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FLUOROMETRIC PROCEDURES FOR DYE TRACING

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BOOK 3

APPLICATIONS OF HYDRAULICS

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Photomultiplier

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

Range control

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

Sensitivity, or range control, is electronically adjusted in the FLM/AMINCO fluorocolorimeter by use of multiplier and fine-adjust controls. The multiplier control is used to select one of seven ranges of sensitivity available in the measuring circuit. These scales of relative intensity are as follows: ×100, ×30, ×10, ×3, ×1, ×.3, and ×.1. All scales are exact multiples. For example, when the 0 to 0.1 (×.1) scale is selected, 0.1 is full scale. When the multiplier switch is changed to the 0 to 1.0 (×1) scale, 0.1 is one-tenth of full scale and indicates the same relative intensity. This allows background to be suppressed on the most sensitive scale and to remain suppressed on all scales. This, along with the fact that scales are exact multiples, is very useful during calibration when performing precision analysis.

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

Preferred systems

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

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

Accessory equipment

Temperature-control apparatus

Because fluorescence depends on temperature, all fluorometer readings must be either

taken at or adjusted to a common temperature. Except for very precise work—discharge measurements, for example—special equipment for controlling temperature usually is not necessary. The procedures for minimizing the need for temperature corrections and for making such corrections are discussed elsewhere in this manual.

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

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

For temperature control in a laboratory setup with the Turner model 111 fluorometer, the constant-temperature door should be used when analyzing samples that have been brought to room temperature. For best results, the temperature of the circulating water in the door should be adjusted to that of room temperature. Another excellent setup is to combine the constant-temperature door with a constant-temperature bath: Samples can be stored in the bath before testing and the bath water circulated through the fluorometer door.

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

Portable power supplies

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

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

the FLM/AMINCO fluorocolorimeter or the Turner Designs model 10.

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

A constant-voltage transformer should always be used between the converter and the Turner model 111 fluorometer. However, the drain on the battery is considerable, and even with fresh batteries and constant attention to conservation, readout trouble and a dead battery may occur. An alternating-current voltmeter is a very useful accessory for monitoring the condition of the system. When a drop of 2 volts or more in transformer output is observed, the system should be shut down immediately and the battery recharged. If the battery of an automobile is used, running the vehicle's engine during operation of the fluorometer will help, but it will not keep up entirely with the drain on the battery. Intermittent use of the system is the best procedure. The data obtained using this procedure can be considered only approximate because of inadequate fluorometer warmup time. The Turner model 111 requires at least a 1-hour warmup time for analytical stabilization, the Turner Designs model 10 about 5 minutes, and the FLM/AMINCO fluorocolorimeter about 10 minutes.

Some fluorometers require less power and are more readily adapted to field requirements than others. For example, the Turner Designs model 10 fluorometer can operate directly from a 12-volt storage battery, 115 volts, or 230 volts alternating current. The current demand for the fluorometer, when operating from a 12-volt battery, is only 2 amperes. An external power converter and a constant-voltage transformer are not necessary with this piece of equipment.

Continuous-sampling equipment

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

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

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

Water may leak around various fittings if pressures in the flow-through system are high. Discharge lines from the fluorometer should be kept short to reduce back-pressure. Also, pumps should be sized to produce a good rate of flow through the system without creating excessive pressure in the system. The system should be checked for leaks with the pump running, but before the fluorometer has been turned on.

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

Fluorometer Operation

General procedure in the laboratory

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

Preparations

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

1. Prepare samples. For best results, stream samples should be allowed to stand overnight to stabilize the temperature, to settle out any suspended matter, and to allow dissolved oxygen to come out of solution. High turbidity may require centrifuging or a longer settling time. Always protect samples from sunlight. Standards samples and background samples should be treated in the same manner as river samples, being allowed to come to the same temperature as the stream samples.
2. Check lamp type. Use the far-UV lamp or the green T-5 lamp with rhodamine WT or pontacyl pink and the far-UV lamp with acid yellow 7.
3. Check filter placement. The primary filters (green for rhodamine WT and pontacyl pink and dark blue for acid yellow 7) must be placed between the lamp and the sample. The secondary filters (orange and blue, with the blue filter nearest the sample, for rhodamine WT and pontacyl pink, and dark yellow for acid yellow 7) must be placed between the sample and the photomultiplier tube. For consistent orientation, place a mark, if none exists, in a corner of all filters and always position the filters in the fluorometer so that the mark is in the lower right-hand corner.
4. Check location and position of fluorometer. Unless the fluorometer is completely light-shielded by the manufacturer, the fluorometer will need to be externally shielded from direct or strongly reflected sunlight. It should also be placed in a location that is not expected to experience rapid changes in air temperature. Place the fluorometer on a level, firm base. Allow room for air circulation, especially if the instrument has a cooling fan. For example, don't operate a fluorometer on a car seat as the cushion may restrict a bottom cooling fan.
5. Hook up constant-temperature door, if used. Start circulation of cooling water with door removed to avoid damage due to unexpected leakage.
6. Plug in fluorometer cord; have all panel switches off.
7. Turn on fluorometer. Various fluorometers have different means of doing this. The Turner model 111 fluorometer has both a power and a start switch. The power switch must be turned on and then the start switch held in the "up" position for about 10 seconds to turn on the lamp and assure vaporization of the mercury. For this instrument, verification that the lamp is on should be made by observing the lamp through the primary filter. CAUTION—Do not view the lamp directly; ultraviolet rays can injure the eyes. If power is interrupted, even momentarily, the lamp has to be restarted.

   Some fluorometers have a single "on" switch to start the fluorometer. Carefully check the instruction manual for the fluorometer being used for the appropriate startup procedure.
8. Allow adequate time for fluorometer warmup. Different fluorometers require different warmup times. The Turner model 111 fluorometer requires at least 1 hour, and preferably 2 hours, to adequately stabilize the operating temperature. The Turner Designs model 10 fluorometer is supposed to be stabilized for most uses within 5 minutes of startup. The FLM/AMINCO fluorocolorimeter requires about 10 min-

utes of warmup. For high-accuracy work, a longer warmup time should be provided to allow the temperature of the filters to stabilize. When mercury lamps are used, the fluorometer should be allowed to cool off, when the instrument is turned off, before restarting. The analysis of any samples prior to complete warmup will provide only approximate data. Carefully check the appropriate manual for the warmup time required for any specific instrument.

Background readings

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

1. Light emitted by other fluorescent material present in the water—always possible, but not usually a problem with rhodamine WT or pontacyl pink with the recommended filters.
2. Light leakage from outside the fluorometer.
3. Light scattered by turbidity of the sample. Tyndall scatter occurs at about the same wavelength as the light reaching the sample. High turbidity may cause some light to pass the 590 secondary filter; the 23A filter passes more of this kind of light and should not be used with turbid samples.
4. Light from the lamp passing straight through the filter system.
5. Light scattered or fluoresced by the filter or the sample holder. In dye tracing, these problems usually are not significant if proper precautions are taken.
6. Light scattered by the water. Rayleigh scatter occurs at the wavelength of light reaching the sample and is cut off effectively by either the 90 or 23A secondary filters. Raman scatter always occurs at wavelengths longer than those of the exciting light. With excitation at 546 nm, the 590 filter effectively cuts off the Raman spectrum of water; the 23A filter does not.
7. Secondary Rayleigh, Raman, and Tyndall scatter peaks, as well as secondary fluorescence peaks, which occur at wavelengths approximately double those of their respective primary peaks. The 590 filter eliminates the secondary peaks; the 23A filter does not.

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

Sample testing

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

1. Rinse cuvette once with tapwater and (or) with the solution to be tested, then fill to within about 0.25 in (6 mm) of the top with the solution to be tested. Wipe outside of cuvette with laboratory tissue. If air bubbles are visible on the inside of the cuvette, dislodge them by gently tapping the cuvette. Handle cuvettes only near the top. Avoid spilling solution into the cuvette holder. For best results, use the same cuvette for testing all samples.
2. Insert cuvette in the fluorometer. Close the door gently but tightly to avoid spillage or, in the case of the Turner Designs model 10 or the FLM/AMINCO fluorocolorimeter, place the cap back over the cuvette compartment.
3. Observe the dial reading after the dial has stabilized. A jittery dial (±1/4 dial division) is normal for some fluorometers. For the greatest sensitivity, read each sample on the most sensitive scale possible. It is unnecessary to read every sample on every scale, although occasionally it may be desirable to read the same sample on two

scales. Another practice sometimes employed is to use only the most sensitive scale on which all samples can be read. If samples were first analyzed in the field, reanalysis of the one containing the peak concentration will identify the scale that can be used for all of the sample set. This may also preclude calibration of the fluorometer on other than the scales actually used.

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

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

4. Record the reading. The fluorometer resolution is about ±1 percent of full scale; readings to the nearest 0.5 to 1 percent should suffice.
5. Occasionally check and record the distilled-water readings. Check the distilled-water reading again just before turning off the fluorometer.
6. Turn off the fluorometer before other appliances to prevent sudden voltage surges through the fluorometer. Some fluorometers are protected against such surges.
7. Clean and rinse cuvettes for storage. A minute residue of dye may form inside improperly cleaned bottles and cuvettes that have contained high concentrations.

Special procedures in the field

Discrete sampling

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

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

1. The site layout need not be elaborate. If the fluorometer is not internally shielded from external light, the fluorometer will have to be shaded. Tents, trailers, or bridges can be used to provide shade. It is best to protect the fluorometer from adverse weather conditions to the extent possible, even though some fluorometers are watertight.
2. Often the long warmup recommended for the Turner model 111 fluorometer is not possible. A 2-minute warmup may be used, especially for a mobile setup, but a full warmup is desirable if the samples are not to be retested in the laboratory. Data based on insufficient fluorometer warmup may be very inaccurate and should be so treated.
3. A wide range in sample temperatures may be found. Temperature corrections usually are not necessary in the field if sample readings are used only as a guide to sampling. If field

readings are to be considered final, sample temperatures must be taken.

4. Samples from highly turbid streams may have high and variable background readings; fluorescence of the dye may be totally masked by the scattered light. In such a case, the flow-through door may be useless; single samples may have to stand for an hour or two before meaningful readings can be obtained. Air bubbles in water from cold, turbulent streams also may cause temporary interference in both flow-through and single-sample cuvettes.
5. All samples collected should be tested if possible. Retain at least the first and last set of background samples obtained before the dye arrived at each site. Intervening background samples may be discarded and the bottles reused. Usually, all samples containing dye should be retained for retesting in the lab.
6. A neutral-density filter may be used to obtain readings for samples that otherwise would be off the scale; usually the 10-percent neutral-density filter is adequate. Off-scale readings seldom will occur, except at sampling sites very close to injection sites.

Flow-through sampling

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

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

1. For some fluorometers, commercial power or a gasoline-driven generator should be used; the pump and fluorometer are too great a load for sustained use with a battery converter. However, fluorometers with relatively low power requirements operate very well with a 12-volt battery.
2. The recorder is powered through the fluorometer. The fluorometer dial on most or all instruments operates whether or not the recorder is turned on. The recorder reading occasionally should be compared with that of the dial and differences noted on the chart. The manufacturer's instructions on operation of the recorder should be on hand in the field.
3. Use opaque plastic garden hose or polyethylene tubing on the intake side; rubber has a strong affinity for the dye. Light leakage into the fluorometer has occurred when clear hoses were used. Be sure garden-hose connections have washers.
4. Use the most sensitive scale possible; monitor for necessary scale changes.
5. Flow-through setups must be prepared in time to test and record stream background readings before the dye arrives at the intake hose. In streams with very heavy suspended loads, the background count from scattered light may totally mask fluorescence. Under such conditions, the flow-through method should not be used; grab sampling will permit settling of the suspended material and accurate analysis subsequently in the laboratory.
6. Occasionally check and record the water temperature near the intake or at the discharge hose. Temperature corrections for the recorded data may be necessary.
7. Occasionally mark watch time and a dial reading on the recorder chart for possible corrections; some recorders have event markers useful for this purpose.
8. Occasionally collect grab samples from the discharge hose for later retesting and correlation with chart readings. Key the grab samples to the chart record (Hubbard and others, 1982, p. 13). The correlation will be more accurate when concentrations are not changing rapidly.

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

Troubleshooting

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

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

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

1. Is the lamp on?
2. Is the power source adequate? A drop in power input is a constant threat when using a battery and converter.
3. If the dial will not drop from the off-scale position, try a smaller aperture, a neutral-density filter, or a weaker sample. For the Turner model 111 fluorometer, check the reference lamp by following instructions in the operators manual, replace the main lamp, and test all radio tubes (the latter are easily removed from the back of the fluorometer). Replacements should be the slightly more expensive industrial grade tubes, if possible. If the dial runs rapidly upward when the door is opened, the cutoff switch inside the door latch is not working properly.

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

Fluorometer Calibration

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

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

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

be compared only on the basis of readings for known concentrations.

Concentration units

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

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

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

To illustrate, 1 g of pontacyl pink (powder) dissolved in 1 billion mL of final solution (distilled water diluent) is 1 μg/L. One mL of rhodamine WT solution (20 percent by weight, specific gravity 1.19) dissolved in 1 billion mL of final solution is 0.238 μg/L (1 mL×0.20 g/g×1.19×10^{-9} = 0.238×10^{-9}). Concentration values may be rounded for easier handling. A convenient procedure for most applications is to round to three significant figures above 1 μg/L and to the nearest hundredth below 1 μg/L. (Example: 2,380, 238, 23.8, 2.38, 0.24, 0.02.) However, for very precise work such as discharge measurements, use three significant figures for all concentrations.

Preparation of standard solutions

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

Equipment and supplies

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

1. Volumetric transfer flasks *without* stoppers (to be used for measuring diluent amounts for transfer to suitable containers)
   a. 2,000 mL
   b. 1,000 mL
   c. 500 mL
   d. 250 mL
2. Graduated cylinders
   a. 500 mL
   b. 250 mL
   c. 100 mL
3. Volumetric pipets ("to contain" type) with squeeze bulb or length of flexible extension tubing
   a. 10 mL
   b. 20 mL
   c. 25 mL
   d. 50 mL
4. Large-mouth Erlenmeyer flasks in 1,000- and 2,000-milliliter sizes (about a dozen 1-gallon glass condiment jars may be substituted)
5. Wash bottles
6. Glass sample bottles about 1 oz in size
7. Laboratory towels or wipes
8. Laboratory soapless cleaner and brushes
9. Fluorometer and appropriate accessories
10. Disposable laboratory gloves

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

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

The use of volumetric flasks and graduated cylinders as mixing containers is discouraged. These flasks not only are difficult to clean but allow no extra room for agitating and mixing. Volumetric flasks and graduated cylinders should be restricted to measuring the desired quantities of chlorine-free water into separate containers. Large-mouth beakers, Erlenmeyer flasks, or plain 1-gallon (3,785- milliliter) condiment jars, which can be obtained from most restaurants, are large enough to allow easy mixing of dye and water, are strong and durable, can be obtained at little or no cost, and can be easily cleaned with laboratory detergent and water. The mixing of dye and water in these 1-gallon jars can be accomplished by oscillating the jar and contents in a circular manner while keeping it firmly against a table or countertop. This avoids the use of stirrers, which must be cleaned.

Serial dilution procedure

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

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

where

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

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

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

The dilution factor, D_i, is computed as shown following equation 4 and is the amount of dilution of C_i for any one step in the serial-dilution process.

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

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

or

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

where the terms are as previously defined except that

C_f = final concentration of the standard obtained after the fourth step,
C_s = concentration of the dye solution, usually as obtained from the manufacturer,
D_i = a dilution factor at step i equal to $\left[\frac{V_d}{V_w + V_d}\right]$, and

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

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

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

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

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

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

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

The steps in preparing dye standards for the example are described below. If C_s is rhodamine WT 20 percent, the concentration that exists after the third dilution is 100 µg/L.

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

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

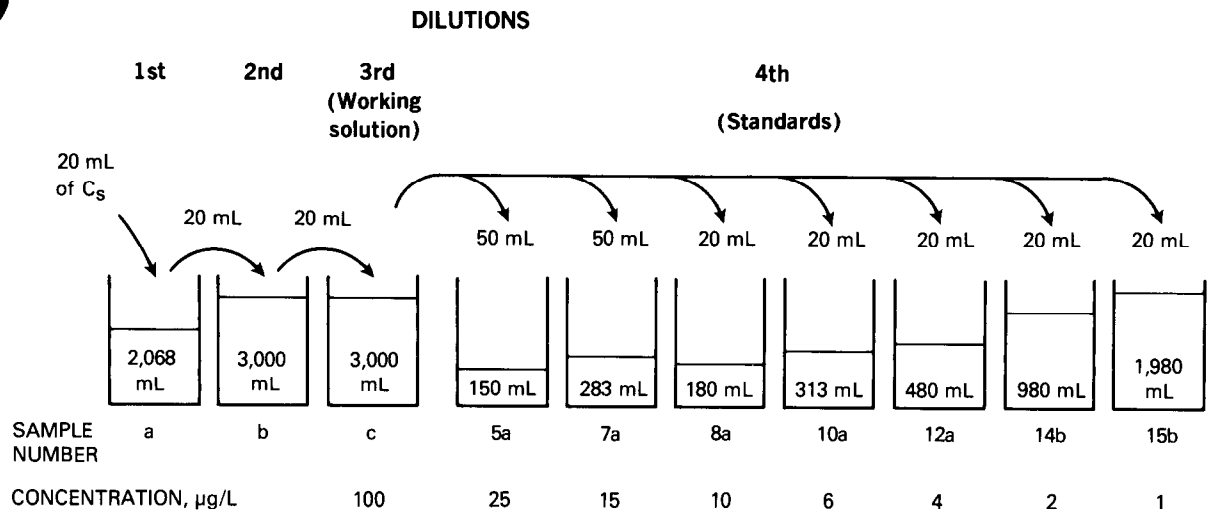

Figure 10.—Example of the use of tables 2 and 3 in preparing a set of standard solutions of rhodamine WT.

This is frequently referred to as the "working solution."

1. Measure all diluent quantities before adding dye.
   a. Based on line c for rhodamine WT, table 2, measure 2,068 mL, 3,000 mL, and 3,000 mL of chlorine-free water into separate containers.
   b. Based on table 3, in a like manner, measure V_w volumes of water into seven separate containers to provide standards of 25, 15, 10, 6, 4, 2 and 1 µg/L as shown in figure 10.
2. Label the containers a, b, c, 5a, 7a, 8a, 10a, 12a, 14b, and 15b, as shown in figure 10, or by the concentration of the contents.
3. Label the sample bottles with the appropriate concentration values for the standard solutions 5a through 15b as listed in step 2 and shown in figure 10.
4. Pipet 20 mL of the rhodamine WT 20-percent dye used in the test into container a and mix thoroughly. Cleanse pipet thoroughly.
5. Pipet 20 mL from container a into container b and mix thoroughly. Cleanse pipet thoroughly. Continue until the "working solution" of 100 µg/L is obtained on the third dilution.
6. Using 50- and 20-milliliter pipets, add these amounts as appropriate to containers 5a through 15b. Note that the same 20-milliliter pipet may be used repeatedly for the fourth-step dilutions without cleaning each time.
7. Agitate all of the final solutions until each is thoroughly mixed. Do no use the pipet to stir solutions as this may introduce contamination. If containers are oscillated with a circular motion while being held firmly against the tabletop, spillage is less apt to occur and mixing will be accomplished.
8. Starting with the standard of smallest concentration, pour into the previously labeled sample bottles.
9. Retain working solution in an airtight bottle that has been clearly labeled; store in a dark place for future use.
10. Analyze standards on fluorometer and record dial readings in conventional manner. Treat standard samples in a manner similar to the river samples. For example, allow the standard samples to stand overnight in the same room or place them in a bath having the same temperature as the river samples.

If desired, the computation of the 1-microgram-per-liter standard may be verified using equation 3:

$$C_f = 20\times10^7 \left[\frac{20\times1.19}{2{,}068+20}\right]_{(a)} \times \left[\frac{20}{3{,}000+20}\right]_{(b)} \times \left[\frac{20}{3{,}000+20}\right]_{(c)} \times \left[\frac{20}{1{,}980+20}\right]_{(15b)} = 1.0\ \mu g/L$$

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

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

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

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

Testing procedure

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

Calibration curves

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

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

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

Calibrations for a few fluorometers are linear except near the origin (below about 5 to 10 percent of full scale), resulting in an apparent intercept on the concentration axis (fig. 11*D*) very rarely, the intercept appears to be on the dial-reading axis (fig. 11*E*). If such an apparent intercept is observed, prepare appropriate standards to define the curvilinear position of

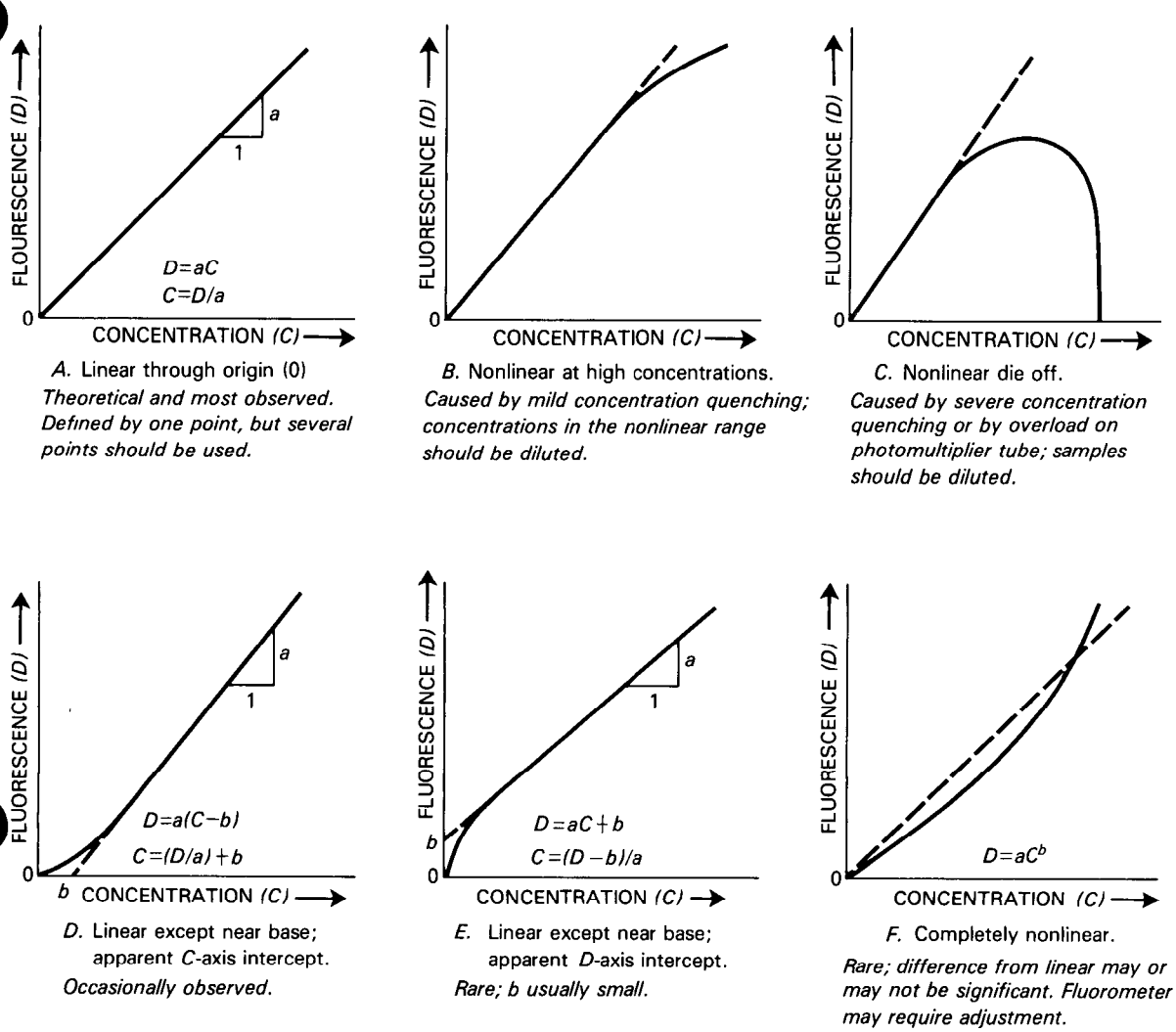

Figure 11.—Types of fluorometer calibration curves.

the curve. G.K. Turner Associates (written commun., 1966) discovered that these two types of curves can result in the Turner model 111 fluorometer from incorrect alignment of the high-sensitivity kit installed on the standard door or as a part of the constant-temperature door. The problem, if serious, can be corrected by factory adjustment.

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

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

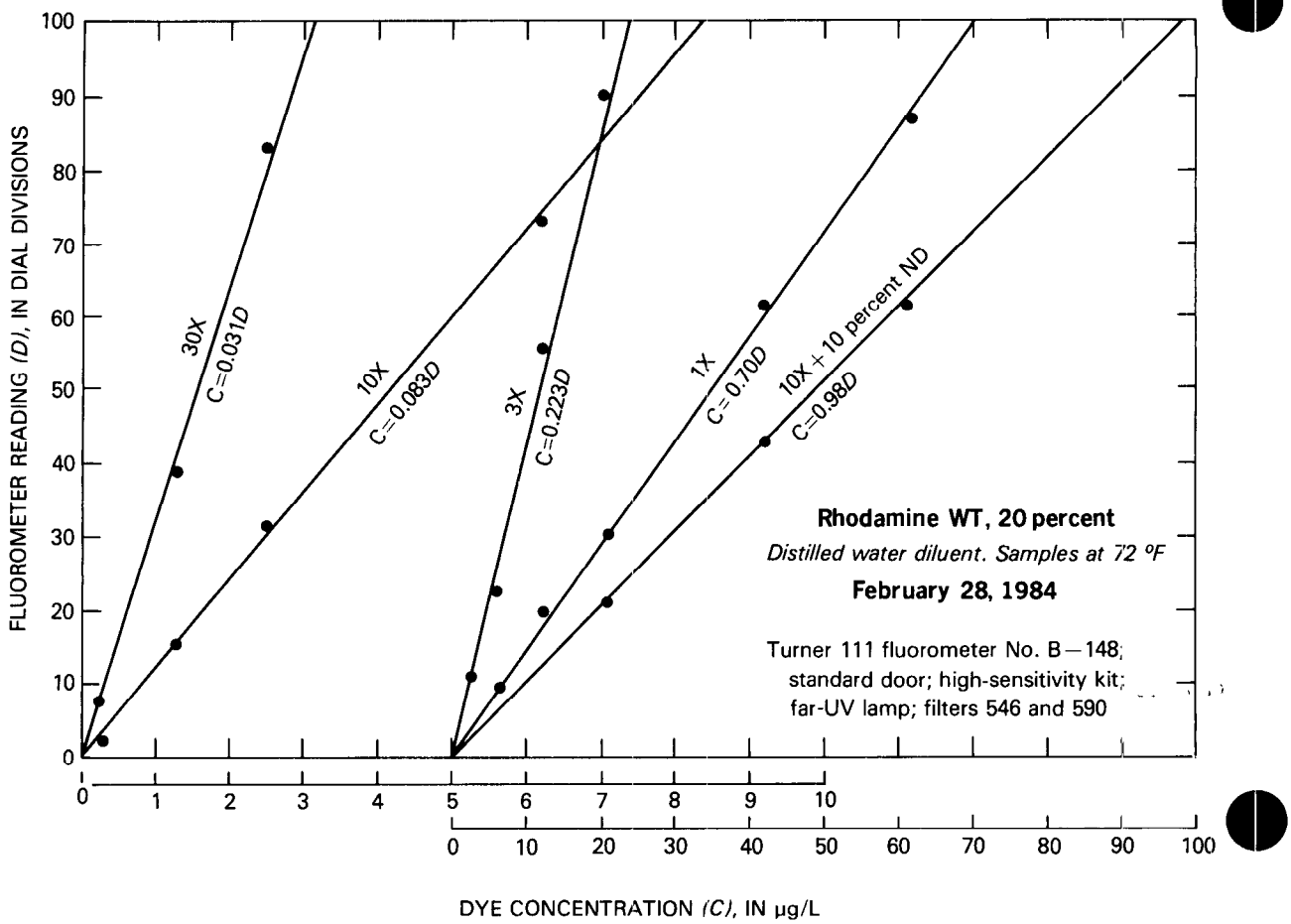

Figure 12.—Typical set of calibration curves.

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

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

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

If the fluorometer is not moved and none of the electronic components are touched, a calibration should remain valid for weeks or months

of normal use. However, spot checks are desirable. A different calibration will be needed for each dye lot used. Some of the more common causes of change in calibration are:

1. Jarring the fluorometer, as might be expected when it is used in the field.
2. Removing the lamp temporarily.
3. Changing the lamp or photomultiplier.
4. Damage to the lamp or photomultiplier.
5. Clouding and deterioration of filters with time.
6. Changes in optical alignment.
7. Changes in temperature of standard samples. (Application of temperature-correction factors will eliminate this problem.)
8. Contamination of the fluorometer with dye.

Calibration of the flow-through cuvette

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

1. Correlate the flow-through readings on the dial or chart with readings for single samples collected at the discharge hose. Determine the concentration of these samples in the laboratory and use the data to develop a calibration curve on or from the chart (Hubbard and others, 1982, p. 9).
2. Prepare large volumes of several standard solutions, pump them through the fluorometer, and record the readings. For this situation, river water may have to be used for both convenience and to have standards at nearly the same temperature as that being pumped through the fluorometer. A container of "working solution" can be carried to the field to expedite preparation of these field standards. If it is desirable to check calibration frequently in the field, the standard solution can be connected to the intake line with a system of T-connections and valves. When switching from stream to standard or back again, be sure to allow time for complete flushing of the water already in the system. Temperature corrections may have to be made in some instances.

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

Determination of Sample Concentration

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

Because a uniform background value for all sampling sites is not likely, background samples should be collected at each site. Time variations in background also may occur but are often impossible to monitor while dye is passing a site. Background readings usually should be subtracted before applying temperature-correction factors because most components of background either are not affected by temperature at all or are affected in a way different from the dyes. If standards and field samples have been brought to a common temperature and if reasonable care is taken, there will be no need for temperature corrections.

Aerial Photography as a Supplement to Fluorometry

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

about the spatial distribution of the dye cloud (Wilson, 1968a).

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

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

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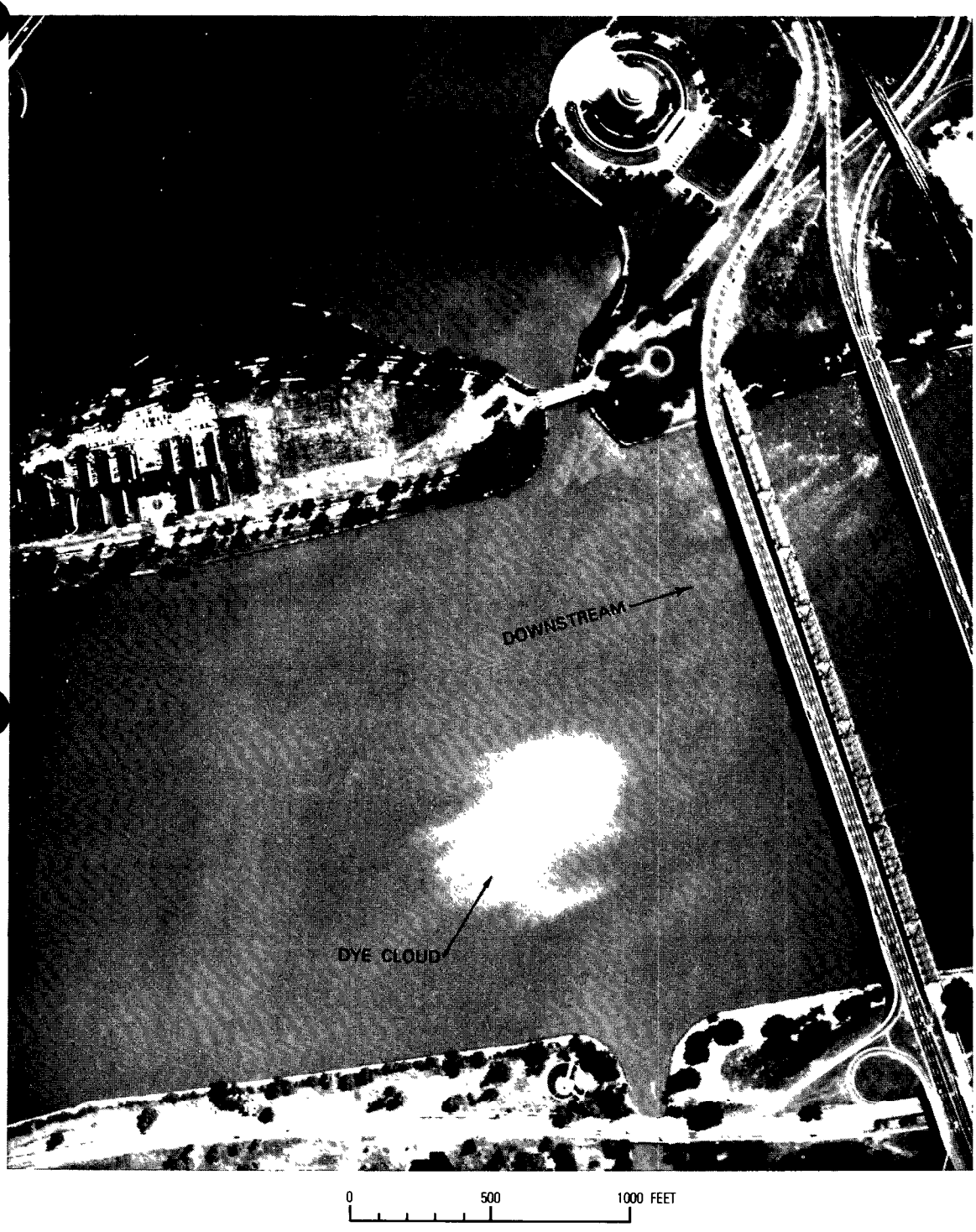

Figure 13.—Aerial photograph of dye cloud, Potomac River estuary at Washington, D.C., August 1965. Taken at low tide with panchromatic film and Wratten 23A filter, from 3,000 feet above terrain.

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