Lab 5: Chlorophyll and CDOM fluorescence 23 July 2021

1. Introduction and Lab Goals

Fluorescence is a property of certain molecules, such as the pigment chlorophyll *a* (chl *a*) and some components of dissolved organic matter (e.g. the amino acids, tyrosine and tryptophan, that are used to biosynthesize proteins), whereby some fraction of absorbed energy is re-emitted at a longer wavelength (lower energy) (Figure 1). Fluorescence can be a powerful tool for studying these compounds in the ocean because, to first order, fluorescence is linearly proportional to concentration of the fluorescing molecule, its spectral signatures are unique, and fluorometers are very sensitive to low concentrations. There are a few caveats to the underlying physical relationships due to measurement constraints and environmental and biogeochemical variability.

Today's laboratory will focus on a few of these issues to highlight the strengths and weaknesses (constraints and opportunities) of the fluorescence protocols for chl *a* and CDOM. Specifically, we will explore:

1. The Androscoggin River CDOM fluorescence
2. How to calibrate a chlorophyll fluorometer and how to understand how the calibration factors vary due to differences in the calibration sample (i.e., what is the factory providing you with when you purchase a fluorometer?)
3. The relative magnitudes of variability in chlorophyll fluorescence across a range of samples with a chlorophyll fluorometer
4. The use of in vitro fluorescence to determine the concentration of chlorophyll across a range of samples and the approach to quantifying uncertainty

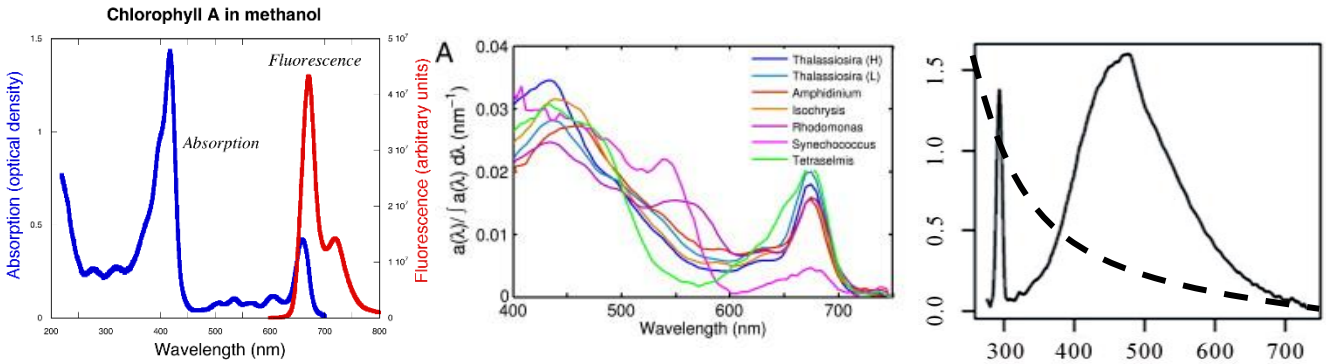

Figure 1. (A) Absorption (blue) and fluorescence (red) spectra for chl a in methanol, (www.yorku.ca/planters /photosynthesis). (B) Absorption spectra for a range of phytoplankton groups (Roesler and Barnard 2013). (C) CDOM fluorescence emission spectrum from Sardinian waters (Sighicelli et al 2014). Schematic CDOM absorption coefficient shown by dashed line.

The WETLabs ECO chl *a* fluorometer excites at 470 nm and measures fluorescence emission at 695 nm. The range of wavelengths that stimulate both chl *a* and CDOM fluorescence (called the excitation spectra) are broad (Figure 1; look at the difference between the excitation for chl *a* in a solvent versus chl *a* in a cell). Think about what this means when the sensor excitation is within a narrow band (indicate the excitation wavelength for these instruments on Figure 1). Additionally, the emission bands of both CDOM and chl *a* are also much broader than the narrow sensor detection bands. Think about how these actual emission bands may impact your measurements (again, look at what these sensors are detecting in the specified emission waveband). Draw a bar on Figures 1B and 1C associated with the ECO Chlorophyll fluorometer excitation (470 nm, 10 nm FWHM). Draw a bar on Figure 1A and 1C associated with the ECO Chlorophyll fluorometer emission (695 nm, 25 nm FWHM). Use these sketches to interpret your work.

2 Activities

Rotations: The lab activity will take place in the teaching lab at multiple stations. Students will be split into four groups of 4 or 5. Unlike the rotations that we have done thus far, this lab will require a little bit of self-organization between student groups. Each group will be doing the exact same activities some of which might take one group longer and one group less time. It will require collaboration and thoughtfulness.

Each group is going to perform a calibration of the chlorophyll fluorometer using their specific water sample by making a 5-point dilutions series (typically we use 9-point but in the interest of time we will do 5), measuring the fluorescence of each dilution, and determining the chlorophyll concentration of each dilution.

Water samples: As with the other labs, we will work with natural water samples collected from the dock in the Harpswell Sound (HS) and the Androscoggin River (AR) in Brunswick as well as 2 phytoplankton cultures. As with lab 4, each group will be assigned a water sample. Each group will be responsible for conducting a fluorometer calibration on their water sample. They will also be responsible for processing their own calibration data. Once each group has their results, the entire class can share group results to answer the synthetic questions. In this way, everyone has the opportunity to fully engage in the process of fluorometer calibration.

3 Steps for a calibration

3.1. Collect your assigned water sample and sample bottles:

Group 1: Harpswell Sound water and clear sample bottles

Group 2: Harpswell Sound water and amber sample bottles

Group 3: Thalassiosira and amber sample bottles

Group 4: Dunalliela and amber sample bottles

3.2. Make your dilutions series

Using label tape, carefully label your sample bottles with your group number and the dilution number (1 is the most concentrated sample, 5 the least). You will need 1-L of each dilution. Here is the recipe; FSW is filtered seawater.

Start with 2-L D1	→ put 1-L D1 into bottle D1	1.0
With remaining 1-L D1, add 1-L FSW = 2-L D2	→ put 1-L D2 into bottle D2	0.5
With remaining 1-L D2, add 1-L FSW = 2-L D3	→ put 1-L D3 into bottle D3	0.25
With remaining 1-L D3, add 1-L FSW = 2-L D4	→ put 1-L D4 into bottle D4	0.125
With remaining 1-L D4, add 1-L FSW = 2-L D5	→ put 2-L D5 into bottle D5	0.0625

3.3. Measure the *in vivo* fluorescence, F_{chl} with the fluorometer. You will measure the fluorescence signal of each dilution, *plus* the fluorescence of filtered seawater. All groups can use the same sample of FSW, which we will put out for you. It is important that each group is using the same chlorophyll fluorometer (so we can compare calibrations). This is going to be one bottleneck in the process.

- Open Hyper-terminal and Start Capture Text. Name your file with your group number.
- Take a dark reading by taping the sensor face and plugging in the battery. Collect 30-60 seconds of data. Remove the battery.
- Next measure the FSW in the 1-L beaker. Submerge the fluorometer into the fluid, taking care not to overflow the beaker, check to make sure there are no bubbles on the sensor head. Slowly move the sensor in a circle to prevent quenching by the sensor. Make sure you keep the sensor vertical and submerged. Once you feel comfortable begin.
- Have one person attach the battery to the test cable to turn the fluorometer on.
- Have another person capture the data stream. Name the file with your group name. We want 30-60 seconds of data collected for each dilution.
- When the data collection is complete, remove the battery. It is not necessary to stop the file, you can parse it on the time stamp.
- Cover the FSW beaker and set aside.
- Pour your least concentrated dilution (D5) into the beaker marked D5. Repeat the measurements as above. When complete, our D5 back into the sample bottle.
- Repeat with each dilution in order of increasing concentration. Do not pour the sample into the beaker until you are ready to measure it as you are trying to minimize exposure to light.

3.4. Prepare the chlorophyll extracts for each dilution. Each member of your team should be responsible for at least one of the dilutions. You will collect triplicate samples for each dilution.

- Place a filter on each of three filter cups. There are 6 spaces so two people can work at the same time.

- Filter 100 ml for D1 and D2, 200 ml for D3 and D4, 300 ml for D5. Record your uncertainty in the filter volume for your method and graduated cylinder: _____ml
- Place each filter into a labeled centrifuge tube (e.g. G1 D3 B for group 1, dilution 3, 2nd of 3 triplicates).
- Add 10 ml of 90% acetone, cap the tube, check to make sure cap is secure and sample won't leak or evaporate. Record your uncertainty in the acetone volume: _____ml
- Vortex the tube for 10 seconds, shake vigorously
- Place tubes in freezer for at least 30 minutes (this is a compromise, we normally leave 24 hours)

3.5. Measure the *in vitro* fluorescence of the extracts using the Turner Fluorometer.

- Remove your samples from the freezer, vortex them again for 10 seconds
- Place in the centrifuge. There is space for 6 vials so pair up with a group member. Set the clock for 3 minutes (this is a compromise, we typically spin down for 5 minutes)
- Take care not to invert tubes as you have just spun them down to remove the filter fibers to the bottom.
- Gently pour supernatant into a fluorometer tube. Cap.
- Wipe the tube with a Kim wipe and place it in the fluorometer, replace the cap
- adjust the sensitivity as you are shown by Meg, so you can read the 0-10 scale between 2 and 9.
- Write down the scale setting and the reading on the log sheet and table 1. Record your uncertainty in the readings.
- Add 3 ml of 10% HCL, cap, invert 3 times and place in the rack.
- Repeat for your other 2 samples, then let your partner read their 3 samples
- Starting with your first acidified tube, record the fluorescence value and scale. Record the uncertainty in your reading.
- Repeat for your other 2 acidified samples, and let you partner read theirs
- Measure the reading for a tube of 90% acetone on the scales you used, enter values in the Table below. You only need to do this once for each of the scales used.
- Empty your fluorometer tubes into the waste container, empty your centrifuge tube and remove the filter. Use the filter to wipe off the label. Rinse all the tubes and caps in the beaker of MQ and let drain by the sink.

Table 1. Fluorometric readings of extracted samples.

	Initial				acidified			
Sample	Scale	R_{blank}	$R_{initial}$	uncertainty	Scale	R_{blank}	$R_{acidified}$	uncertainty
D __A								
D___B								
D___C								

3.6. Things to do while you wait.

- Label your centrifuge tubes
- Measure AR CDOM sample with both the CDOM fluorometer and the Chl fluorometer.
- Measure the HSFSW sample with the CDOM fluorometer
- Measure the absorption of D1 with an acs in spectrophotometer mode
- Measure the absorption of CDOM with an acs in spectrophotometer mode
- Work on the uncertainty propagation, think about source of uncertainty you can identify but not determine, think about sources of uncertainty that you cannot identify
- Set up your data processing steps and code
- Go for a walk
- Have a snack

3.7. Data processing

Extracted [Chl]

1. Compute the blank and scale corrected fluorescence readings, record in Table 2:

$$F_o = \frac{(R_{initial} - R_{blank})}{scale_{initial}} \qquad F_a = \frac{(R_{acid} - R_{blank})}{scale_{acid}}$$

2. Compute the chlorophyll and pheophytin readings using the equations below with Turner calibration coefficients (M, A) provided, enter the values in Table 2.

$$Chl\ (\frac{mg}{m^3}) = M \times (F_o - F_a) \times \frac{V_{acoh}}{V_{filt}} \qquad Pheo\ (\frac{mg}{m^3}) = M \times (A \times F_a - F_o) \times \frac{V_{acoh}}{V_{filt}}$$

3. Compute the mean and standard deviation values of Chl and Pheo from your triplicate readings of dilution 1. Record them in Table 2. This is the Type A uncertainty described below.
4. Compute the propagated uncertainty in your calculation of Chl and Pheo for each dilution. Record them in Table 2. This is the Type B uncertainty described below based upon your uncertainty in each term of the Chl and Pheo equations (Jcgm 2008).
5. Which step in the determination of chlorophyll and pheophytin yields the largest contribution to the propagated uncertainty? Which yields the smallest? What approach would you take in your methodology to reduce the propagated uncertainty?
6. How does the Type A uncertainty compare to the Type B uncertainty for your dilution? How does this impact your methodology? Would you make any changes to your approach?

Table 2. Computed values of chlorophyll and pheophytin and their propagated uncertainty.

Sample	V_{filt}	F_o	F_a	Chl	$Pheo$	$uChl$	$uPheo$
D____A							
D____B							
D____C							
Mean D___						σ=	σ=

Calibration of chlorophyll fluorometer

1. Compute the average and standard deviation of your i*n vivo* fluorescence values (F_{chl}) for your samples (dark, FSW blank, and dilutions).
2. Plot F_{chl} vs [Chl] for your dilution series, including the FSW (assume {Chl] = 0). Use the values of your standard deviations in [Chl] and F_{chl} to plot error bars.
3. Use a type 2 linear regression (which takes into account the standard deviations in X and Y) to estimate the slope of the relationship and estimate the uncertainty in the slope. What are the units of the slope? What are the units of the intercept? Use the information you have collected to provide a calibration equation for your sensor and your water sample of the form:

$$Chl\ (\frac{mg}{m^3}) = \frac{(F_{measured} - F_{dark})}{scale\ factor}$$

4. Where $F_{measured}$ is the measured fluorescence of a sample in digital count units, $F_{dark}$is the sensor dark reading in digital count units, and the scale factor converts digital counts to ($\frac{mg}{m^3}$). This is the form of the equation provided by instrument manufacturers.

F_{CDOM}

1. Recover the AR $a_{CDOM}(370)(m^{-1})$ absorption coefficient that you measured in the spectrophotometer from Lab 2. This is the CDOM absorption at the wavelength that the CDOM fluorometer excites the sample.
2. Compute the F_{CDOM} vs $a_{CDOM}(370)$ ratio. You will use this value to convert F_{CDOM} to $a_{CDOM}(370)$ for the cruise data that you will collect in week 3. (*You will then be able to use the spectral slope of CDOM absorption to model the spectral CDOM absorption* at 370nm for comparison with measured.)
3. Determine the *apparent* chlorophyll fluorescence signal for the CDOM sample using your Fchl reading. We use the term *apparent* because there is no chl a in the filtered CDOM sample. Using your calibration slope to convert the Fchl reading into an apparent chl concentration.

$$[Chl]_{apparent} = \frac{(F_{Chl\_AR} - F_{dark})}{scale\ factor}$$

4. What is the ratio of the $[Chl]_{apparent}$ to F_{CDOM}? This is an estimate of the correction term for F_{CDOM} contamination of F_{Chl}.
5. Calculate the *apparent* $[Chl]$ in the filtered Harpswell Sound water. Is it significant? Do you need to correct the F_{CDOM} signal for contamination by F_{Chl}? Why or why not? What conditions would you look for in the natural environment that would make you consider this correction?

Class-wide comparison of calibration coefficients

How do the chlorophyll scale factors compare across the four water samples? If they are different, how do you explain their difference? If they are the same, does this make sense? Think about the different samples and how that might impact your results.
Look back at Figure 1 to help you think about your answer.

Estimating Uncertainty

First some terminology. The thing you are trying to measure is the **measurand**. The objective of any measurement is to determine the **value of the measurand**; note we do not say *true* or *real* value. Because we are not perfect and even if we were, we could not be sure that we were, the result of a measurement is only an approximation or **estimate** of the value of the measurand. We use the term **accuracy** to describe how close the estimate and actual value of the measurand are to each other. **Precision** is used to describe how clustered a set of measurements are to each other. Imperfect measurements give rise to **errors**. These may be random or systematic. When measuring your filter volume, the **random** error is due to your inability to pour exactly to the 200 ml gradation; the **systematic** error is one that would arise if the graduated cylinder was not used at 25C (the temperature at which it was calibrated).

The uncertainty in the result of a measurement reflects the sum of all of the sources of error you can identify and quantify; it provides us with a guide as to what observed level of variability can be interpreted as natural versus as part of the measurement error. For example, if the measured chlorophyll concentration for two samples was 4.1 mg/m^3 and 7.8 mg/m^3, you might conclude that sample 2 was almost twice the value of sample one. However, if the uncertainty in your measurement was 3.9 mg/m^3, the two values are not statistically different from each other and you could conclude nothing about their relative values. But, if your uncertainty was 0.4 mg/m^3, then you could reasonably conclude that the phytoplankton in sample two were nearly twice as concentrated as in sample 1. This is why you must report uncertainties with every measurement you make, otherwise you should not waste your time making the measurement.

Today you will be dealing with two types of uncertainty. Type A uncertainty is quantified by making repeated measures (i.e., the triplicates of dilution 1). For example, when an *in situ* fluorometer makes 60 sample measurements, we calculate the mean value ($\bar{x}$) and report it along with the standard deviation (σ), mean ± σ. This signifies that 68% of the observations were within σ of the mean. Type B uncertainty is estimated from other information (like how close to the 200 ml gradation you are likely to be able to resolve).

The first step is to identify which terms are measured terms and which are constants, i.e., which terms have known uncertainty. The second step is to quantify the uncertainty for each measured term and then to compute the total uncertainty in the measurement. The goal is two-fold: (1) reporting the mean and uncertainty in our estimated chlorophyll and pheophytin concentrations, and (2) using the exercise to identify which steps in the method are most critical to reduce uncertainty.

Refer to the equations for calculating chlorophyll and pheophytin above. You are going to compute the uncertainty in each of the terms in the equation. This is done by computing the partial differentiation of the equation with respect to each term. This is combined with the uncertainty of each term to construct the Type B propagated combined uncertainty, u_c, in parameter y (e.g., chlorophyll concentration):

$$u_c(y) = \sqrt{\sum_{i=1}^{N} \left(\frac{\partial f}{\partial x_i}\right)^2 u^2(x_i)}$$

where f is the functional relationship (the equation) describing the set of N other quantities, x_i (volume filtered, fluorescence reading, etc.), that are related to measurand y (chlorophyll concentration). $u(x_i)$ is the standard uncertainty for each x_i in f (e.g., uncertainty in filter volume and analog fluorescence readings, or uncertainty in Turner fluorometer calibration coefficients). From the chl equation, this looks like:

$$u_c(Chl)$$

$$= \sqrt{\left(\frac{\partial Chl}{\partial M}\right)^2 u^2(M) + \left(\frac{\partial Chl}{\partial F_o}\right)^2 u^2(F_o) + \left(\frac{\partial Chl}{\partial F_a}\right)^2 u^2(F_a) + \left(\frac{\partial Chl}{\partial V_{filt}}\right)^2 u^2(V_{filt}) + \left(\frac{\partial Chl}{\partial V_{acoh}}\right)^2 u^2(V_{acoh})} \ .$$

where,

$$\frac{\partial Chl}{\partial M} = (F_o - F_a) \times \frac{V_{acoh}}{V_{filt}}$$

$$\frac{\partial Chl}{\partial F_o} = M \times \frac{V_{acoh}}{V_{filt}}$$

$$\frac{\partial Chl}{\partial F_a} = -M \times \frac{V_{acoh}}{V_{filt}}$$

$$\frac{\partial Chl}{\partial V_{filt}} = -M \times (F_o - F_a) \times \frac{V_{acoh}}{V^2{}_{filt}}$$

$$\frac{\partial Chl}{\partial V_{acoh}} = M \times (F_o - F_a) \times \frac{1}{V_{filt}}$$

Plugging those partial differential terms leads to:

$$u_c(Chl)$$

$$= \left(\left((F_o - F_a) \times \frac{V_{acoh}}{V_{filt}}\right)^2 u^2(M) + \left(M \times \frac{V_{acoh}}{V_{filt}}\right)^2 u^2(F_o) + \left(-M \times \frac{V_{acoh}}{V_{filt}}\right)^2 u^2(F_a) + \left(-M \times (F_o - F_a) \times \frac{V_{acoh}}{V^2{}_{filt}}\right)^2 u^2(V_{filt}) \ldots \right.$$
$$\left. + \left(M \times (F_o - F_a) \times \frac{1}{V_{filt}}\right)^2 u^2(V_{acoh}) \right)^{\frac{1}{2}}$$

Make sure you have estimates for each of the u terms in this equation. You can derive a comparable analysis for $u_c(Pheo)$ if you have time. It will be different because the equation for computing pheophytin is different.