Application Note: Preparation of Standards for Dye Studies Using Rhodamine WT

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WHAT IS A STANDARD?

A standard is a known concentration of the dye you are injecting. It is used to calibrate the Turner Designs Model 10 Fluorometer to the desired sensitivity. The fluorometer reading of the standard will be compared with the readings of unknown samples to obtain their concentrations. A known concentration is made by weighing or measuring a sample of tracer and precisely diluting it.

In many cases, the standard will be a known dilution of dye, not a known concentration. For example, in flow rate measurements you are only interested in how much a stream dilutes the dye, not what the actual concentration is. Thus, you do not need to know the concentration of the dye (in parts per billion or other units) when you calibrate your fluorometer. You need only know the dilution factor for the standard and the fluorometer reading, which you will then compare with the fluorometer readings for your unknown samples collected after dye has been injected into the stream. This will allow you to calculate the extent to which the stream dilutes the dye.

Note: For more information on dye studies and whether a known concentration or dilution is required, see the monographs "A Practical Guide to Flow Measurement" and "Flow Measurements in Sanitary Sewers by Dye Dilution," available from Turner Designs.

UNITS OF MEASUREMENT

Use whatever units suit your purpose best. The EPA guidelines on allowable levels will typically be in micrograms per liter (1). Other studies are described in milligrams per cubic meter (mg/m^3) or parts per billion (ppb). The simplest to use when making dilutions is grams per gram, which, for all practical purposes, is the same as grams per milliliter.

The easiest way to record and to think of these units is in exponential notation. If you are not used to this, and are not comfortable with it, move your decimal point around. It is very easy, however, to think in exponential terms:

> 1.3 grams dissolved in 100 ml, makes a 0.013 g/g solution. In exponential notation this is 1.3×10^{-2}. If 1 ml is diluted to 100 ml (100 ml is 10^2 ml), then the new concentration is 1.3×10^{-4}.

Some conversion factors:

1 gram/liter	1 part per thousand (ppt) 10^{-3}
1 milliliter/liter	1 part per thousand (ppt) 10^{-3}
1 milligram/liter	1 part per million (ppm) 10^{-6}
1 microgram/liter	1 part per billion (ppb) 10^{-9}
1 milligram/cubic meter	1 part per billion (ppb) 10^{-9}

LINEARITY

Note: Rhodamine WT comes as a 20% solution in water (meaning it is 20% active ingredient). The EPA - guidelines are in terms of active ingredient. Thus, one microgram of the 20% solution in one liter is 0.2 ppb active ingredient (or 20% of 1 ppb).

Instrument readings for fluorescent dyes are proportional to concentration (linear) from the lowest detectable level up to a certain concentration. Above this concentration, a multipoint calibration curve may be used to obtain concentrations. Then, at a certain concentration (somewhere at about five to ten times the upper limit for the linear range) the curve flattens out and eventually takes a nose-dive. This critical concentration is a function of the compound and of the path length of the flow cell or cuvette.

For practical purposes (using the Turner Designs Model 10 Fluorometer equipped with the 25-mm flow cell or cuvette holder), Rhodamine WT is linear to 0.5 ppm (500 ppb, 5×10^{-7}, or 500 micrograms/liter). In terms of active ingredient (the 20% solution of Rhodamine WT), it is linear to 0.1 ppm (or 100 ppb, 1×10^{-7}, or 100 micrograms/liter).

ACTIVE INGREDIENT OR 20% SOLUTION?

Since Rhodamine WT comes as a 20% solution, a decision should be made at the outset about whether to make the standard as 100% tracer or in terms of active ingredient. The first impulse is to do all calculations on the basis of the active ingredient. However, even to meet the EPA guidelines, you need only keep in mind that 20% of the original solution is active ingredient. For other purposes, it is immaterial whether you take the 20% into account. If you discharge 20 pounds of Rhodamine WT solution, or inject a 10-fold dilution, it doesn't matter that the original material was only 20% pure. If you consider the Rhodamine WT solution to be pure tracer, then all dilutions are relative. For example, if you have made a 100 parts per billion dilution based on 100% tracer, your final dilution will be 20 parts per billion active ingredient. Whatever method you choose, be sure to clearly mark your dilutions as to 100% tracer or active ingredient.

MEASURING BY WEIGHT OR VOLUME

For flow measurements and other studies where dye is to be added to a body of water, if you plan to add dye in pounds, grams, or other weight measure, then your standard must be made by weighing. If you are adding by volume, then the standard must be made by volume measurements. The important thing is to make your standard the same way you make the dye concentration to be injected.

Note, however, that most of the literature cites the specific gravity of Rhodamine WT as 1.2 (sometimes 1.19). Recent literature accompanying the dye says 1.15. Thus, one gram equals 1/(sp.gr.) milliliter. If you add by volume, you could be adding as much as 20% more dye than if you add by weight. Therefore, if you are concerned with absolute concentrations (as with an EPA study), you should make an initial 100-fold dilution by weight (or compensate for the specific gravity). A 100-fold dilution by weight has a specific gravity of 1.002. For the vast majority of studies, errors of 0.2% in doing further dilutions, whether by weight or volume, are acceptable.

If you weigh, it is best to weigh directly into a volumetric flask. (See discussion of flasks below.) This avoids the problem of having to rinse whatever vessel is used for weighing. All of the material must wind up in the flask. Since it will be diluted with water, the rinsing can be done with water.

If you measure by volume, an accurate method is to use a large-tip pipette of at least 10-ml capacity and measure dye into a 1-liter volumetric flask (a 100-fold dilution). Even more accurate is to measure dye with a 20-ml pipette and dilute to 2 liters. The larger the pipette, the smaller the surface area with respect to the volume, hence the smaller the error due to incomplete drainage. The error from a 10-ml pipette will probably be negligible for most work. It certainly should be less than 1%.

The most accurate way to measure the tracer by volume is to fill a 10-ml volumetric flask to the mark, then rinse the tracer into a 1-liter volumetric flask. You can't rinse a pipette, as it is made to deliver the stated volume. Volumetric flasks contain the stated volume, so if you want the stated volume out of the flask, you must rinse.

Note, however, that there is not much point in preparing your standards to an accuracy greater than the means by which the tracer will be added to the system.

THE VOLUMETRIC FLASK

Put your measured sample into the flask, then add water up to the line. The correct level is when the bottom of the meniscus touches the calibration line. The more precise Class A flasks will be accurate to 0.03% for a 1-liter flask, and to 0.16% for a 100-ml one. Less accurate flasks have a tolerance of twice this.

After adding the dye, mix thoroughly. The proper way is to invert the flask (hold the cap on!). When the air bubble has risen, swirl it for a few seconds. Right it; let the bubble rise; then invert and swirl again. Do this ten times.

To care for a volumetric flask, rinse it thoroughly with distilled water. Occasionally, wash it with a mild detergent. To make sure it is clean, put a few milliliters of water in it, swirl, and pour into a cuvette. Then, compare the fluorometer reading with clean water. Rinse until they read the same. The flask does not have to be dry to use it.

THE PIPETTE

There are three choices: a pipettor, a measuring pipette, or a volumetric pipette.

Pipettor. Advantageous for most users. It is not necessary to learn to control the leakage while you adjust to a line, and disposable plastic tips are used. Accuracy is generally about 1%. There are many brands on the market. They can be purchased in fixed volumes, for example, 100 microliters (0.1 ml), 1 ml; or adjustable volumes, 10-100 microliters, 0.1-1 ml.

Pipettors are relatively inexpensive and easy to use: 1) Push a button until you feel a stop; 2) Insert tip in solution; 3) Release slowly to draw up the set volume; 4) Push past first stop to a second one to eject and blow out the last drop. Some pipettors have a further position that ejects the tip.

Measuring Pipette. Available in disposable form. The accuracy of the disposable ones, +/- 2%, is no worse than the nondisposable, and a clean one is always available. When using, let it drain, then blow the last portion out.

Volumetric Pipette. Like the volumetric flask, this is very accurate. First, fill the volumetric flask about 1/2 full with water. Then, fill the pipette to the line, and, holding the pipette vertical, allow it to drain. When it stops dripping, hold it for about 10 seconds longer, then touch the drop on the end to the surface of the water (just once, and only briefly). Remove the pipette.

Problems: A pipette must be dry when you use it, or it must be rinsed with the material you intend to pipette. Volumetric pipettes are difficult to clean and rinse. If you need the accuracy, buy quantities of them, so you can use them, then clean them at leisure (preferably with access to a laboratory pipette washer).

MAKING STANDARD DILUTIONS

For a standard, you need any concentration no greater than the linear range of your tracer (for the rhodamine dyes, approximately 100-ppb (0.1-ppm; 100-micrograms/liter) active ingredient.

To obtain this concentration, you will make serial dilutions. By this, we mean you take your concentrated solution and make a dilution of it. You mix it thoroughly, then make a dilution of that, and so on, until the desired concentration is obtained.

We recommend preparing a higher concentration, i.e., around 0.1-ppm (100-ppb or 10^{-7}) active ingredient. At these high levels (high for fluorescence), contamination will be less of a problem. Contamination from dirt or other things is not a problem, but spurious tracer could be. In preparing the standards, you are handling the pure material and high concentrations, and it is safer to use the highest standard that is convenient.

Note that the dilutions you are after can be achieved in a variety of ways. The easiest way is with 1- and 10-ml pipettors, and a choice of 100-ml and 1-liter flasks. If intermediate concentrations are desired, use an adjustable pipettor, or pipette several shots into one flask, or use intermediate-size flasks (they are available in 200-, 250-, and 500-ml sizes). Generally, all you are after is some concentration not greater than 500 ppb (0.5 ppm)--or 100 ppb (0.1 ppm) if dealing in active ingredient. Since readings are proportional to concentration at or below this point, it is simply a question of convenience.

Don't use **all** clean water. Your last dilution should always be done twice, once in distilled water, and once in the water in which the measurement will be made. This is because sometimes there are substances in the test water that interfere with the reading. This doesn't happen often, but it can invalidate your readings if you don't recognize it.

Your standard will be the dilution in the system water, but first you need to see that it reads the same as the dilution in distilled water -- or make sure you understand any difference.

A. To prepare a 100-ppb (active ingredient) standard of rhodamine WT (20% solution):

1. First, prepare a 100-fold dilution by weight. (See section MEASURING BY WEIGHT OR VOLUME for an explanation.) Using an accurate laboratory scale, weigh 1 gram of dye directly into a 100-ml volumetric flask. The dye may be dripped into the flask with a pipette until 1 gram is obtained. Then dilute to the mark with distilled water. You now have a 10-g/liter (10 ppt, 10^{-2}) concentration of your tracer.

   Note 1: You could obtain the same - concentration by weighing 10 g into a 1-liter flask, or 20 g into a 2-liter flask.

   Note 2: If you intend to inject dye by volume, then pipette 1 ml of dye into a 100-ml volumetric flask and dilute to the mark with distilled water. Or measure 10 ml of dye into a 10-ml volumetric flask and rinse into a 1-liter flask. Then, dilute to the mark with distilled water. This will yield a 10-ml/liter (10-ppt, 10^{-2}) dilution. (Keep in mind the specific gravity factor. See MEASURING BY WEIGHT OR - VOLUME, above.)

2. Next, pipette 1 ml (or weigh 1 gram) of the dilution in #1 (10^{-2} or 10 ppt) into a clean 100-ml volumetric flask and dilute to the mark with distilled water. Mix thoroughly. You now have a 10^{-4}, or 100-ppm, dilution.

3. Now, pipette 5 ml (or weigh 5 grams) of the dilution in #2 (10^{-4} or 100 ppm) into a clean 1-liter volumetric flask and fill to the mark with system water. Mix thoroughly. You now have a 10^{-7} (or 100-ppb; 0.1-ppm) active ingredient standard.

   Note: We measured 5 ml because rhodamine WT comes as a 20% solution (meaning 20% active ingredient). If you are not concerned with active ingredient, then diluting 1 ml 1000-fold yields a 100-ppb dilution of tracer (or 20-ppb active ingredient).

4. Repeat step 3, using distilled water. Compare fluorometer readings of this dilution with that of #3.

B. To prepare a 100-ppb standard of rhodamine B or other dye in powder form:

1. First, prepare a 100-fold dilution by weight. (See MEASURING BY WEIGHT OR VOLUME above, for an explanation.) Using an accurate laboratory scale, weigh 1 gram of dye directly into a 100-ml volumetric flask. Then dilute to the mark with distilled water. (Be sure to mix thoroughly; the powders can be difficult to mix.) You now have a 10-g/liter (10-ppt, 10^{-2}) - concentration of your tracer.

   Note: You could obtain the same - concentration by weighing 10 g into a 1-liter flask, or 20 g into a 2-liter flask.

2. Next, pipette 1 ml (or weigh 1 gram) of the dilution in #1 (10^{-2} or 10 ppt) into a clean 100-ml volumetric flask and dilute to the mark with distilled water. Mix thoroughly. You now have a 10^{-4}, or 100-ppm, dilution.

3. Now, pipette 1 ml (or weigh 1 gram) of the dilution in #2 (10^{-4} or 100 ppm) into a clean 1-liter volumetric flask and fill to the mark with system water. Mix thoroughly. You now have a 10^{-7} (or 100-ppb; 0.1-ppm) standard.

   Note: Rhodamine B is also available in 30% or 40% active ingredient solutions. If you are working with one of these dilutions, then what you actually have is a 30-ppb (30%) or 40-ppb (40%) standard, based on active ingredient.

4. Repeat step 3, using distilled water. Compare fluorometer readings of this dilution with that of #3.

Thus, the necessary concentration for your standard can be achieved in a variety of ways. For example, a 5×10^{-7} or 500-ppb dilution could be achieved in the following cases:

1. You are doing a flow measurement, and have a dye concentration of 5×10^{-3} (5 ppt). To achieve a 5×10^{-7} (500-ppb) dilution, make two 100-fold dilutions of the 5 ppt.

2. You have a 5% solution (5×10^{-2}) of dye; make one 1000-fold dilution and one 100-fold dilution; or a 10-fold, followed by two 100-fold dilutions.

MAKING MORE THAN ONE CONCENTRATION

Whether or not you make more than one concentration depends on such considerations as the confidence you have in your dilution, and how important it is that your standard be exactly right. If this is your first time, it would be important to prepare a range of concentrations and plot a standard curve. This will test your proficiency and make you more confident. The readings, however, should be linear with concentration. You really need only one concentration (and a blank).

The most important thing to do is to be sure there were no errors, i.e., a lapse in counting measurements, an accidental contamination, an air bubble in the pipetted sample, etc.

It is not so much, therefore, the need for more than one concentration as it is a need to duplicate your preparation. This means from the beginning. If you choose to make several concentrations at the final dilution, fine.

pH, TURBIDITY, AND CHLORINE

pH. When you are taking measurements, the most serious, nonvisible problem in test waters is pH. Any pH between 4.5 and 10.5 is fine. Most systems should fall in this range, but if the pH does not, the fluorescence will drop off rapidly. It is, however, reversible. If the dilution in the system water reads very low and there is no obvious reason (intense color, very high turbidity, etc.), check the pH. If you can't check the pH, get some vinegar and some baking soda. Try adding a pinch of the soda to one test tube, and a drop or two of vinegar to another. Neither is capable of taking the pH too far in the other direction. If this causes the reading to increase, add a bit more to see if you have enough, then plan on adding the same amount to all your samples.

If pH is not the problem, then the study probably cannot be done with a fluorescent tracer. This is extremely rare, and it is likely that someone is dumping a high concentration of a very strong oxidizing agent. Investigate.

Turbidity and Color. These are covered in the monograph "A Practical Guide to Flow Measurement." Generally, it takes considerable turbidity or color to interfere with the readings. The interference is a percentage reduction in reading. For example, say 100 ppb reads 900 in clean water. A 10-ppb solution would read 90.0, and a 1-ppb solution, 9.00. Your 100-ppb solution in the system water reads 810 (a 10% reduction). The 10-ppb solution will read 81.0 and the 1-ppb solution, 8.10. In other words, if you use the dilution in the system water as the standard, there will be no error, and no correction needs to be made.

Should you calibrate this way if there is a 90% loss of reading? Theoretically you could, if you were absolutely positive that the turbidity or color would be constant during the study. In practice, it would be much better to increase the dye concentration by a factor of 10, then dilute all samples, the blank, and the standard 10-fold with clean water.

pH and Chlorine. In potable water, we found in lab tests that chlorine appears to destroy rhodamine WT within a few minutes at all pH levels, even with very low levels of chlorine (.1 part per million). There is at least one very thorough published study that showed little effect of chlorine on rhodamine WT in wastewater (2). We speculate that the suspended solids in wastewater have a prophylactic effect.

STORING SAMPLES AND STANDARDS

The tracers will not degrade, and if stored in the dark, are stable for years. After you have completed your study, you may find that something doesn't fit. If you have a sample of the tracer you injected, samples of your dilutions, and your field samples, you can always re-read your dilutions and see what happened. Since the Model 10 Fluorometer 10-030 Cuvette System requires only 4 ml of sample, there is no need to store large amounts. Scintillation vials (discussed in "Flow Measurements in Sanitary Sewers") hold about 20 ml, and 500 of them require very little storage space.

REFERENCES

1. U.S. Environmental Protection Agency (Office of Drinking Water), "Guidance Manual for Compliance with the Filtration and Disinfection Requirements for Public Water Systems Using Surface Water Sources," Report No. EPA 570/9-89-018, Appendix C-9 (October 1989).

2. (0026) D.G. Deaner, "Effect of Chlorine on Fluorescent Dyes," J. of Water Poll. Control Fed., 45:3, 507-514 (1973).

NOTES