**Chlorophyll Analysis: Fluorometer**

**FILTER PREPARATION**

After returning from the field, the chlorophyll filters must be prepared for chlorophyll analysis. Filter preparation should be done in a dark room with as little light as possible. Separate the filter holder into its two halves with input side of filter facing up. Touching only the white ring around the edge of the filter, lift the filter from the holder with the square edged forceps and place input side up (the side that is discolored) on a paper towel to remove excess water. Do not squeeze filter as this may remove chlorophyll concentrated water. Place entire filter including any fragments in a film canister and cap with appropriately labeled lid. Place sample vials in a dark box and freeze for at least 24 hours but no more than 5 days.

1.**Extracting the Sample:**

Note: Because Chlorophyll degrades in the presence of light (and heat), perform the following procedures in low light conditions

A.      **Add 25 mL of methanol to each film canister.** After making sure that the top fits on snuggly, shake each canister for 5 seconds.   If for some reason a different volume of methanol is added to the canister, measure it after reading the sample.

B.      **Put the film canisters in the refrigerator for 24 hours.** The film canisters should be shaken again sometime during the 24 hour period in the refrigerator.

2.**Preparing the Instrument:**

A. **Turn on Turner TD-700 Fluorometer at least 30 minutes before analysis.** Make sure that the proper lamp is installed (Daylight White, marked with a ‘D’) and that the correct filters are lined up in the filter adapter (Position A).   If you change the lamp, be sure to handle it on the black rubber area and not on the bulb itself. Refer to the Owners Manual for more information (Turner Designs Technical Support has been fairly helpful with questions).

B. **Read the solid standard on the high and low setting.** Compare the results to the solid standard measurement at the last primary standard calibration (primary standard calibration should be done at least annually. See calibration notes and manual). If the current readings vary by more than 5%, recalibrate the fluorometer using the solid standard. Use the following settings when calibrating the machine:

-direct concentration, ug/L

-Set the maximum concentration to 250 ug/L

-Set the calibration to read 2 standards

-Set the standard concentrations the same as the solid standard readings during the last primary standard calibration.

Note: It is a good idea to perform the above two steps before extracting the sample to make sure that the fluorometer is functioning properly

3. **Reading the Sample:**

A. **Follow the procedures above for Preparing the Instrument**

B.**Allow the film canisters to sit at room temperature for approximately 15 minutes**to avoid excessive condensation on the glass tubes.

C. **Record the sample information for all of the film canisters on the data sheet**

D**. After shaking the sample for 5 seconds, rinse the pipette tip with 5 mL of sample (and dispose of in a waste container)**

E. **Add 4 mL of sample to a 13 x 100 mL glass tube**

F.       **Insert the sample into the fluorometer and record the reading** in the Fluor Before Acid column. If the sample is over 250 ug/L an OVER message appears and the sample must be diluted. It is a good idea to quickly check the reading of a sample which is suspected to be to high to get an idea if other samples may need to be diluted. If possible read the samples undiluted.

1. **If a sample needs to be diluted**, use an accurate 1000 uL pipette and add 2 mL of methanol to a tube followed by 2 mL of undiluted sample (2x Dilution Factor). Shake the tube well before inserting it into the fluorometer. If the sample still reads OVER, combine 1 mL of undiluted sample with 3 mL of methanol (4x Dilution Factor).   Be sure to record the dilution information on the data sheet. Note that **Dilution Factor** = Total Volume/Sample Volume where Total Volume = Sample Volume + Methanol Volume

G.    **Acidify the sample**by adding 120 uL of 0.1 N HCl. Then gently shake the sample and **wait 90 seconds** before putting the sample into the fluorometer and recording the reading in the Fluor after acid column (30 uL of 0.1 N HCl is added for each 1 mL of sample; 120 uL of acid is added to a 4 mL sample).

1. **To read multiple samples**, it is generally a good idea to group them in categories (by lake or date) and pipette 4 mL of no more than 10 samples into glass tubes. Be sure to keep the samples in the same order that you placed them in the tubes. You will then read and record all of the samples before acidification, acidify all of the samples, shake them all while they are in the glass tube holder, wait 90 seconds, and finally read and record the samples after acidification.

H.   **Double check the results and redo samples which have suspicious numbers.**

1. Make sure that the after acidification values make sense when compared to the before acidification value (the before acid/after acid ratio should be approximately the same for all samples). Note that for samples from Fish Lake in the late summer at depths below the thermocline, the after acid readings have been higher than the before acid readings. In this case, all numbers should be entered into the excel spreadsheet but only the uncorrected chlorophyll a value should be computed.

2. Check that replicate samples are approximately the same.

4.**Clean up:**

Methanol can be disposed of down the drain as long as water is added to make the solution less than 20% methanol and disposal is followed by at least 10 more volumes of water. Disposal down the drain is limited to 10 liters of solvent per day per Principal Investigator.

1. Fill the tubes containing methanol with tap water and dispose of down the drain. Keep the faucet running for a few minutes. Throw the glass tubes in the glass disposal can. Dispose of the remaining methanol in the film canisters and the waste methanol in a similar manner.

2. Rinse the film canisters and lids well with tap water and scrub them out with a bottle brush making sure to remove any remaining filter paper. Give a final rinse with distilled water or Milli-Q and allow to dry.

5.**Calculations:**

The TD700 Fluorometer is calibrated to display readings in ug/L of Chlorophyll (see TD700 Manual). The formulas are in the spreadsheets where the data is entered (from EPA Method 445.0):

**Corrected Chlorophyll a (ug/L)** = Dilution Factor \* (fluor before acid – fluor after acid) \*

(R/(R-1)) \* (mL extraction volume/mL filtered volume)

**Uncorrected Chlorophyll a (ug/L)** = Dilution Factor \* (fluor. before acid) \* (mL extraction volume/mL filtered volume)

**Pheophytin a (ug/L) =**Dilution Factor \* ((R \* fluor. after acid)-fluor before acid) \* (R/R-1) \* (mL extraction volume/mL filtered volume)

where R=acid ratio during primary standard calibration (See Calibration of TD700 Fluorometer)