Lab 5: OVERVIEW for Chlorophyll and CDOM fluorescence lab 10 July 2015

LABORATORY SAFETY ISSUES – acetone, 10% HCl; see SDS 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.

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* and chromophoric dissolved organic material (CDOM). Specifically, you will explore fluorescence in a variety of ways:

1. Sensors for in-water measurement of fluorescence of CDOM and chlorophyll *a*.
2. Solar quenching of chlorophyll *a* fluorescence *in vivo* (living cells).
3. Measurement of chlorophyll *a* concentration in acetone extracts of filtered samples (*in vitro)* in fluorometers with either standard Turner Design filters (broad blue excitation filters) or Welschmeyer narrow band excitation filters.

STATIONS: students divide into six groups of 3 or 4 students per group:

Station 1 – Mitchell Classroom

Station 2 – start in classroom, then to Perry Lab

Lab 5 – Station 1: Fluorescence of CDOM and chlorophyll *a* in living cells

Location: Mitchell Classroom
Focus: understand measurement and calibration of in-water fluorometers

LABORATORY SAFETY ISSUES – general laboratory safety

PRIMARY SENSORS: WET Labs ECOpucks for chlorophyll and CDOM, Turner Designs' Cyclops 7 chlorophyll and CDOM fluorometers.
Black buckets are used to minimize reflection of stray light to the detector; each bucket holds about 12-15 L.

ALL FLUOROMETERS

1) 'Dark counts' and blanks. Compare readings for different conditions.
   Light: lights on, lights off, and pointed toward sun. For WET Labs sensors only – do with black tape.
   Walls of the black bucket – move the sensor around, is it affected by the walls?
   Water blank – RO water and filtered seawater.

2) Linearity test for chlorophyll fluorescence and CDOM.
   - measure signal for filtered seawater
   - add culture of either diatom (first lab) or green alga (second lab) for chlorophyll fluorescence; make sure the culture is mixed first OR tea for CDOM. Add measured aliquots, mix the bucket well, and record fluorescence.
   - does it make a difference if the sensor is stationary or if it is moved in the bucket; for chlorophyll, there can be a quenching effect if the sensor is stationary.
   - for the culture, at end of measurements pour sample into white bucket and deliver to Perry lab. The other group will measure chlorophyll.

   ➔DON'T discard the CODM bucket (see next exercise).

3) Interference on measurement of chlorophyll fluorescence by other components.
   - CDOM bucket:
     - add concentrated culture and assess if values change
     - add inorganic particles (Arizona road dust) and assess if values change
   - chlorophyll bucket – mix a new batch of seawater and culture
     - add tea and assess if values change
     - add inorganic particles (Arizona road dust) and assess if values

4) Measure excitation spectrum for each sensor with a radiometer.

5) Calibration issues – solid standard for Turner Designs fluorometer; discussion of calibration issues.

6) Other fluorometers – discuss other fluorometers such as flow through fluorometers (WET Labs Wet Star as example), combination fluorometers and backscatter sensors, multi-wavelength chlorophyll fluorometers, etc.

Lab 5 – Station 2: Solar quenching of fluorescence; chlorophyll concentration.

Location: Classroom, then to Perry Lab

1) Fluorescence quenching

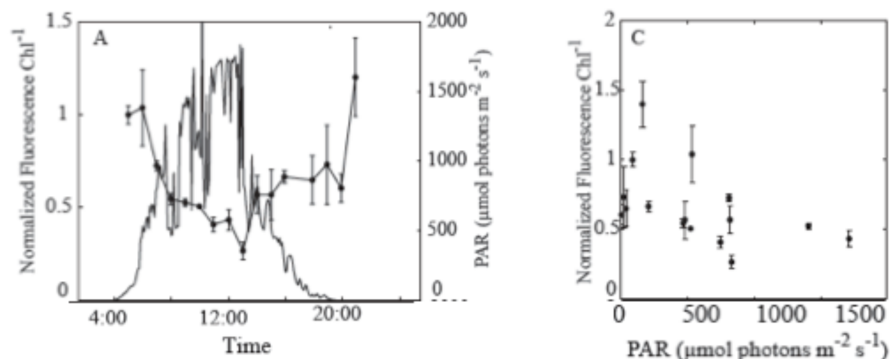
Measurements
Expose 8 tubes of a diatom culture to sunlight over the course of several hours during the afternoon lab (weather permitting and if there is sun). We'll take multiple readings of a sample exposed to bright light or some combination of your choosing. Two tubes will stay in the dark as controls.

1. Set up experiment with ~ 40 mL culture / tube.
2. Read samples in a Turner Designs TD-700 fluorometer and record:
   - solid standard
   - filtered seawater blank in 50-mL tube
   - phytoplankton cultures (invert before reading)
3. Place samples outside in an area without shadows.
4. Read samples every 30 min.
5. PAR will be recorded with 10 min integrations.

Background
Chlorophyll *a* fluorescence is 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.

Because chlorophyll a fluorescence is widely used as a proxy for phytoplankton biomass, variability of its quantum yield of fluorescence is problematic and compromises the linearity of 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 from A. Drzewianowski thesis, 2008; *Fluorescence quenching of phytoplankton from the Damariscotta River Estuary,* http://www.library.umaine.edu/theses/pdf/DrzewianowskiAF2008



2) FLUOROMETRIC ANALYSIS OF CHLOROPHYLL A CONCENTRATION

Chlorophyll is best measured by HPLC; we will filter samples, freeze in liquid N2 and ship to NASA for analysis. The most common way is by fluorometric analysis with either a broad band filter set or the Welschmeyer narrow filter set. We will compare data from all three methods.

MEASUREMENT

Students from Station 1 will provide a sample of a diatom (lab 1) and green alga (lab 2). Triplicate samples will be filtered for acetone extraction and triplicate filters for HPLC; volumes TBD.

Collect one 2 L bottle of DRE water for HPLC. As a class, three DRE samples will be filtered for HPLC. Volume TBD.

Each student will also collect one ~ 280 mL DRE sample from the dock.

Work in dim light. Filter samples through G/FF filters under low vacuum pressure, 5 mm Hg. Don't suck filter dry.

HPLC: Fold HPLC filters in half, place in Al foil envelope, and freeze in liquid N2.

Acetone extracts for fluorometry: Submerge replicates in tubes with 5 mL of 90% acetone; store at -20º C for 24 h to extract chlorophyll from cell. (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.) Read tubes on Saturday afternoon.

Wear goggles and gloves to protect from acetone and HCl.

Turn on both Turner 10AU fluorometers to warm up. Remove tubes from freezer and vortex mix or shake well to complete extraction and to ensure uniform distribution in tube. With forceps or hook, remove filter. Wipe finger prints off tube with Kimwipe. Centrifuge tube 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.

Carefully remove tube from centrifuge; DON'T re-suspend filter pad particles. By now the tubes should be warmed to room temperature. Note and record room temperature. Measure and record reading of secondary standard.

You will have two Turner Designs 10 AU fluorometers – one with broad band filters; one with Welschmeyer narrow filters. Work in dim light and absolutely NO natural sunlight (with UV). Read samples in dim light.

Insert tube in standard filter fluorometer and read Fo.
Read Fo in Welschmeyeter filter fluorometer.

Add two drops HCl, don't mix (density of HCl will take care of gentle mixing), read Fa in both fluorometers.

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. Chlorophyll and pheopigments emit or fluorescence light from the Q band (red wavelengths).

The fluorometer is calibrated with a commercial chlorophyll standard (we use Turner Designs), in units of µg/L. The caveats are :

- **E(λ) must be constant** - do frequent calibration to detect any lamp degradation; as lamps age, the energy emitted decreases, and
- **Φ_f must be constant** - it will be constant for the same solvent, i.e., 90% acetone and at a specified temperature . Φ_f decreases as temperature increases (for chlorophyll in 90% acetone, the decrease is -0.3%/ 1 degree C° increase in temperature). Measurements should be made at the same temperature as the calibration temperature; hence, monitor temperature.

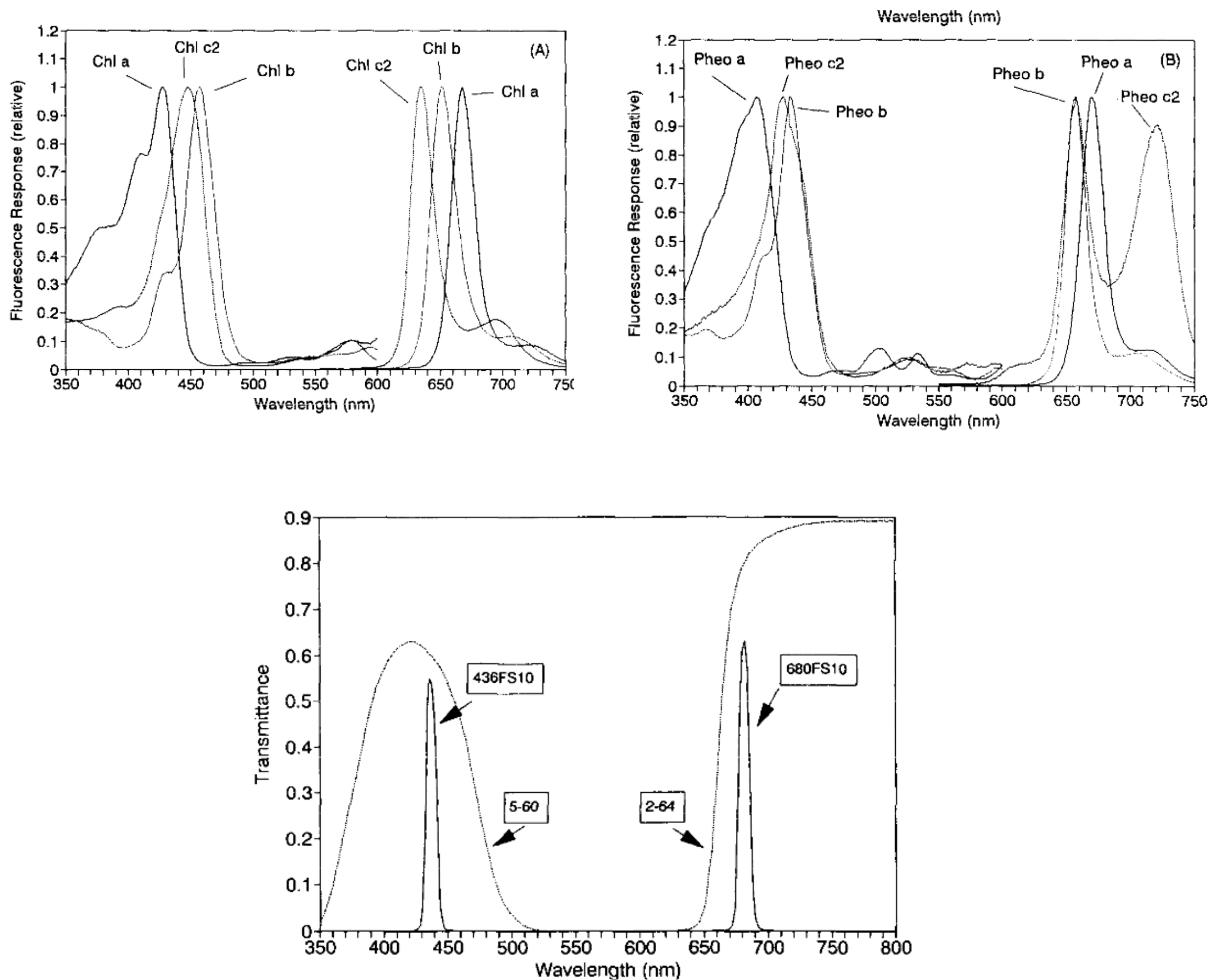
Calibrate with traceable chlorophyll *a* standards twice/year or before/after major field campaigns. Turner Designs sells chlorophyll *a* extracts that are certified. At every use, measure the secondary standard (fluorescent plastic) to detect any drift. Keep temperature constant; as temperature increases, fluorescence is increasing quenched (thermal quenching).

Filter combinations – there are two standard filter combinations:

1) broad band blue excitation filter and long pass red emission and
2) Welschmeyer narrow filters.

All four pigments – chlorophyll *a*, *b* and pheopigment *a*, *b* – absorb light when the broad filters are used and all four fluorescence in the red. The effect of pheopigment *a* can be removed measuring fluorescence before and after acidification (pheopigment *a* has 54% quantum efficiency of chlorophyll *a*). The equation you will use has two measurements – before (Fo) and after (Fa) addition of acid – and two unknowns – chlorophyll and pheopigment *a,* so the Yentsch and Menzel equation to solve for concentrations of both can be satisfied. The excel spread sheet includes the latest calibration factor.

However, the broad filter set and acidification method does not remove the bias due to chlorophyll *b*. Fortunately, there is little chlorophyll *b* in the DRE, but that is not true everywhere. The presence of chlorophyll *b* leads to an underestimation of chlorophyll *a* and an overestimation of pheophytin *a*. With the narrow Welschmeyer filters, only chlorophyll *a* absorbs light.



Calculate chlorophyll concentration using excel spread sheets with calibration coefficients. The fluorometers were calibrated with Turner Design's chlorophyll *a* standards at the same time, several months ago.

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}.

ASSIGNMENTS:

In vivo fluorometers (WET Labs and Turner Designs):

1) Compare the various approaches for dark values and blanks. How do you think a 'blank' should be measured? How would you test for a temperature effect? A pressure effect? Is is there a wall effect? For chlorophyll fluorescence of cultures, is there a difference between a stationary reading vs. reading with a moving sensor (assuming no wall effect)?
2) Linearity: plot various fluorescence readings vs. volume of culture or CDOM. Are these readings linear or do they show saturation at higher volumes?
3) Does the addition of other constituents affect the CDOM or chlorophyll fluorescence signal?
4) Are the excitation wavelengths as advertised by the manufacturer? What pigment(s) do these wavelengths excite?

Chlorophyll concentration in acetone extract:

5) Calculate chlorophyll and pheopigment concentrations; the Excel spread sheet will have the two equations for the standard and Welschmeyer fluorometers.
6) Calculate the fluorescence/chlorophyll for each cultures. Are they the same? Why or why not?
7) Determine the SD for each lab session for the DRE chlorophyll.

Solar quenching:

8) Plot *in vivo* chlorophyll *a* fluorescence vs. time. How would fluorescence quenching affect use of fluorescence as a proxy for chlorophyll (phytoplankton biomass)?

APPENDIX

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).

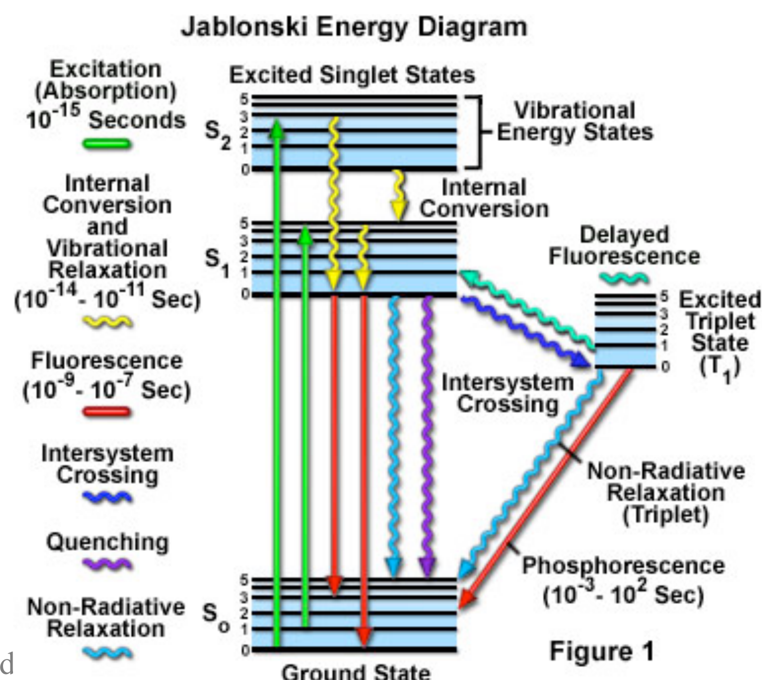
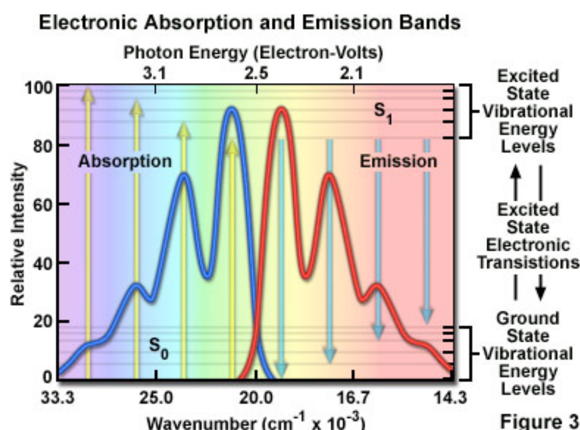


Figure 1

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.



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?

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

Phytoplankton cells 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, where it is not possible to collect discrete samples for chemical analysis of chlorophyll *a,* the uncertainty increases. Solar quenching of fluorescence changes the ratio of fluorescence to chlorophyll *a* concentration both on diel and shorter time scales, creating a BIG problem in sensor calibration.

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.

As discussed in the CDOM lab, 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 µm (nominal) for G/FF filters, although the effective filter size decreases as more material accumulates, and 0.2 µm. 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 or even solar radiation, λ is wavelength denoting the spectrum of the light source, C is concentration of a fluorescent

substance, and Φ_f is quantum yield of fluorescence (= photons emitted/photons absorbed). At high concentrations, the relationship between concentration and fluorescence becomes non-linear, and fluorescence emission decreases. This is sometime called inner-filter effect and is due to attenuation (mostly absorption of both excitation and emission wavelengths along the path.

Another way to represent the fluorescence equation for chlorophyll *a* is to expand the concentration term:

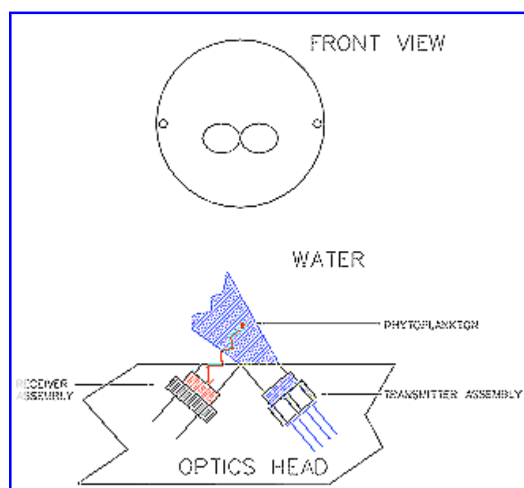
$$C = a \; / \; a^*$$

where a is the measured absorption coefficient (m^{-1}) and a^* is the chlorophyll specific absorption coefficient ($m^2 \; mg^{-1}$); the product of $a \; / \; a^*$ = chlorophyll *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'!

Commercial in-water fluorometers



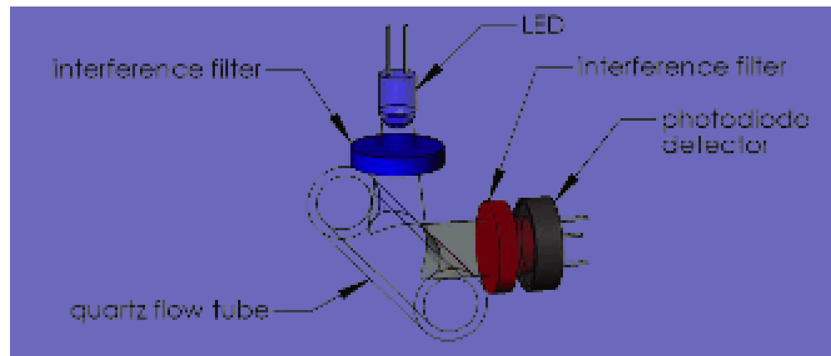
Many fluorometers are available on the market. For a recent comparison of chlorophyll fluorometers, see: http://www.act-us.info/evaluation_reports.php. Fluorometers come with a variety of excitation/emission options that you can select to measure, for example, relative concentrations of chlorophyll, CDOM, uranine, phycocyanin, or phycoerythrin. Converting relative fluorescence (volts or digital counts) to concentration is up to the user (and let the user beware!). 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–

60º with respect to the end face of the unit. Fluoresced light is emitted isotropically and is received by a detector positioned where the acceptance angle forms a 140º intersection with the source beam. An interference filter is used to discriminate against the scattered excitation light. In some applications a wiper is included 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 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 a 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.

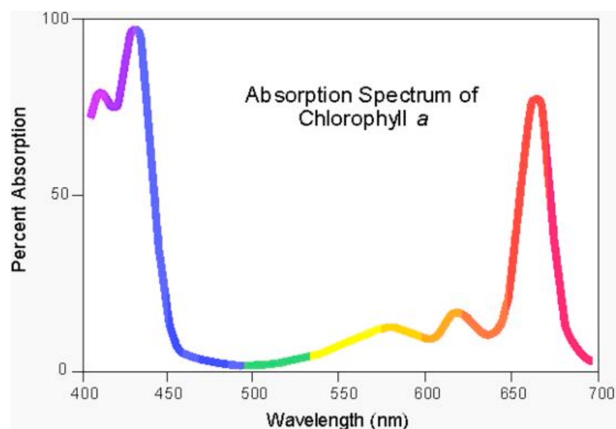


Chlorophyll extraction with simple fluorometric analysis (i.e., standard fluorometric method)

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.



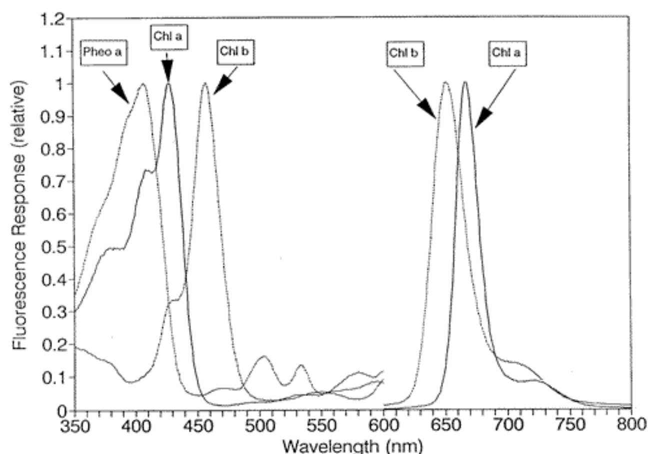
Chlorophyll *a* absorbs in both blue (Soret) and red (Q) bands (figure; source is <http://www.ch.ic.ac.uk/local/projects/steer/chloro.htm>). Chlorophyll *a* has the additional characteristic of fluorescence, a phenomenon by which a fraction of the photons are re-

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>:

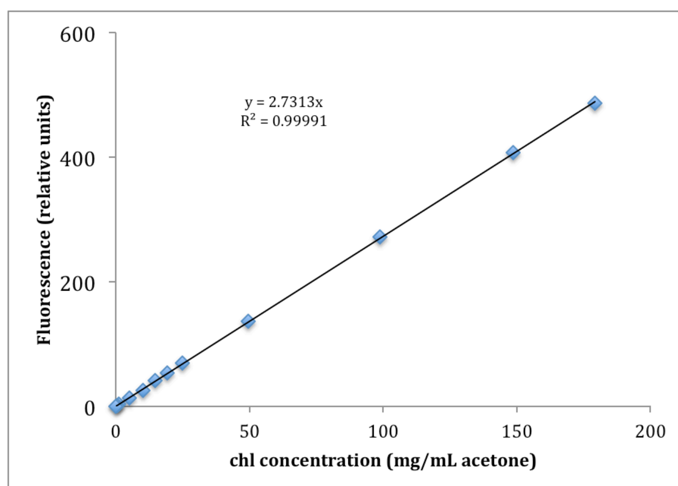
$$\text{chl } a = K\,(Fm/\,Fm-1) \times (Fo - Fa) \times (v/V) \quad (1)$$

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

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}.



Fluorometer calibration with pure chlorophyll *a* standard

REFERENCES

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