Lab 5: OVERVIEW for Chlorophyll and CDOM fluorescence lab 12 July 2013

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 measuring fluorescence of CDMO and chlorophyll *a* (*in vivo)*
2. Standard method for measuring chlorophyll *a* concentration in filtered samples (*in vitro)*

Divide into 2 groups, each with 10 students

	1st session	2nd session
Station 1	Mitchell Lab (Emmanuel & Ali)	Start in classroom, then to MJP Lab (MJ & Nathan)
Station 2	Start in classroom, then to MJP Lab (MJ & Nathan)	Mitchell Lab (Emmanuel & Ali)

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).

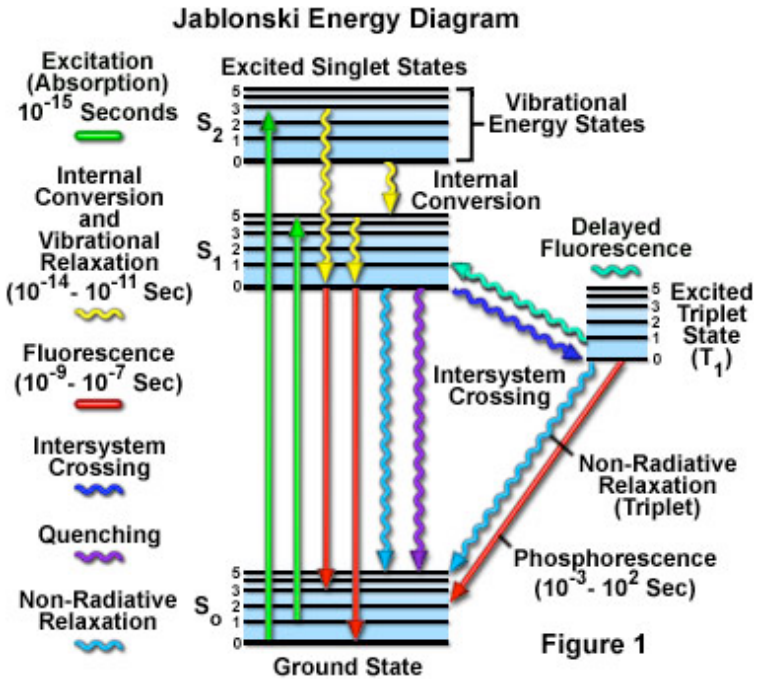


Figure 1

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.

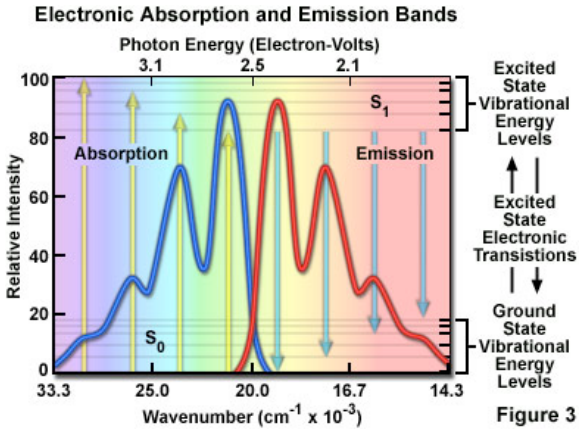


Figure 3

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?

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:

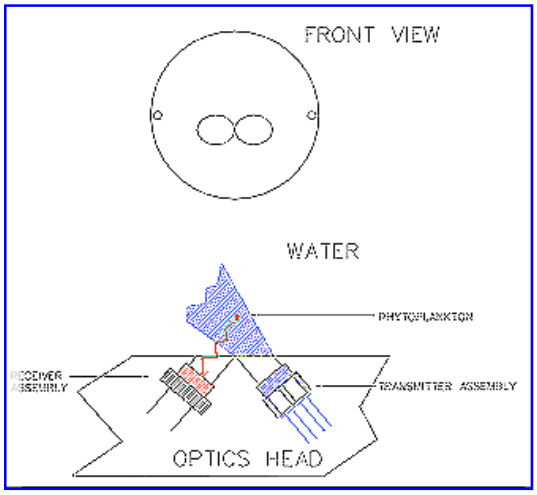
$$C = a / a^*$$

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'!

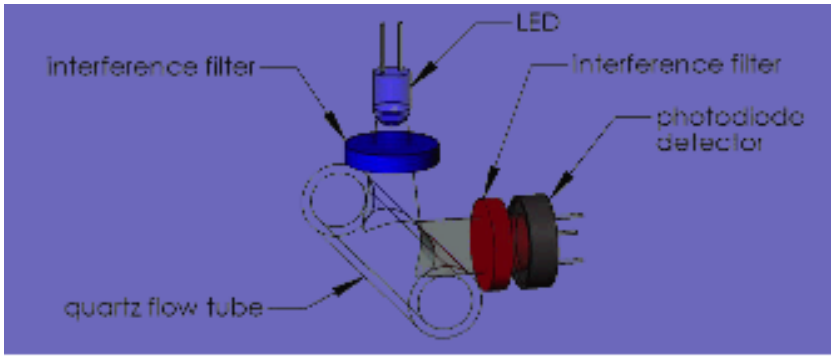
Commercial in-water fluorometers



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>



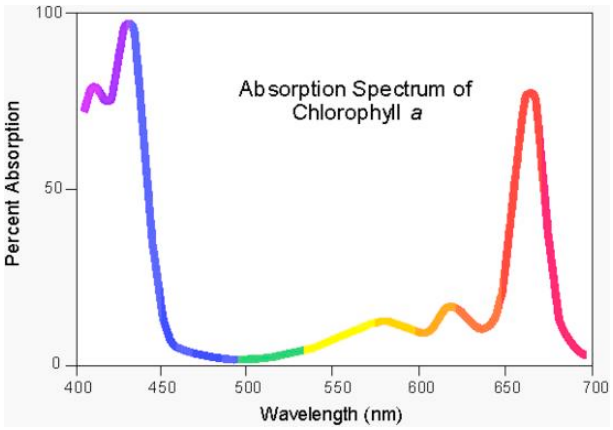
Another fluorometer design is a flow-through system, for example, the WET Labs WETStar or the flow tube attachment in the Turner Designs 10-AU benchtop. The flowthrough design often which works well in a ship's in-line systems. The source and detector are at 90° to each other (see figure below). Note 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.



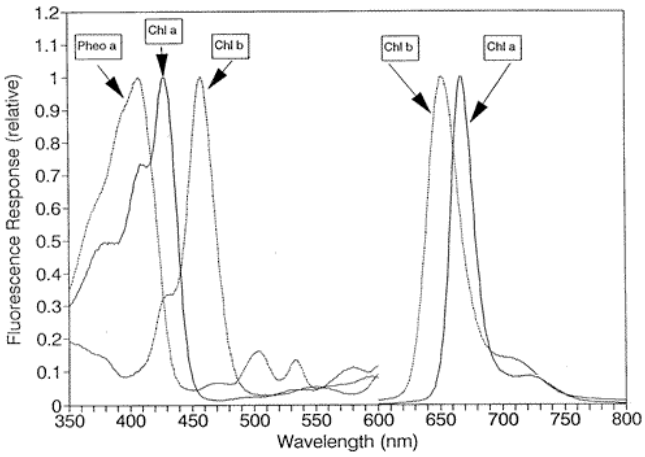
Chlorophyll *a* absorbs in both blue (Soret) and red (Q) bands (figure; source is <http://www.ch.ic.ac.uk/local/projects/steer/chloro.htm>). Chlorophyll *a* has the additional characteristic of fluorescence, a phenomenon by which a fraction of the photons are re-emitted (for chlorophyll, the fluorescence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

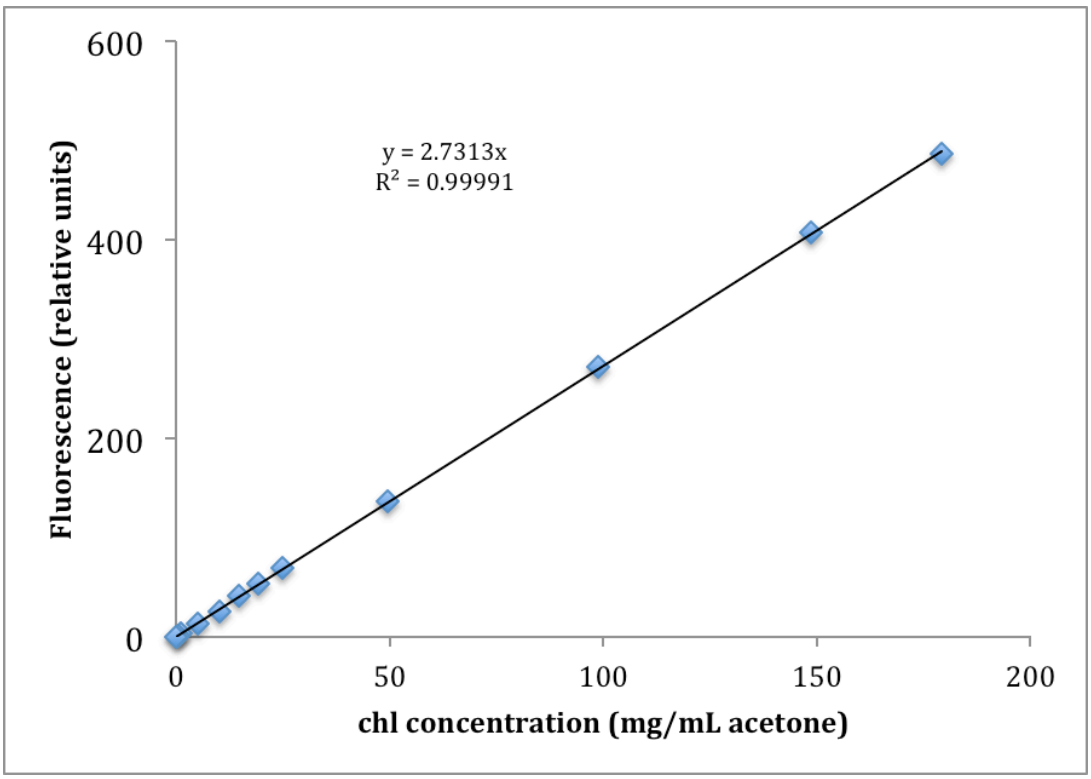
$$\text{chl } a = K\,(Fm/\,Fm\text{-}1) \times (Fo - Fa) \times (v/V) \quad (1)$$

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

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}.



Fluorometer calibration with pure chlorophyll *a* standard

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.

Drzewianowski, A. 2008; *Fluorescence quenching of phytoplankton from the Damariscotta River Estuary,* Univerisity of Maine thesis, http://www.library.umaine.edu/theses/pdf/DrzewianowskiAF2008.pdf

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.

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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 5 – Station 1

Fluorescence of CDMO and chlorophyll *a* in living cells

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

LABORATORY SAFETY ISSUES – general laboratory safety

GOALS

- learn about types of sensors used on ship-based profilers, mobile platforms, moorings and flow through fluorometers on ships
- what is the relationship between fluorescence and concentration of a biogeochemical variable? (i.e., how does CDOM fluorescence relate to CDOM absorption? to dissolved organic carbon concentration? how does chlorophyll *a* fluorescence relate to chlorophyll concentration? to phytoplankton carbon?)
- determination of the blank (dark counts or volts)
- linearity of sensor
- interference – is the signal affected by other variables? i.e, does CDOM attenuate or contaminate chlorophyll *a* fluorescence? does phytoplankton physiology (particularly non-photochemical quenching and photo damage) affect the quantum yield of fluorescence? do scattering particles (minerals, clay or Maalox) affect the fluorescence signal? does sunlight contaminate the signal?
- is the excitation of the LED–filter combination what the manufacturer states? does the spectral emission vary among sensors (manufacturers, models)?
- how are fluorometers calibrated? (to test for lamp and filter stability, to convert to concentration, etc.)

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

CHLOROPHYLL FLUOROMETER

1) 'Dark counts' and blanks. Compare readings for different conditions. For each case, move the sensor around – when is the sensor influenced by the walls of the room or the walls of the bucket?
    a. air with room lights on (note that these are fluorescent lights; might be different with incandescent or LED room lights)
    b. air with room lights off
    c. black electrical tape in air (sensor is not in bucket) – WET Labs only
    d. black electrical tape in tap water (sensor is in bucket) – WET Labs only
    e. tap water (will be particularly interesting for CDOM)
    f. RO water (we should use Milli-Q, but can't produce sufficient volume)
    g. filtered DRE seawater (DON'T discard after measurement; use for next exercise, below)

2) Linearity test for chlorophyll fluorescence of culture and relationship between fluorescence and chlorophyll *a* concentration
   - measure signal of filtered seawater
   - add culture in aliquots, mix and record
     - before taking an aliquot of culture, mix culture with 'Perry swirl technique' to get a uniform suspension. You might want to start with small addition.
     - add more culture; 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.
     - mix sample in bucket frequently (cells can settle).
     - explore moving the sensor around – when is the sensor influenced by the walls of the bucket? hold the sensor stationary, in a location not affected by the walls? Does the signal change with time? (it might for chlorophyll *a* fluorescence)
   - take three replicate water samples for chlorophyll analysis from bucket (note fluorescence values) to determine relationship between fluorescence and chlorophyll concentration (GIVE TO MJ for processing ASAP).
   - DON'T discard the bucket (see next exercise).

3) Interference by CDOM on measurement of chlorophyll fluorescence. Add measured volume of strong tea to bucket. Record measurement – does the fluorescence change? Add more and observe.

4) Interference by particles on measurement of chlorophyll fluorescence. Add measured volume of particles (dust, clay). Record measurement – does the fluorescence change? Add more and observe.
   Ok to discard bucket contents now.

(Effect of solar quenching on chlorophyll fluorescence – covered in Station 2.)

CDOM FLUOROMETER

5) Dark counts' and blanks. Compare readings for different conditions. For each case, move the sensor around – when is the sensor influenced by the walls of the room or the walls of the bucket?
   a. air with room lights on (note that these are fluorescent lights; might be different with incandescent or LED room lights)
   b. air with room lights off
   c. black electrical tape in air (sensor is not in bucket)
   d. black electrical tape in tap water (sensor is in bucket)
   e. RO water (we should use Milli-Q, but can't produce sufficient volume)

6) Linearity test for CDOM fluorescence of tea and relationship between fluorescence and concentration
   - measure signal of water (tap water or filtered seawater)
   - add tea in aliquots, mix and record

- add more tea
- mix sample in bucket frequently (cells can settle)
- explore moving the sensor around – when is the sensor influenced by the walls of the bucket? hold the sensor stationary, in a location not affected by the walls?
- DON'T discard the bucket (see next exercise).

7) Interference by chlorophyll fluorescence on CDOM fluorescence. Add aliquots of tea to bucket. Record measurement – does the CDOM fluorescence change? Add more and observe.

8) Interference by particles on measurement of CDOM fluorescence. Add measured volume of particles (dust, clay). Record measurement – does the fluorescence change? Add more and observe.
Ok to discard bucket contents now.

BOTH FLUOROMETERS

9) Does room light or flashlight or sunlight effect the fluorescence signal? point sensor toward room light and sun. Record.

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

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

12) Other fluorometers – flow through fluorometers (WET Labs Wet Star as example) and combination fluorometers and backscatter sensors.

Lab 5 – Station 2:

1)) solar quenching of fluorescence
2) chlorophyll extraction with simple fluorometric analysis (i.e., standard fluorometric method)

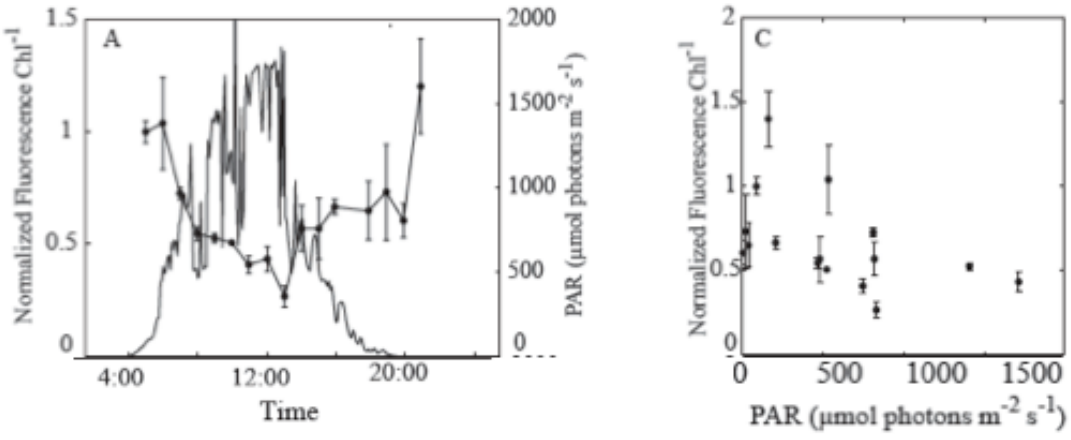
LABORATORY SAFETY ISSUES – acetone, 10% HCl; see MSDS sheets. Wear goggles and gloves when handling solvents and acids. Open solvent containers only under the hood. Pour all wastes into appropriate containers, including seawater. General laboratory safety practices apply.

GOALS – learn about:
sampling, filtration and extraction
measurement of chlorophyll *a* and phaeopigments
potential interference from chlorophyll *b*
calibration with pure chlorophyll *a* standard
record keeping – secondary standards and room's temperature
solar quenching of fluorescence

1) 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 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 below from A. Drzewianowski thesis, 2008; *Fluorescence quenching of phytoplankton from the Damariscotta River Estuary,* http://www.library.umaine.edu/theses/pdf/DrzewianowskiAF2008



MEASUREMENTS

Expose 8 tubes of a diluted phytoplankton 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.

Read samples in a Turner Designs 10-AU fluorometer or TD-700 fluorometer and record:

- solid standard
- filtered seawater blank in 50-mL tube
- phytoplankton cultures

Replace sample outside for exposure to bright light.

2) FLUOROMETRIC ANALYSIS OF CHLOROPHYLL *A CONCENTRATION*

The basic principle of fluorometry is:

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

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.

Fluorometer calibration also you to calculate C from F, if

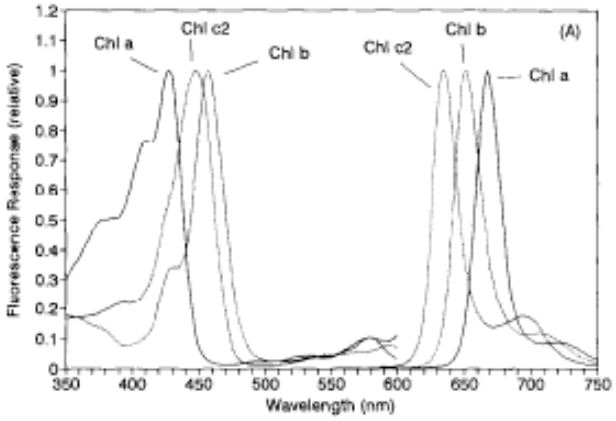
- E(λ) is constant (do frequent calibration to detect any lamp degradation), and
- Φ_f is constant. For the same solvent, 90% acetone, that is true if temperature is also constant. Φ_f decreases as temperature increases (~ 1-2%). Measurements should be made at the same temperature as the calibration temperature; hence, monitor temperature.

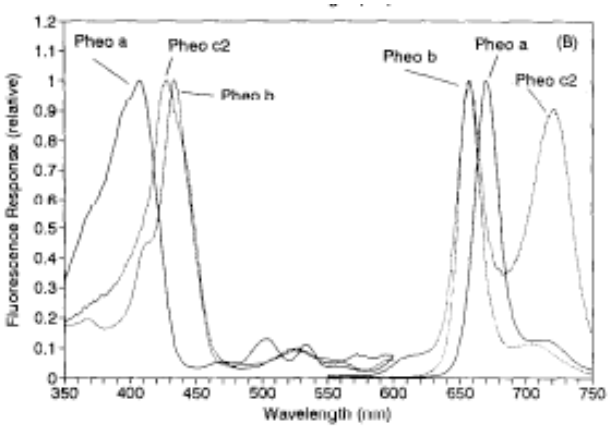
Calibrate with traceable chlorophyll *a* standards twice/year or before/after major field campaigns. Turner Designs sells chlorophyll *a* extracts that are certified. At every use, measure the secondary standard (fluorescent plastic) to detect any drift.

Filter combinations – standard broad band blue excitation filter vs. Welschmeyer narrow filter set. Chlorophyll *a*, *b* and pheopigment *a*, *b* absorb light with the broad filters. The effect of pheopigment *a* can be removed by 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, this method does not remove the bias due to chlorophyll *b.* The presence of chlorophyll *b* gives an underestimate of chlorophyll *a* and an overestimate of pheophytin *a.* With the narrow Welschmeyer filters, only chlorophyll *a* absorbs light. Fortunately, there is little chlorophyll *b* in the DRE.

Best way to measure chlorophyll is HPLC – but it is expensive ($70/sample) and more water must be filtered. So…. the simple method in today's lab remains widely used.





Sample collection:

In real world, collect sample directly into pre-measured bottles – from dock or Niskin bottle or from ship's under way system . (It helps to know approximate magnitude of chlorophyll concentrations, so you adjust sample size to concentration). Rinse bottle 3 times, fill to the top, tap to displace bubbles, and top off. Store in dark and cold until filtration.

Today, we will test your subsampling skills. Work in groups of two and three. Collect water from the dock in a large bottle. Use the 'Perry swirl' method to subsample (swirl bottle 3 times in one direct, reverse direction and swirl once, invert and repeat, pour IMMEDIATELY).

Filtration and extraction in 90% acetone:

Filter three replicates of 100 mL each through G/FF filters under low vacuum pressure, mm Hg. Don't suck filter dry. IN DIM LIGHT chlorophyll in acetone bleaches very rapidly), submerge filter in 5 mL of 90% acetone at -20º C for 24 h to extract chlorophyll from cell. (Test tube is labeled in pencil, not pen – know why?). At sea, you could store filters in Al foil in liquid N_2 and do same for HPLC samples.

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.)

Plan to read tubes on Saturday afternoon.

Fluorescence reading of extract:
Come back Saturday afternoon to read samples. Turn on Turner 10AU to warm up. Work in dim light and absolutely NO natural sunlight (with UV). Read samples in dim light. 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 with Kimwipe. 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.

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. Insert tube in fluorometer and read Fo. Remove tube, add one drop HCl, don't mix (density of HCl will take care of gentle mixing), read Fa. **Wear goggles and gloves to protect from acetone and HCl.**

Calculate chlorophyll concentration using excel spread sheet with calibration coefficient. The fluorometer was calibrated with Turner Design's chlorophyll *a* standards.

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}.

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?
2) For the bucket measurements, 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)?
3) Linearity: plot various fluorescence readings vs. volume of culture or CDOM; are these readings linear or do they show saturation at higher volumes?
4) Does the addition of CDOM (tea) affect the chlorophyll fluorescence signal? Does the addition of phytoplankton affect the CDOM fluorescence signal?
5) Does the addition of scatterers (dust) affect the fluorescence signal? For the WET Labs sensor plot chlorophyll and CDOM 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 natural sunlight (or other light sources) contaminate the signal?
7) Are the excitation wavelengths as advertised by the manufacturer? One very important fact is that most standard chlorophyll fluorometers now use a 470 nm LED. What pigment(s) do wavelengths centered on 470 nm excite?

Solar quenching:

8) Solar quenching of fluorescence: does *in vivo* chlorophyll *a* fluorescence change for cultures exposed to natural sunlight for several hours? How would fluorescence quenching effect use of fluorescence as a proxy for cholorophyll (phytoplankton biomass)?

Replication:

9) Calculate chlorophyll and pheopigment concentration using the Excel spread sheet in the chlorophyll lab folder <chl_10AU.xls>.
10) Determine the SD for your own samples (triplicates).
11) Compare your concentrations with those of your partner (poured from the same bottle).
12) Compare results of all measurements - what's the variability (there will be a total of 30 samples).
13) What's your confidence in literature reports?