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 *p*H 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 adorbed 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

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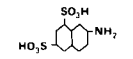
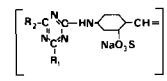
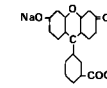
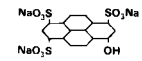
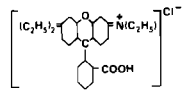
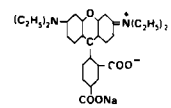
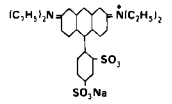
TABLE 1. Generic and Alternative Names and Chemical Structure of the Tracer Dyes

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

*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 reemitted 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, minimize background, 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
Blue Fluorescent Dyes					
Amino G acid	355 (310)	445	7-37*	365	98†
Photine CU	345	435 (455)			
Green Fluorescent Dyes					
Fluorescein	490	520	98†	436	55†‡
Lissamine FF	420	515			
Pyranine	455 (405)	515			
Orange Fluorescent Dyes					
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, *p*H is 7.0.

*Corning filter.

†Kodak Wratten filter.

‡See text.

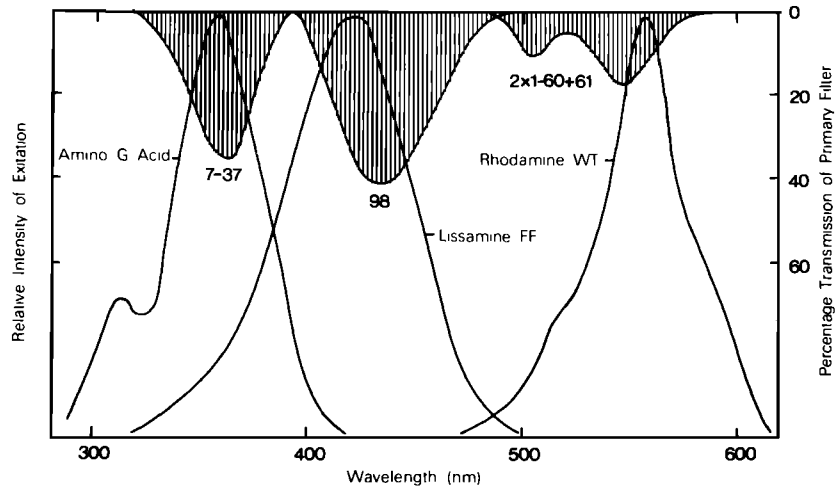


Fig. 1*a*. Excitation spectra of amino G acid, lissamine FF, and rhodamine WT and transmission characteristics of primary filters (shaded).

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 wavelengths 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 *p*H 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 1-56 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

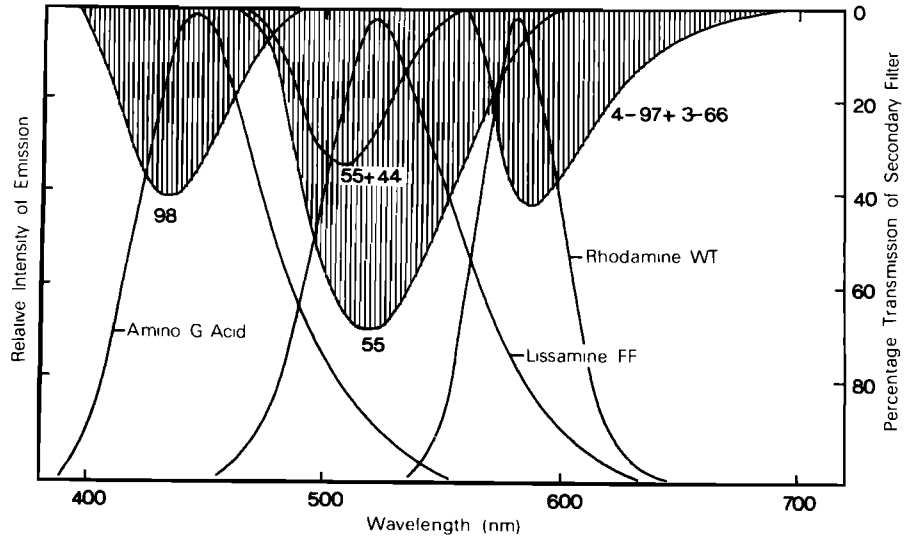


Fig. 1*b*. Emission spectra of amino G acid, lissamine FF, and rhodamine WT and transmission characteristics of secondary filters (shaded).

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

Dye	Sensitivity,* $\mu g\ l^{-1}$/scale unit	Background Reading,† scale units 0–100	Minimum Detectability,‡ $\mu g\ l^{-1}$
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 *p*H 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 $\mu g\ l^{-1}$ 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 [*Abood 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 $\mu g\ l^{-1}$ of rhodamine WT and 60 $\mu g\ l^{-1}$ 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, $°C^{-1}$
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

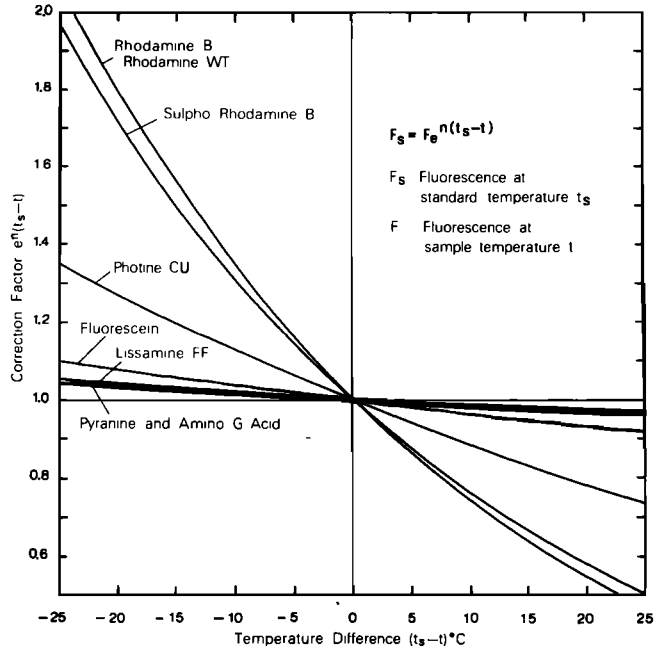


Fig. 2. Temperature correction curves for the tracer dyes.

show no interference until concentrations of lissamine FF exceed 120 $\mu g\ l^{-1}$ and 400 $\mu g\ l^{-1}$, 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-

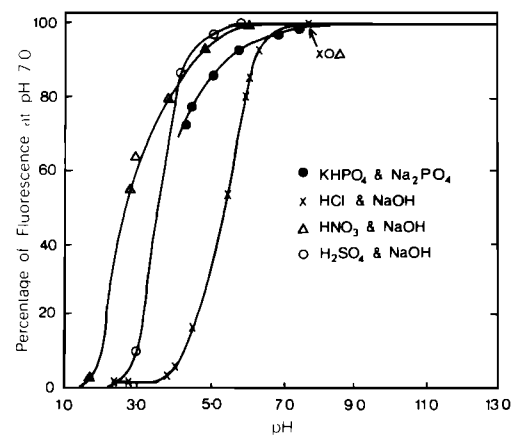


Fig. 3. Effect of *p*H on fluorescence of rhodamine WT, different acids being used.

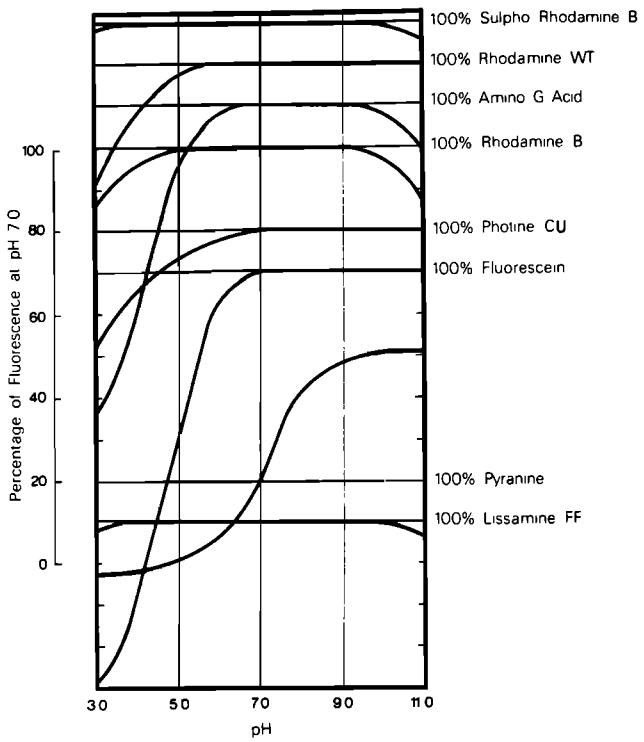


Fig. 4. Effect of *p*H on fluorescence of the tracer dyes.

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.

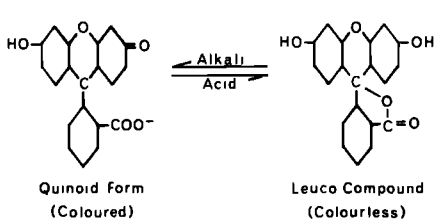


Fig. 5. Structure of fluorescein under acid and alkali conditions.

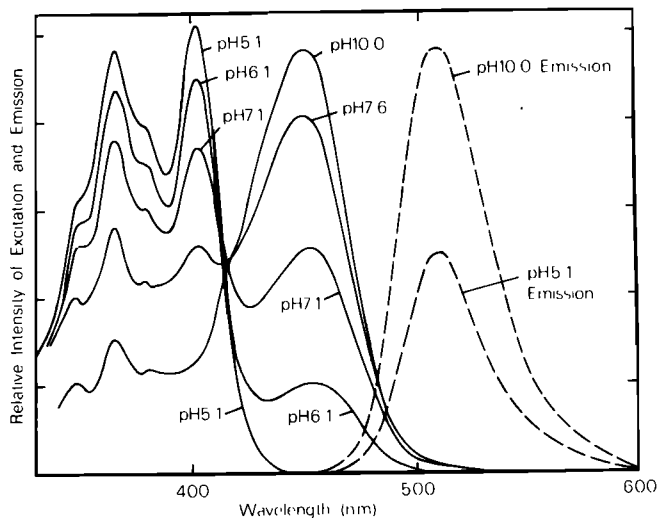


Fig. 6. Effect of *p*H on excitation and emission spectra of pyranine.

The least sensitive, amino G acid, has a minimum detectability such that it can be resolved at a dilution of 1 part in 2×10^9 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°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	98
Rhodamine WT	97	92
Sulpho rhodamine B	100	96

Figures are percentage of fluorescence in distilled water.

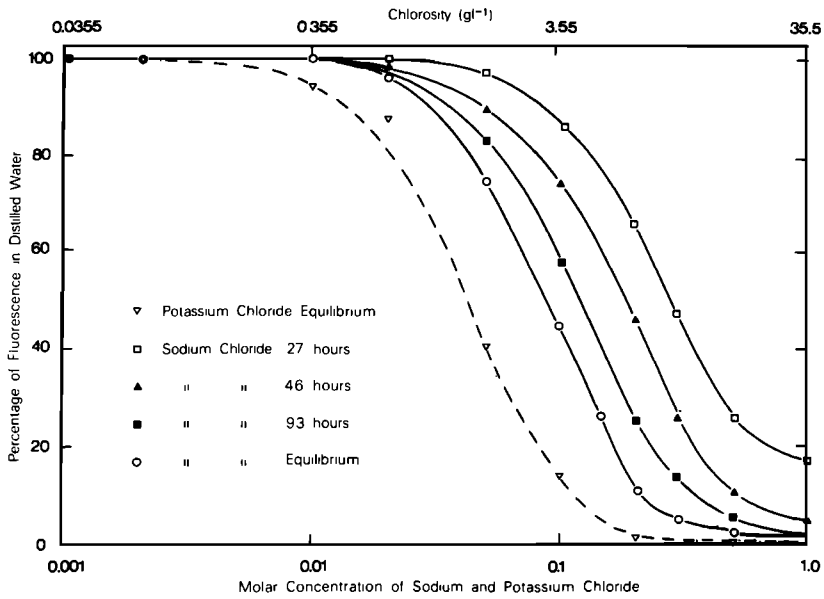


Fig. 7. Effect of sodium and potassium chloride on fluorescence of rhodamine WT.

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 *p*H for rhodamine WT. The curves were prepared by using three different acids and a phosphate buffer sytem to lower the *p*H 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 *p*H/fluorescence curves. It will often be more satisfactory to use natural water samples to prepare these curves when waters of high or low *p*H are to be traced.

The standard curves presented in Figure 4 were prepared by using *p*H 4.0, 7.0, and 9.2 buffer tablets (obtained from British Drug Houses Ltd.) with hydrochloric acid and sodium hydroxide to extend the *p*H range. Figure 4 indicates that *p*H variations between *p*H 4.0 and *p*H 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 *p*H 5.0, amino G acid below *p*H 6.0, and fluorescein and photine CU below *p*H 6.5. Some correction should be considered in waters with a *p*H lower than these values. Pyranine shows excessive variation in fluorescence with *p*H 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

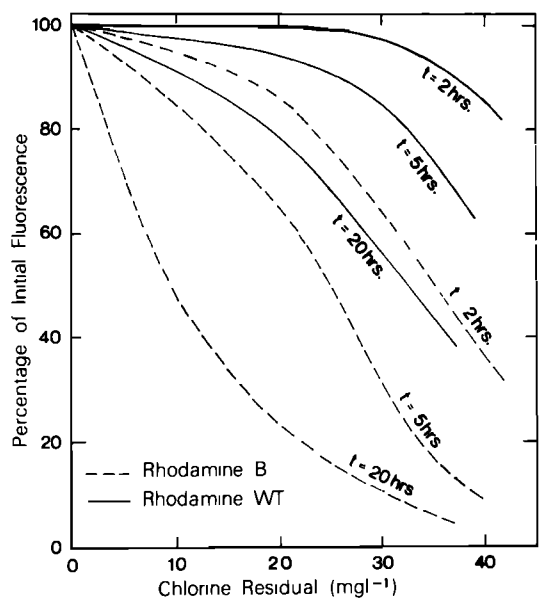


Fig. 8. Effect of chlorine residual concentration on fluorescence of rhodamine WT and rhodamine B in activated sludge (10 μg l^{-1}, initial dye concentration, 22 mg l^{-1}, suspended solids). Data are from *Deaner* [1973, Table 1].

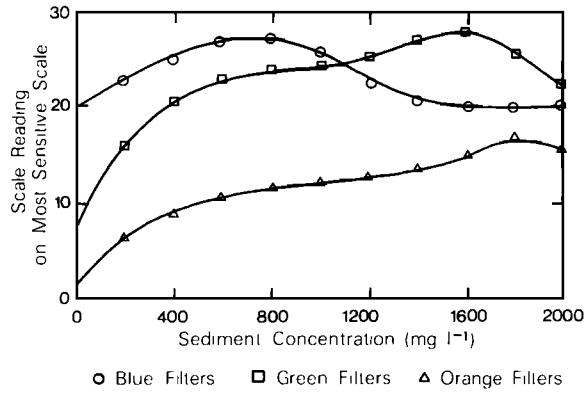


Fig. 9. Effect of suspended sediment concentration on background readings for the blue, green, and orange filter combinations.

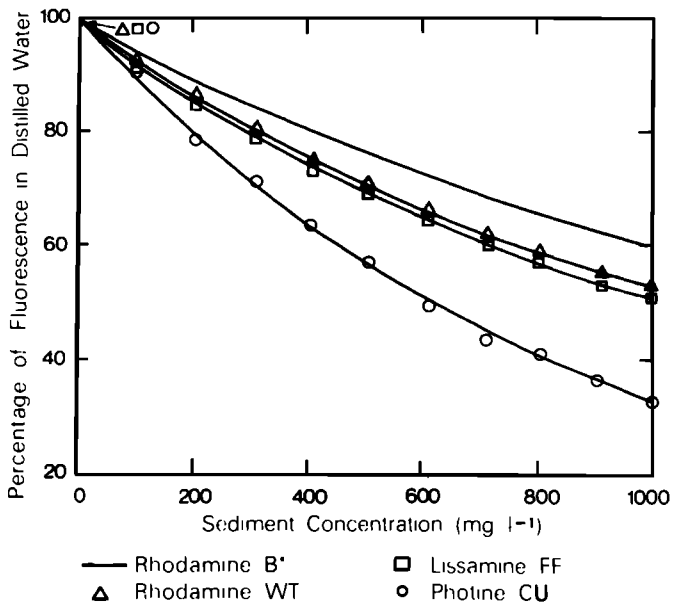


Fig. 10. Effect of suspended sediment concentration on fluorescence of rhodamine WT, lissamine FF, and photine CU.

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 *p*H changes: ionization and structural changes. The dyes examined are anionic (except rhodamine B, which is cationic), and as *p*H 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 *p*H 4 and *p*H 6, compared with *p*H 6–7.5 for phenolic groups and below *p*H 5 for sulphonate groups. Thus the dyes having sulphonate acid groups remain fluorescent to lower *p*H values, as is exemplified by lissamine FF and sulpho rhodamine B.

In some xanthene dyes, structural changes may occur as *p*H 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 *p*H 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 *p*H 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 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 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.

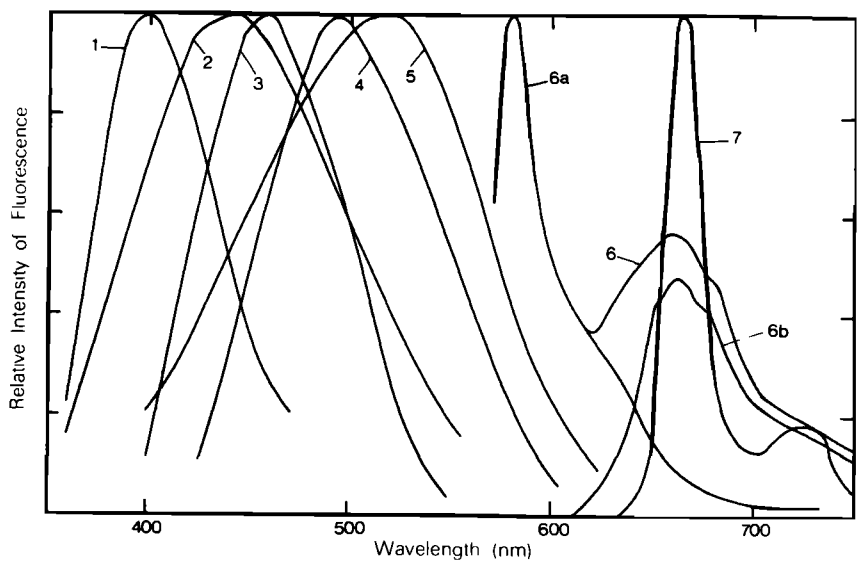


Fig. 11. Emission spectra of some naturally occurring pigments and fluorescent compounds. Curve 1, lignin sulphonates [*Christman and Minear*, 1967, Figure 3]; curve 2, River Frome [*Smart et al.*, 1967, Figure 1]; curve 3, Douglas fir bark extract [*Christman and Ghassemi*, 1966, Figure 3]; curve 4, 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 6*a*) and phycocyanin (curve 6*b*) [*Rabinowitch*, 1951, Figure 23.9B]; and curve 7, chlorophyll *a* [*Rabinowitch*, 1951, Figure 23, 2].

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

Sample Origin	Samples	Blue Filters* $\bar{X}$	Blue Filters* s.d.	Green Filters* $\bar{X}$	Green Filters* s.d.	Orange Filters* $\bar{X}$	Orange Filters* s.d.
Karst Resurgence, Mendips							
46 hours during a storm event	24	...	...	11.9	6.3	...	...
46 hours after a storm event	23	...	...	6.9	0.4	...	...
Agricultural Catchment, South Cotswolds							
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
Karst Area, Central Jamaica West Indies (3 weeks in June 1975)							
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 $\mu g\ l^{-1}$.

†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.

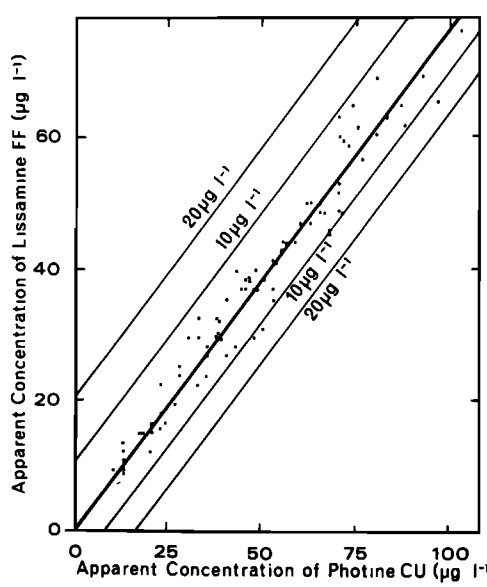


Fig. 12. Correlation between background fluorescence at the green and blue wavelengths expressed as apparent concentrations of lissamine FF and photine CU.

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 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 mg l^{-1} compared with 22 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
Pritchard and Carpenter [1960]								
Artificial light						1.7×10^{-5}		
Sunny			1.3×10^{-1}			1.7×10^{-4}		
Feuerstein and Selleck [1963]								
Cloudy			5.1×10^{-2}			4.5×10^{-3}		2.0×10^{-3}
Sunny			2.6×10^{-1}			2.2×10^{-2}		1.0×10^{-2}
Yates and Akesson [1963]*								
Minimum rate			4.5×10^{-2}	0		3.6×10^{-2}		4.4×10^{-2}
Maximum rate			3.9×10^{-1}	4.6×10^{-1}		1.2×10^{0}		6.4×10^{-1}
Watt [1965]								
Sunny, 10 $\mu g\ l^{-1}$						5.6×10^{-4}		5.6×10^{-4}
Sunny, 100 $\mu g\ l^{-1}$						3.4×10^{-4}		3.4×10^{-4}
Von Möser and Sagl [1967]								
Cloudy			1.5×10^{-2}	8.0×10^{-4}				1.8×10^{-3}
Sunny			2.6×10^{-1}	7.4×10^{-3}				1.0×10^{-2}
Abood et al. [1969]								
Sunny						8.3×10^{-3}	1.5×10^{-3}	5.6×10^{-3}
6 hours, Sunny, 100 $\mu g\ l^{-1}$†	1.6×10^{-2}	$>6.4 \times 10^{-1}$	9.5×10^{-2}	$<1.0 \times 10^{-4}$	1.2×10^{-1}	5.5×10^{-4}	$<1.0 \times 10^{-4}$	3.3×10^{-4}
6 hours under 60-W lamp, 100 $\mu g\ l^{-1}$†	3.7×10^{-4}	5.5×10^{-2}	1.3×10^{-2}	$<1.0 \times 10^{-4}$	1.6×10^{-2}	1.5×10^{-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 absorbence 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 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 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 $\mu g\ l^{-1}$ 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 $\mu g\ l^{-1}$. It is clear that a minimum concentration of 15 $\mu g\ l^{-1}$ of lissamine FF and 20 $\mu g\ l^{-1}$ 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.

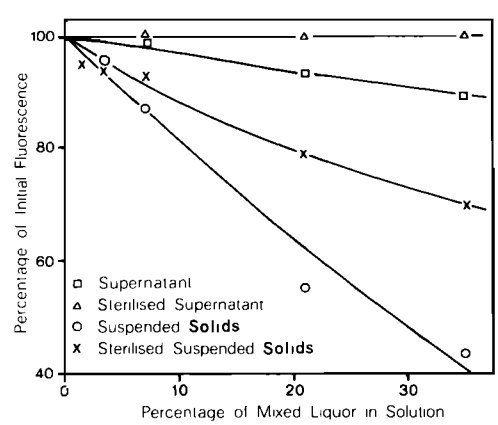


Fig. 13. Comparison of effect of active and sterilized mixed liquor on rhodamine WT.

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 *p*H 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 many field situations but are designed to enable comparison of 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-μm sieve and consisted of an orthoquartzite sandstone (100% quartz), a pure limestone (99% calcium carbonate), and two British Pharmacopeia 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 ($Al_2Si_2O_5(OH)_4$). 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; *p*H, 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 FF 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^{-1} of humus the apparent adsorption loss was negligible, whereas actual losses were over 50%. When the actual

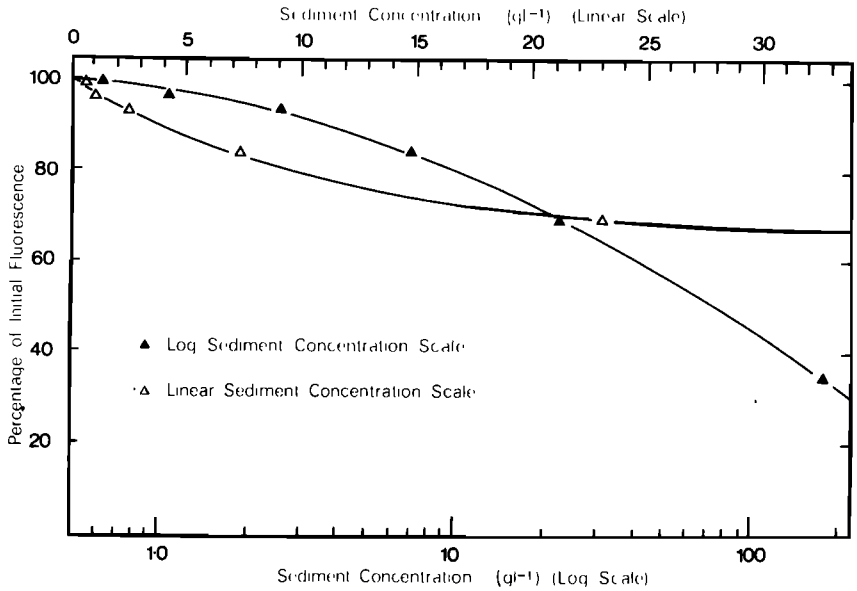


Fig. 14. Effect of sediment concentration on dye adsorption for rhodamine WT. Data are from *Scott et al.* [1969, Figure 2].

dye fluorescence falls below the background fluorescence, the accuracy of the results is dominated by the variability of the background. This was commonly ±10%; therefore for large adsorptive dye losses the error may approach ±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 g l^{-1} of humus gave an apparent concentration of 1.2 $\mu g\ l^{-1}$ of rhodamine WT, 70 $\mu g\ l^{-1}$ of lissamine FF, and 80 $\mu g\ l^{-1}$ 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 adsorptive 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]

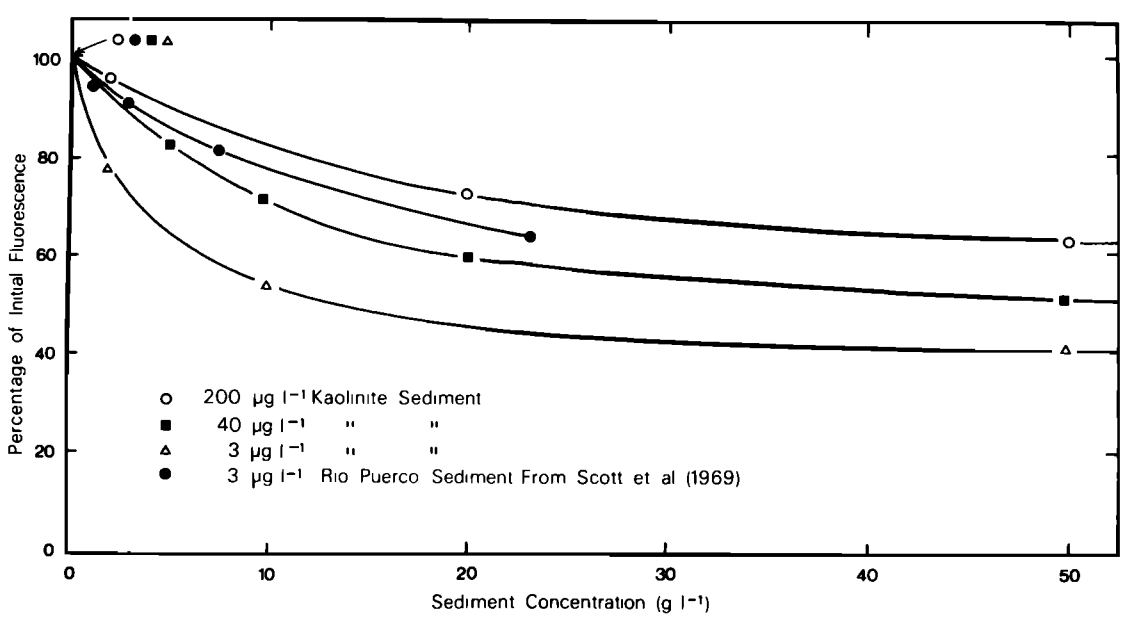


Fig. 15. Effect of dye concentration on dye adsorption for rhodamine WT.

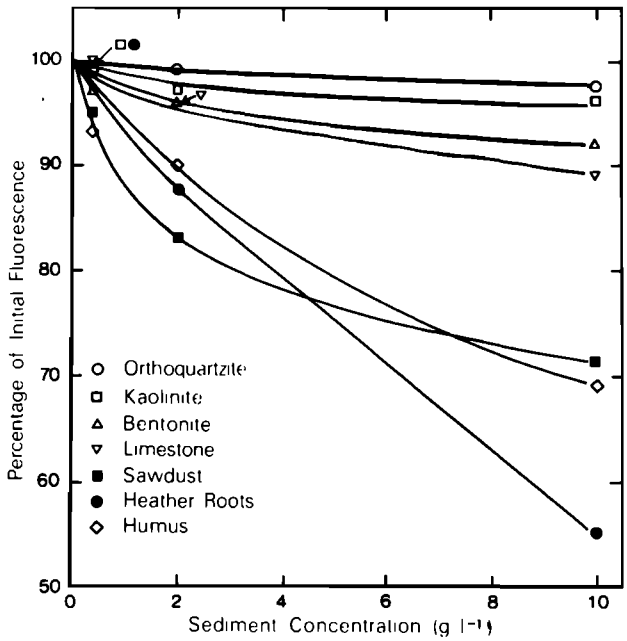


Fig. 16. Effect of sediment type on dye adsorption for lissamine FF. Initial dye concentrations were 200 μg l^{-1}.

containing 'abundances of illite, montmorillonite, and kaolinite materials' adsorbed almost exactly the same amount of rhodamine B at a sediment concentration of 200 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.

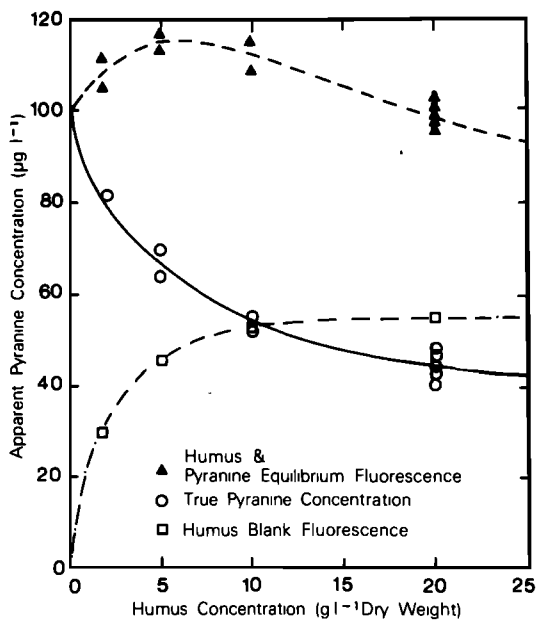


Fig. 17. Effect of humus background fluorescence on apparent concentration of pyranine in adsorption experiments.

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 μg 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 *p*H 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 *p*H 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 *p*H 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 *p*H 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 μg 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

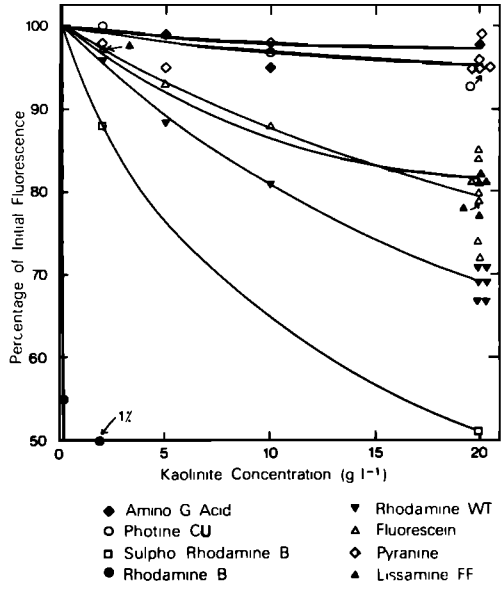


Fig. 18. Comparison of adsorption of the tracer dyes on kaolinite sediment. Initial dye concentrations were 100 μg l^{-1}.

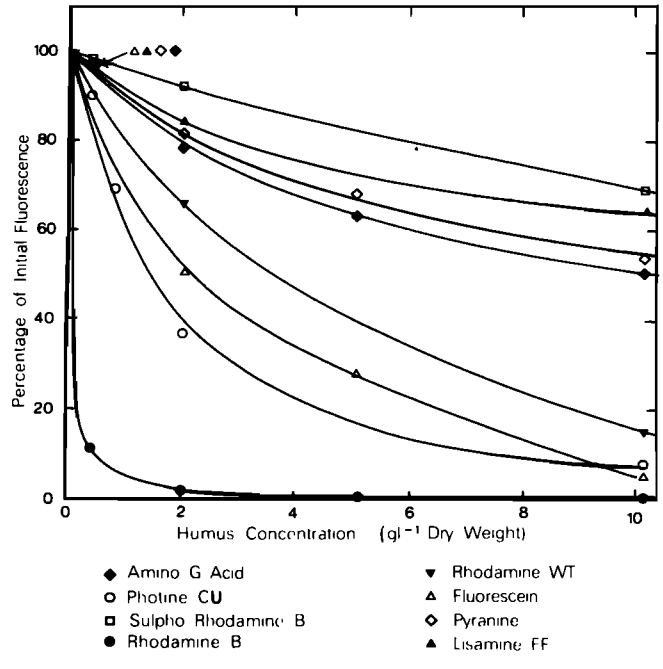


Fig. 19. Comparison of adsorption of the tracer dyes on humus sediment. Initial dye concentrations were 100 μg l^{-1}.

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

Dye	Species	LC_{50}, mg l^{-1}			
		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^{-1} solution. *Bandt* [1957] showed that fluorescein at 100 mg l^{-1} 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_{50}'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 machrochirus*) for 24, 48, and 96 hours at 12°C (Table 9). Rhodamine B was considerably more toxic than fluorescein, and the LC_{50} 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^{-1} for 17.5 hours at 22°C or an additional 3.5 hours at 375 mg l^{-1} [*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^{-1} [*Akamatsu and Matsuo*, 1973]. Ninety-six-hour LC_{50}'s determined for a number of brighteners by *Keplinger et al.* [1974] and *Sturm and Williams* [1975] ranged from 32 to 474 mg l^{-1} for bluegill (*Lepomis machrochirus*), from 108 to 1780 mg l^{-1} for rainbow trout (*Salmo gairdnerii*), and from 86 to 1060 mg l^{-1} for channel catfish (*Ictalurus punctatus*) (cf. Table 9). *Akamatsu and Matsuo* [1973] report LC_{50}'s as high as 2 g l^{-1}, 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].

Panciera [1967] has shown that 2-day-old oyster larvae (*Crassostrea virginica*) died within 2 days at 100 mg l^{-1} concentrations of rhodamine B, showed temporary retardation of growth at 10 mg l^{-1}, and suffered no ill effects at 1 mg l^{-1}. Similarly, at the high concentration, no eggs developed to the larval stage, while 27% had some abnormality at 10 mg l^{-1}, and none were affected at 1 mg l^{-1}. *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^{-1} 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^{-1}, but the dye was rapidly cleared once the clams were placed in dye-free water. At concentrations above 8.4 mg l^{-1} the clams showed avoidance reactions and contact staining. *Woelke* [1972] has observed that sea urchin eggs (species *hemicentrotus*) were affected by rhodamine B at 32 mg l^{-1} but not at 10 mg l^{-1}, 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^{-1} showed no mortality of any species over periods of 48 hours and 1 week compared to the control animals (J. S. Worttley 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 silbene triazine type have LC_{50} 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 $\mu g\ l^{-1}$ 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 μg 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 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 $g\ kg^{-1}$ 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 mg/d, which is unlikely to be exceeded if concentrations at the intake remain below 370 $\mu g\ l^{-1}$. The U.S. Geological Survey recommends that tracer tests aim for a final concentration not exceeding 10 $\mu g\ l^{-1}$ 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 chlorophenols 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 mg l^{-1} of residual chlorine and three different concentrations of rhodamine B. All nine were able to detect the astringent taste at 50 $\mu g\ l^{-1}$, only four at 10 $\mu g\ l^{-1}$, and none at 5 $\mu g\ l^{-1}$.

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 $\mu g\ l^{-1}$. Samples with concentrations as high as 10,000 $\mu g\ l^{-1}$ were found to cause little significant taste, though these were not presented to the whole tasting panel. At concentrations of 100 $\mu g\ l^{-1}$ 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 (0.1–1.0 $g\ l^{-1}$), 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 $\frac{1}{8}$–$\frac{1}{16}$ the amount of pyranine used. *Atkinson et al.* [1973], in contrast, present data indicating a recovery of pyranine 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 *p*H 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 *p*H (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 *p*H 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.

TABLE 10. Comparison of Tracer Dyes in a Peaty Stream

Dye	Background Reading, scale units	Peak Reading, scale units	Peak Concentration, $\mu g\ l^{-1}$	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.

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 *p*H. 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 *p*H in the range normally encountered in natural waters, it cannot be recommended as a quantitative dye tracer unless *p*H 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 11. Cost Effectiveness of the Tracer Dyes

Dye	State	Cost per Kilogram,* £	Volume Labeled per Kilogram, 10^6 m^3 kg^{-1} †	Volume Labeled per £, 10^6 m^3 £$^{-1}$	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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