Lab 5: Chlorophyll and CDOM fluorescence May 23, 2025

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 a component of some 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 the 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 Damariscotta River Estuary CDOM fluorescence,
2. How to calibrate an *in situ* chlorophyll fluorometer and how to understand the variations of calibration factors 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 that were collected and filtered in the same way,
4. The use of in vitro fluorescence to determine the concentration of extracted chlorophyll across a range of samples, and the approach to quantifying uncertainty.

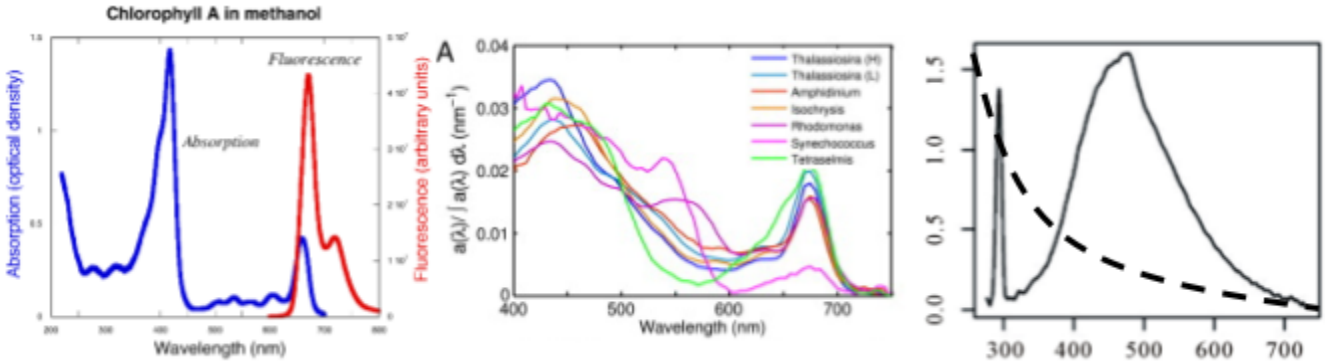

Figure 1. (A) Absorption (blue) and fluorescence (red) spectra for chl a in methanol, (www.yorku.ca/planters /photosynthesis). (B) Absorption spectra for a range of phytoplankton groups (Roesler and Barnard 2013). (C) CDOM fluorescence emission spectrum from Sardinian waters (excitation 266 nm; Sighicelli et al 2014). Schematic CDOM absorption coefficient shown by dashed line.

The Seabird ECO chl *a* fluorometer excites at 470 nm and measures fluorescence emission at 695 nm. The Wetstar chl *a* fluorometer excites at 460 nm, and measures emission at 695 nm. The range of wavelengths that stimulate **both** chl *a* and CDOM fluorescence (called the

excitation spectra) are broad. Think about what this means when the sensor excitation is within a narrow band. 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).

2 Activities

1) **Collect individual water samples at the dock**
2) **With your group's shared sample (not the same as individual samples in #1), make a dilution series**
3) **Measure in vivo chlorophyll and CDOM fluorescence for your dilution series**
4) **Filter chlorophyll samples (dock samples and group dilution series)**
5) **Run chlorophyll samples**

The lab activity will take place at multiple locations. After the dock sampling extravaganza, you will be split into three groups (A(7 students), B(6 students), C(6 students). 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. Additionally, you will be using sampling bottles with precalibrated volumes for all chlorophyll filtrations. Make sure you write down the volume, keep track of your bottle, and think about your labeling and naming.

Each of the three groups is going to perform a calibration of the chlorophyll fluorometer using their specific water sample by making a 4-point dilution series (typically we use 9-point but in the interest of time we will do 4), measuring the in vivo fluorescence of each dilution, and determining the extracted chlorophyll concentration of each dilution.

Water samples: As with the other labs, we will work with natural water samples collected from the Damariscotta River Estuary (DRE) as well as a single phytoplankton culture (Culture 2 in previous labs, *Chaetoceros muelleri*). Group A will have culture samples, exposed to **high light** conditions, Group B will have the same culture samples kept in **low light** conditions, and Group C will use **DRE** water samples.

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.

2.1 Collect individual water sample at the dock

Grab a sampling bottle, and join your group to sample the water. All will sample at the same time - following the directions of the instructor (rinse + sampling). Note the volume of your bottle, and take it with you back to the Mitchell lab where you should LABEL it.

2.2. Make a dilution series with your group's sample

Find your group (A, B, or C), and grab a 2L bottle of your group's sample (D1) and 3 sampling bottles for chlorophyll extraction. Grab 2 more 2L bottles for your serial dilution.

Using label tape, carefully label your 2L bottles with your group number (A, B, or C) and the dilution number (D1 is your concentrated sample, D3 the least). You will need 1-L of each dilution to measure the in vivo fluorescence with the ECO fluorometers, after which you will sample them for extracted chlorophyll analysis. Here is the recipe (FSW is filtered seawater). The exact containers that will be used to hold intermediate dilution steps may be different than in the table; instructors will tell you what to use!

Make the dilution	Store the dilution	Dilution name and value
Start with 2L D1	• Fill up your sampling bottle for chlorophyll extraction (label it) • Put ~800 mL D1 into the D2 bottle	D1 = ? (? meaning you calculate this)
Add 1L FSW to the 800 mL in the D2 bottle and mix	• Fill up your sampling bottle for chlorophyll extraction (label it) • Put ~800 mL D2 into the D3 bottle	D2 = ?
Add 1L FSW to the 800 mL in the D3 bottle and mix	• Fill up your sampling bottle for chlorophyll extraction (label it!)	D3 = ?

After making this serial dilution, make sure your samples for chlorophyll extraction are labeled, and the volume of each bottle is noted in your notes. For Groups B and C, leave your dilution series in Mitchell lab - Group A: give your dilution series bottles to the instructors and we will put it back in the High Light environment.

From here, follow your GROUP SPECIFIC INSTRUCTIONS (A, B, or C).

3. Data processing

3.1 Extracted [Chl]

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

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

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 19-plicates from the dock. 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	$Pheo$	$uChl$	$uPheo$
D1							
D2							
D3							
FSW							
Dock sample							

3.2 Calibration of the ECO chlorophyll fluorometer

1. Compute the average and standard deviation of your i*n vivo* fluorescence values (F_{chl}) for your samples (dock, FSW blank, and dilutions).
2. Plot F_{chl} vs [Chl] for your dilution series, including the FSW (assume [Chl] = 0). Use the uncertainties in [Chl] and F_{chl} to plot error bars.
3. Use a type 2 linear regression (which takes into account the uncertainties 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.

3.3 Analysis of F_{CDOM} and the impacts on F_{Chl}

1. 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.

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

2. 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}.
3. Calculate the *apparent* $[Chl]$ in the filtered DRE 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?

3.4 Class-wide comparison of calibration coefficients

How do the chlorophyll scale factors (calibration slopes) compare across the three 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 (community composition, light exposure) and how that might impact your results. Look back at Figure 1 to help you think about your answer.

3.5 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, so 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 25°C (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., 19 samples you collected at the dock). 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 one 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 if you are using the graduated cylinder to measure your volume).

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:

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

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.