bottle or container, and respiration by the change in carbon in the dark container as follows: Calculate F_2 from pH readings in the lower pH range as

$$F_2 = [\text{antilog}\,(a - \text{pH})] \times [(V_s + v)/V_s]$$

where:

F_2 = antilogarithmic Gran functions for pH change with titrant additions,
a = any convenient number above the pH range (e.g., 5),
V_s = sample volume, mL, and
v = titrant volume, mL.

Plot F_2 against v and fit with a straight line, locating the intersection of the line on the v axis (v_2). Calculate F_1 from pH readings in the higher pH range as

$$F_1 - [\text{antilog}\,(b - \text{pH})] \times (V_s + v) \times (v_2 - v)$$

where:

F_1 = antilogarithmic Gran functions for pH change with titrant additions and
b = any convenient number above the pH range (e.g., 8).

Plot F_1 against v, locating the intersection of the best-fitting straight line with the v axis (v_1). Then,

$$V_1 = v_1 \times \frac{1000}{V_S} \times n$$

$$C_T = V_2 - V_1 = \frac{1000}{V_S} \times n \times (v_2 - v_1)$$

$$V_2 = v_2 \times \frac{1000}{V_S} \times n$$

where:

V_1 = acidity, meq/L,
V_2 = total alkalinity, meq/L,
C_T = total DIC, mmol/L, and
n = normality of the acid titrant.

Use of antilog paper simplifies this procedure. The method can be used for water samples with an initial pH <7 if an equal amount of NaOH is added to all samples.[101,104]

A formulation of a relationship of pH to C_T for a water of constant alkalinity and changing pH (e.g., as a result of photosynthesis or respiration) eliminates the need for titrations to determine the change in C_T in an incubation vessel resulting from macrophyte photosynthesis, as only the initial and final pH would be required.[104]

4. Data Presentation

Express seasonal and annual rates of macrophyte production in units of carbon or dry weight per unit area of colonization or littoral region. Occasionally, energy units (kcal) may be used but are not recommended. Net annual aboveground production, expressed as grams C per square meter per year or grams dry weight (or ash-free dry weight) per square meter per year is used often but variously underestimates total net annual production. Express the results of short-term (minutes, hours, days) measurements of production or photosynthesis as carbon fixed per unit shoot dry weight or per unit chlorophyll *a*. For emergent plants, report as carbon uptake per unit leaf or shoot surface area. The value of data collected depends on clear statements of how the production values are calculated and expressed (e.g., dried at 105°C, dry weight or ash-free dry weight, per unit area colonized or per area lake bottom, etc.) and on the provision of ancillary information to allow the data to be re-expressed and compared with values from other studies (chlorophyll content, ash content, above below-ground weight, etc.).

5. References

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10500 BENTHIC MACROINVERTEBRATES*

10500 A. Introduction

1. Definition

Benthic macroinvertebrates are animals that inhabit the sediment or other substrates on the bottom of freshwater, estuarine, and marine ecosystems. During their life cycles, these organisms may construct attached cases, tubes, or nets that they live on or in; roam freely over rocks, organic debris, and other substrates; or burrow freely in substrates. According to historical definitions, macroinvertebrates are visible to the unaided eye and retained on a U.S. Standard No. 30 sieve (0.595- or 0.600-mm openings).[1] In actuality, however, some are difficult to see without magnification.

The standard sieve for collecting freshwater, estuarine, and marine benthic macroinvertebrates is the U.S. Standard No. 30 sieve; however, some estuarine and marine programs use the U.S. Standard No. 50 sieve (0.300-mm openings) or the U.S. Standard No. 35 sieve (0.500-mm openings). For all aquatic assessment programs, the No. 30 sieve is recommended for collecting benthic fauna from freshwater, estuarine, and marine habitats or from any water transport system. To accommodate some old historical databases (if the study's data-quality objectives permit), investigators might use a U.S. Standard No. 28 sieve (1.0-mm openings). To obtain a more representative sample of the benthos (one that includes smaller forms, early life-stages, and other taxa of macroinvertebrates), they may use a U.S. Standard No. 60 sieve (0.250-mm openings).

The standardization of bioassessment for species composition, taxa richness, diversity, evenness, trophic levels, and major taxonomic spatial and temporal patterns may be enhanced significantly by the conventional use of a U.S. Standard No. 30 sieve.

The major macroinvertebrates found in freshwater are flatworms, annelids, mollusks, crustaceans, and insects. The major macroinvertebrates found in estuarine and marine waters are bryozoans, sponges, annelids, mollusks, roundworms, cnidarians (coelenterates), crustaceans, insects, and echinoderms.

2. Response to Environment

The species composition and density (numbers of individuals per unit area) of macroinvertebrate communities in streams, lakes, estuaries, and marine waters can be uniform from year to year in unperturbed environments. However, life-cycle dynamics produce variations in species composition and abundance either temporally or spatially.

Most aquatic habitats—particularly free-flowing streams and waters with acceptable water quality and substrate conditions—support diverse macroinvertebrate communities in which the total number of individuals is divided into a reasonably balanced distribution of species. Such communities respond to changing habitats and water quality by altering community structure (invertebrate abundance and composition). However, many habitats—especially disturbed ones—may be dominated by a few species.

The responses of macroinvertebrate communities to environmental changes are useful in assessing the effects of municipal, industrial, oil, and agricultural wastes, and of other land uses on surface waters. Investigators have documented macroinvertebrate community responses to increased inorganic nutrients, increased organic loading, substrate alteration, and toxic chemical pollution. Inorganic nutrients and severe organic pollution usually reduce the variety of macroinvertebrates to only the most tolerant ones [usually associated with low dissolved oxygen (DO) concentration] and increase their density. Sometimes severe organic pollution, siltation, or toxic chemical pollution may reduce or even eliminate the entire macroinvertebrate community from an affected area. However, macroinvertebrate responses may be mediated by other environmental (biological, chemical, and physical) conditions.

Assessing the effects of a pollution source generally involves comparing macroinvertebrate communities and their physical habitats in polluted areas with those in adjacent unpolluted sites (e.g., a gradient away from point sources of contamination). The procedure includes sampling and analyzing communities from different sites and subsequently determining whether and how they differ. The basic information required in most structure analyses cases is a count of individuals per taxon. Then, investigators can characterize and compare communities according to community structure, density, diversity, metrics, pollution indicators, or other analyses,[1] including various statistical methods (see 10500D). They also can estimate biomass and productivity.[1–4] Equally important is a characterization of DO concentration, substrate, water depth, sediment type and grain size, total organic carbon (TOC), and other site- and situation-specific characteristics.

While the following macroinvertebrate methods are traditionally used to sample and quantify benthic invertebrate communities, other methods are also being evaluated in an effort to develop and implement narrative biological criteria for surface waters.[5] Not discussed here are U.S. Environmental Protection Agency (EPA)-developed rapid bioassessment techniques[6,7] and Environmental Monitoring and Assessment Program (EMAP) protocols for field operations, as well as methods for sampling macroinvertebrates and assessing the ecological conditions of wadeable streams.[8,9]

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10500 B. Sample Collection

1. General Considerations

Before conducting a benthic survey, determine specific data quality objectives and clearly define the information sought. *Data quality objectives* are qualitative and quantitative statements developed to specify the quality of data needed to support specific decisions and conclusions about the information sought. Discussion with water chemists, hydrologists, limnologists, and individuals from other disciplines will be helpful. Ultimately, the choice of methodology will depend on whether the habitat to be studied is a stream, lake, reservoir, or marine area. For example, investigators only need a few sampling stations upstream and downstream of a discharge to determine whether the macroinvertebrate community downstream is damaged. However, if the objective is to delimit the extent of damage from a discharge or series of discharges, they will need reference stations upstream of, downstream of, and bracketing all discharges. In marine waters, they may need to sample a nearby estuary. In open ocean waters, they may need to sample some distance from the discharge point.

Characterize the physicochemical properties of faunal sampling-station substrate and overlying water. Measure such properties as sediment size class distribution (sand, silt, and clay); organic content and toxic pollutant concentrations; temperature, salinity, hardness, alkalinity, DO, total organic carbon (TOC), ammonia, sulfides, and nutrient (total and dissolved) concentrations; biochemical oxygen demand; water depth; and velocity of flowing streams.

After gaining a thorough understanding of the waterbody's characteristics, select specific areas to be sampled. There is no predetermined number of sampling stations that will be appropriate for all situations. No water-quality survey is routine, nor can one be conducted totally on a "cookbook" basis. However, if investigators adhere to the following basic rules, they can design a sound survey.

a. Always establish a reference station(s) outside the influence of all wastewater discharges of concern (but in the same waterbody). Because most surveys are made to determine the damage that pollution causes aquatic life, this will be the basis for comparing biota in polluted and unpolluted areas. Preferably establish at least two reference stations: one far from the effluent discharge and the other near the discharge, but not subject to its influence. (For example, if the discharge were in a river, one station would be far upstream of the discharge and the other would be immediately downstream.) Whenever feasible, use reference stations with physicochemical characteristics similar to the receiving area's substrate and overlying water.

b. Locate a station immediately downstream of each discharge or in the affected area in its immediate vicinity, as appropriate.

c. If the discharge does not mix completely on entering the waterbody, but instead channels along one side or disperses in a specific direction, then locate stations in the left-bank (looking upstream), midchannel, and right-bank sections of the stream; in concentric arcs in lakes and oceanic waters; or in any other configuration that will meet study objectives.

d. Establish stations at various distances downstream from the last discharge of concern to determine the linear extent of damage. In a marine environment, a nearby estuary may be sampled; in open ocean waters, samples may be taken in a nearby area with comparable currents, depth, sediment characteristics, and salinity.

e. To permit comparison of macroinvertebrate communities, be sure that all sampling stations are ecologically similar. For example, select stations with similar bottom substrate (e.g., sand, gravel, rock, mud, organic content), depth, presence of riffles and pools, stream width, gradient, flow velocity, bank or shore cover, salinity, hardness, TOC, nutrient and DO concentrations, and wave exposure.

f. Collect samples for physical, toxicological (if applicable), and chemical analyses as close to biological sampling stations as possible to ensure correlation of findings; take such samples at

the same time and from the same grab when possible. Collect substrate samples for physicochemical analyses from the upper few centimeters, where most organisms live.

g. Locate macroinvertebrate sampling stations in the best physical habitat [areas not influenced by atypical conditions (bridges, dams, etc.)].

h. Discharges in coastal areas may be subject to various degrees of salt-water intrusion (salt-water wedge). Macroinvertebrate populations may change drastically in such areas; document and/or allow for this effect.

i. When sampling in small, wadeable, first- to third-order streams, begin at the station farthest downstream and proceed upstream to minimize disruptions induced by the sampling itself. This is unnecessary for non-wadeable streams and rivers.

For a long-term biological monitoring program, consider collecting macroinvertebrates at each station at least once during each of the annual seasons, though this may not always be necessary and would depend on the study design.[1] More frequent sampling may be necessary if effluents' characteristics change or spills occur. Make allowance for collections at night where "drift" or night-feeding organisms are of special concern. In general, the most critical period for macroinvertebrates in streams is during periods of high temperature and low flow, whereas in estuarine and marine environments it is the period of maximum stratification and poor vertical mixing. If available time and funds limit sampling frequency, make at least one survey during the critical time.

2. Sampling Design

Some terms have multiple meanings. In biology, for example, a *population* is a group of individuals that are all members of the same species or taxonomic group. In statistics, a *population* is the entire set of values for the characteristic of interest in a whole sampling universe. For example, researchers interested in determining the mean density of worms on a lake bottom might take ten grabs from lake sediments. The number of worms in each grab would be an *observation*, the density of worms would be the *characteristic of interest*, and the contents of each grab would be an *experimental unit* or *sampling unit*. The entire lake bottom would be the *sampling universe* and enough grabs to equate with the area of the entire lake bottom would be the *population* (of units).

Similarly, the term *sample* has two often contradictory uses. In typical studies, observations usually are not made of all possible sampling units; instead, observations are only made of a small fraction of the total. Statistically, this set of observations is called a *sample*. In the example given above, the ten grabs collectively would be a sample. However, in everyday language (and as used in this book and most scientific publications), a *sample* is a portion of the real world that has been selected for measurement (e.g., a water sample, plankton haul, or bottom grab). Therefore, in the example above, each individual grab would be a sample (i.e., "ten samples were taken").

Collecting a representative statistical sample is difficult because of variation in successive scientific samples. Without knowing the sampling variability, investigators cannot know the degree to which the data truly represent the population. Make replicate observations of a population if definitive statistical inferences about the population will be made.[2–11]

Standardize sampling design to consider the following requirements:

a. Approximate the set of all samples that can be selected (i.e., separate the sampling universe into all possible samples). For example, if the location (site) containing the population has an area of 1000 m^2 and the sampling device samples an area of 1 m^2, then 1000 samples could be collected in the sampling universe.

b. Assign each sample an equal probability of being selected. Using the situation above, divide the area to be sampled into 1000 discrete units.

c. Use a table of random numbers to select sites for sampling (i.e., sample randomly, not haphazardly).

d. The sampling design outlined above is known as *simple random sampling*. When using this design, it is often advantageous to determine the number of samples necessary for a certain level of precision:

$$N = \left(\frac{t \times s}{D \times \bar{x}}\right)^2$$

where:

- N = number of samples,
- t = tabulated t value at 0.05 level with the degrees of freedom of preliminary survey (generally $t \approx 2.0$ at larger sampling sizes),
- s = sampling standard deviation of samples, known from a preliminary survey,
- D = required level of precision expressed as a decimal (0.30 to 0.35 usually yields a statistically reliable estimate), and
- $\bar{x}$ = sample mean density of preliminary survey.

To estimate the number of samples needed, analysts first need specific information (the mean and standard deviation) about the population to be sampled. Because this information is unknown (because sampling has yet to occur), estimate the population's mean and standard deviation by one of three ways: conduct a pilot study, use results from an earlier or similar study, or make educated estimates.[3,12] For example, if investigators want to determine the mean chironomid density in relatively homogeneous lake sediments during summer, and they know that six grabs taken the previous summer produced a mean density of 4230 chironomids/m^2 and a standard deviation of 1628 chironomids/m^2, then they can use these data to estimate their study's mean chironomid density [$\pm$30%, with a 5% probability of error ($\alpha = 0.05$)]. Using the formula given above,

$$N = \left(\frac{2.5706 \times 1628}{0.30 \times 4230}\right)^2$$

($t = 2.5706$ at a 5% probability of error and 5 degrees of freedom)

$$N = 10.88 \approx 11$$

Thus, it is estimated that 11 grabs will be necessary.

e. A simple random sampling design is useful when sampling relatively homogeneous areas. However, most taxa are not dis-

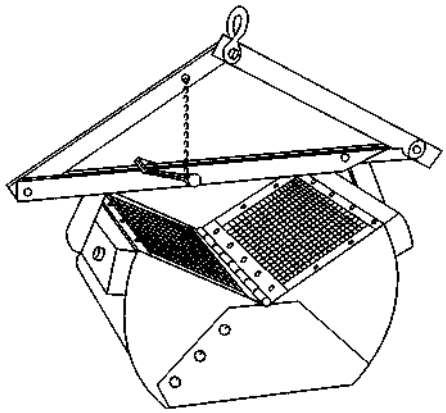


Figure 10500:1. Petersen grab.

tributed uniformly over water bottoms. Different habitats (sand, mud, gravel, or organic material) support different densities and species of organisms. In which case, a stratified random design is more useful.

In a stratified random sampling design, a heterogeneous universe (different bottom substrates, current velocities, depths, temperatures, etc.) is divided into more homogeneous strata. Once the strata are defined, use simple random sampling within each stratum. Stratified random sampling has two important advantages: it provides data on various subsets of a population (e.g., density of benthic invertebrates in each sediment type), and it reduces variability because it deals with more homogeneous subpopulations, allowing for more accurate and precise population estimates.

The data needed to divide the population into various strata usually is acquired via pre-study reconnaissance (a pilot study). A systematic sampling design often is used in such pilot studies. In a systematic-transect design, investigators conduct sampling at equal intervals along a number of transects in a habitat to identify and locate existent strata.[3,12]

f. In descriptive studies, investigators should take at least three replicate sampling units per station.[3,13] If statistical testing is planned, more replicates probably will be needed.

g. Standardize data acquisition and recording when practical. Use metric units.

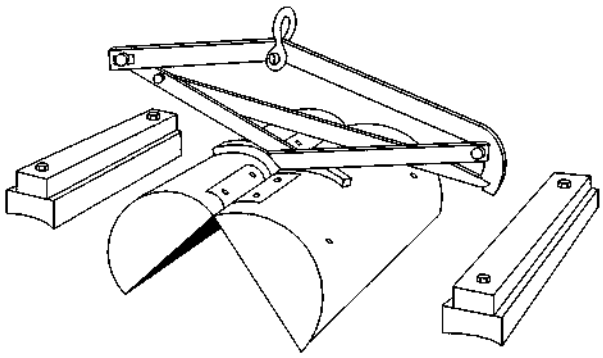


Figure 10500:2. Ponar® grab.

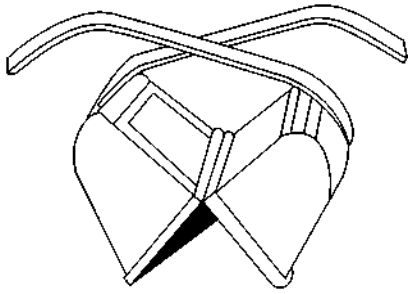


Figure 10500:3. Van Veen grab.

3. Sampling Devices, Quantitative

Quantitative and qualitative samplers have been designed to collect organisms from the bottom of different waterbodies. The most common quantitative sampling devices are the Petersen, Ponar® ,* and Ekman grabs and the Surber or square-foot stream bottom sampler, all described below.

a. Grab samplers:

Before using each grab sampler, calibrate it for actual surface area sampled.

1) *The Petersen grab* (Figure 10500:1) is used for sampling hard bottoms (e.g., sand, gravel, marl, and clay) in swift currents and deep water.[3] It is an iron, clam-type grab manufactured in various sizes that will sample an area between 0.06 and 0.09 m^2. It weighs approximately 13.7 kg, but may weigh as much as 31.8 kg when auxiliary weights are bolted to its sides. The extra weights make the grab stable in swift currents and provide more cutting force in fibrous or firm bottom materials. Modify the sampler by adding end plates, by cutting large strips out of the top of each side, and by adding a hinged 30-mesh screen (as in the Ponar grab).[14]

To use the Petersen grab, set the hinged jaws and lower to the bottom slowly to avoid disturbing lighter bottom materials. Ease rope tension to release the catch. As the grab is raised, the lever system closes the jaws.

2) *The Ponar grab* (Figure 10500:2) is used increasingly in medium to deep rivers, lakes, reservoirs, and estuaries.[15] It is similar to the Petersen grab in size, weight, lever system, and sample compartment, but has side plates and a screen on top of the sample compartment to prevent sample loss during closure. With one set of weights, the standard 23- × 23-cm sampler weighs 20 kg. A 15- × 15-cm petite Ponar may be used. The large surface disturbance associated with a Ponar grab can be reduced by installing hinged (rather than fixed) screen tops, thereby reducing the pressure wave associated with the sampler's descent. This sampler is best used for mud, sand, gravel, or small rocks with mud, but it can be used in all substrates except bedrock.

3) *The Van Veen grab* (Figure 10500:3) is used to sample in open marine waters and in large lakes. The sampler's long arms tend to act as stabilizers without disturbing water at the water–substrate interface. It is basically an improved version of the

* Registered trademark of Morris & Lee, Inc. d/b/a Wildlife Supply Co., Buffalo, NY.

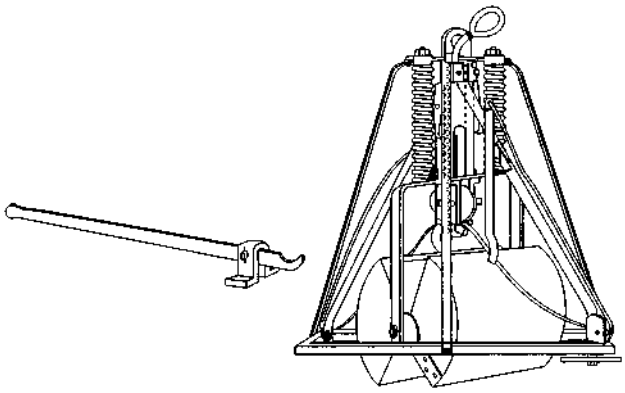


Figure 10500:4. Smith-McIntyre grab.

Petersen grab for mud, gravel, pebble, and sand substrates. The sampler is heavy; lower it from a boat or ship platform via mechanical or hydraulic lifts.

4) *The Smith-McIntyre grab* (Figure 10500:4) has the heavy steel construction of the Petersen, but its jaws are closed by strong coil springs.[16] Its chief advantages are stability and easier control in rough water. Its bulk and heavy weight require operation from a large boat equipped with a winch. The 45.4-kg grab can sample an area of 0.2 m^2,[17–19] but smaller models (0.1 m^2 or 0.05 to 0.06 m^2) are available.

5) *The Shipek®† grab* (Figure 10500:5) is designed to take a sample from virtually any substrate; samples have a surface area of 0.04 m^2 and are approximately 10 cm deep at the center.[3] The sample compartment is composed of two concentric half cylinders. When the grab touches bottom, inertia from a self-contained weight releases a catch and helical springs rotate the inner half cylinder by 180°. The sample bucket may be disengaged from the upper semi-cylinder by releasing two retaining latches. This grab is for special use in marine waters and large inland bodies of water (e.g., in compact substrates).

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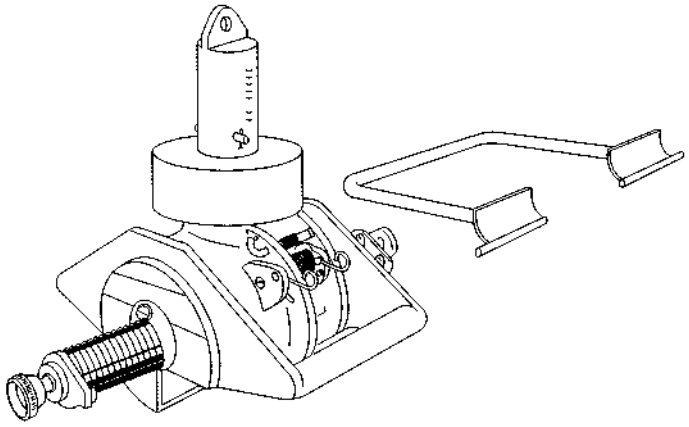


Figure 10500:5. Shipek grab.

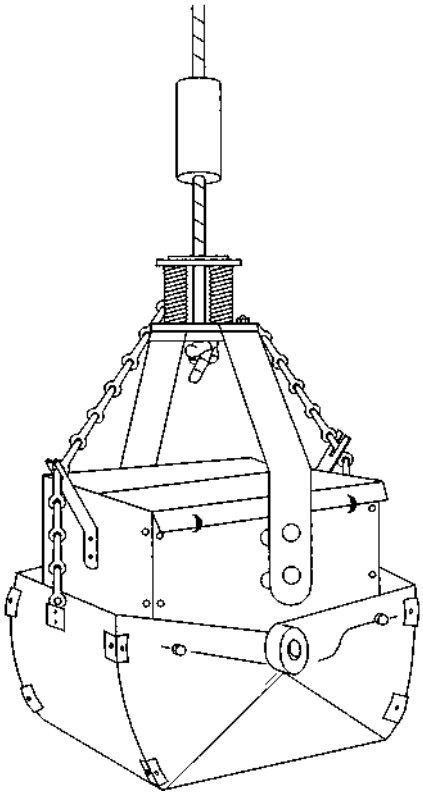


Figure 10500:6. Ekman grab.

6) *The Ekman grab* (Figure 10500:6) is only useful for sampling mud, silt, muck, and sludge in water with little current.[3] It is difficult to use in areas with rocky or sandy bottoms or moderate macrophyte growth because small pebbles or grit or macrophyte stems prevent proper jaw closure. The grab weighs approximately 3.2 kg. The box-like part holding the sample has spring-operated jaws on the bottom, which must be cocked manually (exercise caution when cocking and handling the grab because of possible injuries if jaws are tripped accidentally). At the top of the grab are two hinged overlapping lids that are partially held open during descent by water passing through the sample compartment. These lids are held shut by water pressure when the sampler is being retrieved. The grab is made in three sizes—15 × 15 cm, 23 × 23 cm, and 30 × 30 cm—but the smallest size is usually adequate. A taller model of this sampler (23 cm or 30.5 cm tall) is available. To prevent sample overflow

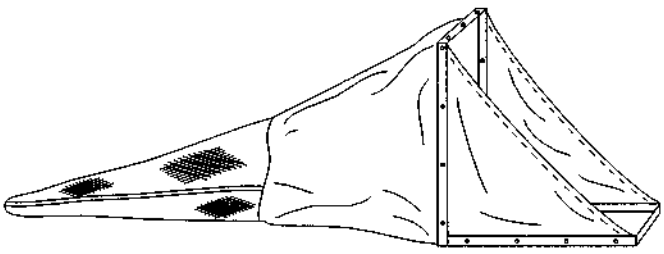


Figure 10500:7. Surber or square-foot sampler.

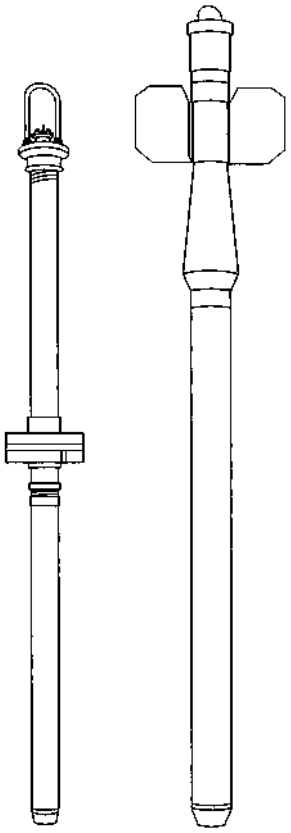


Figure 10500:8. Phleger core sampler.

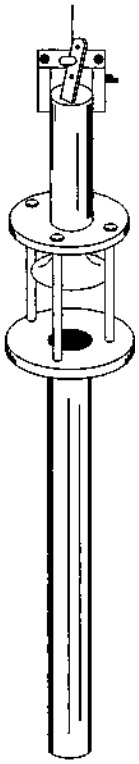


Figure 10500:9. KB corer.

and loss, place a Standard U.S. No. 30 sieve insert in the top of any Ekman grab sampler for deep sediments.

b. Riffle/run samplers:

1) *Surber-type samplers* (Figure 10500:7)[20] consist of two brass frames—each 30.5 cm (1 ft) square—hinged together along one edge. When in use, the two frames are locked at right angles, one frame marking off the area of substrate to be sampled, and the other supporting a net to collect organisms washed into it from the sample area.

The net usually is 69 cm long, and its first few centimeters and its wings are constructed of heavier material (canvas, taffeta) to increase durability. The rest of the net is a standard 30 mesh size (595 to 600 μm). While a finer mesh might collect more of the smaller invertebrates and young instars, it also will clog more easily and resist the current more, possibly resulting in a loss of organisms due to backwashing. This sampler is specific for macrobenthos; many microcomponents of the benthos are not collected.

Use this sampler in shallow, flowing water (no more than 30 cm deep). In deeper water, some organisms may flow over the top of the sampler. Position sampler securely on the stream bottom parallel to water flow, with the net portion downstream. Take care not to disturb the substrate upstream of sampler. Leave no gaps under the edges of the frame that would allow water to wash under the net. Fill any gaps along the back edge of the sampler by carefully shifting rocks and gravel along the outside edge. When sampler is in place (it may be necessary to hold it in place with one hand in a strong current), carefully turn over and lightly hand-rub all rocks and large stones inside the frame to dislodge organisms clinging to them. Examine each stone for organisms, larval or pupal cases, etc., that may be clinging to it before discarding. Scrape attached algae, insect cases, etc., from the stones into the sampler net. Stir remaining gravel and sand with hands or a stick to a depth of 5 to 10 cm (depending on substrate) to dislodge bottom-dwelling organisms. It may be necessary to hand-pick some mussels and snails that the current does not carry into the net.

Remove sample by inverting net into sample container. Carefully examine net for small organisms clinging to it. Remove these—preferably with forceps to avoid damage—and include in sample. Rinse sampler net after each use.

A common problem when using the Surber sampler is that organisms wash under the bottom edge of the sampler. The following modifications have been suggested for different substrates:

- For loose gravel—Extend bottom edge of Surber frame to 5 or more cm so frame can be inserted deeper into substrate. This method works well in soft substrates (e.g., sand and gravel), where the current causes substrate shifting.
- For coarse gravel and rock—Add serrated extension to the back edge of frame to secure it and reduce washing from under this edge. This method is helpful in hard gravel and rock substrates, where sinking the entire frame is impossible.
- For gravel and bedrock—Add a 5-cm band of flexible material to the bottom edge of sampler to create a seal in rocky, uneven substrates. Make band of foam rubber or fine-textured synthetic sponge. Remove organisms that stick to foam and include in sample.

2) *Hess-type samplers* are cylindrical with enclosed sides and an open top. They function much like Surber-type samplers.[3]

c. Core or cylindrical samplers: Use core or cylindrical samplers to sample sediments in depth. Efficient use as surface samplers requires dense animal populations. Core samplers vary from hand-pushed tubes to explosive-driven and automatic-surfacing models.[3,21]

1) *The Phleger corer* (Figure 10500:8) is widely used and operates via gravity.[3] Styles and weights vary among manufacturers; some use interchangeable weights that allow variations between 7.7 and 35.0 kg, while others use fixed weights weighing 41.0 kg or more. Core length will vary with substrate texture.

2) *The KB®‡ core sampler* (Figure 10500:9), or a modification known as the Kajak–Brinkhurst corer, may be useful in obtaining estimates of the standing stock of benthic macroinvertebrates inhabiting soft sediments.[22]

3) *Box core samplers*[23–27] are used to sample soft substrate in large rivers, lakes, and estuaries. They are available in several sizes, can sample a variety of sediments, and are used in marine waters and in the Great Lakes[3,28] to collect benthic macrofauna. The sampler may be deployed from ships or platforms, but diver-collected cores are preferred.

The KC Box Corer frame is made of 60 × 60 × 6 mm, sandblasted, hot galvanized stainless steel square tubes.[27] The 30 × 20 cm or 32 × 32 cm sampler tube is made of 3-mm electropolished stainless steel. The sampling surface area is either 600 or 100 cm^2, and it samples a depth of 20 to 40 cm.

Preferably use a box coring device with a rectangular corer whose cutting arm can seal the sample before retracting from the bottom. To sample enough individuals and taxa, and integrate the patchy distribution of benthic fauna, use a sampler with a surface area of at least 100 cm^2 and a sediment depth of at least 20 cm. A box corer that can sample deeper sediment may be needed to collect deep-burrowing infauna. For sandy sediments, it may be necessary to substitute a grab sampler to adequately penetrate sediment and collect samples. Visually inspect each sample to ensure that an undisturbed, adequate amount of sample is collected.

4) *The Wilding or stovepipe sampler* (Figure 10500:10)[29–30] is made in various sizes and with many modifications.[3] The Wilding sampler is made from any tubular material (e.g., 60- to 75-cm sections of 30-cm-diam stovepipe[30] or 75-cm sections of 30-cm-diam aluminum irrigation pipe fitted with handles). The Maine Department of Environmental Pollution uses a 5-gal bucket with the bottom removed.

The sampler is pressed into the substrate and its contents are agitated. It is especially useful for quantitatively sampling a bottom with dense, vascular plant growth. It may be used to sample vegetation, mud–water interface sediment, or most shallow stream substrates. However, large volumes of vegetation, when sampled in this way, may require a great deal of time for laboratory processing.

d. Drift samplers: Drift samplers, usually in the form of nets (Figure 10500:11), are anchored in flowing water to capture macroinvertebrates that have migrated or been dislodged from the bottom substrates into the current. Drift organisms are important to the stream ecosystem because they are prey for fish and should be considered in the study of fish populations. Drift organisms respond to pollutional stresses (e.g., spills) by increased drift from an affected area so drift is important in water-quality investigations, especially of spills of toxic materials. Drift also is a factor in recolonizing denuded areas and contributes to recovery of disturbed streams.

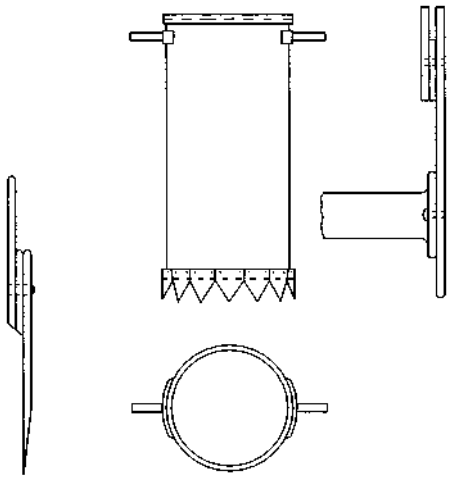


Figure 10500:10. Wilding or stovepipe sampler.

Use nets with a 929-cm^2 upstream opening and mesh equivalent to U.S. Standard No. 30 screen (595-μm pore size). After placing the net in the water, frequently remove organisms and debris to prevent clogging and subsequent diversion of water at the net opening. Use replicate samples, as appropriate, to meet study objectives. Set drift-net samples for any specified time (usually 1 to 3 h) but use the same time for each station. Sampling between dusk and 1 a.m. is optimal.

The total quantity (numbers or biomass) of organisms drifting past a given station is the best measure of drift intensity. Report data in terms of numbers or biomass/m^3.[31–33]

4. Sampling Devices, Qualitative

When sampling qualitatively, search for organisms in as many habitats as possible.[34] Collect samples by any method that will capture representative species.

a. Dip, kick nets are the most versatile collection devices for shallow, flowing water and for lake shorelines. When combined with a standardized kicking technique,[35] these nets are appropriate for quantitatively sampling macroinvertebrates.[36]

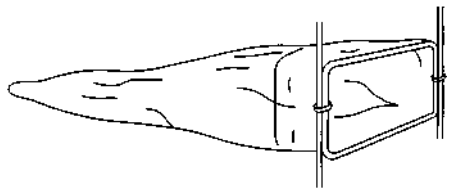


Figure 10500:11. Drift net sampler.

‡ Registered trademark of Morris & Lee, Inc. d/b/a Wildlife Supply Co., Buffalo, NY.

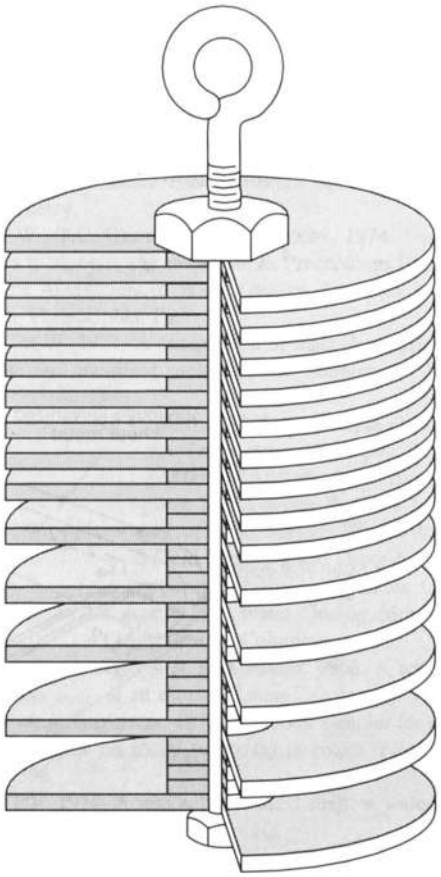


Figure 10500:12. Hester–Dendy artificial substrate unit.

b. Tow nets, dredges, or trawls range from simple sled-mounted nets to complicated devices with teeth that dig into the bottom. Some models feature special apparatus to hold the net open during towing and to close it during descent and retrieval. Available styles have been discussed elsewhere.[21,37,38]

5. Sampling Devices, Artificial Substrate Samplers

Artificial substrate samplers are devices of standard composition and configuration placed in water for a predetermined exposure period to be colonized by macroinvertebrate communities. Because many of the physical variables encountered in bottom sampling are minimized (e.g., depth, light penetration, temperature differences, and species substrate preferences), artificial substrate sampling complements other types of sampling. Like natural submerged substrates (e.g., logs and pilings), artificial substrates are colonized primarily by immature aquatic insects, crustaceans, coelenterates, bryozoans, and to some extent worms, gastropods, and mollusks. In lotic systems, the organisms that colonize artificial substrates are primarily drift organisms (e.g., immature insects and eggs) carried by water currents. Placement conditions should be similar so the numbers and kinds of organisms reflect the capacity to support aquatic life.

Position artificial substrates in the euphotic zone (0.3 m) for maximum abundance and diversity of macroinvertebrates.[13] Optimum time for substrate colonization is 6 weeks in most waters. For uniformity of depth, suspend sampler from floats on a 3.2-mm steel cable. If vandalism is a problem, use subsurface floats or place sampler near the bottom. Regardless of installation technique, use uniform procedures.

At shallow water stations (<1.2 m deep), install samplers so they are midway in the water column at low flow. For samplers installed in July, when water depth is about 1.2 m and the August average low flow is 0.6 m, install 0.3 m above the bottom. Take care not to let samplers touch the bottom or they may become covered with silt, thereby increasing the sampling error. In shallow streams with sheet rock bottoms, secure artificial substrates to 0.95-cm steel rods driven into the substrate or secure to rods mounted on low, flat, rectangular blocks.

Before removing samples from water, it may be necessary to enclose them in an oversized plastic bag (double wrapping) that is tightly sealed to prevent possible organism loss or else remove them via a large dip net (mesh equivalent to a U.S. Standard No. 30 sieve). Disassemble sampler and brush it in a pan of water in the field or add preservative to the bag containing the intact sampler, and disassemble and brush it later in the laboratory.

Although many styles of artificial substrate samplers have been tested,[39] the basket sampler[13] and the Fullner[40] modification of the Hester–Dendy[41] multiplate sampler are widely used.

a. Multiple-plate (modified Hester–Dendy) sampler (Figure 10500:12) is constructed of 0.3-cm-thick tempered hardboard with 7.5-cm round plates and 2.5-cm round spacers with center-drilled holes. The plates are separated by spacers on a 0.63-cm-diam eyebolt, held in place by a nut at the top and bottom. In each sampler, 14 large plates and 24 spacers are used. Separate the top 9 plates by one spacer. Separate Plate 10 by two spacers, Plates 11 and 12 by three spacers, and Plates 13 and 14 by four spacers. The sampler is approximately 14 cm long and 7.5 cm in diameter, has an exposed surface area of approximately 1300 cm^2, and weighs about 0.45 kg. Do not reuse samplers exposed to oils and chemicals that may inhibit colonization. Because it is cylindrical, the sampler fits inside a wide-mouth container for shipping and storage. The sampler is inexpensive, compact, and lightweight.[13,40,41]

Another type of modified Hester–Dendy, multiple-plate artificial substrate sampler is constructed of 0.3-cm tempered hardboard cut into 7.6-cm square plates and 2.5-cm square spacers.[34] Eight plates and twelve spacers are used for each sampler. The plates and spacers are placed on a 1/4-in. (0.64-cm) eyebolt so there are three single spaces, three double spaces, and one triple space between plates. The sampler's total surface area, excluding the eyebolt, is 939 cm^2 (0.9 m^2). Generally, five samplers are used and placed in streams tied to a concrete construction block as anchor. This prevents samplers from coming into contact with natural substrates.

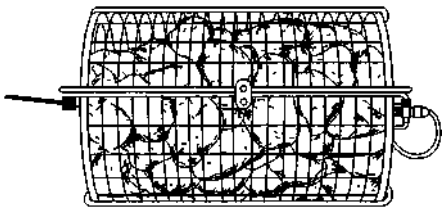


Figure 10500:13. Basket sampler.

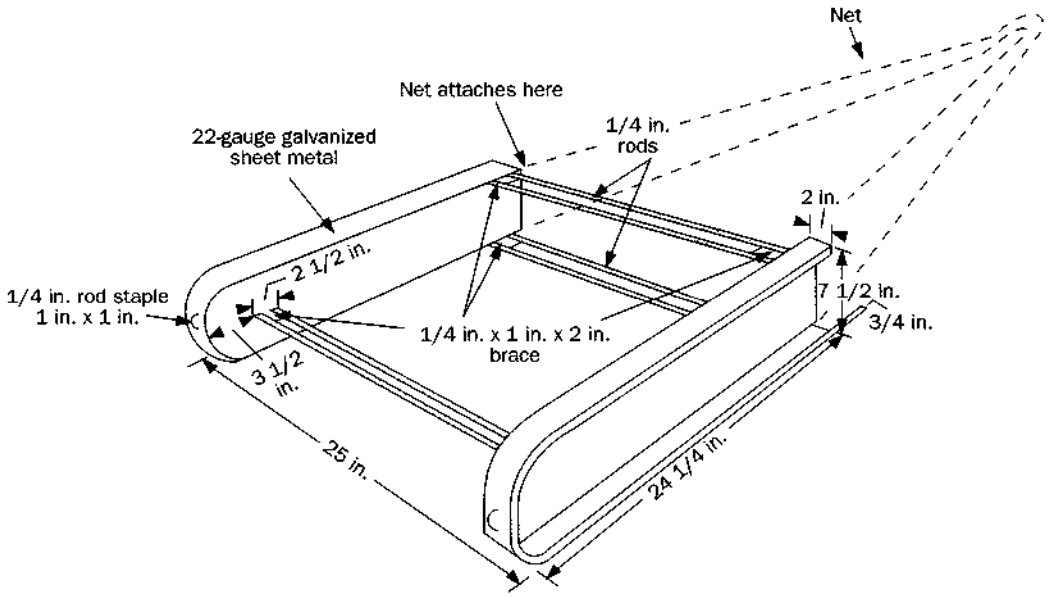


Figure 10500:14. Marsh net sampler.

b. The basket sampler[13] (Figure 10500:13) is a cylindrical "barbecue" basket 28 cm long and 17.8 cm in diameter, filled with approximately thirty 5.1-cm-diam rocks or rocklike material weighing 7.7 kg. A hinged side door allows access to the contents. The sampler provides an estimated 0.24 m^2 of surface area for colonization. The factors governing proper installation and collection are the same as those described for the multiplate sampler. Some investigators prefer using the basket because natural substrate materials are used for colonization.

c. Marsh net sampler (Figure 10500:14) is used for sampling macroinvertebrates in estuarine and marine environments.[42] It can be used in different habitats (e.g., marsh, beach, tidal creek, and tidal flat) of estuarine and marine intertidal zones to depths of 3 m. The metal frame is constructed of No. 22 galvanized sheet metal and 1/4-in. (6-mm) welding rods. A 0.5-m plankton net of nylon monofilament screen is laced to the posterior end of the frame. The net has a bayonet-type cod end for easy removal. The mesh size of the plankton net and cod end is about 1 mm (bar measure). The frame and net weigh 5 kg. The collecting procedures are the same in all intertidal zone habitats. The net is placed at one end of the sampling area, and 30 m of rope is paid out in an arc to prevent the operator from disturbing the sampling site. The net is then retrieved by hand at a rate of about 0.3 m/s. Advantages are that the sampling distance does not have to be measured before taking the sample, the net can be towed at a constant speed, and samples also can be taken over soft mud bottoms.[42]

6. Suction Samplers

Suction samplers are widely used to collect benthic macroinvertebrate samples.[43,44] These samplers can be placed directly on specific sampling sites, but a SCUBA diver is required to collect samples.[45] More accurately located sampling sites and the ability to collect a large number of replicate samples may outweigh the disadvantage of using a diver. Suction samplers have been used widely in sampling marine environments, but they have obvious depth limitations.

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10500 C. Sample Processing and Analysis

1. Sample Processing

After collecting a benthic sample, pour the slurry gradually into a sieve bucket. Gently wash slurry over screen to prevent damaging or losing specimens. Slurries that clog the screen require removal of screened material. A series of one or two coarser screens (e.g., 1-cm and 0.5-cm mesh) will hold back larger materials (e.g., leaves, sticks, shells, and gravel) while permitting organisms and smaller materials to pass through to the bottom sieve. Carefully check rocks, sticks, shells, and other objects for attached or burrowed organisms before discarding. A soft-bristled toothbrush may be used to remove attached invertebrates from rocks, sticks, and similar objects.

Wash residual material on the screen into a container. A cheesecloth bag is useful because it does not restrict the quantity of wash water. Label containers with a collection code but do not affix labels to lids. Similar labels can be written with pencil or indelible ink on high-rag-content paper and placed in the container. Record label code on a field sheet that describes location, date, type of sample, collector's name, and other pertinent information.

Use laboratory elutriation devices,[1,2] as appropriate, to reduce time required to sort benthic organisms from samples containing large amounts of silt, mud, or clay. Wash screened material into a container and fix the contents in a solution of 10% buffered formalin or 70% ethanol.[3–6] If ethanol is used, do not fill more than one-half the container with screened material. Preserve and store animals with calcareous shells or exoskeletons (mussels, snails, crayfish, and ostracods) in 70% ethanol.[6,7]

Some macroinvertebrates (soft-bodied animals) are identified more easily if they are relaxed to prevent constriction during preservation. Common relaxants include carbonated water (soda water) or carbon dioxide added to water. Other relaxants include aqueous solutions of 70% ethyl alcohol, 2% nicotine sulfate, propylene phenoxetol, or 5 to 10% solutions of either chlorotone, chloral hydrate, or magnesium sulfate added gradually to water containing the soft-bodied animals until the degree of relaxation sought is reached. Narcotize organisms before fixing them. Ideally, fix annelid specimens (oligochaetes) in 5 to 10% buffered formalin before preserving them in 70 to 80% ethanol (NOTE: Alcohol is not a satisfactory tissue fixative). Fixation stabilizes tissue proteins to retain characteristics of the soft body (e.g., segmented worms) form.[8,9]

For qualitative samples, place rocks, sticks, and other objects in a white pan partially filled with water. Many animals will float free from these objects and can be removed with forceps.

Assign identification numbers either in the field or at the laboratory and transcribe information from the labels to a permanent ledger. The ledger provides a convenient reference in identifying the number of samples collected at various places, time of sampling, and water characteristics.

Preserve and store in 70% ethanol organisms taken in the field or from artificial substrates and sieved with a U.S. Standard No. 30 sieve. For special studies and to retain anatomical form and structures, fix soft-bodied organisms first with 5 to 10% buffered formalin. **(CAUTION: For health and safety reasons, always take care when using 5 to 10% buffered formalin, or avoid using it to fix or preserve organisms in the field or in the laboratory. Never discard fixatives or preservatives into the environment.)**

2. Sorting and Identification

Whether organisms are sorted in the field or the laboratory, follow consistent procedures. Before processing a sample, transfer information from the label to a data sheet that provides space for scientific names and number of individuals. Place sample directly in a shallow white tray with water for sorting. To facilitate sorting organisms from detritus, the organisms may be stained with rose bengal (200 mg/L or enough to achieve a light pink color) in the formalin or ethanol preservative for at least 24 h.[10] (NOTE: Excessive staining may prevent specific identification of some specimens.) Examine entire sample and separate organisms unless they occur in very large numbers. If a subsample is sorted, take care that rare forms are not excluded. As organisms are picked from the sample, sort under a scanning lens or stereoscopic microscope, separate them into different taxonomic categories, identify to the lowest taxonomic level to meet data quality objectives, and record on the data sheet. Place animals in separate vials according to category and fill vials with 70% ethanol. Inside vials, place labels containing sample tracking number, date collected, sampling location, and names of organisms.

Identify animals in each vial using stereoscopic and compound microscopes (whichever is needed) and available experience and resources. Identify organisms to species level if possible. Additional sources of information on laboratory techniques are available, as well as identification guides and taxonomic keys of macroinvertebrates (see 10500C.4 and Section 10900).

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10500 D. Data Evaluation, Presentation, and Conclusions

There are two basic approaches to evaluating pollution's effects on aquatic life. One approach is to make a qualitative inventory of benthic fauna "above (upstream) and below (downstream)" or "before and after" the suspected or known polluted areas, thereby determining species' presence or absence. Then, via an understanding of various species' responses to certain pollutants and habitat degradation, determine the significance of damage or change. The other approach is to make a quantitative analysis of the numbers of individuals, species, and structure (abundance and composition) of the aquatic community affected by pollution, and then compare that data with reference information. The two approaches are integrated in most pollution surveys because each provides valuable interpretative information.

1. Qualitative Data Evaluation

No two aquatic organisms react identically to a pollutant because of complex relationships between genetic factors and environmental conditions. However, certain taxa are relatively sensitive to certain types of pollution (e.g., siltation and turbidity, organic enrichment, acidity, heavy metals and other industrial toxic wastes, oil production, agricultural products, radioactive wastes, and thermal effects). For example, operculate snails, immature stages of certain mayflies, stoneflies, caddisflies, riffle beetles, hellgrammites, many marine amphipods, mysids, bivalve larvae, and echinoderms are sensitive to many pollutants. Pollution-tolerant macroinvertebrates (e.g., certain sludge worms, midge larvae, leeches, pulmonate snails, and some polychaetes) usually multiply under organically enriched conditions. Facultative organisms (those that tolerate moderate pollution) include most snails, sowbugs, scuds, and blackfly larvae. Tolerant organisms may be found in either clean or polluted situations, so their presence is not definitive. However, a population of tolerant organisms combined with an absence of intolerant ones is a good indication that pollution is present. The same species may well react differently or be present in different numbers in different geographical areas and throughout the year.

2. Quantitative Data Evaluation

Statistical data-evaluation methods and mathematical descriptions of community structure are valuable tools in data analysis. Analyzing biological data commonly begins with the calculation of descriptive statistics (mean, standard deviation, standard error, and confidence intervals). Analysis proceeds by applying robust statistical methods of comparison (Chi-square, Student's *t*, regression, correlation, analyses of variance, or nonparametric equivalents).[1,2]

Mathematical expressions (e.g., numerical indices of community structure) are useful in characterizing and describing aquatic communities. These expressions usually are based on the structural and functional stability of the system.[2]

Diversity indices, although limited, condense considerable biological data into single numerical values.[2–9] Unfortunately, useful information may be lost by condensing biological data.[3,10]

Select methods for analyzing multivariate benthic community data using two important criteria: the methods should test specific effect-related hypotheses suggested by the data quality objectives and study design, and the methods should objectively identify relationships among variables. Use methods that make *a priori* assumptions about relationships among variables only secondarily for presentations, not for primary analysis.

More powerful multivariate statistical analyses generally are less subject to criticism and may be more appropriate for some bioassessment studies.[1,10] Recommended data analyses approaches are regression of species (or taxa) richness on abundance, analysis of variance (ANOVA) followed by linear orthogonal contrasts,[11] various other multivariate approaches (e.g., cluster techniques and ordination, analyzing principal components, ANOVA, discriminate analyses), and macroinvertebrate community metrics[2,12] for assessing biomonitoring data and water quality.

When statistically evaluating data collected in pollution surveys, it always is beneficial to identify the sources of variability commonly found. Variability in macroinvertebrate data comes from sampling methods and organism distribution. Perhaps the major source is sampling error. Organisms generally are clustered in relation to habitat distribution; therefore, random sam-

ples often show high variability among replicates. In statistical analyses of quantitative data, large numbers of samples often are required to detect statistically significant differences. Exercise care when using parametric statistical methods because the basic assumption of normal distribution is not always true. Data often have to be transformed before being tested. Do not assume that a statistically significant difference is ecologically significant. Also, do not assume that lack of statistical significance indicates lack of ecological significance.[13]

3. Data Presentation

Data presentation may take many forms. The basic techniques include tables, bar graphs (horizontal and vertical), pie diagrams, pictorial charts (ideographs), line graphs, frequency distribution tables and graphs, histograms, frequency polygons, and cumulative frequency polygons. These may be superimposed on maps. Several reports that may be useful in analyzing macroinvertebrate data have been included in the bibliography. Methods for interpreting benthic invertebrate data with measures of contamination and toxicity are available.[14]

4. Conclusions

Despite detailed data-quality objectives, field methodology, and laboratory analysis and data presentation, it often requires extensive professional experience, skill, and knowledge of the scientific literature to draw defensible conclusions from a data set. Even in the best circumstances, more than one conclusion can sometimes be drawn from a study. When more than one conclusion is possible, it is appropriate to present all options.

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10600 FISHES*

10600 A. Introduction

1. Ecological Importance

Fishes are a major component of most aquatic habitats; there are thousands of species in streams, rivers, lakes, estuaries, and marine environments. They also are an important source of food and recreation, and are key elements in many natural food webs. They affect the physicochemical properties of the systems in which they occur; interact with plankton, macrophytes, and other aquatic organisms; and can serve as environmental indicators.

Fishes share many physiological processes with mammals and are used in both the laboratory and the field by the environmental manager and health specialist in biological assays.[1] Changes in the composition of a fish assemblage often indicate variation in pH, salinity, temperature regime, solutes, flow, turbidity, dissolved oxygen (DO), substrate composition, or pollution level. The gain or loss of certain species is a common consequence of environmental change. Because fishes are conspicuous, they are often sentinel indicators of the toxification of streams, lakes, and coastal waters. In extreme cases, dead or moribund fishes may adversely affect the potability and recreational use of waters and create foul odors.

In addition, fishes are the focus of economically important sport and commercial fisheries; licensing fees for both private and commercial sectors provide funds for state and federal agencies. Because fishes are both ecologically and economically important, there are often intense commercial and recreational interests surrounding their harvest and study. As a result, there is a need for scientists, often supported by public funds, to arbitrate such competing interests.

2. Terminology

Assemblage (association; community)—a group of several populations sharing a common geographical area. The study of their coordinated activity is critical for understanding the environmental system.

Fish—one organism, several of the same species, or can be used as an adjective (e.g., fish market).

Fishes—two or more species.

Population—a group of individuals of any one species that occupy a particular space. Its study includes definition of taxonomic position, habitat and mobility, diet, numbers of individuals by age, size, weight, sex, fecundity, and sources of mortality.

3. Scope of Analysis

Research often becomes necessary when existing knowledge is inadequate to answer new questions arising from real-world issues. The scope of a research project involves several critical steps that are often overlooked and may lead to findings that lack credibility. Thus time spent in careful planning that recognizes constraints and assumptions is critical for success throughout processes that involve conceptualization, design, data collection, analysis, and interpretation.[2]

The guidelines provided here are directed to general practitioners whose projects may also require the skills of specialists (e.g., fishery biologists, taxonomists, histopathologists, population statisticians, systems ecologists, or toxicologists). Adapting to the particular objectives is necessary for the successful study of fishes in their natural habitat.[3–9]

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* Approved by Standard Methods Committee, 2011.

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10600 B. Data Acquisition

1. Planning and Organization

a. Objectives: Define study goals and objectives clearly before collecting data. Refine the goals or questions into specific objectives that narrow a goal into a tractable issue. Develop testable hypotheses from the objectives, and design the sampling protocol to test these hypotheses. Fishes may be collected for studies of systematics, species or assemblage identification, population dynamics, habitat use, age and growth, feeding habits, reproduction, development, or behavior, or for use in stocking programs. Each type of study requires specific collection and/or analysis techniques.

b. Variables: Before initiating a study, understand the variables that affect fish populations, including the life history characteristics of the species of concern. Environmental variables and habitat characteristics can affect fish abundance and the ability to capture fish. Variables to consider include time of day, season, weather, water height or depth, currents, tidal conditions, bottom type, presence of vegetation, water temperature, salinity, DO, pH, and turbidity. When attempting to measure fish densities, consider the effect of these variables on the catch efficiency of the gear used.

c. Regulations: A detailed understanding of licensing and permit requirements for collecting specimens is essential. Most states have strictly enforced regulations on both collecting and disposing of fish specimens. Effective public relations usually involves the guidance of local residents and waterfront associations on planned activities. Obtain necessary permits for collecting, holding, transporting, and stocking specimens. Provide adequate advance notice of activities to conservation officers, wardens, local law-enforcement agencies, state fish and wildlife agencies, and officers of lake or watershed associations. Understand trespass law for the study area and request access where necessary.

d. Site inspection: Study topographic maps and other relevant data before visiting the site. Make an early and thorough visual examination of the study site. Use glasses with polarized lenses to help examine bottom features and detect fish. Binoculars allow field identification of fishes and help log needed environmental and behavioral information. In clear water, use a faceplate and snorkel or self-contained underwater breathing apparatus (SCUBA) to define habitat, identify fish, and observe behavior. Use wet or dry suits during colder periods and even under ice. (NOTE: Special safety training is essential.)

e. Data forms: Print data forms on good-quality bond paper, waterproof paper, or plastic sheets; use pencil or permanent ink to record data. Electronic media (e.g., data loggers and computer notebooks) allow data to be transferred directly into computer files, saving time and reducing transcription errors. Back up files often and make hard copies to store at a second location. Include the following information in the data form in an order best suited for the study:

- Purpose of activity or project;
- Date and time of collection or observation;
- Exact location using the Universal Transverse Mercator (UTM) system or a local variant; township, range, and section numbers; county and state; physical features (a stream confluence, islands, bays, etc.); and station identification number or code;
- Real-time global positioning system (GPS) coordinates for each sample collected;
- Site conditions for each sample collected [presence of ice, flood state, tidal stage, meteorological information (such as air temperature), the occurrence of storms and rainfall within the last 48 h, water depth, water flow rate, vegetation cover on nearby shores, bottom type, submerged vegetation, etc.];
- Habitat information (e.g., DO, turbidity, pH, substrate type or texture, aquatic vegetation, water temperature, and salinity);
- Description of collections or observations made, including preservatives, photograph numbers, and gear type;
- Information on fishes collected or observed (identity, number, mass, presence of disease, gross external abnormalities or parasites, etc.);
- Personnel and their functions;
- Name of person recording the data; and
- Chain-of-custody signatures and dates.

f. Data collection: Collected materials may define the species of interest, describe the population of a particular species, describe a species assemblage, or characterize the effects of some event (e.g., a chemical spill). The detailed analysis may include

- Preliminary species assignment for each specimen;
- Number of individuals of each species;
- Standard, fork, or total length for each specimen (or a subset of specimens if the number of collected individuals is large);
- Sex, if discernible;
- Maturity as indicated by gonadal condition and coloration;
- Weight of each specimen (or subset of specimens);
- Description of unusual features (e.g., tags, deformities, lesions, tumors, or parasites); and
- Materials taken to determine the age of fish (e.g., scales, otoliths, spines, or opercular bones), stomachs for diet analysis, or tissue samples for genetic, toxin, or stable isotope analysis.

g. Conduct of field workers: Be sensitive to the use of waterfront areas. Avoid, when possible, damage to amenity plantings and capture of favored specimens (e.g., a pair of large smallmouth bass holding a territory next to a dock). Deal pleasantly and in a well-informed manner with the questions of onlookers. Display the name and address of the study group via name tags, arm patches, equipment decals, or temporary signs. Relocate to another sampling area if fishers are near the intended collection site.

Working in, on, and near water involves potential risk. Demonstrate regard for safety. Become proficient with boat safety, proper operation of sampling equipment, and first aid, including cardiopulmonary resuscitation (CPR). Wear life preservers when appropriate. Avoid wearing waders onboard vessels when in deep water. Follow local restrictions on boat speed. Use fail-safe switches on all electrical gear—especially electro-shocking equipment. Ensure that the entire field crew is familiar with safety concerns for fisheries investigations.[1] Be especially careful when working in severe sampling conditions (e.g., temperature extremes, ice, turbulent water, fast currents, high or gusty winds, lightning storms, and at facilities with diversions, dams,

spillways, water intake and discharge structures, and pump stations).

Identify sampling gear with the agency's name, address, telephone number, and permit or license numbers.

Position gear inconspicuously to minimize tampering and vandalism. Avoid navigational channels and other heavily used sites. Whenever appropriate, submerge indicator buoys and mark their location by paired range points (e.g., navigational aids or landmarks). When transferring gear from one locality to another, be careful not to translocate organisms. Maintain gear professionally and handle it proficiently.

Dispose of processed specimens rationally and legally. Consider identifying and counting live specimens in the field and releasing them. Check with regional museums and/or academic institutions that regularly voucher preserved specimens. Obtain accession policies as necessary. Abide by the care and treatment policies and guidelines provided by the Institutional Animal Care and Use Committee (IACUC). Do not overharvest. For example, a 2-h evening set of a gill net may yield enough specimens for a particular study, while an overnight set would be wasteful. Avoid sampling nontarget species.

2. Existing Data

Published and unpublished data already exist for most larger lakes, rivers, and coastal waterbodies. Natural history museums and academic institutions are primary sources of existing data and can often provide preserved materials, as well as the names of local specialists. Members of fishery and ecological professional associations (e.g., the American Fisheries Society, the American Society of Ichthyologists and Herpetologists, and the American Institute of Fisheries Research Biologists) can also provide information. Private engineering and environmental consulting firms often maintain detailed regional files. State agencies (e.g., departments of health, environment, conservation, fisheries, wildlife management, planning) and federal agencies (e.g., U.S. Fish and Wildlife Service, National Marine Fisheries Service, U.S. Environmental Protection Agency, Bureau of Reclamation, U.S. Army Corps of Engineers, and U.S. Geological Survey) are good resources. Large data sets may be available from power utilities, refineries, food producers, and chemical companies with riparian facilities. Cooperative extension services and public colleges and universities provide expertise and information. Other important sources include an area's commercial fishermen and master sport anglers. Local libraries, newspapers, and residents also may provide useful material, including photographs with dates.

3. Collection and Observation Methods

All methods of collecting and observing fishes are selective and, therefore, biased. No single collection method will comprehensively and accurately describe the composition of a fish assemblage, so know each method's inherent biases in order to select one that will best fulfill study objectives. The study's goals will dictate the appropriate method(s). Distinguish between collecting fishes for individual analysis (e.g., gut content analysis vs. age and growth determination using otoliths) and sampling them to determine absolute (fishes per m^2 or m^3) or relative [catch per unit effort (e.g., per minute, per haul, per tow)] population size. Many of the methods listed below are appropriate for collecting fishes but inadequate for determining density or population size.[2–4] For each study, carefully and completely describe the goals, objectives, testable hypotheses, and collecting methods so data users can assess the techniques' adequacy.

a. Angling: The use of hook and line involves relatively simple gear but its effective use is a matter of skill and experience.[5] The services of a competent angler are often valuable. The techniques depend on the angler's resourcefulness and skill, and may be time-consuming and expensive.

b. Set line: A *set line* (also called a *longline* or *trotline*) is a heavy line anchored at each end and bearing regularly spaced leaders [called *gangions* (longline) or *snoods* (trotline)] with baited hooks. Set lines are widely used for commercial and private fishing, are usually fished overnight and on the bottom, and can be used at great depths.

c. Trolling: Hook and line may be towed behind a vessel to collect larger fishes inhabiting open, deep waters. Use metal line with metal weights (downriggers) or wing depressors (planers) to achieve desired depths. Specialized lures that reflect sound navigation and ranging (SONAR) are often used with acoustical electrical gear to determine depth and locate target fish. Trolling may be the most economical means of capturing some species.

d. Spear and bow and arrow: The use of barbed and/or hooked spears (some automated) is of limited utility and often prohibited. Sport bow-and-arrow fishing may yield large numbers of carp, gars, or other larger fishes in shallow water. Spearing through ice may be effective, especially for sturgeon and larger esocids and percids. Efficiency varies seasonally.

e. Nets—general remarks: Netting is used in both static gear (e.g., traps and weirs) and active gear (e.g., seines and trawls). Netting may be made of cotton, plastic, or metal. Natural-fiber nets are subject to microbial decomposition and have been supplanted by other materials but are still valuable if loss of gear is likely. Plastic netting is exceedingly durable but weakened by ultraviolet irradiation; avoid prolonged exposure to sunlight. Netting is available in colors that may provide some sampling advantage. Mesh sizes are measured in terms of *bar* (along the edge of the frame), *diagonal* (from opposite angles of the frame), and *stretched* (from opposite angles when the net is under tension). Knotting varies; some knots are abrasive to captured fish, and knotless netting is also available. Different net designs can be used in different settings and for different target species (e.g., trawls, seines, cast nets, and dip nets).

f. Hoop, fyke, and trap nets: Elongated, tapered nets supported on hoops; variously divided into chambers with secondary funnel net sections; and anchored to the bottom are common. Usually, they are used at depths <3 m. They may be kept in place for a protracted period but usually are visited daily to remove the catch from the inner chamber (cod end). Orient the cod end into the current in slowly moving water.

A basic hoop (ring) net may be converted into a fyke net by adding panels of netting at the open end. Those added to the sides are called *wings*; the panel placed at the center of the mouth may be quite long and is called the *lead*. Wings and leads usually are equipped with floats and weights and are placed to deflect and trap or confuse the normal movements of fishes along shore into the net. The main body of a typical hoop or fyke net may range from 5 to 10 m long and up to 2.5 m in diameter.

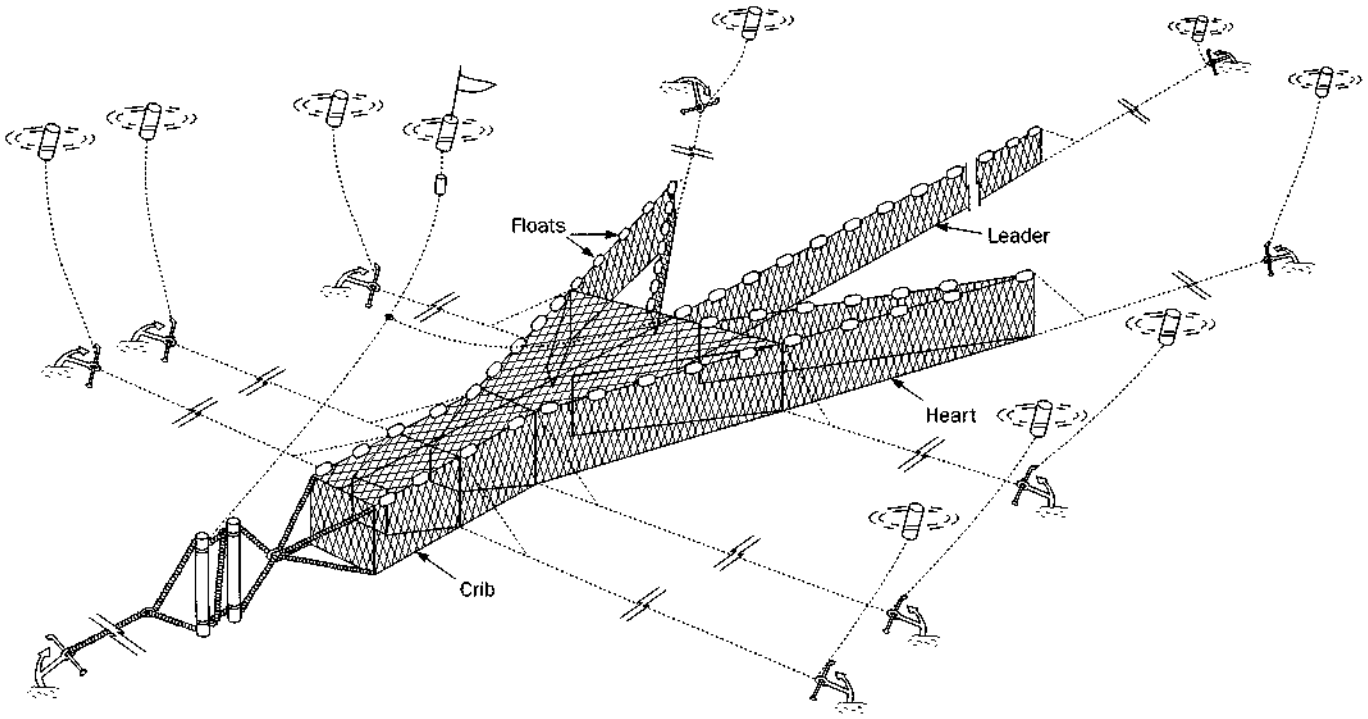


Figure 10600:1. Diagram of a sunken trap net. SOURCE: BAGENAL, T., ed. 1978. Methods for Assessment of Fish Production in Fresh Waters; IBP Handbook No. 3. Blackwell Scientific Publ., Oxford, England.

A net hung on rectangular framing is called a *trap net* (Figure 10600:1). On commercial fishing grounds, researchers usually contract to receive the catches of larger trap nets rather than use their own nets. Another net of this general design is the *pound net*.

Hoop, fyke, and trap nets are effective for larger fishes that are mobile or seek cover. They are especially useful in capturing live fishes for spawning stock or for mark-and-recapture population assessments. The nets can be baited to increase capture efficiency.

Hoop, fyke, and trap nets are available through net supply houses and are commonly built to specification. They usually require a boat to set and inspect. They often are conspicuous, so they attract the public and, therefore, require surveillance and the clear posting of ownership and purpose.

g. Traps: The term *fish trap* is usually reserved for smaller, portable units commonly made of galvanized wire or molded plastic. They are fitted with one or more conical inserts and an opening to remove the catch.

Smaller devices (usually called *minnow traps*) may be useful. Many types are available, including highly durable plastic units that can be easily stacked, stored, and transported. Minnow traps may be placed in difficult-to-sample localities (e.g., in a well, in a cave pool, near hydroelectric facilities, or in shallow wetlands) with good results. They may be more effective when baited. All traps used in the field should have a readily degradable element (e.g., a wooden lathe in a plastic or metal trap) so lost traps do not continue to capture and kill fish.

Pit traps and Breder traps can be used to capture small fishes in marshes that are inundated at high tide.[6–8] Designs vary, but generally pit traps are plastic containers placed into a pit dug into the marsh substrate. Because fishes use natural depressions as refuge when the marsh drains, pit traps can be an effective collection tool.

h. Weirs: Weirs are stationary traps usually installed along the course of a stream, river, or coastal shoreline. Their design is complex, and they may be incorporated into a fish ladder and dam. They guide fishes into a sampling (capture) sector called the *pot.*

i. Gill nets: Gill nets are nets constructed of thin line with mesh large enough for the target species to penetrate just beyond the operculum. Fishes become entrapped while attempting to swim through the net and then are harvested. The nets are composed of panels of netting suspended between a "float line" attached along the upper working edge and a "lead line" attached along the lower working edge. The panels' mesh size may be identical or varied. The float line is stronger and equipped with flotation devices, while the lead line is heavier and equipped with weights. Adjust weights and floats to position the net on the water bottom, surface, or at an intermediate depth. The ends of the gill net are equipped with anchors, tether lines, and buoys.

A gill net may be set under ice through appropriate holes. Gill nets may be used in standing water; they are less successful in flowing water but can be set parallel to the current.

The deployment and recovery of gill nets require special attention. The sampling site must be free of ensnaring objects (e.g., submerged trees). The boat used to set the net must be free of projections that can catch the net during payout, and the net boat must proceed at a speed in harmony with the workers discharging the net. The net must be well pleated and free of snarls to set; the anchors, tethers, and buoys also must be ready

for quick release. Once the net is set, pull the tether to extend it to its full working length. The tether line must be long enough so the buoy is not pulled out of sight. The marker buoy can be set below the surface to reduce vandalism, and paired range points may be used to relocate the site. If the tether line and buoys are lost, attempt retrieval via grappling hook or SCUBA diving. Failure to recover lost gill netting can be environmentally disastrous because it will continue to catch and kill fish. Report lost gill netting to local fisheries authorities.

Overnight setting is common; however, a test set during dawn or dusk or a short night set may be advisable to avoid capturing an excessive number of specimens. Using gill nets can result in high fish mortality. To reduce mortality, the net can be set for a shorter time or checked frequently (e.g., every 2 h) throughout the set so all captured fishes can be removed and processed. Lifting the gill net is best early in the morning during calm weather. If there are relatively few captured fish, they are removed from the net as it is lifted. Otherwise the fish are retained in the net and placed in a sturdy box for subsequent "picking" on shore. Record location, orientation, and mesh size of net. Once "picking" is complete, remove twigs, leaves, and other matter from the net so it may be cleaned and dried. Gill nets, and all entanglement gears, are particularly effective, and selective for fishes with spines or other features that can get caught in netting. If these fishes are abundant, more time will be required to clear the net.

j. Trammel, flag, or tangle nets: Trammel nets typically are composed of at least three panels of netting hung together vertically in the water column. The outer panels have coarse mesh large enough for the target species to swim through, while the central panel(s) is made of much smaller mesh. Fish are trapped by passing through the coarse panel and getting "bagged" in the center net as it is pushed through the larger mesh of the back panel. Trammel nets primarily have commercial applications but are also used in fisheries biology.

k. Trawls: A *trawl* is a towed net. The mouth of the net is maintained by a frame (e.g., the beam trawl or Aggasiz trawl) or with hydraulic planes (called *otter boards* or *doors*) working together with weights and floats (e.g., the *otter trawl*). Trawls are specialized to work at the surface, in midwater, or on the bottom. In a surface trawl, buoying devices predominate; in a midwater trawl, they are balanced against weights; and in a bottom trawl, weights predominate. A bottom trawl may have abrasion skirting on the lower surfaces, rollers that facilitate movement over obstructions, or special chains (ticklers) that run ahead of the lower leading lip of the mouth. Ticklers stimulate fishes to rise off the bottom and into the net. Some trawls have one or more conical inserts before the cod end (terminal end). The cod end is held closed with a cinch line, which can be pulled to release the catch onto a sorting deck or tables.

The trawl is pulled by a bridle and warp worked from a hydraulic winch. Smaller trawls may be worked by hand. The length of warp required depends on sampling speed and depth; a 30° angle of warp to water surface is typical. The towing speed relates to the gear but is often around 2 or 3 knots. The bottom's depth and character may be defined by an echo sounder. The duration of a tow ranges from a few minutes to several hours. Night trawling may yield larger catches but is more difficult in many inland waters because of navigational aids, anchorage buoys, and other obstructions. The fishing effort can be standardized based on the area or volume swept by the trawl or by duration of deployment. (NOTE: In coastal and marine waters, restrictions on turtle-excluder devices, bycatch-reduction devices, and tow duration may be enforced. Contact the National Marine Fisheries Service for current trawling regulations and information on exemption permits for scientific trawling.)



Figure 10600:2. A typical enclosure sampler, the drop sampler, in action. Photo courtesy of the National Marine Fisheries Service Galveston Laboratory.

l. Enclosure samplers: Enclosure samplers include a wide variety of sampling gear used to rapidly enclose a known area. Active enclosure samplers include the encircling/block net, purse seine, drop net, throw trap, drop sampler (Figure 10600:2) , pop net, pull-up net, and bottomless lift net. Once fishes are enclosed, various methods are used to recover specimens, including dip netting, seining, pump filtration, poisoning, and pursing. Enclosure samplers generally catch small fish efficiently, are adaptable for vegetated and structured habitats that cannot be sampled with other gear, and provide quantitative estimates of fish density in different habitats. In tidal estuaries, passive enclosure samplers (e.g., flumes, channel nets and modfied fyke nets) also can be used to collect density data. Enclosure samplers require changing water levels (generally tidal fluctuations) to sample fish distribution patterns. A review compares the efficacy of enclosure samplers and provides recommendations for gear selection in shallow water habitats.[4]

m. Ichthyoplankton sampling: Ichthyoplankton consists of the eggs and very young stages of fish (sacfry, larvae, postlarvae).[9,10] They usually are collected via plankton nets or bulk water sampling.[11] Nets whose mouth diameter is smaller than its main body diameter may be towed faster than the usual 2 to 3 knots.[12] The net's towing bridle affects sample collection, and a number of designs (e.g., the double net or "bongo net") have been devised to reduce this influence; gear comparisons have been made.[13] Other devices may be lowered to a given depth, triggered to open, and then triggered to close again, thereby providing a sample from a known depth. Plankton samplers that can provide depth-specific data include the Clarke–Bumpus, Tucker trawl, light traps, pumps, nets fitted to epibenthic sleds, and multiple opening/closing net and environmental sensing system (MOCNESS).[14] In vertical sampling, the net is lowered to the bottom or some prescribed depth and then pulled upward, sampling the water column. In simple or double oblique sampling, the net is towed between a prescribed depth and the surface, applying approximately equal fishing effort to all depths in the sampled range. Stepped oblique sampling may be used to increase effort at depths of interest. For example, a bottom-midwater-surface stepped oblique tow will provide much more effort at the surface and bottom than a simple oblique tow because the simple oblique tow constantly changes its fishing depth and stops when it reaches the surface.

In all types of deployment, a flow meter is used to estimate the volume of water passing though the net. Mesh size for commercially available plankton netting ranges from 0.158 to 1.000 mm; 0.333 and 0.500 mm are commonly used mesh sizes. Because ichthyoplankton may span a wide range of developmental and sensory capabilities, and may migrate vertically on a diel or tidal basis, note the time of day and/or stage of the tide (in marine and estuarine settings) when sampling occurs. With due consideration for safety, nocturnal sampling may be warranted if vertical migration or gear avoidance is suspected.[15]

Bulk water sampling of ichthyoplankton consists of collecting a known volume of water and filtering out the ichthyoplankton.

n. Seines: A *seine* is a simple panel of netting pulled by a bridle at each end (Figure 10600:3) . In many smaller seines, the bridle is attached to pulling poles (*brails*). The upper line of the seine is equipped with floats and the lower with weights. Some seines are fitted with a central bag of smaller mesh that traps the fish. Seines commonly range from 1 to about 100 m long and 1 to 3 m deep. Mesh size depends on the target species. The seine is an effective device for sampling smaller fishes.

Seines may be worked over shorelines relatively free of obstructions. Pulling may be either parallel, angled, or perpendicular to shore. Two samplers form the net into a gentle "U" while pulling. After a suitable distance, the seine (with the lead line on the bottom) is pulled to and upon the shore. A series of shorter passes may be more productive than one long one.

Small seines may be used over cobbled stream beds by placing the net poles firmly in the bottom and then rolling the cobbles upcurrent of the net; this action dislodges hidden fishes to drift into the net. Benthic species are especially prone to capture in this manner.

Large seines ("beach" seines) usually are set from a boat and may capture large adult fishes. Fix a long warp on shore and pay it out to a set distance via a boat working offshore. Swing the boat parallel to shore and pay the net out. Attach a second warp and return the boat to shore. Draw the seine to shore by hand or draft engine using the two warps.

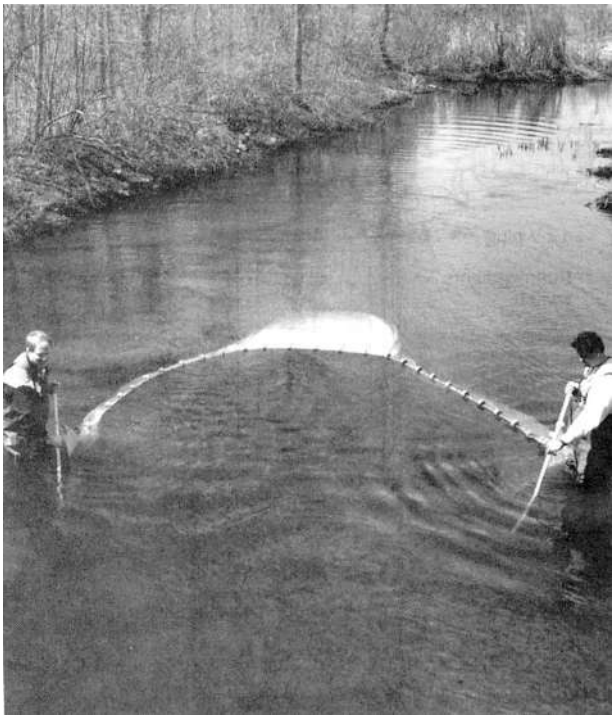


Figure 10600:3. Bag seine in operation in a stream. Photo courtesy of New York State Museum.

A block seine may be used to block the mouth of an embayment. This sampling technique is effective when water elevations change. A flume net is a refinement of this technique for use in marshes and tidal creeks. One method of deploying a flume net is to place parallel walls of netting perpendicular to the marsh creek axis from the creek bank into the high marsh.[16] The net is set at high tide with the cod end staked out into the creek to provide refuge for fishes leaving the marsh at ebb tide. Samples are removed from the cod end when the marsh drains.

A self-contained block net is the closing seine consisting of a circular panel of netting strung on a weighted bottom ring and a floating upper ring. Drop the device onto the bottom enclosing the fishes and remove them with a dip net. The technique is quantitative to a degree but emplacement of the gear can cause fishes to leave the study area.

A commercial purse seine is a larger version of the closing net; it is used in deeper water to capture schooling fish. The lower edge is equipped with a pursing warp (line) that allows the net to be drawn together under the surrounded school. After closure, the fishes are dip netted or hydraulically removed.

o. Lift and dip nets: These nets may be operated by a single worker. The lift net consists of a square panel of net held open by a pair of diagonal braces; it is lifted at the crossing point by a cord. Such nets are sometimes baited and are lifted when enough fishes have been gathered.

Dip or pole nets are conical nets attached to a ring frame, which in turn is attached to a pole. They are effective in capturing salmonids, smelt, and various clupeids (e.g., blueback herring and alewife).

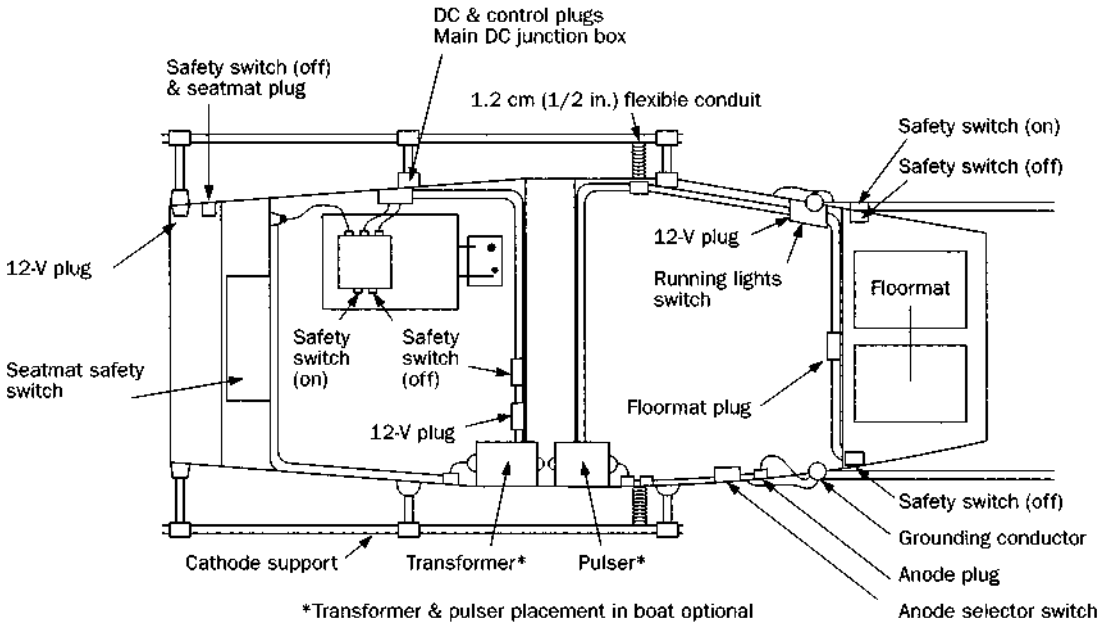


Figure 10600:4. Diagram of electrofishing boat. SOURCE: NOVOTNY, D.W. & G.R. PRIEGEL. 1974. Electrofishing Boats, Improved Designs and Operational Guidelines to Increase the Effectiveness of Boom Shockers; Tech. Bull. No. 73. Dept. Natural Resources, Madison, Wis.

p. Goin dredge: A Goin dredge is a wooden box with a net bottom and one missing wall. Handles or hand holds on the three remaining sides are used to work the device through vegetation.

q. Electrofishing: Electrical current-generating devices also may be used to collect fishes.[17–19] They are particularly useful in areas where uneven bottoms, fast-flowing water, or obstructions make other collection techniques difficult or impossible. The selectivity of electrofishing operations is affected by such factors as water depth and velocity, stream width, the water's conductivity, fish species, fish size, fish morphology, and fish behavior. In this method, an electrical field is produced in the water by passing a current between two submersed electrodes or between one electrode and the ground. Depending on their design, the electrical devices produce either alternating current (AC) or direct current (DC). AC stuns the fishes in its field, allowing them to be dipped from the water, while DC induces galvanotaxis, so fishes move toward one of the poles, from where they are recovered. DC devices are particularly effective in waters that are turbid or have numerous obstructions or heavy vegetation. AC devices are more likely to kill fish. Electrofishing's effectiveness is affected by such environmental factors as water hardness and availability of electrolytes. It may be necessary to add salt to very soft water to raise its conductivity. Effectiveness also increases with fish size.

Electrofishing gear ranges from large, gasoline-motor rigs mounted in a boat (Figure 10600:4) to small, portable backpack units powered by a gasoline motor or a battery. The electric seine consists of a conventional seine in which electrodes of an AC source run along the lead and float lines. **(CAUTION: Be aware of the special dangers associated with this technique. Specially train staff via courses approved by the National Marine Fisheries Service or other fish-management agencies, provide and use protective equipment, and carefully monitor the sampling area before and during the operation.)**

In certain studies (e.g., when assessing stream fish production by habitat type), electrofishing can provide a quantitative sample. In streams with manageable size and flow, sampling areas can be isolated with block nets, allowing capture and processing of most fishes in the area. Also, fish mortality can be carefully controlled. Keep captured fishes in live cans (containers, on occasion with aeration and refrigeration capabilities) during sampling and return immediately after processing.

r. Ichthyocides: Ichthyocides (piscicides) are the least selective collection method but are the most efficient in terms of percentage of total population sampled.[20] They may act as a metabolic poison, a vasoconstrictor (suffocant), or an anesthetic. Most have some serious fault as a fish-collecting tool (e.g., expensive, slow reaction time, only kills at high doses, or deleterious side effects to either the environment or user). Rigorous, time-consuming permitting procedures usually are required. In some places, ichthyocide use is illegal; review ordinances. Historically, cresol, sodium cyanide, sodium hypochlorite, antimycin A and B, quinaldine, tricaine methanosulfonate (MS-222), urethane, and rotenone have been used; of these, only antimycin and rotenone are currently approved for use by the U.S. Environmental Protection Agency.

Widely used as a general collecting tool, *rotenone* is the commercial name of a crystalline ketone ($C_{23}H_{22}O_6$) found in six genera of leguminous plants, particularly in the genus *Derris*. Rotenone is a vasoconstrictor, literally suffocating fishes; it selects for gill-breathing organisms. Fishes collected via rotenone can be eaten, but it is not recommended. It is rapidly detoxified by potassium permanganate, and rapidly breaks down when subjected to light and high temperatures.

Rotenone is available as powder, resin, crystals, or liquid emulsions; crystals are the recommended form. Powdered preparations are the least stable; resins and crystalline products are the most stable. A ready-to-use liquid emulsion is the most

convenient, but relatively expensive. If weight and space problems exist, use crystals or resins.

Rotenone should be used by properly trained personnel. A 5% preparation is the usual strength for collecting fish. Mix resins and crystals with solvents immediately before use. For the resins, mix 100 g fragmented rotenone (broken up before mixing) with 1 L commercial-grade acetone and 100 mL emulsifier. For the crystals, mix 20 g with 3.8 L acetone. The amount of rotenone to be used per collecting station depends on water volume and current, the toxicant's reaction time, water temperature, etc. A sample bioassay may be useful in determining dosage. In general, be conservative because good-quality rotenone is surprisingly effective.

s. Concussion: Concussive methods (e.g., explosive devices and substances) have been used, but most states and the federal government prohibits them. A simple concussive method called *tunking* (*stoning*) consists of striking an emergent boulder with another rock and then turning the boulder over to collect the fishes underneath it. Sculpins and minnows may be captured in this manner.

t. Creel census: Systematically collecting data from sport anglers is a primary means of analysis. A common approach is a field examination of fishes caught, coupled with standard interviews of the anglers. Another method is distributing fishing diaries among anglers and systematically retrieving them at a later date. Statistically rigorous sampling methods and well-designed interview protocols are essential for generating reliable results.

u. Slurp gun: The slurp gun consists of a valved cylinder fitted with a plunger. It is used while SCUBA diving or snorkeling and is especially effective for selectively sampling fish at nesting sites or within otherwise inaccessible interstices.

v. Stomach and gut examination: The contents of fishes' digestive tracts often include examples of additional fish species not otherwise sampled.

w. Serendipity: Useful specimens can be collected after various kinds of fish mortality events. The release of toxic substances, lethal changes in water temperature, anoxic water, construction of cofferdams, dewatering of power-plant flumes, drying up of natural water bodies, and the stranding of fishes after floods exemplify serendipitous events that can yield study materials. Some states have laws prohibiting recovery of fish from catastrophic kill events.

x. Acoustic methods: Acoustic means of fish detection and quantification [e.g., single-beam, multi-beam, and dual-frequency identification (DIDSON) SONAR] involve a series of sound pulses generated by an electroacoustic transducer mounted on a vessel, towed body, or fixed land feature.[21] Vertical and side or horizontal scanning are widely used. For side scanning, one or more transducers are mounted at some depth and oriented horizontally (e.g., on the piers of a bridge crossing a river or on the walls or floor of a lock chamber). The sound impulse is reflected by suspended objects and the bottom. It is received by the same transducer, which reconverts the sound signal into an electrical signal, which is amplified and displayed and/or recorded on a paper tape, video cassette recorder (VCR) tape, liquid display, photographic film, computer file, etc. The printout of the SONAR record provides a profile of the water showing the bottom and the objects suspended above it.

Objects with high "acoustic impedance" (e.g., fishes with air bladders) are especially vivid targets and produce strong signals. Gas bubbles, plankton, particulate, and density differences associated with a thermocline, halocline, floating leaves or seagrass blades, and other objects may produce acoustic traces. In a typical recording, a single, relatively stable fish will produce a chevron-shaped "echogram," with the middle point of the chevron pointing upwards. Several fish usually can be resolved into a corresponding number of such figures, but a school of fish may produce a cloud-like mark, complicating analysis. Under these circumstances, analysts usually estimate biomass.

Verify identity of recorded species via capture or direct observation (e.g., SCUBA). Weighing captured specimens permits extrapolation to biomass estimation.

Apart from quantification, SONAR methods may provide high-quality data on the vertical and horizontal location of fish stocks.

y. Snorkeling and SCUBA: Snorkeling is the process of using a "J"-shaped breathing tube and a transparent faceplate for sustained observation of underwater conditions. A SCUBA consists of a compressed-air tank, a gas-flow regulator, a hose, and a mouthpiece. Ancillary but crucial gear includes a transparent faceplate, a weight belt for adjusting buoyancy, fins, an inflatable life vest, and either a wetsuit or a dry suit.

With good training, snorkeling and SCUBA gear make underwater depths <15 m accessible. For greater depths, more training and experience are necessary.

Underwater study permits precise gear placement, direct observation of gear function, rapid determination of species' resource use, assessment of a chemical spill's effects in deeper water (when dead and moribund specimens may be resting on the bottom), study of fish behavior, and population estimates. A team of divers can swim side-by-side along a defined course (transect) noting fishes present and recording their observations via underwater recording materials. An underwater pad made of sanded vinyl plastic can be marked with a conventional graphite pencil and erased with a conventional rubber eraser. Aluminum foil taped onto a plastic sheet also can be marked with a pencil; it is effective and provides a permanent record.

z. Water intakes: Collecting fish at water intakes is a simple, cost-effective means of procuring fish specimens.[22] Large intakes (e.g., those providing cooling water for electricity-generating stations) typically have onshore screening structures that prevent fish and other items from entering the mechanical system. Screened fish may be returned to the waterbody or disposed, but in either case, they can be intercepted via a dipnet or other suitable collection device. Seek permission from site operators and wear appropriate protective clothing, including a hard hat.

The advantages of water intakes include the large volume of water typically withdrawn and the ability to standardize fish catch per volume of water diverted, allowing comparisons of density changes over time.[23] Although water intakes are fixed locations, their presence in tidal areas may provide an opportunity to obtain a reasonable representation of the fish assemblage within the tidal excursion, depending on species mobility.[24]

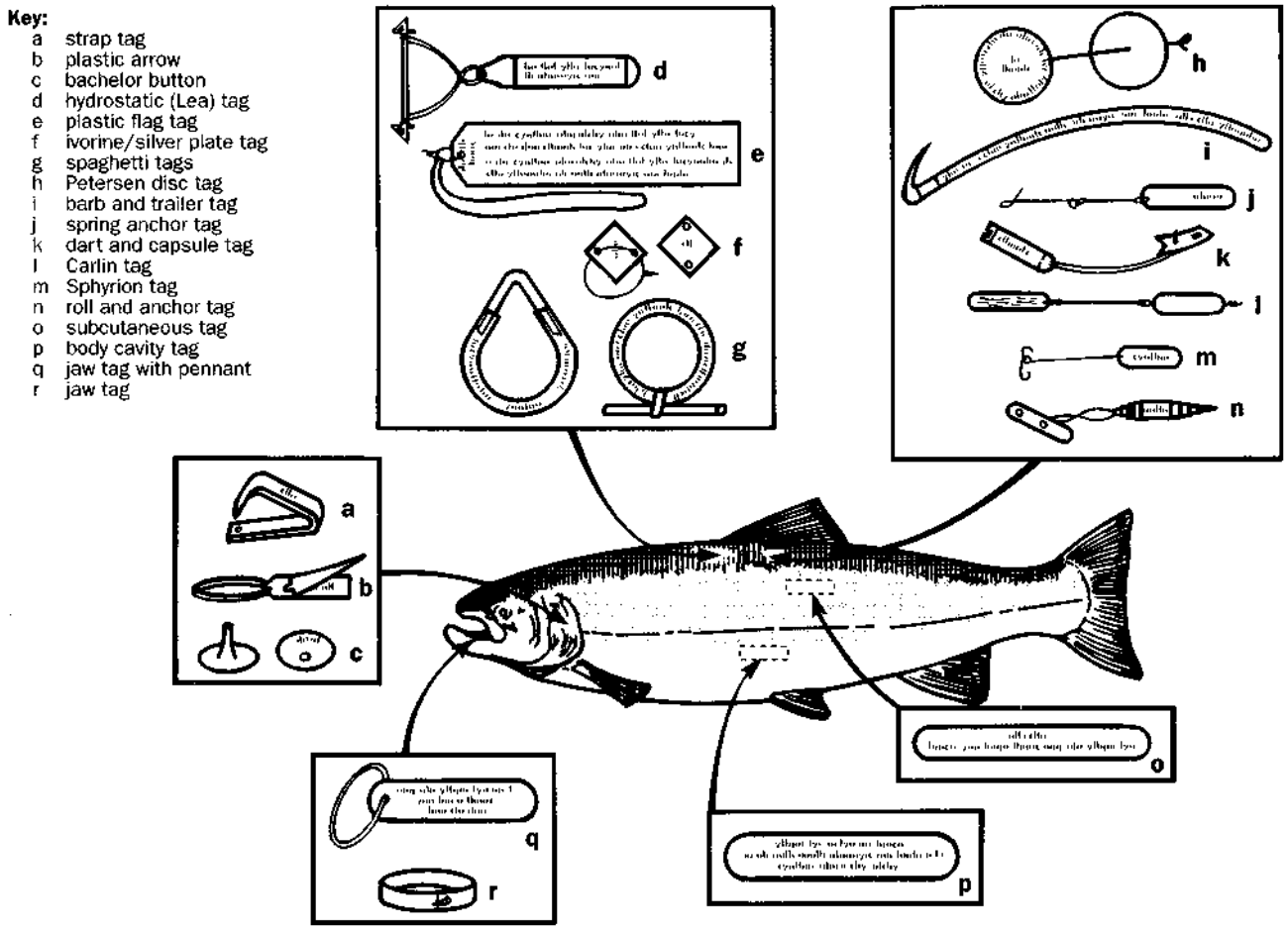


Figure 10600:5. Types of tags commonly used. SOURCE: STOTT, B. 1971. Marking and tagging. *In* T. Bagenal, ed. 1978. Methods for Assessment of Fish Production in Fresh Waters; IBP Handbook No. 3. Blackwell Scientific Publ., Oxford, England.

Fish collected at water intakes tend to be smaller individuals that are less able to resist entrainment. More fish may be col-lected at night or in turbid water because they cannot see the intake and escape from it. These are important considerations when comparing density data for different times or locations.

4. Tags and Tagging

Marking a fish or group of fish, releasing them, and later recapturing them provides information on movement, rates of growth, and population characteristics. Marking methods include dyes and stains (including fluorescent materials), fin clips, tags (Figure 10600:5) , encoded wires, sonic (transmitter) tags, passive integrated transponders (PITs), brands, radioisotopes, and marker chemicals that can be detected easily or influence ageable features (e.g., scales, otoliths, or spines). Chemical marking may be restricted under certain circumstances. Beware of the marker's influence on the fish. For example, jaw tags can suppress growth rate by interfering with feeding; brightly colored tags may make tagged fish more susceptible to predation; large tags may impair swimming or entangle with plants or other objects; infection may occur. Marking is an important tool because it permits analysts to identify genetic strains of hatchery stocks, observe the behavior of individual fish submitted to particular chemicals or other stressors, observe dominance and social rank, etc.

PIT is an innovation with considerable value (Figure 10600:6) .[25] Small (10-mm × 2.1-mm) glass-imbedded electrical units (called *PIT tags*) may be injected into the abdominal cavity or under a fish's epidermis via a syringe-like device. They can be used on fish that are 5 cm or larger. The tag is activated by a handheld excitatory unit that elicits a distinctive signal, permitting recognition of individual fish and immediate data processing via computer. The specimen may be processed with little or no handling via a water vessel or flow-through cylinder (similar to migratory salmon moving through a weir) during tag excitation. The tags last for several years. Salmon smolt can be tagged and individually recognized years later when the adult fish return for spawning. Coded wire tags (CWT) also have been used extensively but require excision of the wire (and, therefore, fish sacrifice) for identification. Visual tags in the form of alphanumeric implants or colored elastomer injections can be used on translucent body parts. Tag loss needs to be assessed for each type of tag used in each situation.

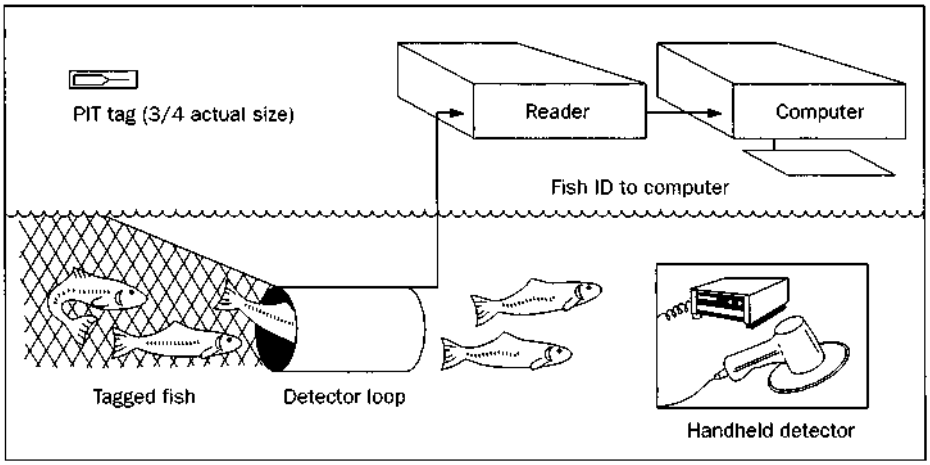


Figure 10600:6. Passive integrated transponder (PIT) tagging system. SOURCE: BIOSONICS, INC., Seattle, Wash.

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10600 C. Sample Preservation

The decision to preserve specimens depends on study objectives. Preserved material may be necessary to confirm a species' identity, to evaluate certain demographic characteristics, to estimate age based on hard parts (otoliths, vertebrae, fin spines, etc.), to analyze diet, or to estimate incidence of parasitic infection or disease. It also may be essential evidence in legal proceedings.

Do not preserve specimens unless there is clear need to do so. Fixatives are toxic, dangerous if used improperly, and subject to hazardous material regulations. Preserved specimens require expensive, time-consuming curation.

Fix specimens in 10% formalin (a 9:1 ambient water dilution of 100% formalin). Fix fishes <10 cm long (total) without opening the visceral cavity. Larger specimens require that preservative either be injected directly into the visceral cavity or that the right ventral body wall be slitted for about 25% of body length before fixing to permit 10% formalin to penetrate specimen. Specimens >25 cm long (especially oily species) usually require injection of concentrated formalin into the dorsal muscle mass.

The placement of fish in the sample container (i.e., head up or down) depends on intended use. A good ratio of specimen mass to preservative is 1:1, or with the level of preservative submerging the specimen by at least an inch.

Fix ichthyoplankton in 5 to 10% formalin. To facilitate sorting, 1 g rose bengal stain/L fixative may be added to stain living tissue. If the sample contains a large amount of biomass (detritus, non-target organisms), then split the sample into two or more sample jars rather than increase the concentration of formalin.

Ideally, use wide-mouthed containers with a durable, screw-type plastic cover. If using metal caps, add about 1 g sodium borate/L to preserved material.

Formaldehyde is highly allergenic and a listed carcinogen; minimize direct contact with skin and avoid breathing fumes. Formaldehyde is best transported in tightly sealed plastic containers.

After several days to 2 or more weeks in the fixative (depending on fish size), transfer specimens to 70% ethyl or isopropyl alcohol for long-term preservation. To reduce shrinkage, distortion, and fin brittleness, lower concentrations of isopropyl alcohol (e.g., 50%) can be used if the storage containers are airtight. A 3 to 5% solution of formalin is a good long-term preservative for ichthyoplankton. If possible, reuse formalin.

It may be harmful and/or illegal to release even small amounts of formalin into wastewater-collection systems. Examine ordinances before disposing of fixatives and preservatives.

Isopropyl alcohol is a less expensive, less flammable substitute for ethyl alcohol, but isopropyl is not a good fixative and may damage the specimens for histology. Both alcohols are highly flammable when stored in bulk. Quantities stored in any building may be limited by codes. If otoliths will be used to determine fish age, do not fix specimens in formalin until

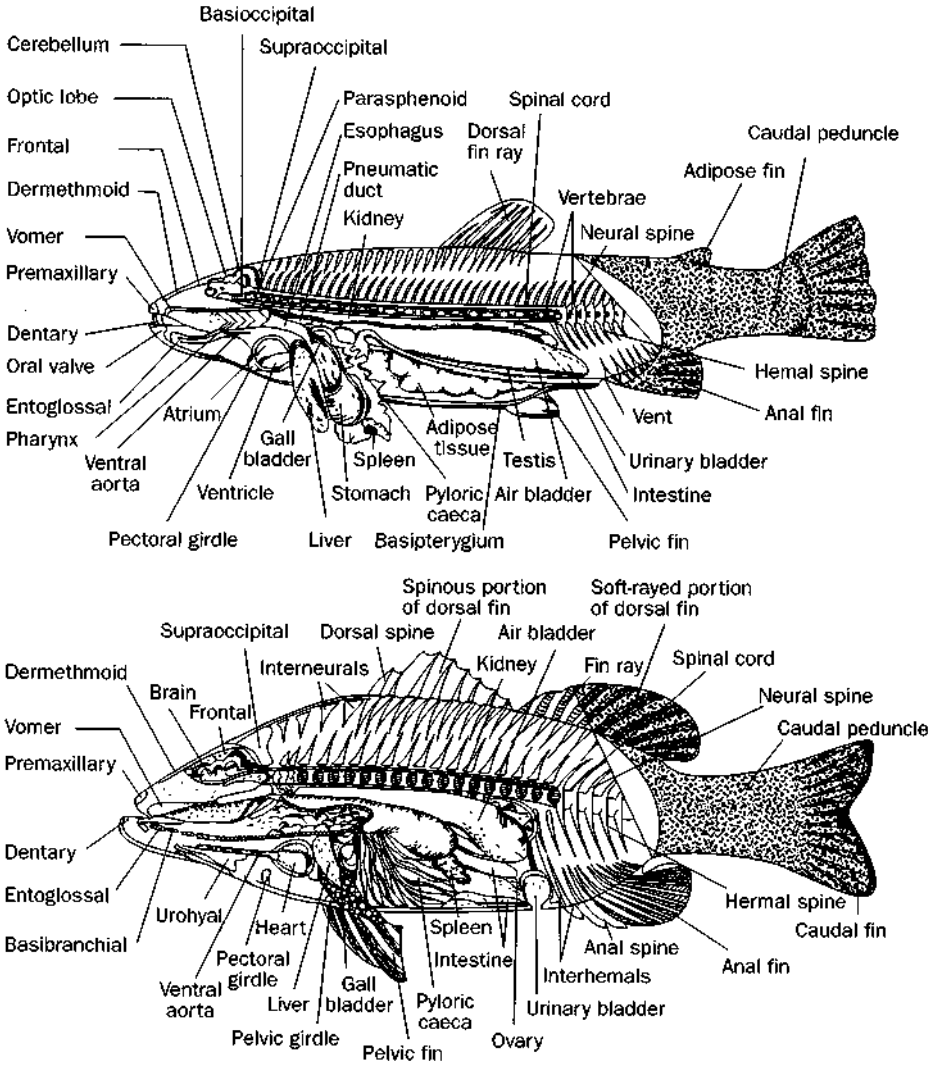


Figure 10600:7. Key organs and external body parts of a soft-rayed (upper) and spiny-rayed (lower) fish. SOURCE: LAGLER, K.F. 1956. Freshwater Fishery Biology, 2nd ed. Wm. C. Brown Co., Dubuque, Iowa.

after the otoliths are removed. If otoliths cannot be removed before long-term preservation, then either fix the specimens in 95% ethyl alcohol (adequate for small fish) or freeze them.

For pathology, fix whole fishes or organs for at least 24 h in neutral buffered formalin (volume = 10 times the volume of the fish or organ) before further processing:

37% Formaldehyde (100% formalin)	100	mL
Distilled water	900	mL
Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$)	4	g
Sodium phosphate dibasic (Na_2HPO_4)	6.5	g

When investigating contaminants, fish must not contact decks or other surfaces and should be bagged separately in plastic bags that do not transfer contaminants among adjacent samples while in storage. If fish is <8 cm long, then fix whole. For larger specimens, fix viscera by either injecting fixative into the body cavity or cutting body cavity from anus to below the head before placing in fixative. Fixation is most effective on live fishes or as soon after death as possible. A delay results in autolysis. In larger fishes that cannot be preserved whole, preserve representative sections of organs about 5 × 5 × 2 mm in size. Use a scalpel or razor blade to obtain such tissue blocks from organ areas with irregular color, size, or consistency, and fix them immediately. Except in large fish, fix the entire brain. See Figure 10600:7 for morphological and anatomical guidance.

Rigorously document preserved materials. Using labels made of highly resistant bond paper, note the lot number, collection date, locality, name(s) of collector(s), and other particulars in graphite or waterproof ink. Place labels inside each container or attach to larger specimens held in plastic bags.

When shipping fixed specimens, pack them in absorbent paper or cloth that has been lightly moistened with the fixative or preservative, seal them in a plastic bag, and then seal the bag in a second (and possibly third) bag. Pad the package and

place both it and a copy of the chain-of-custody documents in a box or canister for transport. NOTE: It is illegal to ship items in bulk fixative or preservative via the U.S. mails.

If preserved specimens are no longer needed once the study is completed, offer them to a regional or national museum. Planning for eventual disposal of specimens is both economical and provides for the best long-term use of the collected materials.

10600 D. Analysis of Collections

1. Identification

a. General remarks: Fishes are identified based on such diagnostic characteristics as body form, color and size, pigmentation patterns, shape and position of fins, dentition, meristic features (e.g., number of rays in a fin or number of scales in a specific series), the presence of distinctive organs (e.g., barbels), or the lateral line and various proportions (e.g., ratio of head length to total body length) (Figure 10600:7). Such characteristics may vary with age, sex, reproductive condition, social status, time of year, and habitat.

Diagnostic keys and other descriptive materials are available for all regions of North America, and a list of selected taxonomic works appears in the bibliography. The common names of North American fishes are listed in a special publication of The American Fisheries Society.[1]

Both fresh and preserved specimens may be identified. Fresh materials are essential for color, although some preserved specimens retain color if a color preservative [e.g., butylated hydroxytoluene (BHT)] was used. Fixed specimens are suitable for determining meristic or mensural characteristics. Use a dissecting binocular microscope with illumination and dissecting tools to examine specimens <10 cm long.

b. Ichthyoplankton: Identifying fish eggs and larval fishes is a special discipline, and selected works are cited in the bibliography. Enumeration often involves knowledge of adult species presence/absence in the survey area, spawning season, and spawning migration. Intensive studies may involve captive spawning, rearing, and documentation of larval development of undescribed species.

c. Rare and endangered forms: Pay special attention to rare and endangered forms that are protected by law. If a rare or endangered form is present, a special permit or memorandum of understanding may be required. Do not intentionally collect rare or endangered forms; if they are accidentally taken and fixed, contact the responsible agency and transfer specimens to a designated museum.

2. Diet

The contents of the digestive tract provide information on the amount and kind of foods eaten. Stomach contents may be extracted from some living fishes by inserting a smooth, moistened glass or metal cylinder or other irrigation device down the esophagus into the stomach and irrigating the stomach contents into a container. Most analyses, however, involve sacrificing the fish. After capture, quickly preserve either the entire fish or the viscera as described above.

To characterize diet, begin by suspending organisms in water in a shallow transparent container or, in the case of microphagous species (e.g., small plantkivorous fishes), distributing ingested organisms in glycerol on a microscope slide and then cover it with a cover slip. Use a microscope of appropriate magnification to identify contents to the lowest practical taxon and express as frequency of occurrence, number (if possible), and bulk (mass or volume). Ingested items (e.g., detritus, grazed algae, and disarticulated small prey) typically cannot be enumerated. Before obtaining wet weights for ingested items, blot them to remove excess water. Ingested-item volume may be estimated via displacement in a graduated cylinder or via calculation using measured dimensions and the appropriate geometric models (e.g., sphere, ellipsoid, cylinder). Volume can be converted to mass if the item's mass density is known.

3. Structure of Populations and Assemblages

a. General remarks: A population's actual properties (e.g., number, average size, and weight) are estimated statistics.[2] For ecological and management purposes, evaluate the numbers of individuals and the biomass (total weight), along with the factors that regulate these. Four key variables define number: natality (number of individuals being added via reproduction), mortality, immigration, and emigration.

Biomass is the net result of dietary ingestion and its conversion efficiency, respiration, defecation, and loss to other compartments of the ecosystem (e.g., predators, parasites, and pathogens).

The ideal static characterization of a population consists of graphs that show by sex:

- age frequency and maturation,
- length frequency,
- weight frequency,
- age versus length,
- age versus weight, and
- length versus weight.

b. Population size: One of the simplest, most practical population-estimation methods is the Petersen ratio based on marked fish. Collect a sample of fish, mark, and release. Then, collect a second (recapture) sample, which includes both marked and unmarked fish. The method assumes random distribution and no immigration or emigration. The larger the number of marked fish, the more reliable the estimate. It is particularly important to mark large numbers of smaller species, if possible, because individuals of such species are less easily recovered. Estimate

population size using the mark–recapture method by the formula:

$$\hat{N} = MC/R$$

where:

$\hat{N}$ = estimate of population size,
M = number of fish marked and released,
C = number of recaptured fish, including both marked and unmarked individuals, and
R = number of marked fish recaptured.

This equation estimates the population size at the time of the first mark-and-release sample, not at the time of recapture.

c. Age and growth: Age and growth rates are useful for determining environmental effects on fish populations. Three methods used to determine fish age are comparing length-frequency, recovering marked fishes of known age, and interpreting layers laid down on hard parts of the fish. Using length-frequency to determine age distribution often is adequate for the first 2 to 4 years of fish life, but usually fails to separate older fish reliably because there is more overlap in length distribution. It also becomes less reliable as one approaches the equator or the poles because breeding seasons are more protracted in warmer areas (making yearly age groups less well defined) and growth rates are often retarded in cold areas (causing age groups to overlap, even in young fish).

Another frequently used method of determining age is interpreting and counting growth zones (growth checks) that appear in the hard parts of fish. Those assumed to be formed once a year are called *year marks, annual marks, annual rings,* or *annuli* (Figure 10600:8); this assumption requires validation.[3,4] The marks form during alternate periods of faster and slower growth (or no growth at all) and reflect various environmental or internal influences. In a temperate region, the period of little or no growth usually occurs only once annually, beginning in winter and extending into spring or early summer. Generally, the more seasons differ with respect to temperature, the sharper the annual marks will be. The most distinct annual rings are developed in temperate climates of the northern and southern hemispheres. Scales and several bony structures have been of value when studying seasonal growth. Otoliths provide the most reliable record of age and growth, but require that the fish be sacrificed.

Still another method of age and growth determination involves marking or tagging fish, releasing them, and later recapturing them. Most tagging methods are not applicable to small fish, or if they are, they may cause mortality, so recaptures are few. Instead of using tags, small fishes may be marked by fin clipping, fin-ray scarring, or injecting dye.

Scales are often used in age determinations to avoid injuring fish. If the fish are scaleless, other structures (otoliths, vertebrae, fin spines) may need to be removed. Take scales from the upper mid-side of the body, where they are large and symmetrical. When taking scales, remove them from the same part of the body in all individuals to be compared. Several scales may be needed for analysis, because an annulus that appears doubtful on one scale may be clear on another. In addition, some scales may have regenerated (i.e., been replaced) and may not show all annuli.

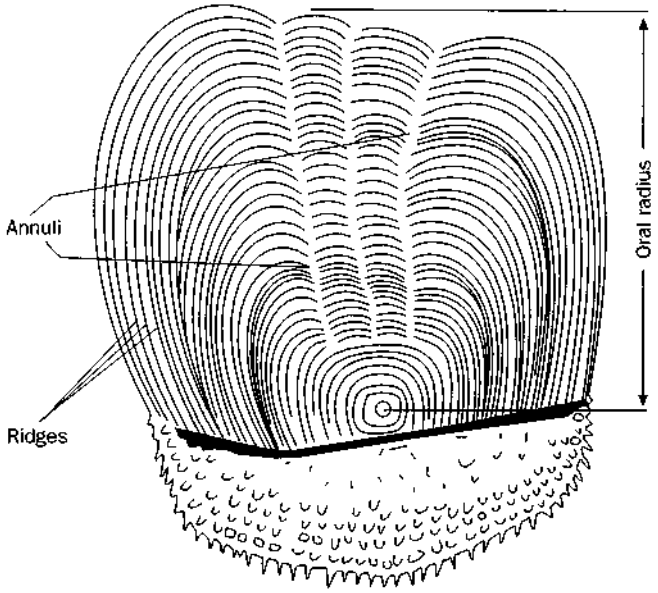


Figure 10600:8. Fish scale. SOURCE: BAGENAL, T., ed. 1978. Methods for Assessment of Fish Production in Fresh Waters; IBP Handbook No. 3. Blackwell Scientific Publ., Oxford, England.

d. Index of condition: The coefficient or *index of condition* (also called *condition factor*) is the length–weight relationship used to express the fish's relative robustness, which is related to environmental conditions. The equation usually used is:

$$K = \frac{(W \times 10^5)}{L^3}$$

where:

K = coefficient or index of condition,
W = weight, g, and
L = length, mm.

Residual deviations from weight–length curves or other defined weight standards may also be used. The *gonadosomatic index* (GSI)—weight of gonads divided by the remaining body weight—is an indicator of spawning preparedness. The *hepatosomatic index* (HSI)—weight of liver divided by the remaining body weight—is an indicator of physiological condition. Be aware of inherent nonlinearities when using condition indices.

e. Assemblage structure: Diversity indices are used to quantify the structure of the species assemblage in a particular habitat over time.[5] Usually, the number of species represented and the relative numbers of each species represented are incorporated into a single number. Margalef's index is illustrative:

$$d = \frac{(S - 1)}{\ln N}$$

where:

d = Margalef index,
S = number of species represented in sample, and
N = total number of individuals in the sample.

The index of biotic integrity (IBI) is another method for assessing community structure. It produces a score based on a composite of indicator metrics for each sample site that allows assessment of environmental conditions at that site by comparing it to scores from other sites. It is a method for comparing changes in environmental conditions at a single site over time or at several sites within a carefully defined geographical area. Reference conditions need to be defined.

The IBI score is generated by using several carefully selected metrics that reflect different aspects of the aquatic system. Typically there are twelve metrics, which fall into three broad categories. The first group assesses the composition of the fish assemblage. These metrics explore the relationship between the number of species and the number of individuals within each species. The second group examines the trophic composition of the assemblage. To score these metrics, some knowledge of the fishes' feeding ecology is required. The third group looks at fish abundance and condition. These metrics deal with the number of individuals caught and their health.

Once the metrics are identified, they are weighted to develop the scoring criteria. Scores are based on the deviation from conditions at a relatively undisturbed reference site. Typically, there are three choices: a high score (5) is assigned if the condition is equal to that found at a similar but undisturbed site (key), a middle score (3) if the condition is worse than that found at the key site, and a low score (1) if conditions are much worse than those at the key site.

The total IBI score is the sum of the metric scores. In the example above, the key site would have an IBI score of 60 (12 metrics, maximum metric score of 5). The scores from all sampled sites would be compared to this maximum. Sites where environmental conditions are relatively undisturbed would score near the maximum, while degraded sites would score near the minimum of 12 (12 metrics, minimum score of 1). IBI's greatest advantage is that comparisons are easy and results are obvious.

IBI metrics and scoring criteria are not universal; metrics and scoring protocols have been or must be developed for a geographic region.[6,7]

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10600 E. Investigation of Fish Kills

Apparent fish kills can vary from a single natural mortality to catastrophic partial or total kills due to natural occurrence or human activity. No single investigative procedure can be appropriate for all situations. The following brief description may serve as an aid in investigating kills. It is vital to get to the scene promptly, before evidence has decomposed or drifted away. If surveillance of a particular body of water or area is involved, have available preset plans and equipment on standby.[1–3]

1. Causes of Fish Kills

Fish kills may be caused by such natural events as acute temperature change, storms, ice and snow cover, decomposition of natural materials causing hypoxia, salinity change, spawning mortalities, parasites, and bacterial and viral epizootics. Human-caused fish kills may be attributed to municipal or industrial wastes, agricultural activities, and water-control activities.

2. Classification of Kills

Although one dead fish in a stream is technically a fish kill, practically speaking, some minimal range in number of dead fish observed should be adopted, plus additional qualifications, in reporting and classifying fish kills. Any fish kill is significant if it affects fishes with sport or commercial value, results from a suspected negligent discharge or malfunctioning waste treatment facility, causes widespread environmental damage, or substantially changes the water body's community structure. The following definitions, based on a stream about 60 m wide and 2 m deep, are suggested as guidelines. For other size streams, lakes, and coastal waterbodies, make proportional adjustments.

a. Minor kill: 1 to 100 dead or dying fishes confined to a small area or stream stretch. If recurrent, it could be significant; investigate.

b. Moderate kill: 100 to 1000 dead or dying fishes of various species in 1 to 2 km of stream or equivalent area of a lake or estuary.

c. Major kill: 1000 or more dead or dying fishes of many species in a reach of stream up to 16 km in length or greater, or equivalent area of a lake or coastal waterbody.

3. Investigation Techniques

When preparing for a field investigation, study maps of the area to determine the fish-mortality zone and access to it. Identify waste discharges and investigate their potential sources. Contact participating laboratories to discuss the number and size of samples that will be submitted, types of analyses required, dates of sample receipt, sample-shipment method, deadline for results, and to whom results will be reported. Use two information record forms for fish-kill investigations: an initial contact form and a field investigation form.

On all fish-kill investigations, take a thermometer, DO test kit or meter, conductivity and pH meters, a general chemical kit, biological sampling gear, sample bottles, fixatives, and other specimen containers. Take a camera to provide a photographic record of the event. Include in the investigating team at least one person who is experienced in investigating fish kills.

The field investigation consists of visual observations; sampling of fish, water, and other biota; and physical measurements of the environment. The first local observer of the kill is a useful guide to the area, which should be reconnoitered initially to establish that a fish kill actually has occurred.

If a fish kill has taken place, immediately start sampling fish because collecting dying or recently dead fishes is critical. Moribund fish usually are preferred. For purposes of comparison, if possible, collect healthy fishes from an unaffected area.

Immediately examine moribund or recently dead animals for external and internal abnormalities. Record all changes in color, size, location, and consistency of organs. Record the location of lesions by organ (Figure 10600:7). Color photographs, with indication of scale, taken onsite are an excellent way of documenting observations and greatly help the consulting pathologist. If photography is impossible, describe exactly what was seen, recording the number and size of abnormalities. Provide healthy fish of the same species when possible.

Do not freeze samples for pathology. If fixatives are unavailable, place samples in plastic bags on ice and rush to the pathologist.

If virology or bacteriology testing is indicated, freeze additional specimens of key organs (e.g., the liver, kidney, spleen, heart, and brain and other parts showing abnormality or lesions), label, and forward for analysis.

Bleed dying fish at collection time to obtain at least 1 mL of blood. Collect a blood sample in a chemically clean, solvent-washed glass bottle with a TFE-lined screw cap.

Identify and count dead fish. In a large river, count dead fishes from a fixed station (e.g., a bridge) for a fixed period of time. Extrapolate to the total time involved. Alternatively, in a large river or lake, make a shore count and project to entire area of kill. In smaller waterbodies, traverse entire area to enumerate dead fish.

Collect water samples representative of unpolluted and polluted areas, in accordance with Section 10200B. At a minimum, measure temperature, pH, DO, and conductivity. Make additional tests depending on suspected causes of the fish kill. Take samples for examination of plankton, periphyton, macrophytes, and macroinvertebrates.

Record observations on water appearance, streamflow, and weather conditions. Color photographs are valuable in recording conditions.

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10700 BENTHIC MEIOFAUNA*

At least 20 of the currently recognized 34 metazoan phyla are represented in the benthic meiofauna: Porifera, Placozoa, Cnidaria, Ctenophora, Platyhelminthes, Orthonectida, Rhombozoa, Cycliophora, Acanthocephala, Nemertea, Nematomorpha, Gnathostomulida, Kinorhyncha, Loricifera, Nematoda, Rotifera, Gastrotricha, Entoprocta, Priapulida, Pogonophora, Echiura, Sipuncula, Annelida, Arthropoda, Tardigrada, Onychophora, Mollusca, Phoronida, Bryozoa, Brachiopoda, Echinodermata, Chaetognatha, Hemichordata, and Chordata. Most are marine, but 14 of these phyla have members found in freshwater systems: Porifera, Cnidaria, Platyhelminthes, Nemertea, Nematoda, Rotifera, Gastrotricha, Entoprocta, Annelida, Arthropoda (Copepoda, Halacaroidea, Ostracoda, Mystacocarida, Tantulocardia), Tardigrada, Mollusca, Bryozoa, Chordata. The contribution of these metazoans to nutrient cycling and other ecological processes is probably substantial, but the details of such interactions are still poorly understood. Benthic meiofauna have an extreme range of morphological and life-history diversity and have free-living, parasitic, and/or symbiotic trophic habits. Comprehensive information about these taxa is available.[1,2]

*Joint Task Group: 21st Edition—Byron J. Adams (chair).

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10750 NEMATOLOGICAL EXAMINATION*

10750 A. Introduction

1. Occurrence and Impact

Nematodes are unsegmented worms with elongated, cylindrical bodies; they are present worldwide in fresh, brackish, and salt waters, and in soil. A freshwater nematode has been defined as "any nematode species inhabiting either fresh water or non-brackish swampy soil below the water table; hence a species that will not drown in fresh water; a species fitted to utilize oxygen dissolved in fresh water."[1] Nematodes are a food source for other invertebrates, small fish, and fungi, and play a fundamental role in cycling carbon and nitrogen through the benthic ecosystem. Predaceous nematodes from the Aporcelaimidae, Diplogasteridae, Dorylaimidae, and Mononchidae families abound in fresh water, devouring other nematodes, oligochaetes, and other small invertebrates. Their role in ingesting algae and diatoms is less clear; however, algae in the gut can occasionally cause dorylaimids to turn bright amber, yellow, or green.

In recent years, examinations of nematode communities in soil or aquatic sediments have been increasingly used to study many aspects of environmental health.[2] The maturity index (MI) originally was developed to measure the ecological succession status of a terrestrial nematode community[3–5]; the concept was later extended to marine, estuarine, and then freshwater nematodes.[6,7] The role of nematodes in wastewater treatment systems is also increasingly being investigated.[8] Bactivorous freshwater nematodes can ingest human enteric pathogens, which then can survive chlorination inside nematode bodies. These nematodes often appear in large numbers in secondary wastewater effluents and have been used as bioindicators of water quality.[9]

2. Nematode Characterization

The most current and frequently cited classification of nematodes is that of De Ley and Blaxter,[10] which incorporates recent molecular and morphological phylogenetic evidence. The following discussion of nematode morphology follows their classification scheme. The body is pierced by six to eight distinct openings and few to many minute apertures. First is the oral aperture at the apex of the anterior end, followed by two amphids on the head or neck region, the excretory pore (usually near the base of the esophagus), the vulva and anus in females, the cloaca in males, and, on some nematodes, two small pores on the tail called *phasmids*. Cuticular ornamentation, such as engravings, pores, spines, or alae (inflations), also may be present.

The digestive system begins at the anterior end with a stoma, which may be armed with teeth or opposable mandibles, unarmed, or modified to form a hollow spear. In some species, the stoma may be closed at rest; in others, it is open and spacious. An esophagus follows the stoma; it terminates in esophageal glands, which range from prominent in many soil-dwelling plant parasitic nematodes to indistinct in most aquatic nematodes. The intestine extends from the base of the esophageal glands to the rectum, which leads to the anus in females or cloaca in males.

The female gonad is single or paired and consists of an ovary, uterus, and vagina. It exits at the vulva. The male gonad consists of one or two testes, vas efferans, and vas deferens, and exits in the cloaca. Males possess spicules (the male copulatory organ) and their guide, the gubernaculum.

The nervous system consists of a nerve ring encircling the esophagus and its connected ganglia and nerve cells. Many of the peripheral nervous system's sensory receptors—especially the sensilla and amphids—are of taxonomic importance. *Sensilla* (sometimes referred to as *setae*) are cuticle projections with chemosensory, tactile, and/or glandular functions.[11] All nematodes have a circlet of six inner labial sensilla surrounding the opening of the stoma, a second circlet of six outer labial sensilla, and a third circlet of four paralabial sensilla (often referred to as *cephalic sensilla*). Sensilla may be setiform (long and filamentous), papilliform (relatively short), or secondarily absent. When all three circlets are separate, they are described as "6+6+4" in taxonomic literature; when the outer labial and paralabial sensilla are in the same circle, they are described as "6+10." Somatic sensilla may be distributed over the entire body or limited to the cervical or caudal regions.

All nematodes have paired chemosensory amphids that are positioned laterally and open to the external environment in the cephalic or cervical region. The shape of the amphid opening is useful taxonomically, especially at higher taxonomic levels (e.g., the ordinal level) and among aquatic nematodes. There are five basic shapes or types of amphids: cyathiform, spiral, circular, blisterlike, or consisting of a tiny pore. Cyathiform (cup-shaped) amphids are found in the primarily aquatic order Enoplida but also in the generally more terrestrial orders Dorylaimida and Mononchida.[11] Spiral amphids are found in members of Chromadorida and Desmodorida, as well as in some members of Araeolaimida and Plectida.[12] (Spirals of a single turn, loops, or shepherd's crook-shape are considered modifications of the spiraled condition.[11]) Circular amphids are found primarily among members of the order Monhysterida. Blisterlike amphids are found only in the marine order Desmoscolecida. Amphids consisting of a simple pore are found among members of the order Rhabditida, which are free-living soil nematodes and parasites of plants and vertebrates.

Although nematodes lack respiratory and circulatory organs, they tolerate large variations in the levels of salts and other environmental chemicals. They also rely on diffusion to

* Approved by Standard Methods Committee, 2011. Formerly numbered 10550. Joint Task Group: 22nd Edition—Ashleigh B. Smythe.

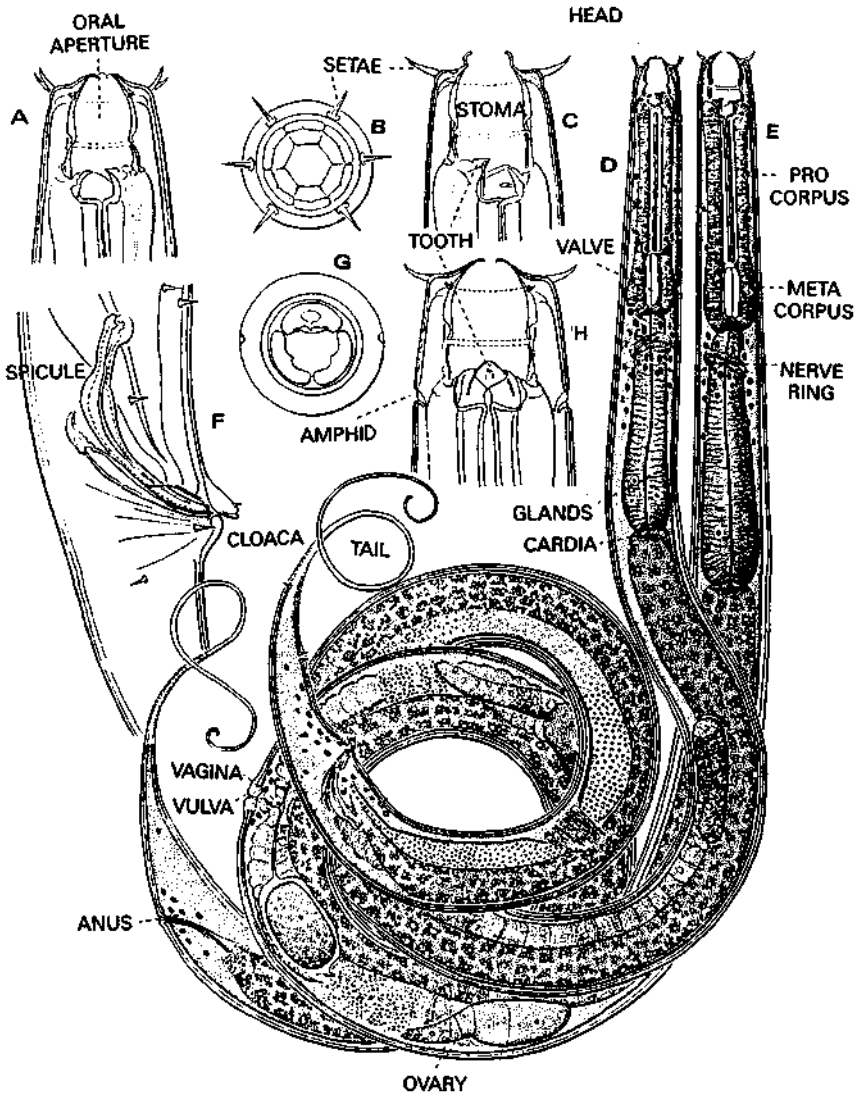


Figure 10750:1. ***Butlerius*** **sp., a freshwater nematode.** A, head; B, *en face* view of head showing six setae and a central oral aperture; C, head lateral view showing stoma and tooth; D, male; E, female; F, male tail portion showing spicules, gubernaculum, and cloaca; G, head section showing amphids; H, ventral view of head showing amphids and tooth. SOURCE: RUSSELL, C.C., Department of Plant Pathology, Oklahoma State University, Stillwater.

translocate nutrients, respiratory gases, and waste products through their tissues. Aerobic metabolism depends on the diffusion of oxygen into their tissues. Most freshwater nematodes have low oxygen requirements; some species' metabolisms may be nearly anaerobic.

While all nematodes can swim, aquatic nematodes are generally found in sediment rather than in the water column. Aquatic nematodes are well adapted to their habitat. Their long, slender bodies are encased by a strong, protective, usually smooth cuticle (skin) inflated by a high-turgor pressure system (Figure 10750:1). Some have a long, filamentous, nonmuscular tail. Tail whipping and undulating sinuous body movement rapidly propels a nematode through water. Many aquatic nematodes have glands in their tails that produce a sticky secretion controlled by a spinneret at the tail tip. These secretions temporarily fasten the nematode to a substrate so it can function without interference by water currents.

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10750 B. Collection and Processing Techniques for Nematodes

1. Samples

Principal samples are tap or well water; free-flowing or standing water without bottom sediment; bottom sediment; and aquatic plants and coarse detritus, such as stones, twigs, or leaves.

a. Sample collection:

1) Tap water—Place a 20-cm, 325 mesh (45-μm pore size) sieve at a 45° angle under the discharge. Adjust water flow to a moderately slow rate, with no splashing, striking upper one-third of sieve. Run for 4 h.

2) Free-flowing or standing water—Take samples from sites where bottom sediments are absent or too deep to be collected. Collect five subsamples as follows: Hold 20-cm, 325 mesh sieve firmly at a 45° angle. Dip 3- to 4-L stainless steel pitcher in water and fill to 1-L mark. Pour contents slowly through top one-third of sieve. Repeat three more times. Collect additional 1 L to wash and concentrate detritus on sieve surface from top to bottom of sieve.

3) Bottom sediment sample—If bottom-to-surface depth is <20 cm, stir bottom with hand garden rake. Scoop up stirred sediments in stainless steel pitcher. Add water to pitcher to within 5 cm of top. Stir, then wait 30 s. Pour contents of pitcher onto an 18 mesh sieve (1-mm pore size) nested in a 325 mesh sieve (45-μm pore size) with the surface held at a 45° angle until dense detritus reaches pitcher lip. (Usually about 9/10 of the pitcher is poured off.)

If bottom-to-surface depth is 20 to 30 cm, collect duplicate samples by holding a 325 mesh sieve (45-μm pore size) at a 90° angle near the bottom. Using hand rake, stir bottom sediments so they roil up in a dense cloud in front of sieve. Let cloud settle about 10 s, then move sieve into cloud about 2.5 to 5 cm above the bottom. Bring sieve out of water while holding it at a 45° angle.

4) Aquatic plants, plant or inorganic debris—Randomly collect one species of live floating or submerged plants from target site and place in 1-L jars filled with collection-site water. Do not fill more than half of jar with plant material. If several plant species are present, take two or more samples. Place plant and inorganic debris (sticks, leaves, pebbles, etc.) in 1-L jar to about half its volume.

b. Sample concentration: Concentrate detritus on sieve into approximately one-quarter of sieve surface by washing tap water across tilted sieve face (45° angle) from top to bottom. Place tilted sieve on lip of a clean, empty 250-mL beaker and wash detritus into beaker by turning sieve upside down and flushing tap water from another beaker or wash bottle through the back side of the sieve—thereby flushing material into the jar.

c. Sample transport and storage: Regardless of collection mode, keep sample jars cool. On very hot days, use ice to cool them. Taxonomic determination is most accurate when nematodes are live and healthy. Because nematode mortality, deterioration, and obfuscation of diagnostic characters begins at time

of collection, process samples for diagnosis within 24 h and complete diagnostic processing within 48 h.

Cold storage retards, but does not halt, deterioration and rot. Plan survey so samples can be processed on the same day they are collected. In an emergency, preserve entire sample indefinitely in 4% formalin (*never* use alcohol). Add equal volume of 8% formalin solution to sample. If sample jar is more than half full, decant excess water after at least 40 min of settling. Preserved specimens will shrink to some degree, and body pores and lumens may become obscure.

2. Sample Processing

For a current review of sample-processing techniques, see Hodda and Eyualem-Abebe.[1]

a. Specialized apparatus:

1) *Custom pipet*, for clean-water samples—Take a 29-cm-long disposable pipet and place a piece of 12-cm-long rubber tube snugly over about 3 cm of the conical pickup end. Add a wire buret clamp on the rubber tube. (Clean by removing clamp and flushing with a syringe.)

2) *Baermann funnel*, for samples containing debris—Use a glass funnel with a 15.5-cm top opening and 1.5-cm tube. Fit a rubber tube to the exit tube and close with a buret wire clamp. Place an 8- to 10-cm-diam, coarse screen (3-mm pores) wire disk in the funnel opening. Add tap water until it lies just above the wire disk. Insert a facial tissue over the disk.

b. Procedure:

1) Clear or relatively clear water—Shake to obtain homogenous mix, then pour slowly onto the surface of a 7.6-cm, 325 mesh sieve (45-μm pore size). Concentrate as indicated in 10750B.1*b*. Pour concentrated residues into 50-mL conical-bottom centrifuge tube or tubes. Let nematodes settle for 40 min. Insert a custom pipet [¶ *a*1) above], with rubber tube closed by finger pressure, to tube bottom. Depress rubber tube to take up the ball of nematodes on bottom of cone. Discharge about 0.05 mL (small drop) of pipet contents onto a microscope slide. Cover drop with a 22-mm cover slip. Use a compound microscope to diagnose nematodes.

2) Samples with much debris—Pour concentrated samples very slowly onto the facial tissue in a Baermann funnel. After 24 h, flush funnel into a 250-mL beaker. Process as directed in ¶ *b*1) above.

3) Samples containing live plants, plant debris, or inorganic material—Process samples immediately on return to laboratory. Shake vigorously and pour contents into beaker. Concentrate samples as directed in 10750B.1*b* and, depending on sample clarity, proceed according to ¶ *b*1) or 2) above. If the laboratory is equipped to process samples with an excess of debris using the centrifugal flotation technique,[2] preferably use this technique.

3. References

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10750 C. Illustrated Key to Freshwater Nematodes

1. General Discussion

The following key was devised for persons trained in biology but not necessarily in nematology. The illustrations include original drawings, photocopies of published drawings, and photocopies on which figures were redrawn. The two most important references were Goodey[1] and Chitwood & Chitwood[2]; other publications used as references and for illustrative material are listed in the bibliography. Other useful, more current keys include a key to subfamilies of commonly occurring marine nematodes (including many estuarine nematodes) by Hope[3] and the keys to many freshwater, terrestrial, and marine nematodes in Eyualem-Abebe, *et al.*[4]

Published literature indicates that several genera in this key contain species predominantly associated with terrestrial habitats. The presence of such nematodes suggests runoff from banks or higher ground in which various plant species (often food sources for these nematodes) are growing. These genera are indicated by an asterisk (*).

2. Key

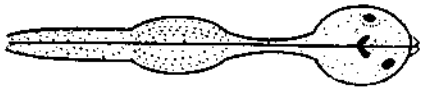
		Refer to Couplet No.
1.	Cephalic setae indistinct or absent	2
	Cephalic setae absent but setae-like head appendages present	64
	Cephalic setae present	69
2.(1)	Stylet present	3
	Stylet absent	38
3.(2)	Base of stylet knobbed or flanged	4
	Stylet knobs or flanges absent	29

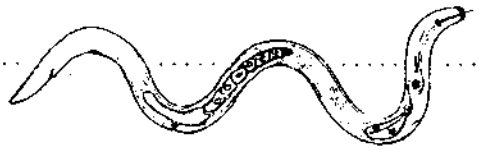
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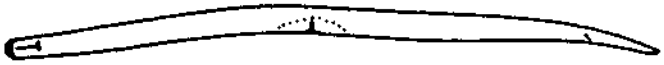
4.(3) Valvate median esophageal bulb present.....  5

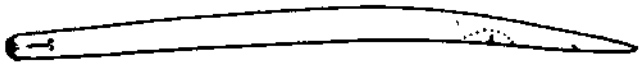
Valvate median esophageal bulb absent 22

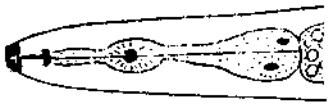
 

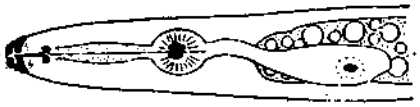
5.(4) Females eel-like.....  6

Females swollen.....  21

6.(5) Vulva at mid-body.....  ... 7

Vulva on lower third of body ....  14

7.(6) Esophagus not overlapping intestine.....  8

Esophagus overlapping intestine.....  11

8.(7) Stylet length less than 50 µm..... 9

Stylet length
greater than 80 µm .....  ..... *Dolichodorus*

9.(8) Tail terminus pointed.....  ..... *Tetylenchus**

Tail terminus not pointed 10

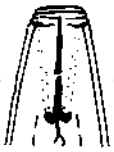
10.(9) Tail terminus knobbed.....  ..... *Psilenchus**

Tail terminus never knobbed or
pointed.....  ..... *Tylenchorhynchus**

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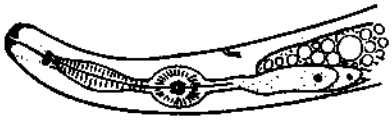
Refer to Couplet No.

11.(7) Labium offset .....  12

Labium flattened, amalgamated or nearly so .....  13

12.(11) Stylet massive, 40–50 μm long .....  ..... *Hoplolaimus**

Stylet long and thin, longer than 90 μm .....  ..... *Belonolaimus**

13.(11) Body 0.5–1.0 mm long, tail tip not mucronate .....  ..... *Radopholus**

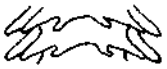
Body 2–3 mm long, tail tip usually mucronate ..... *Hirschmanniella*

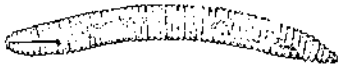
14.(6) Cuticle heavily annulated, stylet elongate .....  15

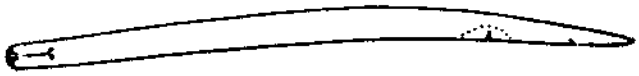
Cuticle not heavily annulated, stylet short .....  ..17

15.(14) Cuticular sheath absent .....  16

Cuticular sheath present .....  ..... *Hemicycliophora*

16.(15) Annules with cuticular spines or scales ..   .. *Criconema**

Annules plain without spines or scales .....   .... *Criconemoides**

17.(14) Body death position straight .....  ...18

Body death position spiral ....  ....  ..... *Helicotylenchus**

		Refer to Couplet No.
18.(17)	Median esophageal bulb distinct but not pronounced	19
	Median esophageal bulb well developed	*Aphelenchoides*
19.(18)	Esophagus overlapping intestine	20
	Esophagus not overlapping intestine	*Tetylenchus**
20.(19)	Median bulb and valves small, stylet usually weak	*Ditylenchus**
	Median bulb, valves and stylet well developed, labium flattened	*Pratylenchus**
21.(5)	Female body soft, white, with few or no internal eggs	*Meloidogyne**
	Female body a rigid brown cyst usually with many internal eggs	*Heterodera**
22.(4)	Stylet short, less than 100 μm	23
	Stylet long, greater than 100 μm	*Xiphinema**
23.(22)	Stylet complex	24
	Stylet simple	25
24.(23)	Stylet with anterior arch-like portion	*Diphtherophora**
	Stylet with dorsal thickening piece	*Tylencholaimellus**
25.(23)	Stylet knobs elongate, flange-like	26
	Stylet knobs round	27

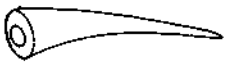
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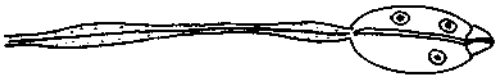
26.(25) Filiform tail .....  ..... *Aulolaimoides*

Round tail .....  ..... *Enchodelus*

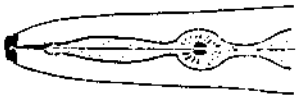
27.(25) Tail rounded .....  28

Tail pointed .....  ..... *Nothotylenchus*

28.(27) Esophagus base elongate .....  ..... *Tylencholoaimus**

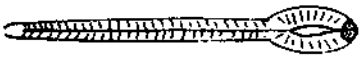
Esophagus base oval .....  ..... *Doryllium*

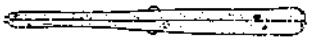
29.(3) Valvate median esophageal bulb absent .....  30

Valvate median esophageal bulb present.....  37

30.(29) Stomal walls not cuticularized .....  31

Stomal walls cuticularized (*Actinolaimus, Metactinolaimus, Paractinolaimus*) .....  .....*Actinolaiminae*

31.(30) Esophagus with basal expansions .....   32

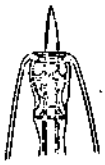
Esophagus expanding uniformly .....   ..... *Oionchus*

32.(31) Terminal fifth or sixth of esophagus an ovoid bulb .....  33

Posterior third of esophagus swollen .....  36

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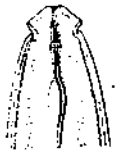
Refer to Couplet No.

33.(32) Stylet axial, positioned centrally .....  34

Stylet not axial, originating from tooth in stoma wall .....  ..... *Campydora**

34.(33) Gonads paired; vulva usually near mid-body .....  35

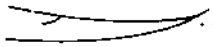
Gonad single, posterior to vulva; vulva anterior to mid-body ..... *Tyleptus**

35.(34) Stylet slender .....  ..... *Leptonchus**

Stylet not slender .....  ..... *Dorylaimoides**

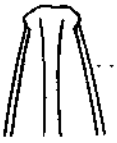
36.(32) Stylet axial, positioned centrally (*Dorylaimus, Eudorylaimus, Labronema, Mesodorylaimus, Thornia, Laimydorus, Prodorylaimus*) .....  ..... Dorylaiminae

Stylet not axial, originating from tooth in stoma wall .....  ..... *Nygolaimus*

37.(29) Tail pointed .....  ..... *Seinura**

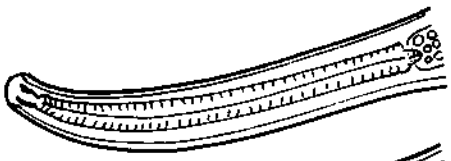
Tail rounded .....  ..... *Aphelenchus**

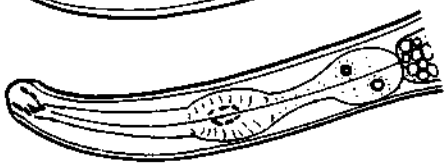
38.(2) Teeth present, prominent .....  39

Teeth absent, minute, or indistinct .....  50

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39.(38) Esophagus without mid-region expansion .....  40

Esophagus expanded at mid-region .....  49

40.(39) Tail pointed or tapering .....  41

Tail rounded .....  47

41.(40) Male tail without setae .....  42

Male tail with setae .....  ..... *Oncholaimus*

42.(41) Stoma with denticles .....  43

Stoma without denticles .....  45

43.(42) Denticles scattered or in longitudinal rows .....  44

Denticles in transverse rows .....  ..... *Mylonchulus*

44.(43) Denticles situated on longitudinal rib of stoma .....  ..... *Prionchulus*

Denticles scattered on stoma wall .....  ..... *Sporonchulus*

45.(42) Tooth anteriorly directed .....  46

Tooth retrorse .....  ..... *Anatonchus*

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46.(45) Tooth in basal part of stoma .....  ..... *Iotonchus*

Tooth in anterior part of stoma .....  ..... *Mononchus*

47.(40) Stoma with prominent medial or apical tooth .....  48

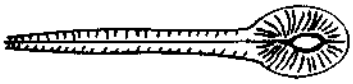
Stoma with small basal tooth .....  ..... *Bathyodontus*

48.(47) Stoma with 3 teeth, without small basal tooth, caudal glands opening terminally .....  ..... *Enoplocheilus*

Stoma with large anterior and small basal tooth, caudal glands opening ventrally .....  ..... *Mononchulus*

49.(39) Lip region with rib-like armature .....  ..... *Mononchoides*

Lip region without rib-like armature .....  ..... *Diplogaster*

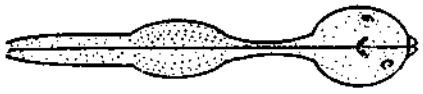
50.(38) Esophagus with basal expansions .....  51

Esophagus uniformly cylindrical .....  60

51.(50) Esophagus without mid-region expansion .....  52

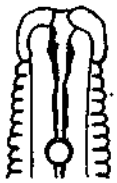
Esophagus expanded at mid-region 55

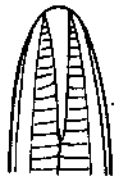




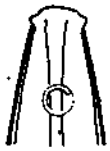
https://doi.org/10.2105/SMWW.2882.213

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52.(51) Amphids distinct .....  53

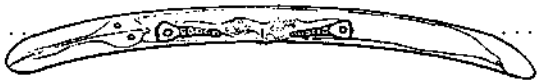
Amphids indistinct .....  54

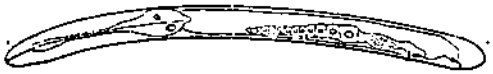
53.(52) Stoma walls anteriorly inflated with minute tooth .....  ..... *Microlaimus*

Stoma walls without tooth and with straight, tapering sides .....  ..... *Leptolaimus*

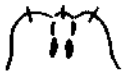
54.(52) Stoma with 3 rod-like thickenings .....  ..... *Rhabdolaimus*

Stoma without rod-like thickenings .....  ..... *Monochromadora*

55.(51) Gonads paired .....  56

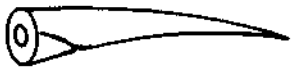
Gonads single .....  58

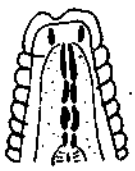
56.(55) Stomal walls straight, amalgamated .....  57

Stomal walls separated, not straight .....  ..... *Alloionema*

57.(56) Moderately swollen metacorpus, stoma not excessively elongate .....  ..... *Rhabditis*

Elongate, cylindrical metacorpus, stoma elongate .....  ..... *Cylindrocorpus*

58.(55) Tail with sharp terminus .....  59

Tail bluntly conical .....  .....  ..... *Cephalobus*

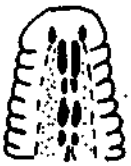
https://doi.org/10.2105/SMWW.2882.213

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59.(58) Anterior part of stoma a broad, open chamber ..... *Panagrolaimus*



Stoma narrow, collapsed ..... *Eucephalobus*



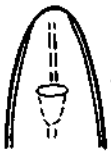
60.(50) Stoma absent or indistinct 61



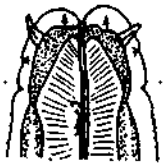
Stoma distinct 63



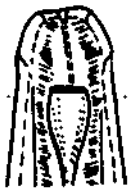
61.(60) Lip region narrow, tooth absent 62



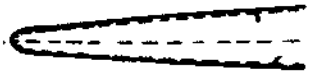
Lip region broad, small denticle apparent in stomal area ..... *Tripyla*



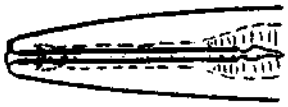
62.(61) Amphid aperture appearing as large slit ..... *Amphidelus*



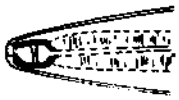
Amphid aperture appearing as minute pores ..... *Alaimus*



63.(60) Stoma narrow and long ..... *Cryptonchus*



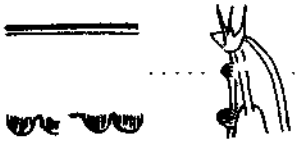
Stoma wide and shallow ..... *Bathyonchus*



64.(1) Body symmetrical 65



Body asymmetrical, bearing series of protuberances on side ..... *Bunonema**



65.(64) Lip appendages not elaborate 66



Lip appendages elaborate 68



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66.(65) Lateral lip appendages thorn-like, directed laterally .....  ..... *Diploscapter*

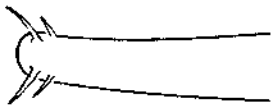
Lateral lip appendages not thorn-like or directed laterally 67

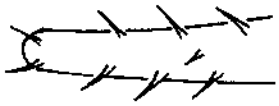
67.(66) Papillae or setae horn-like .....  ..... *Macrolaimus*

Lips flap-like and pointed anteriorly .....  ..... *Teratocephalus*

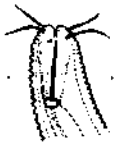
68.(65) Lip appendages forked and elaborately fringed .....  ..... *Acrobeles**

Lip appendages membranous and wing-like .....  ..... *Wilsonema**

69.(1) Post-cephalic setae absent .....  70

Post-cephalic setae present (may be very faint ex. *Tobrilus*) .....  92

70.(69) Stylet absent .....  71

Stylet present .....  91

71.(70) Teeth absent, minute or indistinct .....  72

Teeth usually present, prominent .....  85

72.(71) Esophagus with basal expansions .....  73

Esophagus uniformly cylindrical .....  82

73.(72) Amphids oval, spiral, or stirrup-shaped .....  74

Amphids circular 80

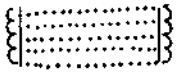
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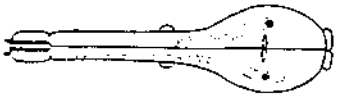
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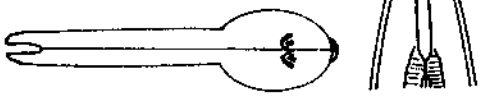
74.(73) Amphids spiral .....  75

Amphids not spiral 79

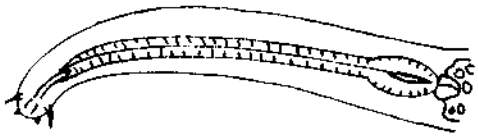
75.(74) Cuticular punctations absent .....   76

Cuticular punctations present .....  78

76.(75) Esophageal bulb without valves .....  77

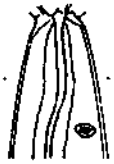
Esophageal bulb valvate .....  ..... *Plectus & Anaplectus*

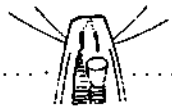
77.(76) Esophageal-intestinal valve elongate .....  ..... *Paraplectonema*

Esophageal-intestinal valve shortened .....  ..... *Paraphanolaimus*

78.(75) Labial region characteristically flap-like .....  ..... *Euteratocephalus*

Labial region not flap-like, lips bluntly rounded .....  ..... *Ethmolaimus*

79.(74) Amphids oval .....   ..... *Greenenema*

Amphids stirrup-shaped .....   ..... *Chronogaster*

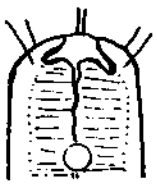
80.(73) Esophageal-intestinal valve shortened .....  81

Esophageal-intestinal valve elongate .....   ..... *Desmolaimus*

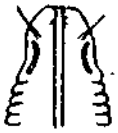
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81.(80) Excretory pore and large excretory gland present .....  ..... *Domorganus*

Excretory pore and gland indistinct or absent .....  ..... *Monhystera*

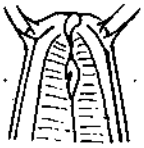
82.(72) Stoma wide and shallow, conspicuous, tail filiform .....  ..... *Prismatolaimus*

Stoma narrow, elongate, collapsed or inconspicuous .....  83

83.(82) Gonad single .....  ..... *Cylindrolaimus*



Gonads paired .....  84

84.(83) Amphids inconspicuous .....  ..... *Tripyla*

Amphids conspicuous .....  ..... *Aphanolaimus*

85.(71) Terminal fifth or sixth of esophagus an ovoid bulb .....  86

Esophagus uniformly cylindrical, stoma with massive teeth .....  ..... *Ironus*



86.(85) Cuticular punctations present .....  87

Cuticular punctations absent .....  89

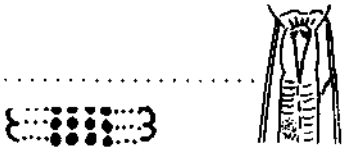
87.(86) Amphids not spiral 88

Amphids spiral .....  .....  ..... *Achromadora*

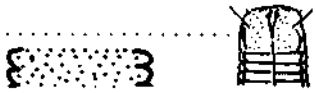
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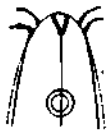
88.(87) Four longitudinal rows of cuticular markings present ..... *Chromadora*



No longitudinal rows of cuticular markings present ..... *Prochromadorella*



89.(86) Amphids distinct 90



Amphids indistinct ..... *Butlerius*

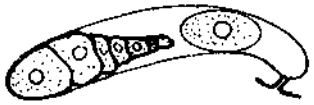


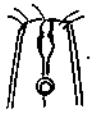
90.(89) Female gonad double, amphids hook-shaped ..... *Anonchus*



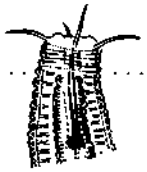


Female gonad single, amphid circular ..... *Monhystrella*

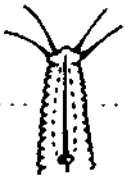




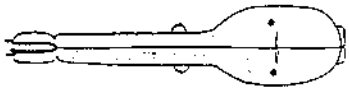
91.(70) Lip region annulated, not set off ..... *Atylenchus*



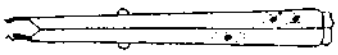
Lip region smooth, set off ..... *Eutylenchus*



92.(69) Esophagus with basal expansion 93



Esophagus uniformly cylindrical 98



93.(92) Cuticular punctation present, amphids not circular 94



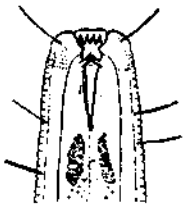
Cuticular punctation present, amphids circular 97



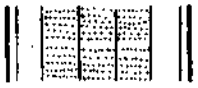
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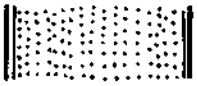
94.(93) Ocelli (eye spots) present .....  95

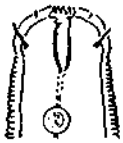
Ocelli absent .....  96

95.(94) Stoma with three equal-sized teeth .....  ..... *Chromadorina*

Stoma with at least one large tooth .....  ..... *Punctodora*

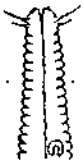
96.(94) Cuticle with lateral longitudinal rows of punctation .....  ..... *Hypodontolaimus*

Cuticle without lateral differentiations .....  ..... *Chromadorita*

97.(93) Esophageal bulb valvate .....  ..... *Prodesmodora*

Esophageal bulb without valves .....   ..... *Odontolaimus*

98.(92) Amphid anterior on body .....  99

Amphid posteriorly located .....  ..... *Bastiania*

99.(98) Amphid spiral .....  ..... *Paracyatholaimus*

Amphid cup-shaped or obscure 100

https://doi.org/10.2105/SMWW.2882.213

Refer to Couplet No.

100.(99) Stomal teeth massive .....  ..... *Oncholaimus*

Stomal teeth small .....  ..... *Tobrilus*

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10900 IDENTIFICATION OF AQUATIC ORGANISMS*

Experienced aquatic biologists will be familiar with most organisms illustrated in Plates 1–40, and seldom will need the assistance of keys to identify organisms to the level illustrated. Because these plates are not intended for critical identification, specific (species) names are not generally cited. Organisms most likely to be observed are illustrated. For the convenience of those less familiar with the organisms referred to in preceding sections, a series of short keys is presented to enable them to identify most organisms to the level illustrated by the plates.

In conformity with preceding sections, organisms are arbitrarily divided into microscopic and macroscopic, depending on whether or not they pass through a U.S. Standard No. 30 sieve (0.5 mm). To study microscopic forms, use a compound or inverted microscope. To examine the smaller macroscopic organisms and to resolve the finer structures of larger forms, use a wide-field stereoscopic microscope.

* Approved by Standard Methods Committee, 2007. Editorial revisions, 2016.
Joint Task Group: 22nd Edition—Donald J. Reish and Lawrence L. Lovell (co-chairs), E.F. Benfield, Donald B. Cadien, Donald G. Huggins, Donald J. Klemm, Vincent H. Resh, Ronald G. Veldarde.

10900 A. Identification Procedure

Critical identification of a specimen often is time-consuming, even for an experienced biologist. Before using any key or other aid to identification, carefully study the specimen. Try to find other examples and compare them with the unknown.

It is important to know where or under what conditions the organism lived before attempting to identify it. For example, did it come from fresh water—a lake or a stream? Is it marine—from the open ocean, shoreline, or estuary? Was it a free swimmer or floater in the water? Was it a bottom organism, attached, crawling, or burrowing? Finally, turn to the following key to major groups.

Only the more common types of aquatic organisms are illustrated here, with special attention to those most frequently used in water quality evaluation. When specimens do not fit obviously into one of the types listed, consult a professional biologist, a microbiologist for the bacteria and fungi, or some of the references provided. Descriptions of color and movement refer to freshly collected or living specimens, or, in the case of microscopic forms, to those preserved as described in Section 10200 in a non-staining preservative.

Sizes of the organisms illustrated in Plates 1–27 are shown in parentheses in the legend. These are intended to represent *common* sizes, not absolute maxima or minima. Exceptional individuals and even whole localized populations may be encountered that are considerably larger or smaller than the sizes cited.

10900 B. Key to Major Groups of Aquatic Organisms (Plates 1–40)

Beginning with couplet 1a and 1b, choose one of the contrasting statements. Proceed to the couplet number indicated at the end of the chosen statement and repeat the process. Continue until an organism name or plate number is cited instead of another couplet number. Additional information is provided in many of the plate legends.

	Refer to couplet No.
1a. Macroscopic: The organism, mass, or colony is easily visible to the naked eye	13
1b. Microscopic: Not readily visible to the naked eye	2

1. Partial Key to Microscopic Organisms

2a. Specimen a single cell or a mass or colony of relatively independent cells (shapeless, rounded, or threadlike)	3
2b. Specimen a many-celled, highly organized plant or animal	7
3a. Cells contain one or more pigments, including chlorophyll *a* (overall color may range through various shades of green, blue, red, brown, or yellow). ALGAE (for details, see 10900D following "Key for Identification of Freshwater Algae")	4
3b. Cells typically colorless, lacking chlorophyll *a*	12
4a. Nuclei present; pigment confined to chloroplasts	5
4b. Nuclei, plastids, or vacuoles absent (aerotopes may be present in certain colonial and filamentous forms). Pigment generally diffused throughout cytoplasm. CYANOBACTERIA (Blue-green algae), Plates 1A and 35, Figures H-1a through H-31.	

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5a. Cell wall permanently rigid, composed of SiO_2, geometrical in appearance, and with regular patterns of fine markings; composed of two essentially similar halves, one placed over the other as a cover. Golden brown to greenish in color. CHRYSOPHYTA-BACILLARIOPHYCEAE (DIATOMS), Plates 1B and 30, Figures C-1a through C-25b.
5b. Cell wall, if present, capable of sagging or bending, rigidity—depending on internal pressure of cell contents and thickness of cell wall. Cell walls usually of one piece 6
6a. Cells, colonies, or filaments nonmotile. Usually some shade of green, yellow-green, or red. PIGMENTED NONMOTILE ALGAE (mixed division), Plates 1A; 1B; 28, Figures A-1 through A-2; 29, Figures B-1 through B-50; 31, Figures D-1 through D-5; 33, Figures F1 through F2; and 40, Figures M-1 through M-4 (in part).
6b. Cells or colonies move by means of one to four relatively long, whiplike flagella. PIGMENTED FLAGELLATE ALGAE (mixed division), Plates 4A; 4B; 29, Figures B-1 through B-50; 31, Figures D-1 through D-5; 32, Figures E-1 through E-2; 33, Figures F-1 through F-2; 34, Figures G-1 through G-2; 36, Figures I-1 through I-4b; 37, Figures J-1 through J-2b; 38, Figures K-1 through K-4; and 39, Figure L-1 (in part).
7a. Body with cilia (hairlike structures used for locomotion)..... 8
7b. Body without cilia..... 9
8a. Body generally covered with cilia, usually somewhat elongate or wormlike, bilaterally symmetrical. Minute FLATWORMS (Platyhelminthes), relatives of *Planaria*, Plate 9.
8b. Cilia confined to one or two crowns at anterior end, which often present the illusion of rotating wheels. Internal jaws present. ROTIFERS (Rotifera), Plate 8.
9a. Long, slender, unsegmented worms that move by sinuous crawling or thrashing motion. ROUNDWORMS (Nematoda), Plate 9.
9b. Possess external skeleton and jointed appendages 10
10a. Crawl about or swim by means of jointed appendages thrust out from between two clamlike shells. All appendages can be withdrawn entirely within shells when disturbed. OSTRACODS (Ostracoda), Plate 11.
10b. Swim rapidly by means of a pair of enlarged jointed appendages (antennae) that cannot be withdrawn inside carapace or shell..... 11
11a. Locomotor appendages (antennae) branched. Microcrustacea, CLADOCERA (Cladocera), Plate 11.
11b. Locomotor appendages (antennae) unbranched; body tapers toward rear. Microcrustacea, COPEPODS (Copepoda), Plate 11.
12a. Ingest and digest food internally (ingested food of various colors may be visible through body wall). Single-celled or colonial, attached or free-living. PROTOZOANS (Protozoa), Plates 4B, 5A, 5B, and 6.
12b. Digest food externally and absorb products through cell wall. Often secrete masses of slime. BACTERIA and FUNGI, Plates 26 and 27.

2. Key to Macroscopic Organisms

13a. Specimen a mass of filaments or a glob of gelatinous or semisolid material containing many tiny units, requiring microscopic examination to determine details of structure 2
13b. Specimen a well-organized unit or colony 14
14a. Organism plantlike; flowerlike structures, if present, do not respond when touched, generally are colored some shade of green, brown, or red..... 16
14b. Organism animal-like; usually responds rapidly when touched, whether attached or free-living 15
15a. Internal backbone present (vertebrates)..... 17
15b. No internal backbone present (macroinvertebrates)*
16a. Plant structure relatively simple. Attachment structures may be present, but no true roots or fibrous tissue. Larger ALGAE, Plates 2A, 2B, 28 (*Chara*, *Nitella*), and 40, Figure M-2 (*Batrachospermum*).
16b. Plant structure usually includes true roots, stems, and leaves. Fibers or vascular tissue usually present; flowers or seeds may be observed. (One atypical group, "watermeal," consists only of tiny roundish masses, 0.5 to 1 mm in diameter, often misidentified as algae.) HIGHER PLANTS, Plates 3A, 3B, and 3C.
17a. Side appendages, if present, are flat fins. FISHES, Plate 24.
17b. Side appendages, if present, are footlike, with separate digits. AMPHIBIANS, Plate 25.

3. Key to Macroinvertebrates

18a. Body bilaterally symmetrical (with right and left sides, but may be superficially coiled into a spiral); animal usually not attached but may live inside an attached cocoon or case, or crawl about; usually solitary..... 23
18b. Symmetry not bilateral..... 19
19a. Body typically radially symmetrical 21
19b. Body or colony nonsymmetrical 20
20a. Body mass generally porous; not a colony, sometimes finger- or antler-like. Freshwater representatives generally are fragile, colored green or brown; marine forms tougher, various colors. SPONGES (Porifera), Plate 7.
20b. Body mass otherwise..... 22

* Invertebrates retained on a U.S. Standard No. 30 sieve (0.5 mm).

21a. Animals with soft smooth bodies and tentacles around a mouth; no anus. Solitary or colonial. Larger colonies usually have rigid limy skeleton of massive, branched, or fan-shaped form. HYDRAS, SEA ANEMONES, JELLYFISHES, CORALS, etc. (Cnidaria), Plate 7.
21b. Body covering usually spiny, soft or rigid, flattened or elongate, typically having five radii, with or without spines or arms; anus present. Solitary. Marine only. STARFISHES and relatives (Echinodermata), Plate 23.
22a. Colony a jellylike mass, a network of branching tubes, a plant-like tuft, or a lacy, limy crust or mass. MOSS ANIMALS (Ectoprocta), Plate 22.
22b. Exclusively marine. Surface of body or colony relatively smooth but tough. Solitary forms, sac-like, with two external openings. Exhibit all degrees of colonialism. Compound forms range from thin slimy masses, with organisms arranged in tiny radial patterns, to huge, shapeless masses resembling tough frozen gelatin. SEA SQUIRTS, SEA PORK (Ascidiacea, Urochorda, Chordata), not illustrated.
23a. Animal living within a hard, limy shell, soft body (Mollusca or Brachiopoda)..... 29
23b. Animal without a limy shell..... 24
24a. Jointed legs present (may not be functional). Body may be hard or soft..... 30
24b. Jointed legs absent, body covering mostly soft, animal pliable (a hardened head capsule may be present)..... 25
25a. Body girded by annulations or creases at regular intervals, dividing it into many small segments much wider than long 26
25b. Segments present or absent; if present, not much wider than they are long..... 27
26a. Body with suction disk at one or both ends, in length usually less than 10 times its width. LEECHES (Annelida, Hirudinea), Plate 9.
26b. Body without suction disks, in length usually more than 10 times its width; hairs or bristles often evident. SEGMENTED WORMS (Annelida), Plates 9 and 10.
27a. Body unsegmented..... 28
27b. Body segmented, slender. TWO-WINGED FLIES (Diptera), Plate 16.
28a. Body long and slender, appearing smooth, evenly tapered to a fine point at one end. ROUNDWORMS (Nematoda), Plate 9.
28b. Body flat, elongate, or oblong; pigmented spots on head; head often spade-shaped. FLATWORMS (Platyhelminthes, Turbellaria), Plate 9.
28c. Body long and slender with a circle of tentacles at one end; lives in a sandy tube, marine. PHORONIDEA, Plate 22.
29a. Shell consisting of two hinged halves. BIVALVES (Pelecypoda) or LAMP SHELLS (Brachiopoda), Plate 21.
29b. Shell entire, usually spiral but may be "coolie hat"-shaped. SNAILS (Gastropoda), Plate 20.
30a. Body with functional legs..... 31
30b. Body without functional legs, mummy- or capsule-like, living in a cocoon. PUPAE (Insecta), Plates 15, 16, and 17.
31a. Body with three pairs of legs. Larvae, nymphs, and some adults (Insecta)..... 42
31b. Body with more than three pairs of legs..... 32
32a. Body compact, spider-like, with four conspicuous pairs of legs (two other pairs of appendages present). WATER MITES (Acari) or SEA SPIDERS (Pycnogonidea), Plates 12 and 22.
32b. Body with at least five conspicuous pairs of legs. CRUSTACEANS (Crustacea)..... 33
32c. Body covering hard; divided into broad head, truncate body, and sharp tail section (marine). HORSESHOE CRAB (Arachnoidea), Plate 22.

4. Key to Crustacea

33a. Paired compound eyes on stalks..... 34
33b. Paired compound eyes, if present, sessile..... 36
34a. Carapace, if present, does not fuse with more than four thoracic segments..... 35
34b. Carapace fused with all thoracic segments; pincers present. DECAPODA, Plate 12.
35a. Carapace covers most of the thoracic segments. MYSIDACEA, Plate 11.
35b. Carapace extends over the anterior segments of abdominal region. LEPTOSTRACA, Plate 12.
36a. Body flattened horizontally..... 37
36b. Body flattened laterally. SCUDS (Amphipoda), Plate 12.
37a. First pair of thoracic legs with pincers. TANAIDACEA, Plate 12.
37b. First pair of thoracic legs pointed and similar to all thoracic legs. ISOPODA, Plate 12.

5. Key to Insect Pupae

38a. Back of pupa with small, paired, hook-bearing plates. CADDISFLIES (Trichoptera), Plate 15.
38b. Back without paired hook-bearing plates but may have knobs or bristles..... 39
39a. Developing wings (pads) held free from body. BEETLES (Coleoptera), Plate 17.
39b. Wing pads closely appressed to body, mummy-like, or appendages not evident..... 40
40a. With one closely appressed pair of wing pads, but not fused to body; or capsule-like, appendages not evident. TWO-WINGED FLIES (Diptera), Plate 16.
40b. Two pairs of wing pads..... 41
41a. First two or three abdominal segments with spiracles (holes for breathing) on each side; body without numerous projections. AQUATIC MOTHS (Lepidoptera), not illustrated.

41b. Body differing from above, may have numerous knobs or other projections on back. HELLGRAMMITES (Neuroptera and Megaloptera), Plate 15.

6. Key to Insect Larvae, Nymphs, and Some Adults

42a. Animal flea-like, with a bifid projecting appendage on the underside. SPRINGTAILS (Collembola), Plate 22.
42b. Animal otherwise 43
43a. Body ending in long segmented filaments. 44
43b. Long filaments absent or, if present, not segmented 45
44a. Two tail filaments, all legs ending in two claws. STONEFLIES (Plecoptera), Plate 13.
44b. Middle and hind legs ending with one claw, three tail filaments (usually). MAYFLIES (Ephemeroptera), Plate 13.
45a. Back of body covered with two hard wing covers, a pair of membranous wings underneath the covers. ADULT BEETLES (Coleoptera), Plate 17.
45b. Back without hard wing covers 46
46a. Body with exposed membranous wings or wing pads on back 47
46b. Body without membranous wings or wing pads (larvae) 49
47a. Membranous wings present; held flat and in a V-shape on back. Mouth parts formed into a long, sharply pointed beak folded underneath body. TRUE BUGS (Hemiptera), Plate 18.
47b. Membranous wings absent, wing pads present. Mouth parts formed into an extendable, scoop-like mask that covers face. (Odonata) 48
48a. Body ending in three oblong, fan-like plates. DAMSELFLIES (Zygoptera), Plate 14.
48b. Fan-like plates absent. DRAGONFLIES (Anisoptera), Plate 14.
49a. Mouth parts formed into slender curved rods nearly half as long as body (<10 mm). SPONGILLA FLIES (Neuroptera), not illustrated.
49b. Mouth parts adapted for biting or chewing 50
50a. Body with five paired knobs on underside of abdominal segments, legs on first three segments short and stubby. Often found on lily pads. AQUATIC MOTHS (Lepidoptera), not illustrated.
50b. Body without paired knobs on underside of abdomen 51
51a. Sides of each abdominal segment with a slender, tapering process 52
51b. Sides of each abdominal segment without a tapering process, but may have hair-like or tubular processes 53
52a. Body ending in a pair of hook-bearing fleshy legs or in a single tapering filament. HELLGRAMMITES and relatives (Megaloptera), Plate 15.
52b. Body otherwise. BEETLES (Coleoptera), Plate 17.
53a. Body covering mostly hard; knobs, hairlike processes, or other special ornamentation may be present on back, or else body is entirely soft except for a hardened head capsule. BEETLES (Coleoptera), Plate 17.
53b. Most of body soft except for a hardened head capsule and with one to three hard plates on the back of first body segments; tubular processes may be present on sides of the body in various arrangements. Body may end in a pair of hook-bearing legs. Most larvae living in portable cases made of bits of sticks, leaves, or sand or in attached fibrous cases. CADDISFLIES (Trichoptera), Plate 15.

ACKNOWLEDGMENTS

Plates 1–40, which follow on succeeding pages, present more than 300 marine, brackish, and freshwater organisms commonly found in oligotrophic, mesotrophic, and eutrophic waters. These plates were drawn for this work by Eugene Schunk of the Cincinnati Art Services, Inc. and by Lhisa J. Reish of Olympia, Washington. Figure 15E was drawn by Dr. Harold Walker; Figures 16A, K, L, and O also are based on his work. The figures in Plate 27 are based on original drawings by Dr. W. Bridge Cooke. Plates 28–40 were provided by Ann St. Amand, Andrew Chapman, Barry Rosen, and Ken Wagner. Chris Carter also provided images that are individually credited. In a number of instances, it would have been impossible to illustrate a certain organism for the purposes of this manual were it not for the courtesy of other publishers, who permitted illustrations from their publications to be incorporated herein. The following organisms were so reproduced:

Plate

1B S—*Achnanthes,* courtesy of Veb Gustav Fischer Verlag, Jena. Source: Die Susswasser—Flora Mitteleuropas, Heft 10, by F. Hustedt, 1930.
U—*Coscinodiscus,* courtesy of E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart. Source: Das Phytoplankton des Susswassers, Die Binnengewasser, Band XVI, Teil II, Halfte II, by G. Huber-Pestalozzi and F. Hustedt, 1942. Plates CVIII-CXVI and CXXIII.
W—*Skeletonema,* courtesy of Academische Verlagsgesellschaft, Leipzig. Source: Die Kieselalgen, by F. Hustedt. *In* L. Rabenhorst, Kryptogamen-Flora von Deutschland, Osterreich und der Schweiz, Band VII, 1930.

8 I—*Notholca* Robert W. Pennak, Fresh-Water Invertebrates of the United States, 1953, The Ronald Press Company, New York. Figure 116*N*, p. 190, adapted for Figure 8, courtesy of The Ronald Press.

11 H—*Diaptomus* (copepod)

12 H—*Cambarus* (crayfish, crawdad) courtesy of Holden-Day, Inc., San Francisco, California. Source: Needham & Needham's Guide to the Study of Freshwater Biology, 1951. Figures 1 and 10, Plate 14, p. 37; Figures 16, 18, and 20, Plate 24, p. 61; and Figure 9, Plate 14, p. 37.

19 E—*Tarebia*,
H—*Lymnaea* (pond snail),
I—Orb snail, and
J—*Ferrissia* (limpet), courtesy of R.M. Sinclair, Advisor for Biological Sampling and Analysis (American Public Health Association), 13th ed.
K—*Lanx* (limpet) courtesy of John Wiley & Sons, Inc., New York. Source: Ward & Whipple, Fresh Water Biology (2nd ed.), W.T. Edmondson, Editor, 1959. Figures 43.62B(E), 43.13(H), 43.20(I), and 43.14(K).

22 C—*Limulus* (horseshoe crab) courtesy of Western Publishing Company, Inc., Golden Press Division, Racine, Wisconsin. Source: Seashores, a Golden Nature Guide, 1955. p. 79.

23 A—*Asterias,* and
C—*Thyrone,* courtesy of Connecticut State Geological and Natural History Survey: Echinoderms of Connecticut, by Wesley Roswell Coe, 1912.

25 C—*Ambystoma* (terrestrial adult), courtesy of Dover Publications, Inc., New York. Source: Biology of the Amphibia, by G.K. Noble, 1931. Figure 147C, p. 471.
D—*Ambystoma* (aquatic larva), courtesy of the New York State Museum and Science Service, Albany, New York. Source: The Salamanders of New York, by Sherman C. Bishop, 1941. Figure 33b, p. 166. [Bulletin 324, New York State Museum, Albany.]
E—*Necturus,* courtesy of Dover Publications, Inc., New York. Source: Biology of the Amphibia, by G.K. Noble, 1931. Figure 35B, p. 99.
G—*Siren intermedia* (siren), reprinted from Sherman Bishop: Handbook of Salamanders, 1943, Comstock Publishing Company, Inc. Used by permission of Cornell University Press.

26 A—Micrococcus,
B—Streptococcus,
C—Sarcina,
D—Bacillus,
E—Vibrio, and
F—Spirillum, courtesy of John Wiley & Sons, Inc., New York. Source: Ward & Whipple, Fresh Water Biology (2nd ed.), W.T. Edmondson, Editor, 1959. Figure 3.1.
K—Actinomycete growth form, Selman A. Waksman, The Actinomycetes, 1957, The Ronald Press Company, New York. Figures 2–6, p. 18, adapted for Figure 26K, courtesy The Ronald Press.
O—*Tetracladium,* and
P, Q—*Achlya,* courtesy of John Wiley & Sons, Inc., New York. Source: Ward & Whipple, Fresh Water Biology (2nd ed.), W.T. Edmondson, Editor, 1959. Figures 4.119 and 4.79.

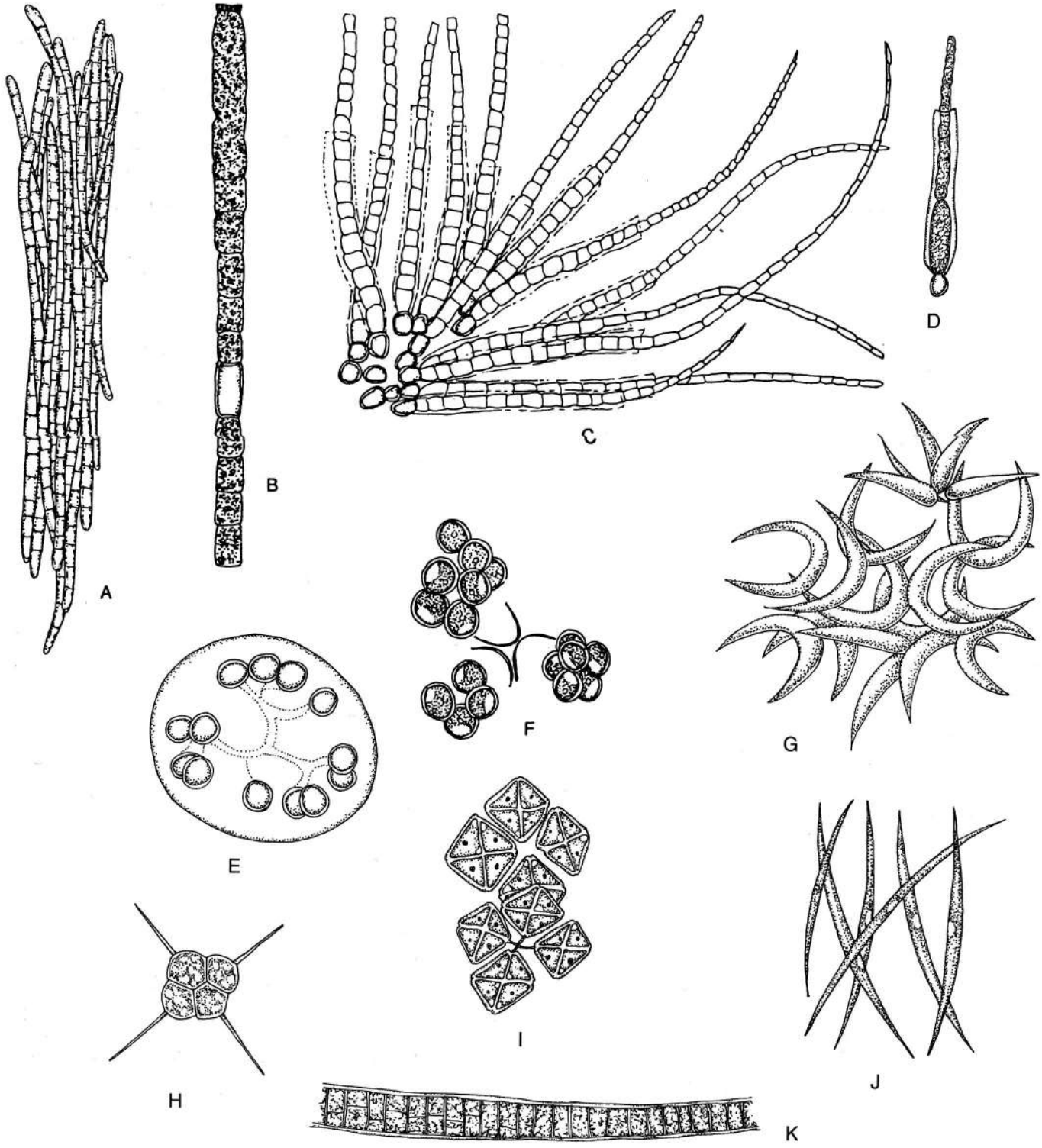


Plate 1A. Cyanobacteria (Blue-green algae) and Chlorophyta (Green algae). Dimensions refer to individual cells or filaments. All organisms inhabit fresh water.

Cyanobacteria (Phylum Cyanophyta):
- A—*Aphanizomenon*, aggregate of filaments (3–6 μm)
- B—*Aphanizomenon*, detail
- C—*Gloeotrichia*, portion of colony (cells 7–9 μm
- D—*Gloeotrichia*, detail diameter)

Green algae (Phylum Chlorophyta):
- E—*Dictyosphaerium* (8–14 μm)
- F—*Westella* (5–7 μm)
- G—*Selenastrum* (6–7 μm)
- H—*Tetrastrum* (5–9 μm)
- I—*Crucigenia* (5–8 μm)
- J—*Ankistrodesmus* (2–3 μm)
- K—*Schizomeris* (12–18 μm)

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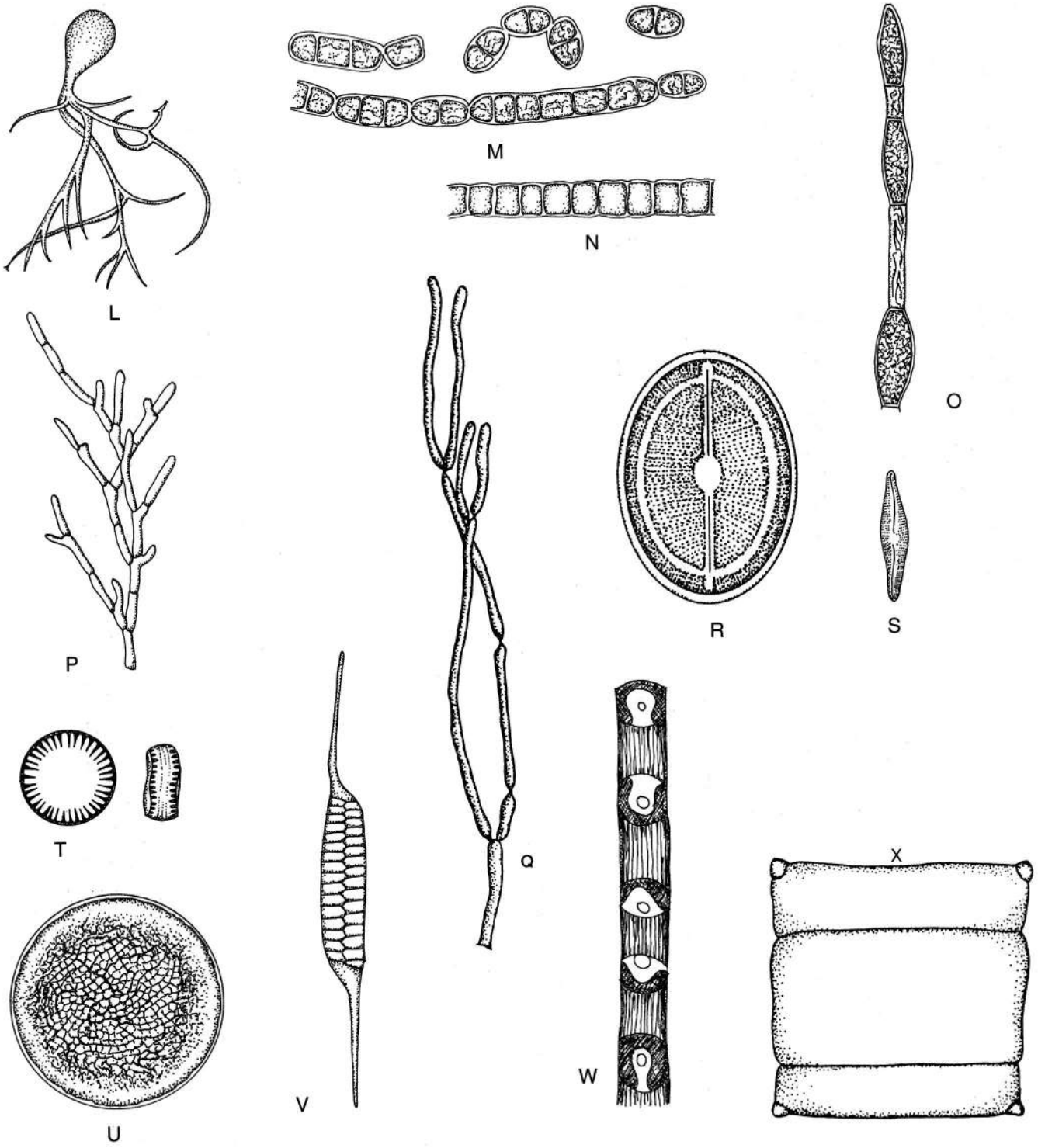


Plate 1B. Chrysophyta (Yellow-green, golden-brown algae) and Chlorophyta (Green algae). Dimensions refer to average individual cells or filaments. All organisms inhabit fresh water.

Yellow-green algae (Phylum Xanthophyta):	
L—*Botrydium*	(1–2 μm)
Green algae (Phylum Chlorophyta):	
M—*Stichococcus*	(3 μm)
N—*Hyalotheca*	(12–30 μm)
O—*Pithophora*	(50–100 μm)
P—*Microthamnion*	(2–4 μm)
Q—*Dichotomosiphon*	(50–100 μm)

Golden-brown algae (Phylum Chrysophyta) (diatoms):	
R—*Cocconeis*	(10 μm)
S—*Achnanthes*	(10 μm)
T—*Cyclotella*	(10 μm)
U—*Coscinodiscus*	(120 μm)
V—*Urosolenia*	(5–15 μm)
W—*Skeletonema*	(5–15 μm)
X—*Biddulphia*	(100 μm)

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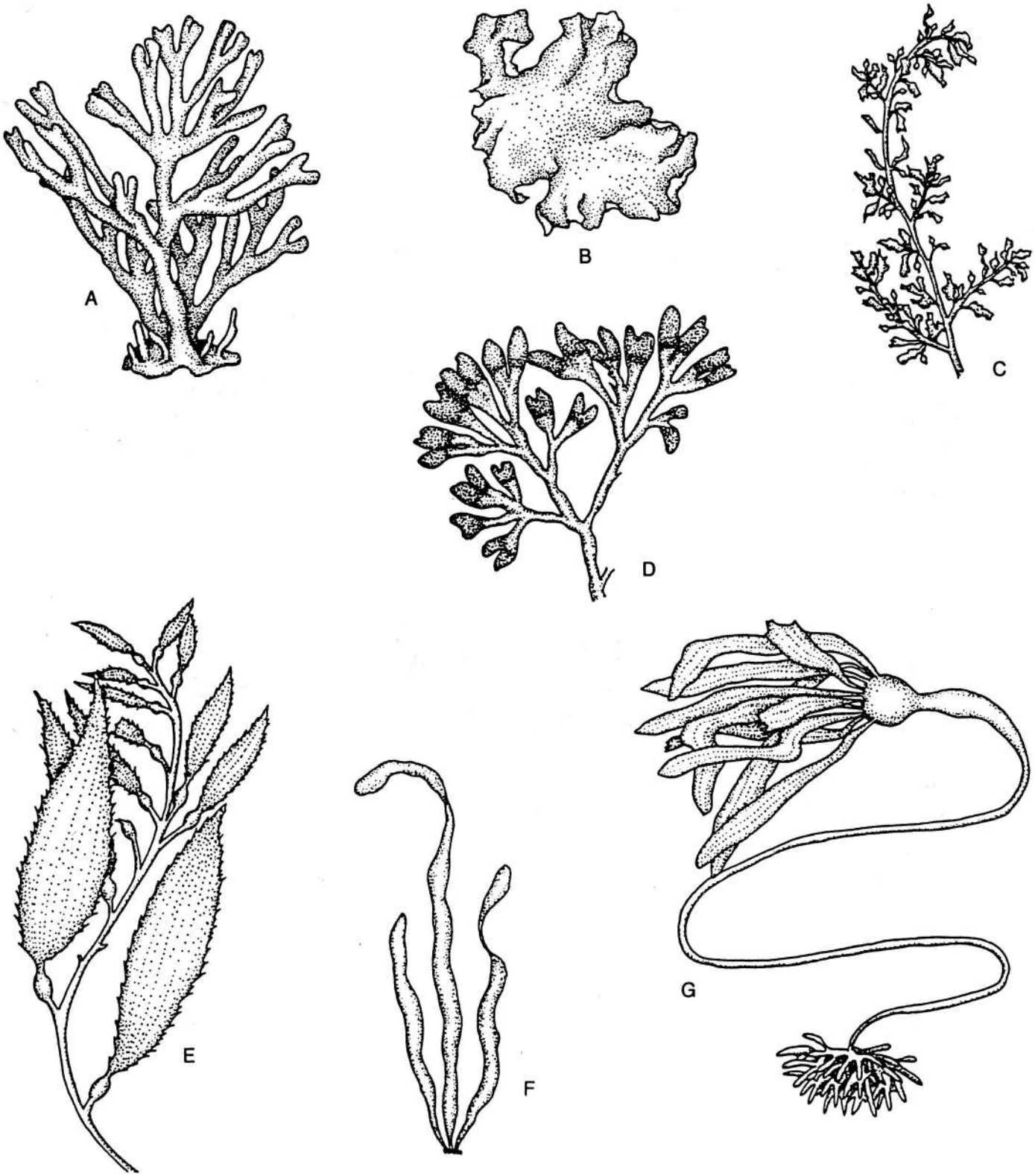


Plate 2A. Types of large marine algae.

Chlorophyta (Green algae):
- A—Sponge weed, *Codium* (30–40 cm)
- B—Sea lettuce, *Ulva* (20 cm)

Phaeophyta (Brown algae):
- C—*Sargassum* (20–100 cm)
- D—Rockweed, *Fucus* (75 cm)
- E—Giant kelp, *Macrocystis* (30 m)
- F—*Enteromorpha* (40 cm)
- G—Bull whip, *Nereocystis* (20 m)

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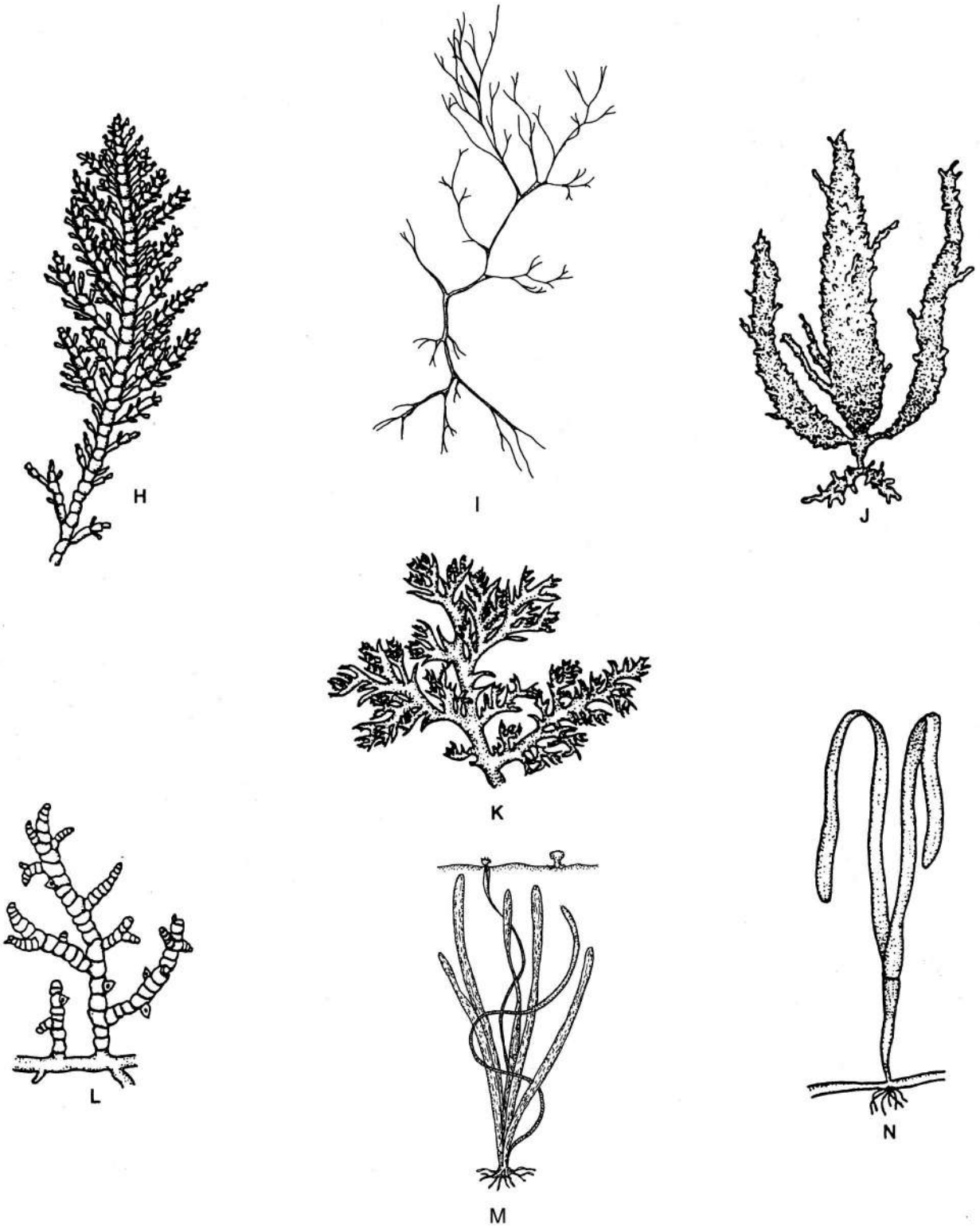


Plate 2B. Types of large marine algae and marine grasses:

Rhodophyta (Red algae):	
H—*Corallina*	(4 cm)
I—*Gracilaria*	(50 cm)
J—*Gigartina*	(30–40 cm)
K—*Plocamium*	(30 cm)
L—*Champia*	(1–6 cm)
Spermatophyta (Marine grasses):	
M—Eelgrass, *Vallisneria*	(50 cm)
N—Eelgrass, *Zostera*	(45 cm)

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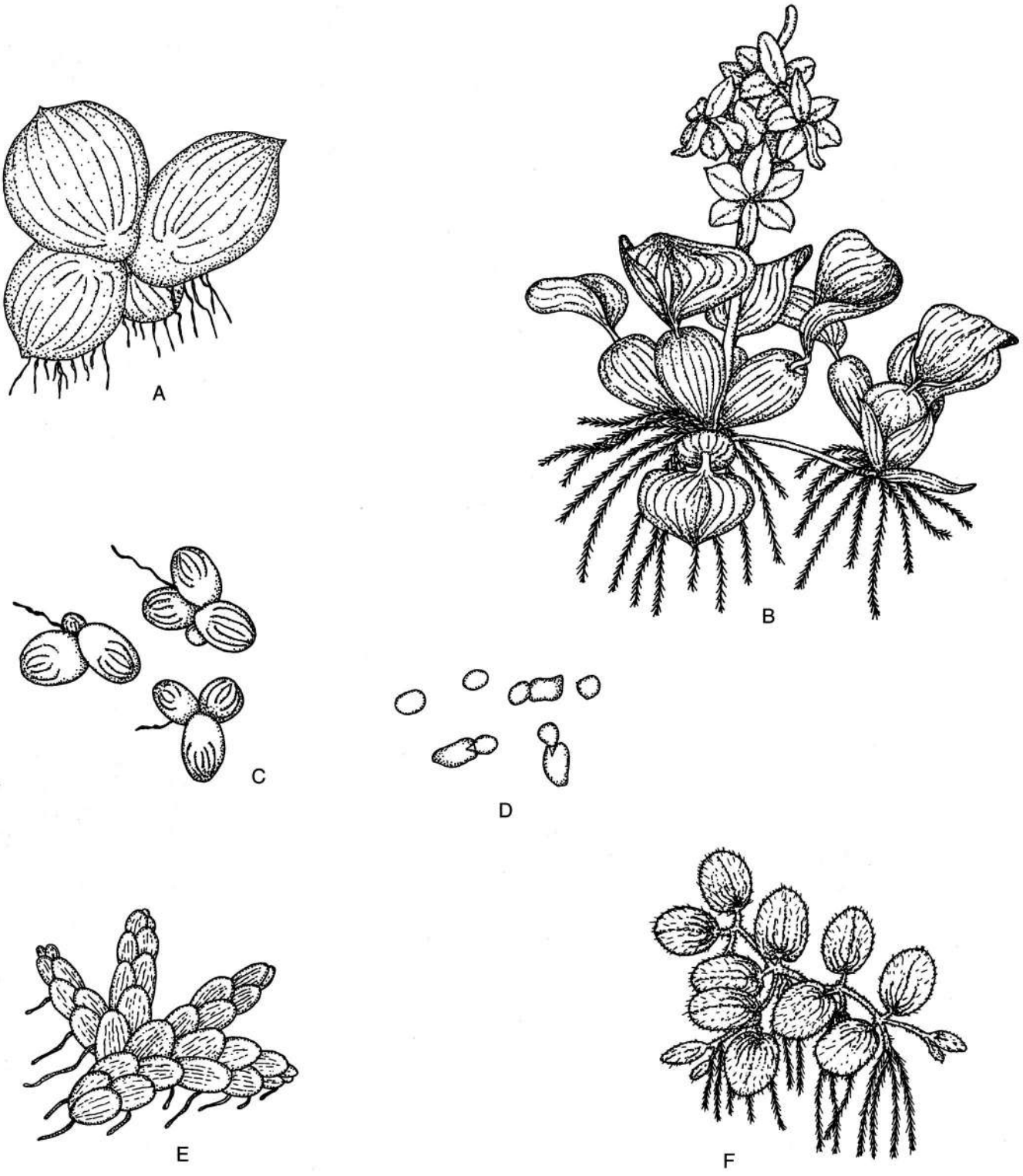


Plate 3A. Higher plants. All floating, freshwater inhabitants.

Phylum Spermatophyta:		D—Watermeal, *Wolffia*	(1–1.5 mm)
A—Great duckweed, *Spirodela*	(8 mm)	Phylum Pteridophyta:	
B—Water hyacinth, *Eichhornia*	(22 cm)	E—Water velvet, *Azolla*	(1 cm)
C—Lesser duckweed, *Lemna*	(5 cm)	F—Water fern, *Salvinia*	(4 cm)

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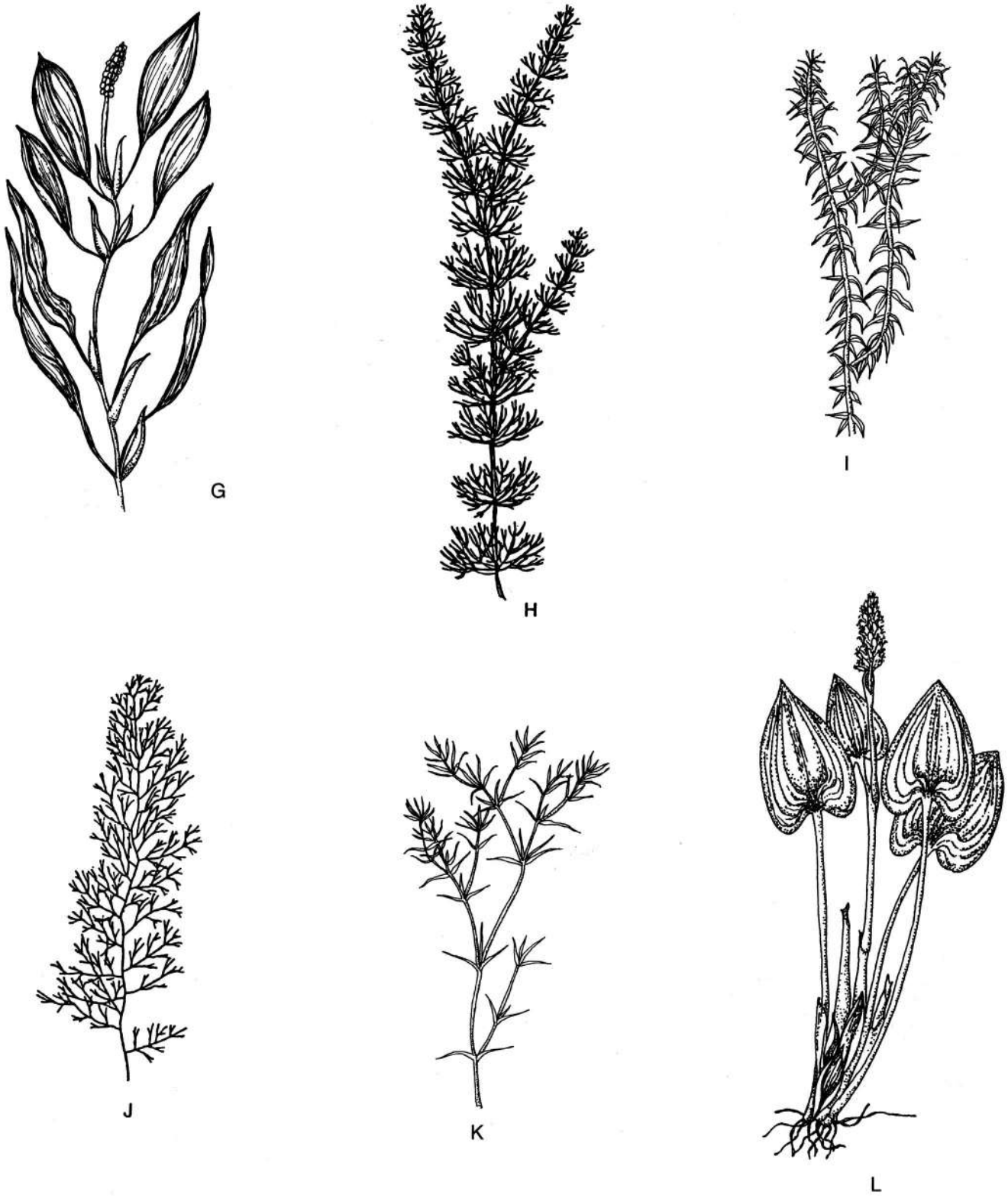


Plate 3B. Higher plants. All freshwater inhabitants (Phylum Spermatophyta).

Submerged:	
G—Pondweed, *Potamogeton*	(30–60 cm)
H—Coontail, *Ceratophyllum*	(30 cm)
I—Waterweed, *Elodea*	(15 cm)
J—Water milfoil, *Myriophyllum*	(30 cm)
K—Naiad, *Najas*	(60 cm)
Emersed:	
L—Pickerelweed, *Pontederia*	(60 cm)

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Plate 3C. Higher plants. All freshwater inhabitants (Phylum Spermatophyta), emersed.

M—Sweet flag, *Acorus*	(30 cm)	O—Cattail, *Typha*	(1–2 m)
N—Spike rush, *Eleocharis*	(30 cm)		

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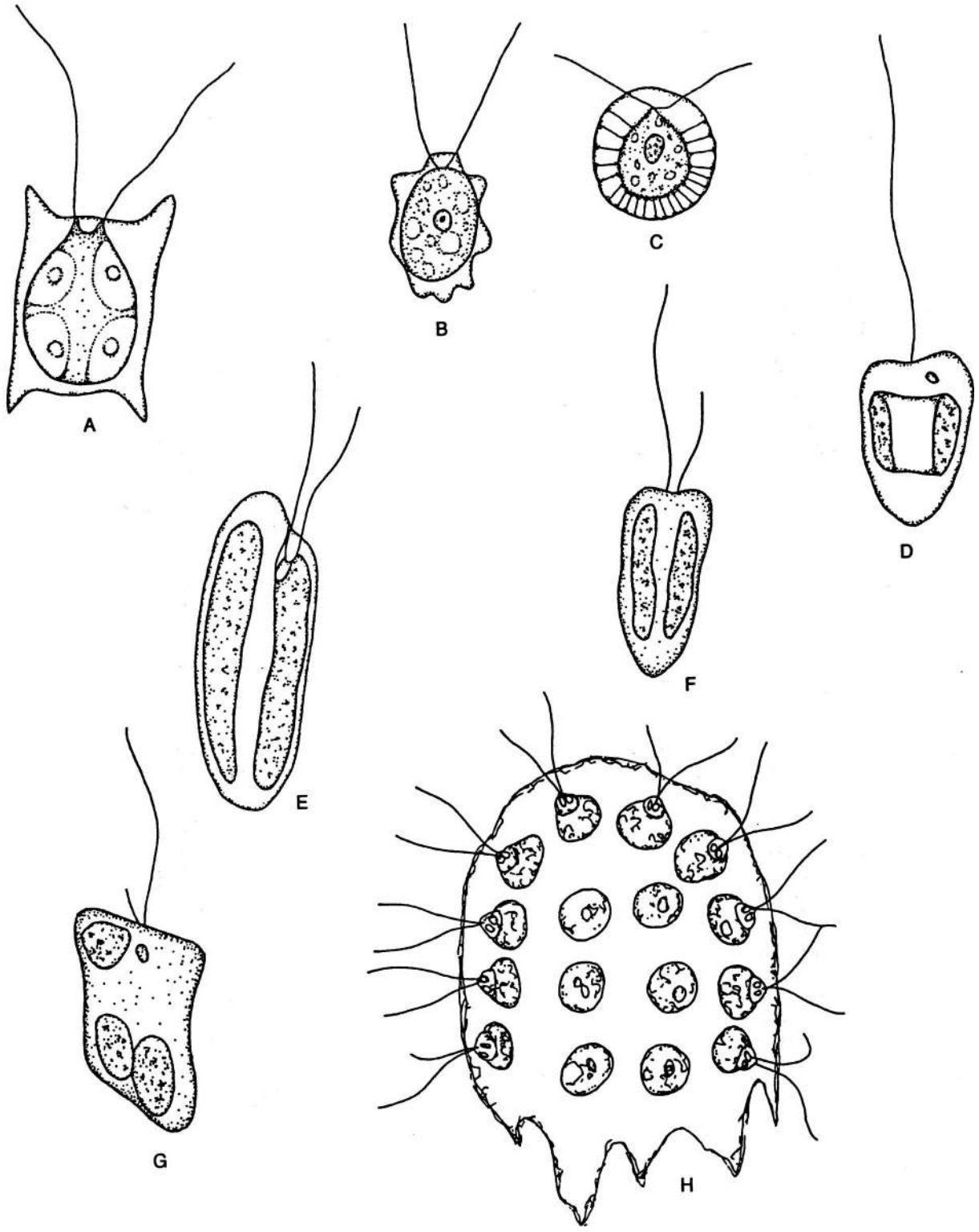


Plate 4A. Flagellates. Pigmented flagellates, various divisions. Freshwater inhabitants.

A—*Pteromonas*	(5–18 μm)	E—*Cryptomonas*	(6–12 μm)
B—*Lobomonas*	(5–14 μm)	F—*Ochromonas*	(7–14 μm)
C—*Haematococcus*	(40–45 μm)	G—*Chloramoeba*	(10–15 μm)
D—*Chromulina*	(4–10 μm)	H—*Platydorina*	(66–70 μm, colony)

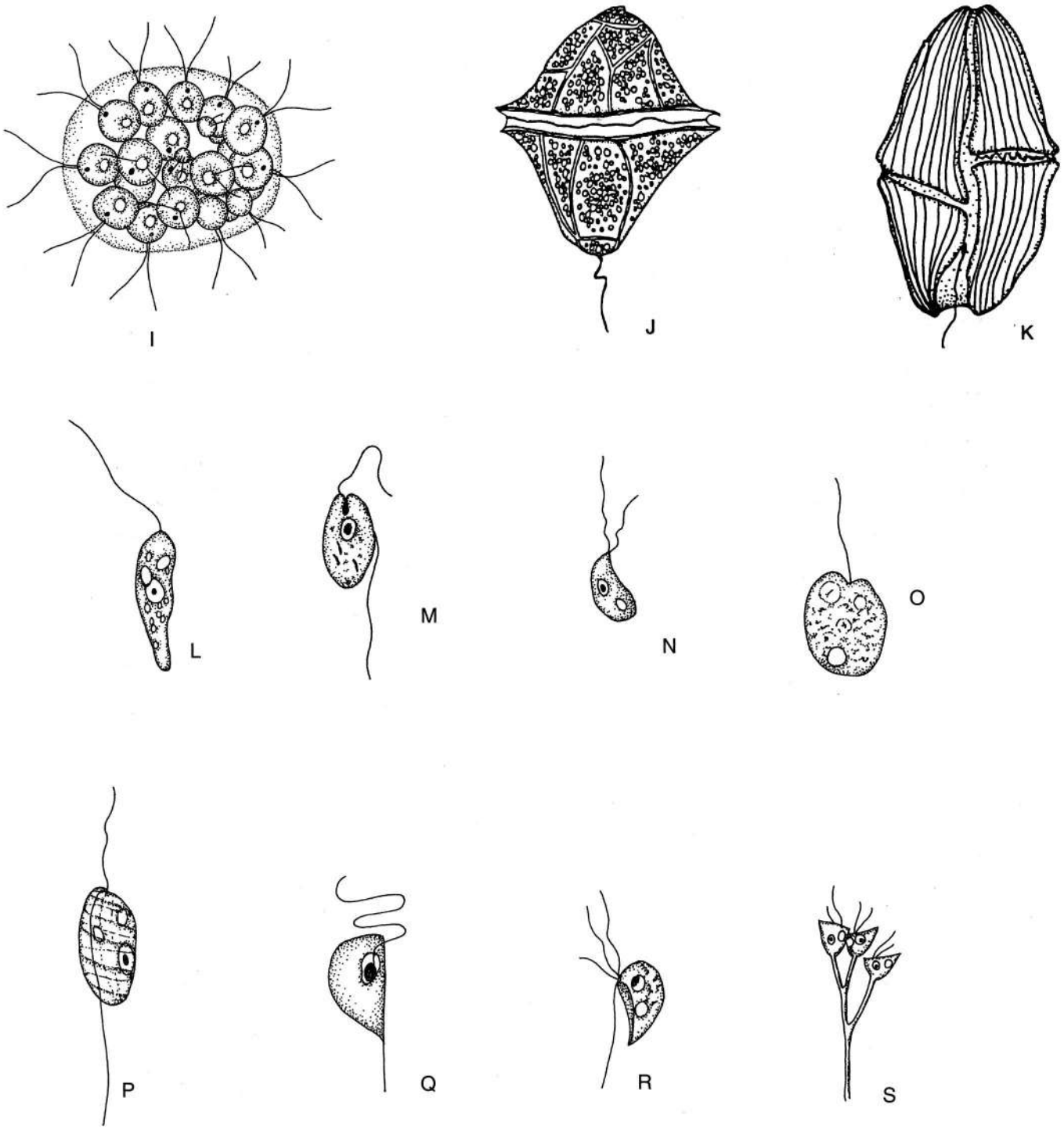


Plate 4B. Flagellates. Freshwater inhabitants except as noted.

Pigmented (various divisions):	
I—*Pleodorina*	(8–10 μm)
J—*Gonyaulax* (marine)	(10 μm)
K—*Gymnodinium* (freshwater and marine)	(10–30 μm)
Nonpigmented (Phylum Protozoa):	
L—*Astasia*	(40–50 μm)
M—*Bodo*	(11–22 μm)
N—*Dinomonas*	(15–16 μm)
O—*Oikomonas*	(5–20 μm)
P—*Anisonema*	(14–60 μm)
Q—*Cercomonas*	(10–36 μm)
R—*Tetramitus*	(11–30 μm)
S—*Dendromonas*	(8 μm)

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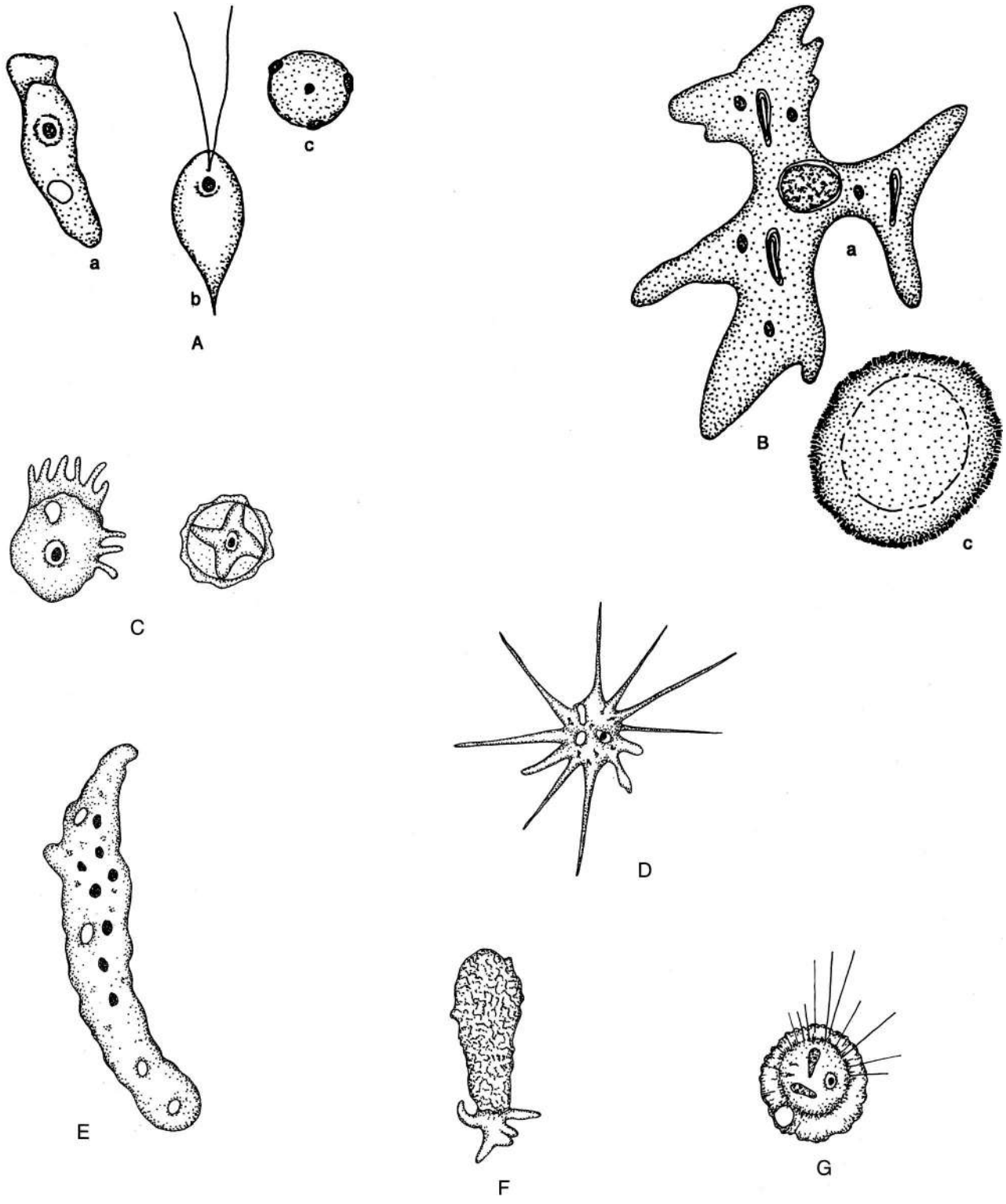


Plate 5A. Amoebas (Phylum Protozoa).

A—*Naegleria*, (a) amoeboid stage, (b) flagellated stage, (c) cyst stage	(10–36 μm)	D—*Amoeba radiosa*	(30–120 μm)
B—*Amoeba*, (a) amoeboid state, (c) cyst stage	(30–600 μm)	E—*Pelomyxa*	(0.25–3 mm)
C—*Acanthamoeba* (*Hartmannella*), (a) amoeboid stage, (c) cyst stage	(15–25 μm)	F—*Difflugia*	(40 μm)
		G—*Actinophrys*	(25–50 μm)

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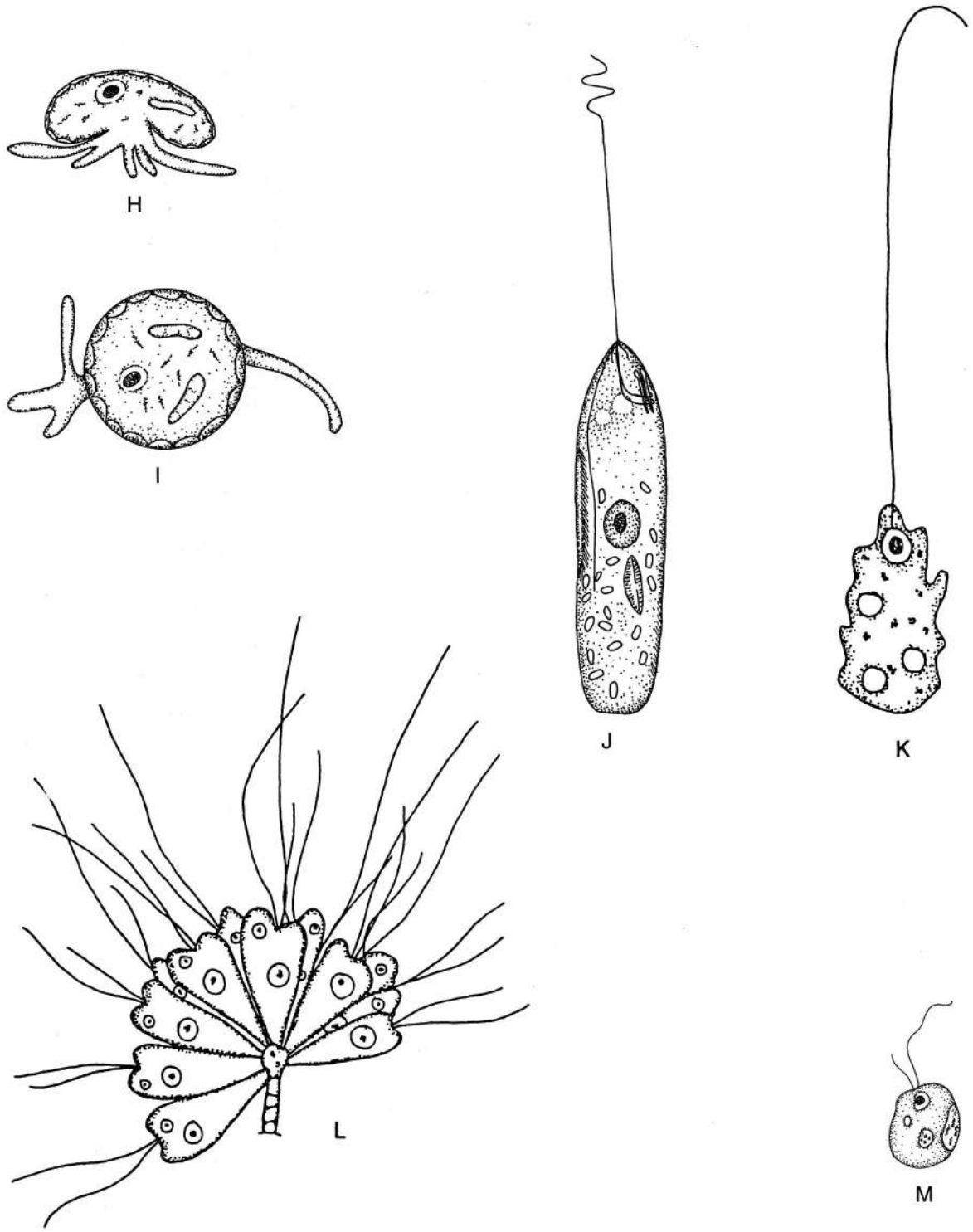


Plate 5B. Amoebas and nonpigmented flagellates (Phylum Protozoa).

Amoebas:		Nonpigmented flagellates:	
H—*Arcella* (side view)	(30–260 μm)	J—*Peranema*	(40–70 μm)
I—*Arcella* (top view)	(30–260 μm)	K—*Mastigamoeba*	(28–200 μm)
		L—*Anthophysa*	(5–6 μm)
		M—*Monas*	(5–16 μm)

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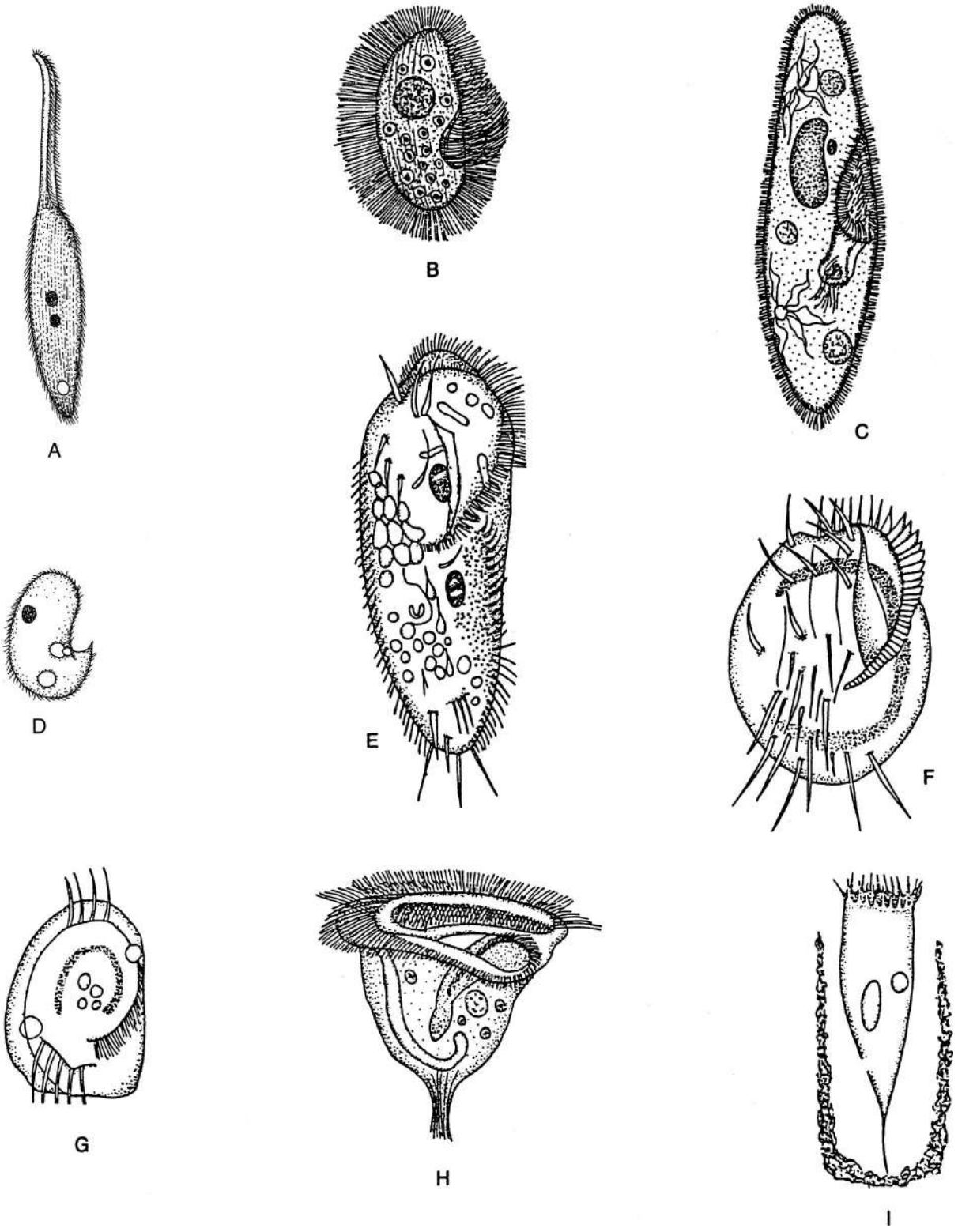


Plate 6. Ciliates (Phylum Protozoa).

A—*Lionotus*	(100 μm)	F—*Euplotes*	(70–195 μm)
B—*Pleuronema*	(38–120 μm)	G—*Aspidisca*	(30–50 μm)
C—*Paramecium*	(50–330 μm)	H—*Vorticella*	(40–175 μm)
D—*Colpoda*	(12–110 μm)	I—*Tintinnidium*	(40–200 μm)
E—*Stylonychia*	(100–300 μm)		

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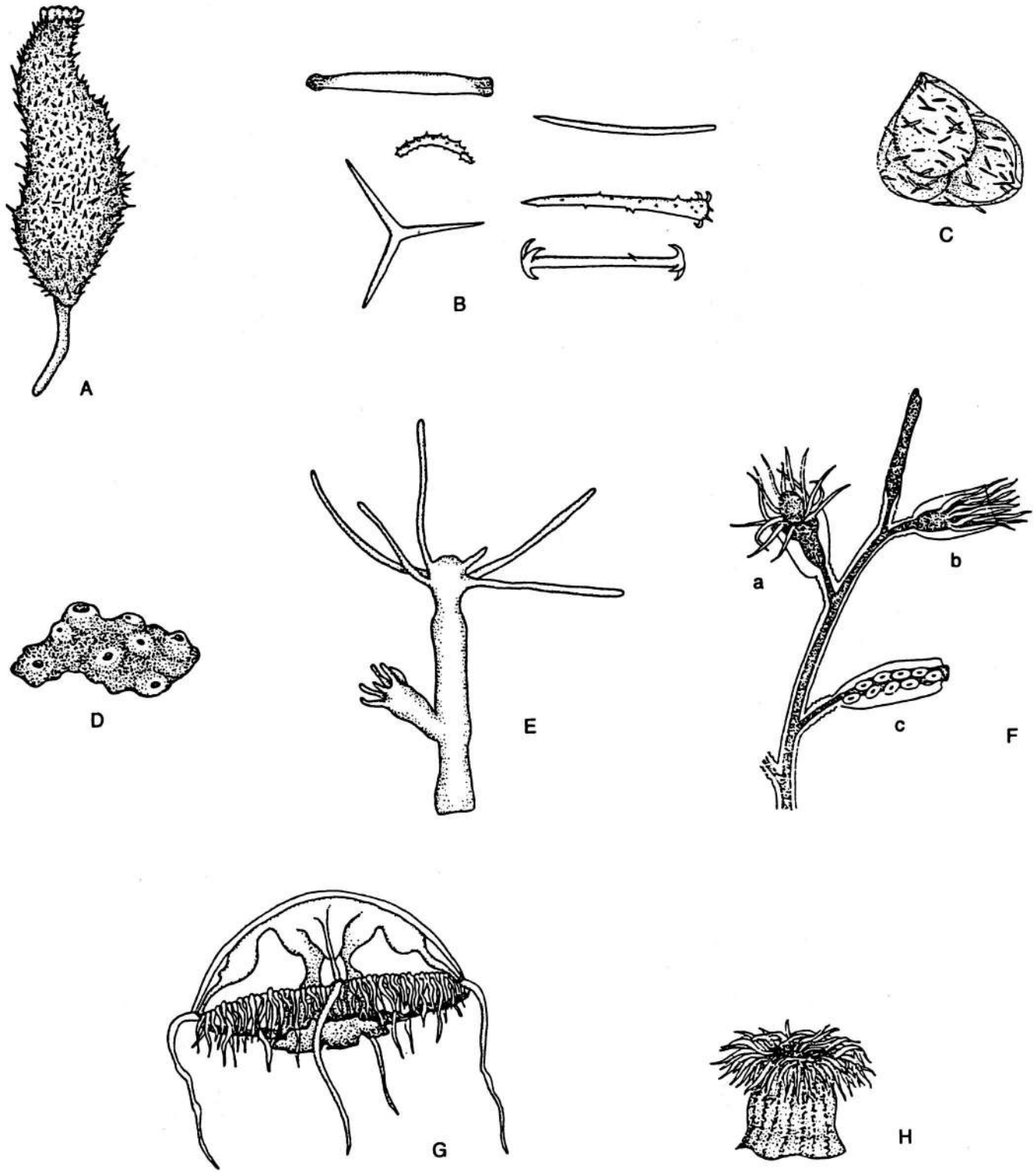


Plate 7. Sponges and coelenterates.

Sponges (Phylum Porifera):

A—Vase sponge, marine	(1 cm)
B—Sponge spicule types	(0.2–0.5 cm)
C—*Trochospongilla*, 3 gemmules, freshwater	(1 mm)
D—Purple sponge, *Haliclona*, marine	(1–10 cm)

Coelenterates (Phylum Cnidaria):

E—*Hydra* extended with bud, freshwater	(2 cm)
F—*Obelia* extended (a) and contracted (b) hydranth and gonotheca (c), marine	(2–10 cm)
G—Jellyfish (medusoid stage), *Craspedascusta*, freshwater	(10–15 mm)
H—Sea anemone, *Anthopleura*, marine	(5–15 cm)

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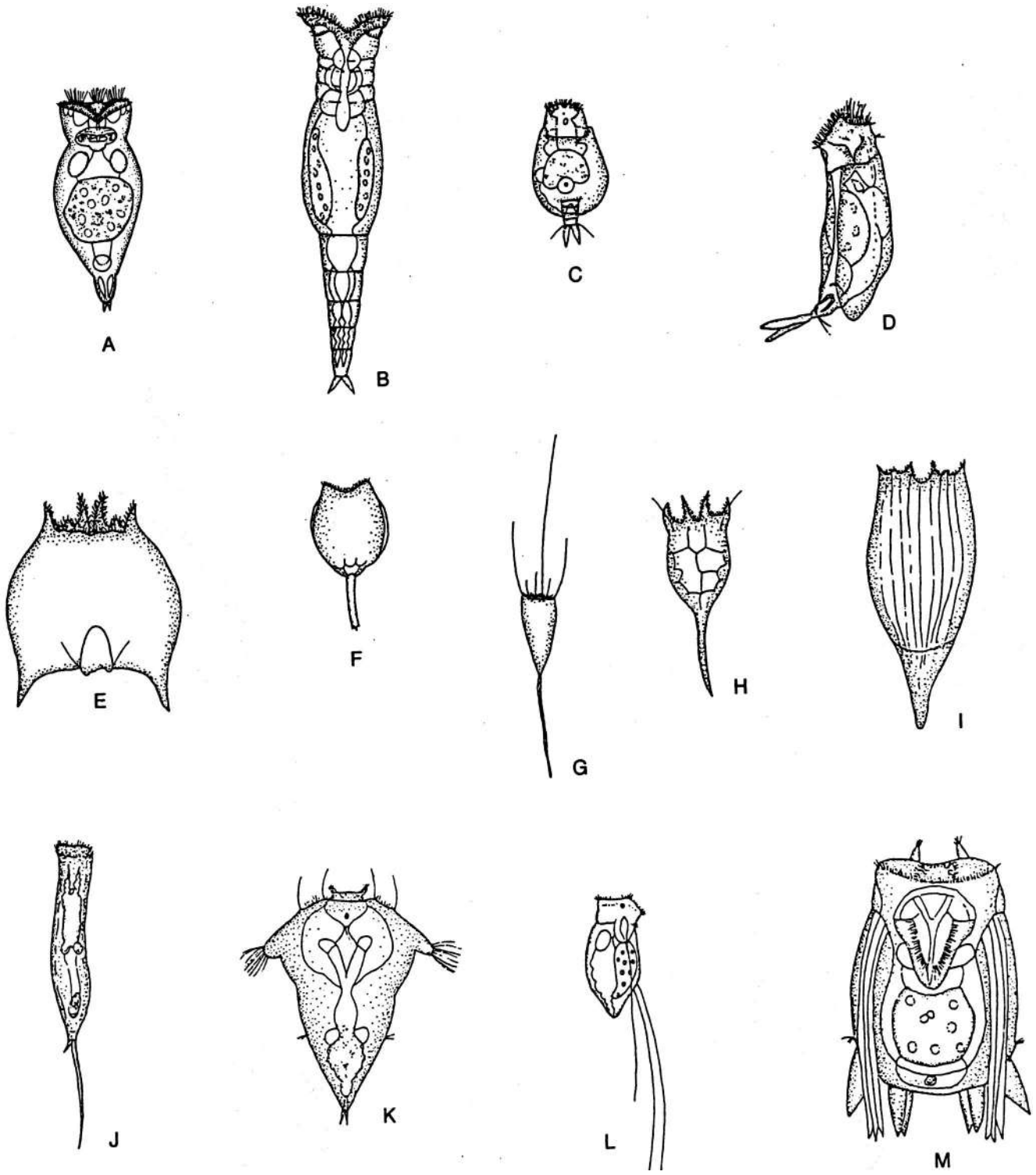


Plate 8. Rotifers (Phylum Rotifera). All freshwater inhabitants. Dimensions include spines.

A—*Epiphanes*	(0.6 mm)	H—*Keratella*	(0.2 mm)
B—*Philodina*	(0.4 mm)	I—*Notholca*	(0.2 mm)
C—*Euchlanis*	(0.25 mm)	J—*Trichocerca*	(0.6 mm)
D—*Proales*	(0.45 mm)	K—*Synchaeta*	(0.26 mm)
E—*Brachionus*	(0.2 mm)	L—*Filinia*	(0.15 mm)
F—*Monostyla*	(0.15 mm)	M—*Polyarthra*	(0.175 mm)
G—*Kellicottia*	(1 mm)		

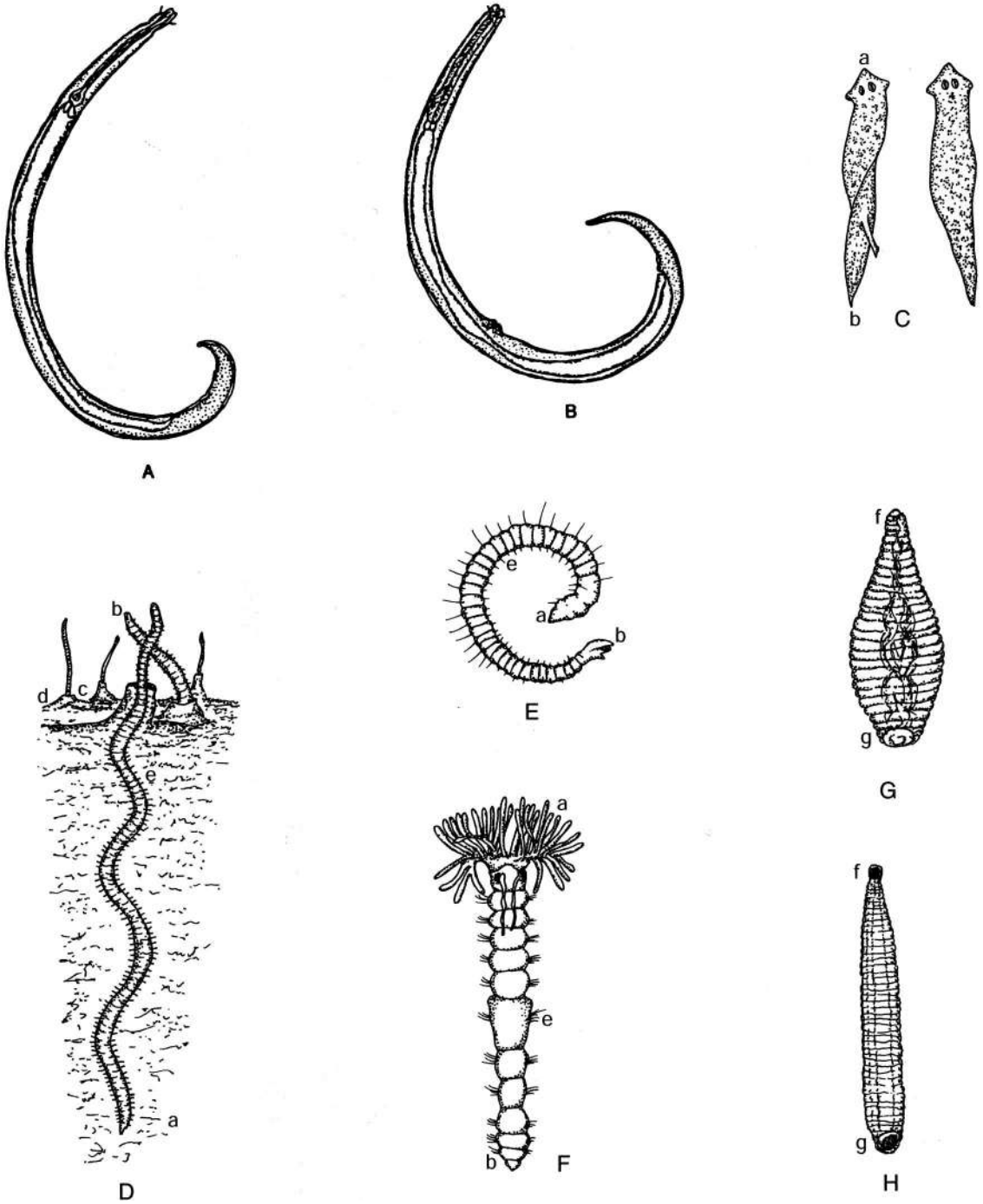


Plate 9. Roundworms, flatworms, and segmented worms. (a) anterior end, (b) posterior end, (c) tubes, (d) mud surface, (e) setae, (f) anterior sucker, (g) posterior sucker. All freshwater inhabitants.

Roundworms (Phylum Nematoda):
- A—*Plectus* (1.0 mm)
- B—*Triplya* (1.0 mm)

Flatworms (Phylum Platyhelminthes):
- C—*Planaria* (5–13 mm)

Segmented worms (Phylum Annelida, Class Oligochaeta):
- D—*Tubifex,* sludgeworm (2.5–5 mm)
- E—*Dero* (3–7 mm)

Segmented worms (Phylum Annelida, Class Polychaeta):
- F—*Manayunkia,* lives in tube (5 mm)

Segmented worms (Phylum Annelida, Class Hirudinea):
- G—Leech (3–6 mm)
- H—Leech (3–6 mm)

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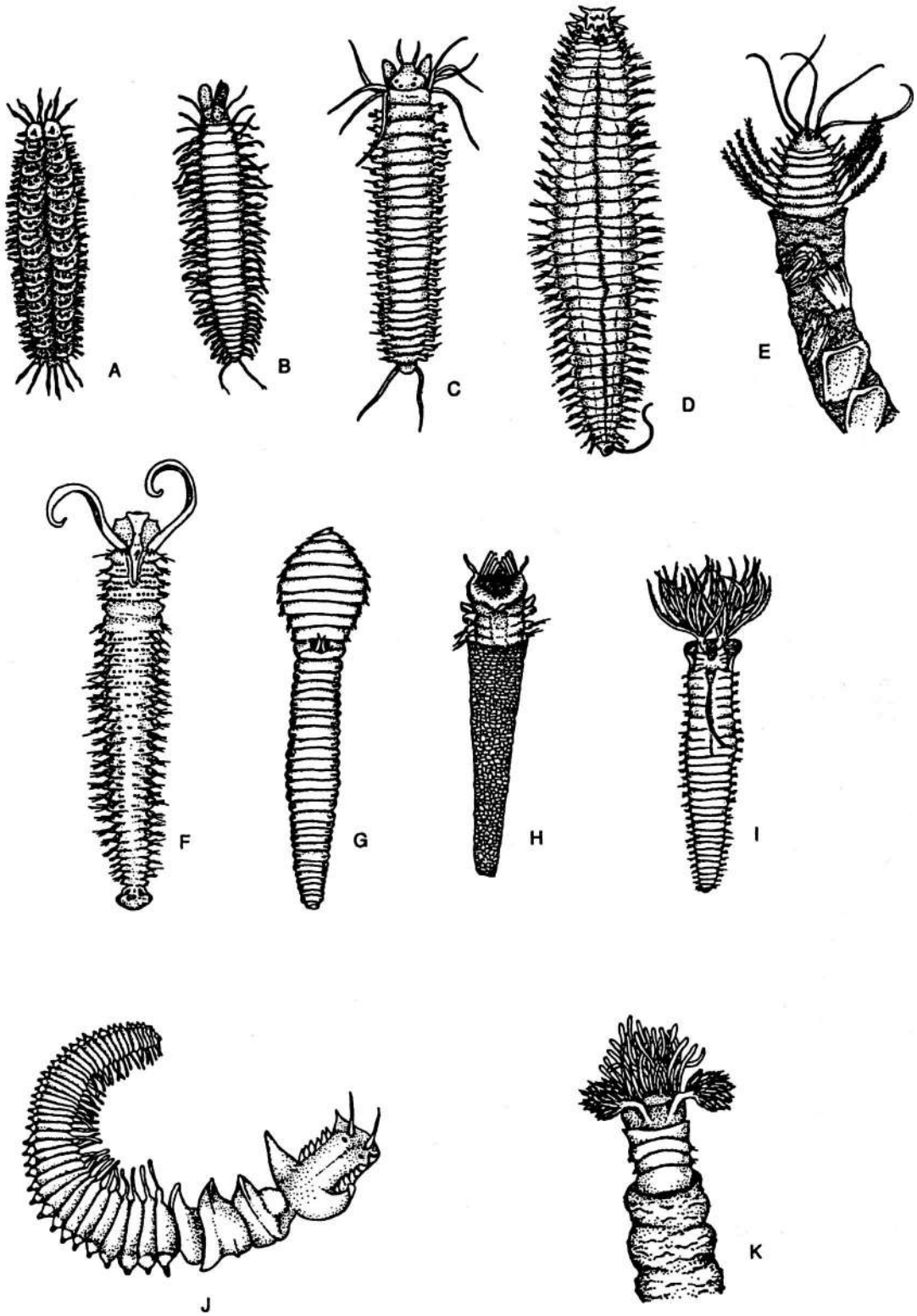


Plate 10. Segmented marine worms (Phylum Annelida, Class Polychaeta).

A—Scale worm, *Halosydna*	(5 cm)
B—Syllids, *Typosyllis*	(2–5 cm)
C—Nereid, *Nereis*	(4–15 cm)
D—Nephtyid, *Nephtys*	(5–25 cm)
E—Onuphid, *Diopatra*, in tube covered with shells	(5–25 cm)
F—Spionid, *Polydora*	(2–4 cm)
G—Capitellid, *Capitella*	(2–4 cm)
H—Ice cream cone worm, *Pectinaria*	(1–3 cm)
I—Feather duster worm, *Sabella*	(10–15 cm)
J—Chaetopterid, *Chaetopterus*, lives in U-shaped tube	(10–20 cm)
K—Terebellid, *Pista*	(10–15 cm)

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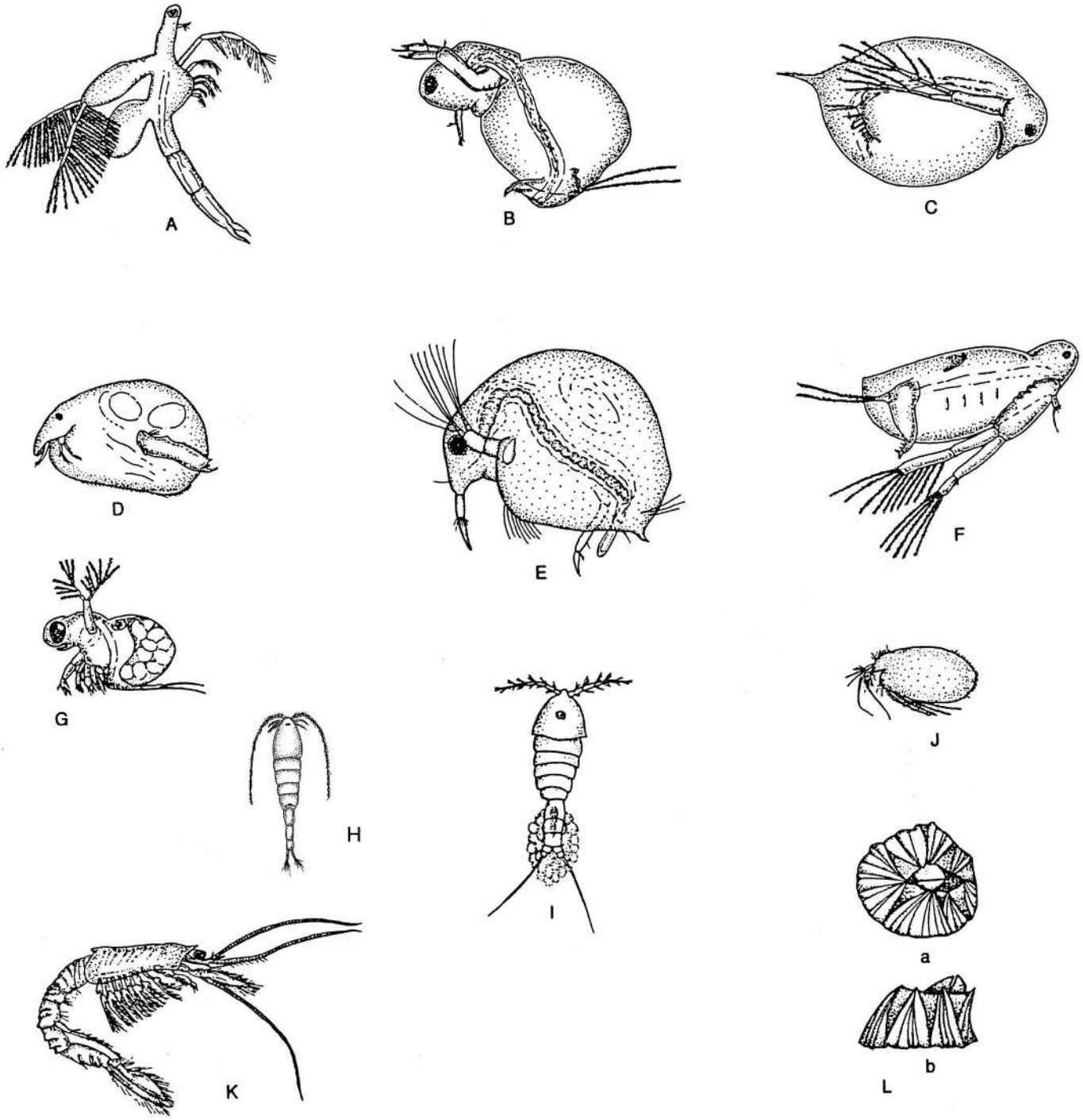


Plate 11. Crustaceans (Phylum Arthropoda)—all freshwater inhabitants, except as noted.

Order Cladocera:	
A—*Leptodora*	(9 mm)
B—*Moina*	(1.5 mm)
C—*Daphnia*	(2 mm)
D—*Alona*	(0.4 mm)
E—*Bosmina*	(0.4 mm)
F—*Diaphanosoma*	(1.5 mm)
G—*Polyphemus*	(1.5 mm)
Order Copepoda:	
H—*Diaptomus*, freshwater	(2 mm)
I—*Tigriopus*, marine	(2–4 mm)
Order Ostracoda:	
J—*Ostracod*, marine	(2–3 mm)
Order Mysidacea:	
K—*Holmesimysis*, marine	(10–20 mm)
Order Cirripedia:	
L—Barnacle, *Balanus*, marine, a. top view, b. side view	(10 mm)

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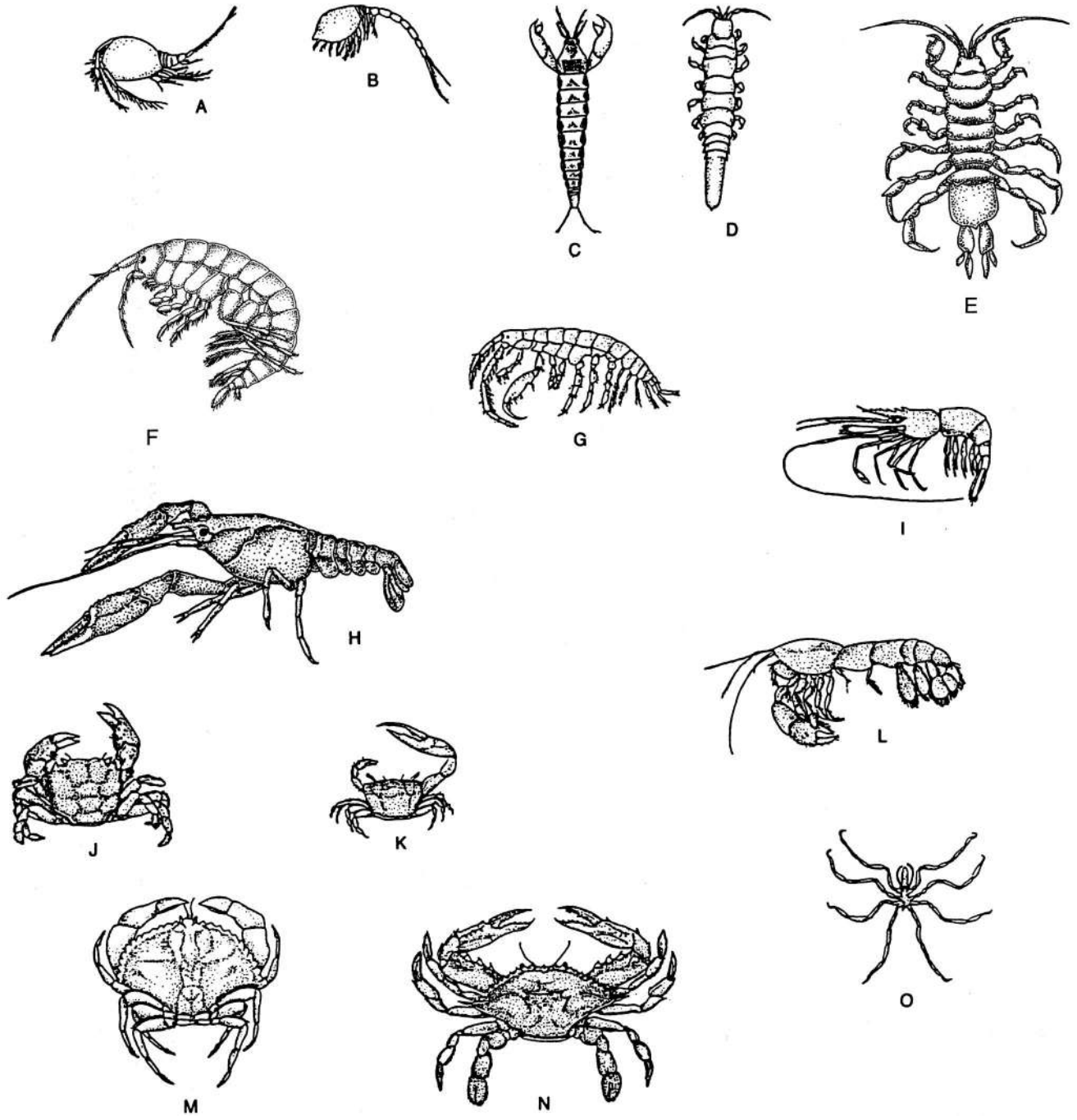


Plate 12. Crustaceans and Pycnogonid (Phylum Arthropoda).

Crustaceans:	
Order Leptostraca:	
A—*Epinebalia*, marine	(10–20 mm)
Order Cumacea:	
B—*Oxyurostylis*, marine	(8–12 mm)
Order Tanaidacea:	
C—*Anatanis*, marine	(8–10 mm)
Order Isopoda:	
D—*Idotea*, marine	(1–3 cm)
E—Sowbug, *Asellus*, freshwater	(2 cm)
Order Amphipoda:	
F—Scud, *Gammarus*, freshwater	(1.5 cm)
G—*Jassa*, marine	(1.5 cm)
Order Decapoda:	
H—Crayfish or crawdad, *Cambarus*, freshwater	(150 cm)
I—Shrimp, *Spirontocaris*, marine	(3–6 cm)
J—Mud crab, *Hemigrapsus*, marine	(3–6 cm)
K—Fiddler crab, *Uca*, marine	(3–6 cm)
L—Ghost shrimp, *Callianassa*, marine	(5–10 cm)
M—Cancer crab, *Cancer*, marine	(3–18 cm)
N—Blue crab, *Callinectes*, marine	(10–20 cm)
Pycnogonid, Class Pycnogonidea:	
O—Sea spider, pycnogonid, marine	(10–20 mm)

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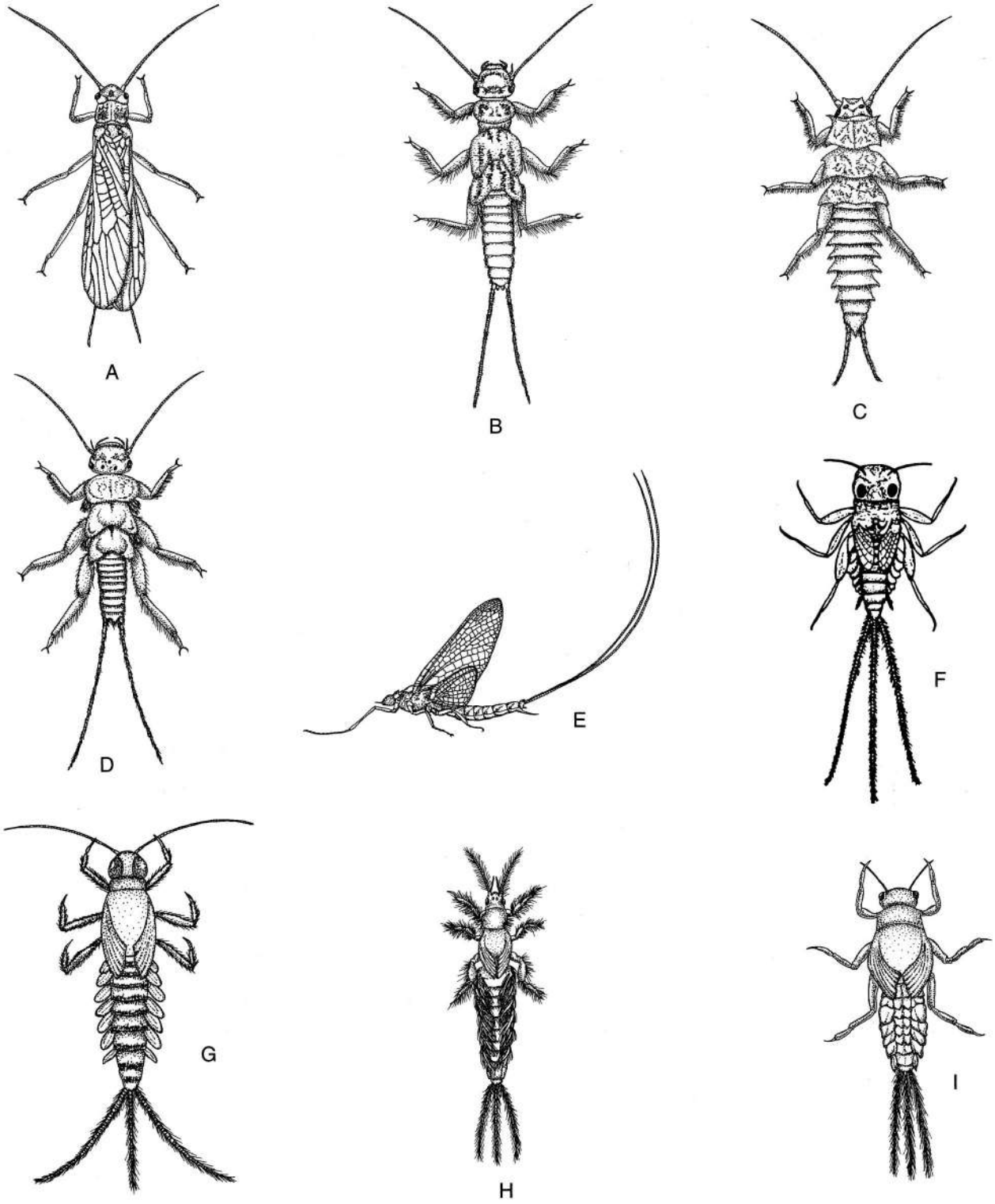


Plate 13. Stoneflies and mayflies. All freshwater inhabitants.

Stoneflies (Order Plecoptera):

A—Adult, *Isoperla*, Family Perlodidae	(14–23 mm)
B—Nymph, *Isoperla*, Family Perlodidae	(10–14 mm)
C—Nymph, *Pteronarcys*, Family Pteronarcidae	(10–40 mm)
D—Nymph, *Acroneuria*, Family Perlidae	(20–30 mm)

Mayfiles (Order Ephemeroptera):

E—Adult, mayfly, Family Heptageniidae	(12–18 mm)
F—Nymph, *Stenonema*, Family Heptageniidae	(10–14 mm)
G—Nymph, *Baetis*, Family Baetidae	(7–14 mm)
H—Nymph, *Hexagenia*, Family Ephemeridae	(20–30 mm)
I—Nymph, *Ephemerella*, Family Ephemerellidae	(8–15 mm)

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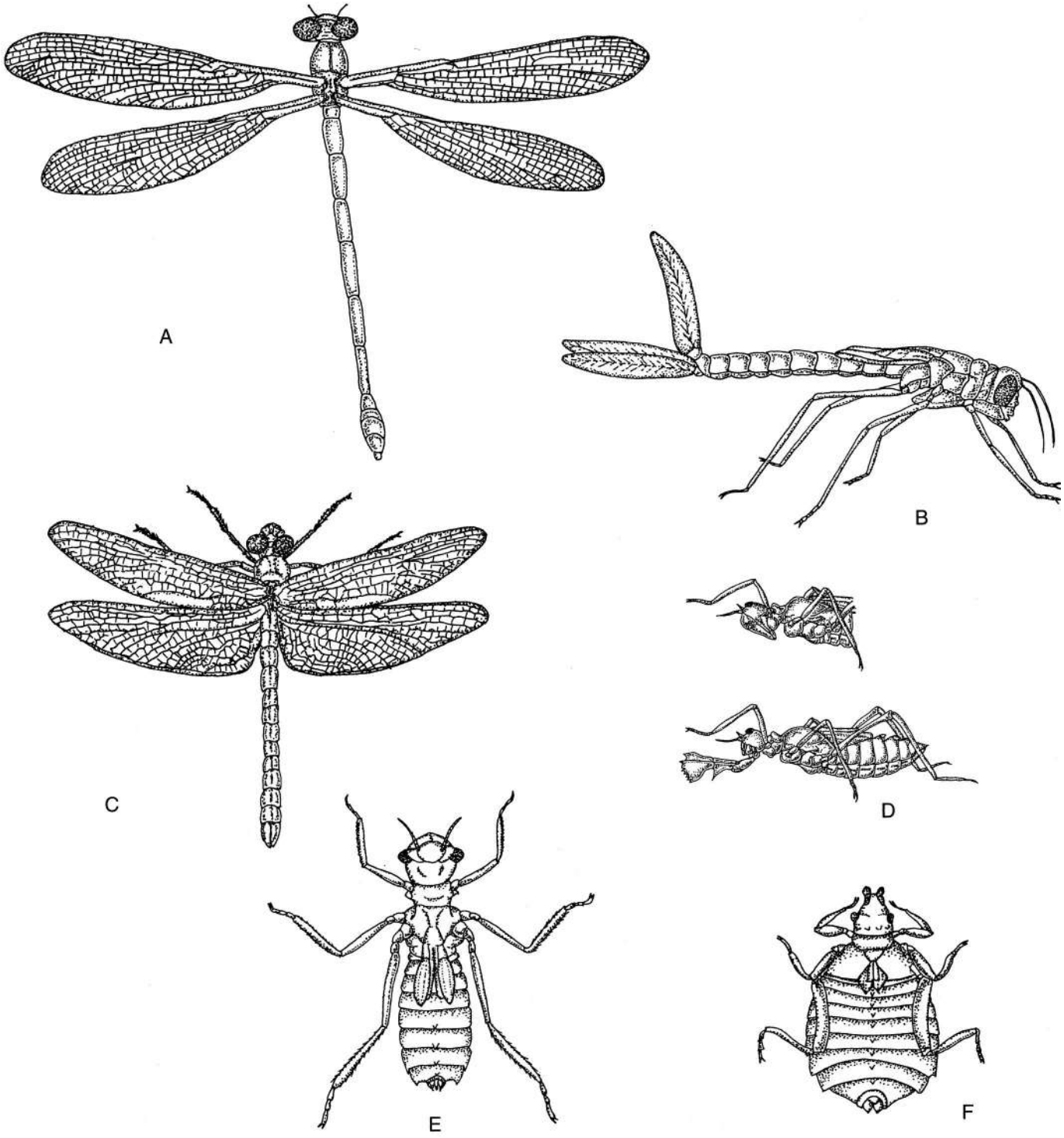


Plate 14. Damselflies, dragonflies (Order Odonata), all freshwater inhabitants.

A—Adult, damselfly	(35–55 mm)	D—Nymph, dragonfly, *Macromia*, Family Macromiidae, showing "mask" both extended and contracted	(15–45 mm)
B—Nymph, damselfly, *Lestes*, Family Lestidae	(20–30 mm)	E—Nymph, dragonfly, *Helocordulia*, Family Libellulidae	(15–45 mm)
C—Adult, dragonfly, *Macromia*, Family Macromiidae	(50–70 mm)	F—Nymph, dragonfly, *Hagenius*, Family Gomphidae	(15–20 mm)

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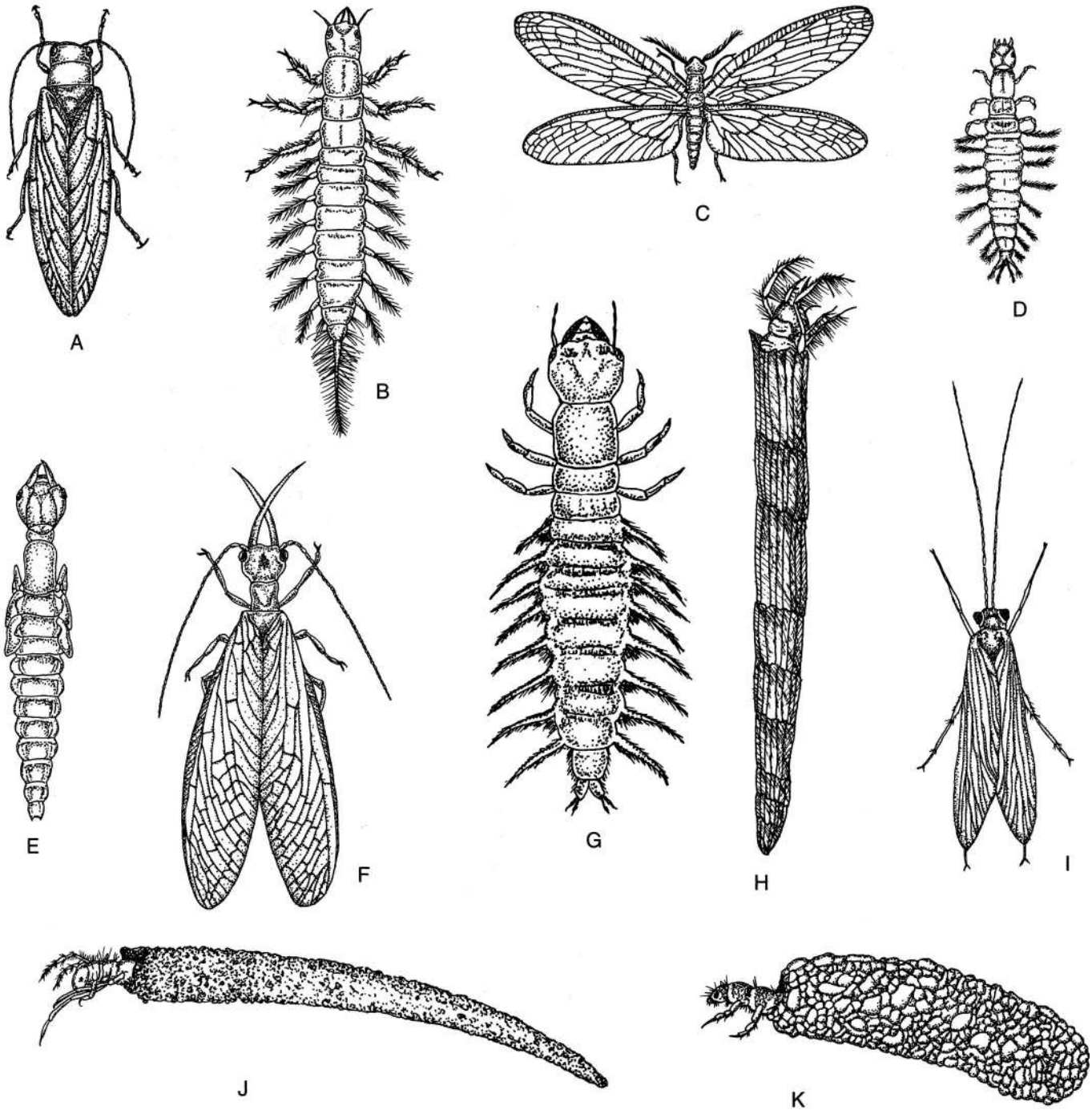


Plate 15. Hellgrammite and relatives, and caddisflies. All freshwater inhabitants.

Hellgrammite and relatives:
- A—Adult, alderfly, *Sialis*, Family Sialidae (9–15 mm)
- B—Larva, alderfly, *Sialis*, Family Sialidae (15–30 mm)
- C—Adult, fishfly, *Chauliodes*, Family Corydalidae (15–30 mm)
- D—Larva, fishfly, *Chauliodes* Family Corydalidae (20–40 mm)
- E—Pupa, hellgrammite, *Corydalus*, Family Corydalidae
- F—Adult, dobsonfly, *Corydalus*, Family Corydalidae (25–70 mm)
- G—Larva, dobsonfly, *Corydalus*, Family Corydalidae (25–90 mm)

Caddisflies:
- H—Larva and case, *Triaenodes*, Family Leptoceridae (10–14 mm)
- I—Adult, *Hydropsyche*, Family Hydropsychidae (20–30 mm)
- J—Larva and case, *Ochrotricha*, Family Hydroptilidae (4–6 mm)
- K—Larva and case, *Leptocella*, Family Leptoceridae (14–18 mm)

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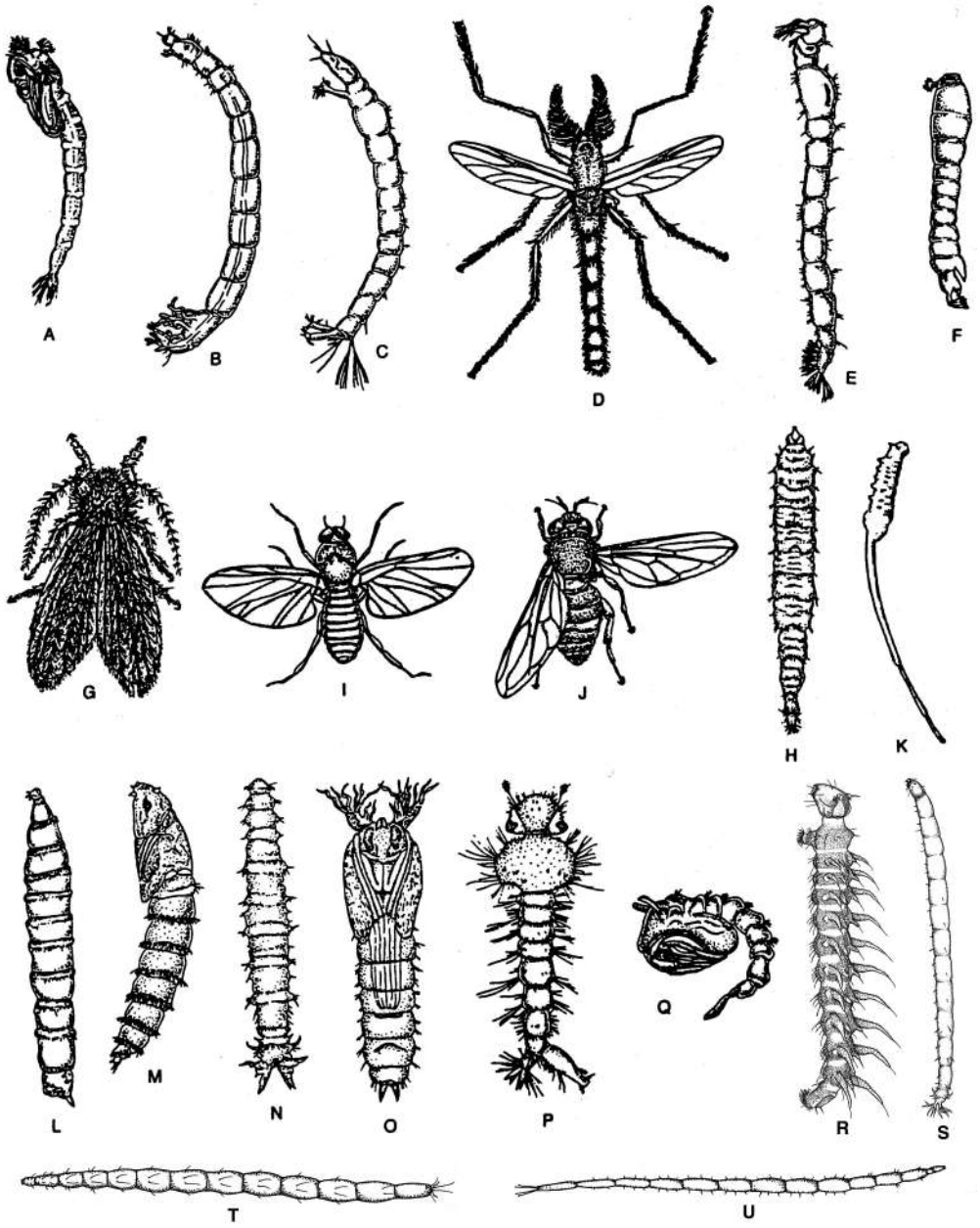


Plate 16. Two-winged flies (Order Diptera). All freshwater inhabitants.

A—Pupa, midge, *Chironomus*, Family Chironomidae	
B—Larva, midge, *Chironomus*, Family Chironomidae	(5–30 mm)
C—Larva, midge, *Ablabesmyia*, Family Chironomidae	(5–10 mm)
D—Adult, midge, Chironomidae	(4–12 mm)
E—Larva, phantom midge, *Chaoborus*, Family Culicidae	(8–12 mm)
F—Larva, housefly, *Musca*, Family Muscidae	(3–8 mm)
G—Adult, sewage fly, *Psychoda*, Family Psychodidae	(2–5 mm)
H—Larva, sewage fly, *Psychoda*, Family Psychodidae	(4–6 mm)
I—Adult, blackfly, *Simulium*, Family Simuliidae	(2–6 mm)
J—Adult, drone fly, Family Syrphidae	(10–15 mm)
K—Rat-tailed maggot, *Eristalis*, Family Syrphidae	(15–30 mm)
L—Larva, *Tabanus*, Family Tabanidae	(30–40 mm)
M—Blowfly, *Tabanus*, Family Tabanidae	
N—Larva, cranefly, *Tipula*, Family Tipulidae	(30–40 mm)
O—Pupa, cranefly, *Antocha*, Family Tipulidae	
P—Larva, mosquito, *Aedes*, Family Culicidae	(10–15 mm)
Q—Pupa, mosquito, *Culex*, Family Culicidae	

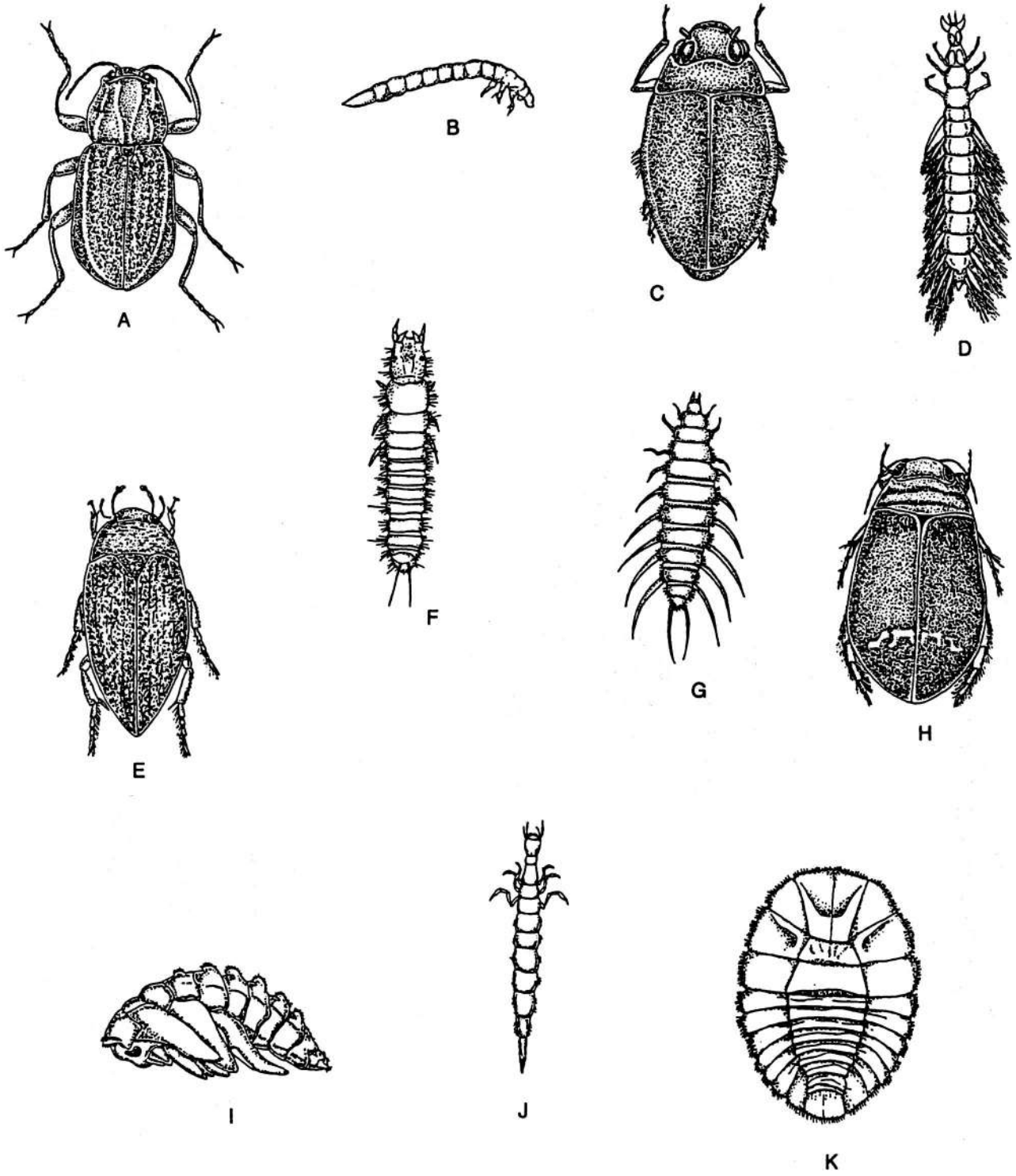


Plate 17. Beetles (Order Coleoptera). All freshwater inhabitants.

A—Adult, riffle beetle, *Stenelmis*, Family Elmidae	(4–10 mm)
B—Larva, *Narpus*, Family Elmidae	(7–15 mm)
C—Adult, whirligig beetle, *Dineutus*, Family Gyrinidae	(10–30 mm)
D—Larva, *Dineutus*, Family Gyrinidae	(2–40 mm)
E—Adult, water scavenger beetle, *Hydrophilus*, Family Hydrophilidae	(5–20 mm)
F—Larva, *Berosus*, Family Hydrophilidae	(2–5 mm)
G—Larva, *Enochrus*, Family Hydrophilidae	(10–25 mm)
H—Adult, predaceous diving beetle, *Dytiscus*, Family Dytiscidae	(2–40 mm)
I—Pupa, *Cybister*, Family Dytiscidae	
J—Larva, *Cybister*, Family Dytiscidae	(10–25 mm)
K—Larva, water penny, *Psephenus*, Family Psephenidae	(3–10 mm)

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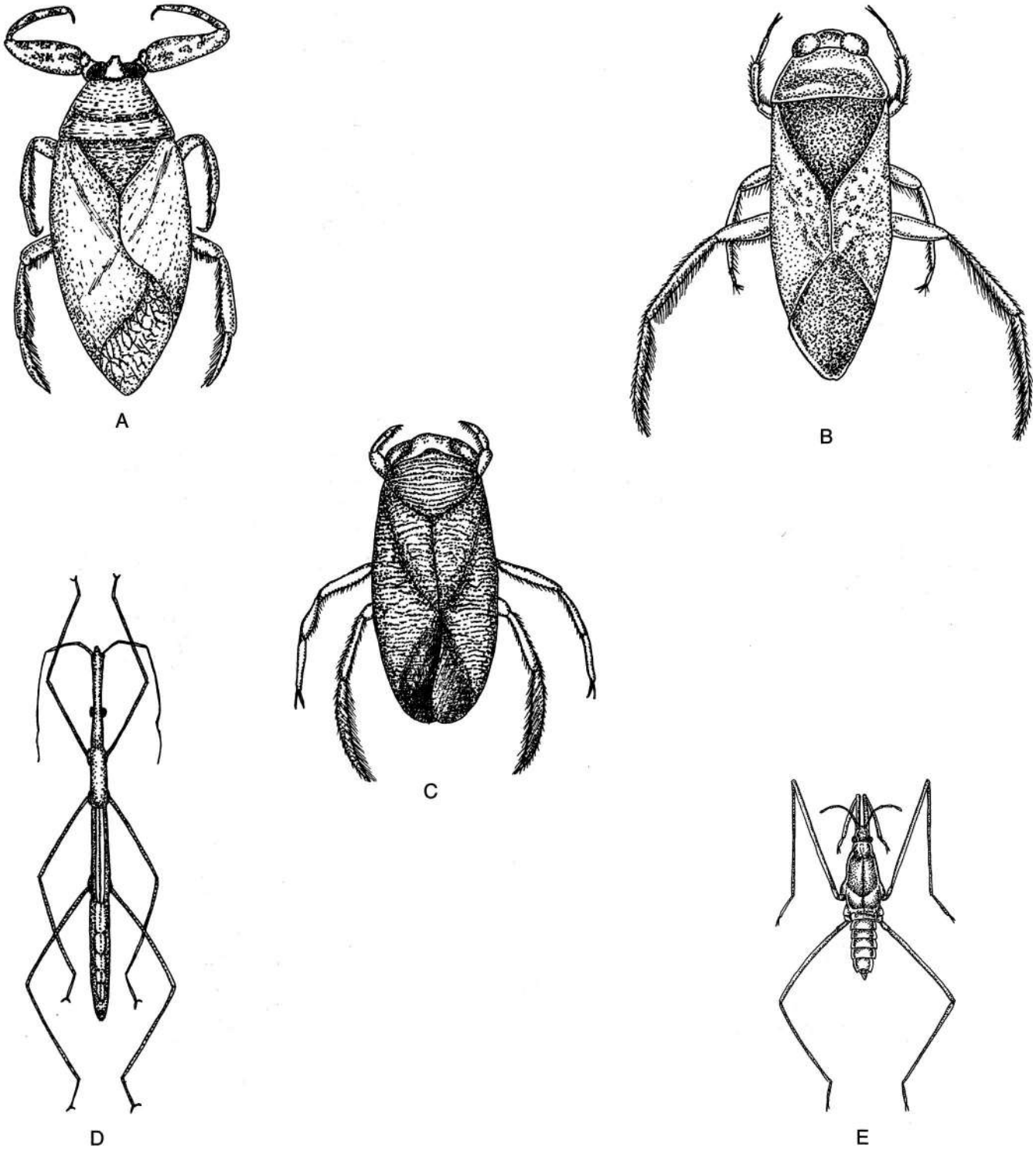


Plate 18. True bugs (Order Hemiptera, all adults). All freshwater inhabitants.

A—Electric light bug, *Lethocerus*, Family Belostomidae (20–70 mm)
B—Backswimmer, *Notonecta*, Family Notonectidae (5–17 mm)
C—Water boatman, *Sigara*, Family Corixidae (3–12 mm)
D—Marsh treader, *Hydrometra*, Family Hydrometridae (8–11 mm)
E—Water strider, *Gerris*, Family Gerridae (2–15 mm)

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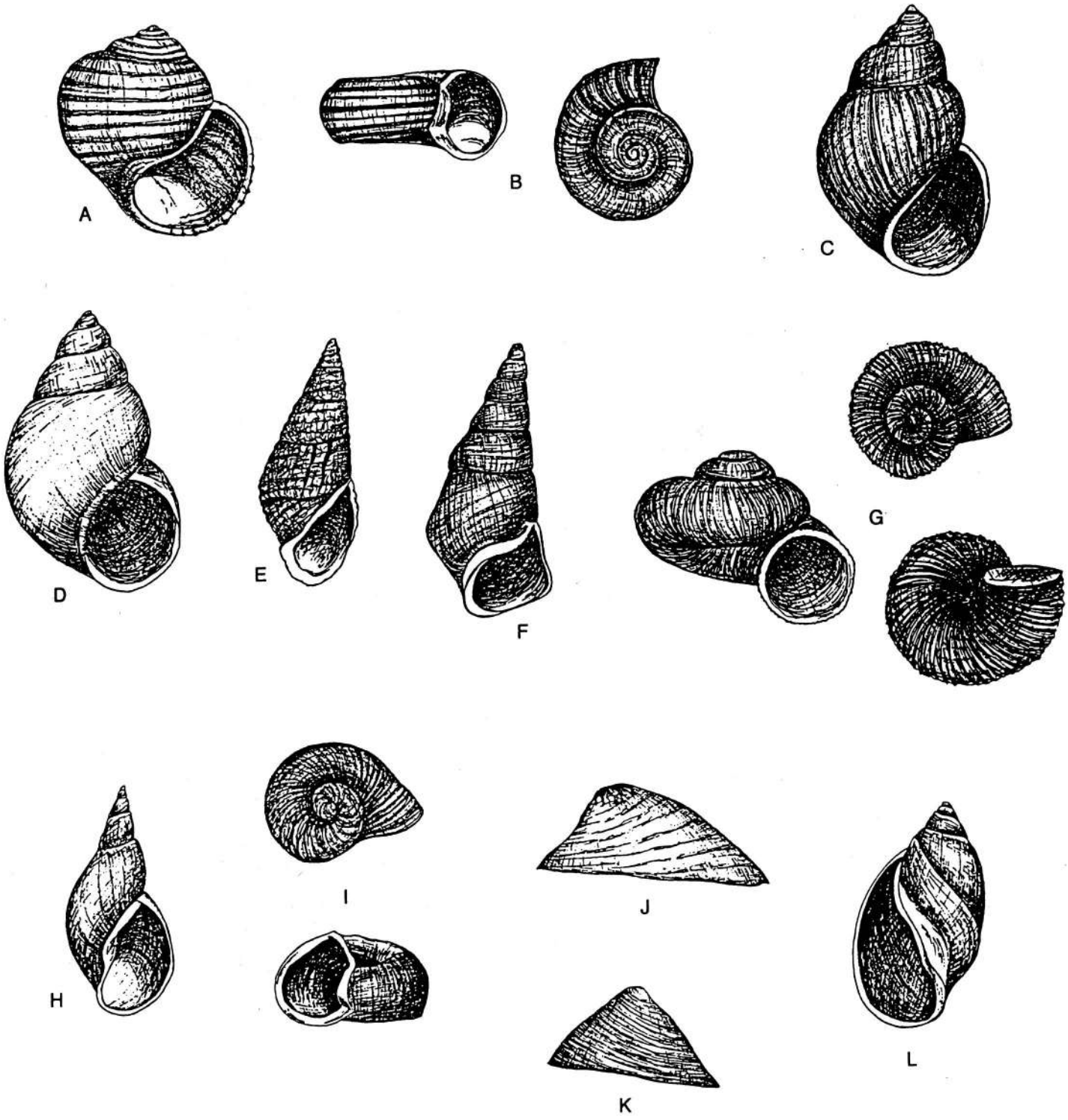


Plate 19. Snails (Phylum Mollusca, Class Gastropoda). All freshwater inhabitants.

A—Apple snail, *Pomacea*, Family Pilidae	(5 cm)	G—*Valvata*, side, top and bottom views, Family Valvatidae	(1 cm)
B—*Marisa*, side and top views, Family Pilidae	(15 mm)	H—Pond snail, *Lymnaea*, Family Lymnaeidae	(15 mm)
C—*Campeloma*, Family Viviparidae	(4 cm)	I—Orb snail, top and side views, *Helisoma*, Family Planorbidae	(1 cm)
D—Faucet snail, *Bithynia*, Family Ammicolidae	(2 cm)	J—Limpet, *Ferrissia*, Family Ancylidae	(2 mm)
E—*Tarebia*, Family Thiaeidae	(15 mm)	K—Limpet, *Lanx*, Family Lancidae	(10 mm)
F—River snail, *Pleurocera*, Family Pleuroceridae	(3 cm)	L—Pouch snail, *Physa*, Family Physidae	(3–5 mm)

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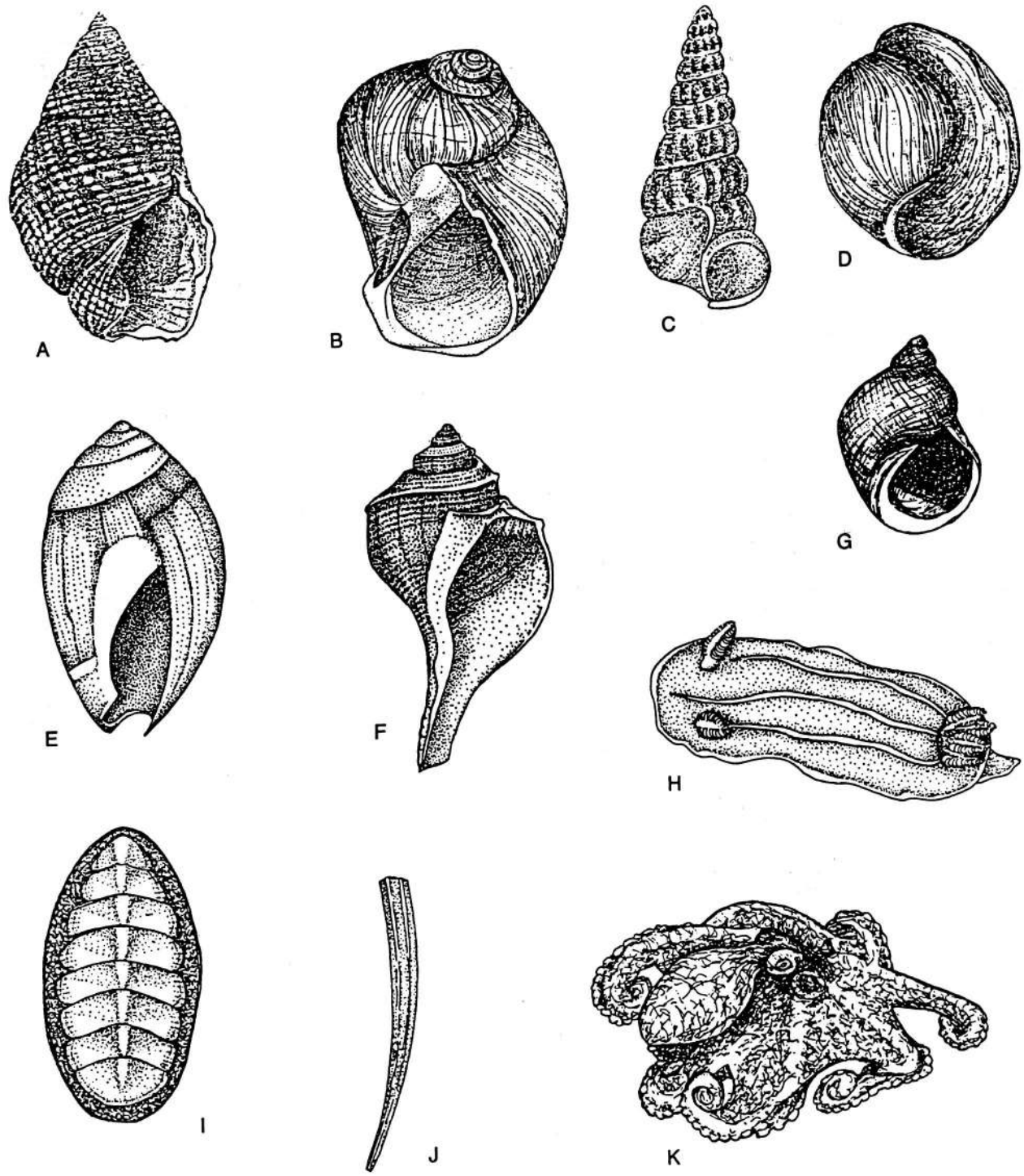


Plate 20. Some marine mollusks.

Snails (Class Gastropoda):	
A—Basket snail, *Nassarius*, Family Nassidae	(2–5 cm)
B—Moon snail, *Polinices*, Family Naticidae	(5–8 cm)
C—Horn snail, *Cerithidea*, Family Cerithiidae	(1–3 cm)
D—Bubble snail, *Bulla*, Family Bullidae	(3–5 cm)
E—Olive snail, *Olivella*, Family Olividae	(2–3 cm)
F—*Busycon*, Family Melongenidae	(5–8 cm)
G—Periwinkle, *Littorina*, Family Littorinidae	(1 cm)
H—Sea slug, *Chromodoris*	
Chitons (Class Polyplacophora):	
I—Conspicious chiton, *Stenoplax*	(3–6 cm)
Tusk shell (Class Scaphopoda):	
J—*Dentalium*	(2–4 cm)
Octopus (Class Cephalopoda):	
K—*Octopus*	(3–10 cm up to 11 m or more)

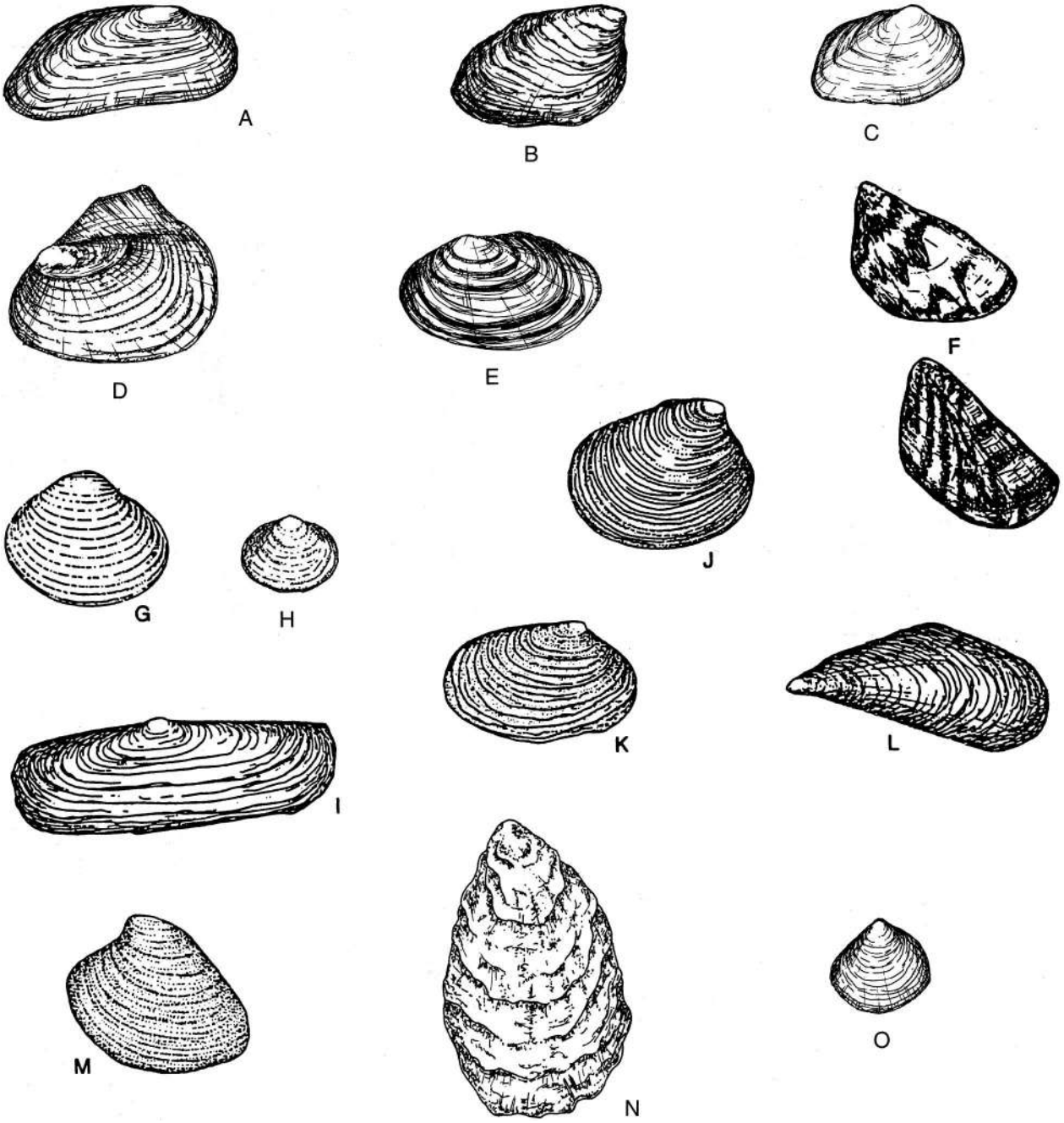


Plate 21. Bivalves (Phylum Mollusca, Class Pelecypoda).

Freshwater species:

A—Spectacle case, *Margaritifera*, Family Margaritiferidae	(10 cm)
B—Pearly mussel, *Pleurobema*, Family Unionidae	(10 cm)
C—Pearly mussel, *Gonidea*, Family Unionidae	(10 cm)
D—Winged lampshell, *Proptera*, Family Lampsilinae	(13 cm)
E—Papershell, *Anodonta*, Family Anodontidae	(14 cm)
F—Zebra mussel (examples of color patterns), *Dreissena*	(5 cm)
G—Asiatic clam, *Corbicula*, Family Corbiculidae	(4 cm)
H—Fingernail clam, *Sphaerium*, Family Sphaeriidae	(1 cm)

Marine species:

I—Jackknife clam, *Tagelus*, Family Psammobiidae	(6–10 cm)
J—Quahog clam, *Mercenaria*, Family Veneridae	(6–10 cm)
K—Soft-shelled clam, *Mya*, Family Myidae	(5–10 cm)
L—Blue mussel, *Mytilus*, Family Mytilidae	(6 cm)
M—Rangia clam, *Rangia*, Family Matricidae	(5 cm)
N—Oyster, *Crassostrea*, Family Ostreidae	(9 cm)
O—Marsh clam, *Polymesoda*, Family Corbiculidae	(4 cm)

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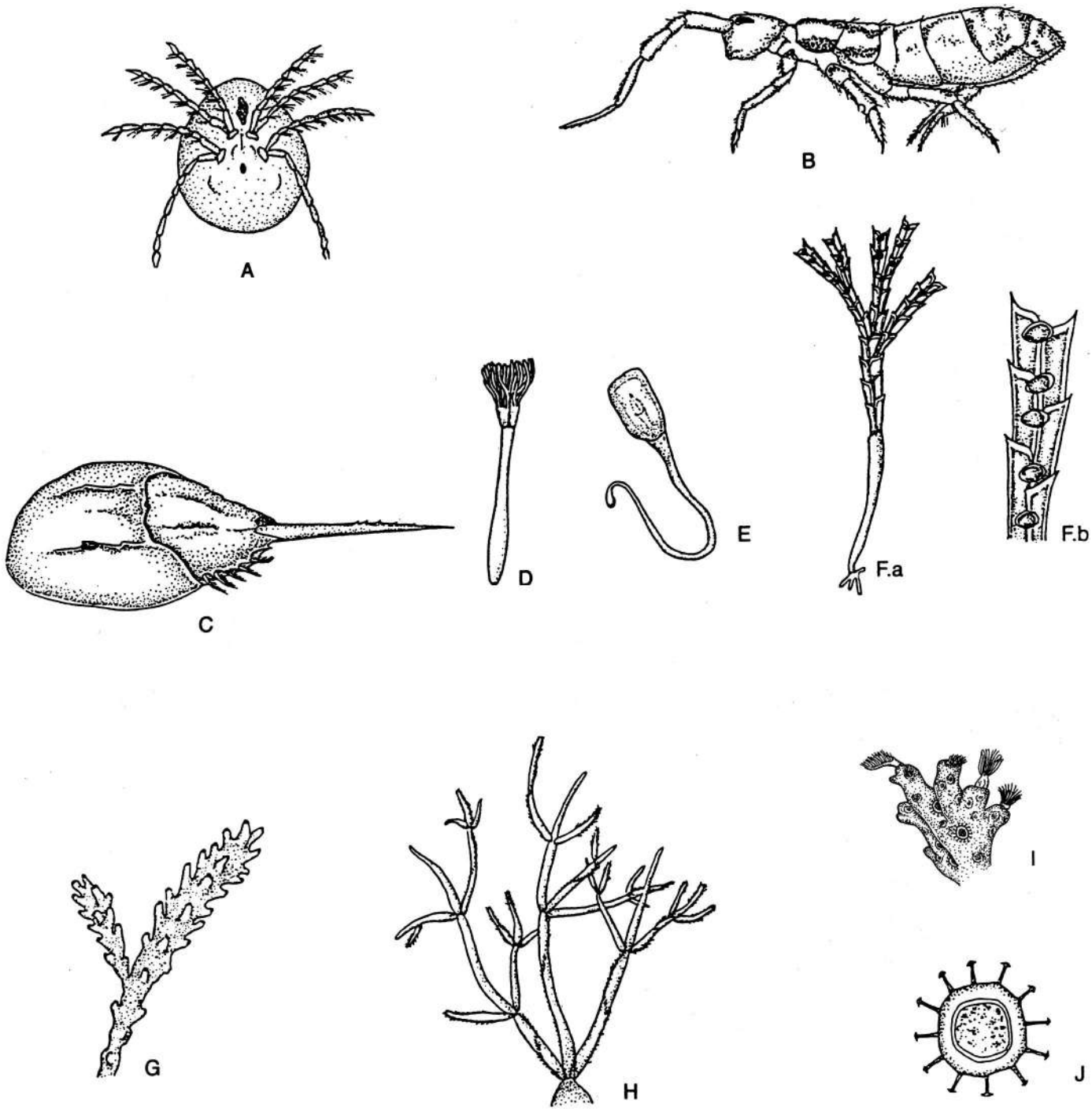


Plate 22. Miscellaneous invertebrates.

Arthropods (Phylum Arthropoda):
- A—Water mite, *Limnochares*, Class Arachnoidea, freshwater (3 mm)
- B—Springtail, *Orchesella*, Class Insecta, Order Collembola, freshwater (2 mm)
- C—Horsehoe crab, *Limulus*, Class Arachnoidea, marine (10–30 cm)
- D—*Phoronis*, Phylum Phoronidea, marine (2–4 cm)
- E—*Glottidia*, Phylum Brachiopoda, marine (3–5 cm)

Phylum Ectoprocta (Bryozoa):
- F—*Bugula*, a. colony, b. enlarged view, marine (1–3 cm)
- G—*Crisulipora*, calcareous colony, marine (1–3 cm)
- H—*Zoobotryon*, gelatinous colony, marine (20–100 cm)
- I—Jellyball, *Pectinatella*, young colony, freshwater (11 cm)
- J—*Pectinatella*, statoblast, freshwater (1 mm)

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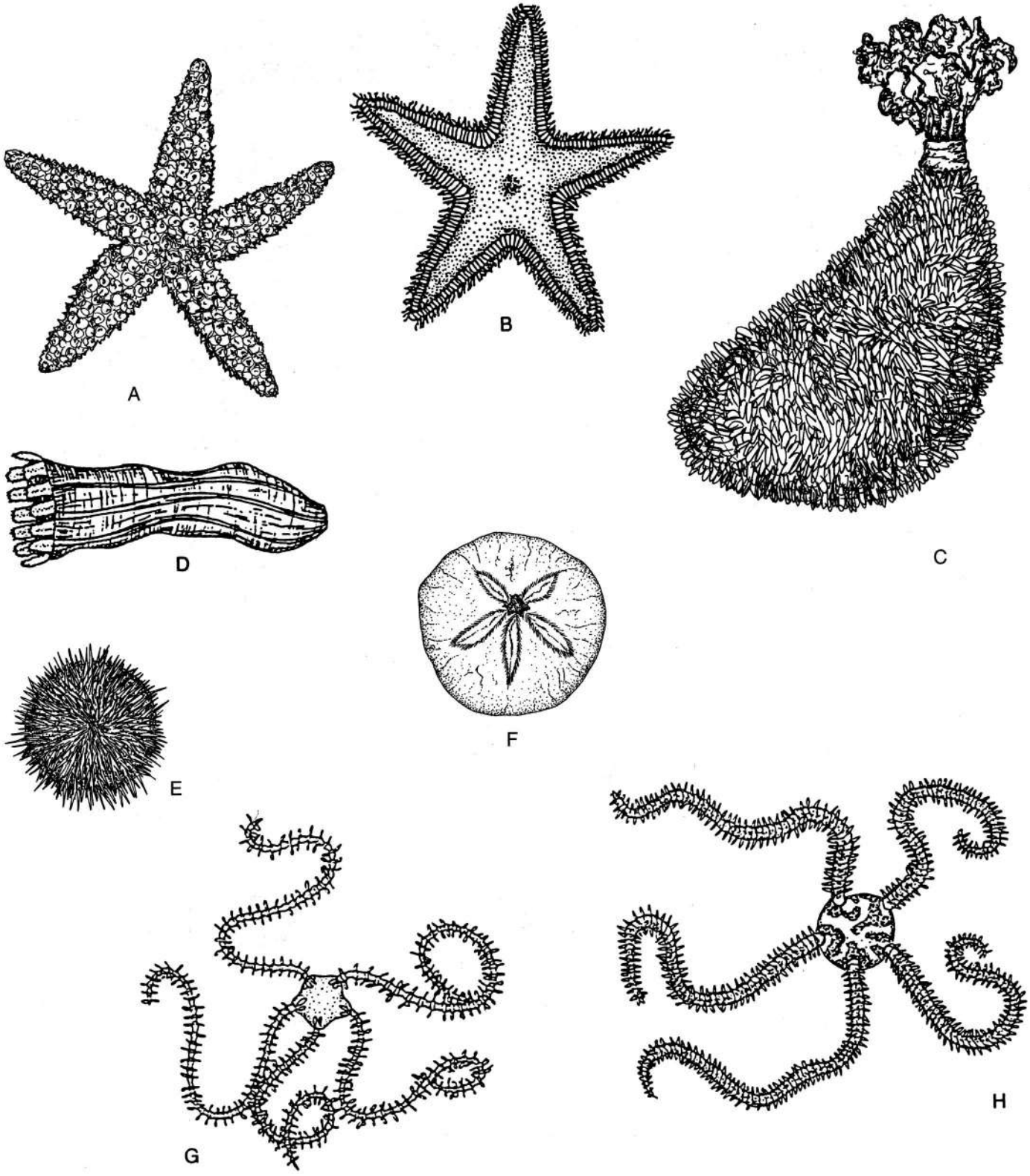


Plate 23. Echinoderm types (Phylum Echinodermata). All marine.

A—Starfish, *Asterias*, Class Asteroidea	(15 cm)
B—Sand starfish, *Astropecten*, Class Asteroidea	(10–20 cm)
C—Sea cucumber, *Thyrone*, Class Holothuroidea	(10 cm)
D—Sea cucumber, *Leptosynpta*, Class Holothuroidea	(5–8 cm)
E—Sea urchin, *Strongylocentrotus*, Class Echinoidea	(6 cm)
F—Sand dollar, *Echinarachnius*, Class Echinoidea	(7 cm)
G—Brittle star, *Amphiodia*, Class Ophiuroidea	(disk 15 mm)
H—Brittle star, *Ophiopholis*, Class Ophiuroidea	(disk 15 mm)

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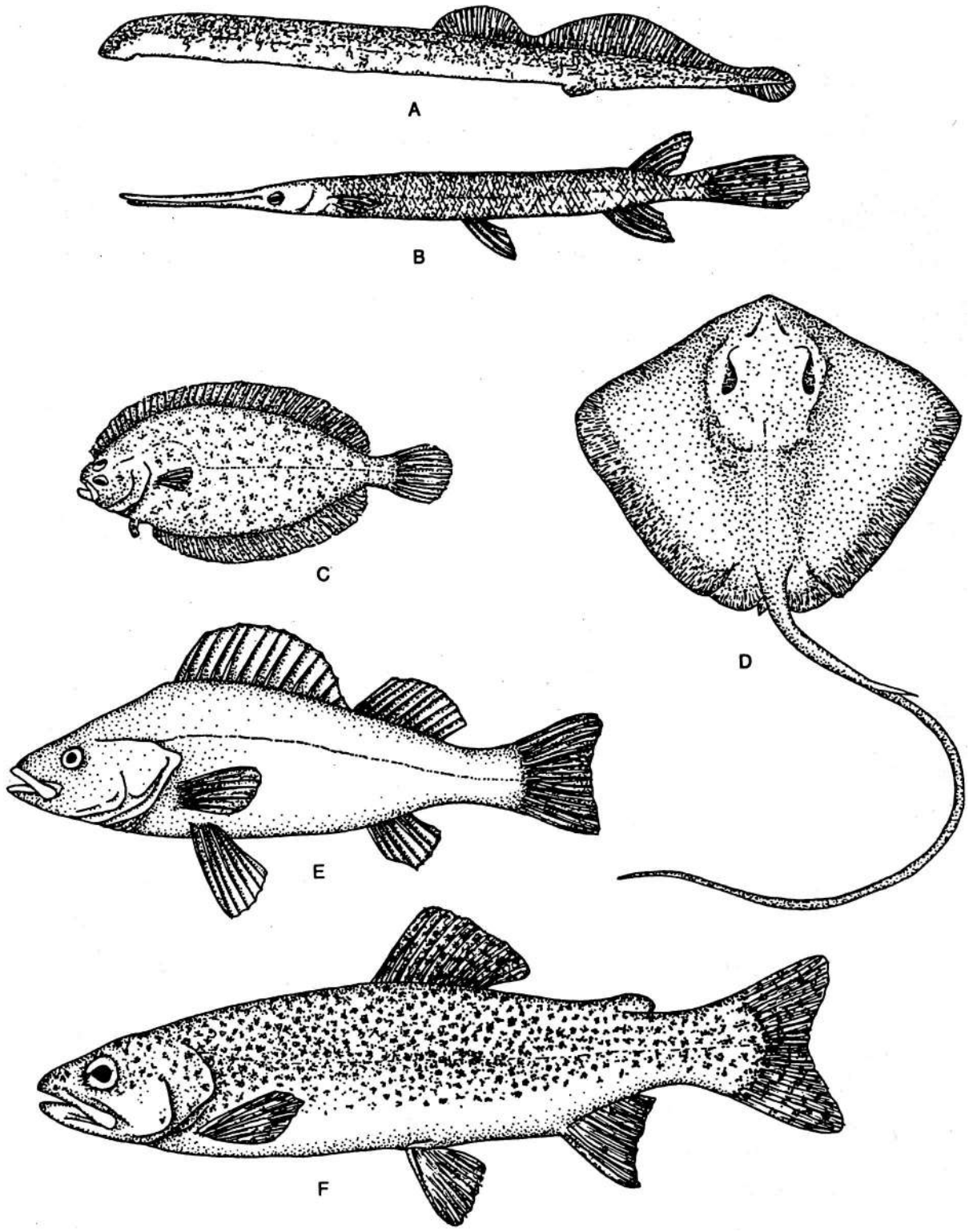


Plate 24. Some types of fishes (Phylum Chordata).

A—Jawless fish, lamprey, *Petromyzon*, Class Agnatha, freshwater (30–45 cm)

B—Ganoid fish, long-nosed gar, *Lepisosteus*, Class Osteichthys, freshwater (2.4 m)

C—Flatfish, flounder, *Paralichthys*, Class Osteichthys, marine (30–60 cm)

D—Cartilage fish, stingray, *Dasyatis*, Class Chondrichthys, marine (2 m)

E—Spiny-rayed fish, perch, *Perca*, Class Osteichthys, freshwater (30 cm)

F—Soft-rayed fish, rainbow trout, *Salmo*, Class Osteichthys, freshwater (30 cm)

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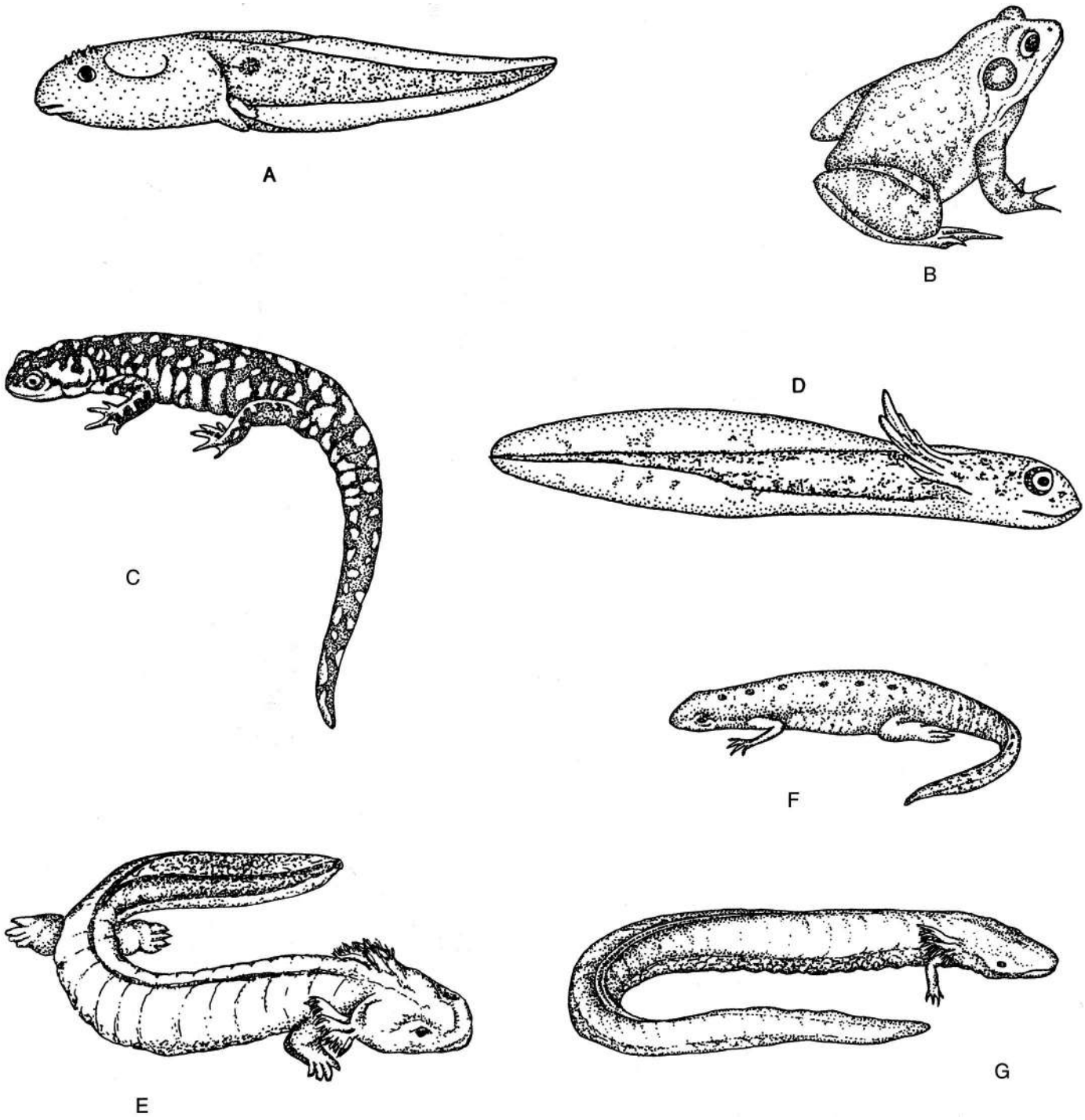


Plate 25. Types of amphibians (Phylum Chordata, Class Amphibia). All freshwater.

Frogs and toads (Order Salientia):
- A—Tadpole larva with hind legs beginning to develop from the body of the tadpole
- B—Frog, *Rana* (30 cm)

Salamanders (Order Caudata):
- C—*Ambystoma*, adult, typically terrestrial (20 cm)
- D—*Ambystoma*, larva, inhabits water (5–15 cm)
- E—Water dog or mud puppy, *Necturus*, gills present in adult (to 30 cm)
- F—Salamander with flat tail, *Notophthalmus* (9 cm)
- G—Salamander, *Siren*, with gills and front pair of legs present (1 m)

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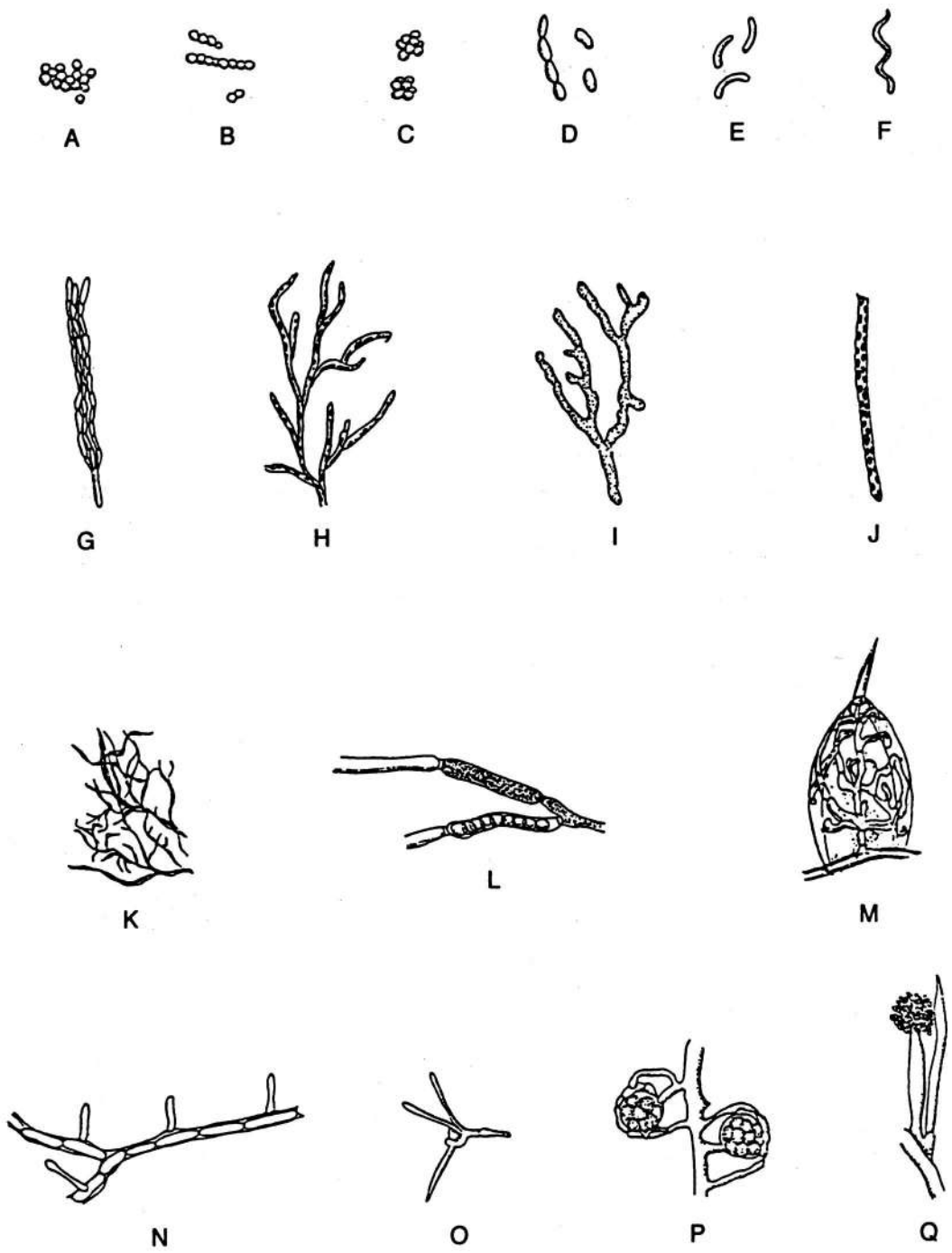


Plate 26. Bacteria and fungi. Diameter of most bacterial cells is <2 μm, although *Beggiatoa* (J) may range up to 16 μm in diameter and be of indefinite length. All freshwater inhabitants.

Bacterial cellular forms and arrangements:
- A—Micrococcus
- B—Streptococcus
- C—Sarcina
- D—Bacillus
- E—Vibrio
- F—Spirillum

Sewage organisms:
- G—*Sphaerotilus* cells
- H—*Sphaerotilus* growth form
- I—*Zoogloea* growth form
- J—*Beggiatoa* (sulfur bacterium)
- K—An actinomycete growth form from compost

Fungi:
- L—*Leptomitus*, showing zoospores and cellulin plugs
- M—*Zoophagus* with rotifer on mycelial peg (diam 3 μm)
- N—*Zoophagus*, showing mycelial pegs
- O—*Tetracladium* (diam 2.5–3.5 μm)
- P—*Achlya*, with oospores
- Q—*Achlya*, with encysted oospores

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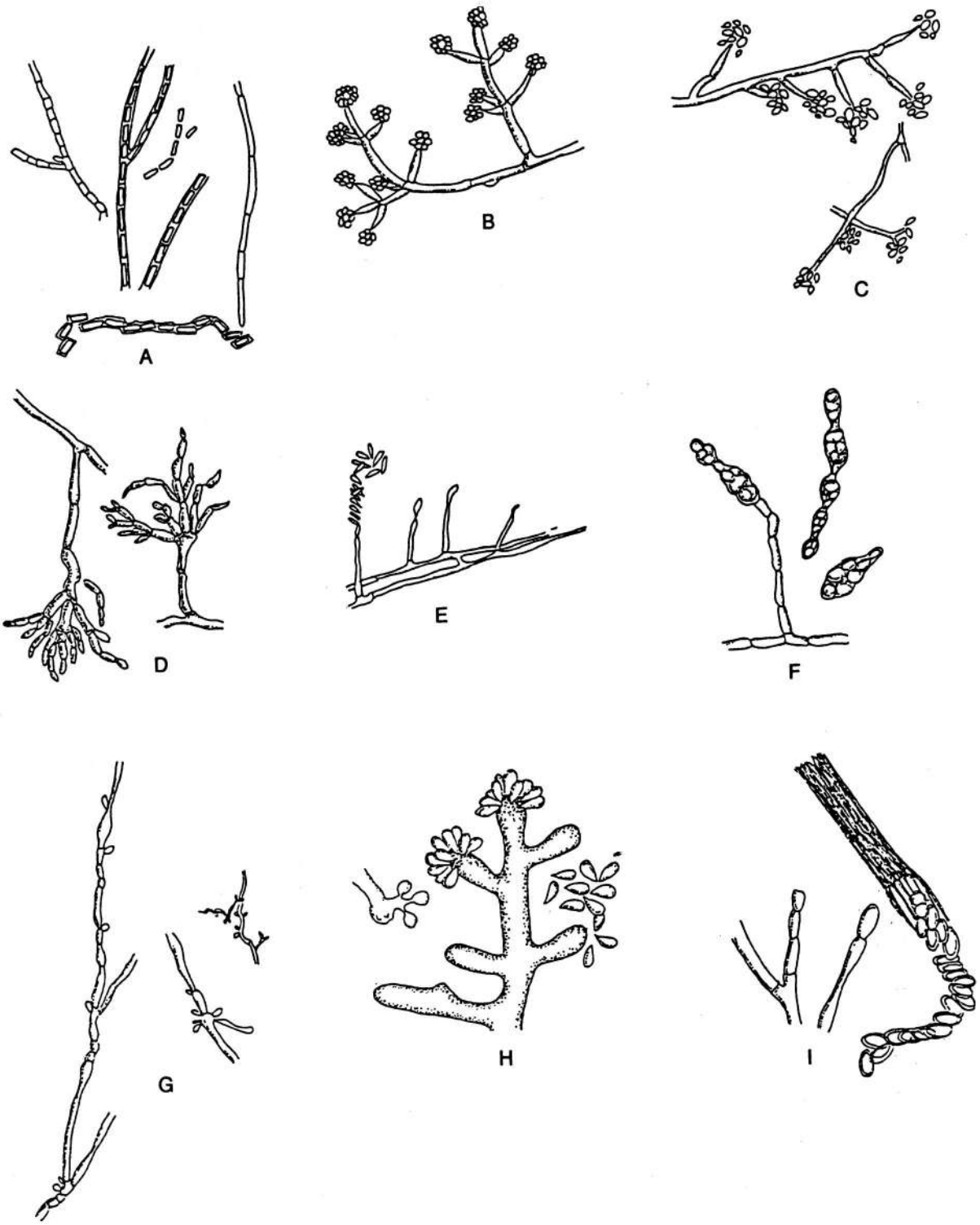


Plate 27. Fungi. All freshwater inhabitants.

A—*Geotrichum candidum*	colonies white	F—*Alternaria alternata*	colonies gray
B—*Trichoderma viride*	colonies green	G—*Trichosporon cutaneum*	colonies white
C—*Exophiala jeanselmei*	colonies black	H—*Pseudodallescheria boydii*	colonies white
D—*Cladosporium cladosporioides*	colonies black	I—*Klasterskaya splendens*	
E—*Fusarium*	colonies orange		

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10900 C. Key for Identification of Common Freshwater Algae (Plates 1A, 1B, 4A, 4B, and 28–40)

Beginning with couplet 1a and 1b, choose one of the contrasting statements. Proceed to the couplet number indicated at the end of the chosen statement and repeat the process. Continue until the alga name is given instead of another couplet number.

	Refer to couplet No.
1a. Plastid (separate color body) absent; complete protoplast pigmented; generally blue-green; iodine starch test* negative (cyanobacteria, blue-green algae)	4
1b. Plastid or plastids present; parts of protoplast free of some or all pigments; generally green, brown, red, etc., but not blue-green; iodine starch test* positive or negative	2
2a. Cell wall permanently rigid (never showing evidence of collapse), and with regular pattern of fine markings (striations, areolae, etc.); plastids brown to green; iodine starch test* negative; flagella absent; wall of two essentially similar halves, one placed over the other as a cover (diatoms)	29
2b. Cell wall, if present, capable of sagging, wrinkling, bulging, or rigidity, depending on existing turgor pressure of cell protoplast; regular pattern of fine markings on wall generally absent; plastids green, red, brown, etc.; iodine starch test* positive or negative; flagella present or absent; cell wall continuous and generally not of two parts	3
3a. Cell or colony motile; flagella present (often not readily visible); anterior and posterior ends of cell different from one another in contents and often in shape (flagellate algae)	51
3b. Nonmotile; true flagella absent; ends of cells often not differentiated (green algae and associated forms)	77

1. Cyanobacteria (Blue-Green Algae)

4a. Cells in filaments or trichomes (or much elongated to form a thread)	5
4b. Cells not in (or as) filaments	23
5a. Heterocytes present	6
5b. Heterocytes absent	14
6a. Heterocyte located at one end of filament	7
6b. Heterocyte at various locations in filament, other than at the end of the filament	9
7a. Filaments radially arranged in a gelatinous bead	*Rivularia*
7b. Filaments isolated or irregularly grouped	8
8a. Filament gradually narrowed to one end	*Calothrix*
8b. Filament not gradually narrowed to one end	*Cylindrospermum*
9a. Filament unbranched	10
9b. Filament with occasional (false) branches	13
10a. Crosswalls in filament much closer together than width of filament	*Nodularia*
10b. Crosswalls in filament at least as far apart as width of filament	11
11a. Filaments found singly or in tight parallel clusters; heterocytes and akinetes cylindric to long-oval in shape. Terminal cells not tapering	*Ahanizomenon*
11b. Filaments found singly, trichomes with terminal cells tapering	*Cuspidothrix*
11c. Filaments not in tight parallel clusters; heterocytes and akinetes often round to oval	12
12a. Filaments in a common, turgid gelatinous mass	*Nostoc*
12b. Filaments planktonic, not in a common, turgid gelatinous mass, aerotopes	*Dolichospermum, Sphaerospermopsis*
12c. Filaments benthic, not in a common, turgid gelatinous mass	*Anabaena* ,
13a. False branches in pairs	*Scytonema*
13b. False branches, single	*Tolypothrix*
14a. Filament or elongated cell attached at one end, with one or more round cells (spores) at the other	*Chamaesiphon*
14b. Filament generally not attached at one end; no terminal spores present	15
15a. Filament with regular spiral form throughout, cells not beadlike	16
15b. Filament not spiral, or with spiral form limited to a portion of filament	17
16a. Filament septate	*Arthrospira*
16b. Filament non-septate	*Spirulina*
17a. Filament very narrow, only 0.5 to 2.0 μm wide, trichomes often in a common sheath	*Schizothrix*
17b. Filament 2.1 to 95 μm wide	18
18a. Filaments loosely aggregated or not in clusters	19
18b. Filaments tightly aggregated and surrounded by a common gelatinous secretion that may be difficult to discern	22
19a. Trichome surrounded by wall-like sheath that frequently extends beyond the ends of the filament of cells	20
19b. Trichome not surrounded by a wall-like sheath;	21
20a. Cells separated from one another by mucilage	*Johannesbaptistia*
20b. Cells tightly connected	*Lyngbya/Planktolyngbya/Leptolyngbya*
21a. All trichomes short, with less than 20 cells; one or both terminal cells sharply pointed	*Cylindrospermopsis/Raphidiopsis*

* Add 1 drop of Lugol's (iodine) solution, diluted 1:1 with distilled water. In about 1 min, if positive, starch is stained blue and later black. Other structures (e.g., nucleus, plastids, cell wall) also may stain, but turn brown to yellow.

21b. Trichome short to long, with short to longer, tightly connected cells; motile or not, and without sharp-pointed terminal cells ..... *Pseudanabaena/Oscillatoria/Planktothrix*
22a. Filaments arranged in a tight, essentially parallel bundle ..... *Microcoleus*
22b. Filaments arranged in irregular fashion, often forming a mat ..... *Phormidium*
23a. Cells in a regular pattern of parallel rows, forming a plate..... *Merismopedia*
23b. Cells not regularly arranged to form a plate..... 24
24a. Cells regularly arranged near surface of a spherical gelatinous colony 25
24b. Gelatinous colony, if present, not spherical..... 26
25a. Cells ovate or spherical to heart-shaped, connected to center of colony by colorless stalks ..... *Gomphosphaeria/Snowella*
25b. Cells round or ovoid, without gelatinous stalks..... *Coelosphaerium,Woronichinia*
26a. Cells cylindric-oval ..... *Aphanothece*
26b. Cells spherical..... 27
27a. Two or more distinct layers of gelatinous sheath around each cell or cell cluster..... *Gloeocapsa*
27b. Gelatinous sheath around cells not distinctly layered..... 28
28a. Cells isolated or in colonies of 2 to 32 cells, aerotopes lacking..... *Aphanocapsa/Chroococcus*
28b. Cells in colonies composed of many cells, aerotopes present ..... *Microcystis*

2. Bacillariophyceae (Diatoms)

29a. Front (valve) view circular in outline; markings radial in arrangement; cells may form a filament (centric diatoms) 30
29b. Front (valve) view elongate, not circular; transverse markings in one or two longitudinal rows; cells, if grouped, not forming a filament (pennate diatoms) 32
30a. Cells in persistent filaments with valve faces in contact; therefore, cells commonly seen in side (girdle) view ..... *Aulacoseira*, *Melosira*
30b. Cells isolated or in fragile filaments, often seen in front (valve) view 31
31a. Radial markings (striations), in valve view, extending from center to margin; short spines often present around margin (valve view)..... *Cyclostephanos*, *Stephanodiscus*
31b. Area of prominent radial markings, in valve view, limited to approximately outer half of circle, marginal spines generally absent ..... *Cyclotella*
32a. Cell longitudinally symmetrical in valve view..... 33
32b. Cell longitudinally unsymmetrical (two sides unequal in shape), at least in valve view..... 49
33a. Raphe at or near the edge of the valve..... 34
33b. Raphe or pseudoraphe median or submedian..... 35
34a. Marginal, keeled raphe areas lie opposite one another on the two valves ..... *Hantzschia*
34b. Marginal, keeled raphe areas lie diagonal to one another on the two valves ..... *Nitzschia*
35a. Cell transversely symmetrical in valve view 36
35b. Cell transversely unsymmetrical (two ends unequal in shape or size), at least in valve view 44
36a. Cell round-oval in valve view, not more than twice as long as it is wide..... *Cocconeis*
36b. Cell elongate, more than twice as long as it is wide..... 37
37a. Cell flat (girdle face wide, valve face narrow) ..... *Tabellaria*
37b. Girdle and valve faces about equal in width 38
38a. Cell with several markings (septa) extending without interruption across the valve face; no marginal line of pores present..... *Diatoma*
38b. Cross-markings (striations or costae) on valve surface, interrupted by either longitudinal space (pseudoraphe), line (raphe), or line of pores (carinal dots) 39
39a. Cells often attached side by side to form a ribbon of several to many cells..... *Fragilaria* (Fragilariaceae)
39b. Cells isolated or in pairs..... 40
40a. Cell narrow, linear, often narrowed to both ends; true raphe absent ..... *Synedra, Ulnaria*
40b. Cell commonly “boat-shape” in valve view; true raphe present on one or both valves..... 41
41a. Cell longitudinally unsymmetrical in girdle view, curved; sometimes with attachment stalk, raphe on one valve ..... *Achnanthes*, *Achnanthidium*
41b. Cell symmetrical in both girdle and valve view; generally not attached 42
42a. Area without striations extending as a transverse belt around middle of cell ..... *Stauroneis*
42b. No continuous clear belt around middle of cell 43
43a. Cell with coarse transverse markings (costae), which appear as solid lines even under high magnification ..... *Pinnularia*
43b. Cell with fine transverse markings (striae), which appear as lines of dots under high magnification..... *Navicula*
44a. Cells attached together at one end only to form radiating colony ..... *Asterionella*
44b. Cell not forming a loose radiating colony 45
45a. Cells in fan-shaped colonies ..... *Meridion*
45b. Cells isolated or in pairs..... 46
46a. Prominent wall markings in addition to striations present just below lateral margins on valve surface of cell ..... *Surirella*
46b. Wall markings along sides of valve limited to striations..... 47
47a. Cell elongate, sides almost parallel except for terminal knobs ..... *Asterionella*
47b. Sides of cell converging toward one end 48
48a. Cells bent in girdle view ..... *Rhoicosphenia*

48b. Cells straight in girdle view ..... *Gomphonema*
49a. Valves with transverse septa or costae ..... *Epithemia*
49b. Valves with no transverse septa or costae 50
50a. Raphe located almost through center of valve ..... *Cymbella/Encyonema*
50b. Raphe excentric, near concave edge of valve ..... *Amphora*

3. Flagellate Algae (Functional, Multi-Division Category)

51a. Cell in a loose, rigid conical sac (lorica); isolated or in a branching colony ..... *Dinobryon*
51b. Case or sac, if present, not conical; colony, if present, not branching..... 52
52a. Cells isolated or in pairs 53
52b. Cells in a colony of four or more cells..... 71
53a. Prominent transverse groove encircles cell 54
53b. Cell without transverse groove..... 56
54a. Cell with prominent rigid projections, one forward and two or three on posterior end ..... *Ceratium*
54b. Cell without several rigid polar projections..... 55
55a. Portions above and below transverse groove about equal ..... *Peridinium*
55b. Front portion distinctly larger than posterior portion..... *Massartia*
56a. Cell with long bristles extending from surface plates..... *Mallomonas*
56b. Cell without bristles and surface plates 57
57a. Cell protoplast enclosed in loose, rigid covering (lorica) 58
57b. Cell with tight membrane or wall but no loose, rigid covering 60
58a. Lorica flattened; cell with two flagella..... *Phacotus*
58b. Lorica not flattened; cell with one flagellum 59
59a. Lorica often opaque, generally dark brown to red; plastid green..... *Trachelomonas*
59b. Lorica often transparent, colorless to light brown; plastid light brown..... *Chrysococcus*
60a. Plastids brown to red to olive or blue-green..... 61
60b. Plastids grass green..... 64
61a. Plastids blue-green to blue..... *Chroomonas*
61b. Plastids brown to red to olive green..... 62
62a. Plastid brown; one or two flagella 63
62b. Plastids red, red-brown, or olive green; two flagella..... *Rhodomonas*
63a. Anterior end of cell oblique; two flagella..... *Cryptomonas*
63b. Anterior end of cell rounded or pointed; one flagellum ..... *Chromulina*
64a. Cell with colorless rectangular wing ..... *Pteromonas*
64b. No wing extending from cell..... 65
65a. Cells flattened; margin rigid..... *Phacus*
65b. Cell not flattened; margin rigid or flexible 66
66a. Pyrenoid present in the single plastid; no paramylon; margin not flexible; two or more flagella per cell 67
66b. Pyrenoid absent; paramylon present; several plastids per cell; margin flexible or rigid; one flagellum per cell 70
67a. Cells fusiform (tapering at each end) ..... *Chlorogonium*
67b. Cells not fusiform, generally almost spherical 68
68a. Plastids numerous ..... *Vacuolaria*
68b. Plastids few, commonly one..... 69
69a. Two flagella per cell ..... *Chlamydomonas*
69b. Four flagella per cell ..... *Carteria, Tetraselmis*
70a. Cell flexible in form; paramylon a capsule or disk; cell elongate..... *Euglena*
70b. Cell rigid in form; paramylon ring-shaped; cell almost spherical ..... *Lepocinclis*
71a. Plastids brown 72
71b. Plastids green 73
72a. Cells in contact with one another ..... *Synura*
72b. Cells separated from one another by space ..... *Uroglena*, *Uroglenopsis*
73a. Colony flat, one cell thick..... *Gonium*
73b. Colony rounded, more than one cell thick..... 74
74a. Cells in contact with one another 75
74b. Cells separated from one another by space 76
75a. Cells pyriform, radially arranged ..... *Pandorina*
75b. Cells all facing one direction..... *Pyrobotrys (Chlamydobotrys)*
76a. Cells more than 400 per colony ..... *Volvox*
76b. Cells less than 75 per colony ..... *Eudorina*

4. Non-Flagelate Green Algae and Associated Forms (Functional, multi-division category)

77a. Cells jointed together to form a net..... *Hydrodictyon*
77b. Cells not forming a net 78

78a. Cells attached side by side to form a plate or ribbon one cell wide and thick; number of cells commonly two, four, or eight and with spines ..... *Desmodesmus*
78b. Cells attached side by side to form a plate or ribbon one cell wide and thick; number of cells commonly two, four, or eight and without spines ..... *Scenedesmus*
78c. Cells not attached side by side 79
79a. Cells isolated or in non-filamentous or non-tubular thalli 80
79b. Cells in filaments or other tubular or threadlike thalli 111
80a. Cells isolated and narrowest at the center because of incomplete fissure (desmids) 81
80b. Cells isolated or in clusters but without central fissure 84
81a. Each half of cell with three spinelike or pointed knobular extensions ..... *Staurastrum*
81b. Cell margin with no such extensions 82
82a. Semicells with a median incision or depression 83
82b. Semicells with no median incision or depression ..... *Cosmarium*
83a. Margin with rounded lobes ..... *Euastrum*
83b. Margin with sharp-pointed teeth ..... *Micrasterias*
84a. Cells elongate 85
84b. Cells round to oval or angular 92
85a. Cell radiating from a central point ..... *Actinastrum*
85b. Cells isolated or in irregular clusters 86
86a. Cells with terminal spines ..... *Schroederia*
86b. Cells without terminal spines 87
87a. Cells with colorless attachment area at one end ..... *Characium*
87b. No attachment area at one end of cell 88
88a. Plastids two per cell; unpigmented area across center of cell ..... *Closterium*
88b. Cell with plastid that continues across the center 89
89a. Cell 5 to 10 times as long as it is broad 90
89b. Cell 2 to 4 times as long as it is broad 91
90a. Pyrenoid absent, or one per cell ..... *Ankistrodesmus, Monoraphidium*
90b. Pyrenoids several per cell ..... *Closteriopsis*
91a. Cells semicircular; cell ends pointed but with no terminal spines ..... *Selenastrum*
91b. Cells arcuate but less than semicircular; cell ends pointed and each with a short spine ..... *Closteridium*
92a. Cells regularly arranged in a tight, flat colony ..... *Pediastrum*
92b. Cells not in a tight, flat regular colony 93
93a. Cells angular 94
93b. Cells round to oval 95
94a. Two or more spines at each angle ..... *Polyedriopsis*
94b. Zero to <2 spines at each angle ..... *Tetraedron*
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96b. Cells oval 98
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97b. Cells in colonies ..... *Micractinium*
98a. Each cell end has one spine ..... *Diacanthos*
98b. Each cell end has more than one spine ..... *Lagerheimia*
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101b. Sphere hollow, regular; short connecting processes between cells ..... *Coelastrum*
102a. Cells round 103
102b. Cells oval ..... *Oocystis*
103a. Cells connected to center of colony by branching stalk ..... *Dictyosphaerium*
103b. No stalk connecting cells ..... *Sphaerocystis*
104a. Oval cells, enclosed in a somewhat spherical, often orange-colored matrix ..... *Botryococcus*
104b. Round cells, isolated or in colorless matrix 105
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106b. No matrix or sheath outside of cell walls ..... *Protococcus*
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109b. Plastid filling ¾ or more of cell ..... *Chlorococcum*
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Spiny-rayed fish	24
Spionid	10
Spirillum	26
Spirodela	3A
Spirogyra	29
Spirontocaris	12
Spirulina	35
Sponges	7
Sponge spicule types	7
Sponge weed	2A
Springtail	22
Starfish	23
Staurastrum	29
Stenelmis	17
Stenonema	13
Stenoplax	20
Stephanodiscus	30
Stichococcus	1B
Stigeoclonium	29
Stingray	24
Stoneflies	13
Streptococcus	26
Strongylocentrotus	23
Stylonychia	6
Surirella	30
Sweet flag	3C
Syllids	10
Synchaeta	8
Synura	32
Syrphidae	16
Tabanidae	16
Tabanus	16
Tabellaria	30
Tadpole	25
Tagelus	21
Tanaidacea	12
Tarebia	19
Terebellid	10
Tetracladium	26
Tetraedron	29
Tetramitus	4B
Tetraselmis	29
Tetrastrum	1A
Thiaeidae	19
Thyrone	23
Tigriopus	11
Tintinnidium	6
Tipula	16
Tipulidae	16
Toads	25
Tolypothrix	35
Trachelomonas	36
Triaenodes	15
Tribonema	33
Trichocerca	8
Trichoderma	27
Trichosporon	27
Tripyla	9
Trochospongilla gemmules	7
Trout	24
Tubifex	9
Tusk shell	20
Typha	3C
Typosyllis	10
Uca	12
Ulnaria	30
Ulothrix	29
Ulva	2A
Unionidae	21
Uroglena	31
Urosolenia	1B
Vallisneria	2B
Valvata	19
Valvatidae	19
Vase sponge	7
Vaucheria	33
Veneridae	21
Vibrio	26
Viviparidae	19
Volvox	29
Vorticella	6
Water boatman	18
Water dog	25
Water fern	3A
Water hyacinth	3A
Water milfoil	3B
Water mite	22
Water penny	17
Water scavenger beetle	17
Water strider	18
Water velvet	3A
Watermeal	3A
Waterweed	3B
Westella	1A
Whirligig beetle	17
Winged lampshell	21
Wolffia	3A
Worms	9, 10
Woronichinia	35
Xanthophyta	1B
Zebra mussel	21
Zoobotryon	22
Zoogloea	26
Zoophagus	26
Zostera	2B
Zygnema	29

10900 E. Selected Taxonomic References

The most useful references for nonspecialists are listed below. These references are primarily regional and will help biologists identify both freshwater and marine plants and animals. Each reference is listed once under the broadest classification (i.e., a general reference to the identification of invertebrates is listed under "Invertebrates, General" and is not repeated under each individual phylum). As a rule, more academic and specialized reports on a single genus or family are not listed; however, these often are listed in the bibliography of a cited reference.

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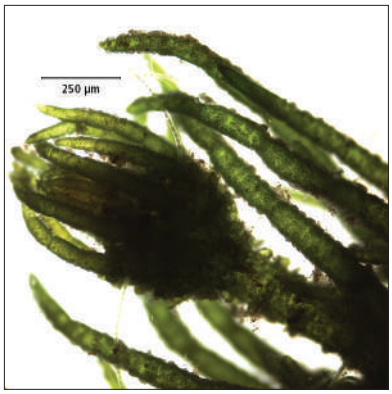
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Plate 28. Charophyta



PE, O/M, TO

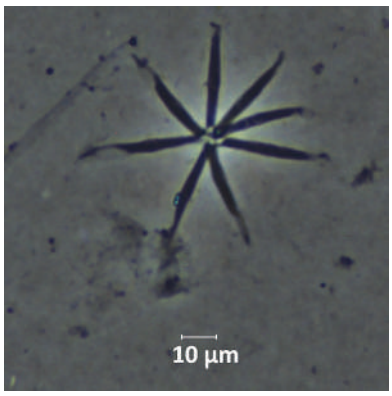
Figure A-1. ***Chara.***



PE, O/M/E, TO

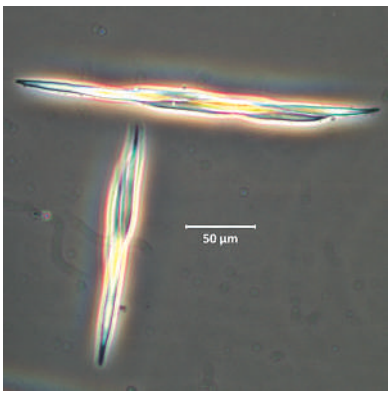
Figure A-2. ***Nitella.***

Plate 29. Chlorophyta



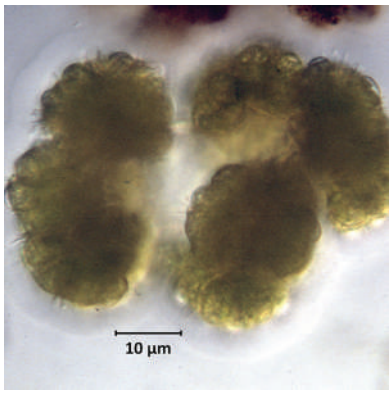
PL, M/E

Figure B-1. ***Actinastrum.***



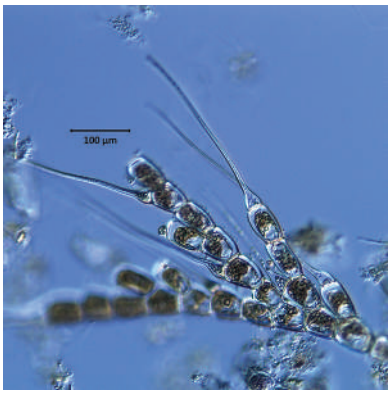
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Figure B-2. ***Ankistrodesmus.***



PL, M/E

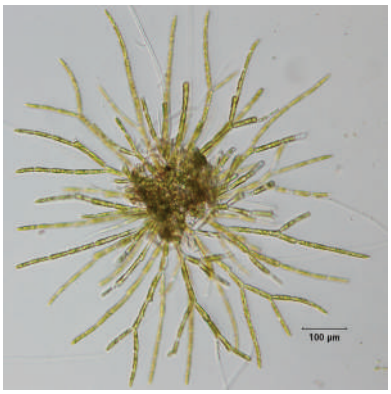
Figure B-3. ***Botryococcus.***



PE, M/E

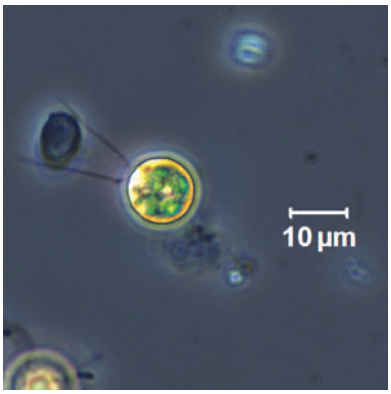
Figure B-4. ***Bulbochaete.***

B = brackish; E = eutrophic; FC = filter clogging; M = mesotrophic; O = oligotrophic; ORG = organically enriched; PE = periphyton; PL = plankton; TO = taste/odor; TP = toxin producer.



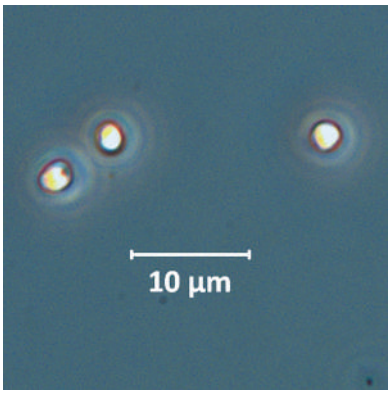
PE, O/M

Figure B-5. ***Chaetophora.***



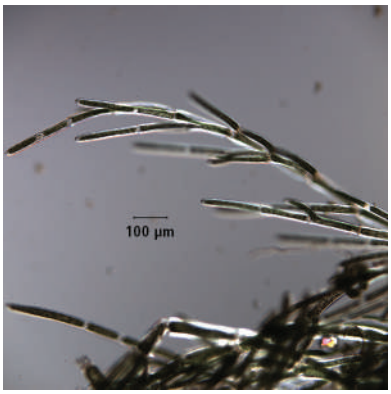
PL, M/E

Figure B-6. ***Chlamydomonas.***



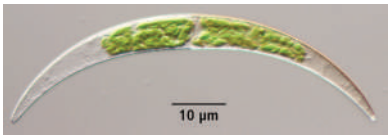
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Figure B-7. ***Chlorella.***



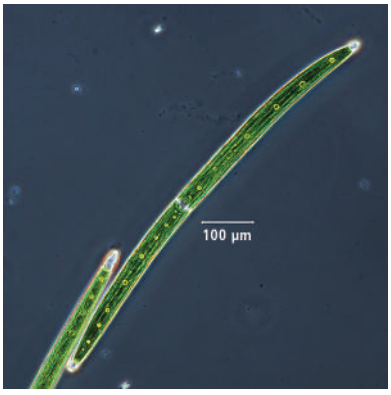
PE, M/E, TO

Figure B-8. ***Cladophora.***



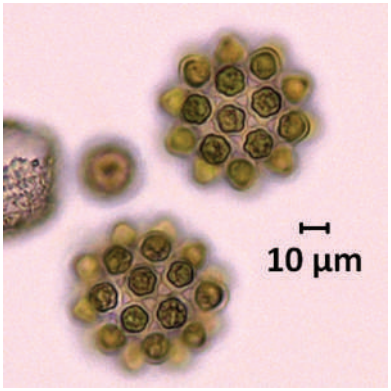
PL, M/E, FC

Figure B-9a. ***Closterium*** **(curved).**



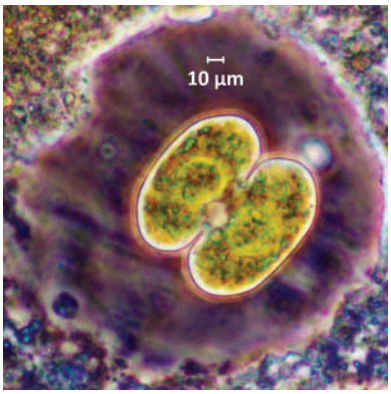
PL, M/E, FC

Figure B-9b. ***Closterium*** **(straight).**



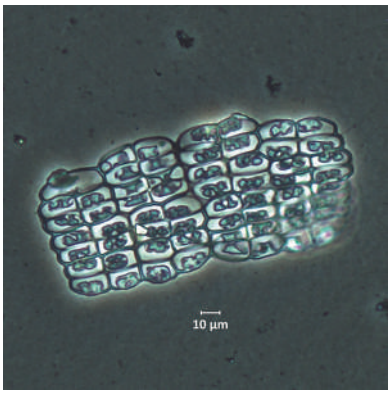
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Figure B-10. ***Coelastrum.***



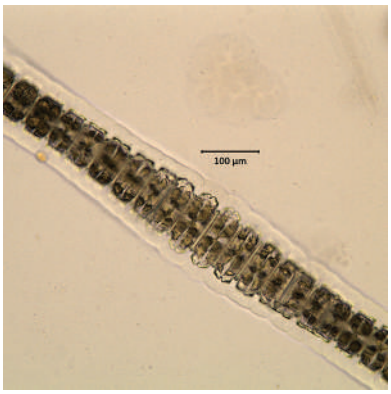
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Figure B-11. ***Cosmarium.***



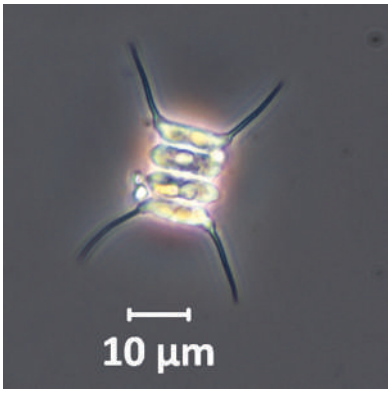
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Figure B-12. ***Crucigenia.***



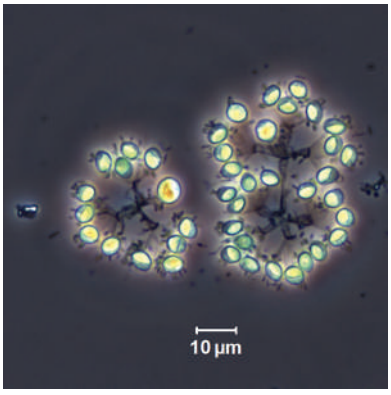
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Figure B-13. ***Desmidium.***



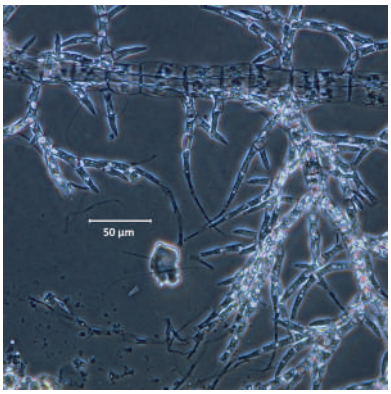
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Figure B-14. ***Desmodesmus.***



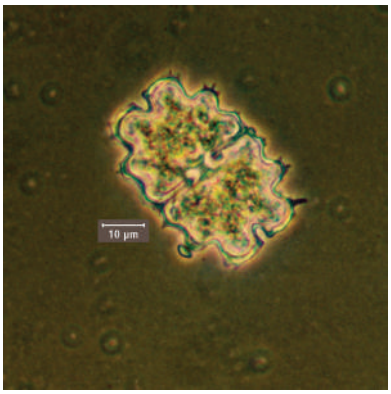
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Figure B-15. ***Dictyosphaerium.***



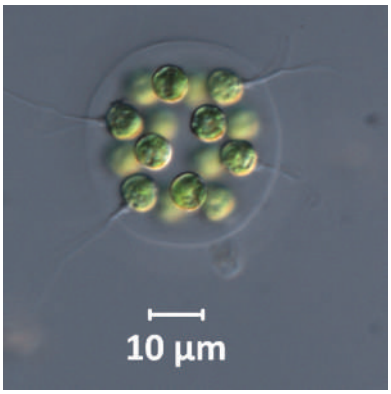
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Figure B-16. ***Draparnaldia.***



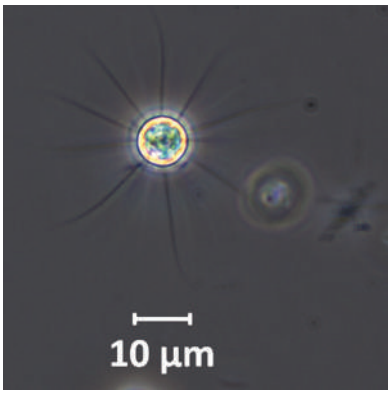
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Figure B-17. ***Euastrum.***



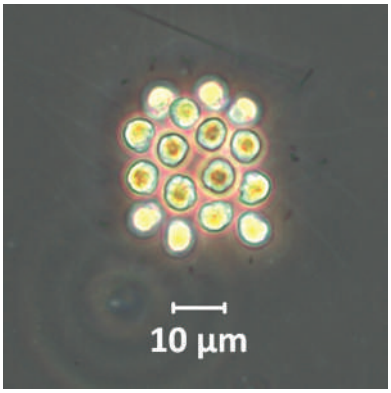
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Figure B-18. ***Eudorina.***



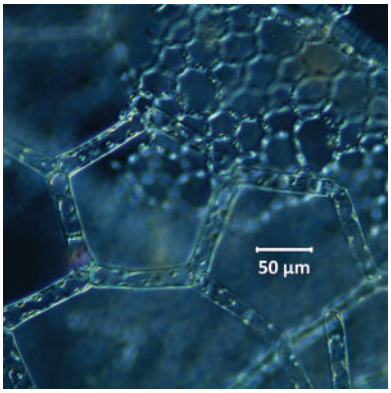
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Figure B-19. ***Golenkinia.***



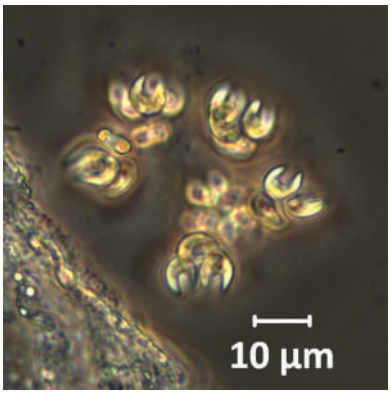
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Figure B-20. ***Gonium.***



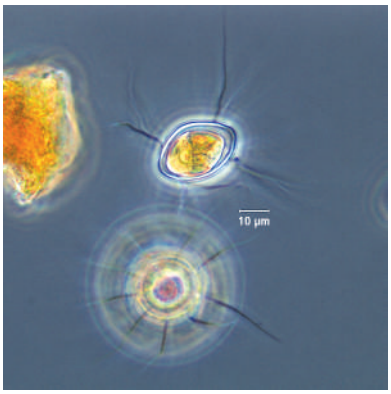
PL, E, FC

Figure B-21. ***Hydrodictyon.***



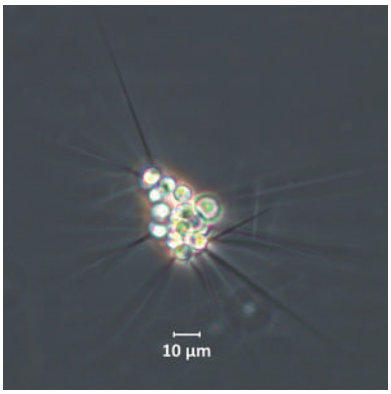
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Figure B-22. ***Kirchneriella.***



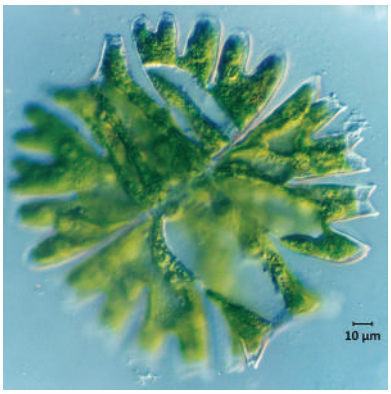
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Figure B-23. ***Lagerheimia.***



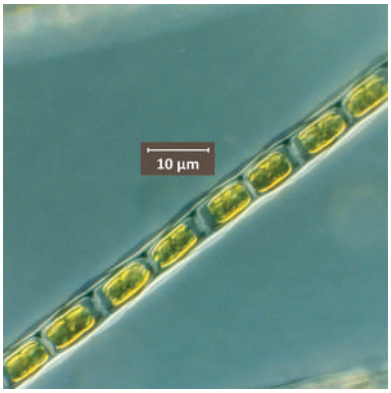
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Figure B-24. ***Micractinium.***



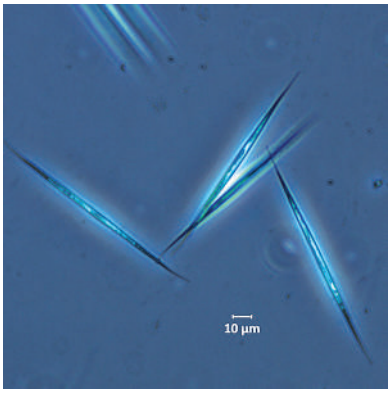
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Figure B-25. ***Micrasterias.***



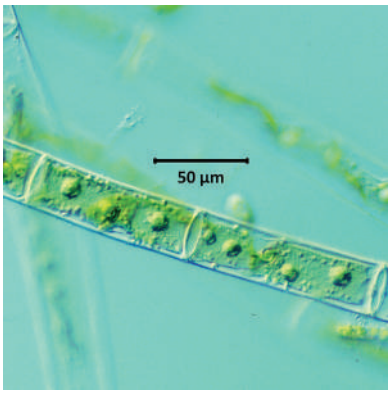
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Figure B-26. ***Microspora.***



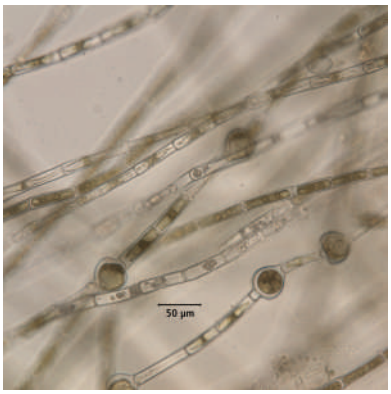
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Figure B-27. ***Monoraphidium.***



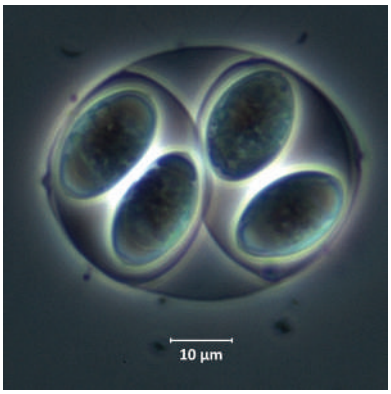
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Figure B-28. ***Mougeotia.***



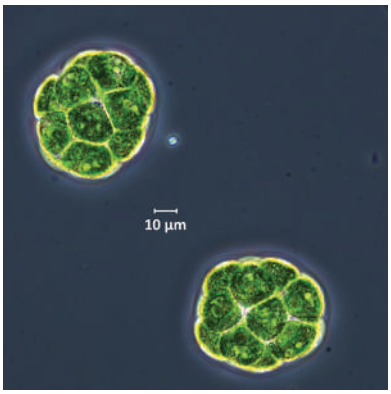
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Figure B-29. ***Oedogonium.***



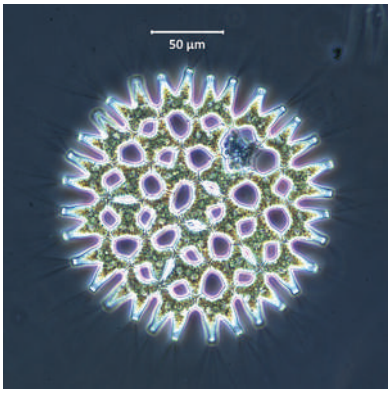
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Figure B-30. ***Oocystis.***



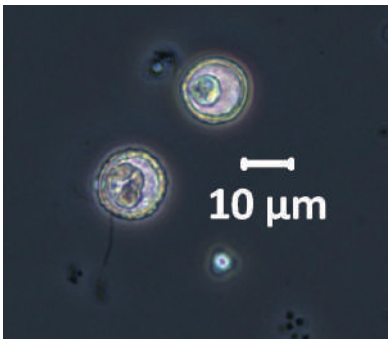
PL, M/E

Figure B-31. ***Pandorina.***



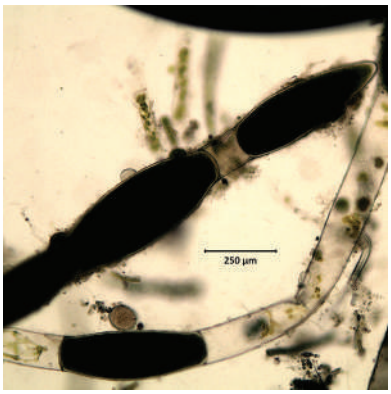
PL, PE, O/M/E

Figure B-32. ***Pediastrum.***



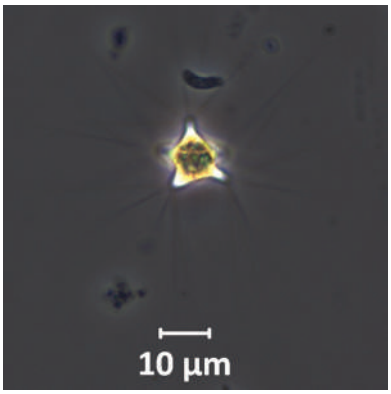
PL, O/M/E

Figure B-33. ***Phacotus.***



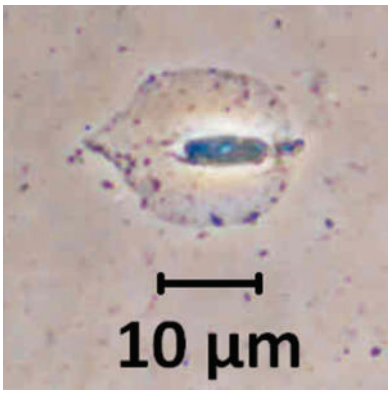
PE, E, TO

Figure B-34. ***Pithophora.***



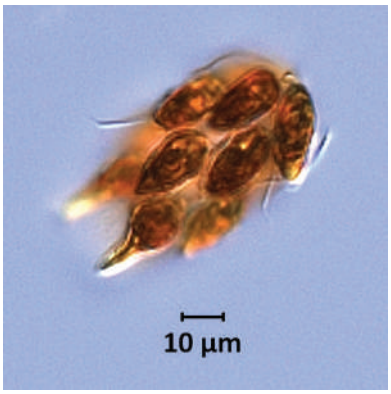
PL, M/E

Figure B-35. ***Polyedriopsis.***



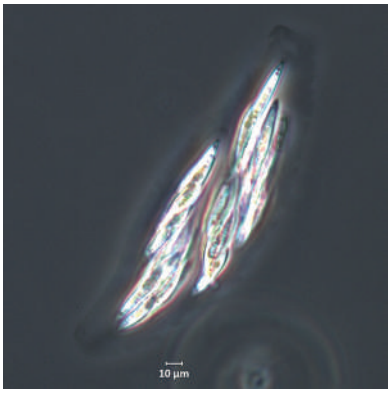
PL, M/E

Figure B-36. ***Pteromonas.***



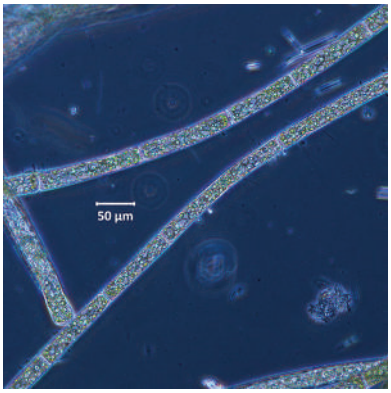
PL, O/M

Figure B-37. ***Pyrobotrys.***



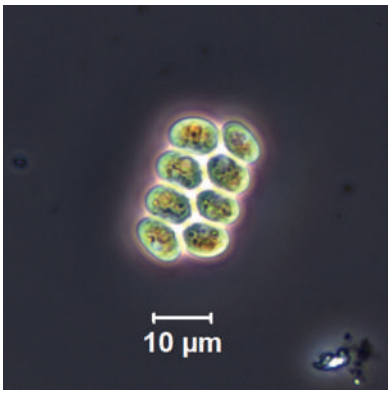
PL, M/E

Figure B-38. ***Quadrigula.***



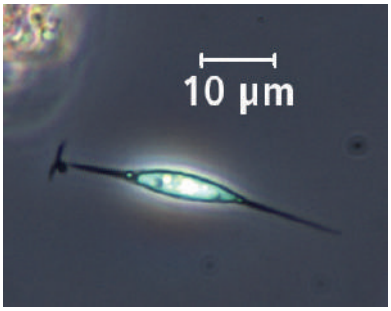
PE, M/E

Figure B-39. ***Rhizoclonium.***



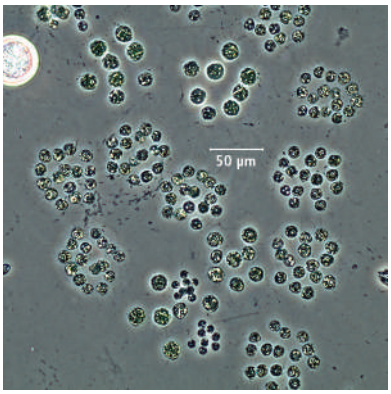
PL, O/M/E

Figure B-40. ***Scenedesmus.***



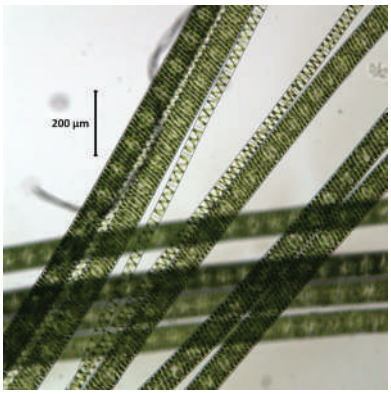
PL, M/E

Figure B-41. ***Schroederia.***



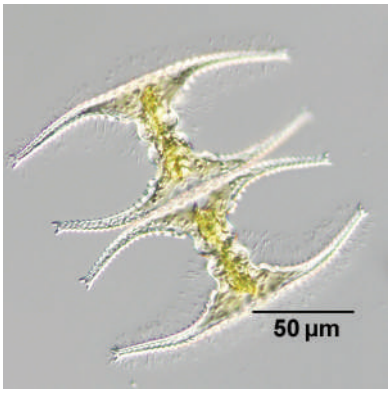
PL, M/E

Figure B-42. ***Sphaerocystis.***



PE, PL, M/E, FC

Figure B-43. ***Spirogyra.***



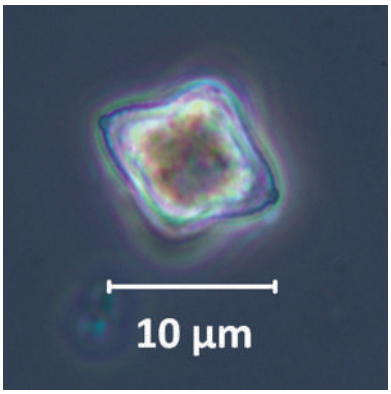
PL, M/E

Figure B-44. ***Staurastrum.***



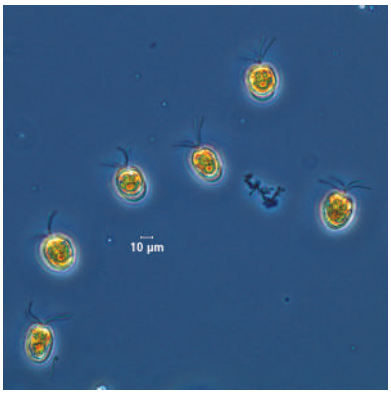
PL, PE, M/E

Figure B-45. ***Stigeoclonium.***



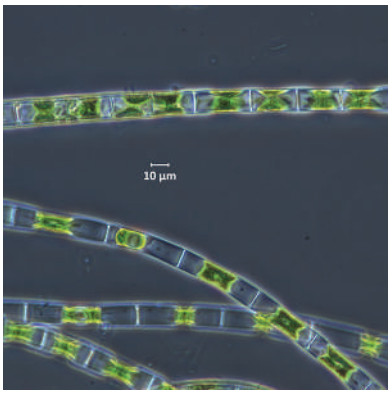
PL, M/E

Figure B-46. ***Tetraedron.***



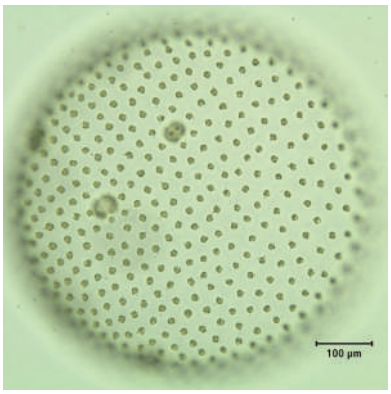
PL, M/E

Figure B-47. ***Tetraselmis.***



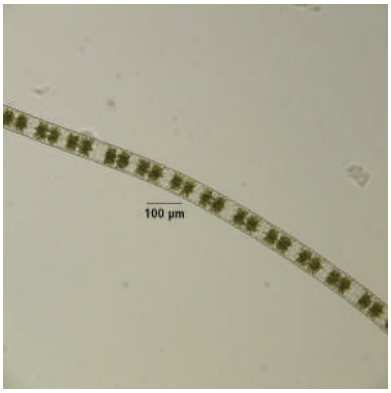
PL, PE, O/M/E

Figure B-48. ***Ulothrix.***



PL, M/E, TO

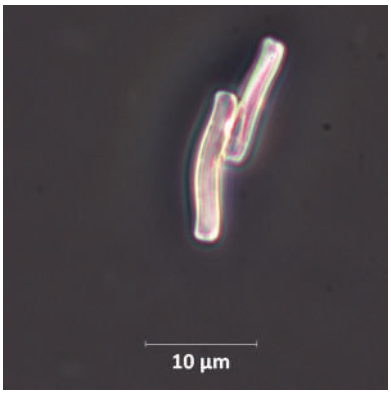
Figure B-49. ***Volvox.***



PL, M/E

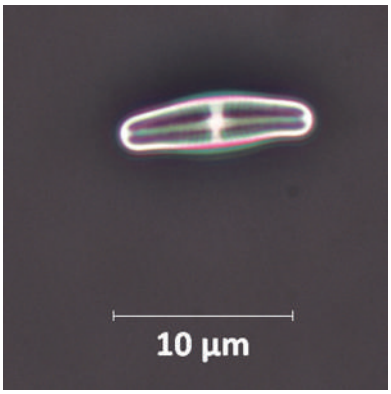
Figure B-50. ***Zygnema.***

Plate 30. Chrysophyta—Bacillariophyceae



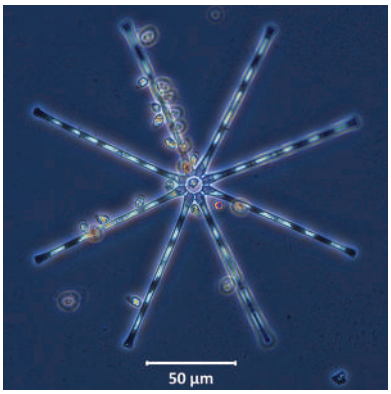
PE, O/M/E

Figure C-1a. *Achnanthidium* (girdle).



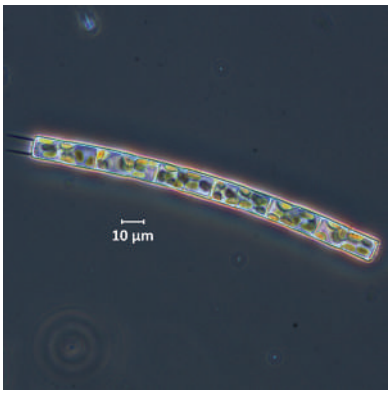
PE, O/M/E

Figure C-1b. *Achnanthidium* (valve).



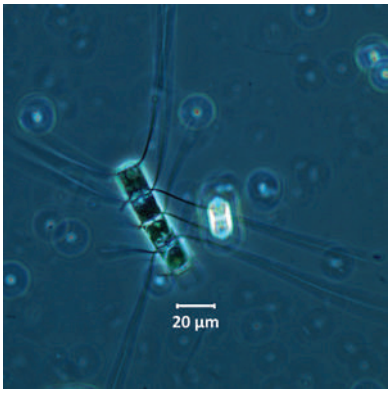
PL, O/M/E, TO, FC

Figure C-2. *Asterionella* (live, girdle).



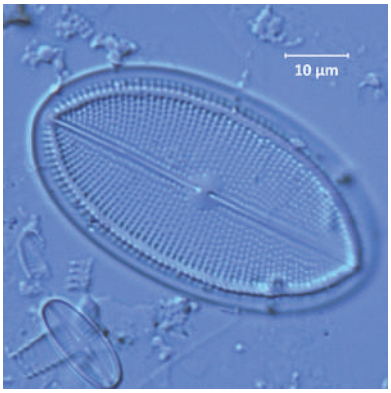
PL, PE, O/M/E, TO, FC

Figure C-3. *Aulacoseira* (live).



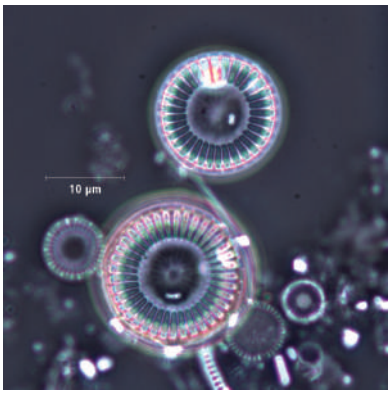
PL, O/M/E, B

Figure C-4. *Chaetoceros* (live).



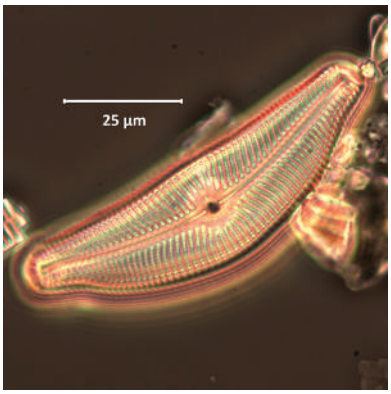
PE, O/M/E

Figure C-5. *Cocconeis* (valve).



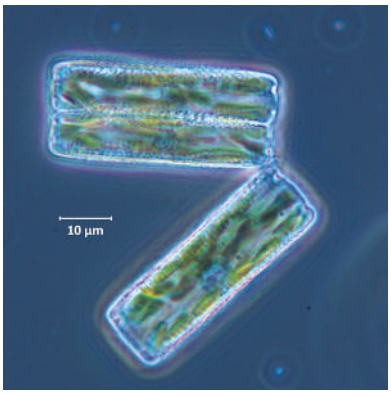
PL, O/M/E, TO, FC

Figure C-6. *Cyclotella* (valve).



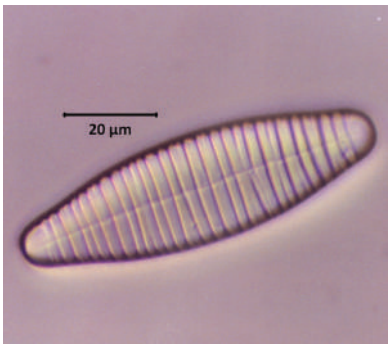
PL, PE, O/M/E, FC

Figure C-7. *Cymbella* (valve).



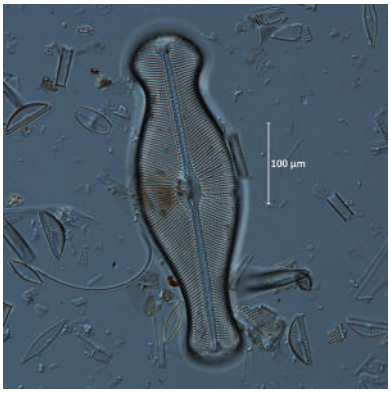
PL, PE, O/M, FC

Figure C-8a. *Diatoma* (live, girdle).



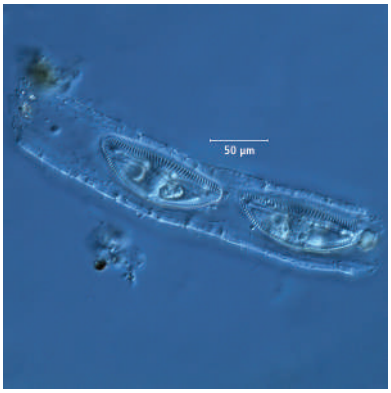
PL, PE, O/M, FC

Figure C-8b. *Diatoma* (valve).



PE, O/M/E, TO, FC

Figure C-9. *Didymosphenia* (valve).



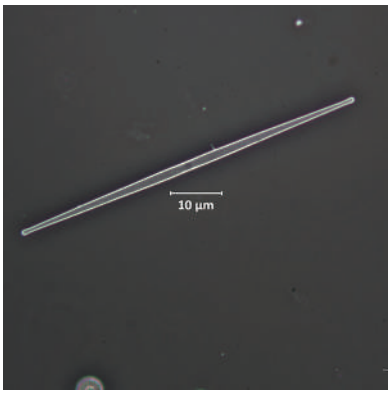
PL, PE, O/M/E, FC

Figure C-10. *Encyonema* (valve).



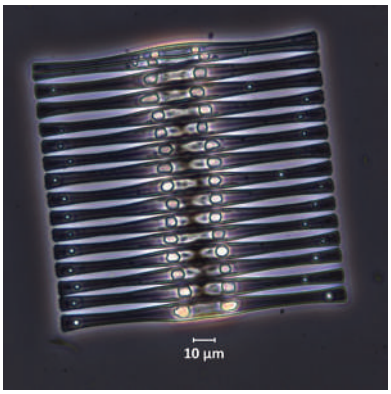
PL, PE, O/M

Figure C-11. *Eunotia* (valve).



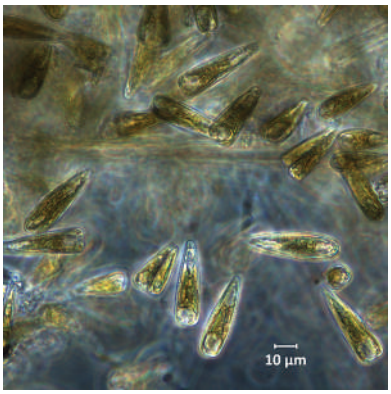
PL, PE, O/M/E, TO, FC

Figure C-12a. *Fragilaria* (single valve).



PL, PE, O/M/E, TO, FC

Figure C-12b. *Fragilaria crotonensis* (live, girdle).



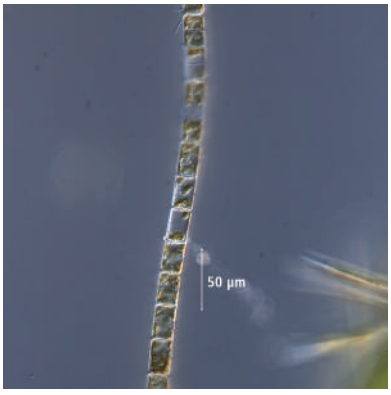
PE, M/E

Figure C-13. *Gomphonema* (live).



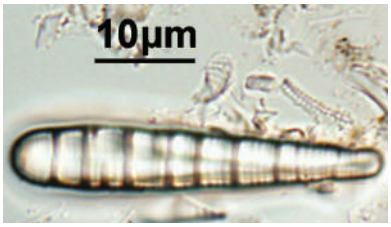
PL, PE, O/M

Figure C-14. *Gyrosigma* (live, valve).



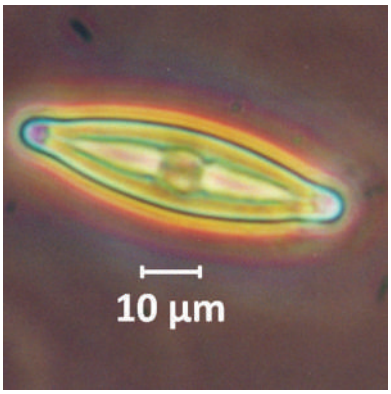
PE, M/E, TO

Figure C-15. *Melosira* (live).



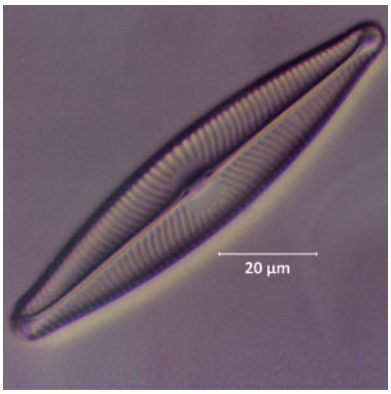
PE, O/M/E

Figure C-16. *Meridion* (valve).



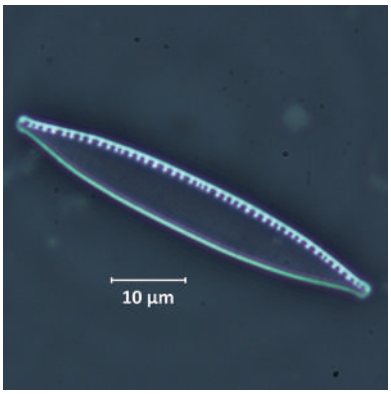
PE, PL, O/M/E

Figure C-17a. *Navicula* (valve, live).



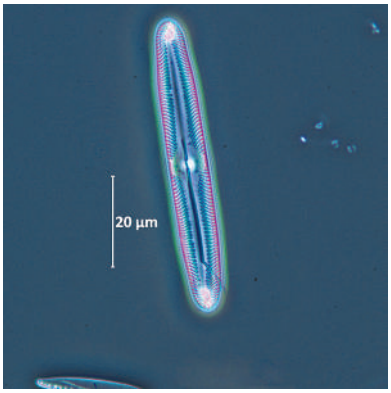
PE, PL, O/M/E

Figure C-17b. *Navicula* (valve).



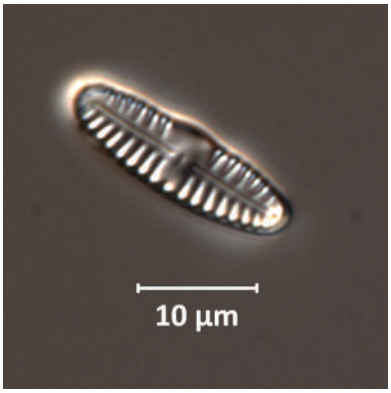
PL, PE, O/M/E, TO

Figure C-18. *Nitzschia* (valve).



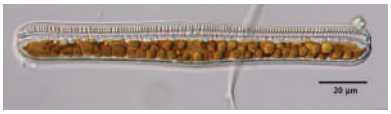
PL, PE, O/M

Figure C-19. *Pinnularia* (valve).



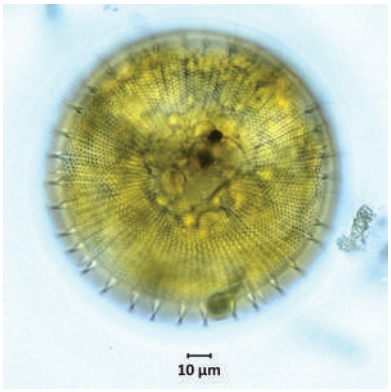
PL, PE, O/M/E

Figure C-20. *Reimeria* (valve).



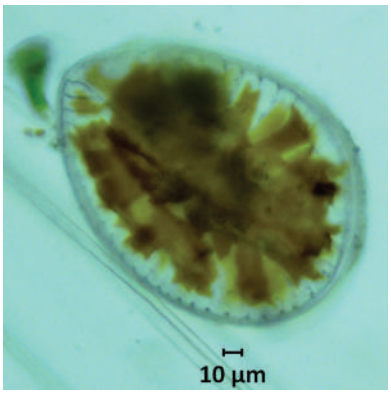
PL, PE, O/M/E

Figure C-21. *Rhopalodia* (live).



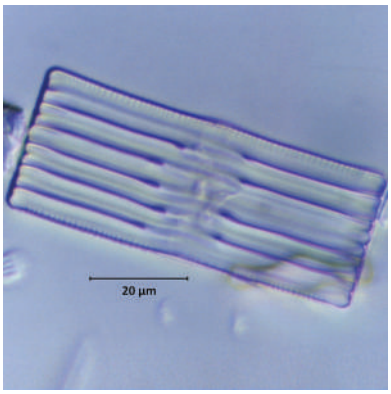
PL, O/M/E, TO, FC

Figure C-22. *Stephanodiscus* (live, valve).



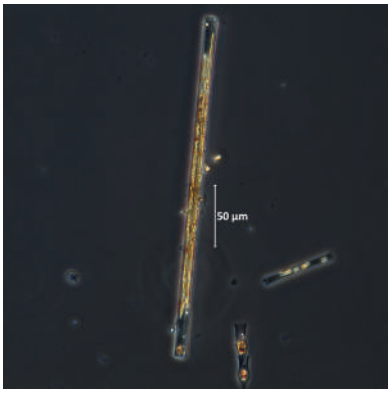
PL, PE, O/M/E

Figure C-23. *Surirella* (live, valve).



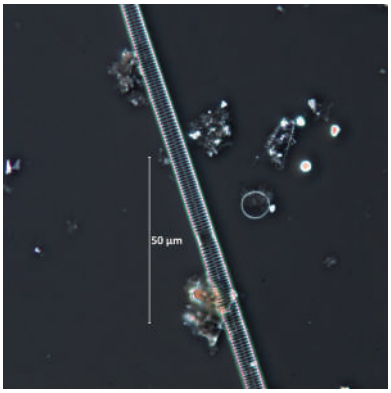
PL, PE, O/M/E, TO, FC

Figure C-24. *Tabellaria* (girdle).



PL, PE, O/M/E, TO, FC

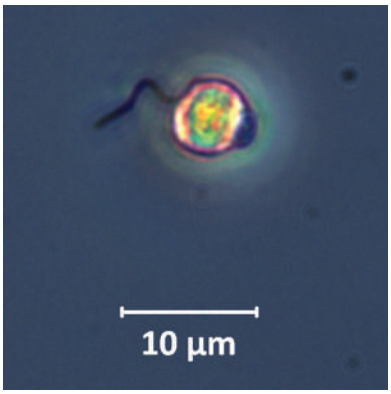
Figure C-25a. *Ulnaria* (live, girdle).



PL, PE, O/M/E, TO, FC

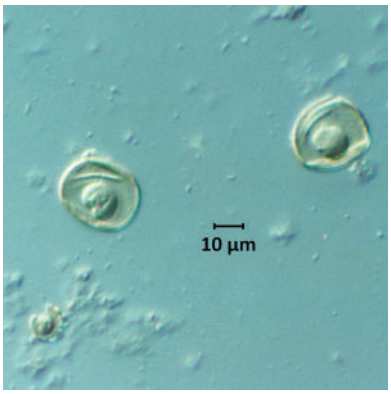
Figure C-25b. *Ulnaria* (valve).

Plate 31. Chrysophyta—Chrysophyceae



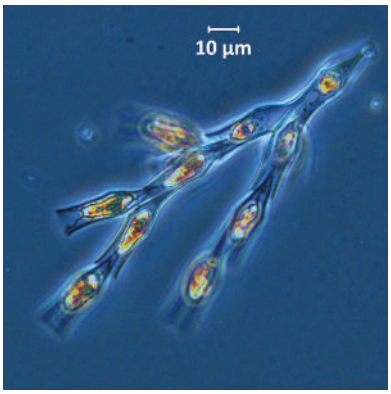
PL, O/M

Figure D-1. ***Chromulina.***



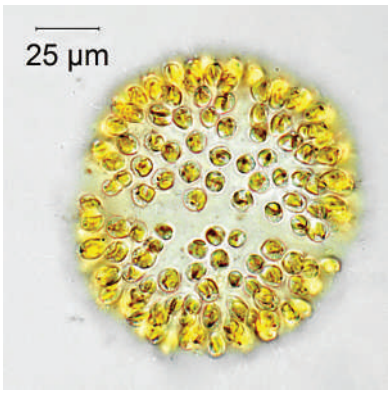
PL, O/M

Figure D-2. ***Chrysococcus.***



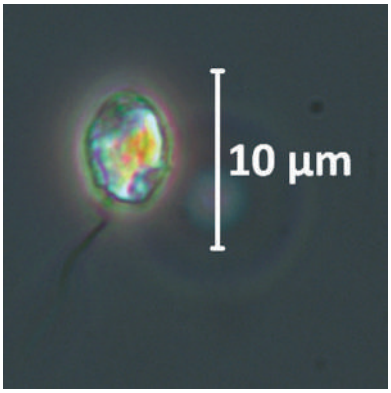
PL, O/M/E, TO, FC

Figure D-3. ***Dinobryon.***



PL, M/E, TO, ORG

Figure D-4. ***Uroglena volvox*** **(low magnification).**
Image contributed by Chris Carter.



PL, M/E, TO, ORG

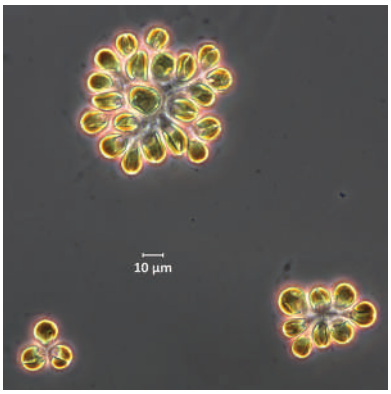
Figure D-5. ***Uroglena*** **(single cell).**

Plate 32. Chrysophyta—Synurophyceae



PL, O/M/E, TO

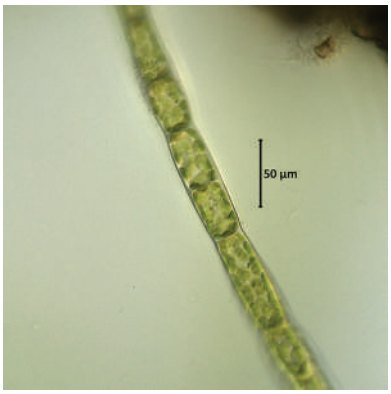
Figure E-1. ***Mallomonas.*** Image contributed by Chris Carter.



PL, O/M/E, TO, ORG

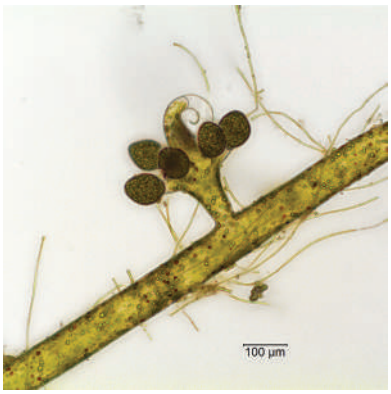
Figure E-2. ***Synura.***

Plate 33. Chrysophyta—Xanthophyceae



PE, PL, O/M/E, FC

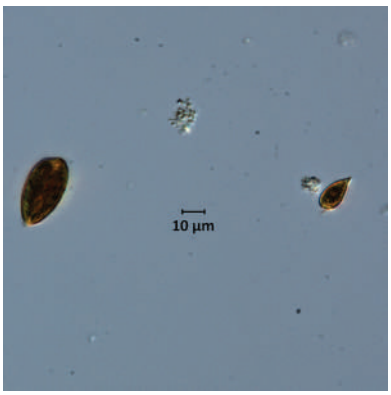
Figure F-1. ***Tribonema.***



PE, O/M

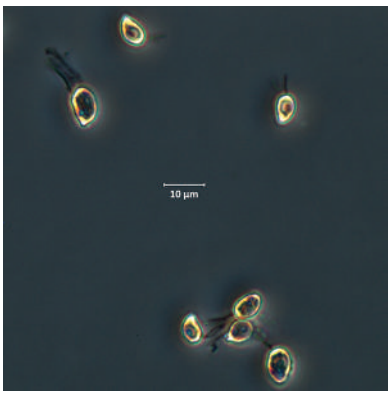
Figure F-2. ***Vaucheria.*** Image contributed by Chris Carter.

Plate 34. Cryptophyta



PL, O/M/E, ORG (*Cryptomonas*)

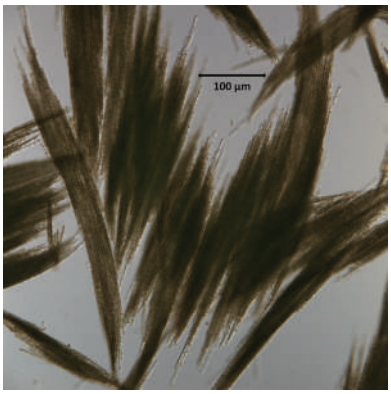
Figure G-1. ***Cryptomonas*** *(l),* ***Rhodomonas*** *(r).*



PL, O/M/E

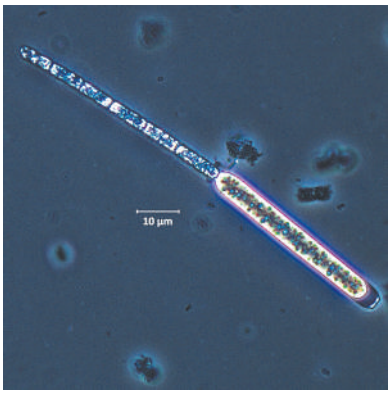
Figure G-2. ***Rhodomonas.***

Plate 35. Cyanobacteria



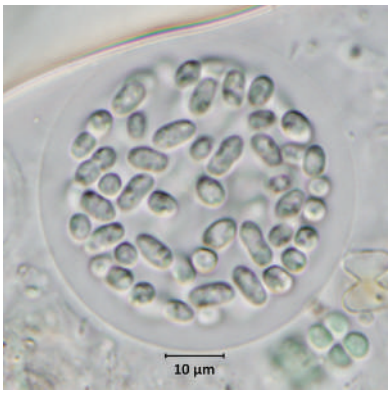
PL, M/E, TO, TP

Figure H-1a. ***Aphanizomenon flos aquae.***



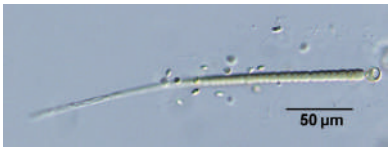
PL, M/E, TO, TP

Figure H-1b. ***Aphanizomenon*** **(single, filament).**



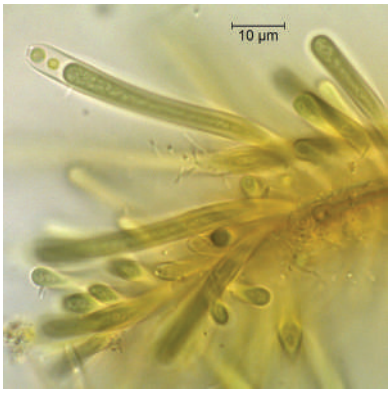
PL, PE, O/M/E

Figure H-2. ***Aphanothece.***



PE, O/M

Figure H-3. ***Calothrix.***



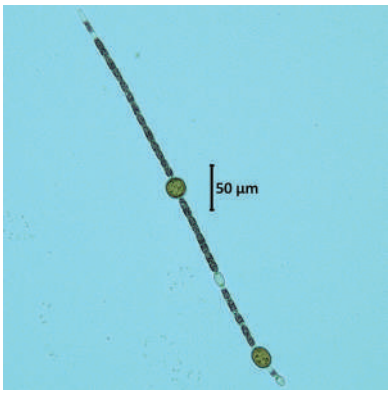
PE, O/M/E

Figure H-4. ***Chamaesiphon.*** Image contributed by Chris Carter.



PL, O/M/E

Figure H-5. ***Chroococcus.***



PL, M/E, TP

Figure H-6. ***Chrysosporum.***



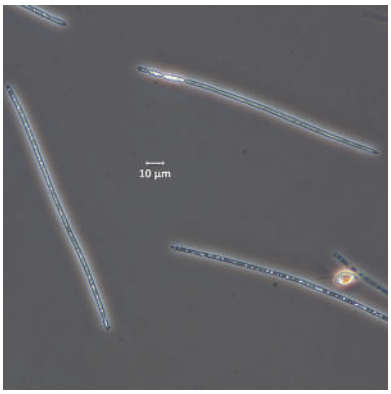
PL, M/E, TO, TP

Figure H-7. ***Cuspidothrix.***



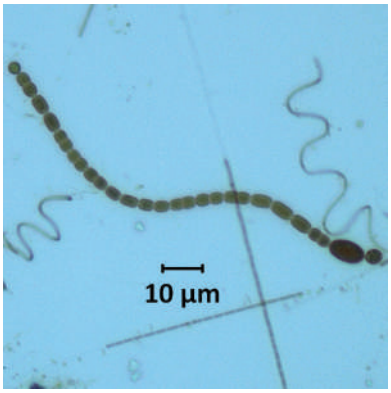
PL, M/E, TP

Figure H-8. ***Cylindrospermopsis*** **(curled).**



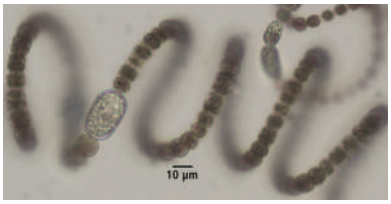
PL, M/E, TP

Figure H-9. ***Cylindrospermopsis*** **(straight).**



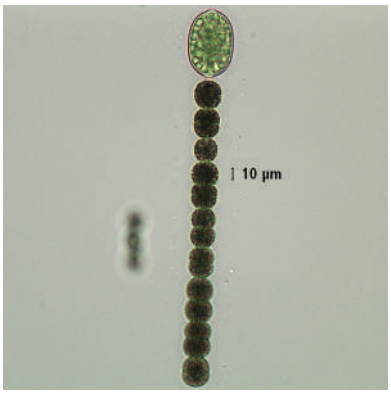
PL, O/M

Figure H-10. ***Cylindrospermum.***



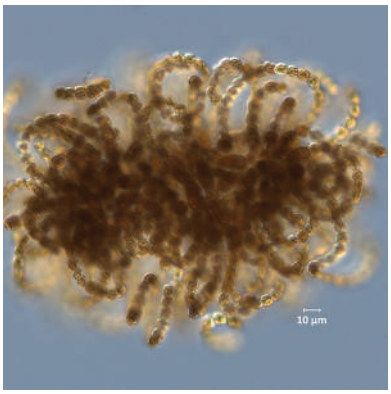
PL, M/E, TO, TP, FC

Figure H-11a. ***Dolichospermum*** **(curled).**



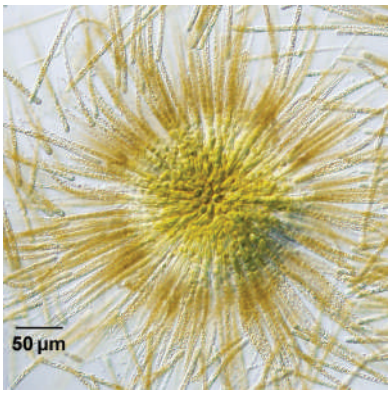
PL, M/E, TO, TP, FC

Figure H-11b. *Dolichospermum* (straight).



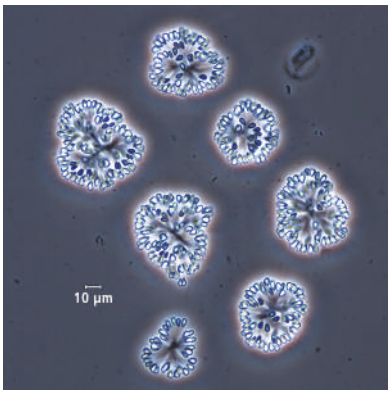
PL, M/E, TO, TP, FC

Figure H-11c. *Dolichospermum* (twisted).



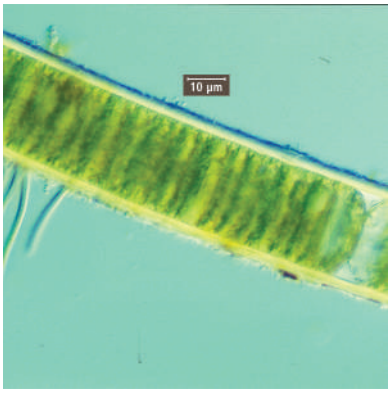
PL, O/M/E, TO, TP, FC

Figure H-12. *Gloeotrichia.*



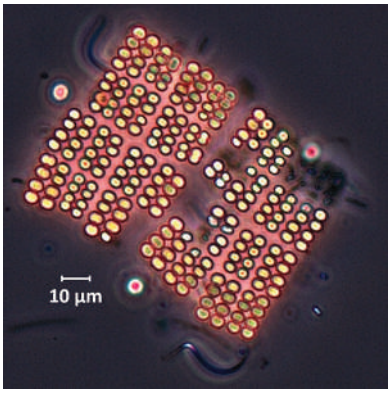
PL, O/M/E

Figure H-13. *Gomphosphaeria.*



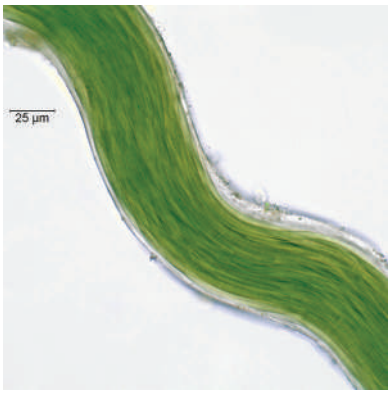
PL, PE, O/M/E, TO, TP, FC

Figure H-14. *Lyngbya.*



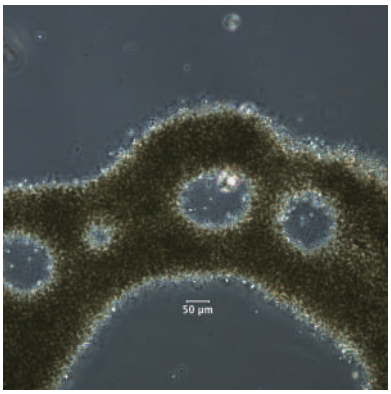
PL, O/M/E

Figure H-15. *Merismopedia.*



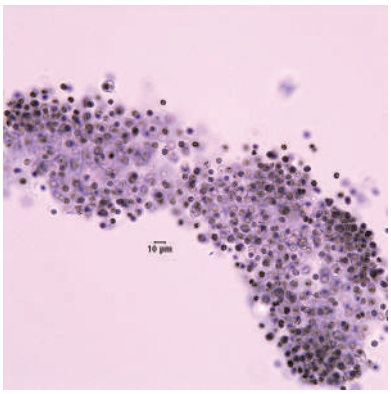
PE, O/M

Figure H-16. ***Microcoleus.*** Image contributed by Chris Carter.



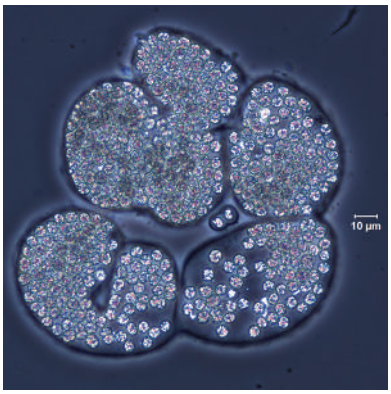
PL, M/E, TO, TP, FC

Figure H-17a. ***Microcystis aeruginosa*** **(calthrate colony).**



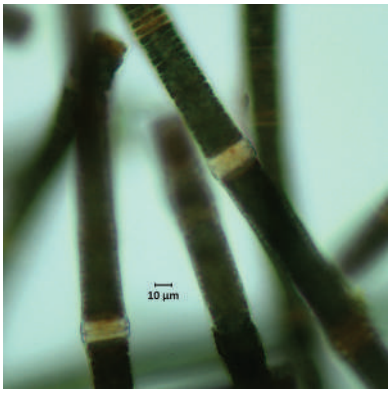
PL, M/E, TO, TP, FC

Figure H-17b. ***Microcystis aeruginosa*** **(young colony*).***



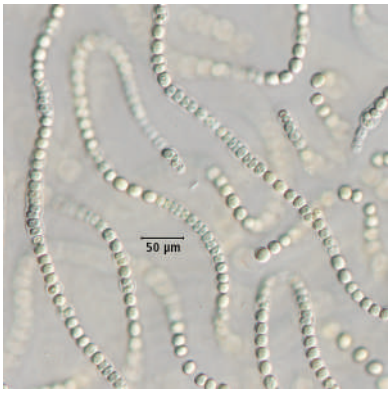
PL, M/E, TO, TP, FC

Figure H-17c. ***Microcystis wessenbergii.***



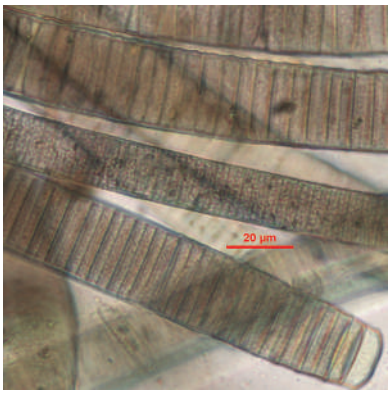
PL, M/E, TO, TP, B

Figure H-18. ***Nodularia.***



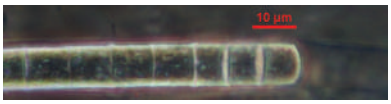
PE, M/E

Figure H-19. ***Nostoc.***



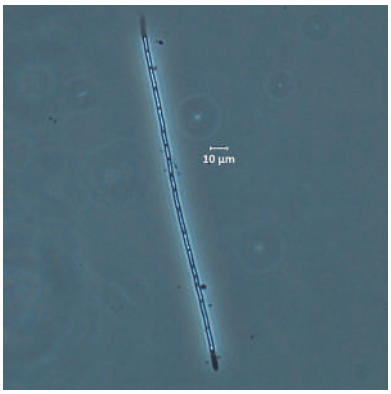
PL, PE, O/M/E, TO, TP, FC

Figure H-20. ***Oscillatoria.***



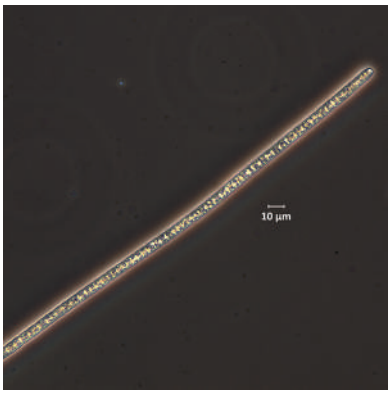
PE, ME, TO

Figure H-21. ***Phormidium.***



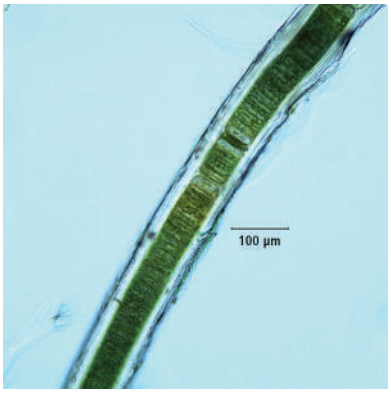
PL, M/E

Figure H-22. ***Planktolyngbya.***



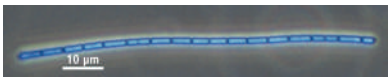
PL, M/E, TO, TP

Figure H-23. ***Planktothrix.***



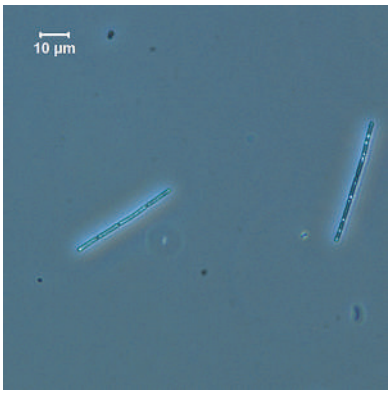
PE, PL, M/E, TO, TP

Figure H-24. ***Plectonema/Lyngbya.***



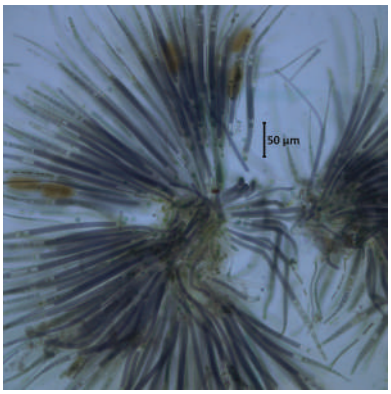
PL, PE, O/M/E, TO, TP

Figure H-25a. ***Pseudanabaena*** **(high magnification).**



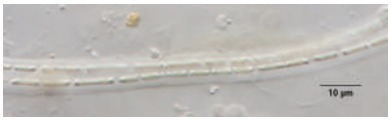
PL, PE, O/M/E, TO, TP

Figure H-25b. *Pseudanabaena* (low magnification).



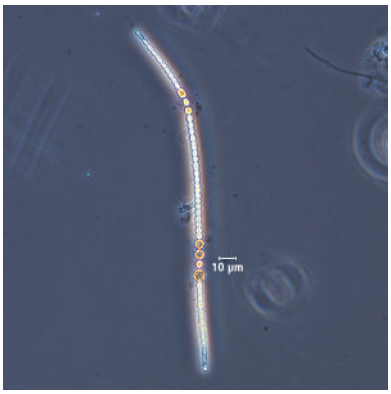
PE, M/E

Figure H-26. *Rivularia.*



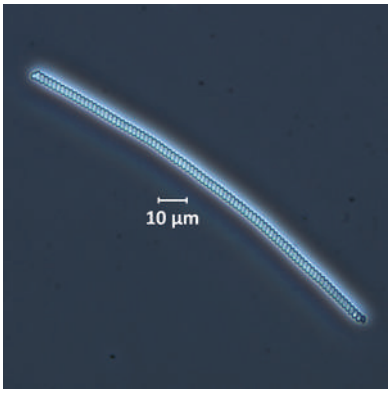
PE, O/M/E

Figure H-27. *Schizothrix.*



PL, O/M/E, TO, TP, FC

Figure H-28. *Sphaerospermopsis.*



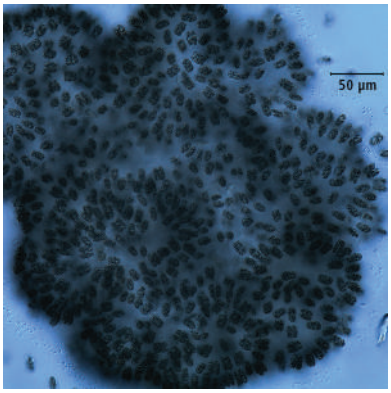
PE, PL, M/E

Figure H-29. *Spirulina.*



PE, O/M/E

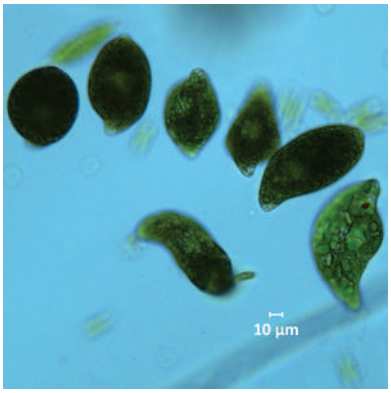
Figure H-30. *Tolypothrix.*



PL, M/E, TO, TP

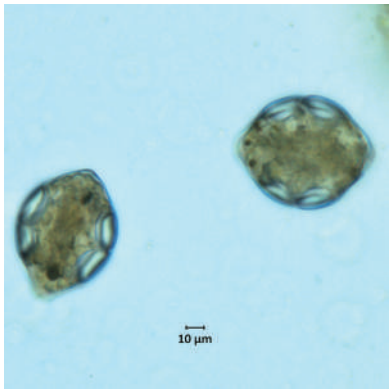
Figure H-31. ***Woronichinia.***

Plate 36. Euglenophyta



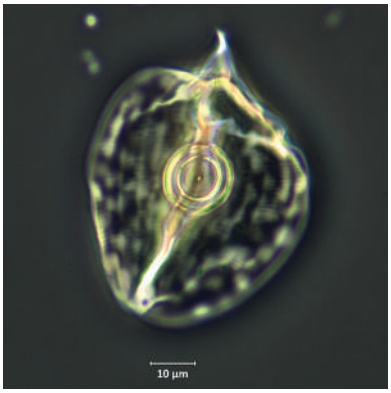
PL, M/E, TO, FC, TP, ORG

Figure I-1. ***Euglena.***



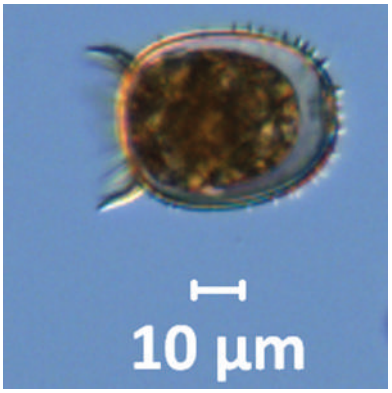
PL, M/E, ORG

Figure I-2. ***Lepocinclis.***



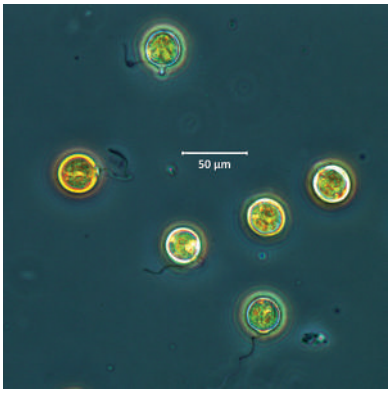
PL, M/E, ORG

Figure I-3. ***Phacus.***



PL, M/E, ORG

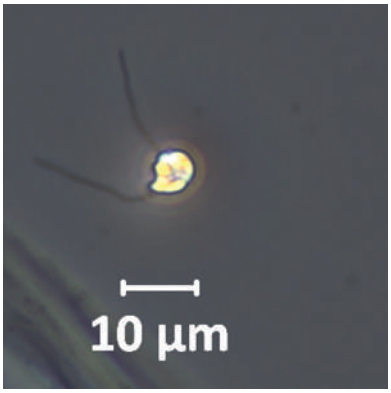
Figure I-4a. ***Trachelomonas*** **(ornamented).**



PL, M/E, ORG

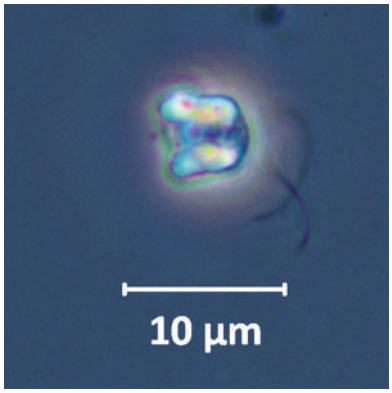
Figure I-4b. *Trachelomonas* (smooth).

Plate 37. Haptophyta



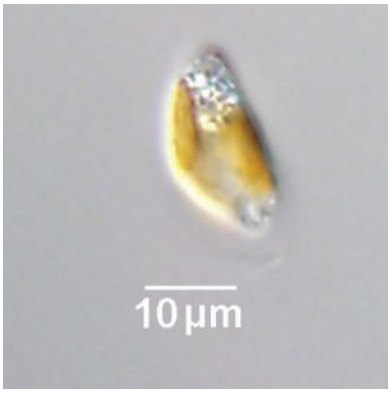
PL, O/M/E, TO

Figure J-1. *Chrysochromulina.*



PL, M/E, TP, B

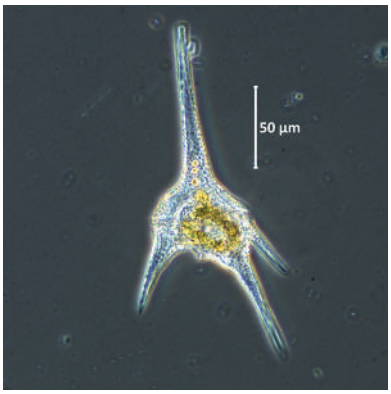
Figure J-2a. *Prymnesium* (preserved).



PL, M/E, TP, B

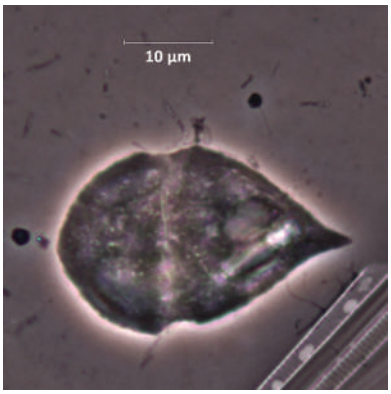
Figure J-2b. *Prymnesium* (live).

Plate 38. Pyrrophyta



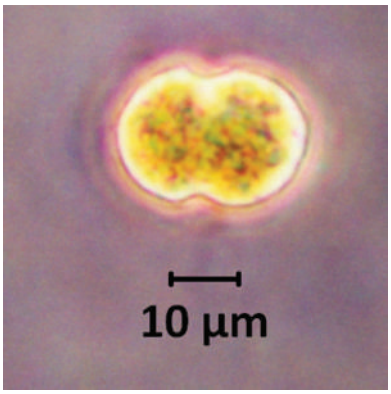
PL, M/E, TO, ORG

Figure K-1. *Ceratium.*



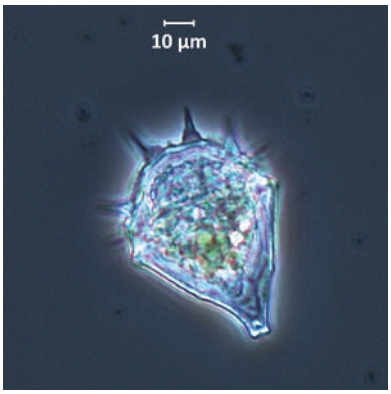
PL, O/M/E, ORG

Figure K-2a. *Gymnodinium* (large).



PL, O/M/E, ORG

Figure K-2b. *Gymnodinium* (small).



PL, M/E, ORG

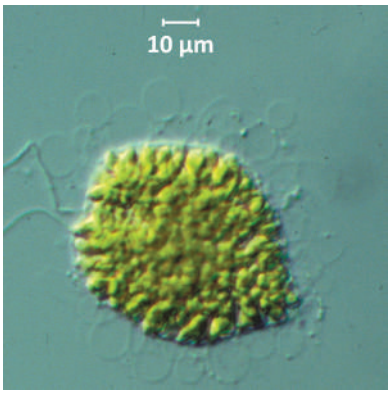
Figure K-3. *Peridiniopsis.*



PL, M/E, ORG

Figure K-4. *Peridinium.*

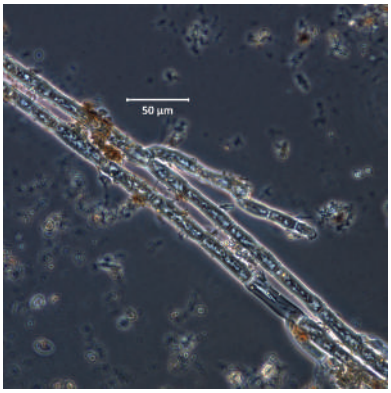
Plate 39. Raphidiophyta



PL, M/E, ORG

Figure L-1. ***Gonyostomum.***

Plate 40. Rhodophyta



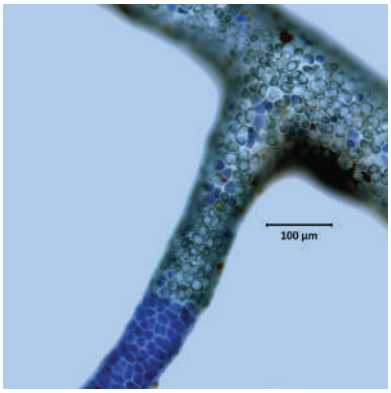
PE, O/M

Figure M-1. ***Audouinella.***



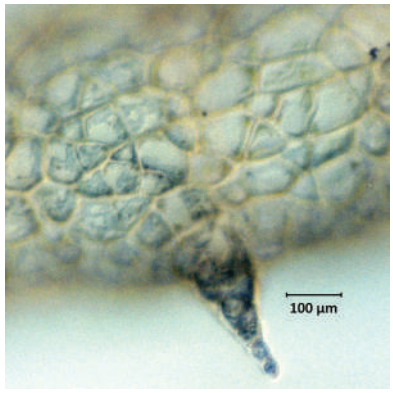
PE, O/M

Figure M-2. ***Batrachospermum.***



PE, O/M

Figure M-3. ***Compsopogon.***



PE, O/M

Figure M-4. ***Lemanea.***

Abbreviations

The following symbols and abbreviations are used throughout *Standard Methods:*

Abbreviation	*Referent*
AA	atomic absorption
A or amp	ampere(s)
AC	alternating current
ACS	American Chemical Society
amu	atomic mass units
APHA	American Public Health Association
ASTM	American Society for Testing and Materials
AWWA	American Water Works Association
BOD	biochemical oxygen demand
°C	degree(s) Celsius
c	counts
Ci	curie(s)
cm, cm^2, cm^3	centimeter(s), square centimeter(s), cubic centimeter(s)
COD	chemical oxygen demand
conc	concentrated
cpm	counts per minute
cps	counts per second
d	day(s)
DC	direct current
diam	diameter
DO	dissolved oxygen
DOX	dissolved organic halogen
dpm	disintegrations per minute
g	gram(s)
g	gravity, unit acceleration of
GC	gas chromatograph
GC/MS	gas chromatograph/mass spectrometer
h	hour(s)
HPLC	high-performance liquid chromatography
IC	ion chromatograph
ICP	inductively coupled plasma
ID	inside diameter
IU	international unit(s)
keV	kiloelectron volt(s)
kg	kilogram(s)
kPa	kilopascal
L	liter(s)
M	mole or molar
m, m^2, m^3	meter(s), square meter(s), cubic meter(s)
MCL	maximum contaminant level
MDL	method detection level
me	milliequivalent(s)
MeV	megaelectron volt(s)
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mm, mm^2, mm^3	millimeter(s), square millimeter(s), cubic millimeter(s)
mol wt	molecular weight
MPN	most probable number
MS	mass spectrometer
mV	millivolt(s)
μA	microampere(s)
μCi	microcurie(s)
μg	microgram(s)
μL	microliter(s)
μm	micrometer(s)
N	normal
nCi	nanocurie(s)
ng	nanogram(s)
NIST	National Institute of Standards and Technology
No.	number
NTU	nephelometric turbidity unit(s)
OD	outside diameter
Pa	pascal
pCi	picocurie(s)
pg	picogram(s)
PTFE	polytetrafluoroethylene
PVC	polyvinyl chloride
rpm	revolution(s) per minute
rps	revolution(s) per second
SD	standard deviation
SDI	sludge density index
s	second(s)
sp., spp.	species
sp gr	specific gravity
ST	standard taper
SVI	sludge volume index
TFE	tetrafluoroethylene
THM	trihalomethane(s)
TOC	total organic carbon
TON	threshold odor number
TOX	total organic halogen
Toxicity terms	*see* Section 8010B
U	unit(s)
USGS	U.S. Geological Survey
USP	United States Pharmacopoeia
UV	ultraviolet
V	volt(s)
v/v	volume ratio
W	watt(s)
WEF	Water Environment Federation
WPCF	*see* WEF

Abbreviations of periodical titles in reference lists and bibliographies are based on those given in *Biosis. List of Serials with Title Abbreviations*, 1970. Biosciences Information Service of Biological Abstracts, Philadelphia, Pa.

General Information

TABLE A: UNIT PREFIXES

Symbol	Prefix	Multiples and Submultiples
M	mega-	10^{6}
k	kilo-	10^{3}
m	milli-	10^{-3}
μ	micro-	10^{-6}
n	nano-	10^{-9}
p	pico-	10^{-12}

TABLE B: METRIC-ENGLISH EQUIVALENTS

Metric Unit	Multipled by	= English Unit
m	3.279	ft
lux	0.0929	ft-c
L	0.2642	gal
cm	0.394	in.
kg	2.203	lb
g	0.0353	oz
kPa	0.145	psi

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