Lab 4 **14 July 2011**

Station 3: standard fluorometric measurement of chlorophyll *a*, *in vitro* (acetone extract)
Location: MJP lab
Focus: provide 'calibration' of fluorescence measurements from a few Station 1s

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 or 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

Filtration:
Filter water sample – issues relating to subsampling, volumes filter, acetone extraction procedure. (Emily will read samples from Collin's lab session 1 and 2 tonight.)

Chlorophyll reading and calculation:
Filter combinations – standard and Welschmyer filter sets
Fluorometer calibration – chlorophyll *a* standards
Mixing and centrifugation of sample
Fo and Fa (before and after acid)
Chlorophyll concentration – excel spread sheet
Interference of chlorophyll *b* – spinach has chlorophyll *b* and should not have pheopigments – what is the calculation?

➔ One group will measure samples from Wednesday (only 9). Other groups will measure replicates of 90% acetone extracts of spinach, *Thaslassiosira* culture, and dock water.

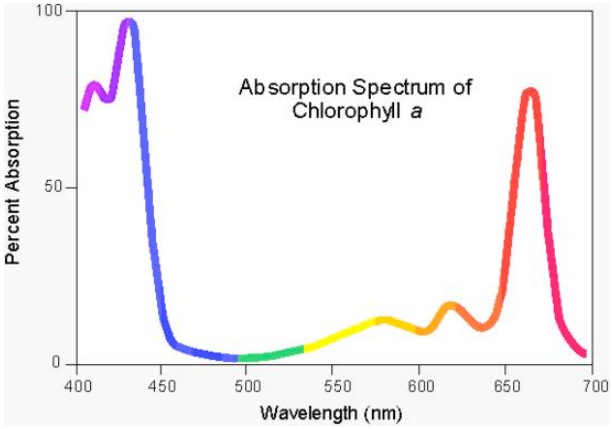
What's the difference between HPLC and fluorometric method? both are on extracts.

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.

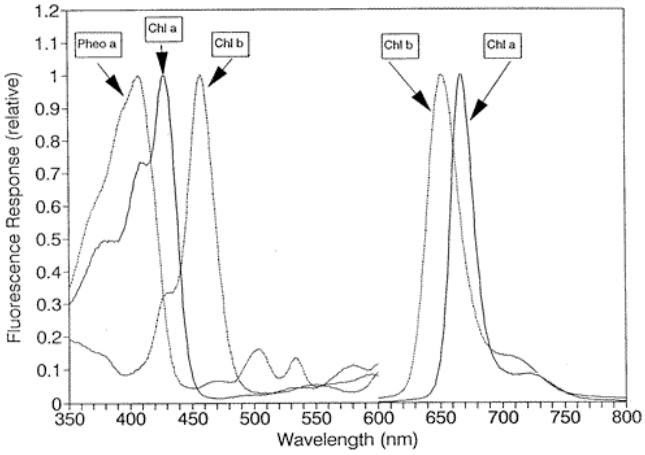


Chlorophyll *a* absorbs in both blue (Soret) and red (Q) bands (figure; <http://www.ch.ic.ac.uk/local/projects/steer/chloro.htm>). Chlorophyll *a* has the additional characteristic of fluorescence, a phenomenon by which a fraction of the photons are re-emitted (for chlorophyll, the fluorescence maximum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

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

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

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}.

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:

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.