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Qualitative Water-Tracing With Dyes In Karst Terranes; Toxicity Of Twelve Fluorescent Dyes  
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Excerpt revised and reprinted from: Quinlan, J. F., ed., 1986. Practical Karst Hydrogeology, with Emphasis on Groundwater Monitoring. National Water Well Association, Dublin, Ohio. 898 p. [Course Manual; not available for purchase. This excerpt is a draft preprint of part of: Aley, T., Quinlan, J. F., and Vandike, J. E., The Joy of Dyeing: A Compendium of Practical Techniques for Tracing Groundwater, Especially in Karst Terranes, which is to be published by the National Water Well Association, 1987.]

## QUALITATIVE WATER-TRACING WITH DYES IN KARST TERRANES

James F. Quinlan

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### INTRODUCTION

The Water Tracer's Cookbook (Aley and Fletcher, 1976) is the most practical, unambiguous manual concerning dye-tracing yet written, but parts of it are out-of-date. Rather than merely reprint the Water Tracer's Cookbook or hastily whip out an unsatisfactory successor to it, we have elected to prepare a new cookbook -- and do it properly. In 1987 the National Water Well Association will publish The Joy of Dyeing: A Compendium of Practical Techniques for Tracing Groundwater, Especially in Karst Terranes (Aley, Quinlan, and Vandike, 1987). Accordingly, this draft preprint will not give coverage as complete as you (and we) might desire. Many dye-related topics are not discussed in this excerpt, but it gives the essential information and rationale for most qualitative procedures. The Joy of Dyeing will be a practical, more comprehensive manual, with specific instructions, written in plain English. In the meantime, refer to the references cited, especially those by Jones (1984b, 1984c).

I estimate that 1300 professionally-run groundwater traces

have been made so far in the U. S. I believe that more than 90% of them were done with dyes, in karst terranes, and that dyes are generally the cheapest, most practical tracers. Therefore, the emphasis here is on dyes rather than other tracers.

A comprehensive general review of groundwater tracers was made by Davis et al. (1985) so no attempt will be made to review all of them now in use. I should add, however, that the discussion of dyes by Davis et al. has many errors, omissions, and misleading statements (Quinlan, 1986).

This text will emphasize facts, procedures for qualitative tracing techniques, practical applications, principles, and sources of dyes and necessary supplies. Some of this material is in the Cookbook but most of it is not in the other recent publications.

Although I enthusiastically extoll herein the general practicality and superiority of qualitative dye-tests to quantitative dye-tests, there are many situations in which quantitative tests give more information which is more useful. (Smart et al., 1986).

I am not saying in these pages that, "This is the way dye-tracing must be done." Rather, I am summarizing what I (and others) have found to consistently give reliable results.

The definitive reference on the properties of dyes used for tracing groundwater was written by Smart and Laidlaw (1977). I won't try to summarize it.

Most traces can be run with one or more of the following four dyes: Fluorescein, Rhodamine WT, certain optical brighteners, and Direct Yellow 96. Additional dyes, other tracing agents, quantitative techniques, and dyeing kinetics will be briefly reviewed in The Joy of Dyeing.

#### DYE NOMENCLATURE

A dye is a substance that can be applied in solution to a substrate, or can be added to a liquid, thus giving the substrate or the liquid a colored appearance. Fluorescent dyes have an advantage over non-fluorescent dyes as tracers. Fluorescent dyes can be easily detected in concentrations which are one to three orders of magnitude less than those at which non-fluorescent dyes can be measured colorimetrically.

Smart and Laidlaw (1977) logically classified water-tracing dyes by color of their fluorescence: blue, green, and orange. To these three groups one must add a fourth, yellow. But I prefer to classify dyes according to the detector (bug) generally used to recover them: activated charcoal and cotton. Before discussing the dyes, however, it is appropriate to discuss dye nomenclature.

The standard industrial reference to dyes is the Colour Index (SDC & AATCC, 1971-1982), an incredible 7-volume compendium of almost 6500 pages which describes an estimated 38,000 dyes and pigments. (Colour Index is abbreviated as CI.) Most dyes listed in it are classified both according to the dyeing method in which they are used to color various types of textiles, leather, paper, or other goods (the CI Generic Name) and according to their chemical structure (the CI Constitution Number). For example, Fluorescein is CI Acid Yellow 73 and CI 45350; it is sold under more than 30 different commercial names such as Soap Yellow F. There are 18 CI Generic Name categories and 29 CI Constitution Number categories for dyes, but they will not be discussed here -- other than to stress that the Generic Name classification is based on industrial use and that the four major generic types of dyes used in water tracing are: Acid, Basic, Direct, and Fluorescent Brightening Agent. [References to discussions of dye nomenclature are given by Quinlan (1986).] Dyes are also classified according to their use in foods, drugs, and cosmetics. For example, water-soluble Fluorescein which has certified purity is designated by the U. S. Food and Drug Administration as D&C Yellow 8. (D&C = Drug and Cosmetic.)

There are numerous commercial names for most dyes. The rules and logic of these commercial names are as chaotic as those for prescription drugs. In order to avoid confusion in publications concerning dye properties and trace results, one should always include specification of dyes used by: the CI Generic Name or CI Constitution Number, manufacturer, and the manufacturer's commercial name.

The first part of the commercial name of a dye should not be confused with the dye itself. Tinopal and Diphenyl, for example, should always be capitalized; they are trade names belonging to the Ciba-Geigy Corporation and used for whole series of chemically unrelated dyes made by that particular company. To use just the first part of a commercial name for a dye, capitalized or uncapitalized, is ambiguous and erroneous. There are, for example, 7 chemically different Tinopals and 20 different Phorwites currently sold in the U. S. by Ciba-Geigy and by Mobay as optical brighteners.

The need for careful identification of dyes used for water-tracing -- particularly if one is discussing their chemical properties, toxicity, or excitation and emission spectra -- and the importance of knowing something about dye nomenclature and classification, are made obvious from inspection of the Colour Index itself. For example, 5 structurally different kinds of Rhodamine are sold in 1986 in the U. S. under 10 different names by 6 manufacturers. This multiplicity of names for what may or may not be the same dye is a result of the fact that until recently, the manufacturer of a new dye or an allegedly new dye did not want customers or the competition to know the nature of the dye. If the manufacturer knew that his product was the same as that of another, he did not say so because such identification could increase competition and force prices lower. Even today, much information about dye structure and composition is propri-

etary; it is not released, partly because development costs must be amortized.

When one publishes the results of tracing studies and gives the name of a dye, it is essential that the full Colour Index Generic Name be given at least once. To refer to, for example, Direct Yellow without also specifying its number, 96 (as some authors have), keeps the reader ignorant as to which of more than 180 direct yellows was used. Omission of this number in the title or text is like dropping just one shoe. Only part of the story is known; the rest is left to the imagination.

The above discussion of dye nomenclature is academic and useless if one is going to use nothing but fluorescein and one or two other dyes. But familiarity with dye nomenclature and classification is essential if one is going to read and understand the dye literature, make justifiable decisions about dye toxicity and equivalencies, find alternative sources of supply (particularly when a supplier has ceased to make a particular dye), and make valid price comparisons.

#### TYPES OF FLUORESCENT DYES USED FOR GROUNDWATER TRACING

There are three fluorescent dyes and one group of fluorescent dyes used for most groundwater tracing in the U. S. Although all of them are sorbed onto activated charcoal, they can be pragmatically classified into two principal groups according to the method conventionally used to detect and recover them. They are:

- A. Dyes recovered on cotton
  - 1. Optical brighteners (organic compounds added to detergents to "make your whites whiter")
    - a. Tinopal 5BM GX (a new, granular, less dusty formulation of Tinopal 5BM Extra Conc; the latter is no longer available)
    - b. Phorwhite BBH Pure (powder)
    - c. Phorwhite AR Solution (13% BBH Pure, in water)
  - 2. Direct Yellow 96 (powder)
- B. Dyes recovered on activated charcoal
  - 1. Fluorescein (powder)
  - 2. Rhodamine WT (sold as a 20% solution, in water)

Sources of supply, costs, concentration, and Colour Index information on these dyes are given in Tables 1 and 2. Toxicity data for all of them but Tinopal 5BM GX is given by Smart (1984). Toxicity data for Tinopal 5BM GX is given by Lyman et al. (1975) and Ganz et al. (1975); in the latter paper, it is identified as DASC-4.

Similar data for other dyes which can be safely used will be given in The Joy of Dyeing, but the major emphasis in it will be on those listed above.

Not all optical brighteners are suitable for water-tracing. Some work best in hot water and are inefficient in cold (54 °F) hard water. Many work best on a specific type of fabric. There



TABLE 2. Suppliers of dyes, chemicals, bug materials, and ultra-violet lamps

CC/D	Chemcentral/Detroit 13395 Huron River Drive Romulus, Michigan 48174	(313) 941-4800
C-G	Ciba-Geigy Corporation Dyestuffs & Chemicals Division P.O. Box 18300 Greensboro, North Carolina 27419	(800) 334-9481
<u>C&amp;K</u>	Crompton & Knowles Industrial Products Division P.O. Box 68 Skokie, Illinois 60076	(800) 323-4383
	Fisher Scientific Co. 541 Creek Road Cincinnati, Ohio 45242	(513) 793-5100 (There are 26 other branches)
	Klein & Thomas, Inc. P.O. Box 387 Saratoga, California 95071	(408) 378-7752
Mobay	Mobay Chemical Corporation Dyes and Pigments Division Mobay Road Pittsburgh, Pennsylvania 15205	(800) 662-2927
Pylam	Pylam Products Co. Inc. 1001 Stewart Ave. Garden City, New York 11530	(800) 645-6096
	Ultra-Violet Products, Inc. P.O. Box 1501 San Gabriel, California 91778	(818) 285-3123

The abbreviations in column 1 are used in Table 1.

are brighteners and there are brighteners.

There are two dyes called Fluorescein. Both of them are CI Acid Yellow 73 but only one of them, sodium fluorescein, is water-soluble and used for tracing. In the U. S. and England this dye is generally (and erroneously) called Fluorescein rather than its European name, Uranine. I will follow this custom. But not all sodium fluorescein is the same. The yield of the dye-manufacturing process varies from lot to lot and has increased as improvements were made in it. In order to achieve a uniform product and, in some cases, in order to enhance the ease of dyeing a product, it is conventional to add diluents (cutting agents) to technical grade dyes. This is standardization, not adulteration. For Fluorescein the diluents commonly used are any one of the following: sodium chloride, sodium sulphate, and Dextrin (a starch product made from corn), in concentrations that range from 10% to 60%; 25% and 50% are the most common. This means that the Fluorescein bought from a company today may have a different diluent composition and a different strength (diluent percentage) from what was bought from the same company last year. It also means that: 1) calculations of dye-concentration (but not dye-recovery) in quantitative tests will be higher than the actual value if no allowance is made for the purity of the dye, and 2) comparison of dye costs must consider diluent concentration as well as cost per pound. A dye which costs more per pound could be cheaper (in terms of active ingredients) than one which costs less. A comparison of Fluorescein prices and concentrations listed in Figure 1 shows that one company offers 50% more dye per pound at about half the cost per pound charged by another. There are Fluoresceins and there are Fluoresceins.

Optical brighteners and Fluorescein are very susceptible to photochemical decay, especially when in low concentrations; Rhodamine WT is less so, but this property is a problem only if bugs are placed in a stream exposed to sunlight. Direct Yellow 96 does not undergo any significant photochemical decay; it is ideal if you are tracing a stream that repeatedly sinks, resurges, and flows along the surface.

All tracer dyes tend to react with the environment through which they flow. Fluorescein, Rhodamine WT (to a lesser extent), optical brighteners, and Direct Yellow 96 (the latter two to a much lesser extent than Rhodamine WT) are all sorbed by clays. Sorption precludes the use of Fluorescein and Rhodamine WT in granular aquifers but it is less important in karst aquifers. Optical brighteners and Direct Yellow 96 are more reactive with cellulose (wood, leaves, etc.) than the other two.

#### DETECTION OF DYES

Dye is recovered with detectors known colloquially as bugs. They are suspended in streams and springs, commonly on a hydrodynamically stable stand known as a gumdrop which is explained in Figure 1.

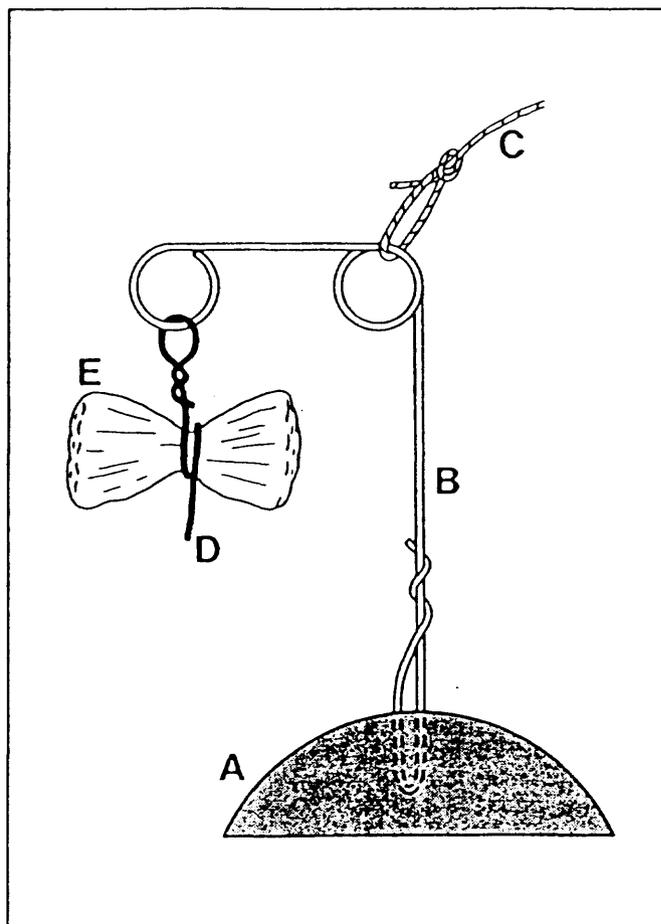


Figure 1. Gumdrop used to suspend dye-detectors (bugs) above stream beds. Total height is about 12 to 14 inches.

- A -- Concrete semi-hemisphere, approximately 6 inches in diameter and 2 to 3 inches high. (Concrete is poured into a hydrodynamically stable plastic cereal bowl lined with Saran Wrap.)
- B -- Galvanized steel wire, #9 gage. Note loops bent into it.
- C -- Nylon cord, 3/32 inches in diameter, tied to loop in wire and to tree or large rock. (Tan or gold color is recommended because it blends with dirt.)
- D -- Vinyl-clad #10 copper electrical wire. It is twisted through the steel loop and snugly around the piece of cotton, E.
- E -- Surgical cotton, 4 inches long x 2 inches wide and 1 inch thick. The cotton swings freely in any current and stays free of sediment that might bury it.

Note: A second detector, for Fluorescein, Rhodamine WT, or another dye sorbed onto charcoal, can be hung with a paper clip onto the same loop to which C is attached. This detector consists of one to two heaping teaspoons of activated coconut charcoal in a packet made of screening, as described in the text.

## Cotton Bugs

Johnson & Johnson's Red Cross brand surgical cotton, purchased in the 1-pound size rather than a smaller size, is highly recommended. Several other brands of surgical cotton are treated with an optical brightener during their manufacture; they are, therefore, unsuitable. Recently, however, Johnson & Johnson has started to use brightener on some of its cotton. Check yours before you buy in bulk or start your test. Always use an ultraviolet lamp during bug fabrication to inspect bugs for brighter or brightened contaminants on the cotton. Tom Aley has found 2.5-inch diameter cotton pads that are useful as bugs. They are sold as Swiss Beauty Pads and may be found in some drug stores. You can order them directly from the manufacturer, Klein & Thomas Inc., at the address given in Table 2 (\$2.99 for 100 pads). Swiss Beauty Pads can be suspended in a packet made of aluminum, nylon, or fiberglass screening, like those made for charcoal bugs and described in the caption for Figure 1.

A cylindrical cotton bug suitable for use in wells is Tampax. I don't know about the fluorescence or non-fluorescence of other brands of tampons. Tampax would also be a good "emergency bug" that could be bought almost anywhere.

When recovering bugs in the field, I prefer to put them into individual pre-marked 4x4-inch Ziploc or Whirl-Pac PVC bags. Before going into the field, label and date each bag with a marking pen which has an aromatic solvent-based ink. Water-based inks will wash off. After you put the bug in the bag, but before you seal it, squeeze the excess water out of the bug and drain it.

After collecting a cotton bug, wash it thoroughly with a high-speed jet of water. (I use a garden hose with a squeeze-operated nozzle.) This removes clay, silt, and trash from the cotton mass. Press it flat and examine with a long-wave UV lamp. I recommend the Ultra-Violet Products model UVL-21 (\$55.00) with its model CC-10 viewing cabinet (\$80.00; overpriced, but extremely convenient and almost essential).

Cotton which has reacted with optical brightener is characteristically blue-white. Cotton which has reacted with Direct Yellow 96 is canary yellow. Cotton which has reacted with both of these dyes is a distinctive white-white. Fluorescence intensity is directly but not linearly proportional to the amount of dye which has reacted with the cotton. Bugs which have not reacted with optical brightener will not fluoresce. Bugs may have a few blue-white flecks that fluoresce; these do not make a bug positive. The flecks are merely brightened fibers from elsewhere which got mechanically trapped in the bug as it sat in the stream of water.

Some practitioners prefer to use cotton cloth rather than surgical cotton as a detector because the cloth can be more easily scanned by a fluorometer or spectrofluorometer, thus partially quantifying the degree of positiveness of a bug. I

don't. My field tests with both types of detectors mounted on the same gumdrop repeatedly show that the surgical cotton is significantly brighter and more sensitive to lower concentrations of dye. This is probably because there are fewer fibers per unit area in the cloth.

### Charcoal Bugs and Elution

A charcoal bug consists of 1 to 2 teaspoons of activated coconut charcoal in a packet made of aluminum, nylon, or fiberglass screening. The packet is about 2.5 inches square and two of them can be folded from a 5 x 7-inch piece of screening. Do not use copper or brass screening. If you use nylon or fiberglass screening, two of the seams can be closed with a sewing machine, preferably with non-cotton thread, and the third can be closed with staples. Alternatively, all the seams can be closed with staples. Fold the ends over to prevent loss of charcoal at the seams. The bug is hung on a gumdrop, as described in Figure 1. Replace paper clips after two to three weeks; they rust and they fail by fatigue where bent.

The following detector and some or all of the indicated reagents are needed:

Activated coconut charcoal, 6-14 mesh (Fisher #5-685-A; cost: \$18.00 per pound). Unless you are going to use hundreds of bugs per year, do not buy charcoal in containers larger than 1-pound. Charcoal loses its sorption ability by reaction with air over a period of weeks to months, depending upon how tightly the can is sealed. Do not use the charcoal designed for water-treatment processes and aquariums.

Isopropyl alcohol (2-propanol): This is rubbing alcohol, a 70% solution, available in any drug store for about \$1.00 per pint. You can also use the 99% solution available from chemical supply houses at a price about 40 times higher, or you can use the inexpensive 99% solution which is sold for cleaning drums on xerox machines, but each has to be diluted with water to 70%. If you don't dilute them, the KOH will not dissolve properly. Make a saturated solution by adding about 6 to 7 grams KOH to 100 ml of the 70% alcohol. Use just the lighter liquid, at the top, which is a saturated solution of about 5% KOH.

Ethanol

1-propanol

Potassium hydroxide

Ammonium hydroxide

Distilled water (Tap water would probably be satisfactory when nothing else is available.)

} Available from any  
chemical supply house

A Fisher address is given in Table 2. Bugs should be changed every couple of days to weekly. In streams with smelly or colored water, the charcoal will be rapidly exhausted; bugs should be changed daily or more often.

If elution of dye from charcoal is not done within 6 to 12 hours after collection, the bug should be dried as soon as possible after collection. This will minimize bacterial reactions that can destroy some of the fluorescein in the charcoal.

Elution of dye is simple. Interpretation of elutants, however, ranges from obvious to subtle and can be difficult when the amount of dye recovered is minimal. One has to develop an "eye" for doing so.

I recommend the following elutants for recovering dye from activated charcoal:

- A. For Fluorescein: 5% KOH in 70% isopropyl alcohol. (This has a limited shelf-life. Do not use elutant more than a few days old.)
- B. For Rhodamine WT: a 5:2:3 mixture of 1-propanol, concentrated NH<sub>4</sub>OH, and distilled water. (This has a shelf-life of several months.)

The choice of which hydroxide to use in which alcohol depends upon what dye is to be eluted, whether two dyes are to be eluted, the adequacy of available ventilation, and one's tolerance for ammonia fumes. As discussed by Atkinson and Smart (1981, p. 177), the best and most efficient elutant for Rhodamine WT is a 5:2:3 mixture by volume of 1-propanol, concentrated NH<sub>4</sub>OH, and distilled water, preferably warmed to about 140 °F (Smart, 1972; Smart and Brown, 1973). Most practitioners, myself included, elute at roomtemperature and we have assumed that the 5:2:3 "Smart Solution" is also optimal for elution of Fluorescein. But it might not be optimal. Staff of the Missouri Geological Survey prefer to use ethanol rather than 1-propanol because the fumes are significantly less when ethanol is used. Their laboratory tests have shown that 5% KOH in ethanol releases significantly more Fluorescein from charcoal than does 5% NH<sub>4</sub>OH in the same alcohol. In contrast, the recovery of Rhodamine WT from charcoal is far greater with 5% NH<sub>4</sub>OH in ethanol than with 5% KOH in the same alcohol. For semi-quantitative tests using activated charcoal, a spectrofluorometer, and both dyes, the Survey has standardized on elution with 5% NH<sub>4</sub>OH in ethanol. This is because the significant decrease in the recovery of Fluorescein when NH<sub>4</sub>OH is used is much less than the great decrease in the recovery of Rhodamine WT when KOH is used (James W. Duley, oral communication, January 1986). Nevertheless, it has not yet been shown whether 1-propanol is more efficient or less efficient than isopropyl alcohol, ethanol, or any other alcohol for elution of Fluorescein from activated charcoal. There is a great need for a systematic study of Fluorescein elution similar to the study by Smart (1972) of Rhodamine WT elution. Such a study should start with laboratory tests and be extended to include bugs which have been subjected to the vagaries of exposure in actual springs and streams.

It is practical to elute dye in baby-food jars on which an identifying number has been scribed. Similarly-sized jars which can be sealed are also suitable.

Wash bugs with a high-speed jet of water; this will remove clay and silt which interferes with the analysis. Pour about half of the charcoal into the numbered jar and cover the charcoal with about 1/8" to 1/4" of elutant. Wait. [Keep the other half of the charcoal in case it is needed for evidence, or in case there is a foul-up such as dropping a jar, etc.]

The kelly-green color of Fluorescein is distinctive; but interpretation of weakly positive tests is complicated by confusion between this color and the color of algae and organic matter (humic acid, agricultural waste, etc.) which can occur as background. All of them are sorbed by the charcoal and released from it by the elutant. Many a test has been falsely called positive by well-meaning people who hadn't yet learned to distinguish the green of this background from that of Fluorescein. The human eye is better able to distinguish between them than is the fluorometer or spectrofluorometer.

The following is a semi-quantitative scheme proposed by Aley and Fletcher (1976, p. 16) for assessing the degree of positiveness of Fluorescein recovery on charcoal:

1. Very strongly positive: Dye can be seen distinctly with the naked eye in sunlight or in an artificially lighted room within 15 minutes of the time that KOH and alcohol are added to the charcoal.
2. Strongly positive: Same as above, but after 15 minutes and before 3 hours.
3. Moderately positive: Dye can be seen with the naked eye in sunlight or in an artificially lighted room, but not until 3 to 24 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 jar.
4. Weakly positive: Dye cannot be detected by the naked eye in sunlight or in an artificially lighted room until more than 24 hours after adding KOH and alcohol. Dye can be distinctly seen by the naked eye when a light is beamed through the sample jar.
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 distinctly seen by the naked eye when a light is beamed through the sample jar.

The light-beaming technique increases the detectability of Florescein; it can be seen in concentrations as low as one part per billion. The technique is simple and can be done in either of two ways:

1. Use the focussed beam from a microscope lamp.
2. Use sunlight and a 4-inch reading glass.

The "green beam" is more visible if it is viewed against a black background.

Do not shake the jar. If you do, and if there is any suspended clay that was on the charcoal and is then in the elutant, the beam will be white; you will have to wait, perhaps as long as

several hours, until it settles.

Elution of dye from activated charcoal does not give reproducible, reliable results on a fluorometer or spectrofluorometer. There can be dye-losses due to microbiological reactions and diffusion of adsorbed dye onto internal high-energy sites in the charcoal; not all dye is eluted or the time necessary for attainment of equilibrium between elutant, charcoal, and dye is long, variable, and temperature-sensitive (Smart and Friederich, 1982, p. 108-111).

Jars used for elution should be thoroughly washed between uses. Some practitioners prefer to include a bit of Clorox in the rinse water, in order to eliminate the possibility of contamination from tests to another. The use of Clorox, however, is probably not necessary.

#### PLACEMENT OF DETECTORS

Place the bugs in a stream or spring and at a location which maximizes the amount of water which passes through them. Anchor a bug to a rock, use a gumdrop (Figure 1), or a Chaney-pin (described in the subsequent section on principles of dye-tracing). In my opinion, gumdrops offer the greatest convenience and flexibility where there is much suspended sediment and when bugs must be recovered at sites where water levels may fluctuate more than a few feet. In the Mammoth Cave area we commonly have a fluctuation of 10 to 20 ft; 50 ft is possible. Accordingly, the non-gumdrop end of the cord should be tied at an elevation not likely to be flooded during the duration of the test being run. If flow velocities are very high, put the bugs in a more sheltered area. Otherwise cotton may be washed away and charcoal may be washed through the screen; the test can be lost.

Bugs can also be placed in wells or, preferably, in a stream of water continuously or regularly pumped from a well. They can even be put in the tank behind a toilet but, since the degree of positiveness of a bug is directly related to the amount of dye-laden water that has passed through it, pumped wells are more sensitive and reliable than toilet tanks as an indicator of the presence of dye. Recent observations in Arkansas by Tom Aley suggest that even though most wells do not intercept trunk conduits or their major tributaries, continuous pumping of a well can reverse the normal flow of water. Pumping can draw dye-laden water from the conduits and into the joints and bedding-plane enlargements that normally feed them. He has had success by continuously pumping wells for a week or more at rates up to 5 gpm (7200 gpd). Water flows through a garden hose and to an opaque container which has an opaque cover. This protection minimizes the likelihood that sunlight will enhance the growth of green algae which can interfere with interpretation of elutant color. Research needs to be done on the extent to which wells in karst areas can monitor the conduits they don't intercept.

Pilfering of gumdrops can be minimized by using a plastic-

laminated tag similar to the one shown in Figure 2. It works.

#### HOW MUCH DYE TO USE

This is a matter of experience. Equations and a nomograph may be given in The Joy of Dyeing but, for now, I'll state some rules of thumb. For the average Kentucky spring (whatever that is), under average conditions (whatever they are), I would start with the following and adjust amounts downward or upward, as initial results suggest:

Fluorescein -- one pound per mile, up to five pounds

Direct Yellow 96, Tinopal 5BM GX, and Phorwite BBH Pure -- twice as much as for Fluorescein

Phorwite AR Solution -- one gallon per mile, up to four gallons

Rhodamine WT -- not recommended for most qualitative tests because the tea-like color of any organic matter commonly present can mask the pink tint of the dye unless relatively large quantities are used. If you must, try 0.5 gallon per mile.

For quantitative tests, using a fluorometer or spectrofluorometer, use one tenth to one hundredth as much dye. Rhodamine WT is the best dye to use for quantitative traces.

It is always a goal to limit the amount of dye to a quantity which is insufficient to impart a visible coloration to water. This goal is inspired by health and aesthetic (public relations) considerations, but there are times when, for public confidence in results (or for rabble-rousing), it is advisable to have the resurgence of the dye vividly obvious to all. The limit of visibility of Fluorescein is about 0.03 ppm; 0.3 ppm can be easily seen with the naked eye.

The U.S. Geological Survey has a policy of limiting the maximum concentration of fluorescent dyes at water-user withdrawal points to 0.01 ppm (Hubbard *et al.*, 1982, p. 15), but this is an arbitrary, conservative, non-obligatory limit rather than

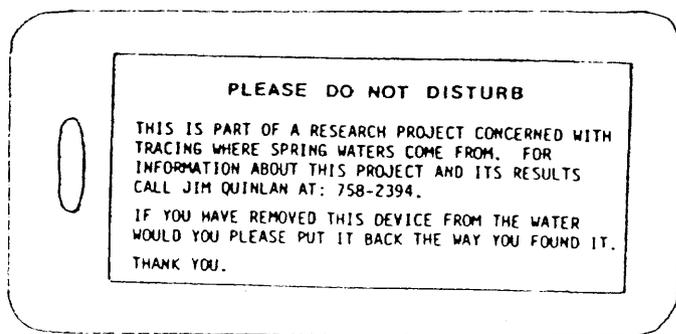


Figure 2. Laminated tag sometimes attached to a gumdrop in order to discourage removal of it from water by people who are curious as to what it is. (Shown actual size)

one based on a comprehensive assessment of toxicity.

I urge caution in the use of Direct Yellow 96. If you use too high a concentration in a potable water supply you might dye someone's laundry pale yellow. People will wonder where the yellow came from. There will be no injury to their health but they might be irked. And you might have to buy some clothes.

#### INJECTION OF DYE

This too is an art. Whenever possible, use dye pre-mixed in water. It can be stored and carried in gallon milk jugs. I recommend using 1 pound per gallon of water for Fluorescein and 2 pounds per gallon for Direct Yellow 96 and optical brightener. Warning: Do not store Direct Yellow 96 in a plastic milk jug for more than a month or two. Direct Yellow 96 eats plastic; the jugs crack and leak. The timing and location of the consequent disaster follows Murphy's Law.

Although you can easily inject Fluorescein powder and Tinopal 5BM GX where there is little or no wind, never inject Direct Yellow 96 or Phorwite BBH Pure in powdered form -- unless you are prepared to have contamination of clothes and everything you come in contact with. When injecting Fluorescein powder and there is a wind, always be upwind of the powder; keep your equipment and all onlookers upwind and well away from it. Try to be to the side of or upstream from dye being injected into a stream. I find Wellington-type rubber boots to be ideal and I always wear long, electrician-type rubber gloves. [Playtex gloves aren't long enough.]

A tank-truck of water can be used for dye-injection into the subsurface where natural flow, is not available. I have had success with a "primer" of 1000 gallons before dye is injected. The injection is followed by a "chaser" of about 1000 gallons. More water or less water could be used, as circumstances seem to require, but it is important to have a primer and a chaser.

#### RECORDING TEST DATA

The results of a dye test should be recorded on a data sheet similar to that shown in Figure 3. I fill them in with an ordinary pencil but record all positive results with a red pen. This makes positive results less likely to be overlooked. I record a weakly positive test with a single dotted red cross.

Daily tasks associated with a dye-trace are recorded on the bug sheet shown in Figure 4. Note the column for recording the results of the dye tests. The jar number is always recorded on the bug sheet, checked, and rechecked before any elutant is poured into the jar.





## RECENT ADVANCES IN THE INTERPRETATION OF DYE TRACES

Significant advances have been made during the past few years in the interpretation of dye-trace results in karst terranes. Repeated traces in the same swallet-to-spring system show that the travel times in several British systems show an inverse, non-linear (hyperbolic) relation with discharge (Smart, 1981; Stanton and Smart, 1981). They showed that the breakthrough curve (time vs. dye concentration) indicates increasing dispersion with decreasing discharge, a consequence of the increasing "dead volume" of the conduit. Multiple dye-peaks suggest that branched flow routes are present and that the relative significance of each branch changes as spring discharge changes. Where the travel time shows a 1:1 inverse relationship to discharge, a system is interpreted to be phreatic; this characteristic of simple phreatic streams allows graphic analysis to distinguish them from vadose and complex phreatic streams. They also showed that the amount of Rhodamine WT which is sorbed in transit from swallet to resurgence is appreciably greater at low flows than at high flows. A pulse-test (a powerful technique explained and reviewed by Smith, 1977, p. 99-101) made during low flow in one of the systems indicates that only a small proportion of the conduit is vadose (Smart and Hodge, 1980). Significant advances in quantitative dye-tracing have also been made by C. C. Smart (1983a, 1983b) and Smart and Ford (1982) in a glacierized Canadian karst and by Lang et al. (1979), Collins (1982), Burkimsher (1983), and Brugman (1986) in glaciers.

Interpretation of results of intensive tracing, especially when done with complementary chemical analyses and discharge measurements, has made it possible to construct mental models that enable visualization and comprehension of water and pollutant movement through karst terranes -- as shown by Jones (1973), Quinlan and Rowe (1977), Aley (1977, 1978), Quinlan and Ray (1981), Crawford (1984), Friederich and Smart (1982), C. C. Smart (1984a, 1984b), Hallberg et al. (1985), Quinlan and Ewers (1985), Vandike (1985), Gunn (1986), and many others. A recently proposed mathematical technique for analysis of transport of material through any kind of natural system examines residence-time distributions and can relate tracer studies to empirical models (Buffham, 1985; discussed by Woods, 1985). Perhaps this technique and one proposed by Rathor et al. (1985) can be used for karst studies.

### PRINCIPLES WHICH MAXIMIZE THE COST-EFFICIENCY, SUCCESS, RELIABILITY, AND UTILITY OF DYE-TRACING STUDIES

I have had more than twelve years of experience in running and directing hundreds of dye-tests in carbonate rocks. Tom Aley and I have had more experience in doing so than anyone else in the U. S., but we are still learning. This experience has taught us much about how to and how not to run dye tests. The following are some of the lessons I've learned and their application to the study of karst hydrology. The principles are not listed in any

particular order of significance.



PRINCIPLE 1. Dye-tests should be designed so that there is always a positive result -- somewhere.

DISCUSSION: Negative results to a monitored site are always questionable. But if they are accompanied by strongly positive results to another site they can be considered reliable for the flow conditions during the test -- and they can be used. [The results might be different when the flow is much lower or higher.] Also, until one knows where a tracer has gone, that tracer can not be used in the same groundwater basin for a long time. If one has not accounted for the dye from the first test, and if the same dye is used for a second, third, or even a fourth or fifth test, one never knows whether the positive results of a given test are a result of dye injection from the first, second, fourth, or fifth test.

PRINCIPLE 2. Always run background tests before tracing with optical brightener.

DISCUSSION: This is always necessary with any dye if there is a possibility of litigation over interpretation of tracer results, or if it is possible that another investigator may be tracing in the same area. Litigation aside, many karst areas have a slight to moderate brightener background because brighteners are in laundry detergents; laundry wastewater is part of the effluent from septic tanks. Brightener can still be used, and the background can be overridden, but a standard for comparison is needed. [One can use anomalously high background for brighteners as a prospecting guide to locating effluent from sewage treatment plants, sewer lines, and septic field systems (Quinlan and Rowe, 1977; Aley, 1985).] Direct Yellow 96 can be ideal for use in areas where there is a high background for brighteners. Routine qualitative tests with other dyes generally do not require determination of background but quantitative tests do. If there is a possibility that someone else might have injected dye recently, check for it. Cave explorers, for example, sometimes run tests, generally with Fluorescein. Similarly, if you know of someone who may be doing some tracing in your area of interest, check with him. The courtesy is appreciated and may be mutually beneficial. Also, he might have (and be willing to share) data that will be useful to you. Return the favor.

PRINCIPLE 3. Generally speaking, never follow a qualitative test to a given recovery point with the same dye if the second test might also go to the same recovery point -- unless a major storm has occurred after the first test is over and before the second is started.

DISCUSSION: For traces to a given recovery site one should always alternate from one dye to another -- unless a major rainstorm has occurred. This is because a rain that occurs after the first test may flush dye-laden water which was locally left stranded in the system (either in pools or as coatings on passage walls), thus giving a falsely positive



 result. The rain that occurs after the first dye-test may also flush dye which had been temporarily sorbed onto clays and left "stranded" by receding floodwaters, thus also giving a second maximum in a plot of dye-concentration vs. time. My preference is to switch from a dye recovered on one type of bug to a dye recovered on the other type.

PRINCIPLE 4. If possible, always try to run two tests simultaneously -- to the same possible recovery site or to adjacent sites.

DISCUSSION: This doubles productivity and cuts labor costs per test to approximately half. For a given potential recovery site, and depending upon circumstances, one should use one dye detected on charcoal and another detected on cotton. For example, use Fluorescein and optical brightener. This can be followed by Rhodamine WT and Direct Yellow 96. (Of course one can start with Fluorescein and Direct Yellow 96 and follow it with Rhodamine WT and Tinopal 5BM GX or Phorwite BBH Pure or AR Solution.) In some aquifers, however, where dilution (and perhaps also sorption) is high, Fluorescein is the only dye which can be used for qualitative tests; Rhodamine WT could be used for quantitative tests.

PRINCIPLE 5. Qualitative dye-tests give useful results much more rapidly and cost-efficiently than quantitative tests; often, they are all that is needed.

DISCUSSION: Qualitative tests, using activated coconut charcoal and/or surgical cotton as detectors can be run with a detector cost of less than \$0.20 each. The detectors work 24 hours per day and need to be changed only once or twice per week. (Of course, samples can be taken much more frequently if there is a need for doing so.) Although the materials cost is trivial, the preparation, setting, changing, elution, and analysis of bugs is very labor-intensive. Much extremely useful information can be gained by using grab-samples (or an automatic sampler) and a fluorometer or a spectrofluorometer. Fluorescein is the most practical dye to use with activated charcoal; Rhodamine WT is not recommended for general qualitative use. Each of the other three dyes is suitable for quantitative use, but activated charcoal will not give accurate quantitative results with any of them. Optical brightener and Direct Yellow 96 work equally well with cotton detectors.

PRINCIPLE 6. Set bugs at all the likely places to which dye might flow, many of the unlikely places, and a few of the stupid, incredibly impossible places.

DISCUSSION: Use enough dye to reach the most distant of the above places. Dye is expensive, but it is cheap relative to the time and labor costs necessary to re-run a trace which isn't done properly the first time. The cost of charcoal and cotton detectors is miniscule.

PRINCIPLE 7. Delineation of a groundwater basin by dye-tracing should generally involve partial delineation of its neighboring basins.



DISCUSSION: This is a corollary of Principle #1. You should generally set bugs in the adjacent groundwater basins. By having most dye flow to them, by simultaneously working opposite sides of the basin to be delineated, and by alternating the dyes used, one can work more rapidly and efficiently. Both sides of a suspected divide should be tested by tracing, but the concept of an imaginary line that neatly separates one basin from another doesn't always apply in karst terranes. In most, dye which is injected near the center of a basin goes to a spring or to a distributary group of springs. But in some terranes, dye injected close to the assumed boundary between two basins flows to each of them. Smart (1977) has suggested that, when it is desirable to compute a water balance, the basin boundary should be chosen so as to coincide with dye-inputs in which the dye was divided evenly between the springs of two adjacent basins. The "neatness" of basin boundaries is also shattered by results in West Virginia described by Jones (1984c). Dye from a sinking stream flows about a mile in 24 hours to a particular spring. But a total of only about 5% of the dye goes there; the remainder of the dye was recovered a month later at a spring 12 miles away. Although calculation of basin area for a water balance may seem like an interesting but merely academic exercise, delineation of the boundary, and determination of whether it is fuzzy or sharp, is extremely practical and necessary if one must monitor a site or make an emergency response to spillage of toxic agents. Such a determination could only be done if springs outside the basin to be delineated were also bugged.

PRINCIPLE 8. Always, always, always replace one set of bugs with another, even if you are sure that the test is over.

DISCUSSION: Bugs are cheap relative to labor costs and the risk of losing a test. Also, sites should be monitored until you know the dye is out of the system. Meanwhile, the dye may also show up elsewhere in either distributary flow or radial flow.

PRINCIPLE 9. Be paranoid about the possibility of contamination of samples, tampering, or removal of bugs by people curious as to what they are.

DISCUSSION: Contamination happens, sometimes in the strangest ways. Anticipation of its possibility is the key to its prevention. Recognition of possible tampering is made easier if: 1) extra, well-hidden bugs are set, 2) the dye (or dyes) used and the bugged locations are kept secret until the test is over, 3) control bugs are set upstream from tributary junctions, and 4) optical brightener is secretly used with, for example, Fluorescein, as a check. Also, careful monitoring of the timing, location, and dye-concentration of the positive results will detect tampering by all but the most skillful attempt at manipulation of the test results. I minimize loss of bugs by using 3/16" goldline cord on gumdrops; it "blends" with dirt. I even rub dirt onto new cord. Sometimes a monofilament fishing line is best. If there is any possibility of public access to the bugs (by



hikers, fishermen, etc.), it is necessary to set an extra bug or two at each site. Tom Aley has had extensive experience with steel "Chaney pins" which are driven into a stream bottom and used for holding bugs without the aid of telltale cords. If necessary, when Chaney pins are buried by alluvium, they can be recovered with the aid of a metal detector. The use of laminated tags like the one shown in Figure 2 is effective.

PRINCIPLE 10. If you have a choice, inject dye during moderate flow conditions, while streamflow is in recession.

DISCUSSION: Dilution will probably be minimal and flow times will be average. This is the optimum time for tracing. See Principle 11.

PRINCIPLE 11. Although the first dye-tests should be run when conditions seem optimum, they should also be run at both low-flow conditions and high-flow conditions -- in many situations, but not all.

DISCUSSION: Flow-routes that function only during moderate- and flood-flow conditions may divert some of the water to springs in adjacent groundwater basins. During such conditions dye (or leachate from a site) can go to springs which it does not reach during low flow. In the Mammoth Cave Region the flow time between two points about 5 miles apart (Parker Cave and Mill Hole) ranges from 18 days to less than 24 hours. The average flow time is 3 to 5 days.

PRINCIPLE 12. Do not use a dye unless you know what it is.

DISCUSSION: There are many dyes sold as "leak-tracers" which are carcinogenic, mutagenic, etc. The companies selling them disclaim responsibility for their use, but you can't -- morally or legally. See the toxicity review by Smart (1984). In brief, Fluorescein, Rhodamine WT, and optical brighteners are the safest dyes to use. Available data for Direct Yellow 96 does not suggest any problems with its use but it has not been tested for mutagenicity. Rhodamine B is a known carcinogen and possible mutagen; it should never be used unless you can give a rigorous justification for using it rather than a different dye. It should be stressed, however, that the toxicity of Rhodamine B has been shown to be due to impurities (dilutents) within some batches of technical grades of the dye, not the dye itself (Smart, 1984). According to Smart, no water-tracing dye is acutely hazardous because of short exposures to the locally very high concentrations of it which can briefly occur where dye is injected. A concentration of 1 ppm for 48 hours can be endured by the more sensitive organisms.

PRINCIPLE 13. Always set bugs before you handle or dump dye.

DISCUSSION: This lessens the probability of contamination of bugs. Also, carry the dye containers in plastic bags and in a separate part of your vehicle (such as in the trunk or in the back of a pickup truck). If you are working by yourself and must load your dye after making up the bugs and loading them, wash your hands and inspect them under an ultraviolet lamp after you load the dye. [I don't recall how many times

we have had to decontaminate spots of optical brightener on the steering wheel of a pickup truck. Keep rubbing it with cotton swabs until no more brightener transfers to cotton.] Ideally, two people and different vehicles could be used, but this is not necessary. Be careful, very careful.



PRINCIPLE 14. If there are enough accessible wells, if there is no perched water above the main water mass, and if project needs can justify and afford it, make a map of the potentiometric surface, preferably before dye-tracing is started or completed.

DISCUSSION: The map can pay for itself, many times over. It is a useful supplement which can greatly aid decision-making in designing dye-tests and it can greatly decrease the number of tests otherwise needed. Such a map can be almost useless, however, if there are not enough wells to allow reliable contouring, or if there are siltstones or shales within the carbonate section; they tend to perch the water and make the data misleading and difficult to interpret.

PRINCIPLE 15. Know your dye.

DISCUSSION: Play with it in the office or laboratory, before you start running tests for the first time. Make up a Fluorescein solution that is approximately 1 ppm by mixing 1 gram of dye in 1000 ml of water and dilute it to 1 ppm. Pour approximately 500 ml of this 1 ppm solution through a bug at such a rate that it takes about 15 to 20 seconds to pass through the bug. (Alternatively, and with a pair of tongs, swish a bug through about 500 ml of the 1 ppm solution for several minutes.) Rinse the bug in clean tap water for about a minute and elute. You should get a very strongly positive result. Repeat, using 500 to 1000 ml of 0.1, 0.01, and 0.001 ppm solutions. Results from these elutions are only semi-quantitative, partly because not all of the dye will be released from the charcoal. But it will give you a feeling for how to elute and how to recognize Fluorescein. Having run this series of tests, leave a "background" bug in a stream for a week and run the same series of tests on the elutant from it when you repeat the above series on a 1 ppm solution of Fluorescein. Compare the results.

As with love-making, there is only so much you can learn from the printed word. There is no substitute for experience or experimentation.



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#### SELECTED BIBLIOGRAPHY

Aley, T. 1985. Optical brightener sampling; a reconnaissance

- tool for detecting sewage in karst groundwater. *Hydrological Science and Technology: Short Papers*. v. 1, p. 45-48.
- Aley, T., and M. W. Fletcher. 1976. *The Water Tracer's Cookbook*. *Missouri Speleology*. v. 16, no. 3, p. 1-32.
- Aley, T., J. F. Quinlan, and J. E. Vandike. 1987. *The Joy of Dyeing: A Compendium of Practical Techniques for Tracing Groundwater, Especially in Karst Terranes*. National Water Well Association, Dublin, Ohio. (in prep.)
- Brugman, M. 1986. Dye-tracing events at Variegated Glacier, Alaska. Ph. D. dissertation (Geology), California Institute of Technology. (in prep.)
- Buffman, B. A. 1985. Residence-time distributions in regions of steady-flow systems. *Nature*. v. 314, p. 606-608.
- Burkimsher, M. 1983. Investigations of glacier hydrological systems using dye tracer techniques: Observations at Pasterzengletscher, Austria. *Journal of Glaciology*. v. 29, p. 403-416.
- Collins, D. N. 1982. Flow-routing of meltwater in an alpine glacier as indicated by dye tracer tests. *Beitraege zur Geologie der Schweiz - Hydrologie*. v. 28, pt. 2, p. 523-534.
- Crawford, N. C. 1984. Karst landform development along the Cumberland Plateau escarpment of Tennessee, in LaFleur, R. G., ed. *Groundwater as a Geomorphic Agent*. Allen & Unwin, Boston. p. 294-339.
- Davis, S. N., D. J. Campbell, H. W. Bentley, and T. J. Flynn. 1985. *Ground Water Tracers*. National Water Well Association, Worthington, Ohio. 200 p. [Available from NWWA for \$15 to members and \$18.75 to non-members]
- Friederich, H., and P. L. Smart. 1982. The classification of autogenic percolation waters in karst aquifers: A study in G. B. Cave, Mendip Hills, England. *University of Bristol Speleological Society, Proceedings*. v. 16, p. 143-159.
- Ganz, C. R., J. Schulze, P. S. Stensby, F. L. Lyman, and K. Macek. 1975. Accumulation and elimination studies of four detergent fluorescent whitening agents in Bluegill (Lepomis Macrochirus). *Environmental Science and Technology*. v. 9, p. 738-744.
- Gunn, J. 1986. A conceptual model for conduit flow dominated karst aquifers. *International Symposium on Karst Water Resources (Ankara, 1985), Proceedings*. 8 p. [in press]
- Hallberg, G., R. D. Libra, and B. E. Hoyer. 1985. Nonpoint source contamination of ground water in karst-carbonate aquifers in Iowa, in *Perspectives in Nonpoint Source Pollution*. U. S. Environmental Protection Agency, EPA 440/5-85-001, p. 109-114.
- Hubbard, E. F., F. A. Kilpatrick, L. A. Martens, and J. F. Wilson, Jr. 1982. Measurement of travel time and dispersion in streams by dye tracing. *U. S. Geological Survey, Techniques of Water Resources Investigations*. Book 3, Chapter A9. 44 p.
- Jones, W. K. 1973. Hydrology of limestone karst in Greenbriar County, West Virginia. *West Virginia Geological and Economic Survey, Bulletin* 36. 49 p.
- Jones, W. K., ed. 1984a [1986]. *Water Tracing -- Special Issue*. *NSS Bulletin*. v. 46, no. 2, p. 1-48.
- Jones, W. K. 1984b [1986]. Dye tracer tests in karst areas. *NSS*

- Bulletin. v. 46, no. 2, p. 3-9.
- Jones, W. K. 1984c [1986]. Analysis and interpretation of data from tracer tests in karst areas. NSS Bulletin. v. 46, no. 2, p. 41-47.
- Lang, H., C. Liebundgut, and E. Festel. 1979. Results from tracer experiments on the water flow through the Aletschgletscher. Zeitschrift fur Gletscherkunde und Glazialgeologie. v. 15, p. 209-218.
- Lyman, F. L., J. Schultze, C. R. Ganz, P. S. Stensby, M. L. Keplinger, and J. C. Calendra. 1975. Long-term toxicity of four fluorescent brightening agents. Food and Cosmetic Toxicology. v. 13, p. 521-527.
- Quinlan, J. F., 1986. Discussion of "GROUND WATER TRACERS" by Davis et al. (1985), with emphasis on dye-tracing, especially in karst terranes: Ground Water. v. 24, no. 2, p. 253-259 and no. 3, p. 396-397. [Reply: p. 398-399]
- Quinlan, J. F., and R. O. Ewers. 1985. Groundwater flow in limestone terranes: Strategy rationale and procedure for reliable, efficient monitoring of ground water quality in karst areas. National Symposium and Exposition on Aquifer Restoration and Ground Water Monitoring (5th, Columbus), Proceedings. p. 197-234.
- Quinlan, J. F., and D. R. Rowe. 1977. Hydrology and water quality in the Central Kentucky Karst: Phase I. University of Kentucky, Water Resources Research Institute, Research Report no. 101. 93 p.
- Rathor, M. N., L. G. Gibilaro, and B. A. Buffman. 1985. The hopping model for residence time distributions of systems with splitting and merging streams. American Institute of Chemical Engineers, Journal. v. 31, p. 327-329.
- SDC & AATCC [Society of Dyers & Colourists and American Association of Textile Chemists]. 1971-1982. Colour Index, 3rd ed. SDC & AATCC, Bradford. 7 v. 6460 p.
- Smart, C. C. 1983a. Hydrology of a glacierized alpine karst Castleguard Mountains, Alberta. Ph. D. dissertation (Geography), McMaster University. 343 p.
- Smart, C. C. 1983b. Hydrology of the Castleguard karst, Columbia Icefields, Alberta, Canada. Arctic and Alpine Research. v. 15, p. 471-486.
- Smart, C. C. 1984a. Glacier hydrology and the potential for subglacial karstification. Norsk geografiske Tidsskrift. v. 38, p. 157-161.
- Smart, C. C. 1984b. Overflow sedimentation in an alpine cave system. Norsk geografiske Tidsskrift. v. 38, p. 171-175.
- Smart, C. C., and D. C. Ford. 1982. Quantitative dye tracing in a glacierized alpine karst. Beitrage zur Geologie der Schweiz - Hydrologie. v. 28, pt. 1, p. 191-200.
- Smart, P. L. 1972. A laboratory evaluation of the use of activated carbon for the detection of the tracer dye Rhodamine WT. M.S. thesis (Geography), University of Alberta. 188 p.
- Smart, P. L. 1977. Catchment delimitation in karst areas by the use of quantitative tracer methods. International Symposium on Underground Water Tracing (3rd, Ljubljana-Bled, 1976), Papers. v. 2, p. 291-198.
- Smart, P. L. 1984 [1986]. A review of the toxicity of twelve

- fluorescent dyes used for water tracing. NSS Bulletin. v. 46, no. 2, p. 21-33.
- Smart, P. L., T. C. Atkinson, I. M. S. Laidlaw, M. D. Newson, and S. T. Trudgill. 1986. Comparison of the results of quantitative and non-quantitative tracer tests for determination of karst conduit networks: an example from the Traligill basin, Scotland: Earth Surface Processes and Landforms. v. 11, p. 249-261.
- Smart, P. L., and M. C. Brown. 1973. The use of activated carbon for the detection of the tracer dye Rhodamine WT. International Speleological Congress (6th, Olomouc, Czechoslovakia), Proceedings. v. 4, p. 285-292.
- Smart, P. L., and H. Friederich. 1982. An assessment of the methods and results of water-tracing experiments in the Gunung Mulu National Park, Sarawak. British Cave Research Association, Transactions. v. 9, p. 100-112.
- Smart, P. L., and P. Hodge. 1980. Determination of the character of the Longwood sinks to Cheddar resurgence conduit using an artificial pulse wave. British Cave Research Association, Transactions. v. 7, p. 208-211.
- Smart, P. L., and I. M. S. Laidlaw. 1977. An evaluation of some fluorescent dyes for water tracers. Water Resources Research. v. 13, p. 15-33.
- Smith, D. I. 1977. Applied geomorphology and hydrology of karst regions, in Hails, J. R., ed. Applied Geomorphology. Elsevier, Amsterdam/New York. p. 85-118.
- Vandike, J. E. 1985. Movement of shallow groundwater in the Perryville area, southeastern Missouri. Missouri Geological Survey, Miscellaneous Publication no. 4. 53 p.
- Woods, J. 1985. Residence times of water masses in regions of the ocean. Nature. v. 314, p. 578-579.

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# A REVIEW OF THE TOXICITY OF TWELVE FLUORESCENT DYES USED FOR WATER TRACING

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*Toxicological information is reviewed for twelve fluorescent dyes used in water tracing, Fluorescent Brightener 28, Tinopal CBS-X, Amino G Acid, Diphenyl Brilliant Flavine 7GFF, Pyranine, Lissamine Yellow FF, Fluorescein, Eosine, Rhodamine WT, Rhodamine B, Sulphorhodamine B and Sulphorhodamine G. Mammalian tests indicate a low level of both acute and chronic toxicity. However, only three tracers could be demonstrated not to provide a carcinogenic or mutagenic hazard. These were Tinopal CBS-X, Fluorescein and Rhodamine WT. Rhodamine B is a known carcinogen and should not be used. In aquatic ecosystems, larval stages of shellfish and algae were the most sensitive. Persistent dye concentrations in tracer studies should not cause problems provided they are below 100 µg/l.*

## INTRODUCTION

Fluorescent dyes have been widely employed in the tracing of water because of their high detectability. There is, however, increasing concern on the effects of chemicals introduced into natural waters. This prompted a previous review of the toxicity of fluorescent dye tracers (Smart, 1982). This paper updates the earlier work and also incorporates material not previously included due to limitations on space. Information is provided on the 12 fluorescent dyes listed in Table 1. The chemical structures of these dyes are given in Figure 1. Dyes for which the exact structural formula is not known, such as CI Fluorescent Brightener 15, are not included. In order to update this data base, I would appreciate receiving details of any toxicological information which has not been included, or which has become available since publication.

Before examining the data, it is important to remember that differences in test protocols and methods, in test species, route of administration and dose, all make comparison of toxicity data from different studies difficult. Furthermore, the standard of toxicity tests has improved with time, and some of the earlier studies may follow protocols no longer acceptable in modern toxicology. A more difficult problem is that the toxicity of dyes may well vary with manufacturer, or even the batch of dye tested, due to the presence of impurities. In the case of biological stains, for instance, Marshall and Lewis (1974) have shown that many substances other than the stated dye can be present. This has been confirmed for several of the dyes discussed in this

Table 1. Colour index (3rd ed.) designations, dye type and bibliographical code used for the fluorescent dye tracers.

Name	CI Number	Dye Type	Code
Calcophor White ST			
CI Fluorescent Brightener	28	Stilbene derivative	FB28
Tinopal CBS-X			
CI Fluorescent Brightener	351	Sulphostyryl derivative	FB351
Amino G Acid	—	Dye intermediate	AGA
Diphenyl Brilliant Flavine 7GFF	DY 96	Stilbene derivative	DY96
Pyranine	CI 59040	Pyrene	P
Lissamine Yellow FF	CI 56205	Aminoketone	LYFF
Fluorescein Sodium	CI 45350	Xanthene	FL
Eosine Sodium	CI 45380	Xanthene	E
Rhodamine WT*	—	Xanthene	RWT
Rhodamine B	CI 45170	Xanthene	RB
Sulphorhodamine B	CI 45100	Xanthene	SRB
Sulphorhodamine G	CI 45220	Xanthene	SRG

\*Acid Red 388

paper by Luty (1978) and Nestman et al. (1979), who quote purities as low as 75%. The presence of impurities explains why some workers reported Rhodamine B to be mutagenic in the *Salmonella typhum*—mammalian microsome test, while the pure dye was demonstrated to be non-mutagenic (Nestman et al., 1979). There are also differences in the concentration of commercial dyestuffs due to incorporation of additives, which makes comparison of different products difficult. No attempt had been made in this review to deal

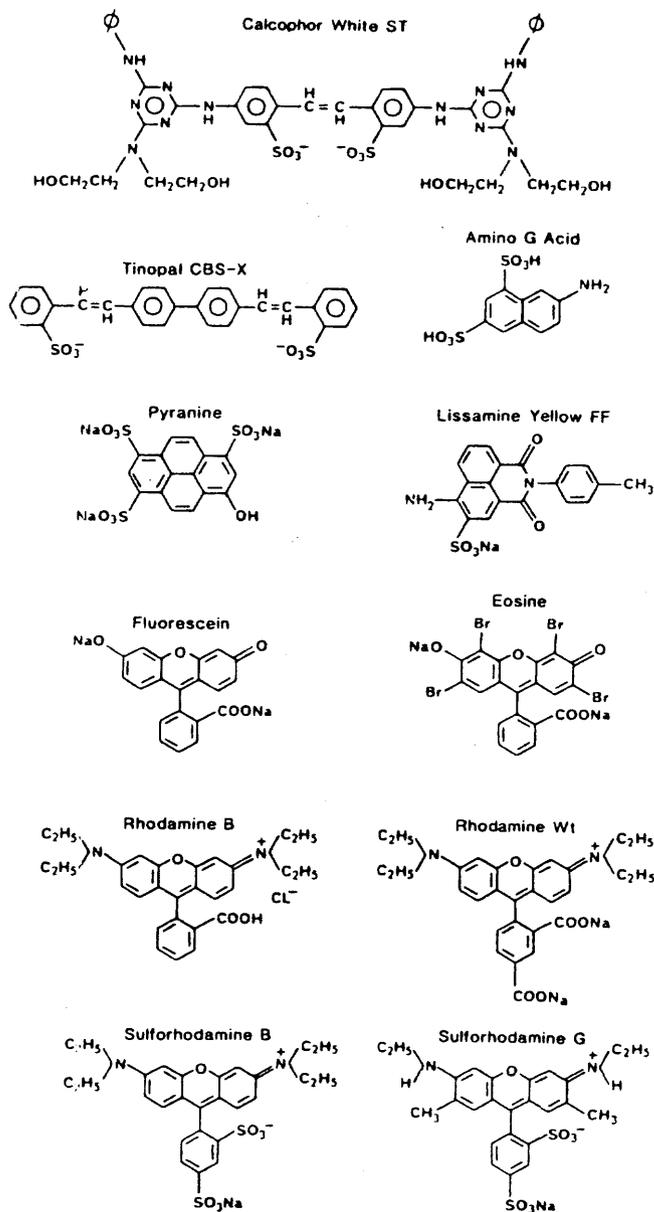


Figure 1.

with these problems, but referral back to the original source often clarify these points. There are clearly advantages in purchasing a product for which a hazard data sheet detailing toxicity information is available from the manufacturer.

The paper is divided into three sections, corresponding to the three main toxicological areas of concern in using tracer dyes. First, the toxicity of dyes in mammal systems is discussed in order to assess the acute and chronic effects of dye ingestion in man, and the possible hazards associated with handling the dyes. The second section reviews the data on carcinogenicity and mutagenicity, hazards which may be caused by long-term low-level exposure. Finally, the possible toxicity of tracers introduced into aquatic ecosystems is discussed. The toxicity criteria and measures included in the

Table 2. Toxicity of fluorescent tracer dyes in mammals.

Test	Animal	FB28	FB351	AGA	DY96	P	LYFF	FL	E	RWT	RB	SRB	SRG	Source
Acute Oral LD50 g/kg	Rat	14.5	5.58	> 15.0	> 15.0	> 15.0	8.56	6.72	> 1.0	> 25.0	> 0.5	> 10.0	> 10.0	16, 19, 23, 27, 28 43, 53, 106, 118. 15, 53, 94.
Acute Intravenous LD50 mg/kg	Mouse	> 10.0	> 5.0	—	—	—	—	4.74	—	—	0.89	—	—	—
No Effect Acute Intraperitoneal mg/kg	Mouse	—	—	—	—	1050	110	300	550	430	a	b	b	48, 66, 71.
No Effect Chronic Oral. Dose (%)	Rat	375	—	—	—	> 50	> 50	> 91	> 50	> 167	5	> 75	> 75	24, 25, 57, 67. 57, 94.
Time (Weeks)	Rat	400	> 1.0	> 0.5	—	—	—	500	250	—	< 95	—	—	68.
	Dog	106	12.9	—	—	—	—	—	18	—	0.1	—	—	—
	Hampster	> 1.0	—	—	—	—	—	< 0.75	—	18	—	—	—	—
No Effect on Reproduction or Teratogenicity Dose/Time	Chronic Rat	1%	0.1%	—	—	—	—	—	—	—	c	—	—	68, 93.
Irritation c	Rat	18m	3 gen	—	—	—	—	—	—	—	—	—	—	74, 96.
Photoactivity d	Eyes	m	c	—	—	—	—	—	—	—	—	—	—	16, 19, 27, 39, 43, 106.
	Skin	o	s	—	—	—	—	—	—	—	—	—	—	31, 32, 33, 43, 56 77, 78, 102, 107.
	Acute Rat	o	v	—	—	—	—	—	—	—	—	—	—	—

Notes: a. LD50 Less than RWT. b. LD50 Greater than RB. c. o = none, s = slight, m = moderate, se = severe, e = extreme.  
d. + = toxicity increased with light exposure. 0 = no effect. v = toxicity decreased with light exposure.  
e. 'Highest no effect level in prior two-year study.' Exact dose not quoted.

summary tables have been selected to permit wide comparisons of all the dyes. Where several sources of information are available, these have been checked for conformity and the most toxic result recorded. The tables provide a summary only, and reference should also be made to the original sources from which the information has been abstracted. These sources are coded according to the numbers given in the bibliography.

#### TOXICITY IN MAMMALS

Of the acute lethal data reported (Table 2), the LD50 (Lethal Dose—50) value for oral administration is the best general indication of dye toxicity. Although the maximum experimental values for Eosine and Rhodamine B are lower than desirable, the LD50 for all twelve dyes is very high. None of the dyes would be regarded as toxic using this criterion, the corresponding value for common salt being 8 to 10 g/kg. The intravenous and intraperitoneal administration routes provide a test of the most severe situation, where there is no barrier to movement of dyes from the gut into the body. There is again no indication of substantial toxicity, and Luty (1978) concluded that Pyranine, Lissamine Yellow FF, Eosine and Rhodamine WT, could be safely used for angiography in the human eye. Fluorescein is already widely used for this purpose.

Given the higher intraperitoneal toxicity of Rhodamine B compared to the other tracer dyes (Table 2), the more severe effects observed for this dye in both acute and chronic oral studies suggest that it is readily adsorbed in the gut. This is confirmed by metabolic studies (Table 3). The two optical brighteners, Fluorescein and Eosine are adsorbed to only a limited extent, but information is not available for the other tracer dyes. Eosine is discharged from the body via the bile duct, a pattern associated with its bromine substituents (Iga et al. 1971). All other dyes are cleared via the kidneys into the urine. Pathological examination of rats fed high dietary levels of Rhodamine B, showed enlargement of both the liver (where metabolism occurs) and the kidneys (Webb et al. 1961), but the metabolites were markedly less toxic. In man, Fluorescein is metabolised predominantly to the monoglucuronide (Sheng-Chin Chen et al. 1980), with the basic fluoran structure remaining intact, as is also the case in the metabolism of Rhodamine B and Eosine.

No teratogenic or other effects on reproduction were observed in multi-generation tests on Calcophor White ST, Tinopal CBS-X, Fluorescein and Rhodamine B. Rhodamine WT and Tinopal CBS-X are severe irritants to the eye and moderately irritating to the skin. Both Fluorescein and Eosine are more toxic when contact is combined with exposure to light. In the case of Eosine, this may be due to release of halogen atoms during photo-decomposition (Tonogai et al., 1978), but Takashi and Kobayashi (1977) have demonstrated the importance of the singlet oxygen formed on light excitation of both these dyes. Rhodamine B does not show evidence of enhanced phototoxicity. This has also been demonstrated by an extensive series of tests for the two fluorescent brighteners.

All personnel handling dyes should wear protective gloves and clothing. Excessive inhalation of dust should be avoided, or a face mask employed during repacking of bulk supplies. All skin areas inadvertently contaminated by dye should be washed immediately with soap and water. Any splashes in the eyes should be flushed with copious quantities of water.

Based on the experimental results reviewed above, there is no evidence of either a short or long term toxic hazard to dye users or those drinking water containing tracer dyes. Even those employing tracers routinely in their work would not be likely to ingest sufficient dye to cause concern. For Rhodamine B (the most toxic of the tracers), the long term oral feeding studies yielded 'safe' continuous ingestion levels of 0.75 mg per day (U.S. Department of Health Education and Welfare, 1966), equivalent to 370 µg/l for normal consumption of drinking water. Comparable levels of the other fluorescent tracer dyes would therefore be acceptable.

#### MUTAGENICITY/CARCINOGENICITY

Early experiments by Umeda (1956) demonstrated development of Sarcoma (cancer) in long-term experiments with rats fed 0.2% of Rhodamine B, Eosine and Fluorescein. However, these studies were found lacking with respect to numbers of animals surviving, provision of adequate control, and experimental duration, when reviewed by IARC (1977 and 1978).

Table 3. Uptake and excretion of the tracer dyes in mammals.

	FB28	FB351	P	LYFF	FL	E	RWT	RB	SRB	SRG	Source
Gut adsorption <sup>a</sup>	O	O	—	—	O	O	—	+	—	—	16, 26, 79, 113
Excretion <sup>b</sup>	—	—	U	U	U	B	U	U	U	U	48, 49, 57, 66 67, 98, 104, 105, 110, 112, 113, 114.
Metabolism <sup>c</sup>	—	O	O	M	M	O	O	M	—	M	11, 43, 66, 67, 112, 113

Notes: a. O = Less than 10% uptake      + = Greater than 10% uptake  
 b. U = In urine via kidneys            B = In faeces via bile  
 c. O = Not metabolised                M = Metabolised

Table 4. Carcinogenicity data for the tracer dyes

Test	FB28	FB351	AGA	DY96	P	LYFF	FL	E	RWT	RB	SRB	SRG	Source
Dominant Lethal Mutagenicity													
Test Rat and Mouse—No effect													
g/kg	1.0	1.5	—	—	—	—	—	—	—	—	—	—	4, 16, 53, 64, 80.
Carcinogenicity—rat													
	0/3	0/3	—	—	—	—	0/1	0/3	—	1/3	—	—	7, 13, 16, 33, 42, 46,
mouse	—	—	—	—	—	—	?/1	?/1	—	3/4	—	—	47, 68, 110, 115.

Key: O/n = noncarcinogenic; ?/n = possibly carcinogenic; x/n = carcinogenic in x tests out of n reported

Subsequent experiments (Anonn, 1981) have confirmed that Rhodamine B is carcinogenic. Results of more recent feeding studies, with mouse and rat (US National Toxicology Program) are not yet available for Fluorescein, while no further testing of Eosine has been undertaken.

Fluorescent whiteners have been extensively tested in mammals because of their widespread use and large production. Both Calcophor White ST and Tinopal CBS-X have been demonstrated to be non-carcinogenic in long-term feeding studies in rat, and in the Dominant Lethal Mutagenicity test in rat and mouse (Table 4). Information on carcinogenicity is not available for the remaining dyes, and an evaluation of their safety must be based on mutagenicity screening tests, which are rapid and less costly (Hoffman, 1982). These frequently employ micro-organisms or *in vitro* cultures of mammalian cells, although several *in vivo* tests are also available. There are strong correlations between the mutagenicity of chemicals in short-term tests and their carcinogenicity in mammals (Bartsch et al., 1980).

The three mutagenicity test results reported for Tinopal CBS-X are negative (Table 5), as would be expected from the carcinogenicity data discussed above. Amino G Acid and Lissamine Yellow FF have only been subject to a preliminary screening using the Ames Test (Kilbey, pers. comm., 1981). Amino G Acid was non-mutagenic, confirming the statement of Combes and Haveland-Smith (1982) that sulphonation of amino-naphthalenes generally renders them non-mutagenic. Lissamine Yellow FF yielded uncertain results.

Fluorescein has been extensively tested in a variety of micro-organism tests for mutation and DNA alteration (Table 5). The results of the DNA-cell binding (DCB) test suggested that this dye was a possible mutagen on metabolic activation (Kubinski et al., 1981), but the authors were unable to confirm this result using gel-electrophoresis. Yoshikawa et al. (1978) reported no inactivation of transforming DNA *in vitro* using *Bacillus subtilis*. Other systems were also negative for mutagenic activity, with the exception of the results using photoactivation reported by Nishioka (1976) for unspecified mixtures of xanthene dyes. Photoactivation was not demonstrated in the Rec-Assay or inactivation of transforming DNA in *Bacillus subtilis* (Yoshikawa

et al., 1978). Fluorescein has not been tested in mammalian systems for its effects on chromosomes, but the balance of the test results indicates it does not constitute a mutagenic hazard.

Eosine has also been widely tested and, unlike Fluorescein, demonstrates consistent photoactivation of mutagenicity in three different systems and four tests (Table 5). It is, however, uncertain to what extent these *in vivo* results can be applied to *in vitro* systems. No clastogenic effects were observed in tests using Chinese hamster ovary cells (Au and Hsu, 1979), nor were mutations induced *in vivo* in *Allium apa* or *Vicia faba* (Landa et al., 1965). Dye concentrations in the latter tests were, however, rather low, although a mutagenic effect was obtained for Rhodamine B.

Rhodamine WT has been the subject of extensive study by Douglas et al. (1983). Nestmann and Kowbel (1979, reported that the dye was mutagenic in the *Salmonella typhum*/mammalian microsome Ames test. However, in a battery of *in vitro* and *in vivo* tests in mammalian systems, Douglas et al. (1983) were only able to demonstrate a weak *in vitro* mutagenicity on using very high dye concentrations. No evidence of *in vivo* genetic activity was observed in terms of sperm abnormalities or bone marrow micro-nuclei in mice. They conclude that 'Rhodamine WT appears not to represent a major genotoxic hazard.'

Rhodamine B has been widely tested for mutagenicity as it is a known carcinogen. Out of twelve systems investigated, mutagenic activity was demonstrated in only five, including *in vivo* in *Drosophila melanogaster* (Clark, 1953)—although these experiments have been criticized by Lee et al. (1983). This demonstrates the limitations of mutagenicity tests and emphasizes the need for a wide suite of test results before deciding on the status of test materials. Furthermore, in the Ames test different results were reported for dye from a number of sources, with only two out of seven tests demonstrating mutagenic activity. Nestman et al. (1979) and Douglas et al. (1980) demonstrated that the mutagen was an impurity in the technical grade dye employed. This is an important finding because the concentration of impurities in commercial dyes may well vary with both manufacturer and dye batch. The advantages of utilising dyes from a manufacturer who has obtained relevant toxicity test information are thus clear.

Table 5. Mutagenicity data for the tracer dyes

Test	FB128	FB351	AGA	DY96	P	LYFF	FL	E	RWT	RB	SRB	SRG	Source
Rec-assay— <i>Bacillus subtilis</i>	—	—	—	—	—	—	0.3	1/2 <sup>b</sup>	—	1/1	0.1	—	52, 103, 109, 119
— <i>Escherichia coli</i>	—	—	—	—	—	—	0.1	0/1	—	0/1	—	—	51
Disc test— <i>Escherichia coli</i>	—	—	—	—	—	—	0	?	—	?	—	—	65
Pette mutations and gene conversions—Yeast	—	0/1	—	—	—	—	0/1	1/2 <sup>b</sup>	—	0/1	—	—	54, 56, 82, 84, 102
Ames test— <i>Salmonella typhimurium</i>	—	0/1	0/1	—	—	?	1/4 <sup>b</sup>	1/4 <sup>b</sup>	A1-1	2/6 <sup>a</sup>	?	1.2	26, 38, 55, 56, 75, 83, 85, 86, 87, 88, 92, 97, 101
Chromosome aberrations	—	—	—	—	—	—	—	—	—	—	—	—	—
—Chinese hamster cells	—	0/1	—	—	—	—	—	0.1	0/1	3/3	1/1	—	8, 25, 26, 52, 62
—Human bone cells	—	—	—	—	—	—	—	—	—	0/1	0/1	—	52, 97
Sister Chromatid exchange	—	—	—	—	—	—	—	—	—	—	—	—	—
—human or hamster cells	—	—	—	—	—	—	—	—	0/1	+1.2	0/1	—	25, 52, 92, 97
DCB Test for DNA alteration	—	—	—	—	—	—	A?/1	—	—	0/1	—	—	5, 92
Inactivation of transforming DNA	—	—	—	—	—	—	—	—	—	—	—	—	119
DNA damage Chinese hamster cells	—	—	—	—	—	—	0.1	1.1 <sup>b</sup>	—	—	—	—	—
Chromosome alterations in vivo in bone marrow—rat	—	—	—	—	—	—	0/1	—	—	0/1	0/1	—	25, 52, 92
—mouse	—	—	—	—	—	—	—	—	0/1	0/1	—	—	—
Mutations in vivo—silk worm	—	—	—	—	—	—	0/1	—	—	0/1	0/1	—	52

Key: 0/n = nonmutagenic; ?/n = possibly mutagenic; x/n = mutagenic in x tests out of n reported;  
 A = activation required  
 Notes: a. Mutagenic impurities present in some commercial products (14, 47)  
 b. Photoactivation required to produce mutagenicity (S, Y, AC, 7)

effect of Sulphorhodamine B in fetal rat hepatocytes, although this was significantly smaller than for halogen-bearing xanthene dyes. There is therefore some evidence from *in vitro* systems that Sulphorhodamine B is a mutagen, but this has not been demonstrated *in vivo*.

Table 6. Status of the tracer dyes with respect to carcinogenicity and mutagenicity.

Dye	Source	Status
FB28	Non-carcinogenic	No data
FB351	Non-carcinogenic	Non-mutagenic
AGA	No data	Probably non-mutagenic
LYFF	No data	Possibly mutagenic
FL	Non-Carcinogenic	Non-mutagenic
E	Uncertain	Possibly mutagenic
RWT	No data	Possibly non-mutagenic
RB	Carcinogenic	Possibly mutagenic
SRB	No data	Possibly mutagenic

No information on the mutagenicity of Direct Yellow 96, Pyranine or Sulphorhodamine G has been obtained.

The discussion above illustrates the difficulty of assessing the status of chemicals when a complex and differential genotoxic response is shown, as appears to be the general case with rhodamine dyes. This difficulty is enhanced when the products tested are of technical grade and may contain impurities. Table 6 summarises the findings of this review. Three dyes can be identified as causing minimal carcinogenic and mutagenic hazard; Tinopal CBS-X, Fluorescein, and Rhodamine WT. Conversely, Rhodamine B is known to be carcinogenic and possibly mutagenic and should not be used.

AQUATIC ORGANISMS

There is a considerable body of data on the toxicity of the tracer dyes to fish. Most comparative data is available for the 48 and 96 hour LC50 (Lethal Concentration—50) in rainbow trout (*Salmo gairdneri*) (Table 7). In these tests, Rhodamine B and Tinopal CBS-X were the most toxic tracers, a finding supported by studies with bluegill (*Lepomis macrochirus*) and channel catfish (*Ictalurus punctatus*). However, the LC50 values of these two tracers are still relatively high and they would not generally be considered toxic. Furthermore, dye concentrations of over 100 mg/l would only be achieved during the injection phase of a tracer test, and would be very unlikely to persist for several days. Data for Calcophor White ST and Pyranine is limited to a single species but, again, indicates low acute toxicity.

The only long-term fish exposure experiments are those of Benoit-Guyod et al. (1979) using guppy (*Lebistes reticulatus*) and are particularly useful as they relate to the most widely used tracers. After thirty days, there was some evidence of continuing mortality with additional exposure, but the TLM values at this time are the most useful guide to long-term toxicity in fish. Rhodamine B, Eosine and the two

Sulphorhodamine B has been tested in six systems. It shows no *in vivo* chromosome alteration in rat or *in vitro* in human bone-marrow cells, findings similar to those for Rhodamine B. However, unlike Rhodamine WT, it does cause chromosome aberrations *in vitro* in Chinese hamster cells. Sako et al. (1980) have also demonstrated a cytotoxic

Sulphorhodamine dyes are significantly more toxic than Rhodamine WT or Fluorescein. However, the TLM values, even for the more toxic tracers, are still three orders of magnitude greater than the visible dye concentration and five orders of magnitude in excess of those commonly expected in long-term tracer experiments. Indeed, as pointed out by Abram and Rhodes (1978), aesthetic considerations relating to the visible colouration of natural waters are more likely to limit tracer concentrations than dye toxicity. These considerations, however, would not apply to the blue fluorescent tracers which are colourless in solution.

Data are also presented in Table 7 for three aquatic invertebrates. *Asellus aquaticus* (the water hog louse) is generally considered a robust organism, while *Daphnia magna* (water flea) is more sensitive, and has been widely employed in bioassay work. The 72 hour LC50 values for *Daphnia* are generally lower than those for fish (as is also the case for *Artemia salina*). However, the egg and larval stages of organisms are even more sensitive as demonstrated by the limited data for a number of shellfish (Table 7). Concentrations of 1 to 10 mg/l of Rhodamine WT, Rhodamine B and Fluorescein (depending on the test organism) do not affect development or cause mortality in shellfish eggs and larvae after forty-eight hours' exposure. Whilst further data are needed for the other tracer dyes in these sensitive systems, it can be concluded that dye concentrations as high as 1 mg/l can be tolerated for two days without damage to aquatic organisms.

Several studies have been conducted to examine the degree of uptake and elimination of Rhodamine B by shellfish. Waugh and Key (1967) and Geckler and Wandstrat (1964) found that no staining occurred at concentrations of 0.02 mg/l in European flat oysters (*Ostrea edulis L*) and quahog clams (*Mercenaria mercenaria*) respectively. Even at much higher initial concentrations, the dye was eliminated within twenty-four hours on transfer to clean water, although Galassi and Canzonier (1976) demonstrated retention for up to three days in the blue mussel (*Mytilus edulis galloprovincialis*). Comparable data is not available for the other tracers, although Feron and Hitz (1975) and Ganz et al. (1975) found that Tinopal CBS-X was not significantly accumulated and was rapidly expelled from bluegill (*Lepomis macrochirus*), even after prolonged exposure at 0.1 mg/l. Smart and Smith (in prep.) observed that the strong staining of trout (*Salmo gairdneri*) exposed to 500 mg/l of Rhodamine B declined on transfer to clean water.

The bioaccumulation tendency of dyestuff can be indicated from the partition coefficient in n-octanol/water (Anliker et al., 1981). This coefficient has been determined for several tracers by Benoit-Guyod (1979) (Table 8). As expected, the high water solubility and anionic character considered desirable in a tracer, give low partition coefficients. Also shown in Table 8 is a rating for protein binding, which is derived from *in vitro* experiments (Lutty, 1978; Lutty,

Table 7. Toxicity of tracer dyes in aquatic organisms.

	FB128	FB351	AGA	DY96	P	LYFF	FL	E	RWT	RB	SRB	SRG	Source
<b>Fish</b>													
TL50 mg/l—	—	—	—	—	—	—	752	138	1360	24	116-58	88	12.
LC50 mg/l—	—	130	>1000	—	—	>1000	1372	>100	>320	155	—	—	9, 53, 70.
96 hr. <i>Salmo gairdneri</i>	—	—	>1000	>1000	—	>1000	3420	>100	>320	506	450	—	100, 19, 70.
48 hr. <i>Salmo gairdneri</i>	—	—	—	—	—	—	3433	—	—	379	—	—	100, 107, 16.
96 hr. <i>Lepomis macrochirus</i>	>1000	241	—	—	—	—	2267	—	—	526	—	—	70, 53, 70.
96 hr. <i>Ictalurus punctatus</i>	—	126	—	—	—	—	3000	1800 <sup>a</sup>	—	—	>3000	—	107, 108.
48 hr. <i>Oryzias latipes</i>	—	—	>3000	—	—	—	—	—	—	—	—	—	—
Maximum Non-48 hr. <i>Leuciscus idua</i>	—	—	—	—	>500	>500	—	—	—	—	—	>500	11, 43.
lethal Con-96 hr. <i>Idua melanthes</i>	—	—	—	—	—	—	—	—	—	—	—	—	11, 43.
centration mg/l													
<b>Aquatic Invertebrates</b>													
LC50 mg/l	—	—	>3000	—	—	>1000	—	—	>2000	550	—	—	100, 117.
96 hr. <i>Asellus aquaticus</i>	—	—	—	—	—	—	100-300	—	—	180	—	—	89.
24 hr. <i>Artemia salina</i>	—	—	386 <sup>c</sup>	—	—	>1000 <sup>b</sup>	165	90	170	29	139	88	12, 100.
72 hr. <i>Daphnia magna</i>	—	—	—	—	—	—	—	—	—	—	—	—	—
<b>Shell Fish</b>													
No effect	—	—	—	—	—	—	—	—	—	1.0	—	—	90.
48 hr. <i>Crassostrea virginica</i>	—	—	—	—	—	—	—	—	10.0	—	—	—	91.
Development of	—	—	—	—	—	—	10.0	—	—	10.0	—	—	89.
48 hr. <i>Hemienterolus pulcherrimus</i>	—	—	—	—	—	—	1.0	—	—	3.2	—	—	89.
eggs mg/l	—	—	—	—	—	—	—	—	—	—	—	—	—
LC50 mg/l	—	—	—	—	—	—	10 < > 1.0	—	—	>500	—	—	18
96 hr. <i>Corbicula manilensis</i>	—	—	—	—	—	—	—	—	—	10 < > 1.0	—	—	76.
120 hr. <i>Australorbis glabratus</i>	—	—	—	—	—	—	—	—	—	—	—	—	—

<sup>a</sup> a. 1200 when bromine released during photodecomposition. <sup>b</sup> 36 hr. <sup>c</sup> hr.

1979; Tonogai et al., 1979b; Tatsuji et al., 1971; Gangolli et al., 1972). These results relate well to the partition coefficients, although Eosine appears to be bound to a greater extent than Fluorescein, despite its somewhat smaller partition coefficient. They also indicate that Lissamine Yellow FF has some potential for bioaccumulation. However, even in the case of Rhodamine B, the most lipophilic of the dyes tested, the bioaccumulation factor would be below 100 and further testing *in vivo* would not be considered necessary.

**Table 8. Partition coefficients (n-octanol/water) and protein binding of the tracer dyes.**

	Partition Coefficient	Protein binding <sup>a</sup>
P	—	0
LYFF	—	+
FL	$4.1 \times 10^{-1}$	$\pm$
E	$4.7 \times 10^{-2}$	$\pm$
RWT	$4.7 \times 10^{-2}$	$\pm$
RB	$1.9 \times 10^{+2}$	+
SRB	$6.2 \times 10^{-3}$	0
SRG	$9.5 \times 10^{-3}$	0

Notes: a + = Protein bound.  $\pm$  = Weakly bound. 0 = Not protein bound.

**Table 9. Effect of tracer dyes on algae (dye concentration 10 mg/l, exposure period 7 days).**

	FB2B	FL	E	RB	Source
<i>Selanastrum capricornutum</i>	++	—	—	+0	63
<i>Chlorella sp.</i>	—	++	++	++	72
<i>Scenedesmus sp.</i>	—	0	0	+	72
<i>Chlorococcum sp.</i>	—	0	+0	+	72
<i>Nostoc sp.</i>	—	0	0	+0	72
<i>Anabaena sp.</i>	—	0	++	+0	72
<i>Oscillatoria sp.</i>	—	0	0	+0	72

Key: ++ = No growth retardation  
+ = Some growth retardation  
+0 = Severe growth retardation  
0 = No growth

In practice, the empirical studies of the elimination of Rhodamine B from shellfish and trout discussed above indicate that there is little possibility for bioaccumulation of this dye once environmental concentrations fall on completion of a tracer test.

Only two studies have reported the effects of tracer dyes on algal growth (Table 9). Little and Chillingworth (1974) showed that whilst Calcophor White ST caused no growth inhibition in *Selanastrum capricornutum* over a fourteen-day exposure period, Rhodamine B caused severe growth retardation at 10 mg/l. There was no effect for Rhodamine B at 1 mg/l. In an earlier study, Mason-Williams (1969) showed a differential response to dye exposure for a number of species found in Welsh streams. Surprisingly, Rhodamine B was less toxic overall to algae than Eosine, which was marginally less toxic than Fluorescein, the reverse of the order in higher organisms. Algal growth appears to be affected at similar concentrations by tracer dyes as the sensitive larval stages of shellfish. This reinforces the conclusion drawn

above that enduring tracer concentrations as high as 1 mg/l would not be detrimental to aquatic ecosystems.

The toxicity of dyes to bacteria has been investigated primarily to determine the possible suppression of bacterial decomposition in sewage works. At present, only limited data is available, but following the adoption of a test protocol by ETAD (Brown et al., 1981), more comparable data should become available. No data is available for Lissamine Yellow FF or Rhodamine WT. However, as only the basic dye Rhodamine B appears to affect aerobic decomposition (Table 10), a finding supported by the wider survey of Hunter (1974) for other acid and basic dyestuffs, no detrimental effects would be expected for these dyes. The limited data for *Salmonella typhum* employed in the Ames test protocol suggest that there are no large differences in the toxicity of the various tracers to bacteria. It is not possible to convert the plate concentrations to environmental levels, but overall the data do suggest that bacteria are less sensitive to the tracer dyes than either algae or shellfish eggs and larvae.

In conclusion, there is no evidence of significant bioaccumulation for any of the tracer dyes in fish. The most sensitive aquatic organisms to the dyes are the developmental stages of shellfish, and algae. These, therefore, determine the maximum prolonged dye concentration which can be recommended. This limit is set at 1 mg/l, well above the persistent dye concentrations commonly used in tracer tests, and at least one order of magnitude above the visible threshold. There is no evidence that short-term exposure to concentrations in excess of 1 mg/l, such as could occur transiently at injection sites, are harmful, but prior dilution should be employed if rapid dispersion and dilution of the tracer dye is not expected.

## DISCUSSION

Before discussing the toxicity information described above, attention must be drawn to the possibility that chemical transformation of the tracer dyes may occur after release, producing compounds which are intrinsically more toxic.

Information on the toxicity of photo-decomposition products of tracer dyes has been reviewed above, with Eosine appearing to exhibit both photo-activation of mutagenic activity and phototoxicity due to release of bromine atoms. Smart and Smith (in prep.) showed in acute tests using *Asellus aquaticus* that for Lissamine Yellow FF the photodegradation products were similar in toxicity to the parent dye; but for Amino G Acid, they were significantly less toxic. In other tests, photo-decomposition products may well have been present and are therefore incorporated into toxicity figures in a non-systematic manner. The eventual photo-decomposition products of Fluorescein (and, by analogy with metabolism, of rhodamine dyes) are phthalic acid and resorcinol (Ishibashi 1965). The latter was found to be non-mutagenic when test data were reviewed by Hed-

**Table 10. Effect of tracer dyes on bacterial decomposition (dye concentration 100 mg/l, exposure period 3 hours), and in *Salmonella typhum* (Ames test).**

	FB28	FB351	DY96	P	FL	E	RWT	RB	SRB	SRG	Source
Activated sludge <sup>a</sup> (Aerobic)	+ <sup>b</sup>	+	+	+	+	+	+	0	+	+ <sup>c</sup>	11, 14, 16, 19, 29, 43, 45, 72
<i>Salmonella typhum</i> <sup>d</sup>	—	>2	—	—	10	>1	21	>1	—	—	56, 83, 86, 87

Notes: a. Upper line = effect on bacterial decomposition (+ no effect, 0 significant effect)

Lower line = Biodegradation of dye (+ biodegraded, 0 not biodegraded)

b. Aerobic and anaerobic decomposition.

c. Anaerobic decomposition only.

d. Concentration of dye retarding growth (mg / plate).

dle et al. (1983). Therefore, photo-decomposition product toxicity appears only to be a problem for Eosine.

A particular hazard has been identified by Abidi (1982) due to the production of diethylnitrosamine (DNA) from the reaction of nitrites with the diethylamino moieties present in all rhodamine dyes. DNA is a potent animal carcinogen (Magee and Barnes, 1967). Abidi (1982) demonstrated the production of DNA in both natural and distilled water systems with nitrite levels in excess of 11 µg/l and Rhodamine B with WT concentrations greater than 1 µg/l. It is, however, difficult to reconcile the high reaction yields (up to 96%) quoted in the laboratory experiments, with the known long-term persistence of rhodamine dyes in tracer tests. The DNA formed in the laboratory experiments was found to photo-decompose relatively slowly under ultraviolet irradiation. In the presence of ascorbic acid nitrosation was inhibited. Thus the observed persistence of anionic rhodamine dyes may be related to competing reactions which retard nitrosation of the dye, reducing the DNA hazard correspondingly. Determination of DNA levels during field trials with rhodamine dye tracers is urgently needed.

The third problem associated with the chemical transformation of tracer dyes is related to the production of chlorophenol compounds on chlorination of water for domestic supply. No experimental work has been conducted on this problem, although taste experiments with Rhodamine B performed by Wilson (1968) suggest that this reaction does occur. This confirms the statement of Murphy et al. (1975) that ring structures with electron-activating substituents (primarily OH and amino groups) are likely to be chlorinated. Further work is needed on this problem.

The acute and chronic toxicity of all the tracer dyes in mammal systems is sufficiently low that no danger should result in their use, providing normal precautions are observed during dye handling. However, only three tracers can be demonstrated to cause minimal carcinogenic and mutagenic hazard, Tinopal CBS-X, Fluorescein and Rhodamine WT. Conversely, Rhodamine B is known to be carcinogenic and should not be used. The status of the remaining tracers is uncertain and they should be used only after careful consideration of the probable exposure risk, both to tracing personnel and to the wider population. In aquatic ecosystems, bioaccumulation is not a problem with any of the dyestuffs.

There is no acute hazard associated with short exposures to very high dye concentrations, such as can occur on injection. For longer exposures, the development stages of shellfish and algae appear to be more sensitive than fish. Even in these systems, a concentration of 1 mg/l for 48 hours can be endured. It should not be a problem to keep persistent dye concentrations well below 100 µg/l, particularly as this is well above the visible threshold where aesthetic considerations may become dominant.

Further information is needed both on the mutagenicity of tracer dyes, and on the effects of long-term exposure in sensitive larval stages of aquatic organisms. There is also a need to assess the toxicological significance of compounds formed in the environment by reactions with dyestuffs.

## REFERENCES

- Abidi, S. L. (1982)—Detection of diethylnitrosamine in nitrite-rich water following treatment with rhodamine flow tracers: *Water Res.* 16:199-204. (RWT, RB). (1)
- Abram, F. S. H. and Rhodes, S. (1978)—The toxicity to fish of dyestuffs used in the manufacture of paper: Water Research Centre Tech. Rept. ER654. (2)
- Akamatsu, K. and Matsuo, M. (1973)—Safety of optical whitening agents. *Senryo to Yakuhin* 18(2):2-11. (English translation available from British Library, Boston Spa, Yorkshire, England. Translation RTS 9415, June 1975, p.18). (FB28). (3)
- American Cyanamid Co. Ltd. *Product Data Sheets.* (FB28). (4)
- Anliker, R., Clarke, E. A. and Moser, P. (1981)—Use of the partition coefficient as an indicator of bioaccumulation tendency of dyestuffs in fish: *Chemosphere* 10:263-274. (5)
- Annon (1950)—Summary tables of biological tests: *Chemical Biological Coordination Center Review* 2:1-77. (RB). (6)
- Annon (1981)—Increased incidence of thyroid neoplasms reported in D and C Red 19 tests: *Food Chemical News* Oct. 5, 1981:44-45. (RB). (7)
- Au, W. and Hsu, T. C. (1979)—Studies of the clastogenic effects of biological stains and dyes: *Env. Mutagen* 1:27-35. (E, RB). (8)
- Bandt, H. J. (1957)—Giftig oder ungiftig für Fische: *Deutsche, Fischerei Zeit* 4:170-171. (FL, E). (9)
- Bartsch, H., Malaveille, C., Camus, A. M., Martel-Planche, G., Brun, G., Hautefeuille, A., Sabadie, N., Barbin, A., Kuroki, T., Drevon, C., Piccoli, C., Montesano, R. (1980)—Validation and comparative studies on 180 chemicals with *S. typhum* stains and V79 Chinese hamster cells in the presence of various metabolising systems: *Mutat. Res.* 76:1-50. (10)
- Bayer (1981)—*Product Data Sheet.* (P). (11)
- Benoit-Guyod, J. L., Rochat, J., Alary, K., Andre, C. and Taillandier, G. (1979)—Corrélations entre propriétés physico chimiques et écotoxicité de

- tracers fluorescents xanthéniques: *Toxicol. Eu. Res.* 11:241-246. (FL, E, RWT, RB, SRB, SRG). (12)
- Bonser, G. M., Clayson, D. B. and Jull, J. W. (1956)—The induction of tumours of the subcutaneous tissues, liver and intestine in the mouse by certain dye-stuffs and their intermediates: *Brit. Jour. Cancer* 10:653-667. (RB). (13)
- Brown, D., Hitz, H. R. and Schafer, L. (1981)—The assessment of the possible inhibiting effect of dyestuffs on aerobic waste-water bacteria experience with a screening test: *Chemosphere* 10:245-261 (DY96, SRB). (14)
- Brown, J. P., Dietrich, P. S. and Baker, C. M. (1979)—Mutagenicity testing of some drug and cosmetic dye lakes with the Salmonella/mammalian Microsome assay: *Mutat. Res.* 66:181-185. (E, RB). (15)
- Burg, A. W., Rohovsky, M. W. and Kensler, C. J. (1977)—Current status of human safety and environmental aspects of fluorescent whitening agents used in detergents in the United States: *CRC Crit. Rev. Env. Control* 7:91-120. (FB28, FB351). (16)
- Burnett, C. M., Agersborg, H. P. K., Barzelleca, J. F., Eagle, E., Ebert, A. G., Pierce, E. C., Kirschman, J. C. and Scala, R. A. (1974)—Teratogenic studies with certified colours in rats and rabbits. (Abstract): *Toxicol. Appl. Pharmacol.* 29:121. (RB). (17)
- Chandler, J. H. and Marking, L. L. (1979)—Toxicity of fishery chemicals to the Asiatic clam, *Corbicula manilensis*: *Prog. Fish Cult.* 41:148-151. (RB). (18)
- CIBA-GEIGY, U. K. Ltd. (Pers. comm.) (DY96, SRB). (19)
- Clark, A. M. (1953)—Mutagenic activity of dyes in *Drosophila melanogaster*: *Am. Nat.* LXXXVII 295-305. (RB). (20)
- Combes, R. D. and Haveland-Smith, R. B. (1982)—A review of the genotoxicity of food, drug and cosmetic colours and other azo triphenylmethane and xanthene dyes: *Mutat. Res.* 98:101-248. (FL, E, RWT, RB). (21)
- De Marco, T. J. and Sokolof, M. Y. (1973)—Effect of sodium fluorescein on the mucosa of the hamster: *Jour. Periodontol* 44:640-644. (FL). (22)
- DeWitt, J. B., Bellack, E., Klingensmith, C. W., Ward, J. C. and Treichler, R. (1953)—Relationship between chemical structure and toxic action on rats: *Chemical-Biological Coordination Center Review* 5. (RB). (23)
- Dougherty, T. J. (1968)—Activated dyes as anti-tumour agents: *Journ. Nat. Cancer Inst.* 52:1333-1336. (FL). (24)
- Douglas, G. R., Grant, C. E., Bell, R. D. L., Salamone, M. F., Heddle, J. A. and Nestman, E. R. (1983)—Comparative mammalian *in vitro* and *in vivo* studies on the mutagenic activity of Rhodamine WT: *Mutat. Res.* 118:117-125. (RWT). (25)
- Douglas, G. R., Grant, C. E., Bell, R. D. L., Wytmsa, J. M., Nestman, E. R. and Kowbel, D. J. (1980)—Effect of impurities and metabolic activation on the mutagenic activity of Rhodamine B in bacterial and mammalian cells: *Env. Mutagen.* 2:289-290. (Abstract). (RB). (26)
- DuPont de Nemours and Co. (pers. comm.) and Compton and Knowles Tertre S. A. Product Data Sheet. (RWT). (27)
- Emerson, G. A. and Anderson, H. H. (1934)—Toxicity of certain proposed anti-leprosy dyes: Fluorescein, eosin, erythrosin and others: *Int. Jour. Leprosy* 2:257-263. (FL, E). (28)
- Etzel, J. E. and Grady, C. P. L. (1973)—Effects of dyes on an anaerobic system: Ch. VII IN *Dyes and the Environment* Vol. 1. American Dye Manufacturers Inst. Inc. (FB28). (29)
- Feron, J. P. and Hitz, H. R. (1975)—Ch. V1/3, Measurement of the uptake of a <sup>14</sup>C labelled fluorescent whitening agent by fish from water and through a model food chain. IN Anliker, R. and Müller, G. (eds.) *Fluorescent Whitening Agents* Thieme, Stuttgart, 157-164. (FB351). (30)
- Fondren, J. E. and Heitz, J. R. (1978)—Xanthene dye induced toxicity in the adult face fly *Musca autumnalis*: *Env. Entomol.* 7:843-846. (FL, E, RB). (31)
- Fondren, J. E. and Heitz, J. R. (1979)—Dye-sensitized house fly toxicity produced as a function of variable light sources: *Env. Entomol.* 8:432-435 (FL, E, RB). (32)
- Forbes, P. D. and Urbach, F. (1975)—Experimental modification of photocarcinogenesis.
- I: Fluorescent whitening agents short-wave UVR.  
 II: Fluorescent whitening agents and simulated solar UVR.  
 III: Simulation of exposure to sunlight and fluorescent whitening agents:
- Fd. Cosmet. Toxicol.* 13:335-337, 339-342, 343-345. (FB351). (33)
- Galassi, S. and Canzonier, W. J. (1976)—Uptake and elimination of Rhodamine B by mussels: *L'Igiene Moderna* 69:112-118. (RB). (34)
- Gangolli, S. D., Grasso, P., Golberg, L. and Hoosen, J. (1972)—Protein binding by food colourings in relation to the production of subcutaneous sarcoma: *Fd. Cosmet. Toxicol.* 10:449-462. (E, RB). (35)
- Ganz, C. R., Schulze, J., Stenby, P. S., Lyman, F. L. and Macek, K. (1975)—Accumulation and elimination studies of four detergent fluorescent whitening agents in Bluegill (*Lepomis macrochirus*): *Environ. Sci. Technol.* 9(8), 738-744. (FB351). (36)
- Geckler, J. R. and Wandstrat, T. A. (1964)—Uptake and retention of Rhodamine B by Quahog clams, *Merienaria mercenaria*: *Chesapeake Science* 5:134-137. (RB). (37)
- Green, M. R. and Pastewka, J. V. (1980)—Mutagenicity of some lipsticks and their dyes: *Jour. Nat. Cancer Inst.* 64:665-669. (38)
- Griffijj, J. F. (1973)—Fluorescent whitening agents—tests for skin sensitizing potential: *Arch. Dermatol.* 107:728-733. (39)
- Hansen, W. H., Fitzhugh, O. G. and Williams, M. W. (1958)—Sub-acute oral toxicity of nine D and C coal-tar colors: *Jour. Pharmacol. Exp. Ther.* 122:29A (Abstract). (E, RB). (40)
- Heddle, J. A., Hite, M., Kirkhart, B., Mavournin, K., MacGregor, J. T., Newell, G. W. and Salomere, M. F. (1983)—Induction of micronuclei as a measure of genotoxicity—a report of the US EPA Gene-Tox Program: *Mutat. Res.* 123:61-118. (41)
- Hillier, F. K. (1962)—Prüfung des Tetrabromfluoresceins (Eosin-säure) auf Verträglichkeit und Cancerogenität: *Arzneimittel. Forsch.* 12:587-588. (E). (42)
- Hoechst U. K. Ltd. *Product Data sheets.* (LYFF, SRB, SRG). (43)
- Hoffman, G. R. (1982)—Mutagenicity testing in environmental toxicology: *Environ. Sci. Technol.* 16:560A-574A. (44)
- Hunter, J. V. (1973)—Effect of dyes on aerobic systems: Ch. VI IN *Dyes and the Environment*, Vol. 1. American Dye Manufacturers Inst. Inc. (FB28). (45)
- IARC (1977)—Eosin and Eosin Disodium salt: *IARC Monograph* 15:183-193. (E). (46)
- IARC (1978)—Rhodamine B: *IARC Monograph*, 16:221-230. (RB). (47)
- Iga, T., Awazu, S. and Negami, H. (1971)—Pharmokinetic behaviour of biliary excretion. III. Comparison of excretion behaviour in xanthene dyes, fluorescein and bromosulphthalein: *Chem. Pharm. Bull.* 19:297-308. (FL, E). (48)
- Iga, T., Awazu, S. and Negami, H. (1972)—Pharmokinetic studies of biliary excretion. V. The relationship between the biliary excretion behavior and the elimination from plasma of xanthene dyes and bromosulphthalein in rat: *Chem. Pharm. Bull.* 20:349-356. (FL, E, RB). (49)
- Ishibashi, T. (1965)—Hygienic studies on the fading and the decomposed products of coal-tar dyes used for food: *Jap. Jour. Pub. Health* 12:613-627. (FL). (50)
- Kada, T., Tutikawa, K. and Sadaie, Y. (1972)—*In vitro* and host mediated 'rec-Assay' procedures for screening chemical mutagens, and phloxine, a mutagenic red dye detected: *Mutat. Res.* 16:165-174. (E, RB, SRB). (51)
- Kawachi, T., Yahagi, T., Kada, T., Tazima, Y., Ishidate, M., Sasaki, M. and Sugiyama, T. (1980)—Cooperative programme on short-term assays for carcinogenicity in Japan: *IARC Sci. Pub.* 27:323-330. (FL, RB, SRB). (52)
- Keplinger, M. L., Fancher, O. E., Lyman, F. L. and Calandra, J. C. (1974)—Toxicological studies of four fluorescent whitening agents: *Toxicol. Applied Pharmacol.* 27:494-506. (FB351). (53)
- Kilbey, B. J. (1977)—A review of genetic studies with fluorescent whitening agents using bacteria, fungi and mammals: *Mutat. Res.* 39:177-188. (FB28, FB351). (54)

- Kilbey, B. J. (1981)—*personal communication*. (AGA, LYFF, RWT, SRB). (55)
- Kilbey, B. J. and Zetterberg, L. G. (1975)—Mutagenicity assays on fluorescent whitening agents using micro-organisms: Ch. VII/11 IN Anliker, R. and Müller, G. *Fluorescent Whitening Agents*, Thieme, Stuttgart, 246-277. (FB351). (56)
- Klassen, C. D. (1973)—Comparison of the toxicity of chemicals in newborn rats to bile duct—ligated and sham-operated rats and mice: *Toxicol. Appl. Pharmacol.* 24:37-44. (FL). (57)
- Kobayashi, N., Taniguchi, N., Sako, F. and Takakuwa, E. (1977)—A screening method for the toxicity of food dyes using *Artemia Salina* larvae: *Jour. Toxicol. Sci.* 2:283-390. (58)
- Kubinski, H., Gutzke, G. E. and Kubinski, Z. O. (1981)—DNA-cell-binding (DCB) assay for suspected carcinogens and mutagens: *Mutat. Res.* 89:95-136. (FL). (59)
- Landa, Z., Klouda, P. and Pleskotova, D. (1965)—The mutagenic effects of fluorochromes: IN Veleminsky, J. and Gichner, T. (eds.) *Induction of Mutations and the Mutation Process. Proc. Symp., Prague 1963*. Czechoslovak Acad. Sci., Prague, 115-122. (E, RB). (60)
- Lee, W. R., Abrahamson, S., Valencai, R., von Halle, E. S., Wurgler, F. E. and Zimmering, S. (1983)—The sex-linked recessive lethal test for mutagenesis in *Drosophila melanogaster*: *Mutat. Res.* 123:183-279. (RB). (61)
- Lewis, I. L., Patterson, R. M. and McBay, H. C. (1981)—The effects of Rhodamine B on the chromosomes of *Muntiacus muntiac*: *Mutat. Res.* 88:211-216. (RB). (62)
- Little, L. W. and Chillingworth, M. A. (1974)—Effects of 56 selected dyes on growth of the green alga *Selenastrum capricornutum*. Ch. II IN *Dyes and the Environment*, V.II. American Dye Manufacturers Institute Inc. 5-14. (FB28, RB). (63)
- Lorke, D. and Machefer, L. (1975)—Testing mutagenic properties with the dominant lethal test on the male mouse. Ch. VII/9 IN Anliker, R. and Müller, G. (eds.), *Fluorescent Whitening Agents*, Thieme, Stuttgart, 239-246. (FB28, FB351). (64)
- Lück, van H., Wallnöfer, P. and Bach, H. (1963)—Lebensmittelzusatzstoffe und mutagene Wirkung VII Mitteilung Prüfungseiner Xanthen-Farbstoffe auf mutagene Wirkung on *Eschenchia coli*: *Path. Microbiol.* 26:206-224. (65)
- Lutty, G. A. (1978)—The acute intravenous toxicity of biological stains, dyes and other fluorescent substances: *Toxicol. Appl. Pharmacol.* 44:225-249. (LYFF, P, E, RWT). (66)
- Lutty, G. A. (1979)—An intraperitoneal survey of biological stains, dyes, and other fluorescent substances. *Bull. Nippon Kankoh-Shikiso Kenkyusho*, 50:26-50. (LYFF, P, FL, E, RWT, RB, SRB, SRG). (67)
- Lyman, F. L., Schulze, J., Ganz, C. R., Stensby, P. S., Keplinger, M. L. and Calandra, J. C. (1975)—Long-term toxicity of four fluorescent whitening agents: *Fd. Cosmet. Toxicol.* 13:521-527. (FB351). (68)
- Magee, P. N. and Barnes, J. M. (1967)—Carcinogenic nitroso compounds: *Advances in Cancer Res.* 10:163-246. (69)
- Marking, L. L. (1969)—Toxicity of Rhodamine B and Fluorescein Sodium to fish and their compatibility with Antimycin A: *Prog. Fish-Culturist* 31:139-142. (FL, RB). (70)
- Marshall, P. N. and Lewis, S. M. (1974)—Batch variations in commercial dyes employed in Romanowsky-type stainings: a thin-layer chromatographic study: *Stain Technol.* 49:351-358. (E). (71)
- Mason-Williams, A. (1969)—A note on the effects of tracer dyes on microbial populations of streams: *Proc. 4th Int. Cong. Speleol., Ljubljana*, V.4-5: 167-172. (FL, E, RB). (72)
- McDonald, T., Kaster, K., Hervey, R., Gregg, S., Robb, C. A. and Bergman, A. R. (1974)—Acute and sub-acute toxicity evaluation of intravenous fluorescein in mice, rats and dogs. *Toxicol. Appl. Pharmacol.* 29:97-98. (Abstract). (FL). (73)
- McEnerney, J. K., Wong, W. P. and Peyman, G. A. (1977)—Evaluation of the teratogenicity of fluorescein sodium: *Am. Jour. Ophthalmol.* 84:847-850. (FL). (74)
- McGreggor, D. B. and Ainsworth, L. (1976)—Lack of mutagenic activity in *Salmonella typhum* of four optical brighteners: *Mutat. Res.* 40:169-172. (FB351). (75)
- Michelson, E. H. (1964)—Shell fluorescence in *Austialorbis glabratus* and other aquatic snails exposed to tetracyclines: *Jour. Parasitol.* 50:743-747. (FL). (76)
- Miyata, M. and Tanaka, K. (1972)—Toxicity of fluorescein: *Nippon Ganka Gakkai Zasshi* 76:1601-1607. (FL). (77)
- Morikawa, F., Fukuda, M., Naganuma, M. and Nakayama, Y. (1976)—Phototoxic reaction to xanthene dyes induced by visible light: *Jour. Dermatol.* 3:59-67. (E). (78)
- Muecke, W., Dupuis, G. and Esser, H. O. (1975)—Metabolic behaviour of water-soluble fluorescent whitening agents in the rat and bean plant: CH. VII/5 IN Anliker, R. and Müller, G. (eds.) *Fluorescent Whitening Agents*, Thieme, Stuttgart, 179-179. (FB351). (79)
- Müller, D., Fritz, H., Langauer, M. and Strasser, F. F. (1975)—Nucleus anomaly test and chromosomal analysis of bone marrow cells of the Chinese hamster and dominant lethal test in male mice after treatment with fluorescent whitening agents: CH.VII/10 IN Anliker, R. and Müller, G. (eds.) *Fluorescent Whitening Agents*, Thieme, Stuttgart, 247-263. (FB351). (80)
- Murphy, K. L., Zaloum, R. and Fulford, D. (1975)—Effect of chlorination practices on soluble organisms: *Water Res.* 9:389-396. (81)
- Murthy, M. S. S. (1979)—Induction of gene conversion in diploid yeast by chemicals: correlations with mutagenic action and its relevance in genotoxicity screening: *Mutat. Res.* 64:1-17. (FL). (82)
- Muzall, J. M. and Cook, W. L. (1979)—Mutagenicity test of dyes used in cosmetics with the *Salmonella*/Mammalian Microsome test: *Mutat. Res.* 67:1-8. (FL, E, RB). (83)
- Nagai, S. (1973)—Effect and after-effect of eosin and related dyes on the cells of *Saccharomyces* and *Torulopsis*: *Proc. 3rd Specialised Symp. Yeasts Part I*:111-112. (E). (84)
- Nestmann, E. R., Douglas, G. R., Matula, T. I., Grant, C. E. and Kowbel, D. J. (1979)—Mutagenic activity of rhodamine dyes and their impurities as detected by mutation induction in *Salmonella* and DNA damage in Chinese hamster ovary cells: *Cancer Res.* 39:4412-4417. (RB). (85)
- Nestmann, E. R. and Kowbel, D. J. (1979)—Mutagenicity in *Salmonella* of Rhodamine WT, a dye used in environmental water-tracing studies: *Mutat. Res.* 68:389-392. (RWT). (86)
- Nestmann, E. R., Kowbel, D. J. and Ellenton, J. (1980)—Mutagenicity in *Salmonella* of fluorescent dye tablets used in water tracing: *Water Res.* 14:901-902. (FL, RB). (87)
- Nishioka, H. (1976)—Detection of carcinogenicity of color cosmetics in bacterial test systems (Abstract): *Mutat. Res.* 38:34. (FL, E). (88)
- Okura, K. and Okubo, T. (1962)—Study of the bio-assay method for the evaluation of water pollution. II—Use of fertilised eggs of sea urchins and bivalves: *Bull. Tokai Regional Fisheries Res. Lab.* 32:131-140. (FL, RB). (89)
- Panciera, M. (1967)—The toxicity of Rhodamine B to eggs and larvae of *Crossostrea virginica*: *Proc. Nat. Shellfish Assoc.* 58:7-8. (Abstract). (RB). (90)
- Parker, G. G. (1973)—Tests of Rhodamine WT dye for toxicity to oysters and fish: *Jour. Res. U.S. Geol. Surv.* 1:449. (RWT). (91)
- Parodi, S., Zunino, A., Ottagio, L., DeFerris, M. and Santi, L. (1983)—Lack of correlation between the capability of inducing sister-chromatid exchanges *in vivo* and carcinogenic potency of 16 aromatic amines and azo derivatives: *Mutat. Res.* 108:225-238. (RB). (92)
- Pierce, E. C., Agersborg, H. P. K., Borzelleca, J. F., Burnett, C. M., Eagle, E., Ebert, A. G., Kirschman, J. C. and Scala, R. A. (1974)—Multi-generation reproduction studies with certified colors in rats (Abstract): *Toxicol. Appl. Pharmacol.* 29:121-122. (93)
- Rochat, J., Demenge, P. and Rerat, J. C. (1979)—Contribution à l'étude toxicologique d'un traceur fluorescent: la Rhodamine B: *Toxicol. Eu. Res.* 1:23-26. (RB). (94)

- Sako, F., Kobayashi, N., Watabe, H. and Taniguchi, N. (1980)—Cytotoxicity of food dyes on cultured fetal rat hepatocytes: *Toxicol. Appl. Pharmacol.* 54:285-292. (SRB). (95)
- Salem, H., Loux, J. J., Smith, S. and Nichols, C. W. (1979)—Evaluation of the toxicological and teratogenic potentials of sodium fluorescein in the rat: *Toxicol* 12:143-150. (FL). (96)
- Sasaki, M., Sugimura, K., Yoshida, M. A. and Abe, S. (1980)—Cytogenic effects of 60 chemicals on cultured human and Chinese hamster cells: *La Kromosomo* 11-20:574-584. (RB). (97)
- Sheng-Chin Chen, Nakamura, H. and Tamura, Z. (1980)—Studies of the metabolites of fluorescein in rabbit and human urine: *Chem. Pharm. Bull.* 28:1403-1407. (98)
- Smart, P. L. (1982)—A review of the toxicity of 12 fluorescent dyes used for water tracing: *Beit. Geol. Schweiz-Hydrol* 28:101-112. (FB28, FB351, AGA, DY96, P, LYFF, FL, E, RWT, RB, SRB, SRG). (99)
- Smart, P. L. and Smith, R. (in prep.) The toxicity of some fluorescent dyes used for water tracing: (AGA, LYFF, FL, RWT, RB). (100)
- Sugimura, T., Sato, S., Nagao, M., Yahagi, T., Mtauahim, T., Seine, Y., Takeuchi, M. and Kawachi, T. (1976)—Overlapping of carcinogens and mutagens: *IN Magee, P. N., Takayama, S., Sugimura, T. and Matsushima, T. (eds.) Fundamentals in Cancer Prevention*, 191-215, Tokyo: Univ. Tokyo Press, (FL). (101)
- Takashi, I. and Kobayashi, K. (1977)—A survey of *in vivo* photodynamic activity of xanthenes, thiazines and acridines in yeast cells: *Biochem. Photobiol.* 26:581-587. (E, RB). (102)
- Tanooka, H. (1977)—Development and applications of *Bacillus subtilis* test systems for mutagens, involving DNA—repair deficiency and suppressible auxotrophic mutations: *Mutat. Res.* 42:19-32. (FL). (103)
- Tatsui, I., Awazu, S. and Negami, H. (1971)—Pharmokinetic study of biliary excretion: comparison of excretion behaviour in xanthene dyes, fluorescein and bromosulphthalein: *Chem. Pharm. Bull.* 19:297-308. (FL). (104)
- Tatsui, I., Awazu, S. and Negami, H. (1972)—Pharmokinetic studies of biliary excretion V. The relationship between biliary excretion behaviour and the elimination from plasma of xanthene dyes and bromsulphthalein in rat: *Chem. Pharm. Bull.* 20:349-356. (105)
- Thomas, P. and Krüger, L. (1976)—Acute oral, dermal and inhalation studies: Ch. VII/2 In Anliker, R. and Müller, G. (eds.). *Fluorescent Whitening Agents*, Thieme, Stuttgart, 193-197. (FB28, FB351). (106)
- Tonogai, Y., Iwaida, M., Tati, M., Ose, Y. and Sato, T. (1978)—Biochemical decomposition of coal tar dyes and their decomposed products: *Jour. Toxicol. Sci.* 3:205-214. (AGA, FL, SRB). (107)
- Tonogai, Y., Ito, Y., Iwaida, M., Tati, M., Ose, Y., Sato, T. (1979a)—Studies on the toxicity of coal-tar dyes. I. Photodecomposed products of four xanthene dyes and their acute toxicity to fish: *Jour. Toxicol. Sci.* 4: 115-126. (108)
- Tonogai, Y., Ito, Y., Iwaida, M., Tati, M., Ose, Y. and Sato, T. (1979b)—Studies of the toxicity of coal tar dyes. II. Examination of the biological reaction of coal-tar dyes to vital body: *Jour. Toxicol. Sci.* 4: 211-220. (FL, E, SRB). (109)
- Umeda, M. (1956)—Experimental study of xanthene dyes as carcinogenic agents: *Gann.* 47:51-78. (FL, E, RB). (110)
- U.S. Dept. of HEW (1966). (111)
- Waugh, G. D. and Key, D. (1967)—Experiments with Rhodamine B and European flat oysters (*Ostrea edulis* L): *Jour. Cons. per. int. Explor. Mer.* 31:272-278. (RB). (111)
- Webb, J. M., Fonda, M. and Brouwer, E. A. (1962)—Metabolism and excretion patterns of fluorescein and certain halogenated fluorescein dyes in rats: *Jour. Pharmacol. Exptl. Ther.* 137:141-147. (FL, E). (112)
- Webb, J. M. and Hansen, W. H. (1961)—Studies of the metabolism of Rhodamine B: *Toxicol. Appl. Pharmacol.* 3: 86-95. (RB) (113)
- Webb, J. M., Hansen, W. H., Desmond, A. and Fitzhugh, O. G. (1961)—Biochemical and toxicological studies of Rhodamine B and 376 diamino fluoran: *Toxicol. Appl. Pharmacol.* 3:696-706. (114)
- Willhelm, R. and Ivy, A. C. (1953)—A preliminary study concerning the possibility of dietary carcinogenesis: *Gastroenterol.* 23:1-11. (E, RB). (115)
- Wilson, J. F. (1968)—Fluorometric procedures for dye tracing: *Techniques of Water Resources Investigations of the US Geol. Surv.* Book 3, Ch. A12. (116)
- Wortley, J. S. and Atkinson, T. C.—*personal communication*. (RWT). (117)
- Yankell, S. L. and Loux, J. J. (1971)—Acute toxicity testing of Erythrosine and sodium fluorescein in mice and rats: *Periodontol.* 48:228-231. (FL). (118)
- Yoshikawa, K., Kurata, H., Iwahara, S. and Kada, T. (1978)—Photodynamic action of fluorescent dyes in DNA-damage and *in vitro* inactivation of transforming DNA in bacteria: *Mutat. Res.* 56:359-362. (FL, E). (119)

## NOTES ADDED IN PROOF

- 1) Theiss et al. (1981) have reported a weak tumorigenic response in mice for Amino G Acid. In view of this finding, one of the other blue fluorescent tracer dyes should be used in preference to Amino G Acid.
- 2) A draft report detailing the toxicology and carcinogenesis trials on Fluorescein has now been prepared (National Toxicity Program Technical Report Series 265, available from National Toxicity Program, P.O. Box 12233, Research Triangle, Park, NC 27709, USA). The study found equivocal evidence of carcinogenicity in male rats, but no evidence in female rats or in male or female mice. Fluorescein was also found to be non-mutagenic in the *Salmonella typhum* mammalian microsomal assay, confirming the earlier results of Nestmann et al. (1980).
- 3) Crompton and Knowles Corporation have obtained expert opinion on the probable cancer risks arising from formation of diethylnitrosamine from Rhodamine WT. They conclude that incremental lifetime risks due to diethylnitrosamine exposure from Rhodamine WT would be far less than  $10^{-6}$  (considered by the EPA as acceptable).

## Reference

- Theiss, J. C., Shimkin, M. B. and Weisburger, K. (1981)—Pulmonary adenoma response of strain A mice to sulphonic acid derivatives of 1- and 2- Naphthylamines. *Jour. Nat. Cancer Inst.* 67: 1299-1302. (AGA).

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# An Evaluation of Some Fluorescent Dyes for Water Tracing

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Eight fluorescent dyes (amino G acid, photine CU, fluorescein, lissamine FF, pyranine, rhodamine B, rhodamine WT, and sulpho rhodamine B) were compared in laboratory and field experiments to assess their utility in quantitative tracing work. The properties considered included sensitivity and minimum detectability, the effect of water chemistry on dye fluorescence, photochemical and biological decay rates, adsorption losses on equipment and sediments, toxicity to man and aquatic organisms, and cost. The orange fluorescent dyes are more useful than the blue and green because of the lower background fluorescence at the orange wave band, which permits higher sensitivities to be obtained. Pyranine fluorescence is strongly affected by pH over the range encountered in natural waters, which precludes its simple use in quantitative work. Amino G acid, photine CU, pyranine, and fluorescein all have high photochemical decay rates. Pyranine, lissamine FF, and amino G acid are the dyes most resistant to adsorption, but rhodamine WT, fluorescein, and sulpho rhodamine B also have moderately high resistance. Rhodamine B is readily adsorbed by most materials. Rhodamine WT (orange), lissamine FF (green), and amino G acid (blue) are the three tracer dyes recommended; they may be used simultaneously to trace three injection sites with the filter combinations suggested.

## INTRODUCTION

Fluorescent dye tracing techniques are now widely used in hydrology. In surface waters they are commonly used for dye dilution gaging [Cobb and Bailey, 1965], in particular for the calibration of structures [Kilpatrick, 1968] and where current metering is difficult, for instance, under an ice cover [Kilpatrick, 1967] or in steep rocky channels [Church and Kellerhals, 1970]. Dyes are also used for time of travel studies [Buchanan, 1964] and for dispersion experiments in rivers [Yotsukura et al., 1970] and in marine/estuarine environments [Pritchard and Carpenter, 1960]. The tracing of karst groundwater has frequently been carried out by using fluorescent dyes [Drew, 1968; Brown et al., 1969], though applications in other aquifers have been largely limited to oil fields [Sturm and Johnson, 1950]. Dyes have also been employed for point dilution studies in wells [Lewis et al., 1966]. Reynolds [1966] reports the use of fluorescent dyes for tracing soil water, while Robinson and Donaldson [1967] have studied water uptake in plants, using these tracers. There are also significant applications of dye tracing techniques in engineering, for instance, circulation studies in chlorine contact chambers [Deaner, 1970] and infiltration measurements in foul water sewers [Smith and Kepple, 1972].

Of the commonly used fluorescent dyes, fluorescein (*Colour Index* (CI) 45350 [Society of Dyers and Colourists, 1971]) has been used since the end of the nineteenth century [Dole, 1906]. It is visibly detectable in low concentrations but has very poor stability under sunlight. Thus in the early 1960's, when workers in the United States and Japan were assessing fluorescent dyes for quantitative tracing work in surface waters, they adopted the equally fluorescent dye rhodamine B (CI 45170 [Pritchard and Carpenter, 1960]). However, it became apparent that rhodamine B was readily adsorbed onto sediments, and subsequently, sulpho rhodamine B (CI 45100) was introduced. Although this dye was resistant to adsorption, it was comparatively expensive and was later replaced by the cheaper

dye rhodamine WT, which was developed specifically for tracing work (U.S. patent 3, 367, 946). Reynolds [1966] used the green dye pyranine (CI 59040) for tracing percolation water because it was very resistant to adsorption. Recently, a group of blue fluorescent dyes, known as optical brighteners because of their use in whitening paper, textiles, and other off-white products, have been applied to water tracing [Glover, 1972].

There has been a little previous work on the suitability of rhodamine WT for water tracing, but considerably more data are available for fluorescein, sulpho rhodamine B, and rhodamine B. However, much of this information cannot be directly compared between one study and another because of differences in experimental techniques. Furthermore, little work has been presented on the use of pyranine, the optical brighteners, or two dyes used for aerosol tracing, lissamine FF (CI 56205 [Yates and Akesson, 1963]) and amino G acid [Dumbauld, 1962]. Available details of the names, structures, and suppliers of all the dyes studied are given in Table 1. This paper evaluates the existing information on the above dyes and presents the findings of an extensive series of tests on their usefulness as water tracers. Finally, recommendations are made on the utility of the different dyes for quantitative and other tracing work.

## ANALYSIS

*Instruments.* Although it is possible to determine the concentration of fluorescent dyes in solution by using a spectrofluorometer, the expense, complexity, and delicacy of these instruments rule out their general application. Most water tracing work is carried out by means of filter fluorometers such as the Turner 111 or the Aminco Bowman fluoro/colorimeter. These machines are only moderately expensive, simple to use, and sufficiently robust for operation in the field with a portable generator. Furthermore, their sensitivity is comparable to that of a spectrofluorometer, although they are considerably less specific unless interference filters are employed.

In a filter fluorometer, excitation energy is provided by a replaceable light source, commonly a low-pressure mercury lamp with or without a phosphor coating. The light passes

TABLE 1. Generic and Alternative Names and Chemical Structure of the Tracer Dyes

Name in Text	Colour Index No.	Generic Name	Alternative Names	Chemical Structure
<b>Blue Fluorescent Dyes</b>				
Amino G acid <sup>a,d</sup>			7-amino 1,3 naphthalene disulphonic acid	
Photline CU <sup>a</sup>		CI fluorescent brightener 15		
<b>Green Fluorescent Dyes</b>				
Fluorescein	45350	CI acid yellow 73	Fluorescein LT <sup>i</sup> Uranine <sup>d,f</sup> Sodium fluorescein	
Lissamine FF	56205	CI acid yellow 7	Lissamine yellow FF <sup>i</sup> Brilliant sulpho flavine FF <sup>i</sup> Brilliant acid yellow 8G <sup>d</sup>	
Pyranine	59040	CI solvent green 7	Pyranine <sup>e</sup> D&C green 8 <sup>i</sup>	
<b>Orange Fluorescent Dyes</b>				
Rhodamine B <sup>a,d,f,h</sup>	45170	CI basic violet 10	Pontacyl brilliant pink B <sup>a,h</sup> Lissamine red 4B <sup>i</sup> Kiton rhodamine B <sup>i</sup> Acid rhodamine B <sup>i</sup>	
Rhodamine WT <sup>a</sup>				
Sulpho rhodamine B <sup>a,f</sup>	45100	CI acid red 52		

\*Discontinued.

All Colour Index numbers refer to 3rd edition of the Colour Index [1971]. Superscript letters refer to manufacturer: a, L. B. Holiday Ltd.; b, Hickson and Welch Ltd.; c, ICI Limited; d, Allied Chemical Corporation (Specialty Chemicals Division); e, Farbwerke Hoechst A. G.; f, CIBA-Geigy U.K. Ltd.; g, Farbenfabriken Bayer A.G.; h, Du Pont de Nemours and Co. Ltd.; i, GAF Corporation; j, H. Kohnstanns and Co. Inc.

through a primary filter before entering the sample compartment, where it is absorbed by the dye sample to be re-emitted at a longer wavelength as fluorescence. This emitted light passes through a secondary filter, which is opaque to light passing the primary filter and is normally at 90° to the primary light path. The amount of light passing through the secondary filter is measured on a photomultiplier and compared with a reference light path to produce a readout. Sensitivity may be controlled by changing the amount of excitation energy or by varying filter transmittances using neutral density filters. Further details of the operation and construction of fluorometers are given by Wilson [1968], Udenfriend [1962], and the literature of fluorometer manufacturers.

**Filters and lamps.** Careful selection of the primary and secondary filters is necessary in order to maximize sensitivity,

and permit the analysis to be sufficiently specific. Normally, the primary and secondary filters are chosen to have peak transmission at the maximum excitation and emission wavelength of the selected dye. However, where overlap of the primary and secondary filters occurs, light scattered in the sample may enter the photomultiplier and produce an apparent fluorescence reading. Because some fluorescent dyes have only 50 nm between the excitation and emission maxima, this can be a particular problem. In such cases it may be necessary to excite the sample at other than the excitation maximum. This will also be necessary if a noncontinuous light source is used, for example, the mercury lamp emitting only at the mercury lines. Such a lamp will simplify filter selection considerably because it is only necessary to select a specific line(s) for the assay, and a filter

TABLE 2. Excitation and Emission Maxima of the Tracer Dyes and Filter Combinations for Their Analysis

Dye	Maximum Excitation, nm	Maximum Emission, nm	Primary Filter	Mercury Line, nm	Secondary Filter
<b>Blue Fluorescent Dyes</b>					
Amino G acid	355 (310)	445	7-37*	365	98†
Photline CU	345	435 (455)			
<b>Green Fluorescent Dyes</b>					
Fluorescein	490	520	98†	436	55‡
Lissamine FF	420	515			
Pyranine	455 (405)	515			
<b>Orange Fluorescent Dyes</b>					
Rhodamine B	555	580	2 × 1-60* + 61†	546	4.97* + 3.66*
Rhodamine WT	555	580			
Sulpho rhodamine B	565	590			

Figures in parentheses refer to secondary maxima. For all spectra, pH is 7.0.

\*Corning filter.

†Kodak Wratten filter.

‡See text.

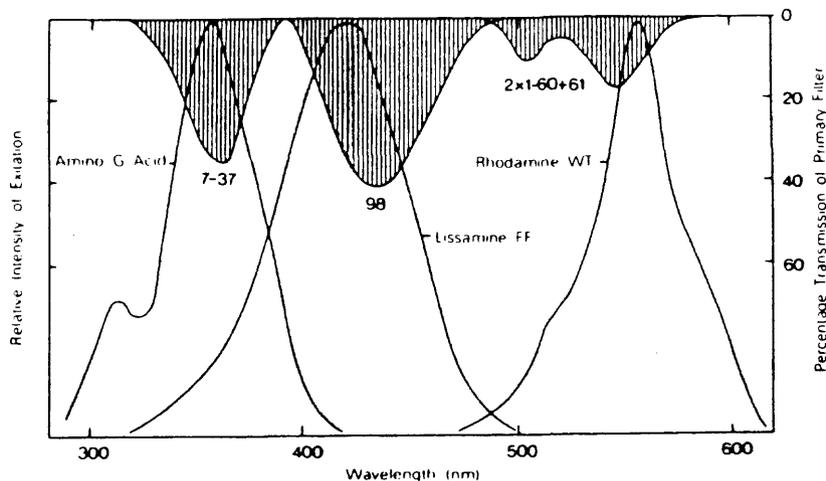


Fig. 1a. Excitation spectra of amino G acid, lissamine FF, and rhodamine WT and transmission characteristics of primary filters (shaded).

overlap is thus permissible at wavelengths other than those emitted by the source. Furthermore, it may reduce the background emission of other fluorescent compounds present in the sample.

There are two commonly available types of color filter, dyed in the glass and gelatin filters (interference filters are not considered here because of their relatively low transmission). Dyed in the glass filters are extremely stable under high light intensities, but because of the limited number of dyes available, they tend to have a broad transmission wave band with a long 'tail' toward longer wavelengths. Gelatin filters are much sharper in their resolution but less stable to light. Furthermore, if they are used unmounted, they scratch readily and are badly affected by heat. This has proved to be a specific problem with the Turner 111 fluorometer, where the primary filters are in close proximity to the light source and may become very hot. Gelatin filters should therefore be glass mounted, either on purchase or by using photographic slide cover plates.

Table 2 presents maximum excitation and emission wave-

lengths for the dyes considered here and the filter combinations recommended for their analysis, the spectra of which are given in Figure 1. Other filter combinations will also give satisfactory results. In all cases a low-pressure mercury lamp (General Electric Company G4T4.1) having significant emission only at the mercury lines has been employed. The orange filters are those recommended by G. K. Turner Associates for the Turner 111 fluorometer, which have proved to be very sensitive and to produce a low background. The green filters use the 436-nm mercury line, but a primary transmitting the 405-nm and 436-nm lines (e.g., a Wratten 36) would give similar results and reduce the pH sensitivity of the pyranine analysis. The secondary filter, a Wratten 55, has a relatively high transmittance, and therefore a neutral density filter (about 30-40% transmittance) should be used to provide a convenient working range. A Corning I-56 filter with a broad transmission in the visible wavelengths has been used in this study in place of the neutral density filter. A sharp-cut secondary filter composed of a Wratten 55 in combination with a

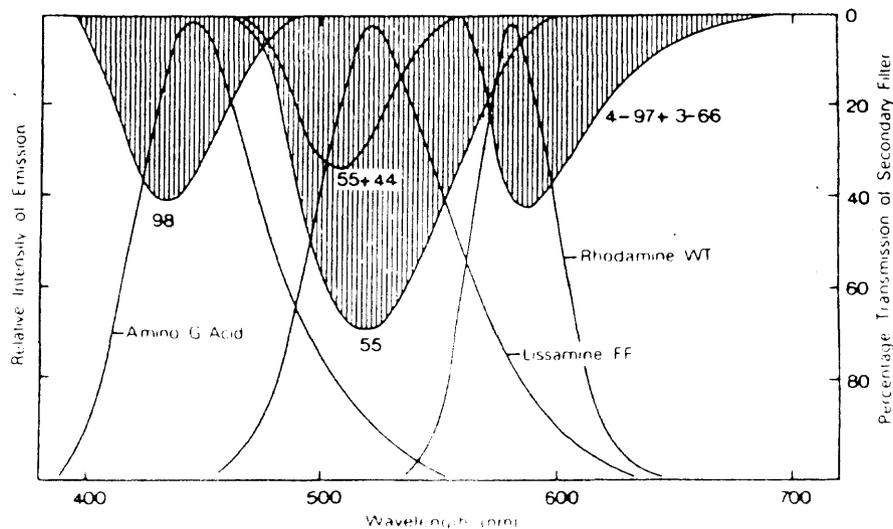


Fig. 1b. Emission spectra of amino G acid, lissamine FF, and rhodamine WT and transmission characteristics of secondary filters (shaded).

TABLE 3. Sensitivity and Minimum Detectable Concentrations for the Tracer Dyes

Dye	Sensitivity,* $\mu\text{g l}^{-1}/\text{scale unit}$	Background Reading,† scale units 0-100	Minimum Detectability,‡ $\mu\text{g l}^{-1}$
Amino G acid	0.27	19.0	0.51
Photine CU	0.19	19.0	0.36
Fluorescein	0.11	26.5	0.29
Lissamine FF	0.11	26.5	0.29
Pyranine	0.033	26.5	0.087
Rhodamine B	0.010	1.5	0.010
Rhodamine WT	0.013	1.5	0.013
Sulpho rhodamine B	0.061	1.5	0.061

For a Turner 111 filter fluorometer with high-sensitivity door and recommended filters and lamp at 21°C.

\*At a pH of 7.5.

†For distilled water.

‡For a 10% increase over background reading or 1 scale unit, whichever is larger.

Wratten 44 will minimize interference from orange fluorescent dyes. The Corning 7-37 primary and Wratten 98 secondary combination has been found excellent for the blue fluorescent dyes.

To date, no quantitative work using two or more dyes simultaneously has been reported. (However, *Rochat et al.* [1975] have described the separation of rhodamine dyes by both chromatographic and solubility techniques. A minimum sensitivity of 1-2  $\mu\text{g l}^{-1}$  was claimed from laboratory studies, but no analyses were reported from field tests. The technique could also be used for the separation of fluorescein and lissamine FF or pyranine, so that five fluorescent dyes could be determined in the same sample.) Simultaneous use is desirable because a single set of water samples can be used, for instance, to define the flow paths of two or three sinking streams in a karst aquifer. Fluorescent dye techniques have therefore been limited in this respect in comparison with microbiological [*Wimpenny et al.*, 1972], radioactive [*Aboud et al.*, 1969], and lycopodium spore [*Drew and Smith*, 1969] tracing methods. This disadvantage may be overcome by using a blue, a green, and an orange fluorescent dye with the recommended filter combinations. These filters minimize the additive interference from the fluorescence of the other two dyes, even at moderately high concentrations. The green filter combination is most sensitive to this interference; however, concentrations of at least 65  $\mu\text{g l}^{-1}$  of rhodamine WT and 60  $\mu\text{g l}^{-1}$  of amino G acid are required to produce a 10% increase in the distilled water background fluorescence with the Wratten 55 and 44 secondary combination. The blue and orange filter combinations

TABLE 4. Temperature Exponents for the Tracer Dyes

Dye	Temperature Exponent, °C <sup>-1</sup>
Amino G acid	-0.0019
Photine CU	-0.012
Fluorescein	-0.0036
Lissamine FF	-0.0020
Pyranine	-0.0019
Rhodamine B	-0.027
Rhodamine WT	-0.027
Sulpho rhodamine B	-0.029

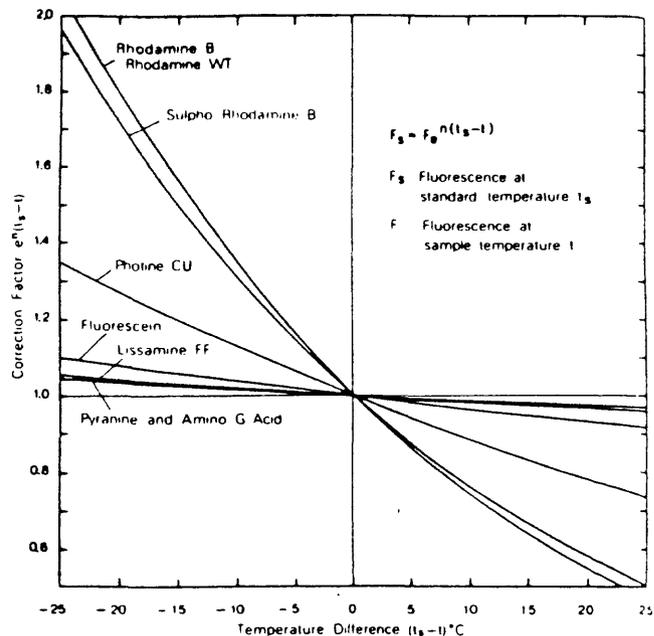


Fig. 2. Temperature correction curves for the tracer dyes.

show no interference until concentrations of lissamine FF exceed 120  $\mu\text{g l}^{-1}$  and 400  $\mu\text{g l}^{-1}$ , respectively. With concentrations in excess of these values, cross calibration will be required to correct for the interference. This will be necessary at much lower concentrations if less selective filter combinations are adopted.

**Sensitivity and detectability.** The sensitivity of the fluorometric analysis depends on both the efficiency of the dye in converting excitation energy into fluorescence and the transmission of the filter combination. However, the detectability also depends on the background or blank fluorescence value. Background fluorescence in natural waters is variable in both space and time; therefore detectabilities are reported here for a distilled water blank. It is best to use distilled or deionized water to prepare general calibration curves, the dye concentration in natural waters being determined by subtraction of the difference between the higher natural background values and those in distilled water. This procedure eliminates the necessity for specific calibration curves for water of different quality.

Table 3 presents sensitivities and minimum detectable con-

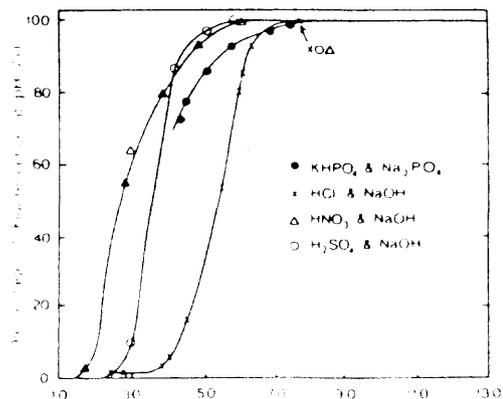


Fig. 3. Effect of pH on fluorescence of rhodamine WT, different acids being used.

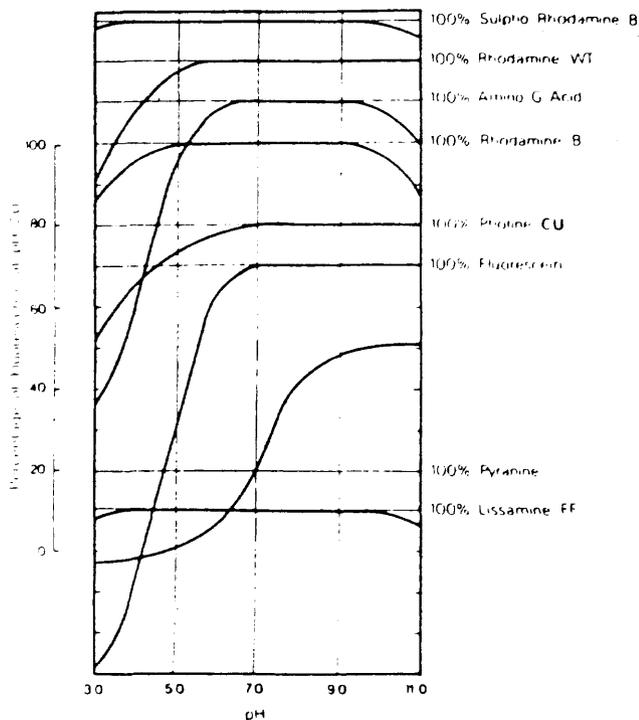


Fig. 4. Effect of pH on fluorescence of the tracer dyes.

centrations for the eight dyes under study (a Turner 111 filter fluorometer with a high-sensitivity door, a far ultraviolet lamp, and the recommended filter combinations being used). The instrument is readable to 0.5% of full scale and is linear to 1%. The minimum detectability in Table 3 is taken as being a reading 10% in excess of background fluorescence for distilled water or 1 scale unit, whichever is greater. Sensitivity is the gradient of the calibration curve for the most sensitive scale. These values will all vary slightly from one fluorometer to another.

The orange dyes have considerably lower background readings than the blue, which are smaller than the green. By the use of suitable neutral density filters the background readings could be reduced to similar absolute values, though there would be a corresponding reduction in sensitivity. Rhodamine WT and rhodamine B have the lowest minimum detectability. The minimum detectability for sulpho rhodamine B is better than that for pyranine, despite the latter's higher sensitivity. This illustrates the influence of the absolute value of the background reading on the detectabilities quoted. The remaining four dyes have detectabilities of a similar order of magnitude.

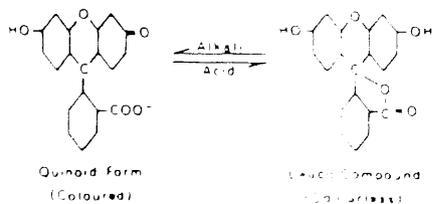


Fig. 5. Structure of fluorescein in acid and alkali conditions.

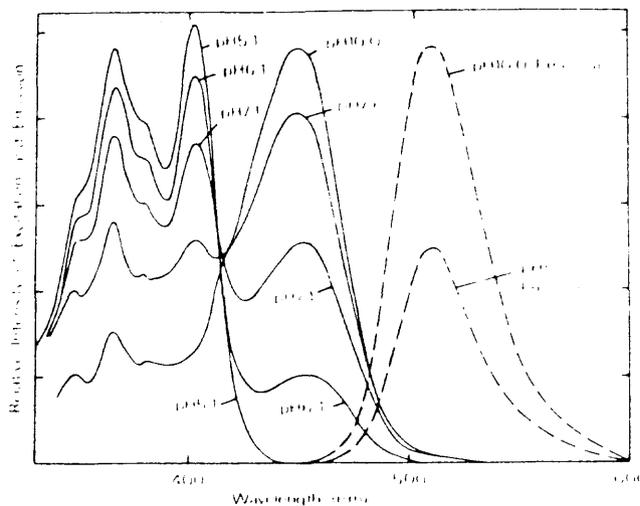


Fig. 6. Effect of pH on excitation and emission spectra of pyranine.

The least sensitive, amino G acid, has a minimum detectability such that it can be resolved at a dilution of 1 part in  $2 \times 10^6$  of distilled water.

**Temperature.** Fluorescence intensity varies inversely with temperature, though this rate depends on the dye. The experimental data, consisting of fluorescence readings at a number of different temperatures, were fitted by a curve of the form

$$F = F_0 \exp nt$$

where  $F$  is the fluorescence reading at temperature  $t$ ,  $F_0$  is the fluorescence at  $0^\circ\text{C}$ , and  $n$  is a constant for a given dye. The exponents obtained for each dye are given in Table 4. The fluorescences of the rhodamine dyes and photine CU are significantly affected by temperature variations, and corrections may therefore be necessary in quantitative studies. Figure 2 presents temperature correction curves for the eight tracer dyes derived from the temperature exponents by the procedure of Feuerstein and Selleck [1963] and Wilson [1968]. The curves agree closely with those previously reported for fluorescein, rhodamine B, rhodamine WT, and sulpho rhodamine B.

Dunn and Vaupel [1965] have shown that temperature variations may also affect the operation of filter fluorometers. This is well illustrated by the gradual increase in fluorometer efficiency which occurs as the machine warms up after switching on. These authors have presented a method for the correction of fluorometer readings based on the sample compartment temperature. However, it will often be simpler to prepare a calibration curve at a selected room temperature and continue to use this room temperature for all analyses. When continu-

TABLE 5. Effect of Sodium Chloride on the Tracer Dyes

Dye	Sodium Chloride Concentration	
	0.1 M	0.5 M
Amino G acid	100	100
Photine CU	100	100
Fluorescein	100	100
Lissamine FF	100	100
Pyranine	100	100
Rhodamine B	100	95
Rhodamine WT	97	92
Sulpho rhodamine B	100	96

Figures are percentage of fluorescence in distilled water.

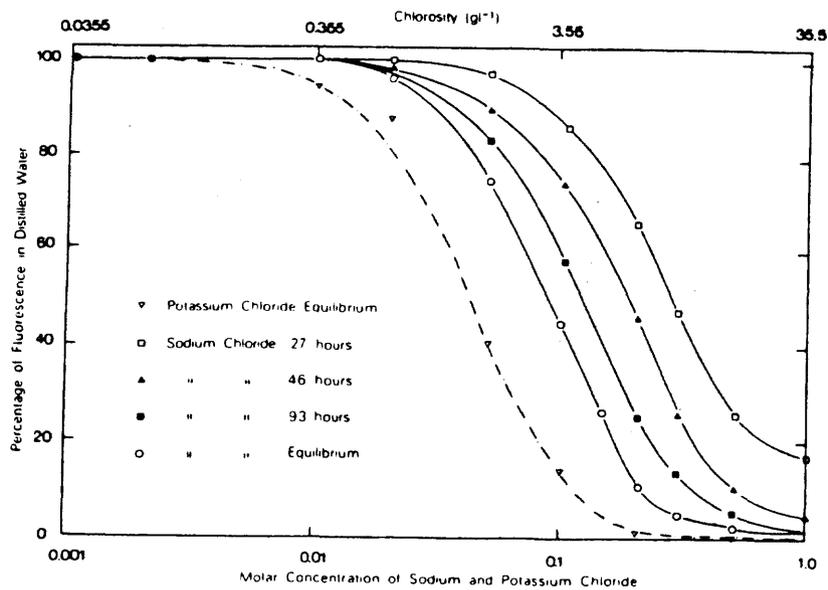


Fig. 7. Effect of sodium and potassium chloride on fluorescence of rhodamine WT.

ous monitoring work is being carried out in the field, it is relatively simple to take occasional discrete samples for later laboratory analysis. These may be used to check the continuous record and also to correct for both machine and sample temperature differences from the values used during calibration. Furthermore, if the samples are allowed to come to laboratory air temperature or are placed in a water bath, it will often be necessary to check only two or three sample temperatures for a whole batch of samples.

#### EFFECT OF WATER QUALITY

##### pH

Figure 3 presents data showing the variation in fluorescence with pH for rhodamine WT. The curves were prepared by

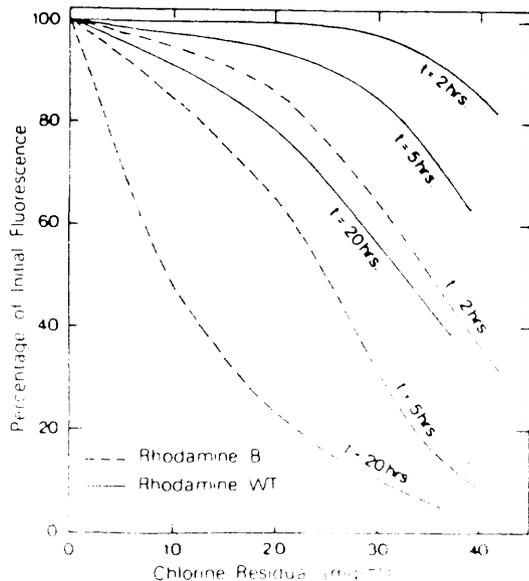


Fig. 8. Effect of chlorine residual concentration on fluorescence of rhodamine WT and rhodamine B in activated sludge (0.1 g l<sup>-1</sup>, initial dye concentration, 22 mg l<sup>-1</sup>, suspended solids). Data from Deane (1973, Table 1).

using three different acids and a phosphate buffer system to lower the pH of the dye solution. It is clear that different curves are obtained for each particular anion, and that specific interactions may therefore complicate the determination of pH/fluorescence curves. It will often be more satisfactory to use natural water samples to prepare these curves when waters of high or low pH are to be traced.

The standard curves presented in Figure 4 were prepared by using pH 4.0, 7.0, and 9.2 buffer tablets (obtained from British Drug Houses Ltd.) with hydrochloric acid and sodium hydroxide to extend the pH range. Figure 4 indicates that pH variations between pH 4.0 and pH 10.0 present no significant problems with lissamine FF and sulpho rhodamine B. Rhodamine B and rhodamine WT fluorescence is affected to a significant extent below pH 5.0, amino G acid below pH 6.0, and fluorescein and photine CU below pH 6.5. Some correction should be considered in waters with a pH lower than these values. Pyranine shows excessive variation in fluorescence with pH changes in the range normally encountered in natural waters. This would prove a severe problem for quantitative applications in waters of variable quality. These results are in good accord with the findings of Feuerstein and Selleck [1963] for fluorescein, rhodamine B, and sulpho rhodamine B: those

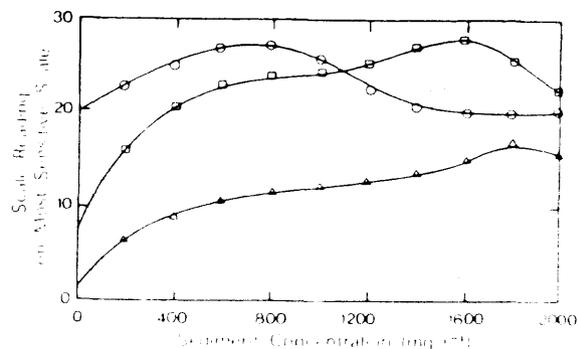


Fig. 9. Effect of suspended sediment concentration on background readings for the blue, green, and orange filter combinations.

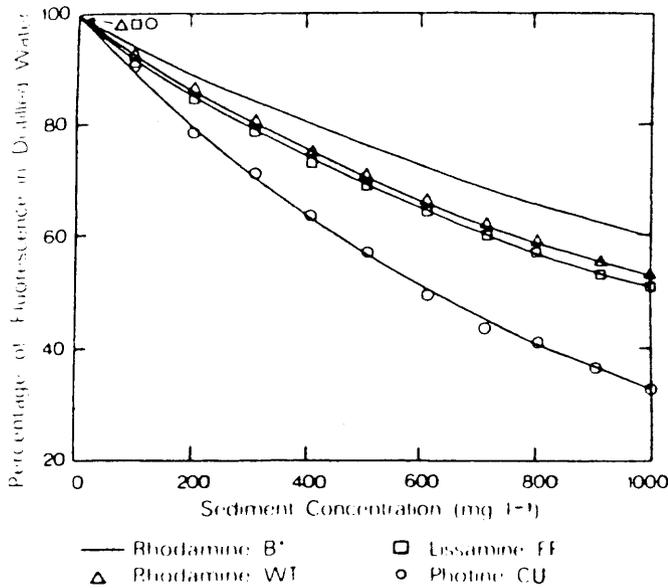


Fig. 10. Effect of suspended sediment concentration on fluorescence of rhodamine WT, lissamine FF, and photine CU.

of Von Möser and Sagl [1967] for sulpho rhodamine B and fluorescein; and those of Abood *et al.* [1969] for rhodamine WT.

There are two possible reasons for the response of fluorescent dyes to  $pH$  changes: ionization and structural changes. The dyes examined are anionic (except rhodamine B, which is cationic), and as  $pH$  decreases, the acid functional groups become protonated. This affects the degree of resonance in the molecule and reduces the amount of fluorescence. The change will be instantaneous and directly related to the dissociation constant of the dye. For carboxylic groups, dissociation occurs between  $pH$  4 and  $pH$  6, compared with  $pH$  6–7.5 for phenolic groups and below  $pH$  5 for sulphonate groups. Thus the dyes

having sulphonate acid groups remain fluorescent to lower  $pH$  values, as is exemplified by lissamine FF and sulpho rhodamine B.

In some xanthene dyes, structural changes may occur as  $pH$  decreases. Fluorescein, for example, changes from a quinoid structure under alkali conditions to a colorless leucocompound under acid conditions (Figure 5). The quinone ring in the quinoid structure is fluorescent, while the lactone ring of the leucocompound is not. Similar changes, which are often reversible, an 'indicator effect' thus being given, may also occur with the other dyes.

For pyranine the very sharp change in fluorescence at  $pH$  7.0 is due to the ionization of the phenolic OH group, which causes a change in the absorption spectrum (Figure 6) but not in the emission, which remains at 510 nm. The use of the 405-nm mercury line in addition to the 436-nm line in the primary filter combination will reduce the magnitude of this effect. Either a  $pH$  correction must be applied when pyranine is used, or the dye should be calibrated in the water under study.

#### Salinity

When tracers are being used in estuarine and marine environments or in brackish groundwater, high salinities will be encountered which may affect tracer performance. Feuerstein and Selleck [1963] have reported that rhodamine B and sulpho rhodamine B were only slightly affected by chlorosities of up to  $18 \text{ g l}^{-1}$  but that there was a marked effect for fluorescein. Unfortunately, the full data were not presented, nor were the experimental methods. Table 5 shows the effect of two concentrations of sodium chloride on the tracer dyes; these correspond to chlorosities of 3.6 and  $17.8 \text{ g l}^{-1}$ . Fluorescein exhibited no decrease in fluorescence with increasing salinity, but sulpho rhodamine B, rhodamine B, and rhodamine WT were all affected. Earlier experiments have given much more significant reductions in the fluorescence of rhodamine WT (Figure 7). No explanation can be offered for the differences in behavior between the two tests, which were conducted several years apart in different laboratories and on different batches of dye.

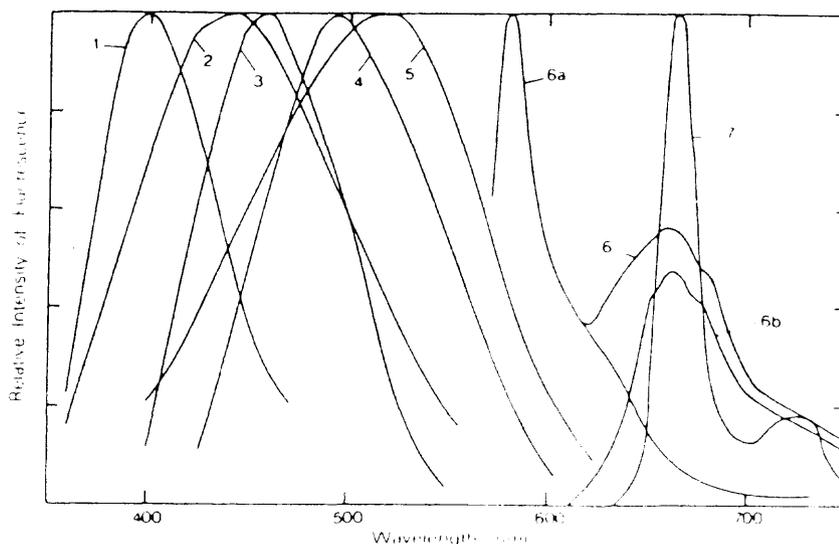


Fig. 11. Emission spectra of some naturally occurring pigments and fluorescent compounds. Curve 1, loam sulphates [Christman and Minear, 1967, Figure 3]; curve 2, River Frome [Smart *et al.*, 1967, Figure 1]; curve 3, Douglas fir bark extract [Christman and Ghassemi, 1966, Figure 3]; curve 4, Suwannee River black and Christman, 1963, Figure 3]; curve 5, fulvic acid extract from soil [Seal *et al.*, 1964, Figure 1]; curve 6, water extract of red algae, differentiated into the pigments phycoerythrin (curve 6a) and phycoerythrin (curve 6b) [Rubinowich, 1951, Figure 23.9B]; and curve 7, chlorophyll *a* [Rubinowich, 1951, Figure 23.2].

TABLE 6. Background Fluorescence Expressed as Apparent Dye Concentration for Selected Field Situations

Sample Origin	Samples	Blue Filters*		Green Filters*		Orange Filters*	
		$\bar{X}$	s.d.	$\bar{X}$	s.d.	$\bar{X}$	s.d.
<i>Karst Resurgence, Mendips</i>							
46 hours during a storm event	24	...	...	11.9	6.3	...	...
46 hours after a storm event	23	...	...	6.9	0.4	...	...
<i>Agricultural Catchment, South Cotswolds</i>							
28 days in October 1974, low flow	90	36.5	16.5	11.0	5.5	0.05	0.04
21 days in July 1974, summer storm	63	51.5	22.5	21.0	8.3	0.07	0.04
23 days in January 1975, high flow	50	47.2	25.0	23.6	6.4	0.06	0.05
<i>Karst Area, Central Jamaica West Indies (3 weeks in June 1975)</i>							
Groundwater from production well	16	1.2	7.6	1.5	1.9	-0.01†	0.008
Southern springs	34	11.4	12.2	2.4	2.0	0.008	0.013
North coast springs	32	14.4	13.2	4.0	3.4	0.013	0.023
Surface streams	36	23.2	9.8	7.2	2.5	0.029	0.052
Spring polluted by bauxite effluent	7	85.4	14.9	21.0	3.3	0.10	0.014

\*Expressed as apparent concentration of photine CU, lissamine FF, and rhodamine WT, respectively, in  $\mu\text{g l}^{-1}$ .

†Fluorescence less than distilled water used for calibration.

Figure 7 presents detailed data for the effect of sodium and potassium chloride concentrations on this dye. It is clear that there is a significant difference between the two salts, perhaps related to the alkali in which rhodamine WT is dissolved. Furthermore, the effect of the salts was not instantaneous, a gradual decay occurring over a period of up to 300 hours. This may explain dye losses in tracer tests in saline environments, which have previously been attributed to adsorption. Further work is clearly needed if rhodamine WT is to be used for quantitative work in saline waters.

### Chlorine

In some specialized applications, particularly high concentrations of other compounds may be present. In such cases it is necessary to evaluate the behavior of the tracer in that system.

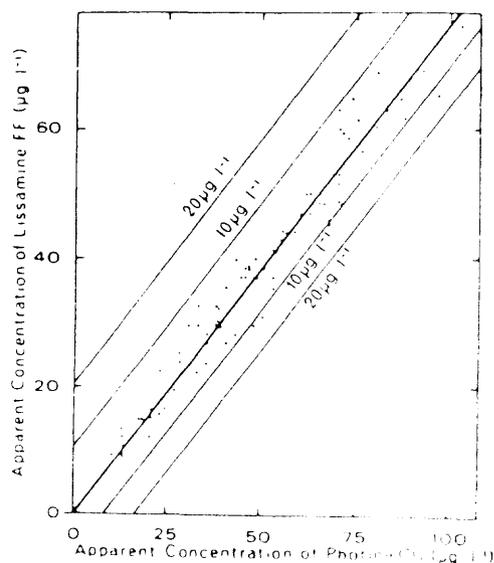


Fig. 12. Correlation between background fluorescence at the green and blue wavelengths expressed as apparent concentrations of lissamine FF and photine CU.

Deaner [1973] has investigated the effect of chlorine on rhodamine B and rhodamine WT for use in chlorine contact chambers. He showed that there was a progressive loss of fluorescence which was independent of dye concentration and most rapid at high chlorine residuals, though no single rate could be obtained for a given residual because of the continuous loss of chlorine from the samples. Figure 8 is a plot of the data in Deaner's Table 1 showing the effect of chlorine after exposure times of 2, 5, and 20 hours. The data indicate that rhodamine WT is more resistant to chlorine than rhodamine B. However, the data in Deaner's Figure 5 show a 31% reduction in fluorescence after 5 hours at  $22 \text{ mg l}^{-1}$  initial residual chlorine concentration, compared with only an 8% reduction interpolated from the results in his Table 1. This may be related to the higher suspended solid concentration for the Figure 5 samples ( $40 \text{ mg l}^{-1}$  compared with  $22 \text{ mg l}^{-1}$  for Table 1), indicating that dye adsorption may also be contributing to the reduction in fluorescence. For short-duration tests at normal chlorine dosage there will not be a significant reduction in the fluorescence of rhodamine B or rhodamine WT, but for durations over 2 hours, apparent dye losses must be taken into consideration.

### Background

An apparent or real fluorescence background in water samples taken for dye analysis can cause several problems in tracer studies. It may mask very low concentrations of the tracer or cause apparent recoveries to be in excess of 100% in quantitative work. The two major sources of background are natural fluorescence and suspended sediment.

*Suspended sediment.* The presence of suspended sediment raises apparent background fluorescence and reduces effective dye fluorescence because of light absorption and scattering by the sediment particles. Figure 9 shows the effect of a silt size sediment suspended in distilled water on fluorescence readings for the blue, green, and orange filter combinations. The effect is relatively small when it is compared to the other sources of background, the maximum increases being 7, 20, and 15 scale divisions on the most sensitive scale for the blue, green, and orange filters, respectively. All three curves exhibit maxima,

TABLE 7. Photochemical Decay Coefficients for the Tracer Dyes

Source and Conditions	Amino G Acid	Photine CU	Fluorescein	Lissamine FF	Pyranine	Rhodamine B	Rhodamine WT	Sulpho Rhodamine B
<i>Pritchard and Carpenter</i>								
[1960]								
Artificial light						$1.7 \times 10^{-5}$		
Sunny			$1.3 \times 10^{-4}$			$1.7 \times 10^{-4}$		
<i>Feuerstein and Selleck</i>								
[1963]								
Cloudy			$5.1 \times 10^{-4}$			$4.5 \times 10^{-4}$		$2.0 \times 10^{-4}$
Sunny			$2.6 \times 10^{-4}$			$2.2 \times 10^{-4}$		$1.0 \times 10^{-4}$
<i>Yates and Akesson</i>								
[1963]*								
Minimum rate			$4.5 \times 10^{-4}$	0		$3.6 \times 10^{-4}$		$4.4 \times 10^{-4}$
Maximum rate			$3.9 \times 10^{-4}$	$4.6 \times 10^{-4}$		$1.2 \times 10^{-4}$		$6.4 \times 10^{-4}$
<i>Watt [1965]</i>								
Sunny, $10 \mu\text{g l}^{-1}$						$5.6 \times 10^{-4}$		$5.6 \times 10^{-4}$
Sunny, $100 \mu\text{g l}^{-1}$						$3.4 \times 10^{-4}$		$3.4 \times 10^{-4}$
<i>Von Mäuser and Sagl</i>								
[1967]								
Cloudy			$1.5 \times 10^{-4}$	$8.0 \times 10^{-4}$				$1.8 \times 10^{-4}$
Sunny			$2.6 \times 10^{-4}$	$7.4 \times 10^{-4}$				$1.0 \times 10^{-4}$
<i>Aboud et al. [1969]</i>								
Sunny						$8.3 \times 10^{-4}$	$1.5 \times 10^{-4}$	$5.6 \times 10^{-4}$
6 hours, Sunny, $100 \mu\text{g l}^{-1} \dagger$	$1.6 \times 10^{-4}$	$>6.4 \times 10^{-4}$	$9.5 \times 10^{-4}$	$<1.0 \times 10^{-4}$	$1.2 \times 10^{-4}$	$5.5 \times 10^{-4}$	$<1.0 \times 10^{-4}$	$3.3 \times 10^{-4}$
6 hours under 60-W lamp, $100 \mu\text{g l}^{-1} \dagger$	$3.7 \times 10^{-4}$	$5.5 \times 10^{-4}$	$1.3 \times 10^{-4}$	$<1.0 \times 10^{-4}$	$1.6 \times 10^{-4}$	$1.5 \times 10^{-4}$	$<1.0 \times 10^{-4}$	$<1.0 \times 10^{-4}$

\*These decay rates refer to exposure of dry dye analyzed as a solution.

†Expressed as half the actual decay rate to correspond to environmental rates, 12 hours of darkness being included.

indicating that the increased absorbance due to suspended sediment becomes more important than the scattering. As would be expected, this effect occurs at lower sediment concentrations for the shorter wavelengths, and in fact, the fluorescence readings for the blue filter combinations are reduced to the blank values at quite low sediment concentrations.

Figure 10 gives the reduction in fluorescence of a dye solution for sediment concentrations of up to  $1000 \text{ mg l}^{-1}$ . Above this concentration, adsorption of the tracer onto the sediment may become an experimental problem. The effect was found to be independent of dye concentration. The blue emission wavelength is clearly affected much more than the green and orange. The average of the data presented by *Feuerstein and Selleck* [1963, Figure 7] for the orange filters supports the general trend of this data but not the absolute magnitude. This is probably due to the different sediment used. Fine white sediment may in fact increase apparent fluorescence even at sediment concentrations which are very turbid, while this is never the case with dark-colored sediments.

In most cases, if the suspended sediment is allowed to settle out for a period of 10–20 hours, substantially correct dye concentrations may be obtained from the decanted sample. For sediment concentrations below  $1000 \text{ mg l}^{-1}$ , adsorption effects will not be a problem unless the sediment is extremely fine or contains much organic matter. For such cases or when readings are required immediately, the sample may be centrifuged to remove the sediment. When tracer dyes are being monitored in the field, with either individual samples or a continuous flow sampling system, it is often necessary to obtain information immediately. In such cases where it is not possible to centrifuge the samples, dilution using distilled water has been found to give excellent results, even with sediment concentrations of several thousand milligrams per liter [*Petri and Craven*, 1971]. Dilution of the turbid samples by 1:5 with

distilled water was found to increase readings by 50% for a peak concentration of  $8 \mu\text{g l}^{-1}$  of rhodamine WT. Furthermore, the technique allowed satisfactory determination of the low-concentration tail of the tracer pulse, which was completely obscured by the very high sediment concentrations.

**Background fluorescence.** Unlike background problems caused by suspended sediment, those caused by natural fluorescence are widely reported [*Feuerstein and Selleck*, 1963; *Wright and Collings*, 1964; *Knochenmus*, 1967; *Drew*, 1968; *Brown and Ford*, 1971]. The cause of this fluorescence has frequently been wrongly ascribed to the fluorescence of algae, especially *Chlorella*, and to other natural plant pigments. The majority of algae and phytoplankton contain the green pigment chlorophyll, which has a strong red fluorescence peaking at 650 nm (Figure 11, curve 7). Clearly, this will cause very little fluorescence interference even when the orange filter combination is used. Some red algae do contain phycoerythrin, which has a fluorescence maximum at 580 nm, coincident with the rhodamine emission peak (Figure 11, curve 6). However, it has been widely recognized that the background fluorescence at the green wave band is many times stronger than that at the orange, which is rarely a major problem. Therefore it may be concluded that the algal pigments are not an important cause of background fluorescence.

Most natural waters contain dissolved and colloidal organic matter which when it is sufficiently concentrated produces a marked yellow/brown coloration [*Black and Christman*, 1963]. This material consists of complex polymeric hydroxy-carboxylic and aromatic acids [*Lamar*, 1968], which frequently contain known fluorescent structures. Figure 11 presents fluorescent emission spectra reported in the literature for a number of natural waters and soil and plant extracts. Emission maxima occur at wavelengths from 420 to 520 nm for natural waters and at 400 nm for pulp mill effluent. In all cases, the fluores-

cence was strong. Furthermore, recent work has shown that the total organic carbon (TOC) concentration in a range of polluted and natural waters correlates linearly with fluorescence measured over a wide wave band between 400 and 600 nm [Smart *et al.*, 1976].

The mean and standard deviation for background fluorescence values during a series of dye tests conducted in unpolluted limestone basins in the southern Cotswolds and Mendips (United Kingdom) and in a karst area in central Jamaica are presented in Table 6. As would be expected, the blue and green background values are much higher than the orange, as is their standard deviation. The variation is due to both between-site variations caused by differences in the water sources (illustrated by the Jamaica values) and temporal variations at a site caused by storm flow (illustrated by the Mendip resurgence data). Highly variable values are found for soil water, surface runoff from clay soils, and rivers receiving sewage or agricultural effluent, while lower more constant figures are obtained from groundwater bodies sampled in pumping wells and springs.

Because of the high background at the green and blue wavelengths the sensitivity of the analysis for these dyes has been reduced from that obtained by other workers [Feuerstein and Selleck, 1963; Von Möser and Sagl, 1967]. The apparent increased discrimination of a high-sensitivity filter combination is negated by the corresponding increase in the variability of the background. Attempts to separate background from dye fluorescence by physical and chemical techniques have proved unsuccessful because of the chemical similarity of the compounds producing the background to the dye itself. Thus a thorough knowledge of the range of background variation is required when green and blue fluorescent dyes are being used. This is rarely necessary for the orange dyes because of the much lower background readings.

Given the spectral uniformity of background fluorescence, it is sometimes possible to graph intercorrelations of blue, green, and orange fluorescence values. Any increase in the background value at a given wavelength will be reflected by a parallel increase at the other wavelengths. Thus a sample containing dye will have a significant positive deviation from the mean correlation line between readings at that wavelength and those at both of the others. There is often considerable scatter in such plots, which limits their sensitivity. The technique may not work if the sample is positive for both filter combinations plotted, but by using the third wavelength it is often possible to prove this case. Figure 12 shows the correlation obtained between background fluorescence at the blue and green wavelengths for 76 water samples from several sample locations over a period of 10 days. The correlation coefficient is 0.54, which is statistically significant at the 99.5% level. Also shown are lines representing the deviation from the best fit line through the data produced by the presence of one dye in concentrations of 10 and 20  $\mu\text{g l}^{-1}$ . It is clear that a minimum concentration of 15  $\mu\text{g l}^{-1}$  of lissamine FF and 20  $\mu\text{g l}^{-1}$  of photine CU will be necessary in the sample to be readily separable from background fluorescence.

#### NONADSORPTIVE DYE LOSS

**Photochemical decay.** When compounds absorb light energy, the molecules become excited and raised to a higher energy state. Fluorescence is caused when the molecules revert to the lower energy state by the emission of light. The high-energy state will also take part in chemical reactions more readily than the base state; thus as compounds fluoresce, they

often decompose owing to oxidation and other chemical changes. The rate of this decay will depend on the energy of the incident light beam. Thus photochemical decomposition is dependent on both light intensity and wavelength, ultraviolet light causing more rapid decomposition than longer wavelengths.

It is very difficult to obtain photochemical decay rates which have direct application to field conditions because decomposition is dependent on dye concentration and light intensity. Table 7 gives decay coefficients reported in the literature for an exponential decay of the form

$$F = F_i \exp -kt$$

where  $F_i$  is initial fluorescence,  $F$  is fluorescence at time  $t$ , and  $k$  is the decay coefficient. The decay rates are very high for fluorescein, which rapidly loses its fluorescence under bright sunlight conditions. Sulpho rhodamine B is less affected than rhodamine B, but lissamine FF appears to be an order of magnitude better than these two. The rates presented probably represent maximum values for field conditions, where water depth and turbidity will considerably reduce the average light intensities.

Comparable values for the dyes evaluated in this study are also given in Table 7. The very fast decay of photine CU under all light conditions precludes its use as a quantitative water tracer. This also applies to fluorescein [Feuerstein and Selleck, 1963] and pyranine, which was previously thought to be reasonably stable [Drew, 1968]. The orange fluorescent dyes and lissamine FF exhibit low photochemical decay rates such that no correction will be required for tests of up to 1 week in duration. It is significant that the difference between the decay rates for a 6-hour exposure to sunlight and a 6-hour exposure to artificial light becomes progressively higher for dyes absorbing at shorter wavelengths. This ratio varies from 3-4 times for the orange dyes to over 40 times for the blue dyes, as would be expected given the much greater ultraviolet absorption of blue dyes together with the significant ultraviolet content of sunlight. Amino G acid has a low photochemical decay rate compared with other blue fluorescent dyes.

**Chemical decay.** Feuerstein and Selleck [1963] and Watt [1965] have both reported that vigorous agitation of dye solutions may cause reduction in fluorescence even under dark conditions. Watt attempted a systematic study of this effect but experienced considerable experimental difficulties, finally concluding that rhodamine B was more susceptible than rhodamine WT or sulpho rhodamine B to this type of decay.

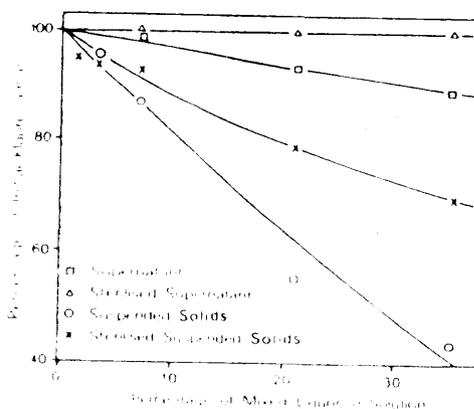


Fig. 13. Comparison of effect of active and sterilized mixed liquor on rhodamine WT.

He reported little or no significant loss for the dyes over a 3-day period of agitation or after a similar period with oxygen bubbling through the sample.

The experimental blank solutions used in all the experiments reported here have been found stable, even when they are agitated. Changes in the pH of unbuffered pyranine samples may cause variation in fluorescence, and in common with photine CU and fluorescein, its high photosensitivity may result in significant decay over the short periods when these samples are removed from dark conditions. G. K. Turner Associates reports the reduction of rhodamine B fluorescence in contact with metals.

**Biodegradation.** The susceptibility to biodegradation of a tracer dye is significant because experiments may be carried out in biologically hostile environments, for instance, activated sludge systems [Scalf *et al.*, 1968], when the tracer must be conservative. However, over the long term it must not persist in the environment, although because of the low dosages normally used, this should not be a problem except for continuous dilution flow gaging [Goodell *et al.*, 1967].

Feuerstein and Selleck [1963] reported adsorption isotherms for a high-rate sewage oxidation pond effluent, but over the 1-hour period used for equilibration, biodegradation was probably small. Pritchard and Carpenter [1960] report that rhodamine B in a sample which contained a large algal population showed no measurable decrease in fluorescence over a period of 4 days.

Experiments on the biodegradation of these tracers using biologically active materials are difficult to conduct because the relative magnitudes of adsorption and biodegradation losses are not known. Furthermore, the populations present are not stable through time, and very high background values may be encountered. Figure 13 shows data for adsorption on two different components of a mixed liquor, subsamples of which had been sterilized before addition of the dye. The marked difference between the curves for the sterilized and live subsamples indicates that there was a significant non-adsorptive loss, which probably represents biodegradation of the dye. Certainly, other dyes are known to be biodegradable in both aerobic and anaerobic systems [Hunter, 1973; Etzel and Grady, 1973], including stilbene triazine optical brighteners similar to photine CU.

Therefore for tracing work in systems with large populations of microorganisms it is likely that biodegradation will be a significant cause of dye loss. In the majority of surface waters it will be unnecessary to consider biodegradation of the tracer dyes because bacterial populations will be very much lower than those used in these experiments.

#### ADSORPTIVE DYE LOSS

Adsorption of dye onto sediment surfaces is mainly irreversible, and therefore a high resistance to adsorption is of paramount importance for a dye tracer. Consequently, a large number of laboratory experiments, normally using batch techniques but sometimes using elution through a column, have been reported in the literature [Dole, 1906; Feuerstein and Selleck, 1963; Wright and Collings, 1964; Watt, 1965; Scanlan, 1968; Talbot and Henry, 1968; Scott *et al.*, 1969]. However, because of variations in dye concentration, equilibration time, experimental technique, and the sediments used, it has proved difficult to extend the results of one study to those of more recent studies on newly introduced tracers. The experiments usually employ higher sediment concentrations than occur in natural situations but are designed to enable comparisons

dyes or sediments rather than to simulate environmental conditions closely.

**Experimental methods.** The inorganic adsorbents used in the tests were all ground to pass a 100- $\mu$ m sieve and consisted of an orthoquartzite sandstone (100% quartz), a pure limestone (99% calcium carbonate), and two British Pharmacopoeia clays, bentonite and kaolinite, obtained from Evans Medical (Liverpool, United Kingdom). The bentonite was identified as a low-silica member of the Montmorillonite series by X ray diffraction techniques, and the kaolinite as a pure kaolinite ( $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$ ). The organic adsorbents used were an acid peat (humus), finely shredded heather roots and stems (*Calluna vulgaris*), and a marine ply sawdust.

A known weight of adsorbent and a dye solution of selected concentration were sealed in a flask and shaken for 2 hours. Adsorption was substantially complete within this time, but several days were allowed for a true equilibrium to be established. The samples were centrifuged, and the equilibrium dye concentration determined. Blanks of both dye alone and sediment alone were also prepared. The blanks of dye alone were used to correct for any decay of the dye solution with time, while those of sediment alone permitted subtraction of the fluorescence background due to leaching from the adsorbent (particularly important with organic substrates). Blanks constituted about 25% of all samples run in the experiments.

A number of variables control dye adsorption in a batch system; pH, temperature, water quality, and degree of agitation are environmental factors which were held constant during the experiments. The four variables sediment concentration, dye concentration, sediment type, and dye type were experimental variables. The effects of sediment and dye type are discussed below. Scott *et al.* [1969] have illustrated the effect of sediment concentration on the percentage of rhodamine WT adsorbed on a fine fluvial sediment from the Rio Puerco (Figure 14). The arithmetic scale shows that at high sediment concentrations the substrate is a considerably less efficient adsorbent than it is at low sediment concentrations, though the converse may be inferred from the logarithmic plot presented in the original (their Figure 3).

The effect of varying the dye concentration is illustrated in Figure 15. There is a marked decrease in the percentage of dye loss with increasing initial dye concentration, though the actual weight of dye adsorbed increases. This is important in quantitative applications because percentage dye loss, and therefore error in discharge determination, will be higher for low dye concentrations than for high dye concentrations in a given situation. Talbot and Henry [1968] examined the effect of dye and suspended sediment concentration on adsorption losses. They presented correction curves for rhodamine B dye based on their experimental results but were only able to achieve a moderate fit with test data. In practice, attempts to correct dye concentrations for adsorption losses are liable to considerable error, and a specific correction curve would be needed for each field trial. Clearly, it is more desirable to select a dye with a high adsorption resistance than to rely on such procedures.

**Effect of sediment type on adsorption.** Figure 16 presents adsorption data for lissamine B on seven different sediments, four inorganic and three organic. For the inorganic materials, natural background fluorescence was relatively small, but for the organic materials at high concentrations it was similar in magnitude to the dye blank fluorescence. Figure 17 shows that at 20 g/l of humus the apparent adsorption loss was negative 2.5%, whereas actual losses were over 50%. When the natural

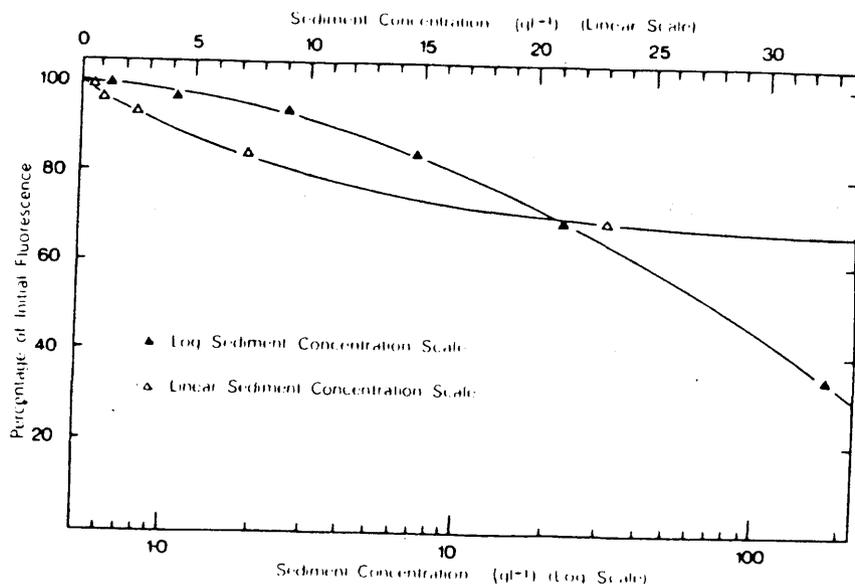


Fig. 14. Effect of sediment concentration on dye adsorption for rhodamine WT. Data are from Scott et al. [1969, Figure 2].

dye fluorescence falls below the background fluorescence, the accuracy of the results is dominated by the variability of the background. This was commonly  $\pm 10\%$ ; therefore for large adsorptive dye losses the error may approach  $\pm 100\%$ , though it was much less in the majority of the experiments. For the orange fluorescent dyes the error was considerably less than it was for the blue and green dyes because the blank fluorescence was lower ( $10 \text{ g l}^{-1}$  of humus gave an apparent concentration of  $1.2 \mu\text{g l}^{-1}$  of rhodamine WT,  $70 \mu\text{g l}^{-1}$  of lissamine FF, and  $80 \mu\text{g l}^{-1}$  of amino G acid). These limitations on experimental accuracy should be considered when the adsorption data are being examined.

In Table 8, adsorption losses for all eight dyes tested are presented. At the bottom of each sediment type column a mean rank is given; this is derived by ranking the sediments for each dye at the two different concentrations in order of ad-

sorptive efficiency and averaging for the total number of cases. The organic sediments adsorb far more dye than the inorganic (see also Figure 16). This is expected because of the extremely large surface area of organic material and the large number of broken bonds present on these surfaces. This finding has been widely reported for the adsorption of organic pesticides on soils; for example, adsorption of the acid pesticide Pichloram correlates highly with soil organic matter content but not significantly with percentage of clay [Grover, 1971]. The undecayed organic material of the heather roots and sawdust has an adsorptive efficiency very similar to that of the decomposed humus material [cf. Pauli, 1961].

The orthoquartzite adsorbs markedly less dye than the other three materials, which are not significantly different from each other despite the larger total surface area of the clays. The fine San Francisco Bay sediment of Feuerstein and Selleck [1963]

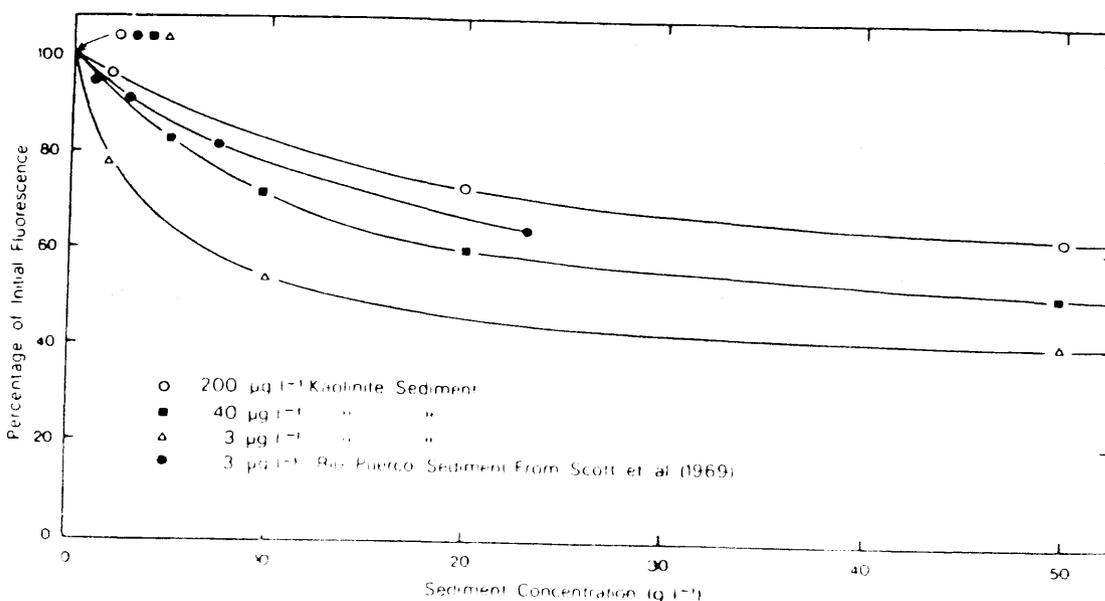


Fig. 15. Effect of dye concentration on dye adsorption for rhodamine WT.

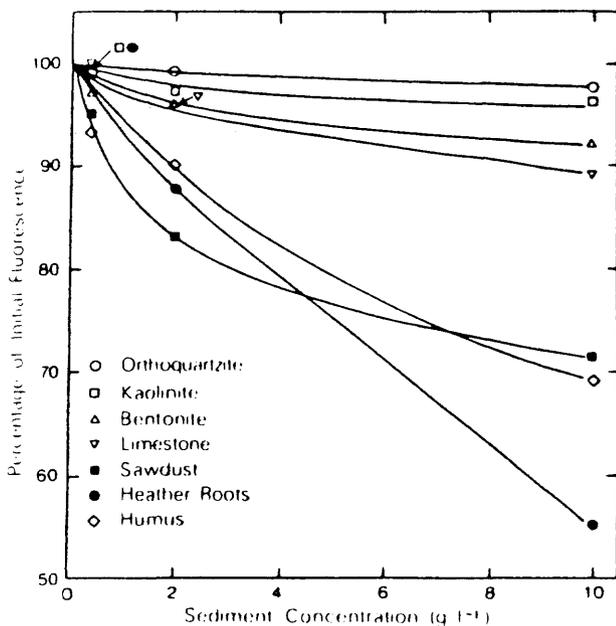


Fig. 16. Effect of sediment type on dye adsorption for lissamine FF. Initial dye concentrations were  $200 \mu\text{g l}^{-1}$ .

containing 'abundances of illite, montmorillonite, and kaolinite materials' adsorbed almost exactly the same amount of rhodamine B at a sediment concentration of  $200 \text{ mg l}^{-1}$  as did the kaolinite in this study. The 'filter sand' of Watt [1965] was very similar to the orthoquartzite used in these experiments, but the Rio Puerco sediment of Scott *et al.* [1969] fell between these two. It may be concluded that adsorption losses under field conditions will be much more of a problem in environments containing abundant organic matter, such as soil or sewage ponds, than it will be in even the most turbid inorganic fluvial system.

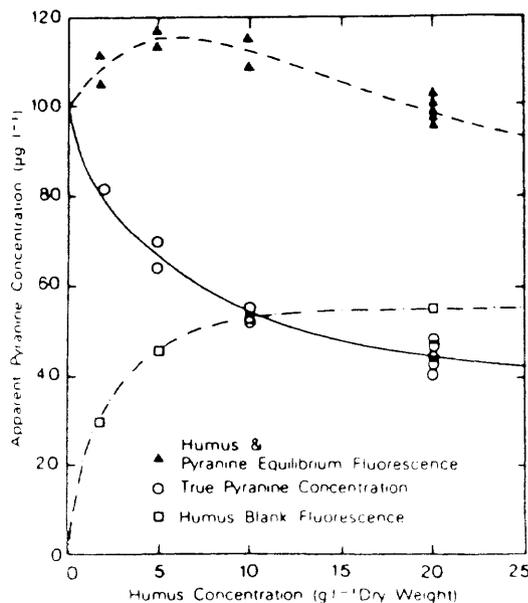


Fig. 17. Effect of humus background fluorescence on apparent concentration of pyranine in adsorption experiments.

**Resistance to adsorption of the tracer dyes.** Figure 18 shows adsorption of the tracer dyes on a range of kaolinite sediment concentrations using a constant initial dye concentration of  $100 \mu\text{g l}^{-1}$ . Rhodamine B is conspicuously the least resistant to adsorption owing to its cationic nature. It is clear from the replicate values plotted that even with inorganic substrates there is some experimental variance. Sulpho rhodamine B exhibits the highest losses of the anionic dyes, twice as large as those for rhodamine WT, the next most resistant. Lissamine FF and fluorescein exhibit very similar values, while amino G acid, photine CU, and pyranine show very small losses, which would be negligible under field conditions. Comparison of the adsorption losses in Table 8 with those in Figures 18 and 19 shows that different adsorption runs do not give the same absolute results, although the ranking of the dyes remains essentially unchanged.

Humus was also used to study the relative resistance of the tracer dyes (Figure 19). Again rhodamine B showed poor resistance. Photine CU, fluorescein, and rhodamine WT were considerably more resistant, but much less so than amino G acid, pyranine, lissamine FF, and sulpho rhodamine B. Because of the high humus concentration used, the pH of the solution was as low as 4 for several of the runs. Adsorption of the anionic dyes is retarded by repulsion from the negatively charged surface of the organic matter. Therefore greater adsorption would be expected at low pH values when the dye molecules become protonated. Thus the increased dye losses at the high humus concentrations may be augmented by this effect, which has been widely reported for organic pesticides [Frissel and Bolt, 1962; Boardman and Worrall, 1966; Grover, 1971].

The four dyes most resistant to adsorption on organic matter all have sulphonic acid functional groups: three for pyranine, two each for sulpho rhodamine B and amino G acid, and one for lissamine FF. This strong acid group does not protonate until relatively low pH values, and the dyes therefore maintain their resistance to adsorption. Photine CU also has sulphonic acid groups, but because of its planar molecule, which has a high affinity for cellulose surfaces, it exhibits poor resistance to adsorption on humus compared to its good resistance to adsorption on inorganics. Both rhodamine WT and fluorescein have carboxyl acid groups, which are liable to protonation at a higher pH than the sulphonic acid groups, their lower resistance to adsorption thus being explained. Corey [1968] has previously suggested that more than one sulphonic acid group was a useful indicator of a resistant dye. The data support this conclusion, though the good performance of lissamine FF with only one such acid group shows that this should not be the only criterion employed in dye selection.

Rhodamine B is clearly of little use as a quantitative tracer because of its very poor resistance to adsorption. Pyranine and amino G acid are resistant to adsorption on both organic and inorganic materials, while lissamine FF and sulpho rhodamine B show a relatively higher resistance to adsorption on organic material. Rhodamine WT and fluorescein have a moderate performance on both types of substrate, but photine CU, which is resistant to adsorption on inorganic sediments, shows a marked affinity for organic materials.

**Adsorption losses onto equipment.** Yotsukura *et al.* [1970] have reported adsorption of rhodamine B dye onto glass sample bottles during a dispersion study, though the problem was not encountered when rhodamine WT was used. Rhodamine B is cationic and is therefore attracted by the negative

TABLE 8. Comparison of Tracer Dye Adsorption on Mineral and Organic Materials

Dye	Sediment Concentration, $g\ l^{-1}$	Mineral				Organic		
		Kaolinite	Bentonite	Limestone	Orthoquartzite	Sawdust	Humus	Heather
Amino G acid	2.0	99	...	95	...	66	75	...
	20.0	97	...	96	...	17	39	...
Photine CU	2.0	93	80	93	58	48	60	57
	20.0	90	38	40	83	...	14	23
Fluorescein	2.0	98	98	98	98	86	83	41
	20.0	93	87	94	98	11	17	0
Lissamine FF	2.0	97	96	96	99	83	90	88
	20.0	96	92	88	95	70	68	54
Pyranine	2.0	95	100	96	100	70	76	74
	20.0	95	98	85	87	30	31	18
Rhodamine B	2.0	1	4	8	10	12	3	4
	20.0	4	8	2	8	4	2	1
Rhodamine WT	2.0	89	92	93	98	81	82	81
	20.0	67	79	66	90	42	11	18
Sulpho rhodamine B	2.0	88	98	97	...	92	92	...
	20.0	51	...	76	...	...	63	...
Average ranking of best adsorbents		5.2	5.3	5.4	6.2	2.7	2.3	1.8

Figures are percentage of dye remaining in solution from a  $100\ \mu g\ l^{-1}$  initial dye concentration.

charges present on most solid surfaces. The other dyes studied here are anionic and are repelled by such surfaces; hence adsorption losses are considerably less. No significant losses of any of the anionic dyes tested were observed on soft or hard glass (Pyrex) containers for periods of up to 10 weeks. Furthermore, no losses were found for rhodamine WT and lissamine FF stored in polythene bottles or in contact with rubber bungs or 'Parafilm' laboratory sealing film over the same period.

When blue fluorescent dyes are used, care should be taken that the samples do not come into contact with cotton wool, paper, textiles, or other materials treated with blue fluorescent optical brighteners. It is particularly important that non-fluorescent laboratory detergents be used because most domestic products contain 0.1–0.6% by weight of brightening compounds. One particular source of contamination was found to

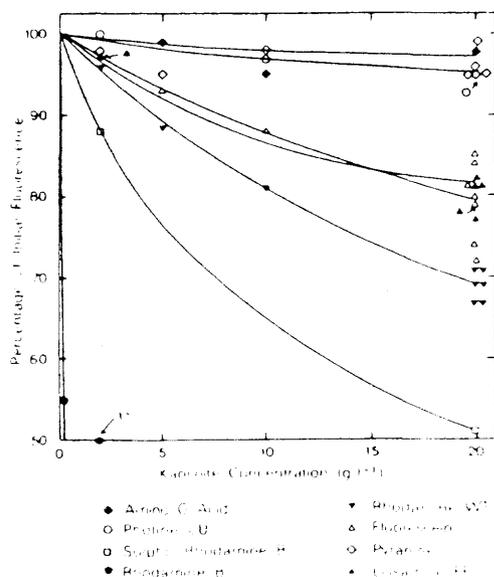


Fig. 18. Comparison of adsorption of the tracer dyes on kaolinite sediment. Initial dye concentrations were  $100\ \mu g\ l^{-1}$ .

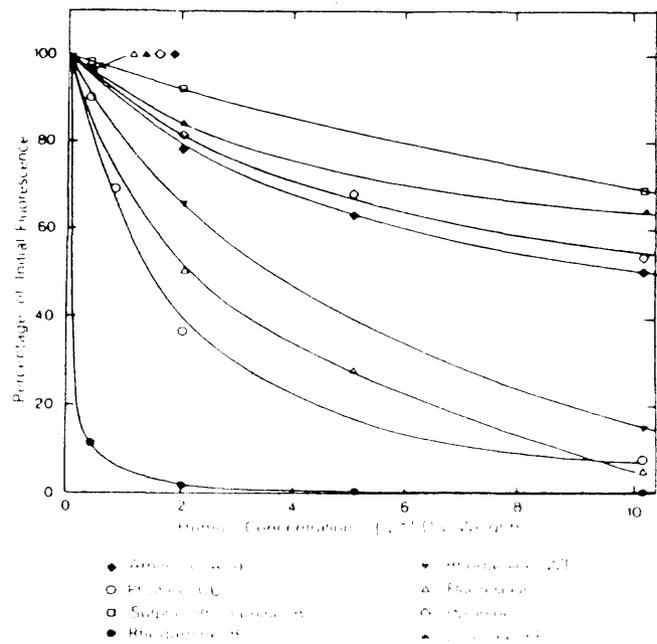


Fig. 19. Comparison of adsorption of the tracer dyes on humus sediment. Initial dye concentrations were  $100\ \mu g\ l^{-1}$ .

TABLE 9. Toxicity of Rhodamine B and Fluorescein to Fish at 12°C

Dye	Species	LC <sub>50</sub> , mg l <sup>-1</sup>			
		6 Hours*	24 Hours	48 Hours	96 Hours
Rhodamine B	rainbow trout	...	736	306	217
	channel catfish	...	962	647	526
	bluegill	1176	754	700	379
Fluorescein	rainbow trout	6410	4198	3420	1372
	channel catfish	...	3828	2826	2267
	bluegill	...	5000	4898	3433

\*Estimated from other data.

Data are from *Marking* [1969].

be transparent polythene tubing, which contained very large quantities of brightener, readily leached by dye solutions passing along it. It is relatively simple to check such gross sources of contamination by using sample blanks or a hand-held ultraviolet lamp. In general, contamination at the green and orange wavelengths is minimal.

#### TOXICITY

Two aspects of dye toxicity are important: first, possible deleterious effects on aquatic and marine life and second, the limitations which should be considered where human consumption of the labeled water is a possibility. *Pritchard and Carpenter* [1960] reported on the toxicity of rhodamine B to an unnamed species of fish which survived without ill effects for 2 months in a 100 mg l<sup>-1</sup> solution. *Bandt* [1957] showed that fluorescein at 100 mg l<sup>-1</sup> was not toxic to trout and roach, while *Sowards* [1958] observed that visible concentrations of this dye did not affect the toxicity of Pronoxfish, a fish toxicant, to longnose dace (*Rhinichthys cataractae*). *Marking* [1969] determined LC<sub>50</sub>'s, the concentration in solution which causes 50% mortality in the test species after a specified exposure period, for rhodamine B and fluorescein by using rainbow trout (*Salmo gairdnerii*), channel catfish (*Ictalurus punctatus*), and bluegill (*Lepomis macrochirus*) for 24, 48, and 96 hours at 12°C (Table 9). Rhodamine B was considerably more toxic than fluorescein, and the LC<sub>50</sub> decreased with exposure. The dyes had little effect on the toxicity of antimycin A, rhodamine B slightly increasing it and fluorescein slightly decreasing it. Smolt of both silver salmon and Donaldson trout experienced neither mortalities nor respiratory problems in concentrations of rhodamine WT of 10 mg l<sup>-1</sup> for 17.5 hours at 22°C or an additional 3.5 hours at 375 mg l<sup>-1</sup> [Parker, 1973]. No abnormalities in body length or weight were observed for goldfish (*Carassius auratus*) maintained in several stilbene triazine optical brighteners at concentrations of 10 and 20 mg l<sup>-1</sup> [Akamatsu and Matsuo, 1973]. Ninety-six-hour LC<sub>50</sub>'s determined for a number of brighteners by *Keplinger et al.* [1974] and *Sturm and Williams* [1975] ranged from 32 to 474 mg l<sup>-1</sup> for bluegill (*Lepomis macrochirus*), from 108 to 1780 mg l<sup>-1</sup> for rainbow trout (*Salmo gairdnerii*), and from 86 to 1060 mg l<sup>-1</sup> for channel catfish (*Ictalurus punctatus*) (cf. Table 9). *Akamatsu and Matsuo* [1973] report LC<sub>50</sub>'s as high as 2 g l<sup>-1</sup>, though experimental details are not given. Although body levels of optical brightener above the concentration present in the water have been observed in long-term aquarium studies with goldfish and bluegills, these concentrations rapidly fell once the fish were placed in freshwater [Jensen and Pettersson, 1971; Ganz et al., 1975].

*Pancieria* [1967] has shown that 2-day-old oyster larvae (*Crassostrea virginica*) died within 2 days at 100 mg l<sup>-1</sup> concen-

trations of rhodamine B, showed temporary retardation of growth at 10 mg l<sup>-1</sup>, and suffered no ill effects at 1 mg l<sup>-1</sup>. Similarly, at the high concentration, no eggs developed to the larval stage, while 27% had some abnormality at 10 mg l<sup>-1</sup>, and none were affected at 1 mg l<sup>-1</sup>. *Parker* [1973] showed that for rhodamine WT, development continued normally in Pacific oyster (*Crassostrea gigas*) eggs and larvae up to concentrations of 10 mg l<sup>-1</sup> for 48 hours at 24°C. The flesh of quahog clams (*Mercenaria mercenaria*) was rapidly stained by rhodamine B at dye concentrations in excess of 0.09 mg l<sup>-1</sup>, but the dye was rapidly cleared once the clams were placed in dye-free water. At concentrations above 8.4 mg l<sup>-1</sup> the clams showed avoidance reactions and contact staining. *Woelke* [1972] has observed that sea urchin eggs (species *hemicecentrotus*) were affected by rhodamine B at 32 mg l<sup>-1</sup> but not at 10 mg l<sup>-1</sup>, while embryos of the bay mussel (species *mytilus*) were affected at concentrations 10 times lower than this. Toxicity experiments conducted at 10°C with a number of fresh and brackish water invertebrates including water flea (*Daphnia magna*), shrimp (*Gammarus zaddachi*), log louse (*Asellus aquaticus*), may fly (*Cloeon dipterum*), and pea mussel (species *pisidium*) at a maximum concentration of rhodamine WT of 2000 mg l<sup>-1</sup> showed no mortality of any species over periods of 48 hours and 1 week compared to the control animals (J. S. Wortley and T. C. Atkinson, personal communication, 1975).

From the data presented, it is apparent that rhodamine B is more toxic to aquatic organisms than both rhodamine WT and fluorescein, probably because it is readily adsorbed on living tissues owing to its cationic nature [Little and Lamb, 1973]. Nevertheless, concentrations sufficiently high to be a problem are so transient under normal field applications, because of rapid dilution following injection, that the dye will not cause any ill effects to aquatic life. However, in view of the greater toxicity of rhodamine B compared with anionic dyes, it is recommended that rhodamine B not be used as a water tracer. No specific information has been obtained on any of the other anionic dyes, but it is probable that they have toxicities similar to those reported for fluorescein and rhodamine WT. Optical brighteners of the stilbene triazine type have LC<sub>50</sub> values in the same range as those for the other dyes, and it is thus likely that photine CU has a similarly low toxicity.

The toxicity of compounds to man is normally investigated on laboratory animals; safe dosage levels are then scaled up, and an additional safety factor is incorporated. Rhodamine B has been investigated by a number of workers and is generally recognized to be by far the most toxic of the xanthene dyes because it is readily adsorbed on body tissue [Webb et al., 1962]. *Webb and Hansen* [1961] studied the metabolism of this dye and, as is true for fluorescein, found that the basic fluoran structure was not broken down in the body, though the me-

tabolites were significantly less toxic than the original dye [Webb *et al.*, 1961]. Umeda [1956] reported that sarcoma was caused in some rats following subcutaneous injections of rhodamine B but that this was much less frequent for injections of fluorescein. At dietary levels below 0.2%, neither dye caused tumors in feeding studies. Hansen *et al.* [1958] noted 100% mortality within 42 days after feeding rhodamine B at a 2% dietary level. The dye retarded growth and caused liver damage; liver enlargement was also evident after 90 days in rats fed at a 1% level. D. Donaldson (unpublished data, 1971) studied the effects of rhodamine B, sulpho rhodamine B, and rhodamine WT in oral feeding studies at  $10 \mu\text{g l}^{-1}$  in drinking water in the rat. He reported that all test animals showed loss of body weight compared to a control group and that rhodamine B and sulpho rhodamine B caused the greatest liver enlargement. Subcutaneous injections of  $50 \mu\text{g}$  of sulpho rhodamine B caused inflammatory sores at the injection sites and a marked loss in body weight, whereas rhodamine WT and rhodamine B appeared to cause no traumatic ill effects even after 56 days of this treatment.

Akamatsu and Matsuo [1973] have reviewed a large number of studies on the toxicity of optical brighteners and conclude that they do not present any toxic hazard to man even at excessive dosage levels. The  $\text{LD}_{50}$ , the dose per unit body weight which causes 50% mortality in the test species, for oral feeding studies in mice and rats averaged  $7 \text{ g kg}^{-1}$  of body weight for stilbene triazine brighteners, while continuous feeding studies indicate that a 60-kg man could ingest 1–2 g/d for a considerable period without any ill effects. Hickson and Welch, Ltd. (personal communication, 1975) have confirmed that the brightener used in photine CU has been subjected to acute and long-term feeding studies, which have indicated a satisfactory toxicity level.

Rhodamine WT, fluorescein, and photine CU have relatively low toxicity levels, while those of rhodamine B and sulpho rhodamine B appear to be slightly higher. No data are available for the other dyes studied, but the manufacturers have indicated that lissamine FF is unlikely to cause any unusual toxic hazards. Fluorescein and pyranine have been certified for use in externally applied drugs, lipsticks, and cosmetics in the United States by the Food and Drug Administration, while no applications have been made for the remaining dyes except for rhodamine B, which was decertified in the early 1960's. Rhodamine B and fluorescein have been placed in toxicological classification C111 by the Food and Agriculture Organization/World Health Organization. The use of rhodamine B in water which may pass into supply is generally avoided in the United States. Permitted continuous ingestion levels for rhodamine B, sulpho rhodamine B, and rhodamine WT have been set at  $0.75 \text{ mg/d}$ , which is unlikely to be exceeded if concentrations at the intake remain below  $370 \mu\text{g l}^{-1}$ . The U.S. Geological Survey recommends that tracer tests aim for a final concentration not exceeding  $10 \mu\text{g l}^{-1}$  and preferably below this level. Until more information is available on the toxicity of tracer dyes, it is recommended that these levels be adhered to and that local water undertakings and river authorities be informed prior to the running of any tracer test. (This is in fact required by law in Great Britain.) However, because of the relatively short duration of most tracer tests, the very high detectabilities available with fluorescent dyes, and the low toxicities of the dyes themselves, no problems should be encountered with any of the dyes.

An additional consideration where water may enter a domestic supply which is chlorinated is the production of chloro-

phenols in water containing dye molecules. Chlorophenols impart a bitter metallic taste to water even at very low concentrations and are thus extremely undesirable. Wilson [1968] reports the results of a taste test in which nine tasters sampled water containing  $0.75 \text{ mg l}^{-1}$  of residual chlorine and three different concentrations of rhodamine B. All nine were able to detect the astringent taste at  $50 \mu\text{g l}^{-1}$ , only four at  $10 \mu\text{g l}^{-1}$ , and none at  $5 \mu\text{g l}^{-1}$ .

Taste tests were carried out with the anionic dyes by using four untrained tasters and dye samples prepared in Sheffield tap water, the residual chlorine concentration of which was low. No chlorophenol or other taste was detected for any of the dyes at concentrations of 10 and  $100 \mu\text{g l}^{-1}$ . Samples with concentrations as high as  $10,000 \mu\text{g l}^{-1}$  were found to cause little significant taste, though these were not presented to the whole tasting panel. At concentrations of  $100 \mu\text{g l}^{-1}$  the dyes are readily visible, and it is therefore probable that water color will be a more important constraint on dye concentration than taste. However, if higher chlorine residual concentrations are present, the threshold level for taste may be lower. Because the blue fluorescent dyes adsorb in the ultraviolet wave band, they are colorless in solution until they reach very high concentrations ( $0.1\text{--}1.0 \text{ g l}^{-1}$ ), when they are detectable by a blue fluorescent sheen. In situations where the aesthetics of a water body are to be considered, this may prove an extremely useful property.

#### FIELD EXPERIMENTS

Many authors have reported on the relative merits of different dyes under field conditions. Watt [1965] showed that rhodamine B gave losses of 25% averaged over 24 tests in mountain watersheds in Colorado. In one stream the average losses were as high as 53% for rhodamine B and 32% for sulpho rhodamine B, though no check was made on the accuracy of the gaging structures used in these tests. Kilpatrick *et al.* [1967] reported losses of 49% for rhodamine B, 25% for sulpho rhodamine B, and 7% for rhodamine WT for gaging tests made at the same location under different discharge conditions. An average loss of 5.4% for sulpho rhodamine B (six tests) and 1.1% for rhodamine WT (24 tests) was reported by Kilpatrick *et al.* [1967]. Kilpatrick [1970] also presented the results of a large number of dye gagings conducted by the U.S. Geological Survey and concluded that less rhodamine WT is needed for a given injection than rhodamine B, despite the greater minimum detectability of rhodamine B. Yotsukuro *et al.* [1970] have also emphasized the clear advantages that rhodamine WT has as a tracer over rhodamine B in time of travel and dispersion measurements.

Fewer tests have been conducted with green fluorescent dyes because they are widely recognized as being inferior to the orange dyes for use in surface waters [Feuerstein and Selleck, 1963]. Batsche *et al.* [1966] present results which show that fluorescein and sulpho rhodamine B exhibit comparable losses under similar test conditions, although they were not directly compared. Mather *et al.* [1969] have reported very large apparent losses of fluorescein under acid conditions in a sandstone aquifer, while salt injected simultaneously was not affected. W. I. Stanton (personal communication, 1974) found that pyranine failed to trace connections in a karst aquifer later proved by the use of rhodamine WT in amounts of  $1\text{--}2 \text{ g}$  the amount of pyranine used. Atkinson *et al.* [1973], in contrast, present data indicating a recovery of about 130% in a quantitative test, despite careful calibration of the dye using springwater from the sampling site, though discharge was not

known exactly. Similarly, *Abood et al.* [1969] report several cases where dye recoveries have been greater than 100%, probably owing to variation in the background fluorescence. *Smart and Smith* [1976] have recently tested a number of dyes in a surface river in Jamaica. They concluded that lissamine FF was the most resistant to adsorption, though rhodamine WT and fluorescein also gave high recoveries. Pyranine and the optical brighteners photine CU and photine CSP experienced large losses in both surface water and groundwater tests, recovery values being under 50%.

Tests conducted during this work have shown that rhodamine WT is generally the most satisfactory dye tracer, while pyranine and photine CU have proved to have severe limitations. A comparison of these three dyes in a surface stream containing a large growth of weed gave recoveries of 100%, 95%, and 30% for rhodamine WT, pyranine, and photine CU, respectively, after a mean residence time of 3.5 hours and 98%, 88%, and 11% at a second site after another 7.4 hours. The pyranine results were carefully corrected for pH variation, and it is clear that moderate recovery figures can be obtained. Better recovery figures are obtained for photine CU in underground tests because photodecomposition losses are eliminated. In a groundwater trace in a karst area in Great Britain, lissamine FF and rhodamine WT gave directly comparable concentrations after correction for the different amounts injected. Exact recoveries were not computed.

In most comparison tests it is usual to use quantities of tracer which produce comparable percentage increases over background readings. Table 10, however, presents data derived from a test in which 10-mg solutions of dye were injected sequentially into a small peaty stream with a low pH (5.0). It is clear that the accuracy for the determination of the orange dye concentrations is much greater than that for the green and blue dyes because of the low background combined with high instrumental sensitivity, as was discussed previously. Sulpho rhodamine B, rhodamine WT, fluorescein, and lissamine FF have comparable recovery figures, while rhodamine B and amino G acid were better than photine CU and pyranine (not pH corrected). In practice, percentage losses would be much lower for the blue and green dyes because larger initial injections would be used, raising the final concentrations and decreasing the percentage loss.

#### RECOMMENDATIONS FOR DYE TRACER APPLICATIONS

The presence of a significant fluorescence background at both green and blue wavelengths is probably the most important factor affecting selection of a tracer dye. For a single

injection there is thus a definite preference for the use of an orange dye, though clearly both blue and green dyes will be necessary for multiple injections. Rhodamine WT and rhodamine B are 3 times as fluorescent as sulpho rhodamine B and will therefore label a larger volume of water per unit weight (Table 11). Similarly, pyranine is more fluorescent than lissamine FF and fluorescein, which are in turn more fluorescent than the blue dyes.

Although temperature corrections are easily applied, for continuous monitoring it may be necessary to obtain a simultaneous record of temperature variations. While the green and blue dyes have low temperature sensitivities, those for the orange dyes are fairly high. Consequently, it is normally necessary to standardize temperatures, for instance in a water bath, or to measure sample temperature on analysis. Of the other water quality parameters examined, salinity does not appear to have a significant effect on dye fluorescence, though there is some evidence that long-term exposure of rhodamine WT may lead to some losses. Little information on the relative behavior of these dyes in contact with chlorine is available, though it is known to affect dye fluorescence. For work at high chlorine levels, specific investigation of dye performance is recommended. The anionic dyes are stable under alkali conditions but show a reduction of fluorescence at low pH. Sulpho rhodamine B and lissamine FF are most stable under these conditions, while fluorescein and photine CU would exhibit large losses. Because pyranine shows excessive variation of fluorescence with pH in the range normally encountered in natural waters, it cannot be recommended as a quantitative dye tracer unless pH is carefully monitored.

Pyranine, fluorescein, and photine CU have extremely high photochemical decay rates, a reduction of fluorescence under both natural and artificial illumination thus being caused. Amino G acid has a moderate decay rate and when it is exposed continuously to bright sunlight would therefore exhibit significant losses. For the three orange dyes and lissamine FF, photochemical decay would only be important in tests lasting several days. Biodegradation will not be a problem under most natural conditions, though for work in biologically hostile environments it is probably a significant cause of dye losses, which should be considered in dye selection. Rhodamine B suffers from enormous losses due to adsorption on many surfaces and is not recommended because of this problem. It is also significantly more toxic than other dyes, and its use should therefore be avoided. Pyranine and amino G acid are both very resistant to adsorption on both mineral and organic surfaces, while fluorescein and rhodamine WT exhibit moderate resistance. Although sulpho rhodamine B is not readily adsorbed by humus, it suffers significant losses on mineral surfaces, the converse of the adsorption characteristics of photine CU.

The volume of water labeled per unit cost is given for the tracer dyes in Table 11 on the basis of the minimum detectabilities in Table 3 and prices quoted for 10 kg of tracer delivered in the United Kingdom. Although rhodamine B appears to be the most economical tracer, because of its very large adsorption losses this figure will effectively be much reduced in practice. Furthermore, these losses preclude its use for quantitative applications. Rhodamine WT, the second most cost effective dye in the table, has no serious disadvantages, although it was not the most conservative tracer of those examined. In environments with much organic matter, sulpho rhodamine B might be considered because of its good adsorption resistance, though it is more expensive than rhodamine

TABLE 10. Comparison of Tracer Dyes in a Peaty Stream

Dye	Background Reading, scale units	Peak Reading, scale units	Peak Concentration, $\mu\text{g l}^{-1}$	Dye Recovery, %
Amino G acid	22.5	25.6	12.5	52
Photine CU	22.5	23.2	5.0	27
Fluorescein	33.5	49.2	22.0	80
Lissamine FF	33.5	46.3	20.5	84
Pyranine	33.5	38.9	3.0	12
Rhodamine B	0.9	54.4	15.4	72
Rhodamine WT	0.9	92.4	21.2	86
Sulpho rhodamine B	0.9	18.6	22.0	100

For dye injection of 50 mg

TABLE 11. Cost Effectiveness of the Tracer Dyes

Dye	State	Cost per Kilogram,* £	Volume Labeled per Kilogram, 10 <sup>6</sup> m <sup>3</sup> kg <sup>-1</sup> †	Volume Labeled per £, 10 <sup>6</sup> m <sup>3</sup> £ <sup>-1</sup>	Supplier
Amino G acid	powder	3.50	2.0	5.7	L. B. Holliday Ltd., Huddersfield, U. K.
Photine CU	20% solution	1.00	2.8	5.6	Hickson & Welch Ltd., Castleford, U. K.
Fluorescein	powder	4.00	3.5	8.8	Brico Ltd., London, U. K.
Lissamine FF	powder	13.50	3.5	2.6	L. B. Holliday Ltd., Huddersfield, U. K.
Pyranine	powder	13.00	12.0	9.2	Bayer U. K. Ltd., Richmond, U. K.
Rhodamine B	powder	5.00	100.0	200.0	Brico Ltd., London, U. K.
Rhodamine WT	20% solution	6.50	77.0	24.0	Du Pont U. K. Ltd., Altringham, U. K.
Sulpho rhodamine B	powder	8.50	16.0	19.0	Brico Ltd., London, U. K.

\*For 10-kg lots delivered, October 1975.

†Based on minimum detectabilities in Table 3.

WT. Lissamine FF, which is extremely stable and resistant to adsorption losses, is unfortunately the least cost effective of the dyes considered, being over 9 times more expensive to use than rhodamine WT. However, because of its superior properties it is recommended as the best quantitative tracer of the three green dyes tested. In nonqualitative work, especially for groundwater tracing, where photochemical decay is not a problem, the economy of using fluorescein, which is only 2.6 times more expensive than rhodamine WT, may be considered where more than one dye is to be injected simultaneously. Of the two blue dyes, whose cost effectivenesses are very similar, amino G acid has superior photochemical and adsorption characteristics and is therefore the most useful.

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#### REFERENCES

- Ahoad, K. A., J. P. Lawler, and M. D. Disco, Utility of radioisotope methodology in estuary pollution control study, I, Evaluation of the use of radioisotopes and fluorescent dyes for determining longitudinal dispersion, *Rep. NYO-3961-1*, p. 197, U.S. At. Energy Comm., New York, 1969.
- Akamatsu, K., and M. Matsuo, Safety of optical whitening agents (in Japanese), *Senryo To Yakuhin*, 18(2), 2-11, 1973. (English translation, *Transl. Programme RTS 9415*, Brit. Libr., Boston Spa, Yorkshire, England, June 1975.)
- Atkinson, T. C., D. I. Smith, J. J. Lavis, and R. J. Whitaker, Experiments in tracing underground waters in limestones, *J. Hydrol.*, 19, 323-349, 1973.
- Bandt, H. J., Giftig oder ungiftig für Fische?, *Deut. Fisch. Rundsch.*, 4(6), 170-171, 1957.
- Batsche, H., et al., Vergleichende Markierungsversuche im Mittelsteirischen Karst, 1966, *Steirische Beitr. Hydrogeol.*, 18/19, 331-403, 1966.
- Black, A. P., and R. F. Christman, Characteristics of coloured surface waters, *J. Amer. Water Works Ass.*, 56(6), 753-770, 1963.
- Boardman, G., and W. E. Worrall, Adsorption mechanisms of certain dyes on clays, *Trans. Brit. Ceram. Soc.*, 65, 343-362, 1966.
- Brown, M. C., and D. C. Ford, Quantitative tracer methods for investigation of karst hydrology systems, with reference to the Maligne basin area, Canada, *Trans. Cave Res. Group G. Brit.*, 13(1), 37-51, 1971.
- Brown, M. C., T. L. Wigley, and D. C. Ford, Water budget studies in karst aquifers, *J. Hydrol.*, 9(1), 113-116, 1969.
- Buchanan, T. J., Time of travel of soluble contaminants in streams, *J. Sanit. Eng. Div. Amer. Soc. Civil Eng.*, 90(SA3), 1-12, 1964.
- Christman, R. F., and M. Ghassemi, Chemical nature of organic colour in water, *J. Amer. Water Works Ass.*, 58(6), 723-741, 1966.
- Christman, R. F., and R. A. Minear, Fluorometric detection of lignin sulphates, *Trend Eng.*, 19(1), 3-7, 1967.
- Church, M., and R. Kellerhals, Stream gauging techniques for remote areas using portable equipment, *Tech. Bull. 25*, 90 pp., Dep. Energy Mines and Resour. of Can., Inland Waters Br., Ottawa, Can., 1970.
- Cobb, E. D., and J. F. Bailey, Measurement of discharge by dye dilution methods, in *Surface Water Techniques of the U.S. Geological Survey*, vol. 1, chap. 14, 26 pp., U.S. Geological Survey, Washington, D. C., 1965.
- Corey, J. C., Evaluation of dyes for tracing water movement in acid soils, *Soil Sci.*, 106(3), 182-187, 1968.
- Deaner, D. G., A procedure for conducting dye tracer studies in chlorine contact chambers to determine detention times and flow characteristics, *Reprint 11269*, 3 pp., G. K. Turner Associates, Palo Alto, Calif., 1970.
- Deaner, D. G., Effect of chlorine on fluorescent dyes, *J. Water Pollut. Contr. Fed.*, 45(3), 507-514, 1973.
- Dole, R. B., Use of fluorescein in the study of underground waters, in *U.S. Geol. Surv. Water Supply Pap. 160*, edited by M. C. Fuller, 73-83, 1906.
- Drew, D. P., Tracing percolation water in karst areas, *Trans. Cave Res. Group G. Brit.*, 10(2), 103-114, 1968.
- Drew, D. P., and D. I. Smith, Techniques for the tracing of subterranean drainage, *Brit. Geomorphol. Res. Group Tech. Bull.*, 2, 36 pp., 1969.
- Dumbauld, R. K., Meteorological tracer technique for atmospheric diffusion studies, *J. Appl. Meteorol.*, 1(4), 437-443, 1962.
- Dunn, B., and D. E. Vaupel, Effect of sample and fluorometer compartment temperatures on fluorometer readings, *U.S. Geol. Surv. Prof. Pap. 525D*, 225-227, 1965.
- Etzel, J. E., and C. P. L. Grady, Effect of dyes on an anaerobic system, in *Dyes and the Environment*, vol. 1, chap. 8, American Dye Manufacturers Institute, New York, 1973.
- Feuerstein, D. L., and R. E. Selleck, Fluorescent tracers for dispersion measurements, *J. Sanit. Eng. Div. Amer. Soc. Civil Eng.*, 89(SA4), 1-21, 1963.

- Frissel, M. J., and G. H. Bolt, Interaction between certain ionizable organic compounds (herbicides) and clay minerals, *Soil Sci.*, 94, 284-291, 1962.
- Ganz, C. R., J. Schulze, and P. S. Stensby, Accumulation and elimination studies of four detergent fluorescent whitening agents in bluegill (*Lepomis macrochirus*), *Environ. Sci. Technol.*, 9(8), 738-744, 1975.
- Glover, R. R., Optical brighteners—A new water tracing reagent, *Trans. Cave Res. Group G. Brit.*, 14(2), 84-88, 1972.
- Goodell, B. C., J. P. C. Watt, and T. M. Zorich, Stream flow volumes and hydrographs by fluorescent dyes, *Int. Union Forest. Res. Org.*, 1, 325-348, 1967.
- Grover, R., Adsorption of Pichloram by soil colloids and various other adsorbents, *Weed Sci.*, 19(4), 417-418, 1971.
- Hansen, W. H., O. G. Fitzhugh, and M. A. Williams, Subacute oral toxicity of nine D & C coal tar colours, *J. Pharmacol. Exp. Ther.*, 122, 29A, 1958.
- Hunter, J. V., Effect of dyes on aerobic systems, in *Dyes and the Environment*, vol. 1, chap. 6, American Dye Manufacturers Institute, New York, 1973.
- Jensen, S., and O. Pettersson, 2, 5-di-(benzoxazole-2-yl)thiophine, An optical brightener contaminating sludge and fish, *Environ. Pollut.*, 2, 145-155, 1971.
- Keplinger, M. L., O. E. Fancher, F. L. Lyman, and J. C. Calandra, Toxicological studies of four fluorescent whitening agents, *Toxicol. Appl. Pharmacol.*, 27, 494-506, 1974.
- Kilpatrick, F. A., Dye-dilution discharge measurements made under total ice cover in Laramie River at Laramie, Wyoming, *U.S. Geol. Surv. Water Resour. Div. Bull.*, 41-47, July/Dec. 1967.
- Kilpatrick, F. A., Flow calibration by dye dilution measurement, *Civil Eng.*, 38(2), 74-76, 1968.
- Kilpatrick, F. A., Dosage requirements for slug injections of Rhodamine BA and Rhodamine WT dyes, *U.S. Geol. Surv. Prof. Pap. 700B*, 250-253, 1970.
- Kilpatrick, F. A., W. W. Sayre, and E. V. Richardson, Discussion—Flow measurements with fluorescent tracers, *J. Hydraul. Div. Amer. Soc. Civil Eng.*, 93(HY4), 298-308, 1967.
- Knochenmus, D. D., Tracer studies and background fluorescence of groundwater in the Ocala, Florida area, open file report, 35 pp., U.S. Geol. Surv., Washington, D. C., Nov. 1967.
- Lamar, W. L., Evaluation of organic colour and iron in natural surface waters, *U.S. Geol. Surv. Prof. Pap. 600D*, 24-29, 1968.
- Lewis, D. C., G. J. Kriz, and R. H. Burghy, Tracer dilution sampling technique to determine hydraulic conductivity of fractured rock, *Water Resour. Res.*, 2(3), 533-542, 1966.
- Little, L. W., and J. C. Lamb, Acute toxicity of 46 selected dyes to Fathead minnow (*Pimephales promelas*), in *Dyes and the Environment*, vol. 1, chap. 5, American Dye Manufacturers Institute, New York, 1973.
- Marking, L. L., Toxicity of rhodamine B and fluorescein sodium to fish and their compatibility with antimycin A, *Prog. Fish Cult.*, 31, 139-142, 1969.
- Mather, J. D., D. A. Gray, and D. G. Jenkins, The use of tracers to investigate the relationship between mining subsidence and groundwater occurrence of Aberfan, South Wales, *J. Hydrol.*, 9, 136-154, 1969.
- Panciera, M., Toxicity of Rhodamine B to eggs and larvae of *Crassostrea virginica*, *Proc. Nat. Shellfish. Ass.*, 58, 7-8, 1967.
- Parker, G. G., Tests of Rhodamine WT dye for toxicity to oysters and fish, *J. Res. U.S. Geol. Surv.*, 1(4), 499, 1973.
- Pauli, F. W., Fluorochrome adsorption studies on decomposing plant residues, 2, Adsorption studies, *S. Afr. J. Agr. Sci.*, 4(3), 281-292, 1961.
- Petri, L. R., and J. L. Craven, Dilution helpful in measuring fluorescence of samples containing much sediment, *U.S. Geol. Surv. Water Resour. Div. Bull.*, 24-25, April/Sept. 1971.
- Pritchard, D. W., and J. H. Carpenter, Measurement of turbulent diffusion in estuarine and inshore waters, *Bull. Int. Ass. Sci. Hydrol.*, 20, 37-50, 1960.
- Rabinowitch, E. I., *Photosynthesis*, vol. 2, Interscience, New York, 1951.
- Reynolds, E. R. C., The percolation of rainwater demonstrated by fluorescent dyes, *J. Soil Sci.*, 17(1), 127-132, 1966.
- Robinson, D. W., and D. Donaldson, Pontacyl brilliant pink as a tracer dye in the movement of water in phreatophytes, *Water Resour. Res.*, 3(1), 203-211, 1967.
- Rochat, J., J. Alary, J. Molinari, and R. Charrière, Séparations physicochimiques de colorants Xanthéniques utilisés comme traceurs en hydrologie, *J. Hydrol.*, 26, 277-293, 1975.
- Scaff, M. R., J. L. Withero, and C. P. Priesing, Iron-59 as a solids tracer in aqueous suspensions, *J. Sanit. Eng. Div. Amer. Soc. Civil Eng.*, 94(SA6), 1195-1211, 1968.
- Scanlan, J. W., Evaluation and application of dye tracing in a karst terrain, M.Sc. dissertation, Univ. of Mo., Rolla, 1968.
- Scott, C. H., V. W. Norman, and F. K. Fields, Reduction of fluorescence of two tracer dyes by contact with a fine sediment, *U.S. Geol. Surv. Prof. Pap. 650B*, 164-168, 1969.
- Seal, B. K., K. B. Roy, and S. K. Mukherjee, Fluorescent emission spectra and structure of humic and fulvic acids, *J. Indian Chem. Soc.*, 41(3), 212-214, 1964.
- Smart, P. L., and D. I. Smith, Water tracing in tropical regions: The use of fluorometric techniques in Jamaica, *J. Hydrol.*, 30, 179-195, 1976.
- Smart, P. L., B. L. Finlayson, W. D. Ryland, and C. M. Ball, The relation of fluorescence to dissolved organic carbon in surface waters, *Water Res.*, 10, in press, 1976.
- Smith, S. A. R., and L. G. Kepple, Infiltration measure in sanitary sewers by dye-dilution method, *Water Sewage Works*, 58-61, Jan. 1972.
- Society of Dyers and Colourists, *Colour Index*, 3rd ed., Bradford, England, 1971.
- Sowards, C. L., Sodium fluorescein and the toxicity of Pronoxfish, *Prog. Fish Cult.*, 20(1), 20, 1958.
- Sturm, P. W., and W. E. Johnson, Field experiments with chemical tracers in flood waters, *Prod. Mon.*, 11-17, Dec. 1950.
- Sturm, R. N., and K. E. Williams, Fluorescent whitening agents: Acute fish toxicity and accumulation studies, *Water Res.*, 9, 211-219, 1975.
- Talbot, J. W., and J. L. Henry, The adsorption of rhodamine B on to materials carried in suspension by inshore waters, *J. Cons. Cons. Perma. Int. Explor. Mer.*, 23(1), 7-16, 1968.
- Udenfriend, S., *Fluorescence Assay in Biology and Medicine*, 517 pp., Academic, New York, 1962.
- Umeda, M., Experimental study of xanthene dyes as carcinogenic agents, *Gann*, 47, 51-78, 1956.
- Von Möser, H., and H. Sagl, Die Direktmessung hydrologischer Farbtracer im Gelände, *Steirische Beitr. Hydrogeol.*, 18/19, 179-183, 1967.
- Watt, J. P. C., Development of the dye-dilution method for measuring water yields from mountain watersheds, M.Sc. dissertation, Colo. State Univ., Fort Collins, 1965.
- Webb, J. M., and W. H. Hansen, Studies of the metabolism of Rhodamine B, *Toxicol. Appl. Pharmacol.*, 3(1), 86-95, 1961.
- Webb, J. M., W. H. Hansen, A. Desmond, and O. G. Fitzhugh, Biochemical and toxicological studies of Rhodamine B and 3,6-diaminofluoran, *Toxicol. Appl. Pharmacol.*, 3(6), 696-706, 1961.
- Webb, J. M., M. Fonda, and E. A. Brouwer, Metabolism and excretion patterns of fluorescein and certain halogenated fluorescein dyes in rats, *J. Pharmacol. Exp. Ther.*, 137(2), 141-147, 1962.
- Wilson, J. F., Fluorometric procedures for dye tracing, in *Techniques of Water Resources Investigations of the U.S. Geological Survey*, vol. 3, 31 pp., U.S. Geological Survey, Washington, D. C., 1968.
- Wimpenny, J. W. T., N. Cotton, and M. Statham, Microbes as tracers of water movement, *Water Res.*, 6, 731-739, 1972.
- Woeleke, C. E., Development of a receiving water quality bioassay criterion based on the 48 hr Pacific oyster (*Crassostrea gigas*) embryo, *Tech. Rep. 9*, Wash. Dep. Fish., 1972.
- Wright, R. R., and M. R. Collings, Application of fluorescent tracing techniques to hydrologic studies, *J. Amer. Water Works Ass.*, 56, 748-754, 1964.
- Yates, W. E., and N. B. Akesson, Fluorescent tracers for quantitative microresidue analysis, *Trans. ASA E.*, 6, 104-114, 1963.
- Yotsukura, N., H. B. Fischer, and W. W. Sayre, Measurement of mixing characteristics of Missouri River between Sioux City, Iowa and Plattsmouth, Nebraska, *U.S. Geol. Surv. Water Supply Pap. 1899G*, 29 pp., 1970.

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