THE WATER TRACER'S COOKBOOK

A guide to the art and science of water tracing materials with particular emphasis on the use of fluorescent dyes, Lycopodium spores, and bacteriophage in groundwater investigations.

by

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Note: Although this issue is dated as 1976, part of the work included has been done during 1977 and 1978 and published in this issue to bring the series up to date.

FOREWARD

The first version of the *Water Tracer's Cookbook* was prepared in 1971 as an outgrowth of investigations I was conducting for the United States Forest Service. In 1972 it was revised for publication by Cave Research Associates as number 14 in the *Cave Studies* monograph series; unfortunately, the series terminated with number 13 (which may or may not be a reflection on *The Cookbook*). Since that time, numerous xerox copies of *The Cookbook* manuscript have been made; each copy had an increasing number of margin notes, addendum sheets, and better recipes.

In 1974 Mickey Fletcher joined me as co-author of *The Cookbook*, as I felt his general experience with groundwater tracing and, particularly, his work with bacteriophage and *Lycopodium* spores would enlarge the scope of *The Cookbook*. After a total of seven years, *The Cookbook* is finally a publication!

The purpose of *The Cookbook* is to help people do a better job of tracing water. Tracing water is a practical problem; hence, *The Cookbook* is a compilation of practical approaches which we have used with success.

Unfortunately, a substantial portion of the water tracing attempts made in the United States are unsuccessful; this is particularly true with groundwater tracing in cave and karst regions. The major causes of tracing failures are: (a) insufficient hydrologic field work before the tracer is injected, (b) tracing attempts during low flow conditions, and (c) failure to allocate sufficient time to the tracing effort. These are problems which *The Cookbook* cannot solve, but problems which must be kept in mind as one considers possible tracing attempts. Best of luck!!

Tom Aley
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INTRODUCTION

Tracing water, both on the surface and underground, is a valuable and sometimes essential technique in many hydrological investigations. Increasing concern with water contamination has confronted a number of hydrologists with water tracing problems.

Many materials have been used as water tracers. Advantages and disadvantages of the more important tracers will be considered in *The Cookbook*. It is our conclusion that fluorescent dyes are well suited to the general needs of hydrologists. In some cases involving public health, *Lycopodium* spores and bacteriophage may be valuable tracers. In *The Cookbook* we have concentrated our attention on the fluorescent dyes, *Lycopodium* spores, and bacteriophage.

Information in *The Cookbook* is largely based on extensive groundwater tracing work conducted in south central Missouri in the recharge areas for Big, Greer, and Mammoth Springs. The techniques recommended work exceedingly well in the soluble rock lands of Missouri; the senior author has had excellent success using these techniques in tracing work in other states.

MATERIALS USED AS TRACERS

A number of different materials has been used to trace water movement. In the late 1800's, painted ducks were used in French cave systems. Bales of hay, wheat chaff, corn cobs, and geese have been used in Missouri. Revenue agents in Tennessee poured 2,000 gallons of illegal whiskey into a sinkhole and inadvertently learned that liquids from this point flowed to a spring supplying water for a local high school. Fortunately, better tracing materials are now available!

Four classes of tracing agents will be discussed in detail:

1. Soluble chemicals such as salt and phosphates.
2. Radioactive materials
3. Fluorescent dyes. (We recognize that these are soluble chemicals, but we wish to consider them separately from the materials discussed in the first category.)
4. Minute biological materials such as bacteria, *Lycopodium* spores, and bacteriophage.

SOLUBLE CHEMICALS (EXCLUDING DYES)

Common salt (sodium chloride), phosphate, and ammonium chloride have been used in groundwater tracing in the past. Chemical tests are used to detect the tracer, and frequent testing is necessary. The concentrations of tracer necessary are often so great that they degrade water quality and, in some cases, injure aquatic life. In general, these tracers should not be used, especially since other materials (particularly fluorescent dyes) do a better and easier job with less water quality damage.

RADIOACTIVE MATERIALS

A number of isotopes (including Cl^{36}, I^{131}, and H^3) have received some experimentation in water tracing work. The safest and most generally useful is tritium (H^3). Tritium is the only radioactive tracer which we will consider in *The Cookbook*.

Tritium is not readily adsorbed on rock, gravel, clay, or organic material. It deteriorates slowly enough (half-life is 12.3 years) to be an effective groundwater tracing agent. When combined with oxygen, its movement is essentially identical to normal water. However, tritium has the following drawbacks:

1. It is radioactive, which presents problems with permits, handling, and transporting. Merely obtaining the material is difficult.
2. The analysis is difficult and requires expensive equipment.
3. All sampling must be done with grab samples, and a long time often elapses between collection and analysis of the sample.

FLUORESCENT DYES

These include fluorescein, rhodamine B, rhodamine BA, rhodamine WT, and pontacyl pink. These are all xanthine dyes; each has been used to some extent in water tracing work. All of these dyes are water soluble, highly detectable, harmless in low concentrations, inexpensive, and reasonably stable in natural waters. One of the chief attributes of these dyes is that they can all, to some degree, be adsorbed on activated charcoal. By using activated charcoal packets, it is simple to determine if dye has or has not passed a given point. In many groundwater cases, the purposes of the investigation are to determine if water moves from one point to another, and then to get some general idea of travel time. This can readily be done with dyes and activated charcoal packets. Furthermore, the techniques are simple and require only limited equipment and expense.

Based on experience and a literature survey, fluorescein and rhodamine WT are the two fluorescent dyes most useful in general water tracing work. These two dyes will be discussed in detail in *The Cookbook*.

Since the first version of *The Cookbook* was prepared in 1971, other fluorescent dyes have received some attention as possible water tracing agents. Significant among these are Direct Yellow 96 (manufactured by Ciba-Geigy Corporation, Greensboro, N. C., under the brand name "Dipheny Brilliant Flavine 7GFF"), and Fluorescent Brightener 28 (an optical brightener manufactured by American Cyanamid, Bound Brook, N. J., under the brand name "Calcofluor White ST--solution"). Both of these have been used by Quinlan and Rowe (1977); their use is described by these authors as follows (p. 16):

> *"In brief, a piece of unbrightened cotton such as Johnson and Johnson Surgical Cotton is suspended in a stream or spring. If optical brightener is present in the water, it reacts with the cotton and is retained. Detectors are changed every few days, washed under a high-speed jet of water, and examined under a long-wave ultra-violet lamp for the blue-white fluorescence of the brightener.*
>
> *...Direct Yellow 96 is eminently suitable for water tracing. It is used like an optical brightener, but it turns cotton a bright canary yellow."*

We have not had any experience with the use of optical brighteners or Direct Yellow 96, and are thus not able to provide recommendations for the use of these agents. Since optical brighteners are used in detergents to make clothes "whiter," their use in water systems contaminated by domestic sewage may possibly be limited.

MINUTE BIOLOGICAL MATERIALS

Some water tracing has been done with the bacteria *Serratia marcescens* and *Serratia indica* (Omerad, 1964; Winpenny et al., 1972). Bacteria tracing could be useful in some sewage pollution problems, particularly those where dispersal of pathogens is of great importance. Among major disadvantages are the degree of sophistication necessary for culturing the bacteria and the necessity of working exclusively with grab samples. At present, it seems unlikely that bacterial tracers would be essential for the types of investigations normally conducted by hydrologists.

Some interesting work has been done (Glantz and Jacks, 1967) with water tracing using naturally occuring *Escherichia coli*-serotypes. Unfortunately, the authors state that there are only two laboratories in the western hemisphere capable of serotyping this bacteria. Regardless of this, the paper is recommended for those with a major interest in fecal coliform.

Winpenny et al. (1972) considered the use of two species of *Bacillus* and two pigmented yeasts. We would anticipate interference problems from native populations with these materials.

Bacteriophage seems to be a very promising microbial water tracer. Bacteriophages were used successfully in a groundwater trace in the soluble rock lands of Missouri; they will be discussed in detail later in *The Cookbook*.

Lycopodium spores, which are spores of a genus of club mosses, are another minute biological material which has been used in water tracing. *Lycopodium* spores are commercially available through biological supply houses. When stained, the spores are easily identifiable under a microscope.

One of the major advantages of groundwater tracing with spores is the fact that you get an indication of the size of openings through which the water is moving. The mean diameter of the spores is about 33 microns, which is at least an order of magnitude larger than most pathogenic bacteria. Similarly, the spores are about 300 times larger than most viruses, and about 15,000 times larger than hepatitis virus, which can be transported through water systems. If the spores move through groundwater from one point to another, pathogenic material can potentially follow the same route. The value of this type information in water contamination problems is apparent.

Although *Lycopodium* spores may be suitable for work in very open groundwater systems (such as karst aquifers, fractured rock aquifers, and coarse glacial deposits), the size of the spores makes them poorly suited to finer textured aquifers. In some crude tests, we found that the spores would not pass through 0.6 meters of sand. The sand which we used was the sort sold by building supply firms for masonry purposes.

Another disadvantage of water tracing with *Lycopodium* spores is the substantial amount of time required for sample analysis. Furthermore, the analysis requires a centrifuge and microscope; most practicing hydrologists do not have ready access to this sort of equipment. Still, these disadvantages are not so

great as to preclude *Lycopodium* spore tracing in those cases where questions of "natural filtration" (or the lack of it) are important.

FLUORESCENT DYES FOR WATER TRACING

GENERAL PROPERTIES OF FLUORESCEIN DYE

Fluorescein dye has been used since the late 1800's for groundwater tracing in cave regions. Much of the early work with this dye was pioneered by the French because of the waterborne disease outbreaks in that country.

Fluorescein is most commonly sold in powder form. When mixed with water, it has a brilliant yellow-green color which is readily detectable visually by experienced observers at concentrations of 1 part per million or less. However, it is not very suitable for surface water tracing as it deteriorates rapidly in sunlight; sunlight obviously does not affect the dye in groundwater situations.

Much of the distinctive yellow-green color of fluorescein dye is lost when it is used in water with a pH of 5.5 or lower. As a result, fluorescein dye may be poorly suited for groundwater work in coal strip mine areas or other areas with very acidic waters.

There is some debate in the literature on sorption tendencies of fluorescein. It is clear, however, that fluorescein has a higher sorption tendency than rhodamine WT; this helps explain why the activated charcoal packets (to be discussed later) work so well with fluorescein. The packets work less well with rhodamine WT. Based on our experience, adsorption of fluorescein on fine textured materials does not negate the utility of fluorescein dye for tracing water through soils, residual or alluvial materials. We have successfully used fluorescein dye to trace groundwater from septic fields and dumps to springs. Although there may be a significant loss of dye due to adsorption on fine textured materials, at least some of the dye passes completely through the septic fields or dumps and appears at distant springs. It should be noted, however, that our field experience is somewhat biased; most of our tracing work involving septic fields and dumps dealt with those which were hydrologically poorly located, and were suspected of being sources of groundwater contamination.

GENERAL PROPERTIES OF RHODAMINE WT DYE

Rhodamine WT is a du Pont product and is apparently unavailable from any other producer. It is sold as a 20% solution with a specific gravity of 1.19. Rhodamine WT has a moderately low photochemical decay rate, a low sorption tendency, and high detectability. These factors make it a good tracer, particularly for surface water work where fluorescein is generally unsuitable.

Our experience indicates that rhodamine WT is inferior to fluorescein for most groundwater work for the following reasons:

1. It is not as readily adsorbed on activated charcoal as is fluorescein.
2. In comparison with fluorescein, the amount of rhodamine WT which can be elutriated once it is adsorbed on activated charcoal is low.
3. Tracing with rhodamine WT requires the use of a fluorometer; fluorescein detection can be done with the naked eye.

4. The quantity of rhodamine WT required for positive tracing is typically much greater than the quantity of fluorescein required. Furthermore, fluorescein is a less expensive tracer.
5. Rhodamine WT is not as biologically safe a tracer as fluorescein.

Effective tracing with rhodamine WT requires a fluorometer with 546 and 590 millimicron filters. The cost of such equipment is between $2,000 and $3,000. The U. G. Geological Survey, which has done a substantial amount of work with this dye, has established a policy of keeping concentration of the dye to less than 10 parts per billion where water is withdrawn for human consumption. Similar limits for other workers seem prudent.

Rhodamine WT is the dye we recommend for surface water use. We do not recommend this dye for general groundwater work except perhaps in special cases where high adsorption is a problem. We lack information on the effects of pH on rhodamine WT; if it is less sensitive than fluorescein to pH effects, it might be the better dye for groundwater investigations in very acidic waters.

It would be helpful if two or more kinds of dye could be used simultaneously. Since rhodamine WT is a red dye and fluorescein is a yellow-green, it would initially seem that these dyes could be used simultaneously. Unfortunately, this is not the case as the two dyes interfere with one another in the low concentrations typical of tracing situations. In laboratory experiments we have been unable to find any chemicals which would selectively elutriate only one of the dyes. Fluorescein will fluoresce on a fluorometer equipped with rhodamine WT filters (546 and 590 millimicrons), so fluorometric separation does not appear reliable. We were also unable to separate the two dyes with paper chromotography. As a result, we have never simultaneously used fluorescein and rhodamine WT in tracing work.

TECHNIQUES FOR INJECTING FLUORESCENT DYES

When the dye is injected into a stream, lake, or groundwater system, the intention is to get an adequate mix and to place the dye where it will function effectively. Hopefully,the following suggestions will aid in this effort:

Stream Systems

1. Use a riffle or turbulent portion of the stream for injecting dye.
2. Before injecting dye, clean away leaves or other organic material. Often you can redesign a riffle to increase turbulence, and thus produce better mixing.
3. When using fluorescein, pour the powder in slowly to insure adequate mixing.
4. Wind can cause substantial drift of fluorescein powder. Keep your materials clean by placing them upwind of the injection site.
5. It takes about 50 liters of water to mix one kilogram of powdered fluorescein. When possible, avoid mixing in containers as it is difficult to clean them effectively. Lining mixing containers with plastic bags does not work well because lumps of dye will sink to the bottom and cannot be mixed without tearing the bags.
6. You can avoid contaminating charcoal packets if you place them before injecting dye. If you cannot do this, have someone else place the packets.

Lake Systems

1. You cannot color the whole lake. If you are forced to work with lake

situations (as with leaky impoundments), inject the dye at the points where leakage is believed to occur. Dyed water can be run through a hose to the bottom of a lake and successful tracing can result.

2. Rhodamine WT is the best dye for general lake system work. In some cases where you are dealing with discrete leakage zones in the bottom of a lake you can successfully use fluorescein. However, fluorescein is rapidly destroyed by ultraviolet light and should not be used where it will be significantly affected by sunlight.

Groundwater Systems

1. The greater the flow of water at the injection site, the better are the chances of a successful trace.
2. When working with losing or disappearing streams, follow the recommendations for stream systems.

DETERMINING DYE INJECTION QUANTITIES

Determining the quantity of dye needed for successful tracing is crucial to any dye tracing investigation. An insufficient quantity will result in an unsuccessful trace; too much dye wastes materials and degrades water quality. Tracing is frequently conducted in areas where domestic water supplies and recreational water use may be involved. Dye concentrations in these areas must be kept low, and definitely below the visible threshold (approximately 1 ppm for all of the fluorescent dyes). The U. S. Geological Survey has established a maximum allowable concentration of rhodamine WT of 10 ppb at points where the water is withdrawn for domestic use.

Toxicity concentrations for rhodamine B (somewhat similar to rhodamine WT) and fluorescein have been investigated by Lennon (1969). He reports that:

> *"Rhodamine-B and fluorescein are dyes commonly used in tracing flows of surface and ground waters. Concentrations used in tracing are relatively nontoxic to rainbow trout, channel catfish, and bluegills. The 96-hour LC_{50}'s*of rhodamine-B range from 217 to 526 ppm at 12° C and those of fluorescein are 1,372 to 3,433 ppm. In compatibility tests, we found that neither dye increases nor diminishes the toxicity of antimycin to fish."*

Determining the quantity of dye necessary for successful tracing varies between surface and subsurface systems. For this reason, and also because different dyes are used for the two types of application, surface water and groundwater will be considered separately.

Surface Water

The best information available on determining quantities of dye for surface water tracing is found in Dunn (1968). This paper presents nomographs for rhodamine B for time-of-travel studies. Figure 1 is a nomograph which we have adapted from the Dunn paper, except that our nomograph applies to the recommended rhodamine WT 20% liquid, which is the form in which this dye is sold by du Pont.

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*LC_{50} : That concentration lethal to 50% of the study animals.

The rhodamine WT nomograph will give the quantity of dye in milliliters for a peak terminal dye concentration of 2 parts per billion. The empirical equation on which the nomograph is based is:

$$V_d = f(QL/V)Cp$$

where V_d = volume of dye solution in milliliters to be injected into the stream. One ml. dye solution = 1.19 gm.
f = coefficient equal to 0.40 divided by the dye solution strength in ratio form (0.2 for rhodamine WT 20%, which is the common liquid form).
Q = mean discharge of the stream during period of dye run, in cubic meters per second.
L = length of stream reach, in kilometers.
V = estimated mean velocity of stream, in meters per second.
C_p = peak dye concentration, in parts per billion desired at the terminal site. This nomograph is based on 2 ppb.

Use of the rhodamine WT nomograph involves three steps. First, the value for the L/V scale is determined by dividing the length of the stream reach in kilometers by the mean velocity in meters per second. Second, the flow of the stream in cubic meters per second is marked on the Q scale. Third, a line is drawn between the points on the L/V scale and the point on the Q scale. Where this line intersects the V_d scale, read the volume of dye solution needed.

Groundwater

For the purpose of this section, groundwater systems have been divided into concentrated flow and diffuse flow conditions. Concentrated flow is primarily restricted to subsurface channels such as solution channels, fault or fracture zones, or integrated conduits in lava. Diffuse flow occurs in sandstone, sand, gravel, and other similar aquifers. Both concentrated and diffuse flow occur in soluble rock terrains.

Fluorescein is the recommended dye for use in groundwater tracing. If for some reason rhodamine WT is used, the quantity needed will be at least twice the recommended fluorescein amount. If one is dealing with groundwater systems conducive to the use of fluorescein, the quantity of rhodamine WT necessary (as compared to fluorescein) may be as much as ten times greater for a comparable level of detection. Investigators working with rhodamine WT will need to make their own estimates for dye quantities.

Concentrated Flow Conditions

The quantities of fluorescein necessary for groundwater tracing in concentrated flow situations has received some consideration in the literature, but the methods of estimating the dye quantities have major shortcomings. Three methods of calculating dye quantities are the Wilson method (Dunn, 1968), the Cobb-Bailey method (Dunn, 1968), and the Haas method (Haas, 1959). The Wilson and the Cobb-Bailey methods are both designed for surface water use, but have been considered for some groundwater applications. The Haas method was designed for use in cave regions.

Unfortunately, all the above methods greatly overestimate the quantity of dye necessary for successful traces involving large volumes of water, long travel distances, and long travel times. The overestimation of dye quantities

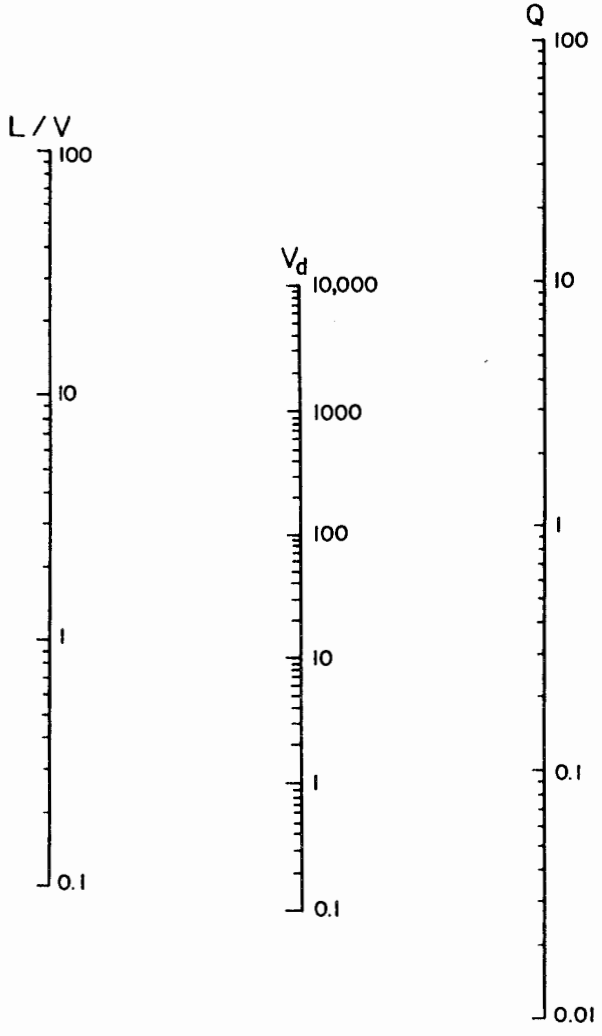

Figure 1. Rhodamine WT nomograph based on the Wilson equation. See text for a description of the equation, units, and details on the use of the nomograph.

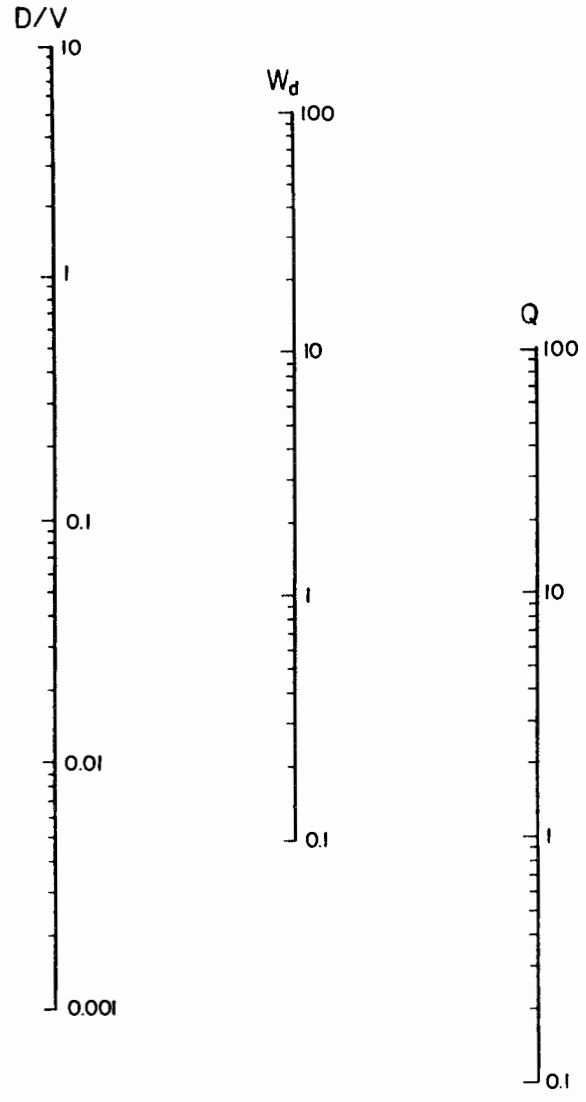

Figure 2. Fluorescein nomograph based on the equation $W_d = 1.478\sqrt{DQ/V}$. See text for description of the equation, units, and details on the use of the nomograph.

occurs because these approaches are designed to produce a particular concentration of dye at the point of resurgence. Successful dye tracing using activated charcoal packets is more dependent upon the total quantity of dye passing through the packet than on the peak concentration of dye in the water. With this in mind, our approach to estimating dye quantities gives primary emphasis to the total quantity of dye passing through the sample packets.

In one successful dye trace from the Hurrican Creek Basin to Big Spring, Missouri (a straight-line distance of 29 km),we used 2.3 kg of fluorescein; this produced a strongly positive dye pulse at the spring. Using the Wilson method of estimating dye quantities, we would have used 91 kg of dye. The Cobb-Bailey method would have required 20 kg, and the Haas method, 45 kg. We have had many similar experiences where successful traces occurred with significantly smaller quantities of dye than were estimated with the Wilson, Cobb-Bailey, or Haas methods.

Figure 2 shows a nomograph for determining quantities of fluorescein to be used in groundwater tracing where concentrated flow conditions prevail. We have developed the nomograph based on successful dye tracing work done in southern Missouri, and have compared the results with successful tracings in Indiana and Arkansas. In our experience, the nomograph is applicable for groundwater tracing where the following conditions are met:

1. The pH of the water is greater than 5.5
2. Water movement is primarily in subsurface conduits such as solution channels, fault zones, or integrated conduits.
3. Activated charcoal packets are used for collection of the dye.

The fluorescein nomograph in figure 2 can be used to estimate the quantity of dye necessary to produce a strongly positive test with an activated charcoal packet. The empirical equation on which the nomograph is based is:

$$W_d = 1.478 \sqrt{DQ/V}$$

Where: W_d = weight of fluorescein dye in kg to be injected.
D = straight line distance in km from point of injection to the point of resurgence.
Q = quantity of flow at the resurgence in cubic meters per second.
V = velocity of flow in meters per hour. The velocity is based on the straight line distance between the point of injection and the resurgence.

To use the nomograph, determine values for the factors D/V and Q. Place a straight-edge on the D/V value on the left scale and the Q value on the right scale. Where the straight-edge intersects the W_d scale, read the quantity of fluorescein dye needed in kilograms.

The estimation of D and Q is simple; the estimation of V, the mean velocity of the groundwater, is a more difficult matter. In an attempt to help in the estimation of groundwater velocities, we have prepared Table 1 which summarizes groundwater velocities encountered in water tracing investigations in the soluble rock regions of the midwestern United States. The data is drawn primarily from southern Missouri, but we have also incorporated information from northern Arkansas and southern Indiana.

Table 1. Groundwater velocities encountered in water tracing work in the soluble rock lands of the midwestern United States. Velocities are based upon straight-line distance between point of injection and point of recovery.

Tracing Distance (km)	Number of Traces	Mean Distance (m.)	Mean Velocity (m/hr.)	Maximum Velocity (m/hr.)	Minimum Velocity (m/hr.)
less than 0.2*	3	110	6.1	8.8	0.7
0.2 to 3.0	5	1,610	10.0	19.7	3.0
3.0 to 65.0**	27	30,000	104.0	230.0	3.0

*All of these traces were at Blanchard Springs Caverns, Arkansas, where water was successfully traced from a parking lot into the ceiling drippage of the cave. Dye and water were injected in the parking lot drains; the rates of flow of all ceiling drippage zones were less than 0.5 liters/second. These cases involved groundwater recharge; many of the conduits utilized by the water were probably air filled.

**This range in tracing distance is substantial, however the mean velocity of 105 m/hour is generally reflective of all groundwater traces within this range of tracing distance.

Diffuse Flow Conditions

Diffuse flow conditions are common in many aquifers, including those in alluvium. One common situation where hydrologists could use water tracing is in relation to stream or lake contamination from septic fields or pit toilets located in alluvial materials. Based on our experience, fluorescein dye can be used in some of this tracing. Diffuse flow conditions require more dye than is indicated by the nomograph developed for concentrated flow conditions. In the absence of adequate data for developing a nomograph for dye quantities in diffuse flow situations, we tentatively recommend 2.5 kg of fluorescein for distances of less than 50 meters from point of injection to point of resurgence, and 5 kg for distances between 50 and 150 meters. When trying to trace dye into a lake, failure will often result unless the lake level can be lowered in conjunction with the investigation. With a stream or river, a better chance of success exists since charcoal packets can be placed into flowing water to adsorb dye.

ACTIVATED CHARCOAL PACKETS FOR DYE DETECTION

The use of activated charcoal packets is the key to successful water tracing with fluorescent dyes. The packets are easily made and analyzed, and cost about twenty-five cents each. Proper construction, placement, and analysis of the packets is essential for successful dye tracing; this section will discuss these aspects in detail.

Construction of Packets

1. Packets should be made of fine mesh screen wire such as that used for window screen. Nylon screen is best, but aluminum screen is acceptable.

2. Packets should be pieces of screen wire 12.5 cm by 15 cm. The screen is folded to form an envelope 12.5 cm by 7.5 cm; the long edge and one short edge are stapled shut. Staples do not need to overlap, but must be close enough to form a reasonably tight packet.
3. Add approximately 12 grams of fresh activated charcoal to each packet and staple shut. This is about 2 heaping teaspoons, or about 3½ heaping small plastic coffee spoons. There is no need to weigh the charcoal.
4. Always use fresh, high quality chemicals. The charcoal specified in the materials section of *The Cookbook* should be used. The activated charcoal used in fish aquariums or in water treatment plants is not suitable. Assume that your charcoal, even when stored in a cool place, has a shelf life of one year. Supplies older than this should be tested before use by pouring 500 ml of water containing 1 ppm fluorescein through a sample packet. Pour at a rate so that it takes 15 to 20 seconds to pour the solution. Follow the directions given later in *The Cookbook* for analysis. A rapidly positive visual test indicates the charcoal is good.
5. Always transport charcoal packets in a clean, closed container. A clean two pound coffee can with a plastic lid makes an ideal field container; plastic bags can also be used.
6. Activated charcoal packets should never be made more than a day or two in advance. The charcoal can must be kept tightly closed.
7. Never re-use screen wire or charcoal.

Placement for Sampling

The activated charcoal packets adsorb dye from the water passing through them. Carefully placed packets will reduce the amount of dye needed for successful tracing.

1. Place each packet to maximize the amount of water passing through it. The packets can often be wedged between rocks on stream riffles. Do not wire a packet flat against a rock as this will decrease the velocity of water passing through it. In some of our dye tracing work, we have measured stream velocities of 1 meter per second passing through charcoal packets.

   In swift currents, we commonly attach packets to rocks as follows: First, we tie a flexible wire around the center of the packet, penetrating the packet in at least one place so that the packet cannot be separated from the wire. This forms an hourglass-shaped packet, which we then wire directly to a rock (preferably, a rock with a hole in it). When attached in this manner, water can readily pass through the packet, but the packet cannot easily be torn loose by the current.

   One technique for anchoring packets which works well on sand, mud, or moderate sized gravel stream bottoms is to bend a 50 cm piece of number 9 galvanized wire into a U-shape. Each leg of the U shaped wire is run through an end of the charcoal packet. The wire legs are then pushed into the stream bottom; the packet is stretched between the legs and is at a right angle to the current.

2. Do not attach packets in such a manner that they will flop against the stream bottom or rocks. This can damage the screen and cause a substantial loss of charcoal.

3. Particularly when using fluorescein, protect the packets from direct sunlight. A flat rock suspended above the packet works well, so long as it does not interfere with water flow through the packet.

4. Always place several packets at sampling points. Packets are occasionally lost, particularly in recreation areas. Conceal your packets from curious people who pull them out of the water and throw them on the shore.

5. Place collected packets in plastic bags until they are analyzed. Use a separate bag for each collection site. Keep the packets moist; it makes analysis easier. Include a label, filled out in pencil, with each packet. Do not use ink pens; some inks fluoresce and others blur.

6. Avoid contaminating your charcoal. When collecting packets,do not wear the same clothes as when you injected the dye. Do not let your boots or clothes come in contact with dye containers. A good procedure in changing charcoal packets in a stream is to always change them from the downstream side. If you have any dye on your boots or on your clothes, it washes downstream instead of into the packets. Contamination of charcoal packets is generally not a major worry, but prudence dictates reasonable precautions.

7. The sampling interval is an important part of the experimental design. The following should be considered when planning sampling intervals:

   A. Adsorbtion of dye on charcoal is a cumulative process. If charcoal packets are changed frequently, very low concentrations of dye could be missed.
   B. New charcoal is more efficient in adsorbing dye than old charcoal. Changing charcoal packets every week is probably better than changing them every two weeks. Based on our Missouri experience, we can have successful traces even when packets are changed at two-week intervals.
   C. Fluorescein adsorbed on charcoal packets can be replaced by longer chain organic compounds. This could be a significant problem in some contaminated waters (for example, those contaminated with gasoline*). To our knowledge, we have never encountered significant problems of this type.

8. Occasionally,it is necessary to sample for dye in domestic water systems. Charcoal packets can be used effectively in the toilet reservoir tank since a substantial portion of daily household water use is through the toilet.

9. Never re-use the screen wire or charcoal from a packet.

10. Control packets free of fluorescein (negative control packets) placed at all sample stations for several days prior to the trace, and then collected prior to the dye injection, help insure that fluorescein from other sources or traces is not present.

11. Control packets spiked with small amounts of fluorescein (positive control packets) can be placed downstream of actual sample packets.

*Note: In a successful trace in 1978, fluorescein was used in a situation in which gasoline from a broken drain contaminated a well. The fluorescein was injected into the drain and was recovered at the well.

These positive control packets should be collected in separate containers; they are helpful in giving the observer a "feel" for positive samples at the particular sampling station. We add several small pieces of wire with colored insulation to each positive control packet to help avoid any chance of confusion.

Analysis for Rhodamine and Pontacyl Pink Dyes

Activated charcoal packets can be used in the detection of rhodamine and pontacyl pink dyes. Visually positive tests may occur, but in most cases the detection of a positive test comes from a fluorometric sample. The activated charcoal packets do not adsorb these dyes as well as they adsorb fluorescein. The best test procedure we have found for rhodamine and pontacyl pink dyes is as follows:

1. Empty contents of the activated charcoal packet to be tested into a baby food jar or other similar small glass container which can be sealed.

2. Barely cover the charcoal with ammonium hydroxide, pour off the excess, then add sufficient methyl alcohol to cover the charcoal with approximately 1 cm of liquid.

3. Since this testing is quantitative, you must use a standard quantity of all materials.

4. Let the charcoal and alcohol solution sit approximately one hour, then pour off the liquid into a small beaker. Filter the liquid through filter paper to remove as much charcoal dust as possible.

5. Test the resulting liquid with a fluorometer. We have found that a far UV lamp G4T 4.1 with primary filter 546 millimicrons and secondary filter 590 millimicrons is most suitable for rhodamine and pontacyl pink dyes.

6. Fluorescein will also fluoresce with the above filters and lamp. We have been unable to develop a system which would let us use any two of the fluorescent dyes simultaneously.

7. If packets are believed to have adsorbed rhodamine or pontacyl pink dye, do not allow the packets to sit in their elutriating solutions for any extended time prior to analysis. Rhodamine WT can withstand this waiting period much better than either of the two fluorescent dyes from Pylam which we tested. These dyes were fluorescent red (probably rhodamine B) and fluorescent pink (probably pontacyl pink). A one-day delay in analysis with these two dyes resulted in a ten-fold decrease in the fluorometric readings on our Turner Model 111 fluorometer. An identical delay with rhodamine WT decreased the fluorometer value by approximately 25%. A five-day delay resulted in the loss of 95% of the fluorescence of rhodamine WT; after five days we could not detect fluorescent pink, and fluorescent red was only barely detectable.

Analysis for Fluorescein Dye

Fluorescein is readily adsorbed on activated charcoal, and visual tests of

the elutriated solution can be made. The suitability of fluorescein for visual tests is one of the primary reasons we recommend this dye for groundwater tracing work. The analysis of the packets is conducted as follows:

1. Empty contents of the activated charcoal packet to be tested into a baby food jar or other similar small glass container which can be sealed.

2. Cover the charcoal with a 5% solution of potassium hydroxide (KOH) in 70% isopropyl alcohol. Ethanol can also be used, but it is more expensive and does not provide better results. Seventy percent isopropyl alcohol is often sold as rubbing alcohol; do not use ethyl or methyl rubbing alcohols, as they are not suitable for fluorescein analysis. Occasionally,one encounters 99% isopropyl alcohol; it must be diluted with water to make 70% alcohol. Without this dilution, the KOH will not dissolve properly.

   The solution of KOH and alcohol must be mixed in a container separate from the charcoal. It is not essential that the solution be exactly 5% KOH. If we are doing qualitative work, we often estimate the amount of KOH necessary and mix it with the alcohol.

3. Do not shake the charcoal and KOH solution.

4. If you are color blind or have trouble distinguishing shades of green, get someone to help you. If you are not sure of your color abilities, go to a paint store and see if you can distinguish between all their shades of green. If not, you need a helper.

5. If fluorescein has been adsorbed by the charcoal,it will be replaced by the KOH and alcohol solution and will appear as a distinctive green haze above the charcoal. In cases where there is a substantial quantity of dye adsorbed on the charcoal, the entire solution will turn the distinctive fluorescein green. Fluorescein dye has a very distinctive color.

6. Naturally occurring organic materials can cause interference in the analysis. These materials can be difficult to separate from weak fluorescein concentrations under ultraviolet or indirect light. They can readily be separated if you work in a darkened room and beam a light into the test solution. For best results,the light beam should be at right angles to the path of your viewing. A flashlight or a small, high intensity tensor type desk lamp provides a very suitable beam.

7. Sample packets with small amounts of fluorescein in them will give you a feel for fluorescein analysis; they can also insure that the activated charcoal being used is fresh and suitable for fluorescein tracing work. A good sample test run can be done as follows:

   A. Make a solution of approximately 1 ppm by weight fluorescein in water. Start with a gram of dye and 1,000 ml of water,and then dilute to 1 ppm.
   B. Pour approximately 500 ml of 1 ppm fluorescein solution through a test charcoal packet; pour at such a rate that it takes 15 or 20 seconds for the liquid to pass through the packet.

C. Rinse the packet in clean water for 30 seconds, then test for the presence of fluorescein. You should get a strongly positive test.

8. A fluorometer can be used in the detection of fluorescein, but in natural water systems, the human eye is more definitive. Using fluorometers, we have been unable to separate the fluorescence of naturally occurring organic materials from the fluorescence of fluorescein; they can readily be separated by eye in a darkened room when a light is beamed through the test solution.

Reliance upon fluorometers for fluorescein determination has resulted in erroneous conclusions that fluorescein was present. For a period of approximately a year, at one week intervals, we pulled and replaced activated charcoal packets from Greer Spring, Missouri. All of the elutriated samples were run on a fluorometer; the analysis approach for each packet was essentially identical. We discovered that fluorescence values, as determined on the fluorometer, would occasionally increase as much as ten fold from one week to another. During our year of investigation, there were three major increases in fluorescence which we might have interpreted as pulses of fluorescein dye. However, there was no dye tracing being done, and visual examination of the elutriated solutions from the packets showed that fluorescein dye was not present.

Natural fluorescence can also mask positive fluorescein dye pulses. We have run small quantities of fluorescein dye through charcoal packets, and then placed these packets in a stream for a period of one or two weeks. Control packets, which were not subjected to the dye, were also placed in the stream. When the packets were elutriated, fluorescein dye could be seen in a light beam passed through the appropriate solution; dye was obviously absent in the control packet. When the elutriated solution samples were run on a fluorometer, total fluorescence in the sample containing fluorescein was about ten percent greater than in the control samples. In practice, a ten percent change in fluorescence from one sample to another is far too minor to attribute to a fluorescein dye pulse. Reliance on a fluorometer could thus result in missing positive traces.

OTHER ADSORBANTS FOR DYE DETECTION

A number of materials have been used for the adsorption of fluorescein and rhodamine dyes, but in our experience all of these are inferiour to high quality activated charcoal. One material which we have not tried is gelatin coated film (Dry Bimat Transfer Film, Type 2436-A; Product of Eastman Kodak Co., Rochester, New York) (Steppuhn and Meiman, 1971).

The film has been used to provide a continuous measure of rhodamine WT concentration in a stream; it must be used in conjunction with a fluorometer which means a total instrumentation cost of perhaps $4000. The detection threshold for rhodamine WT is claimed to be 1 ppb. The gelatin film system would be about as good as having a recording fluorometer in the field.

If someone is conducting investigations where he must use rhodamine WT, it might be interesting to put some of the film in screen wire packets and see if it adsorbed more dye than the activated charcoal. It seems unlikely to us that the film would be as effective as the charcoal.

QUALITATIVE ASSESSMENT OF FLUORESCEIN DYE CONCENTRATIONS

In describing positive dye tests with fluorescein, we have found it helpful to have some qualitative means of comparing the concentrations of dye in packets. We use the following five categories for describing positive fluorescein dye traces as determined by analysis of activated charcoal packets:

1. Very strongly positive: Dye can be seen distinctly with the naked eye in sunlight or in an artifically lighted room within 15 minutes of the time that KOH and alcohol are added to the charcoal.

2. Strongly positive: Same as above, only the time limit is three hours.

3. Moderately positive: Dye can be seen with the naked eye in sunlight or in an artificially lighted room 3 hours after adding KOH and alcohol. The dye is indistinct, and the observer feels it is necessary to verify the results by beaming a light into the sample bottle.

4. Weakly positive: 24 hours after adding KOH and alcohol, dye cannot be detected by the naked eye in sunlight or in an artificially lighted room. Dye can be distinctly seen by the naked eye when a light is beamed through the sample bottle.

5. Very weakly positive: The appearance of the dye is similar to weakly positive tests, but the dye cannot be seen until more than one, but less than 10 days after adding KOH and alcohol. The dye can be distinctively seen by the naked eye when a light is beamed through the sample bottle.

FLUOROMETERS AND QUANTITATIVE DYE DETERMINATIONS

Fluorometers can be used to quantitatively determine the concentration of dye passing a given point. They are useful with any of the fluorescent dyes, and are not difficult to operate. Good descriptions of work with fluorometers are found in the publications by Wilson (1968A, 1968B) and Turner Associates (1970). Anyone seriously contemplating the use of fluorometers should consult these articles. No particular value is seen in attempting to abstract them in *The Cookbook*.

With respect to the types of water-related problems typically encountered by hydrologists in soluble rock terrain, these are several drawbacks to the use of the florometer.

1. They are expensive. In 1972, they cost $2,000 to $2,500 or even more when flow-through attachments were used.

2. Detailed fluorometer analysis of water is time consuming. This can, in part, be overcome by the use of gelatin film samplets described by Steppuhn and Meiman (1971). Setting up for this requires another $1,000 to $1,500 in instrumentation (1972 costs).

3. In most cases, hydrologists do not need the type and precision of data available with a fluorometer. This is particularly true in groundwater situations.

There are situations where a fluorometer would be essential. The detection of rhodamine WT requires a fluorometer or at least an ultraviolet light. Similarly, the approximation of a flow-through hydrograph also requires the quantitative data available from a fluorometer.

LYCOPODIUM SPORES FOR WATER TRACING

GENERAL PROPERTIES OF *LYCOPODIUM* SPORES

The spores of *Lycopodium* are semi-spherical. Based on a sample of twenty spores (half colored, half uncolored), the mean diameter of *Lycopodium* spores available on the market is 33 microns with a standard deviation of 2.9 microns; the range is from 27.5 to 37.5 microns. Since we do not know the species of *Lycopodium* spores purchased, it is possible that spore size may vary between shipments.

When *Lycopodium* spores are added to water, they travel in suspension. If the spores are added to a bottle of water, and the bottle is left undisturbed for a few hours, many of the spores will sink to the bottom. We have little information on the threshold velocities necessary to keep the spores in suspension, but we have successfully used spores in a groundwater tracing where the mean straight-line travel rate of the water was approximately 8 meters per hour.

Based on limited tests, we believe the spores are not a good agent for tracing water through small lakes. McCormack Lake, south of Winona, Missouri, has a surface area of approximately 4 hectares. It is approximately 400 meters from the point where McCormack Creek enters the lake to the dam. We injected 2.25 kilograms of colored *Lycopodium* spores; our recovery of the spores was negligible. We believe the spores settled out of suspension during their time in the lake.

Lycopodium spores are available commercially from biological supply houses; one of their common uses is in the pharmaceutical industry as a dry lubricant on condoms. The spores normally sold belong to species which have thick spore walls. Germination time in some of these species, even under suitable conditions, is as much as seven years. We have kept colored spores in water for 70 days without any noticeable deterioration. Germination or deterioration of the spores does not appear to be a limiting factor in using the spores for water tracing.

PREPARATION OF *LYCOPODIUM* SPORES

In order to distinguish the *Lycopodium* spores used in tracing from those occurring in nature (and to make them easier to find under the microscope), the spores should be colored with a biological stain. Although not a difficult process, the preparation of the spores takes several hours. Never prepare spores in a laboratory which will be used for subsequent *Lycopodium* analysis as the laboratory will be contaminated with the spores.

To prepare 5 kilograms of *Lycopodium* spores (dry, uncolored weight--final moist weight will be approximately 7.5 kilograms):

1. Add 9 liters of water and 110 grams of detergent to the spores to wet them. We do our preparation in a large wash tub. Wear a resp-

irator and use extreme care to avoid powder explosions. Small quantities of *Lycopodium* spores have been used to demonstrate the hazards of powder explosions. It is advisable to weigh the unstained spores the day before the actual staining, particularly if an open flame is to be used in the heating. Stirring does not cause many spores to become airborne, yet prudence dictates that it also be done carefully.

2. Heat slowly, stir, and add water if necessary.

3. Bring to a boil and when mixed (creamy texture without lumps) boil for about 10 minutes.

4. Dissolve 45 grams of biological stain in 0.9 liters of denatured alcohol. This is to dissolve the stain and penetrate the spore wall. It takes several minutes to dissolve the stain. We have used crystal violet and Safranin O for staining spores; any bacteriological stain should be satisfactory if the color is not naturally found in the environment. Individual spores may vary noticeably in color from the stain.

5. Withdraw heat from the spores.

6. Add dye mixture slowly while stirring. Use caution as there appears to be an exothermic reaction.

7. Let the stained spores sit for one hour without letting the temperature fall below 80 or 90 degrees C. This will allow the stain time to thoroughly penetrate spore walls.

8. After an hour, add the following to the hot spores while stirring:
   A. 300 grams urea
   B. 880 grams of formalin (10% aqueous formaldehyde)
   C. 485 grams of 10% potassium hydroxide
   The purpose of the mixture is to permanently fix the color in the spores and preserve them.

9. Dry the spores in an oven at low temperature being careful not to burn them. Moist spores are easier to handle than are dry spores. We keep our spores refrigerated until we are ready to take them to the field; this keeps them from molding. Never prepare more spores than will be used in two or three months.

TECHNIQUES FOR INJECTING *LYCOPODIUM* SPORES

One problem with *Lycopodium* spores is the hazard of contaminating your ultimate sample. The use of moist spores greatly reduces the danger of airborne contamination of equipment or personnel. In addition, the moist spo· mix very readily with water. Techniques suggested for injecting dyes are adequate for spores.

DETERMINING QUANTITY OF SPORES FOR INJECTION

Since *Lycopodium* spores travel in suspension rather than solution, the estimation of quantities is more difficult than for the fluorescent dyes. The quantity of spores necessary depends on a number of factors, of which

the following are particularly important:

1. The amount of filtration in the system

2. Time and distance of travel

3. Velocity of travel. Velocities of a few meters per hour are apparently sufficient to keep the spores in suspension.

4. The sampling interval used. The shorter the interval, the more spores must be used for quantitative resolution.

5. The amount of material caught in sampling nets. An investigator is typically forced to analyze only a portion of the total sample caught in a *Lycopodium* trap. The amount of material caught in the nets will vary with season, water temperature, nutrient level, flow rate, and individual characteristics of the spring or stream. Working with clean streams in the Ozarks, we catch four or five times more material in the summer than in the winter.

Basic data from two successful traces with *Lycopodium* spores in the soluble rock terrain of Missouri may provide a general idea of the quantity of spores necessary for successful tracing.

Case 1.

Trace from a sinkhole dump near Dora, Missouri to Hodgson Mill Spring near Sycamore, Missouri. 5,000 liters of water, 3.2 kilograms of fluorescein dye, and 4.5 kilograms of moist *Lycopodium* spores (equivalent to 3.0 kilograms of dry, uncolored spores) were injected in the bottom of the sinkhole. The floor of the sink was soil and rubble; no solution channels were obvious. After injecting about 2,500 liters of water and tracers, we could hear water cascading below us. The bottom of the sinkhole is approximately 70 meters higher in elevation than the spring; the straight-line distance from the sink to the spring is 9.5 kilometers. The flow of the spring was approximately 0.9 cubic meters per second.

Fluorescent dye began to appear at the spring approximately 19 days after injection at the sinkhole. Beyond 42 days after the data of injection, fluorescein was no longer detectable in charcoal packets placed at the spring. From 42 days to 55 days after injection, a *Lycopodium* sampler was in place at Hodgson Spring. An estimated 2300 spores (7.4 spores/hour) were caught in the trap during this 13 day period. During the time the sampler was in operation, approximately 3% of total spring flow passed through it. Based on information in case 2, it is apparent that peak concentration of the spores was several fold higher.

Case 2.

Trace from Blowing Spring Estavella to Big Spring, Missouri. Blowing Spring povided a continuous flow of approximately 3 liters per second into the estavella. The estavella is located in the bed of Hurricane Creek, and is filled with coarse gravel. During storm periods, when the estavella discharges water, peak discharge flows of 0.6 cubic meters per second have been measured. Approximately 2.3 kilograms of moist colored *Lycopodium* spores (dry, uncolored weight 1.5 kilograms) were injected into the estavella on

Big Spring is 28 km straight-line distance from the estavella. The difference in elevation between the two points is 52 meters. The mean flow of Big Spring during the tracing period was 8.2 cubic meters per second. The *Lycopodium* sampler placed at Big Spring was continuously sampling an estimated 0.3% of the total flow of the spring.

Table 2 summarizes the *Lycopodium* tracing results from Big Spring. Note that half the spores caught in the 17 day tracing period were captured in the first two days of positive results. The spores moved through this groundwater system as a pulse. Based on prior tracing with fluorescein dye between the Blowing Spring Estavella and Big Spring, the pulse of spores emerging from the spring is more attenuated than a pulse of dye. This should be expected when comparing material in suspension (the spores) with material in solution (the fluorescein).

Based on our experience with *Lycopodium* spores in groundwater tracing in cavernous terrain, we see no reason for using large quantities of spores. It seems likely that most groundwater tracing could be done quite adequately with 0.5 to 2 kilograms of colored spores (dry spore weight equivalent 0.3 to 1.3 kilograms).

SAMPLING FOR *LYCOPODIUM* SPORES

Our sampling system is designed to continuously filter large quantities of flowing water. A lake system would require a different sampling approach.

We construct sampling nets of calibrated Nitex nylon monofilament bolting cloth with calibrated mesh openings of 25 microns. This netting readily passes water, but the mesh is smaller than *Lycopodium* spores. The nets are 46 cm long and have a 25 cm diameter opening at one end and a 7.5 cm diameter opening at the other. The larger end has a collar of strong fabric which will not rot when immersed in water for long periods of time. Grommets are placed in the collar for attachment to the culvert cage. The small end is glued inside a sturdy plastic funnel with waterproof glue; 15 cm of surgical tubing extends from the small end of the funnel and is held closed with a sturdy screw clamp.

The net is laced inside a cage built from a one meter piece of 30 cm diameter steel culvert. A hinged cone covered with 60 mm opening hardware cloth is welded to the upstream end of the cage. The downstream end of the trap is also screened with hardware cloth. The conical shape of the upstream end deflects logs and debris and must be ruggedly constructed. A 28 cm diameter circle of screen wire is placed on a number 9 wire frame between the hinged cone and the net to further protect the net from debris. Holes are drilled in the culvert segment 7.5 cm from its upstream end. These match the grommets in the net and permit lacing the net into the trap. A sturdy wire hook supports the funnel near the bottom of the trap to prevent possible tearing of the net.

PLACEMENT OF *LYCOPODIUM* TRAPS

The *Lycopodium* trap should be placed to filter the maximum amount of water possible. The sampler may need to be slightly elevated above the channel bottom to prevent large amounts of mud, sand, and organic debris from being caught in the net. In most Missouri Ozark springs, we can place the sampler directly on the stream channel bottom.

We drive a stake into the channel bottom and lock our sampler in place. It is advisable to conceal the sampler as much as possible to prevent disturbance or vandalism. Samplers could easily be lost during storms unless they are well anchored.

COLLECTION OF *LYCOPODIUM* SAMPLES

Samples are taken from the nets at any interval the investigator desires. The sampler is placed on end in the stream with the conical end up. The net is washed with stream water from the inside to move all sample material into the funnel. Once the washing is completed, the clamp on the end of the tube is removed, and the sample is run into a one liter sample bottle. The net is removed from the sampler, turned inside out, and washed in flowing water for 15 to 20 minutes before being re-inserted in the *Lycopodium* trap. Our experience indicates that this thorough washing removes all *Lycopodium* spores from the nets and permits re-use of the nets.

LABORATORY ANALYSIS OF *LYCOPODIUM* SAMPLES

The laboratory analysis of *Lycopodium* samples is the most time consuming portion of *Lycopodium* tracer studies. It is imperative that the laboratory work be done in such a manner that contamination of the samples by extraneous *Lycopodium* spores does not occur. The room used for spore preparation should not be used for analysis work, and all materials used in the analysis should be scrupulously clean.

The analytical system is based on separation of the *Lycopodium* spore fraction of the sample by filtration to eliminate larger contaminating materials, concentration by centrifugation, and subsequent microscopic analysis.

A typical *Lycopodium* trap sample contains between 200 and 500 ml of material. The sampler nets catch all material larger than 25 microns in diameter. To remove the larger material, which includes both biological and mineral fractions, the entire sample is filtered through a series of calibrated Nitex nylon monofilament bolting cloth filters. The series consists of 102, 53, 44, and 25 micron mesh openings. In samples with a lot of sediment, it would be wise to use all four meshes in the series. The 53 micron mesh screen can be omitted with samples containing little sediment.

A simple filtration system can be set up by obtaining a ring stand; four ring supports (110-130 mm ID), four powder funnels (150 mm top ID, Fisher No. 10-348D), and a large container to catch the final filtrate (should hold at least 2.0 liters). The funnels should be set up so that each is directly over the one below it with the filtrate container at the bottom. Calibrated Nitex cloth filters are placed in the funnels in descending mesh size from the top funnel, i.e., 102 micron cloth in the top funnel and 25 micron cloth in the bottom.

The *Lycopodium* sample should be shaken well and poured into the 102 micron filter at the top of the series. Rinse the sample bottle twice with small volumes of tap water and add the residue to the filter. After all of the liquid has passed through the top funnel, rinse all the residue from the sides of the filter with a squirt bottle. Repeat this procedure on the residue of the remaining filters. Save only the residue from the 25 micron filter. Discard the filtrate and the residue remaining on the other filters. Save the filters for reuse.

An aqueous suspension is made of the residue caught on the 25 micron filter by washing the material into a beaker with a squirt bottle. During this procedure, it is convenient to carefully transfer the filtrate to a piece of aluminum or other metal 35 cm X 45 cm bent in a 90° angle at the middle of the long axis to serve as a filter support. This permits effective washing of the residue from the filter. The resulting suspension includes essentially all of the *Lycopodium* spores plus all other material 25 to 44 microns in size. The suspension is transferred to 50 ml centrifuge tubes and centrifuged for 10 minutes at 3000 revolutions per minute.

After centrifugation, pour off most of the supernatant and discard. The precipitate is resuspended with a laboratory vortex mixer, after which the total volume is determined.

The analysis for *Lycopodium* spores is conducted by removing 1.0 ml of the suspension with a pipet and transferring it to a clean plankton counting chamber (Fisher No. 15-425). (For quantitative results, always be sure to resuspend the sample with the vortex mixer just prior to pipetting). Place a cover slip on the counting chamber and, if a bubble is present, add a small drop of tap water to eliminate it. Sometimes the concentrated suspension may be too dense to analyze microscopically. In such cases, add small amounts of tap water to the final concentrate until the sample can be analyzed.

The counting chamber is scanned at 100 power magnification in a grid-like fashion using a microscope with a mechanical stage. The *Lycopodium* spores are readily discernable from the other material by their stained color and morphology. To confirm spores, 200 power magnification is useful. It takes approximately 30 minutes to mount and scan one slide. We scan a minimum of two, and preferably three, slides per sample. Insight into the range in number of spores per slide can be gained by examining Table 2. One can readily estimate the number of spores in the total sample by knowing what percent of the sample was analyzed.

BACTERIOPHAGES FOR WATER TRACING

BACTERIOPHAGE AS A WATER TRACING AGENT

Bacteriophage are bacterial viruses, and the name literally means "eater of bacteria". Bacteriophage were first evaluated as a water tracer by Winpenny et al. (1972) and were tested in a surface stream. The first apparent use of bacteriophage in tracing groundwater systems was in 1974, and was conducted by Mickey Fletcher at the Ozark Underground Laboratory. Coliphage T-4 was injected in a losing stream (a surface stream which loses water to the subsurface) in a caverous dolomite region. The losing stream was a known tributary of Tumbling Creek, the underground stream in Tumbling Creek Cave.

Sampling for the coliphage was conducted in the cave stream at a point approximately 1,300 meters straight line distance from the point of tracer injection. Based upon grab samples, approximately 2.5 to 3.0% of the injected coliphage T-4 passed the analysis site on the cave stream in viable condition. The coliphage T-4 injection was preceeded by an injection of fluorescein dye; dye concentrations at the analysis site on the cave stream were measured with a fluorometer.

Travel time for the first arrival of the tracers was approximately 16 hours; the majority of the tracers passed the analysis site within 24 hours

of the time of tracer injection. Both tracers moved through the groundwater system in a "slug" type pulse. If the concentration of tracers were plotted agains time, the resulting curve would be similar in shape to a flood hydrograph on a small surface stream.

Bacteriophage have characteristics which make them attractive as a biologic water tracer. Among these are the following:

1. Virulent phages may be used quantitatively.
2. Bacteriophage do not multiply outside their host.
3. Bacteriophage are nonpathogenic to man and domestic animals.
4. Bacteriophage possess many characteristics similar to pathogenic animal viruses.
5. Bacteriophage possess good survival characteristics.
6. Because of host specificity, multiple phage types may be used at different injection sites at the same time.
7. The tracer can be grown in a well equipped lab.
8. Bacteriophage are insensitive to chloroform, which may be used to eliminate sample contaminating fungi and bacteria.

The use of bacteriophages as water tracers has some major disadvantages:

1. Analysis requires the use of grab samples. Our procedures for both fluorescein dye tracing and *Lycopodium* spore tracing are designed to collect cumulative samples; this is not practical with bacteriophage tracing.
2. Analysis is time consuming, and results cannot be determined until a number of hours after the sample has been collected.
3. The concentration of bacteriophage in the water must be substantially greater than the concentration of either fluorescein dye or *Lycopodium* spores in order to insure successful traces.
4. There is a potential for contamination by naturally-occurring phage.
5. Most hydrologists lack experience with microbiological techniques; similarly, most microbiologists lack field experience.

Bacteriophage may be broadly grouped into virulent and lysogenic types. Virulent bacteriophage always produce lysis of the host bacterium following infection. During an assay for bacteriophage, this lysis results in a clear area (plaque) on a confluent lawn of host bacterial growth. Virulent bacteriophage may be used quantitatively in water tracing on the assumption that one bacteriophage will produce one plaque.

Lysogenic bacteriophage cannot be used as quantitative water tracers. The lysogenic phage only produce plaques under special conditions. Following infection of the host bacterium, the phage and bacterial genetic materials integrate and reproduce as a single unit (prophage) until conditions allow the phage to become vegetative and infectious. At that point, the lysogenic phage will produce plaques during an assay. Because of its growth characteristics, we reinterate that the lysogenic bacteriophages are not suited for quantitative tracer studies.

Bacteriophage may be grown relatively easily in a well equipped microbiology lab. A good stock of bacteriophage may be grown in 10 to 24 hours. Similarly, results may be obtained from samples 5 to 12 hours after the initial plating.

Bacteriophage are insensitive to choroform, which may be used to eliminate bacterial or fungal contaminants which might make analysis of the samples difficult. With the microbiological tracer, it is often difficult to obtain counts of the tracer organism without other microbial interference or incorporation of inhibitors into the growth media. Often, the inhibitors reduce the counts of the tracer organism as well. It should be noted that although chloroform eliminates bacterial or fungal contaminants, it does not eliminate contaminating bacteriophage. Natural levels of tracer or contaminating phage should be determined prior to, and after, injection. Low levels of phage can be determined by enrichment, which will be discussed later in *The Cookbook*.

Bacteriophage, although generally morphologically larger, possesses many of the same biochemical features as viruses which infect man and domesticated animals. Therefore, if bacteriophage can pass through a water system, it is generally reasonable to conclude that animal virus may also pass through the system.

Bacteriophage is not the sort of tracer to use for determining flow routes in groundwater systems, or for time of travel studies in surface waters. This work can be done much better and easier with fluorescent dyes.

It appears to us that the primary value of bacteriophage as a water tracer is its similarity to animal viruses. Use of bacteriophage should be seriously considered in cases where one needs to evaluate the movement of viruses in water systems.

PREPARATION OF BACTERIOPHAGE CULTURES

Tracing water with bacteriophage requires competence in microbiology techniques. Careless, improper, or indiscriminate use of microbial water tracers could result in contamination of water supplies.

Bacteriophage will multiply (grow) providing the host is present. Therefore, most of the bacteriophage growth and preparation procedures pertain to the bacterial host. The procedures vary slightly from host to host because of varying nutrient, temperature, pH, and other requirements. Detailed bacteriophage growth procedures may be found in Adams (1963), Billings (1969), and Sargeant (1969).

Tracing water with bacteriophage necessitates large numbers of bacteriophage. This generally means that large culture volumes will be necessary to obtain sufficient quantities. Growth of bacteriophage in large volumes requires special equipment and consideration. Sargeant (1969) considers many of the problems involved in mass culture of bacteriophage; this is a valuable reference.

Once the bacteriophage is grown, it may be desirable to concentrate the suspension to eliminate the transport of large volumes to the field. Bacteriophage suspensions may be concentrated by a variety of methods including centrifugation, molecular sieve or filter separation, and virus concentrators ("Aquella" by Carborundum Co.).

One of the main considerations when working with microbial cultures is maintaining aseptic conditions during all transfers by using sterile equipment, media, etc. One must always be on guard against contamination.

STORAGE OF BACTERIOPHAGE CULTURES

Bacteriophage cultures should be stored at 4° C until used. In addition, bacteriophage stocks should be stored over chloroform to eliminate potential bacterial or fungal contamination.

Injection cultures should be stored at 4° C in sterile Nalgene carboys. If the culture is to be used within 2 days, it is not necessary to store the culture over chloroform. However, it is recommended that the injection culture be used as soon after growth as possible.

Water samples which may contain bacteriophage should be stored over chloroform at 4° C. The samples should be kept refrigerated until analysis. Sample analysis should begin as soon as possible.

DETERMINING QUANTITIES OF BACTERIOPHAGE FOR INJECTION

We recommend that bacteriophage not be used as a primary tracing agent, but, instead, be used as a secondary tracer. Once flow routes, travel times, and attenuation of tracer pulses have been determined by using fluorescent dyes or perhaps *Lycopodium* spores, one is in a position to conduct a bacteriophage tracing.

At present, we do not have sufficient data to effectively recommend quantities of bacteriophage for injection. However, we believe it is prudent to anticipate at least a 97% loss of bacteriophage (this is approximately what we lost in an underground trace lasting between 16 and 24 hours and traversing a straight line travel distance of 1,300 meters). We further recommend that concentrations be based on a minimum detectability of 10 plaque forming units per ml of water at the analysis site.

It should also be recognized that ultraviolet radiation from the sun can have lethal effects on bacteriophage.

LABORATORY ANALYSIS FOR BACTERIOPHAGE IN WATER SAMPLES

Bacteriophage are generally quantified by using serial samples with an agar overly technique. This technique is thoroughly described in Adams (1963) and Billing (1969).

Basically, the agar overlay technique involves adding (a) known host bacterium, and (b) collected water samples at various dilutions (for example the water sample as collected; the water sample diluted ten fold, the water sample diluted 100 fold; etc.) to (c) two or three ml of 0.7% (sloppy) molten 45 degree C. agar. The sloppy agar is then aseptically poured onto a place of agar medium suitable for the growth of the hose bacterium. The top agar is evenly distributed over the bottom layer of agar by tilting and is then allowed to set.

Inoculated plates are incubated upside down at an appropriate temperature and time for the host to grow. After the growth period, the plates should have confluent growth of bacteria except where bacteriophage are present. If a countable number of phage are present, the phage plaques will appear as cleared areas against an opaque background. If too many phage are present, the plate may look like an uninoculated control. If too few phage are present, the plate will look like a bacterial host inoculated control.

When quantitative work is being done, it is desirable to have between 30 and 300 plaques per plate. This is the reason for the serial dilutions of the water sample.

NATURAL PRESENCE OF INTERFERING BACTERIOPHAGES

Prior to conducting a trace with a selected bacteriophage, one should determine whether or not there are wild bacteriophages infectious to the tracer phage's host. This should be determined by sampling at both the proposed injection and recovery sites. Generally, if wild bacteriophages are found which compete with the proposed tracer, the plaque morphology of the wild phages and the proposed tracer phage are distinctly different. Unless the wild phages are abundant, they do not present a significant problem. If, however, the plaque morphologies of the wild and tracer phages are similar, or if the wild phages are abundant; then another bacteriophage tracer-host system must be found. A test to determine the presence of wild interfering bacteriophages is as follows:

1. Obtain samples of injection and recovery site waters in sterile bottles. Add 3 ml chloroform per 10 ml of sample and shake. Store over chloroform at 4 degrees C.

2. Remove aliquots from each water sample, and test initially using the agar overlay technique with bacterial host.

3. To the remainder of the sample volume, add 25 ml of host bacterial cell culture per 75 ml sample. This is done after chloroform has been removed.

4. Incubate samples at appropriate temperatures (with aeration if possible) for 24 to 48 hours.

5. Add 10 to 15 ml of chloroform to each sample and shake. Separate the chloroform and sample fractions.

6. Analyze sample fraction for phage using agar overlay technique with bacterial host.

MATERIALS LIST

The following materials list includes at least one source of supply for each item. For a number of items, there are many unlisted suppliers; some of these may be superior to those listed. We have found that it definately pays to check prices from several suppliers.

Our materials list does not include some minor items which are readily available. One should read the appropriate sections of *The Cookbook* carefully to insure that all materials are on hand or available before any tracing work is begun.

DYE TRACING

Fluorescein Dye: An inexpensive source of supply is Pylam Products Company, Inc., 95-10 218th St., Queens Village, New York 11429. Specify fluorescent yellow dye (fluorescein) in powder form. 1977 prices ranged from $5.75 to $8.00 per pound, depending on total quantity ordered and sizes of containers specified.

Rhodamine WT Dye: E. I. Du Pont,De Nemours and Co., Inc., Dynamic Chemicals Dept., Dyes and Chemicals Division, Wilmington, Delaware, 19898. The dye comes in liquid 20% solution. 1969 price,$2.18 per pound in 25 pound containers.

Other Fluorescent Dyes: Check prices with Du Pont (address above) or Pylam Products (address above). Pylam prices are much lower, but they do not use names such as rhodamine or pontacyl pink. They have price lists for "Leak Tracer Dyes", which we believe to be rhodamine B and pontacyl pink dyes. We have experimented with all of their fluorescent dyes but have been unable to find anything equivalent to rhodamine WT.

Direct Yellow 96 is manufactured by the Ciba-Geigy Corporation, Greensboro, N. C., under the brand name "Dipheny Brilliant Flavine 7GFF". See Quinlan and Rowe (1977). We have no additional information nor recommendations regarding this dye.

Fluorescent Brightener 28 (an optical brightener) is manufactured by American Cyanamid, Inc., Bound Brook, N. J., under the brand name "Calcofluor White ST--solution". See Quinlan and Rowe (1977). We have no additional information nor recommendations regarding this dye.

Activated Coconut Charcoal: Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, N. J. 07410. Specify activated coconut charcoal 6 to 14 mesh, item number 5-685 A. 1977 price was $8.00 per pound in one pound cans, with a minimum order of $25.00. It is advisable not to order more than a one years supply. Store in a cool location. Please note that there are many activated coconut charcoals; we have specified one which we know works well for fluorescein dye adsorption.

Potassium Hydroxide: USP pellets in one pound glass bottles are available from any chemical supply house. Fisher Scientific stocks this as item P-250. 1977 price was $6.45 per one pound bottle.

Isopropyl Alcohol: Seventy percent isopropyl alcohol (rubbing alcohol) can be used in fluorescein tracing. Some isopropyl alcohol is sold as 99%

alcohol; you must dilute this to 70% with distilled water. Do not use ethyl rubbing alcohols, as they generally have a yellow color. Laboratory ethyl alcohol is usable, but is expensive and not really needed.

Methyl Alcohol (Methanol): This alcohol should be used for rhodamine dyes. As this is available from any chemical supply house, Fisher Scientific stocks this as item A-412; 1977 price was $4.38 per pint.

Ammonium Hydroxide: Use household ammonia such as Bo Peep, etc., which is available at grocery stores.

LYCOPODIUM TRACING

Lycopodium spores: Available from Carolina Biological Supply Co., Burlington, North Carolina 27215. Price per pound in 1971 was $11.50.

Biological Stain: Crystal violet, safranin O, and others are available from most biological supply houses. Buy certified biological stain as the color is more uniform and spores will not vary as much in color from one batch to another.

Miscellaneous Chemicals: Denatured alcohol, urea (crystal form), formaldehyde, laboratory detergent (Alconox, Coleo, etc.), are available from chemical or laboratory supply houses.

Plankton Counting Cell: Available from Fisher Scientific, item number 15-425.

Disposable Pipettes: One ml disposable pipettes are available from most chemical or laboratory supply houses. Buy the least expensive ones available.

Powder Funnels: Available from Fisher Scientific, item number 10-348 D.

Nylon Netting: TET/Kressilk Products, 420 Saw Mill River Road, Elmsford, New York 10523, has a wide variety of calibrated netting. We prefer the Nitex Nylon Monofilament Bolting Cloth because of its weave, durability, and relatively reasonable price. The following are 1971 prices per running yard of 40 inch wide bolting cloth:

102 micron mesh opening	$9.30/yard
44 micron mesh opening	16.30/yard
25 micron mesh opening	21.80/yard

BACTERIOPHAGE TRACING

Bacteriophage and Bacteriophage Host Cultures: Bacteriophage and their respective bacterial hosts may be obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852. The phage and host are supplied as freeze-dried specimens contained in glass ampoules. Information for rehydrating the specimens and keys to the preferred growth medium and temperature may be found in the ATCC Catalog of Strains. The approximate cost of cultures is $30 each.

ANNOTATED BIBLIOGRAPHY

The following annotated bibliography contains some of the more important references on water tracing. When publications are readily available, the source of supply is listed.

Adams, Mark H., 1963, Bacteriophages, Interscience Pub. Inc., New York, 591 p.
A classic, detailed and easy to comprehend book on bacteriophages first published in 1959. It has an extensive bibliography.

Aley, Thomas, 1975, A predictive hydrologic model for evaluating the effects of land use and management on the quantity and quality of water from Ozark springs, Ozark Underground Laboratory, 232 p., out of print.
Contains extensive data on groundwater tracing to major karst springs in southern Missouri, including Big, Greer, and Mammoth Springs. Techniques used in The Cookbook for fluorescein dye tracing and Lycopodium spore tracing of subsurface waters rely heavily on the findings of this study.

Billing, Eve, 1969, Isolation, growth, and preservation of bacteriophages, *In* Norris, J. R., and D. W. Ribbons, eds., Methods of Microbiology, Vol. 313, Academic Press, N. Y., pp. 315-329.
General paper on bacteriophages which details many of the techniques and considerations in bacteriophage activation in the laboratory. Contains a good bibliography.

Brown, Michael C., 1972, Karst hydrology of the Lower Maligne Basin, Jasper, Alberta, Cave Studies No. 13, 84 p. Available from Cave Research Associates, 3842 Brookdale Blvd., Castro Valley, California 94546.
A very interesting study of groundwater movement by analysis of flow pulses. Also describes some dye tracing work and gives an excellent comparison of tracer agent characteristics.

Cobb, Ernest D., 1968, Constant-rate-injection equipment for dye-dilution discharge measurements, *In* Selected Techniques in Water Resources Investigations, 1966-67, U. S. Geol. Survey Water Supply Paper 1892, p. 15-22, $1.00 from Supt. of Documents, Government Printing Office.
Discusses equipment used for dye-dilution discharge measurements. This type of equipment has been used to a limited extent in gaging stream flows in mountain streams. There are five other articles in this water supply paper which also deal with dyes.

Collins, M. R., 1968, Selection of dye-injection and meauring sites for time-of-travel studies, *In* Selected Techniques in Water Resources Investigations, 1966-67, U. S. Geol. Survey Water Supply Paper 1892, p. 23-29, $1.00 from Supt. of Documents, Government Printing Office.
"Studies using fluorescent dyes for estimating time of travel in streams may be made by either a single injection method for the entire study reach or a multiple-injection method whereby the study reach is divided into subreaches. The method is governed by the specific prerequisites of the study, time and personnel limitations, and other factors.

The optimum success of a study depends upon proper selection of dye-injection and measuring sites. Site selection should be made after a comprehensive inventory of the study reach. Factors to be evaluated include channel characteristics of the stream, potential loss of dye, muni-

cipal demands, tributary inflow, and stream discharge."--Author

There are five other articles in this Water Supply Paper which also deal with dyes.

Dunn, Bernard, 1968, Nomographs for determining amount of rhodamine B dye for time-of-travel studies, *In* Selected Techniques in Water Resources Investigations, 1966-67, U. S. Geol. Survey Water Supply Paper 1892, p. 9-14.
Nomographs and equations for the Cobb-Bailey and Wilson methods of determining amounts of dye necessary for given concentrations at downstream locations are given. The nomographs are designed for use with rhodamine B, but can be adjusted for other dyes. The nomograph for rhodamine WT used in this cookbook is adjusted from the Wilson equation nomograph in this article. There are five other articles in this Water Supply Paper which also deal with dyes.

Glantz, Paul J., and T. M. Jacks, 1967, An evaluation of the use of *Escherichia coli* serotypes as a means of tracing microbial pollution of water. Penn. State Univ. Water Resources Research Pub. 1-67, p. 57. Available free from Inst. for Research on Land and Water Resources, Penn. State Univ., University Park, Pennsylvania.
A very interesting report on tracing of contaminants. Unfortunately, serotyping the bacteria is very sophisticated. A worthwhile publication for anyone with a major interest in coliform bacteria.

Haas, John L., Jr., 1959, Evaluation of ground water tracing methods used in speleology, Nat. Speleo. Soc. Bull., Vol. 21, Pt. 2, July p. 67-76.
A review paper on groundwater tracing agents prepared prior to the introduction of the rhodamine base dyes. Describes the use of activated charcoal, and recommends using ten grams fluorescein per thousand gallons of water per minute per mile of probable underground travel. This is a linear relationship rather than the non-linear relationship incorporated in the nomograph with The Cookbook.

Lennon, Robert E., 1969, Report from Fish Control Laboratory, La Crosse, Wisconsin. *In* Progress in sport fishery research, 1969, Bur. Sport Fisheries and Wildlife, Resource Pub. 77, p. 115, 117, Supt. of Doc., GPO $2.00.
Reports on the toxicity of fluorescein and rhodamine B to fish. The findings are discussed in The Cookbook in the section on determining dye injection quantities. Toxicity levels are several orders of magnitude higher than concentrations used in water tracing, but as with any chemical, care must be used.

Molitvin, P. V., 1968, Study of the relationship between surface and subsurface fissure-karst waters in the Ay River Basin. Soviet Hydrology, No. 6, May 1968, p. 557-567, In English.
Discusses dye tracing with fluorescein in a karst area. Paper includes a table summarizing 14 dye tracers and gives some groundwater flow velocities. In this karst area, travel distances ranged from 1200 to 3700 meters, and flow velocities ranged from 11 to 80 meters per hour with a mean velocity of 41.4 m/hr. These values are similar to those reported in The Cookbook for groundwater tracing with fluorescein in Missouri, Indiana, and Arkansas.

Ormerod, J. G., 1964, *Serratia indica* as a bacterial tracer for water movement, The Journal of Applied Bacteriology, Vol. 27, No. 2, p. 342-349.
"Methods are described for obtaining suitable liquid cultures of Serratia indica for transporting mature cultures in the field and for viable cell determination on growth curves and on viability of very dilute suspensions of the organism in vitro in distilled and sea water was investigated. The results of a field investigation at a sewage outfall are described."--Author.

Quinlan, James F. and Donald R. Rowe, 1977, Hydrology and water quality in the Central Kentucky Karst: Phase I. Management Report 12, National Park Service, Uplands Field Research Lab., Mammoth Cave, Kentucky, 93 p.
Brief discussion of dye tracing techniques includes discussion of fluorescein, Direct Yellow 96, and Fluorescent Brightener 28 (an optical brightener). No data given for determining quantities of tracers needed. Recommends against the use of rhodamine WT and rhodamine B in groundwater work in karst terrain.

Robinson, T. W., and D. Donaldson, 1967, Pontacyl brilliant pink as a tracer dye in the movement of water in phreatophytes. Water Resources Research, Vol. 3, No. 1, p. 203-211.
Dye was introduced into water surrounding plant roots and was later found in the roots, stems, leaves, and in the transpired water from the plant. If phreatophytes were present, the technique could be used in shallow aquifers to trace groundwater movement. The systems might have some applicability in studies on the dispersion of liquids from septic fields.

Salbidegoitia, Jose Maria, 1970, Trazadores, Kobie (Bilbao), Publication of Grupo Espeleologico, Bull. 2, p. 25-37. In Spanish.
A very good review paper on tracing agents in groundwater systems. Includes some data on fluorescent tracers which are seldom used today and estimates quantities necessary for several types of groundwater conditions. Includes substantial data on radioactive tracers.

Sargeant, K., 1969, The deep culture of bacteriophage. *In* Norris, J. R. and D. W. Ribbons, eds., Methods in Microbiology, Vol. 1, Academic Press, N. Y., pp. 505-520.
Good discussion of the production of large volumes of bacteriophage, which is necessary for water tracing work.

Scanlan, James W., 1968, Evaluation and application of dye tracing in a karst terrain, M. S. Thesis in Civil Engineering, Univ. of Missouri at Rolla, 88 p.
Contains a good deal of general information. Concludes on a small amount of evidence that loss of fluorescein in sands and gravels can be very high; this conflicts with other information in the literature, yet truly comprehensive investigation of this question have not been conducted. Compares many tracing agents in a rather interesting table.

Steppuhn, H. and J. R. Meiman, 1972, Automatic detection of waterborne fluorescent tracers, Int. Association of Sci. Hyd. Bull. 16, No. 4, p. 83-89.
Description of the equipment necessary to automatically detecting rhodamine WT with gelatin film. Method requires the use of a fluorometer, but this instrument need not be located in the field. Suggests

applications for the method and equipment developed.

Stoner, J. D., 1968, Comparison of results using three sampling techniques for a fluorometric dye study in a stream. Selected Techniques in Water Resources Investigations, 1966-67, U. S. Geol. Survey Water Supply Paper 1892, p. 30-33. $1.00 from Supt. of Documents, Government Printing Office.
Paper compares results from grab samples and two different pumping systems. The results from all methods are similar in most cases. There are five other articles in this Water Supply Paper which also deal with dyes.

Turner, G. K., Associates, 1970, Fluorometry in studies of pollution and movement of fluids, Fluorometry Reviews, Acc. No. 9941A, 24 p., Single Copies available free from G. K. Turner Associates, 2524 Pulgas Ave., Palo Alto, California 94303.
This paper is an excellent review of water tracing with fluorescent materials. It contains a wealth of information and ideas. About 60 references are cited; the most recent are abstracted. Since Turner manufactures a fluorometer, there is an emphasis on the review on fluorometric analysis.

Wilson, James F., Jr., 1968A, Fluorometric procedures for dye tracing, Techniques of Water-Resources Investigations of the United States Geological Survey, Book 3, Chapter A12, 31p. Available from the Superintendent of Documents, Government Printing Office, $.35.
An excellent discussion of the use of the fluorometer. Discusses the properties of a number of fluorescent dyes and compares their characteristics. Describes in detail the use of a Turner Model 111 fluorometer and serves as a good field manual for this instrument.

Wilson, James F., Jr., 1968B, Improved dyes for water tracing. *In* Selected Techniques in Water Resources Investigations, 1966-67, U. S. Geological Survey Water Supply Paper 1892, p. 5-8. $1.00 from Supt. of Documents, Government Printing Office.
Discussion of the fluorescent dyes available. Includes a tabular comparison of rhodamine B, rhodamine WT, pontacyl brilliant pink B and fluorescein. Briefly discusses fluorometer use. There are five other articles in this Water Supply Paper which also deal with dyes.

Winpenny, J.W.T., N. Cotten, and M. Statham, 1972, Microbes as tracers of water movement. Water Research, Vol. 6, p. 731-739.
Study evaluating several microorganisms as potential water tracers (two Bacillus species, two pigmented yeasts, Serratia marcescens, and a lambda-like bacteriophage of Escherichia coli K12).

The yeasts multiplied in river water over a period of several days. Interfering microorganisms reduced the utility of Serratia marcescens. The bacteriophage was the most useful tracer evaluated.

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