Application Note: A Procedure for Measuring Extracted Chlorophyll *a* Free from the Errors Associated with Chlorophyll *b* and Pheopigments

(Without Acidification - Using 13 mm Test Tubes)

INSTRUMENT SET-UP

Model TD-700 P/N 7000-000 Fluorometer equipped with:

1. 13 mm cuvette holder (included with the TD-700 Fluorometer)
2. Optical Filter Kit PN 7000-962, which includes:
   P/N 10-113 (436 nm) Excitation Filter
   P/N 10-115 (680 nm) Emission Filter;
3. PN 10-089 Blue Lamp (F4T4.5B2 equivalent).

SUMMARY OF THE METHOD

Conventional fluorescence methods for measuring chlorophyll *a* require samples to be measured twice; once before acidification and once afterwards. Under the most extreme ratio of chlorophyll a/chlorophyll *b* likely to occur in nature (1:1 molar), conventional acidification techniques result in approximately a 60% underestimate of true chlorophyll *a*.

Under these conditions, the new method1 described in these pages yields at most a 10% overestimate of true chlorophyll *a*. In addition, it requires a single fluorescence determination and is sensitive enough for estimates of euphotic zone chlorophyll *a* in all marine and freshwater ecosystems. Filtration of less than 200 mL of water provides adequate sensitivity even in the most oligotrophic environments.

The method requires:

1. Sample preparation.
2. Calibration of the Fluorometer.
3. Reading samples.

SAMPLE PREPARATION

Detailed instructions for extracting chlorophyll *a* and measuring with the Turner Designs analog fluorometer can be found in United States Environmental Protection Agency (EPA) Method 445.0 "In Vitro Determination of Chlorophyll *a* and Pheophytin a in Marine and Freshwater Phytoplankton by Fluorescence." A copy is enclosed for your convenience. Method 445.0 sets forth the conventional fluorescence procedure, requiring two readings for each sample--before and after acidification. (Method 445.0 can be found in the EPA standard methods book, Methods for the Determination of Chemical Substances in Marine and Estuarine Environmental Samples.)

PLEASE NOTE that the procedure described in these instructions is without acidification. The Model TD-700 must be configured with special optical filters and lamp which read chlorophyll *a* in the presence of chlorophyll *b* and pheopigments. These optical filters and lamp should be installed according to instructions in your Model TD-700 User's Manual Sections III and IV (Optical Filter Installation and Removal, and Lamp Installation and Removal). USING THIS METHOD, you must NOT acidify your samples as set forth in EPA Method 445.0, section 11.2.2; and you do not need to perform any calculations as required by section 12.1 of Method 445.0.

CALIBRATION

All you need to do is calibrate the instrument with the following procedure. The calibration should remain stable for some time, and unless you change your blank or standard or want to change from reading very high levels to very low levels (or vice versa), you may not have to calibrate every time you read a new batch of samples. (You will, of course, need to recalibrate if you change the lamp or filters.)

Please note, however, that the standard must be within the linear range for accurate readings (according to the EPA Method 445.0, using the 13 mm cuvettes, chlorophyll *a* is linear to 250 μg/L).

To calibrate:

Have ready a blank of 90% acetone and your standard of known concentration of pure chlorophyll *a* in 90% acetone.

1. Turn on the fluorometer and allow it to warm up for 10 minutes.

Because temperature affects fluorescence, do not allow the blank to remain in the instrument any longer than necessary for a stable reading.

2. Prepare a pure chlorophyll *a* standard and a blank of 90% acetone in a 13 mm test tube. Put the standard in the sample chamber and close the lid. Calibrate3 according to Section VII (Calibration - Raw Fluorescence) or VIII (Calibration - Direct Concentration) in the TD-700 user's manual (whichever you prefer). Remove the standard and insert the blank when the software prompts you to. When the blank reading is stable, press <0>. When finished, remove the blank.

READING SAMPLES

Refer to your user's manual, Section IX (Reading Samples), for additional details.

For your convenience, the Model TD-700 has a "Discrete Sample Averaging" capability, where the instrument averages a reading over a preset period, allowing you to read samples after they have been in the instrument for the same amount of time. This removes the guesswork from reading the digital display and minimizes error due to temperature changes. Defaults for the Model TD-700 are 7 seconds pre-delay for the signal to stabilize, and an averaging period of 12 seconds. To use Discrete Sample Averaging, after putting in your sample, from the HOME screen, press <*> and the instrument will countdown a delay period, average the reading, and then display "END" in the left corner of the screen. The averaged reading will be displayed for 5 seconds. If the fluorometer is not connected to a printer or a computer, write down the reading.

Procedure for running samples:

1. Fill a clean cuvette with a sample, wipe the outside of the cuvette dry with a lab wipe, and place in the instrument. Close the lid.

2. Wait about 10 seconds for the reading to stabilize, and log the reading. (Remember: Because of temperature effects, for greatest accuracy, read all samples after they have been in the fluorometer for approximately the same length of time.) If the display reads "OVER," dilute the sample by 25% (1 part sample to 3 parts 90% acetone solution), and read it. Multiply the reading by 4 to get the actual concentration.

3. Remove the cuvette and put in the next sample.

4. Repeat steps 1 - 3 until all samples are read.

If you calibrated in the direct concentration calibration procedure, these readings are the actual concentration of extracted chlorophyll *a* in the cuvette. To arrive at the environmental chlorophyll *a,* for each sample you must correct for the volume of water filtered and the volume of 90% acetone used in the extraction.

NOTE: It won't hurt the fluorometer to leave it on all day. If you are going to be reading samples off-and-on over the course of a few days, it is better to leave the fluorometer on.

1. The method was developed by Dr. Nicholas A. Welschmeyer of Moss Landing Marine Laboratories, Moss Landing, CA. A paper by Dr. Welschmeyer, Fluorometric Analysis of Chlorophyll *a* in the presence of Chlorophyll *b* and Pheopigments, which details his research, appears in *Limnology and Oceanography*, 39(8), 1994, pp. 1985-1992.

2. Method 445.0 found 250 μg/L to be the upper limit of the linear dynamic range for 13 mm cuvettes using the Turner Designs Model 10 Fluorometer. See section 9.2 of Method 445.0 for procedure for establishing the upper limit of the linear dynamic range for your fluorometer. It will vary somewhat from instrument to instrument.

3. Generally, the standard concentration should be approximately 80% of the maximum concentration you wish to read. This is a rule of thumb and not a rigid requirement. If you are using EPA standards, you can dilute the fluorometric or the spectrophotometric standards of pure chlorophyll *a* with 90% acetone to make the appropriate concentration.