Chlorophyll *a* and Pheophytin *a* (extracted, Optical Kit 10-037R)

EPA Method 445.0 is a standard method for measuring extracted chlorophyll *a* and pheophytin *a* in marine and fresh water algae by fluorescence. It requires extraction with 90% acetone, measurements before and after acidification, and some fairly simple calculations to arrive at the chlorophyll *a* and pheophytin *a* concentrations. Method 445.0 is detailed and straightforward. (If high concentrations of pure chlorophyll *b* are present, see the next section, Optical Kit 10-040R.)

A known concentration of pure chlorophyll *a* (as a standard) is required at least the first time you calibrate the instrument. For greatest accuracy, however, we recommend that you periodically (once every few months), use a known concentration of pure chlorophyll *a* in 90% acetone to recalibrate your instrument. (Liquid Primary and Solid Secondary Chlorophyll *a* standards are available from Turner Designs).

The **only way** to obtain pheophytin *a* concentration is to use the above EPA Method 445.0 to calculate it.

Chlorophyll *a* (extracted, non-acidification, Optical Kit 10-040R)

The Welschmeyer method is a new, simplified way to measure chlorophyll *a* without the need for acidification. It accurately measures chlorophyll *a* even in the presence of chlorophyll *b* and pheophytin. However, you **cannot** obtain a pheophytin *a* measurement with this procedure. Using this method, you extract your samples according to EPA Method 445.0, but skip the acidification step.

Again, you still need to calibrate the instrument the first time using a known concentration of pure chlorophyll *a* in 90% acetone.

Secondary Standard for Extractive Measurements

We recommend the 10-AU Secondary Solid Standard, P/N 10-AU-904, which is stable and fluoresces in the same wavelengths as chlorophyll. This product contains high-level and low-level standards in one housing. No special storage conditions are required and stability is guaranteed for two years.

To use our secondary standard for extracted chlorophyll *a* analysis, first calibrate your instrument using a known standard concentration of chlorophyll *a*. Next, insert your solid secondary standard and note the readings for both high and low standard positions. These two readings can then be used for future analyses to check for instrument drift. The reading can also be used to adjust data if drift occurs until the instrument can be recalibrated with primary chlorophyll standards again.

In Vivo Measurements (Optical Kit 10-037R or 10-096R)

For monitoring algae, *in vivo* chlorophyll *a* fluorescence is a good indicator of algal levels, since all algae contain chlorophyll *a*.

If you wish to measure *in vivo* chlorophyll you can use one of the two methods below. For greatest accuracy of *in vivo* measurements, you should take samples regularly for extraction to correlate with the *in vivo* readings.

Please note that there is no need to use purified chlorophyll *a* to calibrate for *in vivo* studies because extracted chlorophyll *a* in solvent fluoresces differently than chlorophyll *a* in living cells (*in vivo*).

1. Standard Method using traditional chlorophyll filters (Optical Kit 10-037R). In this method, use the 10-050R excitation filter, the 10-051R emission filter, the 10-032 1ND reference filter, and the 10-045 Daylight White Lamp.

   This filter set-up will read chlorophyll *a* and some chlorophyll *b*.

   The calibration method is the same whether using flow through or test tube samples.

2. Chlorophyll *a* in the presence of high blank, humic substances, or chlorophyll *b* (*in vivo*) (Optical Kit 10-096R). If you are going to use the *in vivo* method and background (blank) is very high due to high concentrations of humic substances and/or chlorophyll *b*-containing algae, we recommend the following filter setup: 10-050R excitation filter, the 10-115R emission filter (680 nm interference filter), the 10-032 1ND reference filter, and the 10-089 Blue Mercury Vapor Lamp. Note that the 10-045 Daylight White Lamp is sufficient unless you want to measure very low levels of chlorophyll.

   This filter setup should help reduce interference from these substances.

Procedure for *In vivo* Measurements

The following is one suggested way of doing an *in vivo* study. Note that in this method you are not calibrating at first with a standard of known concentration. This is something that is done later. Refer to the 10-AU User's Manual for details.

1. Place a typical water sample in a 25 mm test tube or start it flowing through the instrument. On the MED range, if possible, adjust the basic operating level using the sensitivity knob to FS% between 40-50% (screen 3.2).

2. Next, set your unknown sample to an arbitrary fluorescent value (i.e. 100) on 10-AU screen 2.2 and calibrate on screen 2.3. **Take a grab sample immediately afterward** for later extraction. You can start taking readings of samples now, but will have to do ratio calculations after you determine the actual concentration through extraction.

   NOTE: You can actually use this method without calibrating. For example, if your grab sample reads 37 on the 10-AU and later, extraction shows it actually contains 6 ppb of chlorophyll *a*, you can do a simple ratio calculation to arrive at the actual concentration of unknowns: i.e., 6/37 x reading of unknown = actual concentration of unknown.

3. Following EPA 445.0, extract the grab sample taken in step 2. Calibrate your fluorometer with a known chlorophyll *a* standard. Then, determine the actual concentration of your grab sample.

4. To estimate the concentration of unknowns, simply do a ratio calculation using the extracted and corresponding *in vivo* data.

   For example (the numbers are for example ONLY!): If your grab sample used for calibration in step 2 was 12 ppb with its reading set to [100] and your unknown sample of water reads 21, you would calculate the actual concentration of the unknown by:

$$\text{Actual Concentration of Calibration Sample Water} \times \frac{\text{10-AU Reading of Unknown}}{\text{10-AU Reading of the Calibration Sample Water}} = \text{Actual Concentration of Unknown}$$

   or 12 ppb/100 x 21 = 2.52 ppb (μg/L)

Secondary Standard for *In vivo* Measurements

At the present, no solid secondary standard exists for use with flow cells. An alternative to solid standards in this sampling mode is the use of Rhodamine WT dye. Concentrations of the dye in the high ppb or low ppm concentration range will result in fluorescence signals equal to natural chlorophyll concentrations. However, a preliminary correlation study is required to determine exactly what dye concentration corresponds to a specific chlorophyll concentration. NOTE: We do not recommend coproporphyrin because it is prepared in an acidic solution. Thus, it should not be used in the flow cell as it can damage the seals, etc. **For your safety**, use caution when handling acidic solutions.