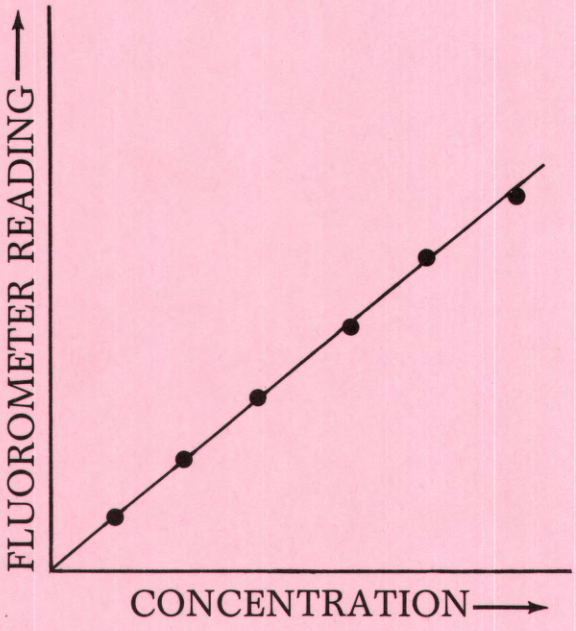
FLUOROMETRIC PROCEDURES FOR DYE TRACING



U.S. GEOLOGICAL SURVEY
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By James F. Wilson, Jr., Ernest D. Cobb, and Frederick A. Kilpatrick

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GLOSSARY

Absorption. The physical assimilation of dye molecules by organic or inorganic solids such as bank, bed, suspended material, or plantlife. Also, the assimilation and conversion to thermal energy of irradiating energy (light) by most substances, including all that are fluorescent.

Absorption spectrum. For a given substance, the characteristic relationship of the intensity of absorbed energy (light) to the wavelength of the incident energy. Related to, but not the same as, excitation spectrum.

Adsorption. The physical adhesion of dye molecules to the surfaces of solids such as bank, bed, suspended material, or plantlife. A primary cause of loss of dye in streams and aquifers.

Background. Fluorometer readings, other than those due to fluorescence of the tracer dye, which result from scattered light, from fluorescence of natural materials or pollutants, or from other causes.

Calibration. The relationship of fluorometer readings to dye concentration.

Concentration quenching. Not true quenching but rather the reduction in the rate of increase of fluorometer readout with increasing dye concentration due to the increasing optical density of the dye itself. A problem only with very high concentrations. (See quenching.)

Converter (inverter). An electronic device used to change direct current from a storage battery into alternating current as required by some fluorometers.

Cuvette (sample holder). A test tube or other device, usually made of Pyrex glass, for containing the water sample in the fluorometer.

Detectability. The extent to which a dye may be identified quantitatively in a water sample; depends on the spectral characteristics of the dye, the potential interference by background materials, and the sensitivity of the fluorometer. (See sensitivity.)

Diluent. A diluting agent or solvent; distilled water is usually the diluent used in preparing standard dye solutions.

Dilution factor. The ratio of the volume of tracer solution to the total resultant solution. Used in computing concentrations of standard solutions.

Dummy cuvette. The opaque cuvette used to set the zero reading on the fluorometer dial before testing water samples.

Emission. The discharge of energy from an excited fluorescent substance. (See fluorescence.)

Emission (fluorescence) spectrum. For a given fluorescent substance, the characteristic relationship of the intensity of emitted energy (light) to the wavelength of the emitted energy; except for magnitude, the relationship is independent of the wavelength of the absorbed light.

Excitation. For fluorescent substances, the state wherein certain electrons are raised temporarily to higher orbits owing to absorption of energy from an external source.

Excitation spectrum. For a given fluorescent substance, the characteristic relationship of the intensity of emitted light to the wavelength of the absorbed light. Related to, but not the same as, absorption spectrum.

Filter fluorometer; fluorimeter; fluorescence meter. An instrument, containing a lamp or other means of exciting fluorescent radiation in a sample, with filters and a detector to measure relative fluorescent intensities caused by variations in concentration of the substance under examination.

Fluorescence. The emission of electromagnetic waves of characteristic energy when atoms or molecules decay from an excited state to a lower energy state. The excitation may be induced by subjecting the substance to radiation of slightly higher energy (shorter wavelength) than that of the characteristic emission, which ceases as soon as the external source is removed.

Fluorescence spectrum. See emission spectrum.

Fluorometer. See filter fluorometer and spectrofluorometer.

Loss of dye. Dye that is lost due to any or all of the following: Absorbtive loss, photochemical decay, quenching, and chemical alteration.

Luminescence. Any emisssion of light not directly ascribable to heat. Fluorescence and phosphorescence are two examples.

Nanometer (nm). A unit of length equal to 10 angstroms, one-thousandth of a micrometer, and one-millionth of a millimeter; used in expressing wavelength.

Neutral-density (ND) (range extension) filter. A filter that is used to reduce the amount of light reaching the photomultiplier tube in the fluorometer. Usually used in addition to other filters when dye concentrations are too high to obtain a reading, the ND filter reduces the intensity of the light, but does not change the spectral distribution of the light.

Photochemical decay (photodecomposition). Degradation of the fluorescence intensity of dye in a stream or sample container by the action of light.

Quenching. The reduction of fluorescence due to any of a number of kinds of interaction of the dye molecules with other chemicals in the water. (See concentration quenching.)

Raman scatter. A spectrum produced when light is scattered as it passes through certain substances such as water. Some of the energy is absorbed; the scattered light has longer wavelengths than the incident light. (See Rayleigh scatter.)

Rayleigh scatter. A spectrum produced when light is scattered as it passes through certain substances such as water; the scattered light has the same wavelengths as the incident light. (See Raman scatter and Tyndall scatter.)

Selectivity. The capability to isolate a narrow spectral band of exciting or emitted light by selection of appropriate fluorometer filters. (See sensitivity and specificity.)

Sensitivity. The extent to which a given fluorometer can detect low concentrations of a given dye; depends on the characteristics and interrelationships of the fluorometer components. (See detectability, selectivity, and specificity.)

Sorption. The process of taking up and holding either by absorption or adsorption. (See absorption and adsorption.)

Specificity. The capability to isolate fluorometrically the fluorescence of a particular dye from that of all other fluorescent substances present in a water sample. (See selectivity and sensitivity.)

Spectral-fluorescence characteristics. The excitation and emission spectra of a particular fluorescent substance, often identified by the wavelengths at which maximum excitation and maximum emission occur. (See excitation spectrum and emmission spectrum.)

Spectral-transmittance characteristics. For a given filter or filter combination, the curve relating transmittance (percent transmission) to wavelength, sometimes identified by the wavelength of maximum transmission. (See transmittance.)

Spectrofluorometer (fluorescence spectrometer). A special type of fluorometer that may be used, among other things, to determine the spectral-fluorescence characteristics of fluorescent substances.

Standard solution. A sample containing a known concentration of dye in distilled water diluent; used to calibrate a fluorometer.

Tracer. In hydrologic tracing, any dissolved, suspended, or floating material used to determine the path and (or) rate of movement and dispersion of similar materials in the water. Tracers include natural materials and pollutants such as sewage, as well as materials intentionally injected, such as floats, salts, radioisotopes, and fluorescent dyes.

Transmittance. The proportion of incident light that emerges from the opposite side of a fluorometer filter. (See spectral-transmittance characteristics.)

Tyndall scatter. A spectrum produced when light is scattered by suspended material in a water sample; the scattered light has about the same wavelengths as the incident light. (See Rayleigh scatter.)

Visible spectrum. The narrow band of the electromagnetic spectrum to which the human eye is sensitive; approximately the interval 380-800 nm, which includes the excitation and emission spectra of most of the tracer dyes.

Xanthene dyes. The group of brilliant fluorescent dyes characterized by the presence of the xanthene nucleus ($C_{13}H_{10}O$). Rhodamine WT and pontacyl pink are xanthene dyes used for water tracing.

SELECTED CONVERSION FACTORS

Factors for Converting International System of Units (SI) to Inch-Pound Units

The following factors may be used to convert the International System of Units (SI) published herein, to inch-pound units.

Multiply SI units	by	To obtain inch-pound units
	Length	
meter (m)	3.281	foot (ft)
	39.37	inch (in)
millimeter (mm)	0.03937	inch
	Volume	
cubic centimeter (cm^3)	0.06101	cubic inch (in^3)
liter (L)	0.2642	gallon (gal)
	0.03531	cubic foot (ft^3)
milliliter (mL)	0.06101	cubic inch
	Mass	
gram (g)	0.002205	pound (lb)
kilogram (kg)	2.205	pound
	35.27	ounce (oz)
	Temperature	
degree Celsius (°C)	°F = 9/5 °C + 32 °C = 5/9 (°F - 32)	degree Fahrenheit (°F)

Other conversion relations which may be helpful to the user of this report are as follows:

1 angstrom (A) = 10^{-10} meters
1 kilometer (km) = 10^3 meters
1 meter = 10^3 millimeters
1 millimeter = 10^3 micrometers (μm)
1 micrometer = 10^3 nanometers (nm)

SYMBOLS AND UNITS

Symbol	Explanation	Units
C_f	Final concentration	µg/L
C_i	Initial concentration	µg/L
C_n	New concentration after a dilution	µg/L
C_s	Concentration of stock solution	µg/L
D_i	Dilution factor	--
D_T	Total dilution factor in a serial dilution	--
S_G	Specific gravity	--
V_d	Volume of the dye solution	L
V_w	Volume of added diluent	L
W_d	Weight of the dye	g

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ABSTRACT

This manual describes the current fluorometric procedures used by the U.S. Geological Survey in dye tracer studies such as time of travel, dispersion, reaeration, and dilution-type discharge measurements. The advantages of dye tracing are (1) the low detection and measurement limits, and (2) the simplicity and accuracy of measuring dye tracer concentrations using fluorometric techniques.

The manual contains necessary background information about fluorescence, dyes, and fluorometers and a description of fluorometric operation and calibration procedures as a guide for laboratory and field use. The background information should be useful to anyone wishing to experiment with dyes, fluorometer components, or procedures different from those described. In addition, a brief section is included on aerial photography because of its possible use to supplement ground-level fluorometry.

INTRODUCTION

The extensive use of fluorescent dyes as water tracers began in the early to mid-1960's. Prior to this, floats, chemical salts, and actual contaminants had been used as tracers. After World War II, radioisotopes such as tritium (heavy hydrogen) gained favor as tracers, but their use was severely limited by handling problems, the special training required, and a general lack of understanding by the public. A search for a suitable substitute for radioisotopes led to the rediscovery of fluorescent dyes for tracing. Although fluorescein had been used occasionally for more than 50 years (Dole, 1906), Pritchard and Carpenter (1960) were the first to combine rhodamine B, a much better surface-water tracer than fluorescein, and greatly improved fluorometers for large-scale tracing studies.

Within the U.S. Geological Survey, feasibility tests of dyes and fluorometers were made in 1961-62 and reported by Wright and Collings (1964). The initial application of fluorometry--and by far the most-used application to date--was for the measurement of time of travel of solutes in streams, (Buchanan, 1964; Hubbard and others, 1982). The procedures also were adapted to the measurement of stream discharge by dye-dilution methods (Cobb and Bailey, 1965[1/]; and Kilpatrick, 1968). Fluorometry has also been applied to studies for determining reaeration rates of streams (Rathbun and others, 1977). Others in the Geological Survey who have contributed to the development of fluorometric procedures for dye tracing include E. L. Meyer, J. R. Kreider, and B. Dunn.

[1/] Cobb, E. D., and Bailey, J. F., 1965, Measurement of discharge by dye-dilution methods: Unpublished data on file in Branch of Surface Water, U.S. Geological Survey, Reston, Va.

In addition to time-of-travel, dispersion, reaeration, and discharge measurements, hydrologic applications have included studies of waste buildup and flushing in estuaries (Yotsukura and Kilpatrick, 1973; Bailey and others, 1966); circulation and stratification of water in reservoirs; path tracing in cavernous limestone, measurement of ground-water time of travel (Freeze and Cherry, 1979, p. 426-430); determination of well-drilling fluid circulation time; studies of the uptake of irrigation waters by plants (Robinson and Donaldson, 1967); tagging of herbicide spray with dye in a canal to facilitate downstream sampling and testing for traces of the herbicide; and tagging of power plant heated water discharges to facilitate downstream sampling.

The outstanding characteristics of dye tracing are (1) the low detection and measurement limits, and (2) the simplicity and accuracy of measuring dye tracer concentrations using fluorescent techniques. Nearly all applications of dye tracing include the introduction of dye into a water body, the subsequent collection of water samples over time and space to measure the response, and the determination of the concentration of dye in the samples by means of a fluorometer. Dosing and sampling procedures and data analysis vary with each application; fluorometric procedures are generally the same for most applications and are the subject of this manual.

This manual is a revision of the manual by Wilson (1968a) and is intended to be a companion to the manuals on individual applications such as discharge measurements (Kilpatrick and Cobb, U.S. Geological Survey written communication), time-of-travel measurements (Hubbard and others, 1982), and others that may follow. This manual contains necessary background information about fluorescence, dyes, and fluorometers and a description of fluorometric procedures as a general guide in both laboratory and field applications of dye tracing. The background information also should be useful to anyone wishing to experiment with dyes, fluorometer components, or procedures different from those described. In addition, a brief section on aerial photography is included because of the possible use of aerial photography in dye tracing to supplement ground-level fluorometry.

The procedures described are subject to modification, as dyes, equipment, and techniques are continually being improved. Also, accuracy requirements for determining dye concentrations vary with the individual applications and should be established in advance of an investigation in order to use the appropriate fluorometric procedures.

GENERAL DESCRIPTION OF FLUORESCENCE

Fluorometric analysis, or fluorometry, utilizes the physical phenomenon called fluorescence. Because fluorescence is the outstanding property of all the tracer dyes, a general understanding of the phenomenon is necessary to assure its proper utilization in any application of dye tracing.

Basically, fluorescence is a form of luminescence, a broad term for any emission of light not directly ascribable to heat. Fluorescent substances emit radiation (light) immediately upon irradiation from an external source; emission ceases when the source is removed. A similar kind of luminescence is phosphorescence. However, phosphorescent substances store some of the

irradiating energy, delaying emission, which continues after removal of the light source. For a detailed technical discussion of luminescence, see Bowen and Garlick (1966).

The almost instantaneous sequence of events in fluorescence is as follows: (1) absorption of energy from an outside source such as the sun or an ultraviolet lamp, (2) excitation of some of the electrons of the fluorescent substance, resulting in enlarged electron orbits--the "excited state," and (3) emission of energy in the form of photons (light), as the excited electrons return to normal positions--the "ground state." The emitted (fluoresced) energy nearly always has longer wavelengths and lower frequencies than the absorbed energy because some energy is lost in the process (Stokes' law). It is this property of dual spectra--a different specific combination of excitation and emission spectra for each fluorescent substance that is utilized to make fluorometry an accurate and sensitive analytical tool. An excellent reference on the basic theory of fluorescence and fluorometry is Udenfriend (1962, p. 1-124).

In fluorometry, wavelengths are given in either nanometers or angstrom units. Nanometers are used in this manual.

Most substances are at least mildly fluorescent, and most fluorescence occurs in the 200-800 nm range of wavelengths--ultraviolet and visible light (fig. 1). Strongly fluorescent substances convert a high percentage of absorbed energy into emitted energy. Most strongly fluorescent substances fluoresce in the ultraviolet-to-green part of the spectrum. A few substances, including some of the preferred tracer dyes, fluoresce in the yellow-orange range. Dyes that are useful for tracer applications are strongly fluorescent and can be detected easily in small concentrations. The spectral properties of the tracer dyes and the corresponding fluorometer optics are discussed in subsequent sections of this manual.

Fluorescent materials likely to be found in some streams include algae and other naturally occurring organics, certain minerals, manmade pollutants such as paper and textile dyes, certain petroleum products, and laundry-detergent brighteners. Wright and Collings (1964, p. 749-750) and Williams and Bridges (1964, p. 372-376, 387-393) discuss the spectral-fluorescent properties of some common organic substances.

Fluorescence intensity is affected in varying degrees by certain physical and chemical factors such as solvent, concentration, temperature, pH, photochemical decay, and fluorescence quenching. Udenfriend (1962, p. 96-114) and Williams and Bridges (1964, p. 377-385) present excellent general discussions of these and other factors. Studies limited to an evaluation of the properties of fluorescent dyes were reported by Pritchard and Carpenter (1960), Feuerstein and Selleck (1963a), Wright and Collings (1964), and Smart and Laidlaw (1977). These properties are discussed in more detail in the next section.

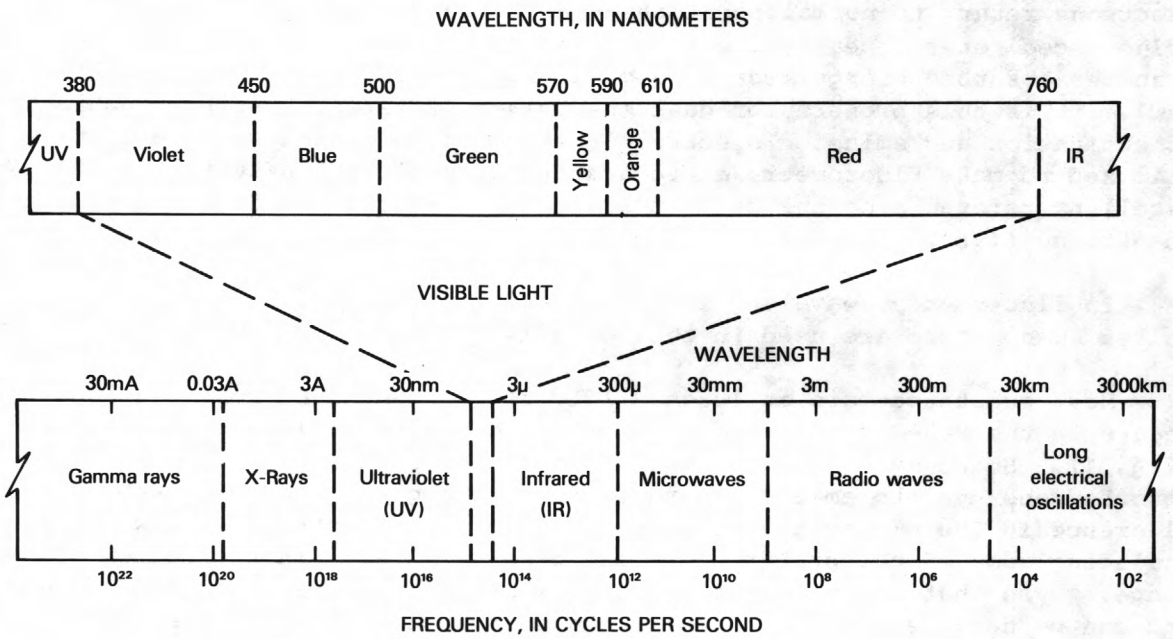

Figure 1.--The electromagnetic spectrum, with an enlargement of the visible spectrum. The fluorescent tracer dyes absorb and emit light at visible wavelengths.

FLUORESCENT DYES

Types Recommended for Tracing

Hundreds of commercial dyes are available in a variety of colors. A great number are strongly fluorescent, but only a few exhibit the combination of properties essential for water tracing. Two dyes, both a variation of the same basic organic structure (xanthene), are preferred for use as water tracers. These are rhodamine WT and pontacyl pink (also known as intracid rhodamine B, pontacyl brilliant pink B, and acid red 52). These are generally good tracers because they are (1) water soluble, (2) highly detectable--strongly fluorescent, (3) fluoresce in a part of the spectrum not common to materials generally found in water; hence, background fluorescence is not a problem, (4) harmless in low concentrations, (5) inexpensive, and (6) reasonably stable in a normal water environment.

A third dye, acid yellow 7 (also known as lissamine FF), lends itself to water tracing in certain environments. Only a small amount of field testing has been performed with this dye. Smart and Laidlaw (1977) reported on the dye's characteristics. It appears to have many good qualities for water tracing. Brian G. Katz (Hydrologist, U.S. Geological Survey, written communication, 1982) reported that background fluorescence was observed to increase with increasing concentrations of total organic carbon for the optical system used with acid yellow 7 dyes. This seems to be one of the principal drawbacks to this dye--that there often is a high natural background in streams and that during unsteady flow, the background may vary with time.

Dennis E. Ford and Kent W. Thornton (Ford, Thornton, Norton, and Associates, written communication, 1982) found in laboratory tests that acid yellow 7 has a solubility of 2.5 g/100 mL. Brian Katz (U.S. Geological Survey, oral communication, 1982) reported that practical upper limits of solubility in his tests were about 1 g/100 mL for acid yellow 7.

Characteristics of the preferred tracer dyes are presented in table 1. It should be noted that for some dyes there are several manufacturers, but only a few are mentioned in table 1.

Rhodamine WT is preferred for most water-tracing uses. It is easy to use and has many other characteristics that are desirable for water tracing. The other dyes may be used to advantage under special conditions.

The acid yellow 7 has not been used extensively in field tests, but it is believed that it is a good dye for use in waters with small or constant concentrations of organic matter.

All of the dyes listed in table 1 are believed to be acceptable for ground-water tracing, although acid yellow 7 has not been used for that purpose. There may be problems with the use of any of these dyes in tracing ground water through clay and silt soils because of sorption on the large surface areas associated with these soils.

Table 1.--Characteristics of preferred tracer dyes

Dye color, formula, and common name	Other names	Cost per pound as specified	Remarks
Rhodamine WT	Intracid rhodamine WT	$9.50[a]. Available in 20-percent solution (specific gravity, 1.19, 9.92 pounds per gallon at 62° F.	High detectability, low sorptive tendency, good diffusity, low acidity. Fluorescence variable with temperature.
Acid red 52 ($C_{27}H_{29}N_2O_4S_2Na$) Often referred to as pontacyl pink	Intracid rhodamine B Pontacyl brilliant pink B	$15.14[a]. Available as powder.	Fairly high detectability, low sorptive tendency, good diffusity, low decay rate, fairly stable at pH extremes. Fluorescence variable with temperature.
Acid yellow 7	Lissamine FF Lissamine yellow FP Brilliant acid yellow 8G Overacid brilliant sulpho flavine FF	$17.85[b]. Available as a powder.	Fairly high detectability, low sorptive tendency, good diffusity, fairly stable at pH extremes, little affected by temperature. Subject to background interference.

[a]Costs provided by Crompton and Knowles Corporation, P.O. Box 68, Skokie, Illinois 60076, phone 312/675-5510, on March 29, 1984, for 25-pound orders.

[b]Costs provided by Organic Chemical Corporation, P.O. Box 4258, East Providence, Rhode Island 02914, phone 401/434-3300, on March 29, 1984, for 25-pound orders.

Note: Costs of dye vary with the amount ordered. Additional charges may be made for handling and shipping.

In the past, rhodamine B and BA have been widely used for water tracing, but because of their high adsorption tendencies, they are not recommended for this purpose and are not referred to further in this manual.

Properties of Dyes

The outstanding property of the dyes, discussed previously, is their strong fluorescence. Additional properties that affect the use of the dyes as tracers are discussed below. Good references on the subject are: Feuerstein and Selleck (1963a, b) and Smart and Laidlaw (1977).

Factors Which Affect Fluorescence

Fluorescence may vary with the solvent used, but in hydrologic tracing the effect is constant because the solvent always is basically water; other chemicals present in the water may affect fluorescence in other ways.

In dilute solutions (solutions in which less than 5 percent of the exciting light is absorbed) and for a given fluorometer setup, fluorescence varies directly with dye concentration; this important fact is discussed in the section on fluorometer calibration.

Aside from concentration, the most significant factor affecting fluorescence of dilute solutions is sample temperature. Fluorescence activity increases (resulting in higher readings) as sample temperature decreases; lower readings are obtained as temperature increases. Temperature effects must be accounted for in data analysis. Temperature-correction curves for rhodamine WT, pontacyl pink, and acid yellow 7 are given in figure 2. It can be seen from figure 2 that acid yellow 7 is relatively insensitive to temperature changes.

If calibration standards and field samples all are brought to a common temperature before the samples are analyzed, temperature effects can usually be ignored unless the samples are allowed to warm up while being tested in the fluorometer.

The pH of the sample also may affect fluorescence intensity. Fluorescence of rhodamine WT is stable in the pH range of 5-10 and decreases outside those limits (see curves prepared by Feuerstein and Selleck, 1963b, p. 13). The fluorescence of pontacyl pink and acid yellow 7 is stable in the pH range of 4 to 10 and decreases outside those limits (Smart and Laidlaw, 1977, p. 19 and 20).

Decreased fluorescence due to pH variation is not usually serious, except possibly in highly acid streams, where apparent recovery of dye could be very small. Feuerstein and Selleck (1963a, p. 24; 1963b, p. 12) indicated that by adjusting the pH of a solution, one can restore fluorescence to full strength. This, however, would not rectify any nonreversible chemical effects due to reaction of the dye with the acidic compound itself.

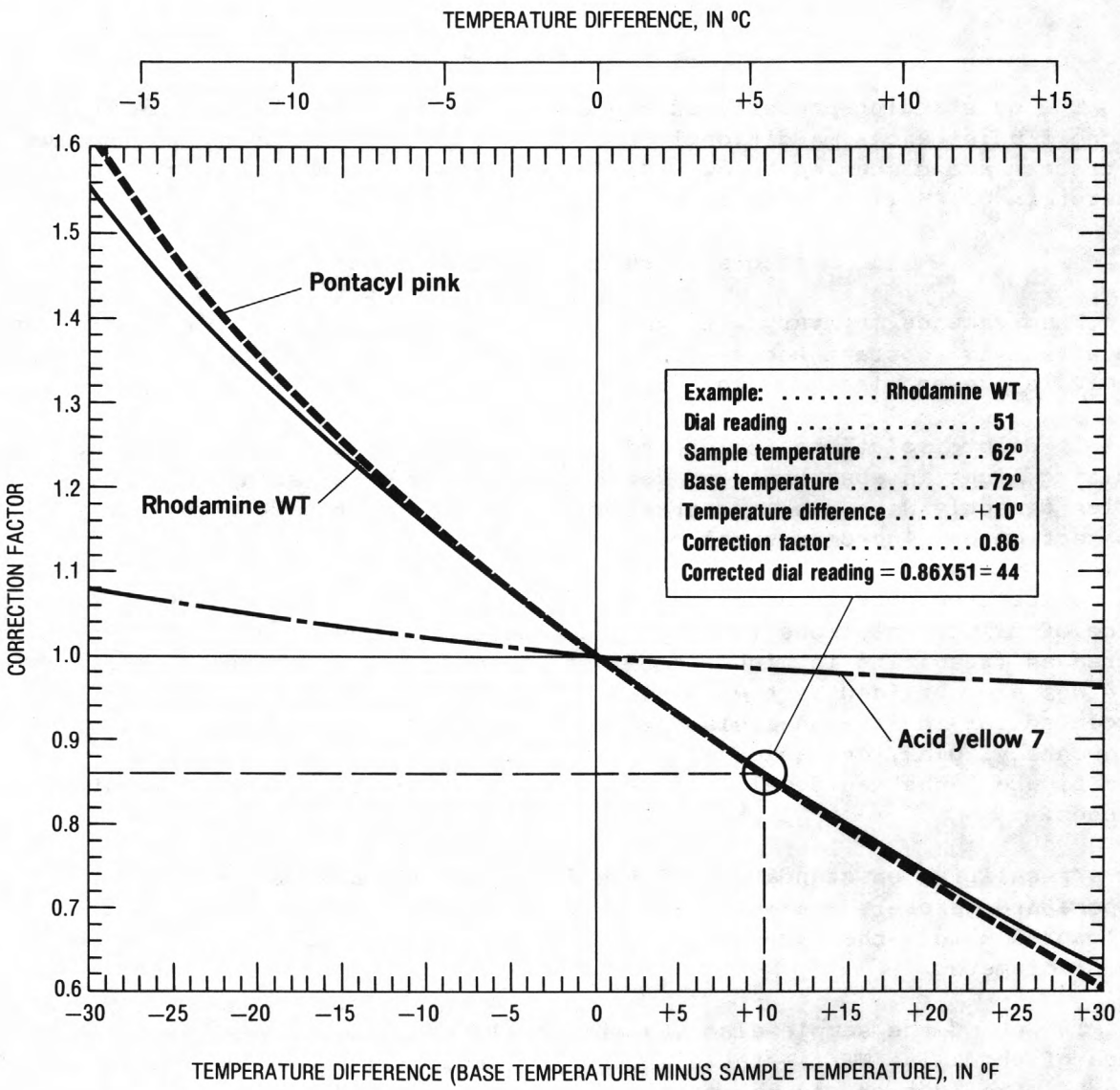

Figure 2.--Temperature-correction curves for rhodamine WT, pontacyl pink, and acid yellow 7 dyes. Curve for acid yellow 7 modified from Smart and Laidlaw (1977, fig. 2).

For tracing purposes, fluorescence also can be adversely affected by quenching, which results from the action of other chemicals in the solution. The quenching agent may do any or all of the following (Williams and Bridges, 1964, p. 383-385): (1) absorb exciting light, (2) absorb light emitted by the dye, and(or) (3) degrade the excited-state energy. A fourth possibility which is nonreversible, is that the quenching agent may chemically change the fluorescent compound. Chlorine, for example, is a known quencher of the fluorescence of rhodamine dyes. For this reason chlorinated tapwater generally should not be used to prepare standard solutions. Where tap water is the only water available, containers of the water that are open to the atmosphere can be allowed to sit for about 12 hours. This will usually remove small concentrations of chlorine. An effect similar to true quenching, concentration quenching, occurs when relatively high concentrations of dye produce a screening effect on both the exciting and emitted light. This problem can be overcome simply by diluting a measured amount of sample with a measured amount of water.

In tests conducted in Baltimore storm sewers using chlorinated hydrant water, Katz (U.S. Geological Survey, written communication, 1982) found no effect of the chlorine on the fluorescence of acid yellow 7, but he did find significant effects on the fluorescence of rhodamine WT. On the other hand, Ford and Thornton (Ford, Thornton, Norton, and Associates, Ltd., written communication, 1982) found sizable decreases in fluorescence of acid yellow 7 when solutions containing 100 and 500 µg/L (micrograms per liter) of acid yellow 7 and 1.0 and 5.25 mg/L (milligrams per liter) of residual chlorine were tested in a laboratory. It may be concluded that chlorinated water should be avoided, if possible, both in field tests and in the preparation of standards.

High levels of oxygen, such as in highly aerated streams, appear to cause the same effect as chlorine. This will not ordinarily be a problem, unless the stream being tested is virtually all "white water." Under these infrequent conditions, unusually large losses of rhodamine WT have been measured.

A permanent reduction in fluorescence can be caused by photochemical decay, or photodecomposition. Bright sunlight has this effect on the rhodamine dyes, and the effect increases gradually with time. Stream depth, turbidity, and cloudy skies tend to minimize photochemical decay, so that the effect usually is significant only for applications requiring recovery of a large percentage of the dye or where the dye is exposed to sunlight for several days. Smart and Laidlaw (1977, p. 24) indicate that the photochemical decay rate of acid yellow 7 appears to be an order of magnitude less than that for pontacyl pink.

Sorption

One of the most important characteristics of dyes used for water tracing is the tendency to adhere to suspended and bed materials, aquatic plants and the like (adsorption), or to be absorbed by such materials. It is important that the sorption tendency of a dye used as a water tracer be as low as possible. Rhodamine WT, pontacyl pink, and acid yellow 7 are only slightly susceptible to adsorption in most situations. Generally, organic sediments adsorb more

dye than inorganic sediments. In laboratory tests reported by Smart and Laidlaw (1977, p. 25-29), acid yellow 7 generally seemed to be more resistant to adsorption on mineral sediments than either rhodamine WT or pontacyl pink. Acid yellow 7 also seems to be more resistant to adsorption by organic matter than does rhodamine WT. There is some evidence in the report by Smart and Laidlaw (1977, p. 25-29) that pontacyl pink is superior or equal to acid yellow 7 in water with significant organic matter.

Although sorption (adsorption and(or) absorption) is not a factor that affects fluorescence, the end results are similar to those from photo-chemical decay or chemical quenching. Like the others, sorptive loss contributes to decreased recovery of dye, but it is impossible to separate quantitatively these three causes of dye loss in a stream. Dye losses have a direct bearing on the accuracy of discharge measurements, but are rarely serious enough to threaten the results of time-of-travel or dispersion measurements. Sorption, of course, is a critical factor in ground-water tracer studies.

Biological Effects

A number of tests have been conducted concerning the effects of rhodamine WT and pontacyl pink on aquatic life and on laboratory test animals. Parker (1973) reported on a test where eggs and 12-day-old larvae of the Pacific oyster *(Crassostrea gigas)* were exposed for 48 hours in water at 24°C with rhodamine WT dye concentrations that ranged from 1 µg/L to 10,000 µg/L. All of the tested eggs developed to larvae without abnormalities and all of the 12-day-old larvae survived with no abnormalities. An additional test was made by Parker (1973) on silver salmon and Donaldson trout. No mortalities or other problems were observed when the fish were exposed to water containing 10,000 µg/L of rhodamine WT for 17.5 hours and then increased to 375,000 µg/L for an additional 3.2 hours.

J. S. Worttley and T. C. Atkinson (reported as personal communication, 1975, in Smart and Laidlaw, 1977) exposed a number of fresh and brackish water invertebrates, including water flea *(Daphnia magna)*, shrimp *(Gammarus zaddachi)*, log louse *(Asellus aquaticus)*, may fly *(Cloeon dipterum)*, and pea mussel (species *pisidium*) to water containing up to 2,000,000 µg/L of rhodamine WT for periods of up to 1 week. No significant differences in mortality were observed between the test and control animals.

D. E. Donaldson (U.S. Geological Survey, written communication cited by Wilson, 1968a, p. 6, and Smart and Laidlaw, 1977, p. 30) prepared a drinking-water solution of 10 µg/L of rhodamine WT that was given to rats. He observed a slight loss of body weight and some effect on certain body organs--especially the liver--when the rats were given this solution for a prolonged time, as compared to a control group of rats.

Smart and Laidlaw (1977, p. 30) reported that subcutaneous injections of 50 µg of pontacyl pink caused inflammatory sores at the injection sites and a pronounced loss of body weight, whereas injections of rhodamine WT caused no traumatic ill effects even after 56 days of this treatment.

No known studies have been made of the effects of acid yellow 7 on test animals. According to Smart and Laidlaw (1977, p. 30), the manufacturers have indicated that acid yellow 7 is unlikely to cause any unusual toxic hazards.

A letter by the Director of the Criteria and Standards Division of the Office of Drinking Water, U.S. Environmental Protection Agency (EPA), dated April 10, 1980, states that "We (EPA) would not object to its (rhodamine WT) use as a tracer in lieu of additional information on human toxicology or a change in the position of the Food and Drug Administration."

S. L. Abidi (1982) reported on laboratory tests showing that when rhodamine WT is mixed with stream water containing nitrites, diethylnitrosamine (DENA), a carcinogen, may be formed. Johnson and Steinheimer (1984 and written commun., 1984) conducted a number of tests relative to DENA formation and persistence. They found that DENA in a simulated stream environment has a half life of less than 3 hours. They also analyzed water samples from four streams taken during rhodamine WT tracer studies and could not detect DENA in any of the samples. Nitrite concentrations in the four streams varied from 2 to 46 µg/L.

Users of rhodamine WT should take special precautions to keep from coming into direct contact with the dye. Rubber or plastic gloves should be worn when handling concentrated dye solutions. When the dye does come into contact with the skin, it should be washed off immediately. Pipetting of dye solutions should be done with a squeeze bulb or by using a long piece of flexible tubing to prevent accidental ingestion of the dye.

The Geological Survey policy for the use of rhodamine dyes by its employees is that the maximum permissible concentration of the dye is 10 µg/L at any water intake that ultimately results in direct or indirect human consumption. Concentrations at water intakes should be kept well below this level: many dye studies can be designed for maximum concentrations of 1 µg/L at such critical points as water intakes.

Spectral Characteristics

It is common practice to define fluorescent substances as those that absorb light at one wavelength and emit light at a longer wavelength. Actually, each fluorescent substance is characterized by a specific excitation spectrum and a specific emission spectrum. The excitation spectrum is the variation in intensity of emitted light with the wavelength of the absorbed light. (The excitation spectrum is equivalent to the absorption spectrum, which relates absorbed light to wavelength.) The emission spectrum is the variation in intensity of emitted light with the wavelength of the emitted light. The characteristic spectra for several tracer dyes are shown in figure 3. Usually the wavelengths corresponding to maximum excitation and emission intensity are given to represent the spectral-fluorescence characteristics of a substance.

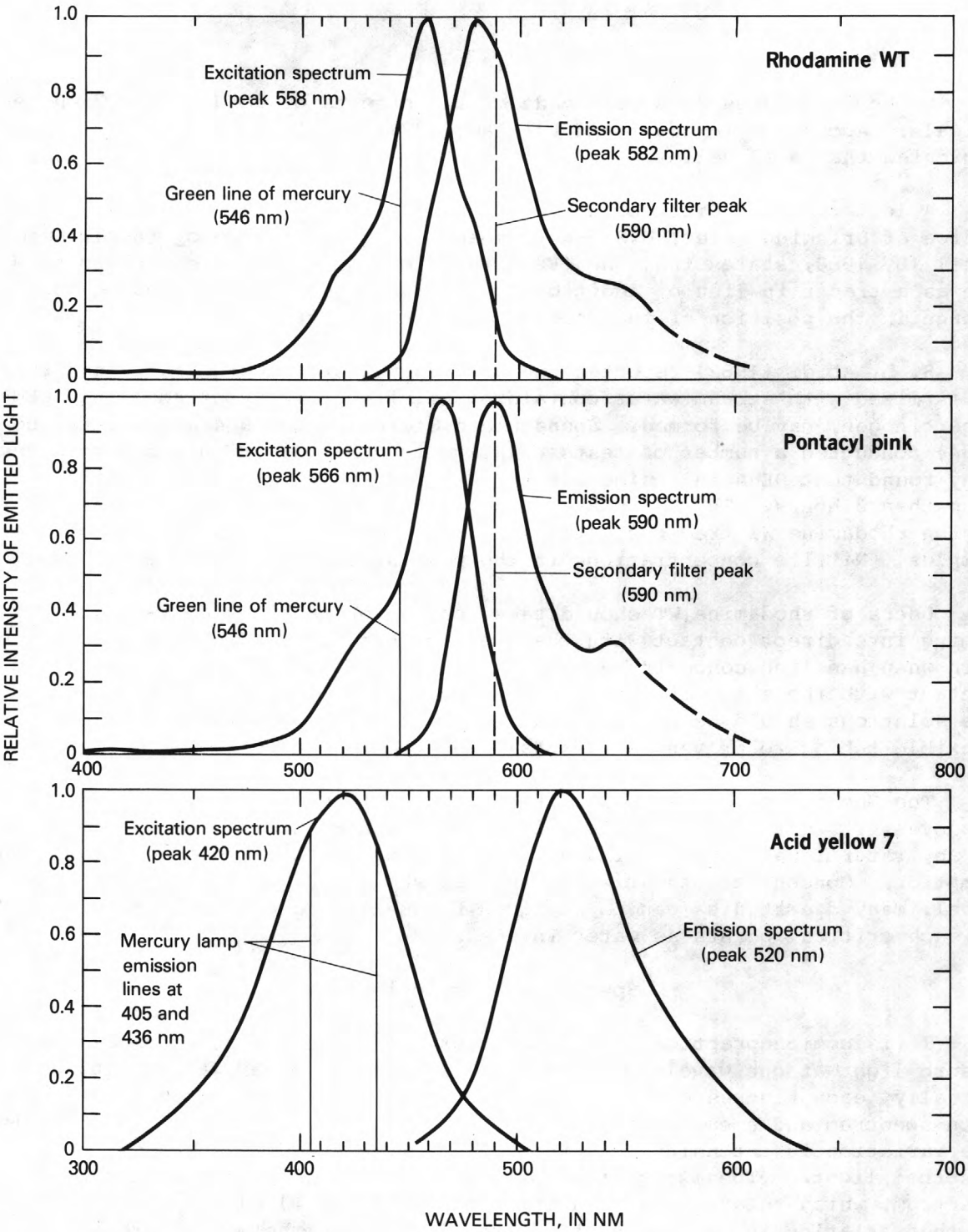

Figure 3.--Excitation and emission spectra of rhodamine WT, pontacyl pink, and acid yellow 7 dyes. Spectrofluorometric analysis for rhodamine WT and pontacyl pink courtesy of G. K. Turner Associates. Spectra for acid yellow 7 adapted from Smart and Laidlaw (1977, fig. 1).

Spectral-fluorescence characteristics of rhodamine WT and pontacyl pink are very similar as can be seen in figure 3. Therefore, it is impossible to differentiate between these dyes in a common solution. Fortunately, however, few other materials exhibit characteristics similar to those of the two dyes. The use of highly selective color filters in a fluorometer permits easy isolation of the fluorescence of the dyes from that of most other materials found in streams.

The spectral-fluorescence characteristics of acid yellow 7 are considerably different from those of the other dyes. The excitation spectrum peaks in the violet color range while the emission spectrum peaks in the green part of the spectrum. It is possible to differentiate quantitatively between acid yellow 7 and the other two dyes shown in figure 3; however, the emission characteristics of acid yellow 7 are somewhat similar to the emission characteristics of other materials which may be found in streams. This can cause interference of background fluorescence with that of the dye. Samples of the water in which acid yellow 7 is to be used as a tracer should be obtained prior to a test and checked for magnitude and variability of the background readings on a fluorometer. The fluorometer filters used to detect acid yellow 7 are different from those used for the other dyes. Filter selection is discussed in the next section.

FLUOROMETERS

General Description

The two fundamental types of fluorometers are (1) fluorescence spectrometers or spectrofluorometers, used for spectral analyses of fluorescent substances (Udenfriend, 1962, p. 62-86), and (2) filter fluorometers, discussed in this section.

A filter fluorometer, or fluorimeter, is an instrument that gives a relative measure of the intensity of light emitted by a sample containing a fluorescent substance; the intensity of fluorescent light is proportional to the amount of fluorescent substance present. However, a fluorometer reading by itself is a number having little meaning until it is compared with readings for samples of known concentrations (standards) on the same fluorometer under the same instrumental and temperature conditions. Generally, a reading for a given sample on one fluorometer cannot be compared directly with a reading for the same sample on a different fluorometer. Every fluorometer is different and must be individually calibrated.

A filter fluorometer consists of six basic components, shown in figure 4. This basic structure is found in all commercial fluorometers (Udenfriend, 1962, p. 62-78).

A number of companies market fluorometers that can be used for water tracing. Reference to a specific fluorometer and components is for the purpose of illustration and is not to be regarded as an endorsement of a particular brand of equipment. In this report three different brands of fluorometers are discussed. There may be other fluorometers that are as useful for dye tracing as those mentioned. The brands discussed are known to be used in the Geological Survey.

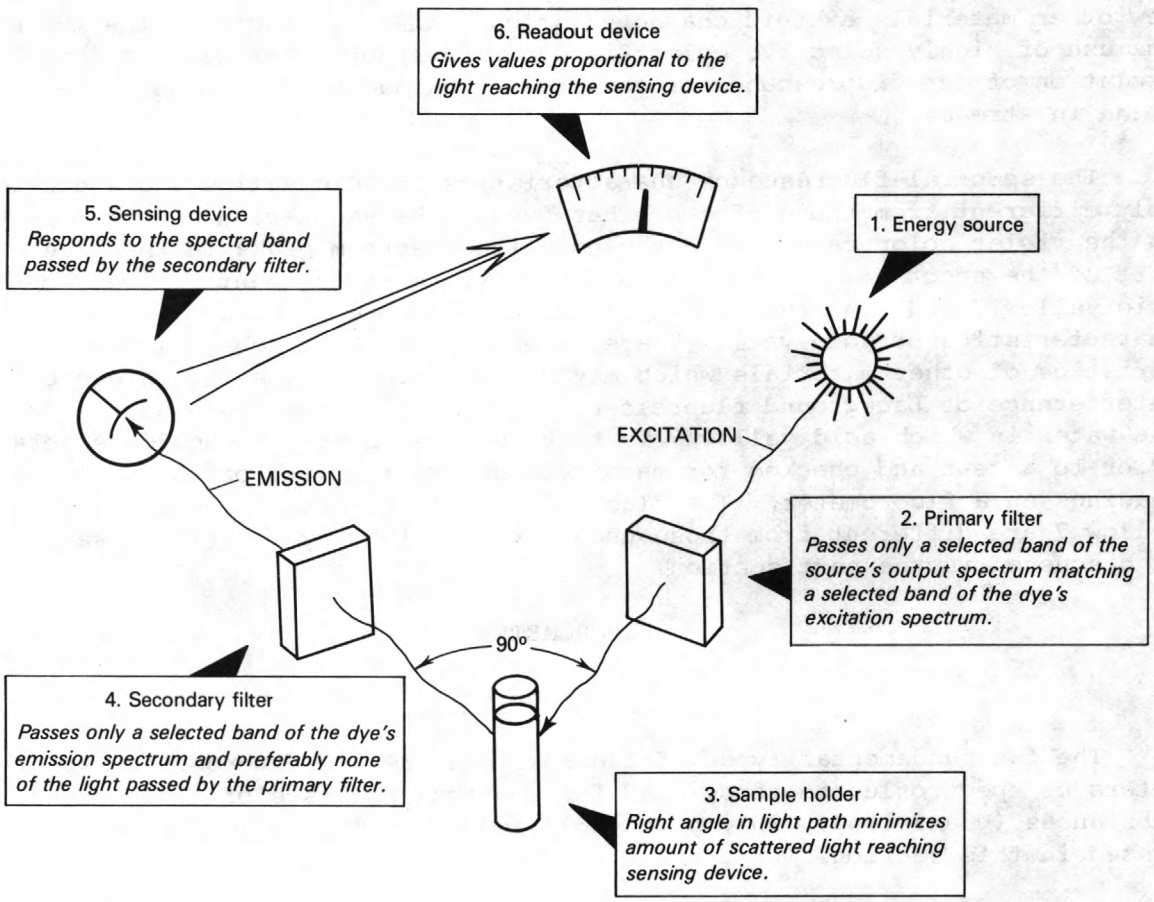

Figure 4.--Basic structure of most filter fluorometers.

Some representative fluorometers of the types that are presently used in the Geological Survey are shown in figures 5, 6, and 7. The Turner Model 111 fluorometer (fig. 5) has been extensively used by the Geological Survey. Figure 8 is a functional diagram of this fluorometer. Other fluorometers generally are based on similar principles. One basic difference however, is that both the FLM/AMINCO fluorocolorimeter and the Turner Design Model 10 fluorometer rely on solid-state electronic amplification of the photomultiplier signal. The Turner Model 111 fluorometer relies on a mechanical servomotor arrangement (fig. 8).

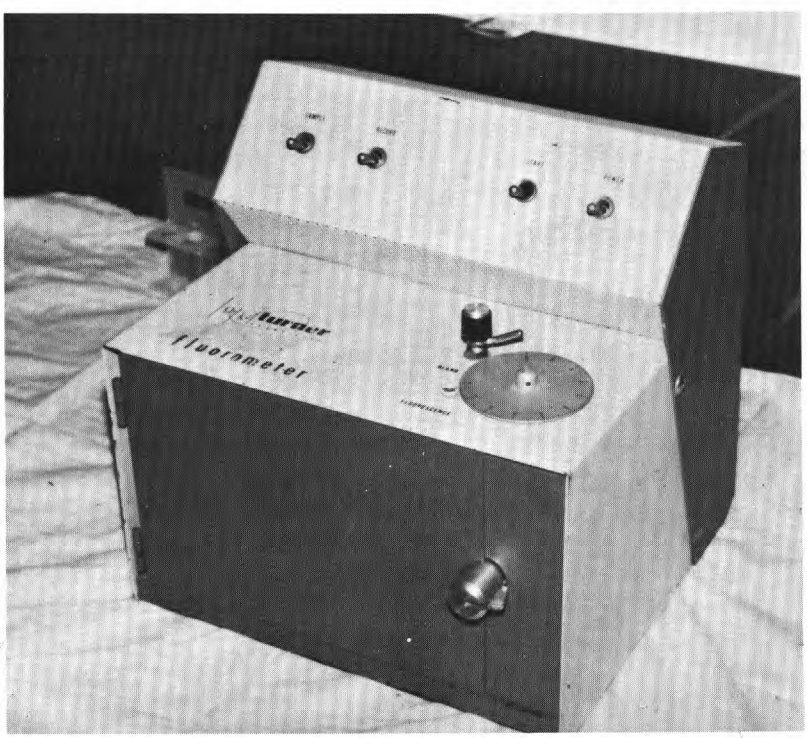

Figure 5.--The Turner Model 111 fluorometer.

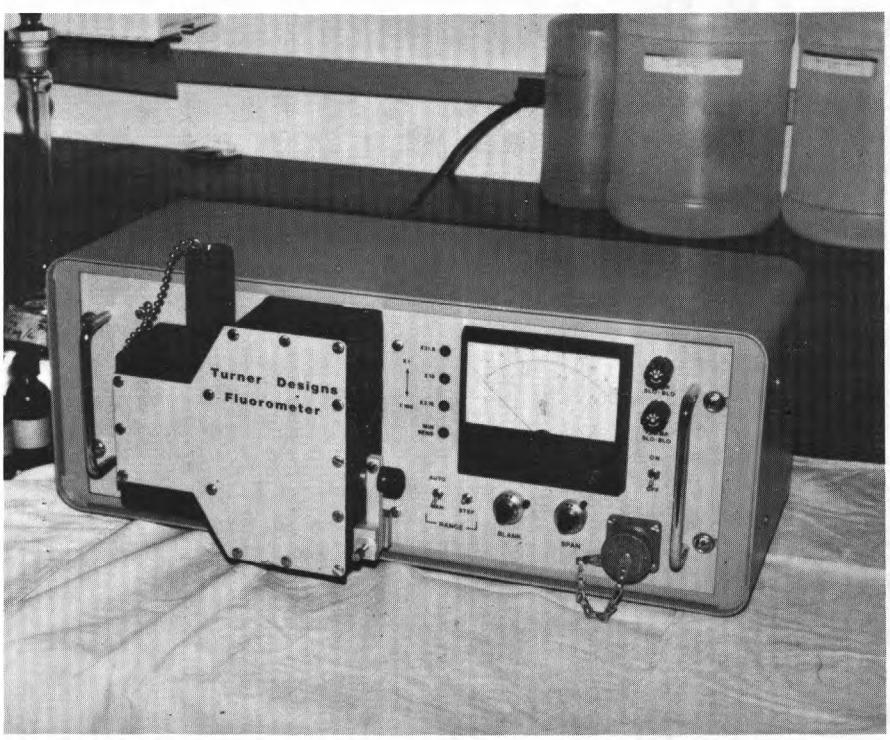

Figure 6.--The Turner Designs Model 10 fluorometer.

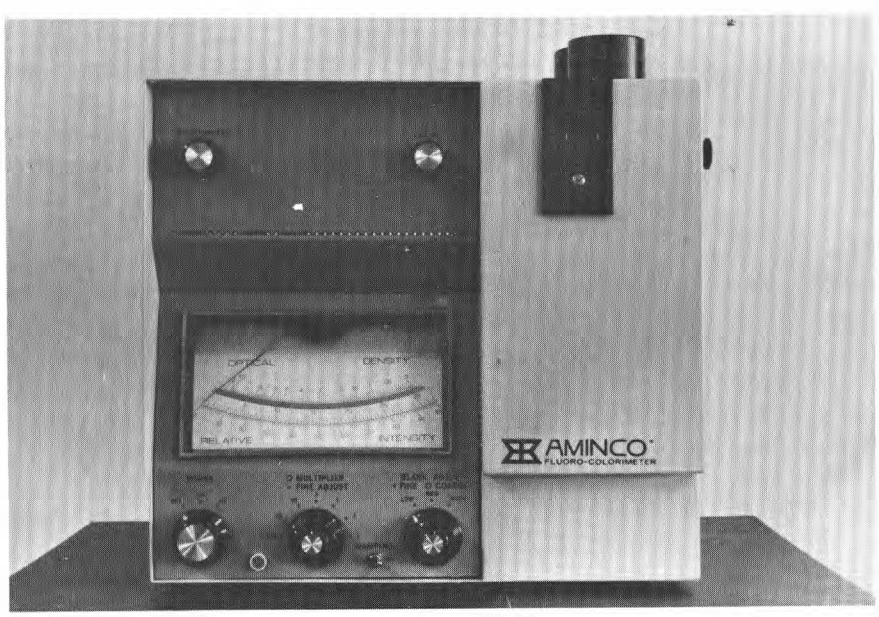

Figure 7.--The American Instrument Company fluorocolorimeter (photograph by Marvin D. Duerk, U.S. Geological Survey).

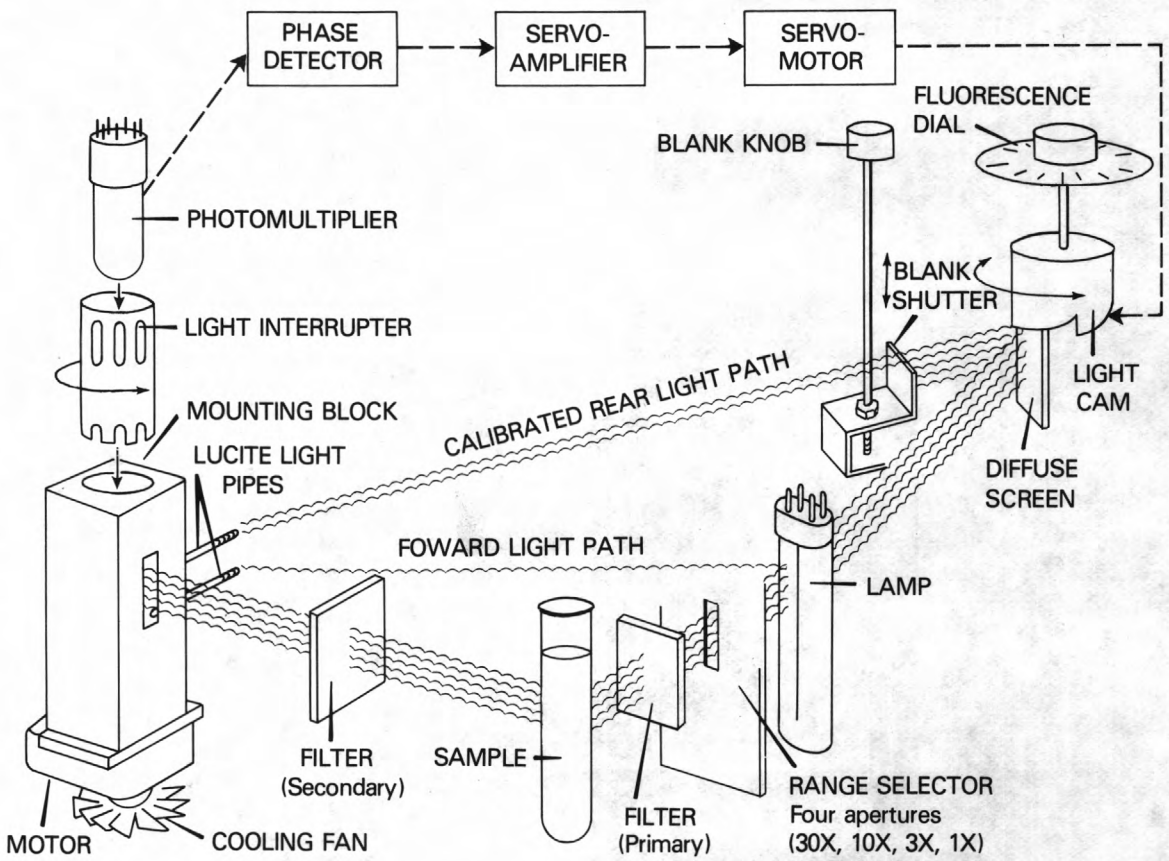

Figure 8.--Functional diagram of the Turner Model 111 fluorometer. Modified from drawing furnished by G. K. Turner Associates.

Selection of Optical Components

The sensitivity of a fluorometer determines the lower limit of detectability of a dye. For a given fluorometer and dye, instrument sensitivity, and hence dye detectability depend upon the characteristics and interrelationships of the optical components of the instrument. For maximum possible sensitivity, all components of the fluorometer's optical system must be properly matched to the dye and physically alined. However, when sensitivity of the instrument is increased, the undesirable effects, such as background interference, also may be increased. The net effect of a contemplated change in optical components must be favorable to dye detectability if the change is to be useful.

Lamps

The objective in both lamp and filter selection is to obtain as much sensitivity to the dye as possible without sacrificing selectivity. Selectivity is the capability of isolating a part of the fluorescence spectrum of the dye from potentially interfering background fluorescence.

There are three lamps that may be used with rhodamine WT and pontacyl pink dyes. The general-purpose ultraviolet (UV) lamp and the far-UV lamp are low-pressure mercury-vapor lamps that emit discontinuous spectra of high-intensity monochromatic lines easily isolated by the proper filters. Most of the mercury lines are in the UV or violet part of the spectrum, but the "green line" at 546 nm is close to the peak excitation wavelengths of rhodamine WT and pontacyl pink (fig. 3). The 546 nm line emitted by the far-UV lamp, which is clear glass, is more than twice as strong as that emitted by the general-purpose UV lamp, which has a white phosphor coating.

A third lamp, the green T-5 envelope lamp, emits a continuous spectral band from less than 520 nm to more than 560 nm, peaking at 546 nm. Because the lamp output is a band instead of a monochromatic line, more light passes the primary filter. The advantage of this form of output is increased sensitivity to the rhodamine WT and pontacyl pink dyes, on the order of tenfold, compared with the far-UV lamp. A disadvantage is a possible increase in background interference from two sources: (1) a small part of the light in the 560-570 nm range passes straight through the filter system to the photomultiplier, and (2) the fluorometer has increased sensitivity to scattered light and to background materials that are not strongly excited by the 546 nm line alone. The first source of interference is constant and presents no great problem. The second source may not be present at all, or it may be great enough to preclude the use of this lamp. In general, this second source has not been found to be a problem. Both the far-UV and the green T-5 lamp have been used extensively with the rhodamine dyes and are recommended for use with the Turner Model 111 fluorometer. The Green T-5 envelope lamp is not needed with the FLM/AMINCO fluorocolorimeter because the range of sensitivity is adequate with the far-UV lamp.

The far-UV lamp or equivalent is recommended for use with acid yellow 7 dye. This lamp has useful outputs at 254, 297, 313, 405, 436, and 546 nm. The outputs at 405 and 436 nm are both near the peak of the excitation spectrum of acid yellow 7 (see fig. 3).

Filters

The purpose of color filters in a fluorometer is to limit, as much as possible, the light reaching the photomultiplier to that fluoresced by the dye. Filter selection must be based on: (1) the useful output spectrum of the lamp, (2) the spectral-fluorescence characteristics of the dye, (3) potential interference from fluorescence of materials present in the stream, and (4) potential interference from light scattered by the sample.

A preferred filter system for use with rhodamine WT and pontacyl pink dyes consists of a primary filter combination peaking at 546 nm and a secondary filter combination that peaks at 590 nm. The primary filter combination can be obtained by the use of two Corning 1-60 and one Wratten 61 filters. The secondary filter combination can be obtained by combining a Corning 4-97 and a Corning 3-66 filter. The spectral-transmittance characteristics of the two filter combinations and of the components of the secondary filter are shown in figure 9.

The loss in transmittance by combining filters is very apparent. It also may be seen in figure 9 that the green line of mercury is completely screened out by the secondary filter, even though there is a slight overlap of the spectra of the primary and secondary filters. This overlap is significant only if the green T-5 lamp is used. The orange filter (Corning 3-66) is always placed closest to the photomultiplier and the blue filter (4-97) closest to the sample to eliminate any fluorescence of the filters themselves.

Although rhodamine WT and pontacyl pink, as shown in figure 3, have slightly different spectral-fluorescence characteristics, the filter combinations shown in figure 9 are good for both dyes. It can be seen in figure 3 that the green line emitted by the far UV lamp and passed by the primary filter is more efficient in exciting rhodamine WT than it is for pontacyl pink. To examine the effect of the secondary filter, one should compare the entire filter transmission spectrum with the entire fluorescence spectrum of the dye.

The 546/590 filter combination is especially preferred when turbidity is present, as the 590 nm secondary filter eliminates most of the scattered light. In the absence of high background levels, sensitivity may be increased by replacing the primary filter with a combination of one Corning 1-60 and one Wratten 58. (This substitution is not recommended if the green T-5 lamp is used.) The secondary filter may be replaced by a Wratten 23A, which has a transmission spectrum very similar to that of the Corning 3-66 shown in figure 9. Background interference (from other fluorescent substances or from scattered light) is a potential source of trouble with the 23A because of the open end of its transmission spectrum. Usually, substitutions in the 546/590 combination are unnecessary. However, when turbidity and background flouresscence are almost totally absent and the experimenter is willing to perform the extra work needed to be assured there is no interference, the substitute mentioned above may be used to advantage.

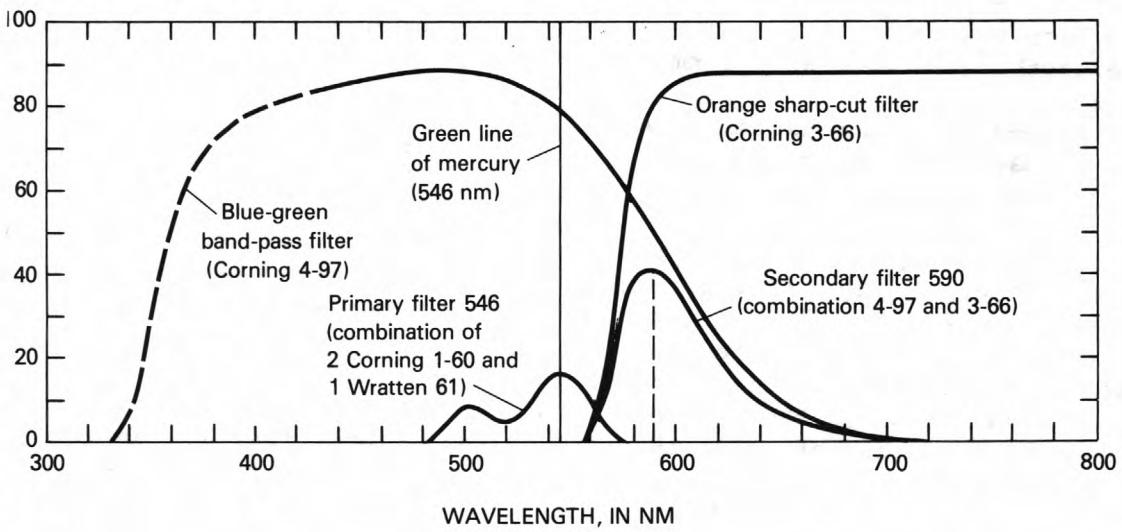

Figure 9.--Spectral-transmittance characteristics of preferred filters for rhodamine WT and pontacyl pink dyes. Sources: Corning Filter Catalog for curves for filters 4-97 and 3-66, Feuerstein and Selleck, 1963a, figure 3, for curve for primary filter combination 546. Separate curves for Corning 1-60 and Wratten 61 not shown. (See Corning Glass Works, 1962, and Eastman Kodak Co., 1965.)

For acid yellow 7 dye the primary filter recommended by Sequoia Turner Corporation (written communication, 1981) is a combination filter consisting of a filter with a color specification 2A and filter number 47B. The 47B filter transmits wavelengths between about 390 and 500 nm with a peak transmittance at about 436 nm. The 2A filter is placed nearest to the lamp. A preferred secondary filter for acid yellow 7 dye is filter number 2A-12, which passes wavelengths greater than 510 nm.

Periodically the filters must be replaced as they become clouded with use. Occasional comparison of fluorometer calibration curves will indicate their deterioration and need for replacement.

Occasionally, very high dye concentrations will necessitate the use of a neutral-density (ND) filter. Although ND filters are available in a wide range of transmission percentages, only one or two are necessary to have on hand. A 10-percent ND filter, which passes only 10 percent of the light reaching it, is recommended. In extreme cases, a 1-percent ND filter may be used, but dilution of the sample can be used as a means of analyzing samples of such high concentration in the rare instances when they are obtained. The ND filter should be placed over the secondary color filter in the fluorometer. Since the FLM/AMINCO fluorocolorimeter has such a wide range of scales, it is seldom necessary to use an ND filter with this instrument.

Sample Holders

The standard cuvette for testing a single sample in the Turner Model 111 fluorometer is glass, 3.5 cubic centimeter in volume, and 12 mm in diameter by 75 mm long. The Turner Designs Model 10 fluorometer uses a 13-mm diameter by 100-mm-long glass cuvette which fits inside a temperature-controlled holder. The FLM/AMINCO fluorocolorimeter uses a 10- by 75-mm cuvette.

The amount of light fluoresced by a given concentration varies directly with the size of the sample holder. Some fluorometers have provision for the use of different sized cuvettes. For example, the flow-through door on the Turner Model 111 fluorometer has provision for a variety of cuvette sizes ranging from 1 to 20 cubic centimeters.

A square cuvette of 4.5 cubic centimeters volume and 10 mm square by 75 mm long can be used in the standard door of the Turner Model 111 without the high-sensitivity kit (the high sensitivity kit requires the use of a round cuvette). This quartz glass cuvettte is designed to increase sensitivity to turbidity but is also useful in producing lower and more consistent background readings where the turbidity is low. In this instance, the green T-5 lamp may be used to acquire the desired sensitivity. The FLM/AMINCO fluorocolorimter does not require modification to increase sensitivity, since it has high-sensitivity capability built into it.

Two methods may be used to improve the sensitivity of the Turner Model 111 fluorometer. Most commonly, a high-sensitivity conversion kit is installed in the standard door when the far UV lamp is used. The kit utilizes mirrors and a glass prism to direct fluorescent light more efficiently toward the

photomultiplier tube, thus increasing the sensitivity of the single-sample cuvette about tenfold. The high-sensitivity kit is a built-in component of the constant-temperature door. These doors are described in the section on accessory equipment. It should be noted that slight misalinement of the high-sensitivity kit can cause calibration difficulties. Most nonlinear calibrations can be traced to this source.

A second method is the use of the green T-5 lamp with the standard door and without the high-sensitivity kit. The high-sensitivity kit and the T-5 lamp should not be used in combination. The use of this lamp eliminates the possibility of misalinement of the high-sensitivity kit, since it is not used, and thus eliminates this cause of nonlinear calibration. Either the more accurate square cuvette or the round cuvette can be used with this combination.

A cuvette itself may be fluorescent or may cause some undesirable scattering of light. The filters used in dye tracing seem to prevent most of this kind of interference. For precision work, the same cuvette or matched cuvettes should be used to avoid any small differences in readings which might be due to light scattered by different cuvettes.

Photomultiplier

A photomultiplier is a special vacuum tube that detects incident radiation and amplifies the resulting electronic signal. The standard photomultiplier tube used in most fluorometers is sensitive primarily to the blue and UV end of the spectrum, while the rhodamine WT and pontacyl pink dyes fluoresce primarily in the orange range. However, some of the standard tubes are very sensitive to the red wavelengths, which accounts in part for a wide variation in sensitivity among instruments. Red-sensitive photomultiplier tubes are available for some fluorometers. The fluorometer manufacturer should be contacted concerning the availability and installation of such tubes if they are desired. The increase in sensitivity with the red-sensitive photomultiplier tube can be as much as three to five times that of the standard tube.

Range Control

Most fluorometers have a means of manually controlling available sensitivity. In the Turner Model 111, this control is accomplished by a range selector between the lamp and the primary filter (see fig. 8). The four positions are called 30X, 10X, 3X, and 1X, indicating their approximate relative sensitivity. For example, the 30X scale gives a reading approximately three times as high as the reading on the 10X scale for the same fluorescent sample. However, the true relationship between the scales varies among instruments. Average values for a group of four instruments were found to be 19X, 7.6X, 2.8X, and 1X when the far UV lamp was used. There is evidence that these ratios vary with intensity of lamp output.

Sensitivity, or range control, is electronically adjusted in the FLM/AMINCO fluorocolorimeter by use of a multiplier and fine adjust control. It is used to select one of seven ranges of sensitivity available in the measuring circuit. These scales of relative intensity are as follows: x100, x30, x10, x3, x1, x.3, and x.1. All scales are exact multiples. For example, when the 0 to 0.1 (x.1) scale is selected, 0.1 is full scale. When the multiplier switch is changed to the 0 to 1.0 (x1) scale, 0.1 is one-tenth of full scale and indicates the same relative intensity. This allows blank and background to be suppressed on the most sensitive scale and to remain suppressed on all scales. This, along with the fact that scales are exact multiples, is very useful during calibration and when performing precision analysis.

Other fluorometers have similar manual controls for sensitivity. Some fluorometers, such as the Turner Designs Model 10 fluorometer, provide for optional automatic selection of sensitivity scale--a useful feature for continuous sampling.

Preferred Systems

For general use with rhodamine WT or pontacyl pink dyes, a preferred system for use with the Turner Model 111 fluorometer, consists of either the standard door, high-sensitivity kit, and far-UV lamp, or the standard door without the high-sensitivity kit, but with the green T-5 lamp. In either system, the 546-590-nm filters should be used. Equivalent lamps and filters should be used with other fluorometers. The Turner Designs "Rhodamine accessory kit" contains the proper lamp and filters for the Turner Designs Model 10 fluorometer. The FLM/AMINCO also has a "Rhodamine kit" for use with their fluorocolorimeter. Other fluorometers may not have highsensitivity kits or may have other means of increasing sensitivities. For maximum sensitivity with the flow-through door the green T-5 lamp is recommended, as the high-sensitivity kit is not part of this door. It is not necessary to modify the FLM/AMINCO's fluorocolorimeter or the Turner Designs Model 10 with a high-sensitivity kit as these instruments have the needed sensitivity built into them.

The high-sensitivity kit should not be used when using acid yellow 7 because the fluorometer is quite sensitive to this dye and the high-sensitivity kit may enhance the background fluorescence too much. For use with acid yellow 7, the far-UV lamp, the 2A and 47B filters (used as a primary filter) and the 2A-12 filter (used as a secondary filter) are preferred.

Accessory Equipment

Temperature-Control Apparatus

Because fluorescence depends on temperature, all fluorometer readings must be either taken at or adjusted to a common temperature. Except for very precise work--discharge measurements, for example--special equipment for controlling temperature usually is not necessary. The procedures for minimizing the need for temperature corrections and for making such corrections are discussed elsewhere in this manual.

For the Turner Model 111 fluorometer, the rather high temperatures generated by the lamp and other components can make it difficult to obtain readings not affected by warming of the samples. The best apparatus for very close control of sample temperature is the constant-temperature fluorometer door. The standard door is easily removed for replacement with the constant-temperature door, which takes the same cuvettes as the standard door and comes equipped with the high-sensitivity kit. Circulating water is used to cool a copper block surrounding the cuvette. Sample temperatures are held to less than 3°F (2°C) higher than the circulating temperature, minimizing the effect of the compartment temperature, which usually is very high (near 100°F or 38°C). Because small pumps tend to warm the circulating water, it is generally preferable to divert water directly from a tap. This procedure also allows for precise regulation of the circulation temperature, but difficulties can arise if the tapwater temperature is unstable. For measuring discharge by dye dilution or for accurate determinations of dye recovery, the constant-temperature door has a distinct advantage. For most time-of-travel and dispersion measurements, the standard door is adequate, provided that significant temperature differences are either avoided or accounted for in the data used.

Both field and standards samples should be brought to a common temperature before laboratory analysis. This can be accomplished by allowing all samples to come to room temperature or by placing all sample bottles in a constant-temperature bath.

For temperature control in a laboratory setup with the Turner Model 111 fluorometer, use the constant-temperature door when analyzing samples that have been brought to room temperature. For best results, adjust the temperature of the circulating water in the door to that of room temperature. Another excellent setup is to combine the constant-temperature door with a constant-temperature bath. Store samples in the bath before testing and circulate the bath water through the fluorometer door.

The sample compartment in many fluorometers, such as the Turner Designs Model 10, and the FLM/AMINCO's fluorocolorimeter are protected from the heat-producing components of the fluorometer. Thus, there is no need to provide temperature control for the sample while it is in the fluorometer, although the Turner Designs Model 10 has an optional sample holder designed for temperature control. Nevertheless, regardless of fluorometer, all samples need to be brought to a common temperature prior to sample analysis.

Portable Power Supplies

Most fluorometers require 115-volt alternating current, 60 cycles per second. For dependable, consistent results, commercial-line power should be used. In the laboratory there is no problem; in the field, outlets may be located sometimes at motels, gas stations, public and private buildings, picnic grounds, boat docks, and at some gaging stations. Often, however, fluorometers must be used at sites where commercial power is not available. Portable power supplies, although to be used as a last resort, can be extremely helpful if properly used.

The best portable power sources for the Turner Model 111 fluorometer and the FLM/AMINCO's fluorocolorimeter are gasoline-driven generators. Generators may be used for either single or continuous (flow-through) sampling, and also for operating additional appliances such as lights or an electric pump. A 1.5-kilowatt generator is usually adequate. If the Turner Model 111 is used, a constant-voltage transformer should be placed on the line between the generator and the fluorometer to smooth out variations in genertor output which might affect fluorometer readout. Such variations are nearly always present even though they are not apparent in the output of other appliances; serious variations have occurred with some generators, preventing consistent fluorometer readings. The constant-voltage transformer should be used only with the fluorometer to protect the fluorometer and to prevent overloading the transformer. A constant-voltage transformer is not needed with the FLM/AMINCO's fluorocolorimeter or the Turner Designs Model 10.

Another portable power source for the Turner Model 111 is a storage battery with electronic converter (inverter), excellent for quick "tailgate" setups of short duration. The converter, which converts direct current to alternating current, is connected to the battery by jumper cables. As a precaution, always keep cigarettes and open flames away from wet-cell batteries to avoid the possibility of igniting battery fumes.

A constant-voltage transformer should always be used between the converter and the Turner Model 111 fluorometer. However, the drain on the battery is considerable, and even with fresh batteries and constant attention to conservation, readout trouble and a dead battery may occur. An alternating-current voltmeter is a very useful accessory for monitoring the condition of the system. When a drop of two volts or more is observed in transformer output, the system should be shut down immediately and the battery recharged. Running the vehicle engine during operation of the fluorometer will help, but it will not keep up entirely with the drain on the battery. Intermittent use of the system is the best procedure to follow. The data obtained using this procedure can only be considered to be approximate because of inadequate fluorometer warmup time. The Turner Model 111 requires at least a 1-hour warm-up time, the Turner Designs Model 10 about 5 minutes, and the FLM/AMINCO's fluorocolorimeter about 10 minutes, for analytical stabilization.

Some fluorometers require less power and are more readily adapted to field requirements. For example, the Turner Designs Model 10 fluorometer can operate directly from a 12-volt storage battery, 115 volts A.C. or 230 volts A.C. current. The current demand for this fluorometer, when operating from a 12-volt battery, is only two amperes. An external power converter and constant-voltage transformer are not necessary with this piece of equipment.

Continuous-Sampling Equipment

Continuous sampling is strictly a field operation and is used primarily to obtain a continuous record of the passage of dye at a fixed sampling point or of the dye profile along a traverse with a boat-mounted fluorometer. In addition to a fluorometer, basic equipment includes flow-through fittings for the fluorometer, intake and discharge hoses, a portable pump, a power supply, and a strip-chart recorder.

A flow-through door is used in place of the standard door on the Turner Model 111 fluorometer. The green T-5 lamp provides needed sensitivity when using the flow-through door. The outside of this door has standard garden-hose fittings to which intake and discharge hoses are attached. Some other fluorometers have other means of converting the unit for flow-through operation. With the Turner Designs Model 10, for example, the front plate is removed to gain access to the single-sample fittings, which are easily replaced by the flow-through fittings. With any fluorometer, opaque plastic garden hose or other nonadsorptive tubing, such as polyethylene, should be used for the intake line.

If line power is available, an electric pump may be used. Alternatives are an electric pump with a generator, a gasoline-driven pump, or a battery-driven DC pump. If a generator is used to power both a fluorometer and a pump, the pump should be connected on a line separate from that for the fluorometer and constant-voltage transformer. It is best to place the pump on the intake side of the fluorometer. This placement will reduce the likelihood of air bubbles forming from dissolved oxygen and passing through the cuvette, a phenomenon often mistaken for air leakage in the system; this is a common problem on cold, oxygen-saturated streams. Air bubbles can cause inconsistent and erratic readings. The use of extremely small hoses and fittings also can cause dissolved oxygen to come out of solution and form bubbles and hence cause operational difficulties.

Leakage of water may occur around various fittings if high pressures exist in the flow-through system. Discharge lines from the fluorometer should be kept short to reduce back pressure. Also, pumps should be sized to produce a good flow rate through the system without creating excessive pressure in the system. The system should be checked for leaks with the pump running, but prior to turning the fluorometer on.

A recorder can be used with most fluorometers. Special electrical outlets are provided on most fluorometers for this purpose.

FLUOROMETER OPERATION

General Procedure in the Laboratory

As a general rule, samples should be analyzed as soon as possible after they are collected to minimize possible deterioration of fluorescence due to other chemicals or substances present in the water. In the absence of such substances, samples may be retained for weeks without loss of fluorescence strength, providing they are in nonsorptive containers protected from light.

Preparations

The basic steps in preparing the samples and fluorometer for sample testing are as follows:

1. Prepare samples.--For best results, stream samples should be allowed to stand overnight to stabilize the temperature, to settle out any suspended matter, and to allow dissolved oxygen to come out of solution. High turbidity may require centrifuging or a longer settling time. Always protect samples from exposure to sunlight. Standards samples and background samples should be treated in the same manner as river samples, being allowed to come to a common temperature with the stream samples.

2. Check lamp type.--Either the far-UV lamp or the green T-5 lamp is to be used with rhodamine WT or pontacyl pink. The far-UV lamp is to be used with acid yellow 7.

3. Check filter placement.--The primary filters (green for rhodamine WT and pontacyl pink and dark blue for acid yellow 7) must be placed between the lamp and the sample. The secondary filters (orange and blue, with the blue filter nearest to the sample for rhodamine WT and pontacyl pink, and dark yellow for acid yellow 7) must be placed between the sample and the photomultiplier tube. For consistent orientation, place a mark, if none exists, in a corner of all filters and always position the filters in the fluorometer so the mark is in the lower right-hand corner.

4. Check location and position of fluorometer.--Unless the fluorometer is completely light-shielded by the manufacturer, the fluorometer will need to be externally shielded from direct or strongly reflected sunlight. It should also be placed in a location that is not expected to experience rapid changes in air temperature. Place the fluorometer on a level, firm base. Allow room for air circulation, especially if the instrument has a cooling fan. For example, don't operate a fluorometer on a car seat as the cushion may restrict a bottom cooling fan.

5. Hook up constant-temperature door, if used.--Start circulation of cooling water with the door removed, at first, to avoid damage due to unexpected leakage.

6. Plug in fluorometer cord; have all panel switches off.

7. Turn fluorometer on.--Various fluorometers have different means of doing this. The Turner Model 111 fluorometer has both a power and a start switch. The power switch must be turned on, then the start switch held in the up position for about 10 seconds to turn on the lamp and assure vaporization of the mercury. For this instrument, verification that the lamp is on should be made by observing the lamp through the primary filter. CAUTION--Do not view the lamp directly; UV rays can injure the eyes. If power is interrupted, even momentarily, the lamp has to be restarted.

Some fluorometers have a single "on" switch to start the fluorometer. Carefully check the instruction manual for the fluorometer being used for the appropriate startup procedure.

8. Allow adequate time for fluorometer warmup.--Different fluorometers require different warmup times. The Turner Model 111 fluorometer requires at least 1 hour and preferably 2 hours to adequately stabilize the operating temperature. The Turner Design Model 10 fluorometer is supposed to be stabilized for most uses within 5 minutes of startup. The FLM/AMINCO's fluorocolorimeter requires about 10 minutes of warmup. For high-accuracy work, a longer warmup time should be provided to allow the temperature of the filters to stabilize. When mercury lamps are used, the fluorometer should be allowed to cool off, when the instrument is turned off, before restarting. The analysis of any samples prior to complete warmup will provide only approximate results. Carefully check the appropriate manual for the warmup time required for any specific instrument.

Background Readings

Fluorometer readings obtained for stream samples must be adjusted for the background readings for the stream water in which the test was made and from the water used for preparing standard solutions for calibration. Although it contains no dye, the background may produce a positive fluorometer reading. This is especially true when filters for acid yellow 7 dye are being used. Background readings may be due to one or more of the following:

1. Light emitted by other fluorescent materials present in the water--always possible, but not usually a problem with rhodamine WT or pontacyl pink with the recommended filters.

2. Light leakage from outside the fluorometer.

3. Light scattered by turbidity of the sample. Tyndall scatter occurs at about the same wavelength as the light reaching the sample. High turbidity may cause some light to pass the 590 secondary filter; the 23A filter passes more of this kind of light and should not be used with turbid samples.

4. Light from the lamp passing straight through the filter system.

5. Light scattered or fluoresced by the filter or sample holder. In dye tracing, the above items usually are not significant if proper precautions are taken.

6. Light scattered by the water. Rayleigh scatter occurs at the wavelength of light reaching the sample and is cut off effectively by either the 90 or 23A secondary filters. Raman scatter always occurs at longer wavelengths than the exciting light. With excitation at 546 nm, the 590 filter effectively cuts off the Raman spectrum of water; the 23A does not.

7. Secondary Rayleigh, Raman, and Tyndall scatter peaks, as well as secondary fluorescence peaks, which occur at wavelengths approximatley double those of their respective primary peaks. The 590 filter eliminates the secondary peaks: the 23A does not.

Set the dial to zero with the water used for preparation of the standards. Check and record the readings on all scales. Background readings for distilled water, even on the most sensitive scale, will usually be quite small. Without changing the zero setting of the fluorometer, test the stream background samples on all scales and record the readings. Some fluorometers have a blank-supression knob, used to reset the background reading to zero. If this is done, background is subtracted automatically from subsequent sample readings. Some users, however, prefer to manually subtract the background component.

Sample Testing

Accurate results will be obtained if consistency in fluorometer procedures are followed. The essential steps in testing samples in a fluorometer are as follows:

1. Rinse cuvette once with tap water and (or) with the solution to be tested then fill to within about one-quarter of an inch (6 mm) of the top with the solution to be tested. Wipe outside of cuvette with laboratory tissue. If air bubbles are visible on the inside of the cuvette, dislodge them by gently tapping the cuvette. Handle cuvettes only near the top. Avoid spilling solution into the cuvette holder. For best results, use the same cuvette for testing all samples.

2. Insert cuvette in the fluorometer. Close the door gently but tightly to avoid spillage or, in the case of the Turner Design Model 10 or the FLM/ AMINCO's flurocolorimeter, place the cap back over the cuvette compartment.

3. Observe the dial reading after the dial has stabilized. A jittery dial ($\pm$ 1/4 dial division) is normal for some fluorometers. For the greatest sensitivity, read each sample on the most sensitive scale possible. It is unnecessary to read every sample on every scale, although occasionally it may be desirable to read the same sample on two scales. Another practice sometimes employed is to use only the most sensitive scale on which all samples can be read. If samples were first analyzed in the field, reanalysis of the one containing the peak concentration will identify which scale can be used for all of that sample set. This may also preclude calibration of the fluorometer on other than the scales actually used.

If the sample is warmed while in the fluorometer, which is the situation with the Turner Model 111 fluorometer without the constant-temperature door, the dial will start to creep downward. The reading should be made before the warming becomes effective. With some fluorometers, the response time may be slow, causing samples having higher dial readings to warm more than others. A degree of consistency can be obtained by always closing the door with the dummy cuvette in place between each sample analysis so that the dial returns to zero each time. Standards having the same dial reading will warm in a like manner, thus the calibration will reflect the tendency for the higher reading samples to warm up. This is why consistency of technique in the analysis of both stream samples and standards usually will produce good results.

In the process of filling the cuvette from the sample bottle, settled solid materials may be agitated into suspension again. Occasionally one or more large particles will cause a high reading which will drop off rapidly as the particles settle again. Such a dropoff will usually appear immediately, whereas a dropoff due to warming of the sample usually follows a brief delay. If the former problem occurs, hold the cuvette outside the instrument (to prevent warming) while the larger particles settle to the bottom.

4. Record the reading. The fluorometer resolution is about ±1 percent of full scale; readings to the nearest one-half to one percent should suffice.

5. Occasionally check and record the blank readings. Check the blank again just before turning off the fluorometer.

6. Turn the fluorometer off before other appliances to prevent sudden voltage surges through the fluorometer. Some fluorometers are protected against such surges.

7. Clean and rinse cuvettes for storage. A minute residue of dye may form inside improperly cleaned bottles and cuvettes that have contained high concentrations.

Special Procedures in the Field

Discrete Sampling

In some applications of dye tracing, some or all samples are tested in the field, primarily as a guide to sampling, and retained for final testing later in the laboratory. The purpose of retesting is to minimize the effects of power fluctuations, turbidity, light leakage into the fluorometer, and sample temperatures, all of which tend to be more variable in the field than in the lab. However, if field tests will be considered as final, special attention should be given to proper fluorometer warmup, sample temperature monitoring, and the other steps described in the preceding section.

Even if the samples are to be retested in the laboratory, the field-testing procedures are basically the same as outlined in the preceding section. The important differences are listed below:

1. The site layout need not be elaborate. If the fluorometer is not internally shielded from external light, the fluorometer will have to be shaded. Tents, trailers, or bridges can be used to provide shade. It is best to protect the fluorometer from adverse weather conditions to the extent possible, even though some fluorometers are watertight.

2. Often the long warmup recommended for the Turner Model 111 fluorometer is not possible. A 2-minute warmup may be used, especially for a mobile setup, but a full warmup is desirable if the samples are not to be retested in the laboratory. Data based on insufficient fluorometer warmup may be very inaccurate and should be so treated.

3. A wide range in sample temperatures may be found. Temperature corrections usually are not necessary in the field if sample readings are used only as a guide to sampling. If field readings are considered as final, sample temperatures must be taken.

4. Samples from highly turbid streams may have high and variable background readings; fluorescence of the dye may be totally masked by the scattered light. In such a case, the flow-through door may be useless; single samples may have to stand for an hour or two before meaningful readings can be obtained. Air bubbles in water from cold, turbulent streams also may cause temporary interference in both flow-through and single-sample cuvettes.

5. All samples collected should be tested if possible. Retain at least the first and last set of background samples obtained before the dye arrived at each site. Intervening background samples may be discarded and the bottles reused. Usually all samples containing dye should be retained for retesting in the lab.

6. A neutral-density filter may be used to obtain readings for samples which are otherwise off scale; usually the 10 percent neutral-density filter is adequate. Off-scale readings seldom will occur, except at sampling sites very close to injection sites.

Flow-Through Sampling

Flow-through, or continuous, sampling can be used in the field for many applications of fluorescence tracing. Flow-through sampling limits the use of a fluorometer to one point in one cross section (possibly more than one point if a system of multiple intakes and valves is used). Flow-through sampling requires a sustained power supply for the fluorometer and pump and continuous surveillance for breakdowns and, for some fluorometers, for needed changes of scale.

The advantage of flow-through sampling is that a continuous strip-chart record is obtained. Continuous sampling is useful where a very complete time-concentration curve is needed or as a guide to sampling. It also is useful for unattended monitoring, provided that the fluorometer has an automatic scale-change feature, such as that of the Turner Designs Model 10. It should be noted that generally the strip-chart record represents both a field test and a final test. Some important points are listed below.

1. For some fluorometers, commercial power or a gasoline-driven generator should be used; the pump and fluorometer are too great a load for sustained use with a battery converter. However, fluorometers with relatively low power requirements operate very well with a 12-volt battery.

2. The recorder is powered through the fluorometer. The fluorometer dial on most or all instruments operates whether or not the recorder is turned on. The recorder reading occasionally should be compared with that of the dial and differences noted on the chart. The manufacturer's instructions on operation of the recorder should be on hand in the field.

3. Use opaque plastic garden hose or polyethylene tubing on the intake side; rubber has a strong affinity for the dye. Light leakage into the fluorometer has occurred where clear hoses were used. Be sure garden-hose connections have washers.

4. Use the most sensitive scale possible; monitor for necessary scale changes.

5. Flow-through setups must be prepared in time to test and record stream background readings before the dye arrives at the intake hose. In streams with very heavy suspended loads, the background count from scattered light may totally mask fluorescence. Under such conditions, the flow-through method should not be used; grab sampling will permit settling of the suspended material, and accurate analysis subsequently in the laboratory.

6. Occasionally check and record the water temperature near the intake or at the discharge hose. Temperature corrections for the recorded data may be necessary.

7. Occasionally mark watch time and dial reading on the recorder chart for possible corrections; some recorders have event markers useful for this purpose.

8. Occasionally collect grab samples from the discharge hose for later retesting and correlation with chart readings. Key the grab samples to the chart record (Hubbard and others, 1982, p. 13). The correlation will be more accurate when concentrations are not changing rapidly.

9. The flow-through fluorometer may be boat-mounted, along with a battery or generator, as needed, for use in spatial sampling. The setup will give continuous longitudinal or lateral profiles of the dye concentration at the depth of the intake. A pipe or hollow airplane wing strut may be attached to the side of the boat to hold the intake hose in place. The length of the intake hose should be kept to a minimum to minimize lag and dispersion in the hose. Special attention will be needed in the field to adequately define the boat location for correlation with the fluorometer readings.

Troubleshooting

The fluorometer is a surprisingly durable instrument, even in the field, although it is less likely to cause trouble if it is never moved from the laboratory bench. Preventive maintenance of the fluorometer is not necessary, but it is good practice to avoid rough treatment. In the field, instruments should be carried on the back seat or floor of a car and padded to prevent sudden shifting.

Excessive heat and frequent starting and stopping of the fluorometer may damage or burn out some electrical components, especially the main lamp. Assure proper ventilation at all times and leave the fluorometer on as long as practical--all day in the laboratory, for example. It also is good practice to keep the operator's manual with the fluorometer at all times and to be sure that all operators are aware of the section on troubleshooting contained in the manual.

Operating difficulties due to malfunction of instrument components seldom occur and are usually not difficult to correct. The most common indicator of trouble is the dial; it may move radically or not at all. Some of the things to check, not necessarily in the order given, are as follows:

1. Is the lamp on?

2. Is the power source adequate? A drop in power input is a constant threat when using a battery and converter.

3. If dial will not drop from the off-scale position, try a smaller aperture, ND filter, or weaker sample. For the Turner Model 111 fluorometer, check the reference lamp by following instructions in the operators manual; replace the main lamp; test all radio tubes; they are easily removed from the back of the fluorometer. Replacements should be the slightly more expensive industrial grade tubes, if possible; if the dial runs rapidly upward when the door is opened, the cutoff switch inside the door latch is not working properly.

If none of the above procedures work, it may be necessary to return the instrument to the manufacturer; some adjustments and repairs are too difficult for most local repair shops; for example, light-pipe adjustment, alinement of optics, or replacement of the photomultiplier tube. The schematic electronic diagram contained in the manual should be provided if the work is done locally. If in doubt concerning the ability of a local shop doing the needed work, return the instrument to the manufacturer.

FLUOROMETER CALIBRATION

Fluorometer readings are relative values of fluorescence intensity. To convert readings to concentrations of a fluorescent solute, it is necessary first to calibrate a particular fluorometer using standards, or prepared solutions of known concentration. A calibration is simply the relation of fluorometer readout units to dye concentration.

Fluorometer readings alone can be used in some applications. The exact relation between each of the fluorometer scales used would have to be known (to convert all readings to a common scale); and for computations of dye recovery or stream discharge, a series of precise dilutions of the injected solution would be necessary to obtain equivalent readings on the scale used.

Although dye concentrations may not be necessary for some types of data analysis, it is necessary that concentrations be determined and reported in accordance with Geological Survey policy. Such data are necessary for assurance that concentration values are below the maximum allowable levels prescribed in current policy statements. Also, because dial readings reflect the individual characteristics of the particular fluorometer used (even among those of the same brand), data from two or more fluorometers can be compared only on the basis of readings for known concentrations.

Concentration Units

The Geological Survey's standard practice in reporting results of water analyses is to use milligrams per liter (mg/L) or micrograms per liter (µg/L) for dissolved constituents. Weight-per-volume units are assumed to be equivalent to weight-per-weight units if the solution contains less than 7,000 mg/L dissolved solids (specific conductance less than about 10,000 micromhos). This relationship is a convenience in preparing standard solutions volumetrically.

Below 7,000 mg/L dissolved solids, 1 milliliter of water is considered to weigh 1 gram. Selected units are defined as follows:

1 kilogram (kg)	= 1,000 grams (g)
1 gram	= 1,000 milligrams (mg)
1 milligram	= 1,000 micrograms (µg)
1 liter (L)	= 1,000 milliliters (mL)
1 mL water	= 1 cubic centimeter (cm^3)
1 mL pure water	= 1 g at 4°C
1 mg per kg = 1 mg per L	= 1 part per million (ppm)
1 µg per kg = 1 µg per L	= 1 part per billion (ppb)

To illustrate, 1 g of pontacyl pink (powder) dissolved in 1 billion mL of final solution (distilled water diluent) is 1 µg/L. One mL of rhodamine WT solution (20 percent by weight, specific gravity 1.19) dissolved in 1 billion mL of final solution is 0.238 µg/L (1 mL x 0.20 g/g x 1.19 x 10^{-9} = 0.238 x 10^{-9}). Concentration values may be rounded for easier handling. A convenient procedure for most applications is to round to three significant figures above 1 µg/L and to the nearest hundredth below 1 µg/L. (Example: 2380, 238, 23.8, 2.38, 0.24, 0.02.) However, for very precise work such as discharge measurements, use three significant figures for all concentrations.

Preparation of Standard Solutions

Dye standards--solutions of known concentration--should be prepared in a laboratory. Although most investigators will prefer to use a volumetric method of preparing standards as described below, a weighing method may also be used. Weighing is required with powdered dyes and may be used with dye solutions. Weights may replace volumes in the dilution equations given later. Specific gravity of the new solution must be considered if the new solution has a concentration greater than 7,000 mg/L.

Equipment and Supplies

Standards are prepared by diluting the dye (liquid or powder) used in the test. Distilled water or chlorine-free tap water should be used for the dilution process in preparing standards. Never use fresh chlorinated tap water. Tap water will normally lose its chlorine if allowed to stand in an open container for about 12 hours. The use of streamwater for standards is advocated by some investigators as a means of canceling any reactions to the dye that may be occuring in the stream due to foreign substances or chemicals. Such reactions are not necessarily duplicatible in the bottles and hence this practice for this purpose is not recommended. In addition to dye and water, the following equipment is suggested.

1. Volumetric transfer flasks without stoppers (to be used for measuring diluent amounts for transfer to suitable containers).

   a. 2,000 mL
   b. 1,000 mL
   c. 500 mL
   d. 250 mL

2. Graduated cylinders

   a. 500 mL
   b. 250 mL
   c. 100 mL

3. Volumetric pipets ("to contain" type) with squeeze bulb or length of flexible extension tubing

   a. 10 mL
   b. 20 mL
   c. 25 mL
   d. 50 mL

4. Large mouth Erlenmeyer flasks in 1,000 and 2,000 mL sizes or about a dozen 1 gallon glass condiment jars may be substituted.

5. Wash bottles.

6. Glass sample bottles about 1 ounce in size.

7. Laboratory towels or wipes.

8. Laboratory soapless cleaner and brushes.

9. Fluorometer and appropriate accessories.

10. Disposable laboratory gloves.

Glass equipment is preferred. Rubber and polyethylene are known to absorb dye; do not use rubber stoppers in flasks. Note whether each pipet used is rated "to contain," which means the solution left in the tip must be included in the measurement by blowing out, or "to deliver," which means the amount remaining in the tip is not included in the measurement. "To contain" pipets are preferred; the viscous nature of the dye solution obtained from the manufacturer may cause an error in the "to deliver" type of pipet.

A squeeze bulb or a piece of flexible tubing attached to the top of the pipet should be used for pipetting to avoid sucking the dye into the mouth.

The use of volumetric flasks and graduated cylinders as mixing containers is discouraged. These flasks are not only difficult to clean but allow no extra room for agitating and mixing. Volumetric flasks and graduated cylinders should be restricted to measuring the desired quantities of chlorine-free water into separate containers. Large mouth beakers, Erlenmeyer flasks, or

plain 1-gallon (3,785 mL) condiment jars, which can be obtained from most restaurants, are large enough to allow easy mixing of dye and water, are strong and durable, obtainable at little or no cost, and can be easily cleaned with laboratory detergent and water. The mixing of dye and water in these 1-gallon jars can be accomplished by oscillating the jar and contents in a circular manner while keeping it firmly against a table or counter top. This avoids the use of stirrers which must be cleaned.

Serial Dilution Procedure

The technique of preparing dye standards for calibrating a fluorometer involves a step-by-step reduction of the stock dye solution, used in the test, by known dilutions until concentrations on the order of those occurring in the stream are reached. This is generally known as a serial-dilution process. For each step of a serial dilution, the new concentration may be computed as:

$$C_n = C_i \left[\frac{W_d}{V_w + V_d}\right] = C_i S_G \left[\frac{V_d}{V_a + V_d}\right] \quad (1)$$

where C_i = the initial concentration,
C_n = the new concentration after one dilution step,
S_G = the specific gravity of the initial dye solution,
V_d = the pipet volume of the dye solution,
V_w = the volume of the added diluent, and
W_d = the weight of the initial solution.

Where the specific gravities are nearly 1.00, the following formula may be used.

$$C_n = C_i \left[\frac{V_d}{V_w + V_d}\right] \quad (2)$$

The dilution factor, D_i, is computed as shown below and is the amount of dilution of C_i for any one step in the serial-dilution process.

Rhodamine WT, as supplied by the manufacturer, has a concentration of 20 percent by weight. At least a four-step serial dilution is required to obtain standard concentrations in the range needed. Thus, the concentrations of final standards obtained by a four-step serial dilution may be computed by the equations:

$$C_f = C_s S_G \underbrace{\left[\frac{V_d}{V_w + V_d}\right]_1}_{\text{Step 1}} \times \underbrace{\left[\frac{V_d}{V_w + V_d}\right]_2}_{\text{Step 2}} \times \underbrace{\left[\frac{V_d}{V_w + V_d}\right]_3}_{\text{Step 3}} \times \underbrace{\left[\frac{V_d}{V_w + V_d}\right]_4}_{\text{Step 4}} \quad (3)$$

or

$$C_f = C_s S_G \, D_1 \times D_2 \times D_3 \times D_4 \quad (4)$$

where the terms are as previously defined except that

C_f = the final concentration of the standard obtained after the fourth step,

C_s = the concentration of the dye solution, usually as obtained from the manufacturer,

D_i = a dilution factor at step i and is equal to $\left[\frac{V_d}{V_w + V_d}\right]$, and

V_d = the pipet volume of the dye solution for each step.

For the various dyes normally used, table 2 provides a range of convenient pipet and diluent volumes to obtain "working solutions" by a three-step serial dilution. It will be noted that volumes, V_w, for the first dilution are uneven, in order to compensate for the specific gravities of the different dyes so as to yield even concentrations for the "working solution." Such a procedure simplifies subsequent dilution computations and lessens chances of errors. This "working solution" is used for all fourth or final standards. It may be retained for future use where the same dye lot is to be used. Thus, the first three serial dilutions need not be repeated every time a dye test is performed from the same dye lot. This "working solution" should be sealed and stored out of direct light.

Because rhodamine WT, as supplied by the manufacturer, is quite viscous, small volume measurements are apt to be in error. For this reason, the smallest pipet volume suggested for the first dilution is 20 ml; use a "to contain" type pipet if available.

It is customary practice to vary the pipet volume, V_d, and diluent volumes, V_w, in step 4 sufficiently to obtain a range in final standard concentrations. Table 3 provides a range of convenient pipet and diluent volumes for the fourth dilution step to provide a complete range of final standard concentrations. Even volumes were employed to the extent possible for both convenience and to lessen potential measurement errors. Normally, all concentrations provided would not be needed; merely those desired would be chosen from table 3. Judicious use of table 3 will often permit the use of only one or two pipets for the fourth-step dilutions, lessening cleaning and handling problems as well as potential errors. For convenience, table 3 also provides for recording fluorometer readings during the calibration process.

The application of tables 2 and 3 in preparing dye standards is illustrated by the example in figure 10. Assume a time-of-travel test using rhodamine WT 20-percent dye has been performed and river concentrations are such that a fluorometer calibration covering concentrations from 1 to 25 µg/L is desired.

The steps in preparing dye standards for the example are described below. If C_s is rhodamine WT 20 percent, the concentration that exists after the third dilution is 100 µg/L. This is frequently referred to as the "working solution."

Table 2.--Convenient three-step serial dilutions for preparation of working solution

Dye used in test		Serial dilutions: First V_d (mL)	First V_w (mL)	Second V_d (mL)	Second V_w (mL)	Third V_d (mL)	Third V_w (mL)	Working solution, in µg/L
Rhodamine WT (20 percent; SG 1.19)	(a)	50	3,792	20	3,500	20	3,500	100
	(b)	25	2,585	20	3,000	20	3,000	100
	(c)	20	2,068	20	3,000	20	3,000	100
	(d)	20	1,158	10	2,000	10	2,000	100
		W_d (g)	V_w (mL)	V_d (mL)	V_w (mL)	V_d (mL)	V_w (mL)	
Pontacyl pink and acid yellow 7 (powder)	(a)	10	3,218	20	3,500	20	3,500	100
	(b)	5	2,188	20	3,000	20	3,000	100
	(c)	5	1,233	10	2,000	10	2,000	100

Table 3.--Convenient fourth-step dilutions for preparing dye standards using a 100 micrograms per liter working solution

Final standard number	V_d (mL)	V_w (mL)	Final standard concentration (µg/L)	Fluorometer Scale			
Background	---	---	--				
1	300	100	75				
2a	200	200	50				
b	250	250					
3a	100	150	40				
b	200	300					
4	100	233	30				
5a	50	150	25				
b	100	300					
6a	50	200	20				
b	100	400					
c	125	500					
7a	50	283	15				
b	100	566					
8a	20	180	10				
b	25	225					
c	50	450					
d	100	900					
9a	20	230	8				
b	25	288					
c	50	575					
10a	20	313	6				
b	25	392					
c	50	784					
11a	20	380	5				
b	25	475					
c	50	950					
12a	20	480	4				
b	25	600					
c	50	1,200					
13a	20	647	3				
b	25	808					
c	50	1,617					
14a	10	490	2				
b	20	980					
c	25	1,225					
d	50	2,450					
15a	10	990	1.0				
b	20	1,980					
c	25	2,475					
16a	5	620	0.8				
b	10	1,240					
c	20	2,480					
d	25	3,100					
17a	5	828	0.6				
b	10	1,657					
c	20	3,313					
18a	5	995	0.5				
b	10	1,990					
19	5	1,995	0.25				

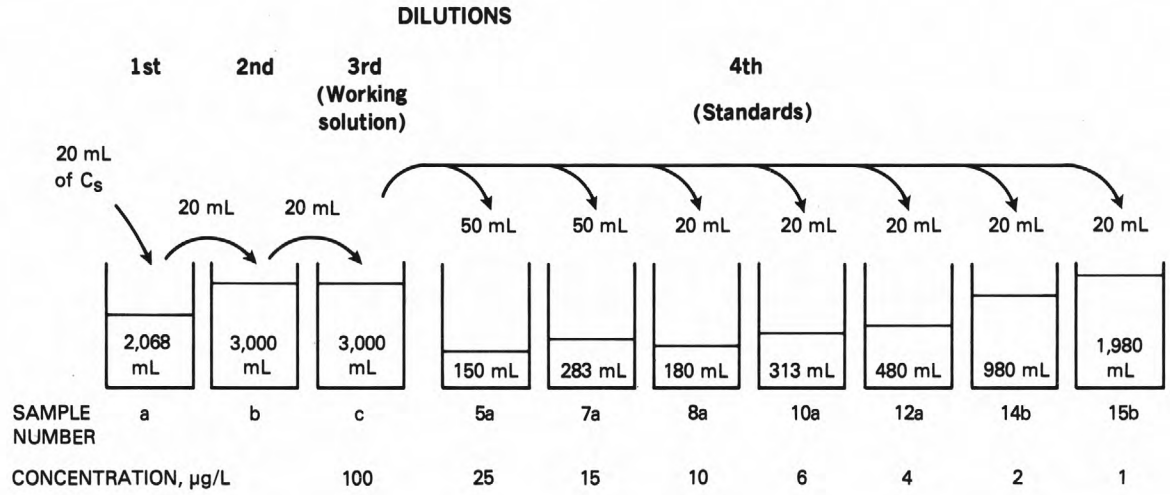

Figure 10.--Example of the use of tables 2 and 3 in preparing a set of standard solutions of rhodamine WT.

1. Measure all diluent quantities first before adding dye.

   a. Based on line c for rhodamine WT, table 2, measure 2,068 mL, 3,000 mL, and 3,000 mL of chlorine-free water into separate containers.

   b. Based on table 3, in a like manner, measure V_w volumes of water into seven separate containers to provide standards of 25, 15, 10, 6, 4, 2, and 1 µg/L as shown in figure 10.

2. Label the containers a, b, c, 5a, 7a, 8a, 10a, 12a, 14b, and 15b as shown in figure 10 or by the concentration of the contents.

3. Label the sample bottles with the appropriate concentration values for the standard solutions 5a through 15b as listed in step 2 and shown in figure 10.

4. Pipet 20 mL of the rhodamine WT 20-percent dye used in the test into container a and mix thoroughly. Cleanse pipet thoroughly.

5. Pipet 20 mL of a into container b and mix thoroughly. Cleanse pipet thoroughly. Continue until the "working solution" of 100 µg/L is obtained on the third dilution.

6. Using 50 mL and 20 mL pipets, add these amounts as appropriate to containers 5a through 15b. Note that the same 20 mL pipet may be used repeatedly for the fourth step dilutions without cleaning each time.

7. Agitate all of the final solutions until each is thoroughly mixed. Do not use the pipet to stir solutions as this may introduce contamination. If containers are oscillated with a circular motion while being held firmly against the table top, spillage is less apt to occur and mixing will be accomplished.

8. Starting with the standard of smallest concentration, pour into the previously labeled sample bottles.

9. Retain working solution in an airtight bottle that has been clearly labeled; store in a dark place for future use.

10. Analyze standards on fluorometer and record dial readings in conventional manner. Treat standard samples in a similar manner as the river samples. For example, allow the standard samples to stand overnight in the same room or place them in the same temperature bath with the river samples.

If desired, the computation of the 1-µg/L standard may be verified using equation (3).

$$C_f = 20 \times 10^7 \left[\frac{20 \times 1.19}{2068 + 20}\right]_a \times \left[\frac{20}{3000 + 20}\right]_b$$

$$\times \left[\frac{20}{3000 + 20}\right]_c \times \left[\frac{20}{1980 + 20}\right]_{15b} = 1.0\ \mu g/L.$$

Experience has indicated that the preparation of standards will be quicker, subject to fewer errors, and less likely to have contamination problems if steps 1 through 10 are followed. Notice that the dye should be handled only after all containers have first been filled with chlorine-free water.

If powdered dye is used in preparing the first dilution, the dye should be weighed on a precision balance. The weighing should not take place in the room in which the solutions are prepared or in which the fluorometer is to be used, to avoid contamination by dye particles carried by air currents.

When using manufacturers' solutions, shake the dye container well before extracting a sample with a pipet. In the field, the sample may be transferred to a small polyethylene or glass bottle for preparing standards later in the laboratory. Use extreme care when handling the dyes. It is difficult to remove dye stains from skin, clothing, floors, and other porous surfaces. Special care should be taken to avoid contact of high-concentration solutions with the hands in order to prevent contamination of sample bottles and equipment to be handled later. Disposable laboratory gloves should be used when handling high-concentration solutions.

Clean all glassware after it has been used. Often this can be accomplished simply by a good rinsing in tap water. For higher concentrations, use of a laboratory soapless cleaner is suggested. The cleanliness of glassware can be readily checked by placing some distilled water into the container and then testing a sample of that water in the fluorometer. If the fluorometer reading is higher than that for distilled water, the container is not yet clean.

Testing Procedure

Let the samples adjust to room temperature or to the temperature of the bath, if used; then test them in the fluorometer using procedures previously outlined. Test all standards samples, including the distilled-water used in their preparation, on all of the scales which will be used for analyzing field samples. Record the readings and make background corrections as necessary. Notes may be arranged in any way convenient to the user. A new set of standards must be made for each new lot of dye.

Calibration Curves

Fluorescence varies linearly with concentration below several hundred micrograms per liter; instrument output is designed to be linear (within about 1 percent) with the amount of light reaching the photomultiplier. It therefore follows that fluorometer dial readings should vary linearly with concentration.

Figure 11 illustrates several possible shapes of curves that have been observed, although curves for most fluorometers are (and should be) straight lines passing through the origin (fig. 11A). Some scatter may be present, but usually not enough to prevent placing a line accurately by eye. If there is doubt, the method of least squares may be used to fit the line.

At very high concentrations--usually above several hundred micrograms per liter--concentration quenching may be observed (figs. 11B and 11C); it is best to keep samples below the level of nonlinearity, diluting them with measured quantities of distilled water, if necessary. For example, if 100 mL of distilled water are added to 100 mL of sample, the resulting observed concentration obtained from a fluorometer should be doubled to obtain the concentration of the undiluted sample.

Calibrations for a few fluorometers are linear except near the origin (below about 5 to 10 percent of full scale), resulting in an apparent intercept on the concentration axis (fig. 11D); very rarely, the intercept appears to be on the dial-reading axis (fig. 11E). If such an apparent intercept is observed, prepare appropriate standards to define the curvilinear position of the curve. G. K. Turner Associates (written commun., 1966) discovered that these two types of curves can result in the Turner Model 111 from incorrect alinement of the high-sensitivity kit installed on the standard door or as a part of the constant-temperature door. The problem can be corrected by factory adjustment if it is serious enough to be warranted.

Complete nonlinearity (fig. 11F) has been found in some instruments; if the deviation from linearity is significant, return the fluorometer to the manufacturer for corrective action. However, if the high-sensitivity kit is used, a solution might be to remove the kit and use a green T-5 lamp, as discussed previously in the section on fluorometers.

Plot the corrected fluorometer readings for the standards against their corresponding concentrations on rectangular coordinates. An example of a set of calibration curves is given in figure 12. It is best to plot the curve for each fluorometer scale on a separate sheet for greater plotting accuracy. On each curve, record the kind of dye, identification of the dye lot, sample temperature, date, and fluorometer components; and label the curves, as shown on the example. Label the axes in such a way that there can be no doubt about the units used.

The user should become familiar with the calibration characteristics of the fluorometer being used. This will help to avoid problems caused by instrument idiosyncrasies. It will also help in selecting standard concentrations for fluorometer calibration.

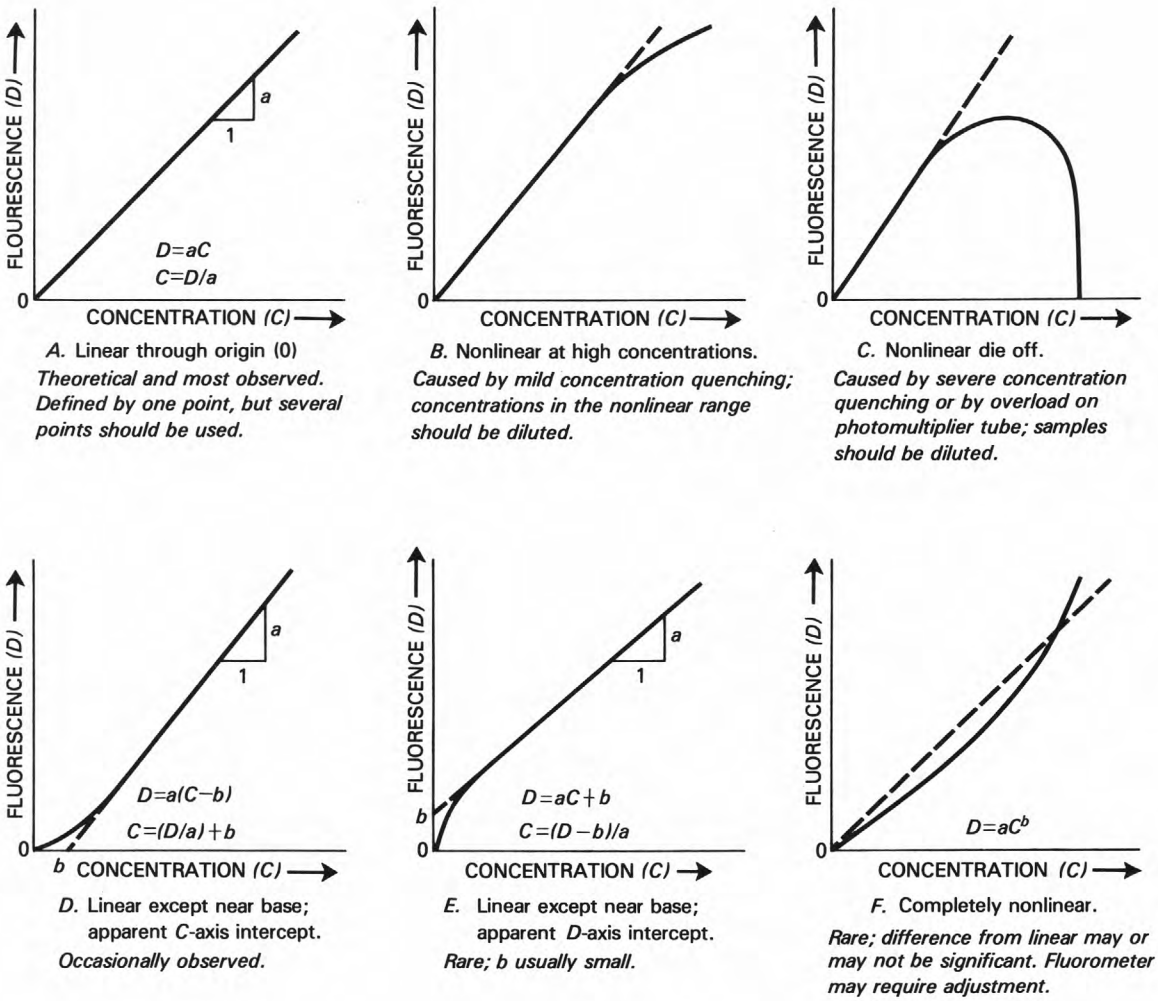

Figure 11.--Types of fluorometer calibration curves.

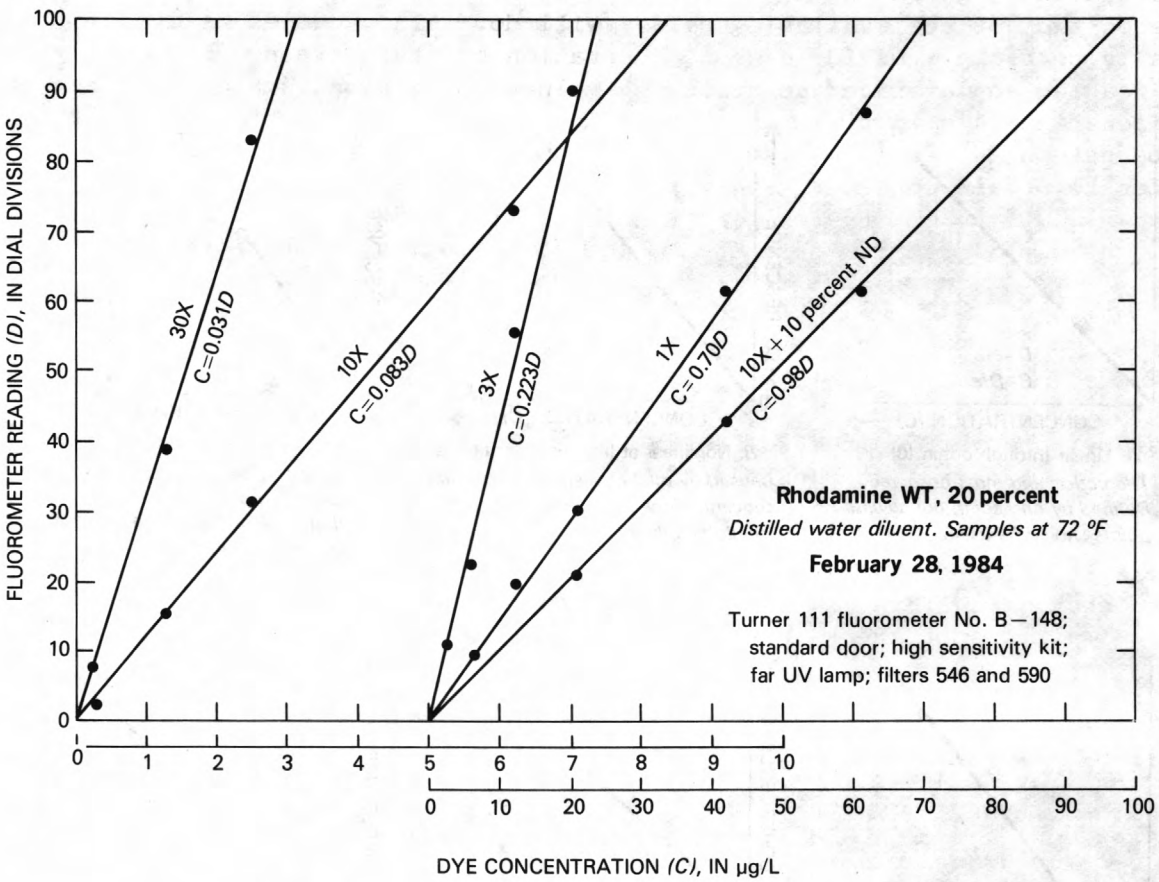

Figure 12.--Typical set of calibration curves.

Once it has been established that the calibration is a straight line through the origin, the number of standards needed is greatly reduced. The averages of two or three replications of each of two or three well-placed concentrations, giving at least one reading between 20 and 80 percent of full scale on each of the available scales, will do. Also, curves need not actually be drawn. Divide each concentration by its corresponding corrected dial reading and average the resulting values for a given scale. The result is a constant (1/a in fig. 11A; 0.031 for the 30X scale in fig. 12) which may be set on a calculator and multiplied by corrected readings for other samples to obtain concentration values. There is no basis for prorating slight variations in the constant with time; the average of such variations should be used (when the same standards are periodically tested during the analysis of a large number of field samples).

The Turner Designs Model 10 fluorometer provides for direct readings of concentration. For example, the reading for a standard sample of 5 µg/L can be set at 5.0 on the upper scale by adjusting the span-control knob. It is necessary, however, to test different concentrations on more than one range setting, to assure that consistent readings are obtained for a given sample on two adjacent range settings.

If the fluorometer is not moved and none of the electronic components are touched, a calibration should remain valid for weeks or months of normal use. However, spot checks are desirable. A different calibration will be needed for each dye lot used. Some of the more common causes of a change in calibration are:

1. Jarring the fluorometer, as might be expected when it is used in the field.

2. Removing the lamp temporarily.

3. Changing the lamp or photomultiplier.

4. Damage to the lamp or photomultiplier.

5. Clouding and deterioration of filters with time.

6. Changes in optical alinement.

7. Changes in temperature of standard samples. (Application of temperature-correction factors will eliminate this cause.)

8. Contamination of the fluorometer with dye.

Calibration of the Flow-Through Cuvette

Continuous sampling using the flow-through cuvette most often is used just as a guide for discrete sampling. If it is necessary to calibrate the flow-through cuvette, there are two ways to do it:

1. Correlate the flow-through readings on the dial or chart with readings for single samples collected at the discharge hose. Determine the concentration of these samples in the laboratory and use to develop a calibration curve on or from the chart (Hubbard and others, 1982, p. 9).

2. Prepare large volumes of several standard solutions, pump them through the fluorometer, and record the readings. For this situation, river water may have to be used for both convenience and to have standards at nearly the same temperature as that being pumped through the fluorometer. A container of "working solution" can be carried to the field to expedite preparation of these field standards. If it is desirable to check calibration frequently in the field, the standard solution can be connected to the intake line with a system of T-connections and valves. When switching from stream to standard or back again, be sure to allow time for complete flushing of the water already in the system. Temperature corrections may have to be made in some instances.

The first method is generally the easiest and most satisfactory of the methods described. Because temperatures and potential contamination are more difficult to control with the last method it is not recommended for most studies. Field calibrations generally should not be used for applications where a high degree of accuracy is required.

DETERMINATION OF SAMPLE CONCENTRATION

The dial readings for final tests of a set of field samples are converted to concentration by three simple steps: (1) subtract background readings from readings for samples (unless background was suppressed with the fluorometer); (2) apply temperature-correction factors to the net readings, if necessary; and (3) convert the corrected readings to concentrations by applying the calibration.

Because a uniform background value for all sampling sites is not likely, background samples should be collected at each site. Time variations in background also may occur, but are often impossible to monitor while dye is passing a site. Background readings usually should be subtracted before applying temperature-correction factors because most components of background either are not affected by temperature at all or are affected in a way different from the dyes. If standards and field samples have been brought to a common temperature and if reasonable care is taken, there will not be a need for temperature corrections.

AERIAL PHOTOGRAPHY AS A SUPPLEMENT TO FLUOROMETRY

In most applications of dye tracing, the dye cloud produced by either rhodamine WT or pontacyl pink remains visible for some time after injection--from a few hours to more than a day--during which it can be photographed. The visible color of acid yellow 7 disappears rather quickly and can be photographed only for a very short time after injection. While rhodamine WT or pontacyl pink is visible (above 25-50 µg/L), it can be photographed at ground level or from an aircraft. Photographs often yield useful supplementary information about the spatial distribution of the dye cloud (Wilson, 1968b).

Color shots are both appealing and useful, but black-and-white photographs may be used to illustrate reports and are best for quantitative analysis. Black and white pictures are taken with panchromatic film and an orange, red, or deep-red filter. The result is a white dye cloud against dark water (fig. 13). Filters which may be used include Wratten 15 (G), 23A, and 25, and Corning 3-66.

Aerial photographs provide instantaneous synoptic views of the dye cloud and reveal details of the shape of the cloud not usually discernible from surface sampling data. Photointerpretation has potential use as a supplement to surface sampling in estuarine and reservoir studies but is limited to visible dye concentrations and to the hours of daylight. Ichiye and Plutchak (1966) demonstrated that there is an excellent correlation between film density and dye concentration using black-and-white photographs of dye clouds. A densitometer is used to measure the relative magnitude of light transmitted through a film negative.

Figure 13.--Aerial photograph of dye cloud, Potomac River estuary at Washington, D.C., August 1965. Taken at low tide with panchromatic film and Wratten 23A filter, from 3,000 feet above terrain.

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