SOP for analyzing Chlorophyll by Turner 10-AU Fluorometer

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Summary of method

The fluormetric method is used for the analysis of Chlorophyll pigments. Which assumers there are two pigments in the extract: Chlorophyll a and Paeopigment. The initial reading of the extract (F_0) is the fluorescence of chlorophyll *a* and any phaeopigment present. Then 1N HCl is added to convert all of the chlorophyll *a* to phaeopigment, and another reading is taken (F_a). All the process should carry under a cool and dark condition, because Chlorophyll is sensitive to heat and light and Fluorescence is temperature sensitive. Fluorescence decreases as the sample temperature increases. Samples are collected and either filtered in the field or upon arrival at the laboratory. The filtered samples are then kept frozen until they are extracted and analyzed.

Apparatus and Materials

All apparatus should be clean and acid free. Suggestion: soaking all reusable labware in laboratory phosphate free detergent for 4 hrs, then rinsing with hot tap water, de-ionized water, and acetone before drying. Place them on rack for air dry (store upside down).

Syringe 60 mL
Syringe filter holder
Whatman GF/F glass fiber filters (25 mm nominal pore size of 0.7 µm)
Filter forceps or tweezers
Aluminum foil
Nalgene centrifuge Tubes with cap 15 mL – rinse with acetone and air dry (acid free)
Centrifuge – Jouan BR4
-70^{o}C freezer for filter storage
Refrigerator 0^{o}C for store samples.
Fluorometer with excitation filter 436 nm and emission 680 nm (narrow band filter: 431-441; 675-685) http://www.fluorimeter.com/t2/doc/appnotes/998_0050/0050_c3.html

Reagents:

90% acetone HPLC grade	http://jtbaker.com/msds/englishhtml/A0446.htm
1N Hydrochloric acid (HCl)	https://fscimage.fishersci.com/msds/95551.htm
	http://www.jtbaker.com/msds/englishhtml/H3880.htm

Safety and Waste Handling

Consult the MSDS for all chemicals used in this experiment and please follow all laboratory protocol for safety procedures. Acetone is very volatile reagent and is highly flammable, please handle Acetone under a fume hood and wear personal protective equipment (PPE) including gloves, lab coat, and safety glasses.
After analysis follow the waste disposal procedure, discard analyzed samples in an acetone waste container.

Samples collection storage and preparation

Triplicate seawater samples from pump house hose are collected once a week, and filtered at the laboratory. 100mL seawater is filtered through GF/F 25mm filter and is used for chlorophyll analysis.

All glass and plastic ware to be used for chlorophyll sample collection, filtration and flurometric analysis must be ***Acid Free***. Acidic residue can lead to the conversion of chlorophyll to phaeopigments, resulting in an underestimate of the chlorophyll concentration and overestimate of phaeopigment concentration. Equipment and sample bottles used to collect for chlorophyll analysis must be thoroughly rinse with type I water at least three time, to ensure that no acid residue is present. A final rinse should be done with acetone followed by type II water before drying.

Filtering Chlorophyll Samples

1. Before sampling, pre-load a filtration rig or rack with filters (alternatively, you can use a syringe - see below). using clean forceps to load a 25mm 0.7μm pore size GF/F glass fiber filters onto the filtration rack. (or GF/C glass fiber filter if not use the filtered seawater for nutrient analysis)
2. The field sample to be analyzed for chl *a* must be well mixed. Vigorously invert bottle several times immediately before filtration.
3. Measure 100mL seawater using a clean graduated cylinder (acid free).
4. Vacuum filter the sample (<150 mm Hg) until the filter is dry.
5. Again using forceps, remove the filter and place in an 8 ml borosilicate test tube preloaded with 6 ml 90% acetone. Make sure the filter is completely submerged in the acetone solution, and only one filter is in each test tube. *Note: if using a repeat dispenser to pre-load test tubes, use a small graduated cylinder to verify 6 ml are being dispensed.*
6. Allow the filters to extract for ~24 h at -20°C in the dark.
7. After 24hr, shake or vortex each tube vigorously, then remove the filter with a small (very clean) metal spatula. Make sure to rinse the spatula with 90% acetone before use and between samples.
8. Centrifuge acetone extracts for 5 minutes at 5000 rpm and 4^{o}C.
9. Keep chlorophyll tubes in the dark after centrifuging and before reading on the fluorometer.

Filtering with a Syringe

1) Using forceps, place a GF/F filter into a clean Swinex-type 25 mm filter holder, making sure to include the Teflon gasket. Carefully screw the filter holder halves together.
2) Find a 60mL syringe with an intact plunger (i.e. double check that the black plastic on the plunger is not dry and cracked).
3) Measure a known sample volume (100mL) with a graduated cylinder, then pour into a syringe attached to the filter holder. Gently filter the sample.
   ** Don't draw back on the syringe while the filter is in place as this might displace or break the filter.*
4) Unscrew the filter holder and remove filter with tweezers, and place the filter in an 8 ml borosilicate test tube. Fill with 6 ml 90% acetone and close it with cap. Make sure the filter is completely submerged in the acetone solution, and only one filter is in each test tube. *Note: if using a repeat dispenser to pre-load test tubes, use a small graduated cylinder to verify 6 ml are being dispensed.*
5) Allow the filters to extract for ~24 h at -20°C in the dark.
6) After 24 hr, shake or vortex each tube and centrifuge acetone extracts for 5 minutes at 5000 rpm and 4^{o}C. (Turn on the centrifuge to let it chill to 4^{o}C before use, and always make sure the centrifuge is balance if you don't have a full load).

7) Keep chlorophyll sample tubes in the dark after centrifuging and before reading on the fluorometer.

In all steps, try to avoid degradation of pigments as much as possible by minimizing sample exposure to light and heat. Suggest: run Chlorophyll Samples within a month to avoid expiration

Reading samples in the fluorometer

1. Fill a test tube with 90% acetone using as blank and wipe tube with a Kimwipe, Read the Fluorometer as blank and record the fluorescence. (cap this blank and for checking instrument drift at end of day)
2. optional - Check solid standard for the High and Low.
3. Carefully pour sample (90% acetone) into culture tubes leaving the filter in the centrifuge tube. Wipe the side of the tube containing your extract, then place it into fluorometer, let the reading stabilize, and read the fluorescence (F_0). Record sample name, volume of seawater filtered, volume of 90% acetone used for extraction, and the fluorescence reading.
4. Without removing the tube from fluorometer, drop in 2 drops of 1N HCl (acidification). Record the fluorescence after the reading stabilized (F_a).

Sample Disposal

The 90% acetone extractant is disposed of into the labeled acetone waste container located in the fume hood. Rinse the test tube with DIW (discard the water to same waste bottle) or wait till Acetone complete dry out before disposal test tubes in the broken glasses boxes. The acetone soaked filters should be placed on a beaker, and left in the fume hood until completely dried. Once filters are dry they are disposed of in the garbage. Centrifuge tubes used for sample extraction are washed and re-used. Wash and rinse several times with DIW and reagent type I DIW. Then final rinse with acetone before put them away.

*Calculations**

Calculate chl and phaeopigment concentration using the following equations (from Lorenzen, 1966):

$$\text{Chl } a \text{ (µg/liter seawater)} = \frac{F_m}{(F_m - 1)} \frac{k\, v(Fo - Fa)(d)}{V_f}$$

$$\text{Phaeopigments } a \text{ (µg/liter seawater)} = = \frac{F_m}{(F_m - 1)} \frac{k\, v(F_m Fa - Fo)(d)}{V_f}$$

where:

Fo = fluorescence before acidification

Fa = fluorescence after acidification

Fm = maximum acid ratio which can be expected in the absence of pheopigments

k = calibration factor relating amount of pigment to fluorescence intensity: [(µg Chl a/ml solvent)/instrument fluorescence unit]

v = volume of acetone used for extraction (ml)

V_f = volume of seawater filtered (liters)

d = extract dilution factor (e.g. if you diluted 1 ml extract by adding it to 4 ml solvent, your dilution factor would be 5)

The factors Fm and k are specific to each fluorometer, and will change over time, as well as when the light bulb or filters are changed or cleaned. These factors must be determined at regular intervals by calibration with pure chorophyll *a*. A secondary (e.g. solid) standard can be used to check for instrument drift between calibrations.

*The above sample analysis procedure and calculations are for use with the "Traditional extractive acidification method". This requires the fluorometer to be outfitted with a daylight white lamp, an excitation filter of 340-500 nm, and an emission filter of >665 nm. When high levels of chlorophyll *b* are present, an alternate, non-acidification method can be used to minimize the interference of chl *b* with the chl *a* determination. This non-acidification method requires a different lamp and filter set-up, and does not provide an estimate of phaeopigment concentration.

Lorenzen, C.J. 1966. A method for the continuous measurement of the in vivo chlorophyll concentration. Deep-Sea Res. 13: 223-227

Using Turner 10-AU Fluorometer

Blanking the Turner 10-AU Fluorometer

- Turn on Fluorometer and allow to at least 15 minutes for system warm up. The "red" on/off button is on the lower right side by the plug.
- Use 10mL disposable culture tubes for samples and the blank.
- Insert 90% acetone blank and replace cap. Wipe off the outside of the culture tube with a Kimwipe before inserting it into the sample holder.
- Press **ENT** button to enter the Main Menu
- Press **2** to enter the Calibration Menu
- Press **1** to enter the Blanking Menu
- Press **1** to Run Blank
- Wait till TC >= 8
- Set the Span % to 99% by pressing the **up or down arrow buttons**. The Fluorometer is at maximum sensitivity when the Span is set to 100%, and the instrument is in low range. The Blank % should be less than 200%, if not a lower Span % setting may be used.
- Allow the Blank % reading to stabilize, and then press **0**. Wait for 15 seconds while it is blanking.
- Once blanking is complete, presses **ESC four times** to back out of the menus and return to normal operation. With the blank still in the Fluorometer the reading should be close to 0.000.
- **Record** this blank number in your notebook, and read the blank at end of day again for checking instrument drift. (Be sure you cap the test tube, so acetone won't vaporized)

Reading the solid standards: L & H (Rhodamine WT: $C_{29}H_{29}N_2O_5ClNa_2$ H: ~66 & L: ~10)

- Use the right size cuvette holder for the solid standard (solid standard is inside a box and keep at left top drawer of Fluorometer bench.
  **When replace the cuvette holder. Be sure to insert it correctly, with the metal prong to the left and clear prong to the right.*
- Facing the instrument, place the solid standard in the sample chamber with the letter "L" (etched on top of standard holder) on your left hand side. Make sure the metal pin is completely seated in the notches of the 13 mm round cuvette adaptor.
- Press <*> to read the value and record this number as the low standard (~10.2).
- Open the cuvette cap and rotate solid standard 180 degrees ("H" will be on left).
- Press <*> to read the value and record this value as the high concentration (~66.4).
- Repeat same step at end of day to check the instrument stability.

If you have a lot of samples to run, read the high and low solid standards every 30 to 40 samples.

Reading a sample

- Remove the blank, insert your sample (after gently inverting the sample to mix), and replace cap.
- The Fluorometer may automatically change concentration settings, if so wait for 5 to 10 seconds.

- Allow the reading to stabilize then press the "*" button on the keypad. This is a discrete sample averaging function which delays for 15 sec. then averages fluorescence over a 10 sec. period.
- If the fluorometer reads "over" the sample must be diluted and re-analyzed.
- Record the averaged fluorescence value on the data sheet. Also make sure to record your sample name, volume of water filtered, and the volume of acetone used for the extraction.
- Without removing the sample from the Fluorometer, place 2 drops of 1N HCL in the culture tube. Allow the reading to stabilize then press the "*" button on the keypad and record the averaged acidified fluorescence value on the data sheet.
- There is no need to re-blank the instrument in between samples, or after the concentration setting has changed.

Check instrument drift

Read the original blank and both High and Low solid standards again. Compare the reading to the beginning to make sure instrument has not drifted.

You can record the results on the Green folder ("Turner 10-AU Solid Standard Log") as a record of the solid standard calibration procedure (see page 6). The numbers recorded should be very close in value. If not (drift a lot), the instrument might not be in good condition.

Turning off the Fluorometer

- Press the red button to turn off the Fluorometer.