Lab 4: OVERVIEW for Chlorophyll and CDOM fluorescence lab 14 July 2011

INTRODUCTION

Fluorescence is a property of certain molecules, including chlorophyll *a* and CDOM, and can be a powerful tool for studying these compounds in the ocean. In principle, the magnitude of fluorescent emission of a compound is linearly proportional to its concentration at low concentrations (at high concentrations, expect non-linearity). In practice, however, there are a number of caveats that compromise this linear relationship. In today's laboratory, you will participate in several activities that will provide you with greater insight into the relationships between fluorescence and concentrations of chlorophyll *a*, phycoerythrin (PE), and chromphoric dissolved organic material CDOM. Specifically, you will explore fluorescence in a variety of ways:

1. *in vivo* chlorophyll *a* fluorescence — is it affected by other variables? Measure *in vivo* fluorescence of chlorophyll *a* with the type of sensors that are used on moorings, ship-based profilers, and mobile platforms, and test for linearity between fluorescence and concentration of phytoplankton, effect of CDOM, and particles (clay) on the fluorescence signal. What is the excitation spectra of the LEDs?
2. *in vitro* chlorophyll *a* fluorescence — filter and extract chlorophyll from a culture of diatoms with 90% acetone, then measure the *in vitro* fluorescence in acetone on a Turner Designs' 10-AU fluorometer to compute chlorophyll *a* concentration.
3. issues related to standardization; CDOM and PE fluorometers; excitation spectra:
    a. solid secondary standards for Turner Designs' fluorometers;
    b. phycoerythrin (PE) fluorescence for a culture of the cryptophyte *Rhodomonas;* and
    c. CDOM fluorescence for DRE and Biscay Pond.
4. other explorations of *in vivo* chlorophyll *a* fluorescence –
    a. look at emission of chlorophyll *a* from live phytoplankton cells, using an epi-fluorescence microscope, equipped with a blue excitation filter and red emission filter;
    b. investigate fluorescence quenching of phytoplankton from exposure to high light (either natural sunlight or in a solar simulator, depending on the weather), and measure the fluorescence over time in a bench top Turner Designs TD-700 fluorometer.

Divide into 6 groups with 3 or 4 students each; there will be two parallel groups for each station:

	1st session	2nd session	3rd session	4th session
Station 1	Group 1 and 2	Group 5 and 6	Group 3 and 4	
Station 2		Group 1 and 2	Group 5 and 6	Group 3 and 4
Station 3 A	Group 3		Group 1	Group 5
3B	Group 4		Group 2	Group 6
Station 4	Group 5 and 6	Group 3 and 4		Group 1 and 2

BACKGROUND

What is fluorescence? Fluorescence is the property of some molecules whereby a fraction of energy absorbed at shorter wavelengths (higher frequency, higher energy) is re-emitted at longer wavelengths (lower frequency, lower energy).

Recall the relationship between energy and wavelength:

$$E = h\nu = hc/\lambda$$

where E is energy, h is Planck's constant, ν is frequency and λ wavelength of the photon, and c is speed of light. As λ increases, E decreases.

The spacing between the ground and higher electronic states of a molecule corresponds to energy in the UV and visible spectrum, while the spacing between electronic states (called vibrational states) corresponds to energy in the infrared spectrum. Absorption occurs if the energy of a photon matches the energy required to move an electron from its ground state (S_0) to a higher electronic state (S_n). Absorption is an "electronic transition" in which energy is rapidly transferred from a photon to an electron ($O(10^{-15}$ s)); this electron is considered to be in an "excited state". The excited electron relaxes or returns to its lower-energy ground state by releasing energy. Energy is typically released as heat to the surrounding environment through vibrational loss (also called radiationless decay). In some molecules, some of the energy can be dissipated by the release of a photon (fluorescence).

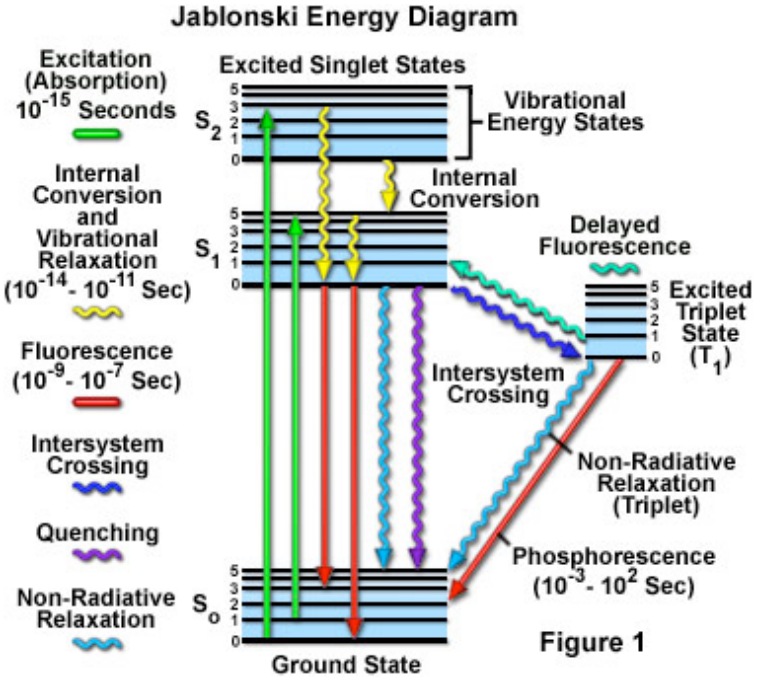


Figure 1

http://www.micro.magnet.fsu.edu/optics/timeline/people/jablonski.html
applet: http://www.micro.magnet.fsu.edu/primer/java/jablonski/lightandcolor/

The emitted photon will always be of lower energy (longer wavelength) than the absorbed radiation (Stokes shift); emission occurs from the lowest vibrational state in the lowest excited state (S_1) because vibrational loss ($O(10^{-12}$ s)) is even more rapid then fluorescence (fluorescent emission is ($O(10^{-9}$ s)). The emission spectrum is a mirror image of the

absorption spectrum, in part due to the probability distribution function for an electron to occupy a higher vibrational level in the ground state.

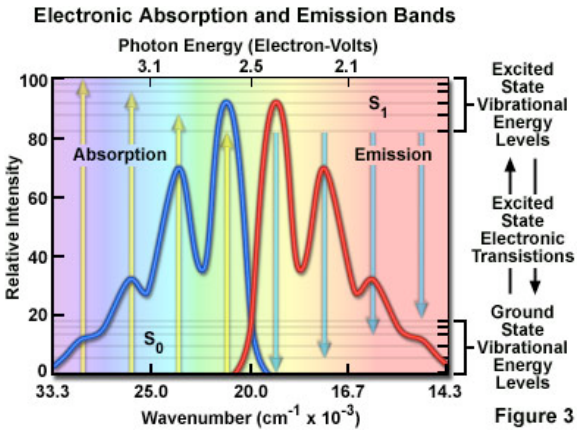


Figure 3

Mirror image: *http://www.olympusconfocal.com/theory/fluoroexciteemit.html*

The fraction of energy released by fluorescence is the quantum yield; on a photon basis, quantum yield (Φ_f) = photons emitted/photons absorbed. The quantum yield will vary with temperature, pH, and competing pathways for dissipation of energy as heat. If (**and only if**) conditions are uniform, the fluorescent emission of a compound is linearly proportional to the concentration of the compound at low concentrations.

Excellent web references and the classic fluorescence text are:
http://www.olympusconfocal.com/theory/fluoroexciteemit.html
http://aic.stanford.edu/jaic/articles/jaic30-01-007_2.html
http://www.micro.magnet.fsu.edu/primer/lightandcolor/index.html
Joseph R. Lakowicz, 1983. *Principles of Fluorescence.* Plenum Press, New York
Fun Java applets:
http://www.micro.magnet.fsu.edu/primer/java/fluorescence/exciteemit/index.html
http://www.micro.magnet.fsu.edu/primer/java/jablonski/lightandcolor/

How is fluorescence used to study phytoplankton and dissolved organic matter?
Phytoplankton contain the photosynthetic pigment chlorophyll *a* which absorbs visible light and re-emits or fluoresces red light. For decades, the concentration of chlorophyll *a* has been widely used as a proxy for phytoplankton biomass, particularly phytoplankton carbon, although the carbon–to–chlorophyll ratio does vary with species, light history, temperature, and nutrient availability. The reason for the wide-spread use of chlorophyll *a* as a biomass proxy are: 1) chlorophyll *a*, or very close derivatives such as divinyl chlorophyll *a*, is in all oxygen-producing photosynthetic organisms, and 2) it is relatively easy to measure both *in vitro* in polar solvent extracts and *in vivo* in living cells in the ocean. Extracted chlorophyll *a* is typically reported in units of mg m^{-3}, and sometimes in units of mg m^{-2} for water-column integrated concentrations, while *in vivo* fluorescence is either reported in relative units, in volts or digital counts, or is converted to absolute units of mg chlorophyll *a* m^{-3}, based on measured or assumed conversion factors. For ship-based profiles with many calibration points, the uncertainty in determining chlorophyll *a* concentration from *in vivo* fluorescence is low; for mooring and mobile platform

applications, the uncertainty increases. Solar quenching of fluorescence changes the ratio of fluorescence to chlorophyll *a* concentration both on diel and shorter time scales.

Note: *in vitro* = outside a living organism; *in vivo* = inside a living organism.

Water samples of CDOM, or chromophoric dissolved organic mater, have characteristic negative exponential absorption spectra (measured in Lab 2) and characteristic fluorescence excitation–emission spectra (or EEMS – excitation emission matrix spectroscopy). Changes in spectral shapes and quantum yields of fluorescence reflect the chemical nature of the chromphores and changes in their structure due to interactions with other molecules, size distribution of the colloidal gels, ionic strength of the water, pH, and effects of photobleaching and microbial/enzymatic breakdown. The magnitude of CDOM absorption has a strong inverse relationship with salinity, reflecting an important terrestrial contribution. From a radiative transfer perspective, CDOM affects the downward penetration of UV and blue/blue-green wavelengths of visible light. From an ecological perspective, high concentrations of CDOM protect marine organisms near the surface from UV damage. From biogeochemical cycling perspective, CDOM is a potential proxy for dissolved organic material, although the relationship between chromophoric and total dissolved organic material varies regionally and seasonally (Vodacek et al., 1997). For this class, we can consider the relationship between CDOM absorption and fluorescence; a fundamental question we cannot directly address is their relationship with total dissolved organics.

In field oceanography, the term "dissolved" is used loosely to describe substances in waters that have been passed through filters. The filter pore size may vary; the two most commonly used are 0.7 mm (nominal) for G/FF filters, although the effective filter size decreases as more material accumulates, and 0.2 mm. In reality, much of the so-called "dissolved" organic material in the ocean exists as colloids or colloidal gels, whose size distribution is not static.

What is the quantitative relationship between fluorescence and concentration?
The relationship between fluorescence and concentration of a fluorescent substance can be described by:

$$F = E(\lambda) \cdot C \cdot \Phi_f$$

where F is fluorescence, E is lamp or LED intensity, λ is wavelength denoting the spectrum of the lamp, C is concentration of a fluorescent substance, and Φ_f is quantum yield of fluorescence (= photons emitted/photons absorbed). At high concentrations, the relationship becomes non-linear and fluorescence emission saturates.

Another way to represent the fluorescence equation is to expand the concentration term:

$$C = a / a^*$$

where a is the absorption coefficient (m^{-1}) and a^* is the specific absorption coefficient (m^2 mg^{-1}); the product of a / a^* = concentration (mg m^{-3}). Both a and a^* can be represented as a spectral average, value at a single wavelength, or spectrally (λ).

In a laboratory setting, if $E(\lambda)$ and Φ_f are constants, the fluorescence of a pure solution will be linearly proportional to the concentration of a fluorescent substance at low concentrations. For an *in vitro* extract of chlorophyll *a* in 90% acetone solvent, the

relationship between F and C is generally robust; Φ_f is high (O(0.33)) and constant at a given temperature. For *in vivo* fluorescence of living phytoplankton, Φ_f is low (O(0.005–0.02) and variable even on the time scale of seconds, reflecting very dynamic regulation of energy dissipative pathways in the photosynthetic apparatus. For CDOM, on the global scale CDOM absorption and fluorescence are positively correlated, but on a finer scale the relationship is variable, as CDOM is a complex mixture of different chromophores whose Φ_f is affected by ionic strength, pH, molecular interactions, and photo-bleaching. The bottom line is – that given the huge dynamic range of CDOM and chlorophyll fluorescence in the environment, the relationships between fluorescence (F) and concentration (C) for both chlorophyll *a* and CDOM are still useful to consider as proxies – but 'let the user beware'!

POOLED ASSIGNMENTS FOR ALL STATIONS:

1) Compare the different versions of 'no' parameter readings for chlorophyll *a*, CDOM or phycoerythrin for black electrical tape vs. tap water vs. filtered seawater (as appropriate) for all fluorometers examined. Is tap water a good blank?
    a. How do you think a 'blank' should be measured?
    b. How would you test a temperature effect? A pressure effect?
2) For the bucket measurements, is there a wall effect? Is there a difference between stationary reading vs. reading with a moving sensor (assuming no wall effect)?
3) Linearity: plot various fluorescence readings vs. volume of culture or Biscay Pond water; are these readings linear or do they show saturation at higher volumes? Are chlorophyll *a* fluorescence and phycoerythrin fluorescence co-linear?
4) Does DRE water have a phycoerythrin signal?
5) Plot fluorescence vs. 700-nm backscatter from clay – did the scatterers change the various fluorescence signals? If so, at what volume of clay and what backscatter signal?
6) Does the addition of Biscay Pond CDOM affect the chlorophyll fluorescence signal?
7) Calculate the fluorescence per volt or digital count (similar to calibration 'scale factor') for the culture and DRE water; units are count or volts/mg chl m^{-3}. Are they similar between the same types of sensors?
8) One very important fact is that most standard chlorophyll fluorometers now use a 470 nm LED. What pigment(s) does (do) that wavelength excite?
9) Calculate a fluorescence scale factor for CDOM (CDOM fluorescence/absorption coefficient) for the water used in the CDOM absorption lab (Lab 2).
10) For chlorophyll concentration, comment on your group's replication (report on Saturday, since you will have to wait until Friday to measure chlorophyll concentration from Station 2).
11) Does *in vivo* chlorophyll *a* fluorescence change as a function of light? Did you see any evidence of fluorescence quenching? How would fluorescence quenching effect use of fluorescence as a proxy for phytoplankton biomass?

Lab 4 – Station 1 **14 July 2011**

Station 1: ***in situ* fluorescence of chlorophyll *a* with WET Labs sensor**
– break into 2 groups
Location: classroom
Focus: measurement of chlorophyll *a* fluorescence with an *in situ* sensors

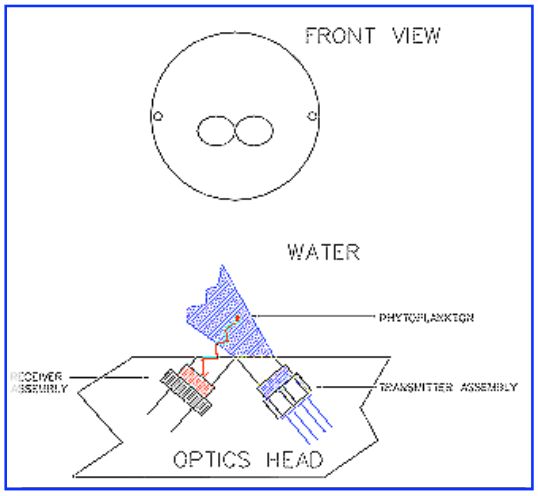
LABORATORY SAFETY ISSUES – general laboratory safety

GOALS

- compare blank measurements using tap water, filtered seawater, and electrical tape;
- determine linearity of signal as a function of phytoplankton concentration;
- determine effect of scatterers on fluorescence signal (clay);
- determine the ratio for fluorescence to extracted chlorophyll for a cultured diatom, *Thalassiosira*;
- compare excitation spectra between sensors.

BACKGROUND:

Many fluorometers are available on the market. For a recent comparison of chlorophyll fluorometers, see: http://www.act-us.info/evaluation_reports.php. We will use two WET Labs fluorometer Eco 'pucks' that also have two channels of backscatter (470 and 700 m); another manufacturer is Turner Designs. Both come with a variety of excitation/emission options that measure, for example, relative concentrations of chlorophyll, CDOM, uranine, phycocyanin, or phycoerythrin. Converting relative fluorescence (volts or digital counts) to concentration is up to the user (and let the user beward). LEDs provide a low power excitation source, important for long-term deployments.

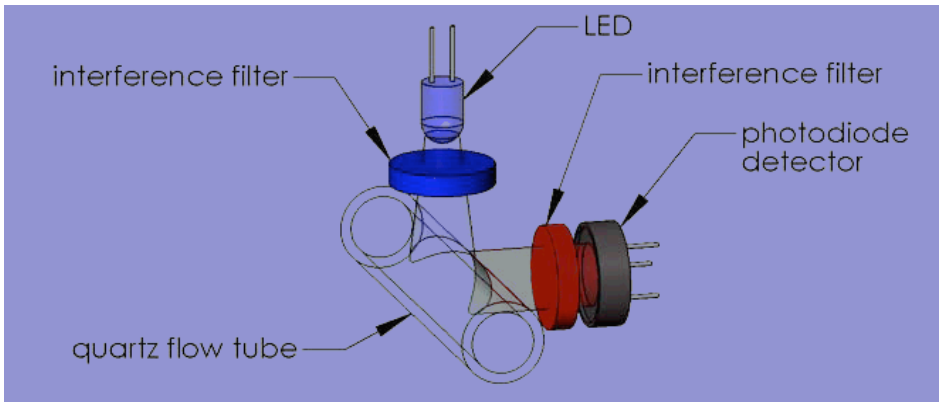


An interference filter is used to reject the small amount of out-of-band light emitted by the LED. The light from the source enters the water volume at an angle; in the figure to right, the angle is approximately 55–60 degrees with respect to the end face of the unit. Fluoresced light is received by a detector positioned where the acceptance angle forms a 140- degree intersection with the source beam. An interference filter is used to discriminate against the scattered excitation light. In some applications a wiper is used to minimize biofouling.

WET Labs ECO (Environmental Characterization Optics) pucks
http://www.wetlabs.com/products/pub/eco/flac.pdf

Turner Designs C3 Submersible Fluorometer and Cyclops-7 Submersible Sensors
<http://www.turnerdesigns.com/t2/doc/brochures/C3_brochure.pdf>
<http://www.turnerdesigns.com/t2/doc/brochures/cyclopsds_3.pdf>
<http://www.turnerdesigns.com/t2/doc/brochures/cyclopsds_3.pdf
<http://www.turnerdesigns.com/t2/doc/manuals/cyclops_manual.pdf>

Another fluorometer design that we will NOT use is a flow-through system, for example, the WET Labs WETStar or the flow tube attachment in the Turner Designs 10-AU benchtop. The flowthrough design often which works well in ship's in-line systems. The source and detector are at 90° to each other (see figure below). Note that if a sample is kept within the flow tube for too long the fluorescence of the sample will change due to the exposure to light.



FLUORESCENCE MEASUREMENTS:
Black buckets are used to minimize reflection of stray light to the detector; each bucket holds about 12-15 L.

1) Try three versions of 'no' chlorophyll samples – and record counts or volts.
    a. black electrical tape (sensor is not in bucket)
    b. tap water (we should use Milli-Q, but can't produce sufficient volume)
    c. filtered seawater (DON'T discard after measurement; use for next exercise, below). Move the sensor around – when is the sensor influenced by the walls of the bucket?

2) Linearity test with culture – measure signal of filtered seawater. Add aliquots of culture of diatom *Thalassiosira pseudonana* (same species as used in the Tuesday lab). Mix culture (Perry swirl method) and remove an aliquot. You might want to start with 1 mL and add to bucket. Mix bucket and measure. Increase addition of the volume of culture added; mix. Figure out an interesting sequence to test linearity of response between concentration of cells and fluorescent response. It might be useful to plot as you go along.
    a. Be sure to mix sample in bucket frequently (cells can settle).
    b. Move the sensor around – when is the sensor influenced by the walls of the bucket?
    c. Hold the sensor stationary, in a location not affected by the walls? Does the signal change with time?
    d. DON'T discard the bucket (see next exercise).

3) Take samples for chlorophyll analysis (Station 2). Mix bucket, submerge a beaker to rinse and fill a 1-L sampling bottle. Put sample on ice and in dark. DON'T discard the bucket (see next exercise).

4) Affect of CDOM on fluorescence. Add aliquots of Biscay Pond water to bucket. Does it affect the reading? Start with 0.5 L; mix bucket and measure. Figure out an interesting sequence to test linearity of addition of high CDOM water. It might be useful to plot as you go along – does fluorescence change with the presence of CDOM? DON'T discard the samples (see next exercise).

5) Affect of scatterers on fluorescence – vigorously mix clay suspension and add 1 mL to bucket, stir and record both fluorescence AND red backscatter (at least for the WET Labs sensor). Figure out an interesting sequence to test affect of scattering on the fluorescence response – does fluorescence change with the presence of scatterers? At what level of scatterers?

6) Thoroughly rinse the bucket to remove cells and clay.

7) Excitation spectrum for each WET Labs puck – to be measured with a radiometer (more details during the lab).

➔ **ASSIGNMENT FOR STATION 1** (see Lab_4_overview for pooled assignments)

1) Compare the three versions of 'no' chlorophyll readings – black electrical tape vs. tap water vs. filtered seawater for both WET Labs sensors. Are they the same? Which would you use for the blank in the field?
2) Is there a wall effect? Is there a difference between stationary reading vs. reading with a moving sensor (assuming no wall effect)?
3) Plot fluorescence vs. volume of culture – is it linear? Remember to take into account the change in volume of water in the bucket.
4) Plot fluorescence vs. 700-nm backscatter – did the scatterers change the fluorescence?
5) Plot fluorescence vs. volume of CDOM-rich Biscay Pond water – did CDOM affect the chlorophyll fluorescence signal? Remember to take into account the change in volume of water in the bucket.
6) Calculate the fluorescence per volt or digital count (similar to calibration 'scale factor') for the culture; units are count or volts/mg chl m^{-3}. You will filter the chlorophyll sample in Station 2 but you will need to measure the chlorophyll extracts on Friday. Are the two fluorometers the same?
7) One very important fact is that most standard chlorophyll fluorometers now use a 470 nm LED. What pigment(s) does (do) that wavelength excite?

Lab 4 **14 July 2011**

Station 2: **standard fluorometric measurement of chlorophyll *a*, *in vitro* (acetone extract)**
Location: MJP lab
Focus: provide 'calibration' of fluorescence measurements from Station 1

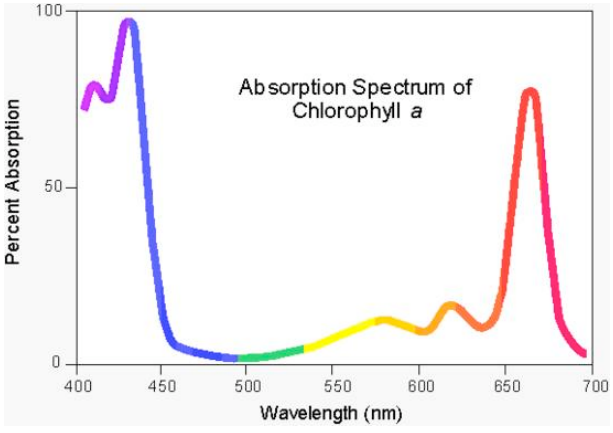
LABORATORY SAFETY ISSUES – acetone, 10% HCl; see MSDS sheets. Wear goggles and gloves when handling solvents and acids. Open solvent containers only under the hood. Pour all wastes into appropriate containers, including seawater. General laboratory safety practices apply.

GOALS – please learn about:
importance of sampling/subsampling – swirl 3X, reverse, swirl 1X, pour
methodologies for measurement of chlorophyll *a* and phaeopigments
extraction – sonication and 24-h in cold; dark to avoid photobleaching
potential interference from chlorophyll *b*
calibration with pure chlorophyll *a* standard
use of secondary standard and record keeping of standard and room's temperature

BACKGROUND:
The basic principle of fluorometry is:

$$F = E(\lambda) \cdot C \cdot \Phi_f$$

Where F is fluorescence, E(λ) is the spectral energy of the excitation light, C is concentration, and Φ_f is the quantum yield of fluorescence. N.B.: Φ_f decreases as temperature increases; hence, fluorescence should be measured at a constant temperature. For 90% acetone, the temperature coefficient is –0.3% per degree C; varies for other compounds.

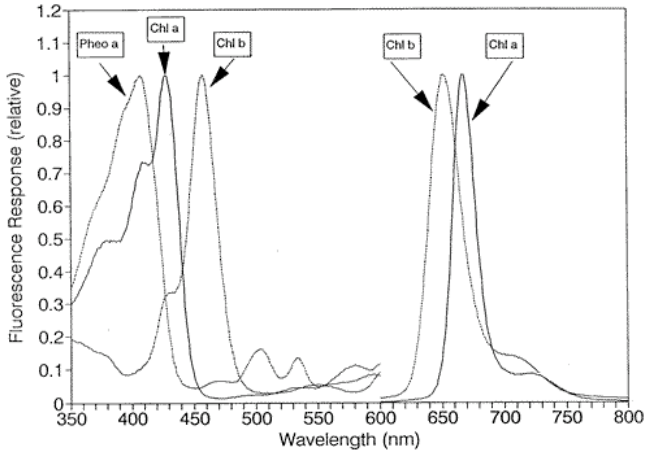


Chlorophyll *a* absorbs in both blue (Soret) and red (Q) bands (figure; <http://www.ch.ic.ac.uk/local/projects/steer/chloro.htm>). Chlorophyll *a* has the additional characteristic of fluorescence, a phenomenon by which a fraction of the photons are re-emitted (for chlorophyll, the fluorescence maximum is at 682 nm in whole cells and 676 nm in acetone extracts. Photons either directly absorbed by chlorophyll *a* itself or transferred to chlorophyll *a* from a photosynthetic accessory pigment (the latter occurs only in the whole cell or in fragments of photosynthetic lamellae), can be re-emitted as red fluorescent photons. The fraction of photons emitted relative to photons absorbed is the quantum yield of fluorescence, Φ_f.

For chlorophyll *a* in a solvent extract, Φ_f is approximately 0.33 (lower at higher temperatures); for live cells, Φ_f varies between 0.005 to 0.03. Chlorophyll *b* fluoresces only *in vitro* (such as in solvent extracts) and not in whole cells. Chlorophyll *c* does not fluoresce.

The standard method for fluorometric analysis of chlorophyll in 90% acetone uses a broad-band blue filter to excite chlorophyll fluorescence and a red filter with a red-sensitive detector to detect fluorescence. However, in field samples both chlorophyll *a* and its principle degradation products (pheophytin *a* and pheophorbide *a*) will also be present. The position of the red absorption peaks is similar for chlorophyll *a* and its degradation products; however, the Soret peak at 440 nm is not present in the degradation products, rather the blue absorption peak is shifted to 412 nm.

In addition, chlorophyll *b* may be present in field samples and it also fluoresces in acetone. A major, and too often ignored, problem with the standard filter set is interference from chlorophyll *b;* this pigment can be present in significant concentrations in some waters masses. The standard filter set is not optimized for excitation of chlorophyll *b*. However, when chlorophyll *b* is acidified, pheophytin *b* is formed and the peak is shifted to the blue; unfortunately, the standard filter set is efficient in exciting pheophytin *b*. The consequence (see equations below) is that chlorophyll *a* concentration is underestimated and pheophytin *a* is overestimated. The Welschmeyer (1994) method avoids the interference of chlorophyll *b* by using narrow band interference filters that excite only chlorophyll *a* (436nm) and detect its emission (680nm). This method, however, is not optimized to measure pheopigment concentration; the preferred method for quantitative measurement of all pigments is by HPLC.



Turner Designs web site has some wonderful technical notes, but not the easiest navigation: <http://www.turnerdesigns.com>.

FLUORESCENCE MEASUREMENT AND COMPUTATION:

By measuring the fluorescence of an extract before (Fo) and after (Fa) acidification, the concentration of both chlorophyll and pheopigment can be determine. Before acidification, a field extract is a mixture of chlorophyll *a* and its degradation product; and after acidification, only degradation products exist because chlorophyll *a* is converted to pheophytin by acid. The method and equations were developed by Yentsch and Menzel

(1963) and Holm-Hansen et al. (1965). This basic method can be found as EPA Method 445.0 (Collins and Arar, 1997) and in the JGOFS (1994) protocols; see also the Turner Designs web site (listed in the References). The equations are given below and in the Excel spread sheet in the chlorophyll lab folder <chl_10AU.xls>:

$$\text{chl } a = K\,(Fm/\,Fm\text{-}1) \times (Fo - Fa) \times (v/V) \quad (1)$$

$$\text{pheo } a = K\,(Fm/\,Fm\text{-}1) \times [(Fm \times Fa - Fo)] \times (v/V) \quad (2)$$

where:

- K = calibration coefficient
- Fm = max acid ratio Fo/Fa of pure chlorophyll *a* standard; the ratio is typically around 2 but is fluorometer specific and depends on the combined spectrum of lamp + excitation filter
- Fo = fluorescence before acidification
- Fa = fluorescence after acidification
- v = 90% acetone extract volume (L)
- V = volume filtered (L)

Assuming no chlorophyll *b*, there are two equations, two measurements (Fo and Fa), and two unknowns (chlorophyll *a* and pheopigment *a*). Units are mg pigment m^{-3} or μg pigment L^{-1}.

PROTOCOL - Wear goggles and gloves to protect from acetone.

Filtration and extraction:

1. Each group should have 1 L of the culture from Station 1.
2. Place G/FF filter on each filter unit; test that the filter cup is secure.
3. Mix sampling bottle (Perry swirl) and dispense 100-mL of sample into one filter cup. (MJP will make an assessment – is that enough or too much volume).
4. Filter samples (xx mL) through G/FF with vacuum below 5 mm Hg pressure. When all water has been filtered, IMMEDIATELY remove filter.
5. Submerge filters in tube with 5-mL 90% acetone. Note tube labeling system.
6. Keep tube in dim light until you are finished (chlorophyll in acetone bleaches very rapidly).
7. Store tubes in freezer for 24-h to allow chlorophyll to extract from cells into acetone. (Alternatively, you could sonicate the tubes with high frequency sound for 7 minutes; do in ice bath to avoid heating. Allow tubes to extract for 15 minutes in cold and dark. Process as below.)

Fluorescence reading of extract:

➔ we will measure samples from Wednesday in today's lab. You will need to measure Thursday's samples on Friday (and if not possible, on Saturday).

8. Read samples in dim light. Turn on Turner 10AU to warm up. Remove tubes from freezer and vortex mix to complete extraction and to ensure uniform distribution in tube. With forecps, remove filter. Wipe finger prints off tube

9. Centrifuge for 5 minutes to remove filter fragments; the new Whatman G/FF filters are much more prone to disintegration. The presence of filter fragments in the light path causes scattering and an artificially high reading.
10. Carefully remove tube from centrifuge; DON'T re-suspend filter pad particles. By now the tubes should be warmed to room temperature.
11. Note and record room temperature.
12. Measure and record reading of secondary standard.
13. Insert tube in fluorometer and read Fo.
14. Remove tube, add one drop HCl, don't mix, read Fa. **Wear goggles and gloves to protect from acetone and HCl.**
15. When finished with all readings, clean up. Turn off fluorometer if no one else will use it. Turn on fan in hood (in chemical lab). Work in hood and empty tubes into 90% acetone waste container. **Wear goggles and gloves to protect from acetone.** Leave fan on and leave tubes in hood.
16. Calculate chlorophyll concentration using excel spread sheet with calibration coefficient. The fluorometer was calibrated this month, with Turner Design's chlorophyll *a* standards.

➔ **ASSIGNMENT** (see Lab_4_overview for pooled assignments)

1. Calculate chlorophyll and pheopigment concentration using the Excel spread sheet in the chlorophyll lab folder <chl_10AU.xls>.
2. Determine SD for your own samples; comment on your group's replication.
3. Use chlorophyll values for calculations for assignments related to Station 1.

REFERENCES

Collins, G.B., and E.J. Arar. 1997. EPA Method 445.0. *In Vitro* Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence. Revision 1.2, September 1997. U.S. Environmental Protection Agency, Cincinnati. 22 pp.

JGOFS. June 1994. Chapter 14. Measurement of Chlorophyll *a* and Phaeopigments by Fluorometric Analysis. JGOFS Protocols. 5 pp.

Holm-Hansen, O., C.J. Lorenzen, R.W. Holmes, and J.D. Strickland. 1965. Fluorometric determination of chlorophyll. J. Cons. Cons. Int. Explor. Mer 30: 3-15.

Jeffrey, S.W., R.R.C. Mantoura, and S.W. Wright. 1997. Phytoplantkon pigments in oceanography. UNESCO Publishing. 661 pp.

Turner Designs – <http://turnerdesigns.com/t2/doc/appnotes/998_5101.html>

Welschmeyer, N.A. 1994. Fluorometric analysis of chlorophyll *a* in the presence of chlorophyll *b*and pheopigments. Limnology and Oceanography 39: 1985-1992.

Yentsch, C.S. and D.W. Menzel. 1963. A method for the determination of phytoplankton chlorophyll and pheophytin by fluorescence. Deep-Sea Res. 10: 221-231.

Lab 4 – Station 3 **14 July 2011**

Station 3A: CDOM fluorescence, then go to Station 3B
Station 3B: phycoerythrin fluorescence, then go to Station 3A
Location: Mitchell Classroom
Focus: measurement of CDOM and phycoerythrin (PE) fluorescence, and discussion of standards

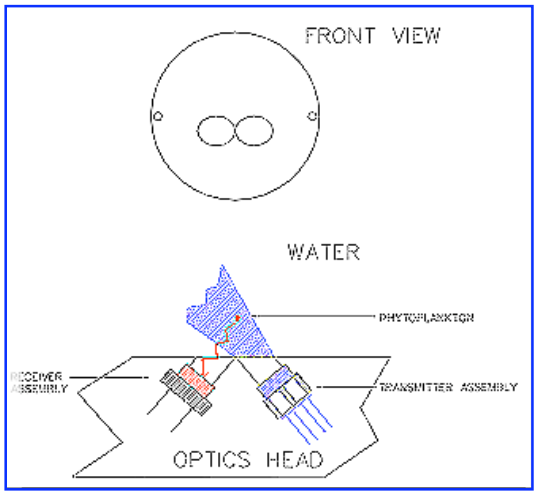
LABORATORY SAFETY ISSUES – general laboratory safety

GOALS

- discussion standards and what they do and do not provide;
- compare measurements using tap or RO water and electrical tape;
- determine linearity of fluorescence as a function of CDOM (CDOM-rich Biscay Pond water) or phycoerythrin (PE) fluorescence as a function of *Rhodomonas* concentration;
- determine effect of scatterers on fluorescence signal (clay)'
- what are the excitation spectra of the various LED/filter combintions.

BACKGROUND (PARTIALLY REPEATED FROM STATION 1)

Many fluorometers are available on the market, and come with a variety of excitation/emission options to measure, for example, relative concentrations of chlorophyll, CDOM, uranine, phycocyanin, or phycoerythrin. We will use Turner Designs' *in situ* fluorometers. Converting relative fluorescence (volts or digital counts) is up to the user. LEDs provide a low power excitation source. An interference filter is used to reject the small amount of out-of-band light emitted by the LED. The light from the source enters the water volume at an angle; in the figure to right, the angle is approximately 55–60 degrees with respect to the end face of the unit. Fluoresced light is received by a detector positioned where the acceptance angle forms a 140- degree intersection with the source beam. An interference filter is used to discriminate against the scattered excitation light. In some applications a wiper is used to minimize biofouling.



Turner Designs C3 Submersible Fluorometer and Cyclops-7 Submersible Sensors
<http://www.turnerdesigns.com/t2/doc/brochures/C3_brochure.pdf>
<http://www.turnerdesigns.com/t2/doc/brochures/cyclopsds_3.pdf>
<http://www.turnerdesigns.com/t2/doc/brochures/cyclopsds_3.pdf
<http://www.turnerdesigns.com/t2/doc/manuals/cyclops_manual.pdf>

Both groups need to go to the dock and collect DRE water for buckets – take 4 carboys.

Each bucket holds about 12-15 L, and will have been 'roughly' calibrations. The black buckets minimize reflection of stray light to the detector; however, the Cyclops 3 has a baffle, and should not see the wall.

STATION 3A: CDOM FLUORESCENCE
measure with Turner Designs' Cyclops 7 (and possibly another CDOM fluorometer)

1) Compare two 'no' CDOM approaches – and record counts or volts.
    a. black electrical tape (not in bucket)
    b. tap or RO water (we should use Milli-Q water, but we can't generate enough water).
2) Measure CDOM fluorescence of G/FF filtered water from Lab 2 (CDOM absorption):
    a. coastal water, collected beyond mouth of DRE
    b. DRE water (dock)
    c. Biscay Pond (freshwater, but CDOM-rich)
    ➔ SAVE WATER FOR NEXT GROUP – replace into white buckets
3) Linearity test – add aliquots of Biscay Pond water to whole (unfiltered) DMR water.
    a. Add unfiltered DRE water to marking on bucket.
    b. Record initial reading.
    c. Add 0.5 L CDOM-rich Biscay Pond; mix bucket and measure. Figure out an interesting sequence to test linearity of addition of high CDOM water. It might be useful to plot as you go along.
    d. Move the sensor around – is there an effect of moving the sensor?
    ➔ DON'T DUMP BUCKET; use in next exercise.
4) Affect of scatterers on CDOM fluorescence – vigorously mix clay and add 1 mL to bucket, stir and record fluorescence. Figure out an interesting sequence to test affect of scattering on the fluorescence response – does it change with the presence of scatterers? At what level of scatterers?
5) Thoroughly rinse the bucket to remove clay.

STATION 3B: PHYCOERYTHRIN (PE) FLUORESCENCE
measurements with Turner Designs' Cyclops 3

1) Compare two 'no' phycoerythrin (PE) fluorescence approaches – and record counts or volts.
    a. black electrical tape (not in bucket)
    b. tap water (we should use Milli-Q water, but we can't generate enough water).
2) Measure PR fluorescence of whole (unfiltered) Damariscotta River Estuary water (dock); is there any PE fluorescence? DON'T DUMP the BUCKET (see next exercise).

3) Linearity test with culture – add aliquots of culture of cryptophyte, *Rhodomonas*, to DRE water.
    a. Add unfiltered DRE water to marking on bucket.
    b. Record initial reading.
    c. Mix culture and add ~ 1 mL to bucket; mix bucket and record both PE and chlorophyll *a* fluorescence.
    d. Figure out an interesting sequence to test linearity of culture and continue. It might be useful to plot as you go along.
    e. Move the sensor around – is there an effect of moving the sensor?
    f. Hold the sensor stationary location? Does the signal change with time?
    ➔ DON'T DUMP BUCKET; use in next exercise.
4) Thoroughly rinse the bucket to remove cells.
5) Check out the calibration cap for the Cyclops 3 (it is only for chlorophyll *a* fluorescence).

➔ Excitation spectrum for each sensor – to be measured with a radiometer (more details during the lab).

ASSIGNMENT FOR STATION 3 (see Lab_4_overview for pooled assignments)

1) Compare the two versions of 'no' CDOM or 'no' phycoerythrin fluorescence readings – black electrical tape vs. tap water. Is tap water a good blank? (recall, it would be better to use Milli-Q water).
2) Is there a difference between stationary reading vs. reading with a moving sensor? Is there a wall effect for Cyclops 7?
3) Does DRE water have a phycoerythrin signal?
4) Plot CDOM fluorescence vs. volume of Biscay Pond water added – is it linear?
5) Plot CDOM fluorescence vs. volume of clay – did the scatterers change the fluorescence?
6) Plot phycoerythrin fluorescence vs. volume of culture – is it linear?
7) Are chlorophyll *a* fluorescence and phycoerythrin fluorescence co-linear?
8) Calculate CDOM fluorescence scale factor (fluorescence/absorption coefficient) for the water used in Lab 2, CDOM absorption lab
    a. coastal water, collected beyond mouth of Damariscotta River Estuary
    b. Damariscotta River Estuary water (dock)
    c. Biscay Pond (freshwater)

Lab 4 – Station 4 **14 July 2011**

Station 4: microscope observation of fluorescing cells and fluorescence quenching.

Location: MJP lab

GOALS : a) View emission of chlorophyll *a* from live phytoplankton cells, using an epi-fluorescence microscope, equipped with a blue excitation filter and red emission filter.

b) Observe how fluorescence in living cells can be quenched (decreased) by exposure to high light (either natural sunlight or a bright lamp, depending on the weather; measure the fluorescence over time in a bench top Turner Designs TD-700 fluorometer.

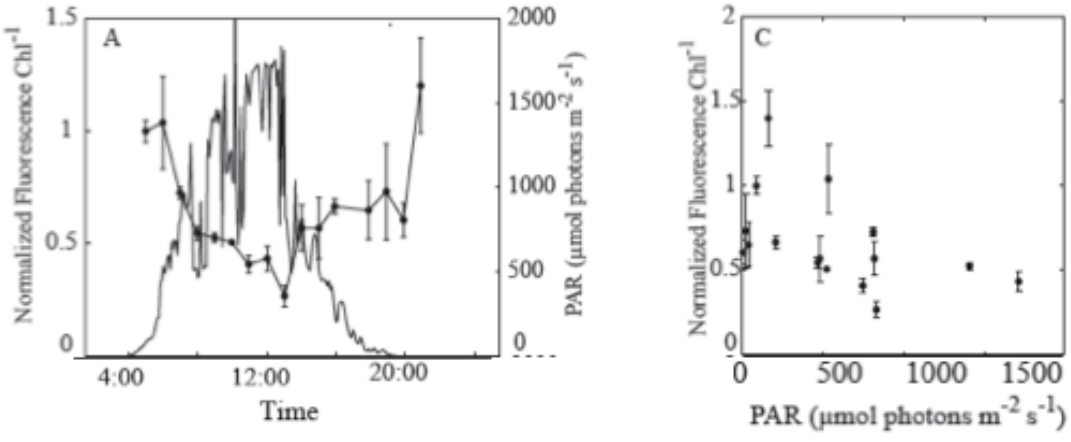
LABORATORY SAFETY ISSUES – general laboratory safety

4A) EPIFLUORESCENCE MICROSCROPHY: Collect phytoplankton with a small mesh net from Damariscotta River Estuary and examine on epi-fluorescence microscope (blue excitation and red emission). Look and admire their beauty.

4B) FLUORESCENCE QUENCHING

Both chlorophyll *a* and CDOM fluorescence are affected by sunlight. *In vivo* chlorophyll *a* fluorescence in the field is affected by physiological state including light history (short term quenching due to energy reallocation within the photosynthetic membrane, intermediate term inhibition due to damage to PS II, and long term photoadaptation), nutrient status, and species composition. CDOM undergoes chemical reactions in sunlight, which can also change its fluorescence quantum yield.

Because chlorophyll a fluorescence is widely used as a proxy for phytoplankton biomass, variability of its quantum yield of fluorescence is problematic, compromising linearity in the relationship between in vivo fluorescence and concentration. One major driver of fluorescence variability is light, particularly high light. Changes in the apparent quantum yield of fluorescence can occur on time scales from seconds to hours. One of the challenges for ecological and biogeochemical studies is how to better constrain this aspect of variability in the relationship between chlorophyll a fluorescence and concentration. Graph below from A. Drzewianowsk thesis, 2008; *Fluorescence quenching of phytoplankton from the Damariscotta River Estuary,* http://www.library.umaine.edu/theses/pdf/DrzewianowskiAF2008.pdf



MEASUREMENTS

Culture of diatom *Thalassiosira pseudonana* (same species as used in the Tuesday lab) will be exposed to sunlight over the course of several hours during the afternoon lab (weather permitting – if there is sun) and on a shorter time scale at high intensities with a bright lamp.

Use Turner Designs TD-700 fluorometer:

Read filtered seawater blank in 50-mL tube.
Read fluorescence solid standard.
Fill three 50-mL tubes with culture. Invert to mix tube.
Record fluorescence and time.
Place sample as directed, for exposure to bright light.

Read again. (We may pool samples from different groups).

➔ **ASSIGNMENT:** (see Lab_4_overview for pooled assignments)

Does *in vivo* chlorophyll *a* fluorescence change as a function of light?
(Plots might be nice.)

Time	**Light conditions** (sunny? cloudy?)	**Blank**	**Solid Standard**	**Sample Fluorescence**	**Who Sampled**