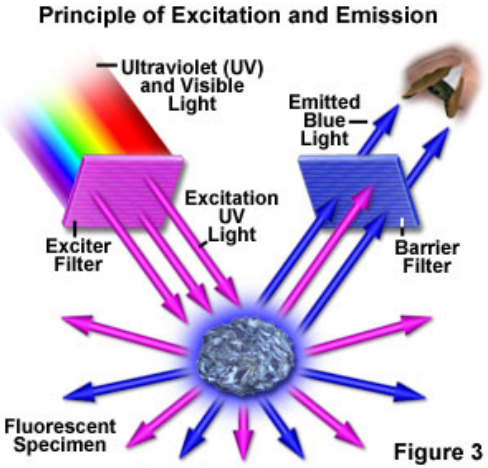
SMS 598 – Sensors

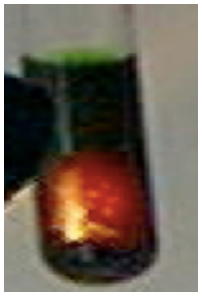
Chlorophyll Fluorescence

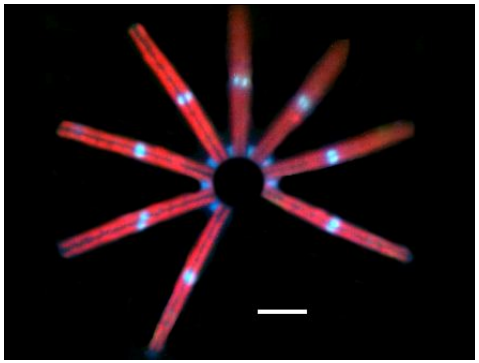
Mary Jane Perry

22 October 2012



http://micro.magnet.fsu.edu/primer/lightandcolor/fluorointroduction.html





Epifluorescence microscope: chlorophyll fluorescence in *Thalassionema* (courtesy of M. Sierackiz0

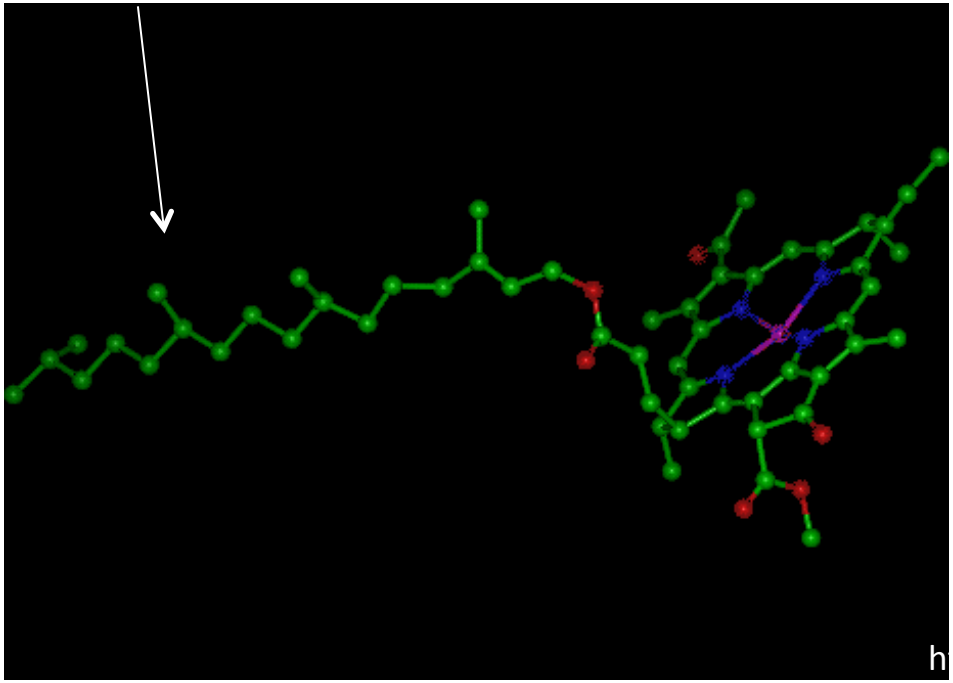
Chlorophyll fluorescence

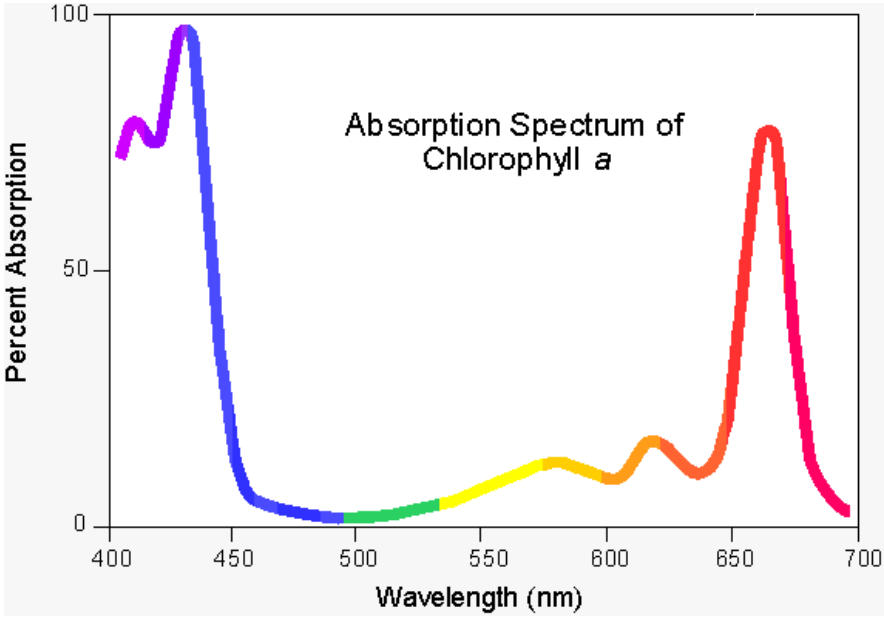
- Who has used chlorophyll *a* fluorescence?
- Big picture – what do we really want to know about phytoplankton?
- Different ways to think about chlorophyll *a* & phytoplankton
- Fluorescence in general; relationship with absorption
- Brief history of measurements related to chlorophyll *a* (context for present measurement of chlorophyll *a* fluorescence; reference for 'calibration' of fluorescence)
- General principles of fluorometric measurement and types of oceanographic fluorometers
- Interpretation of data, and challenges therein
- Synthesis of class
- Back to the lab to measure dock samples – if time

1) What is chlorophyll *a*?

phytol tail

Porphyrin ring w/ 4 N & 1 Mg^{++}





Chlorophyll *a* – the molecule.

Degraded pigments:

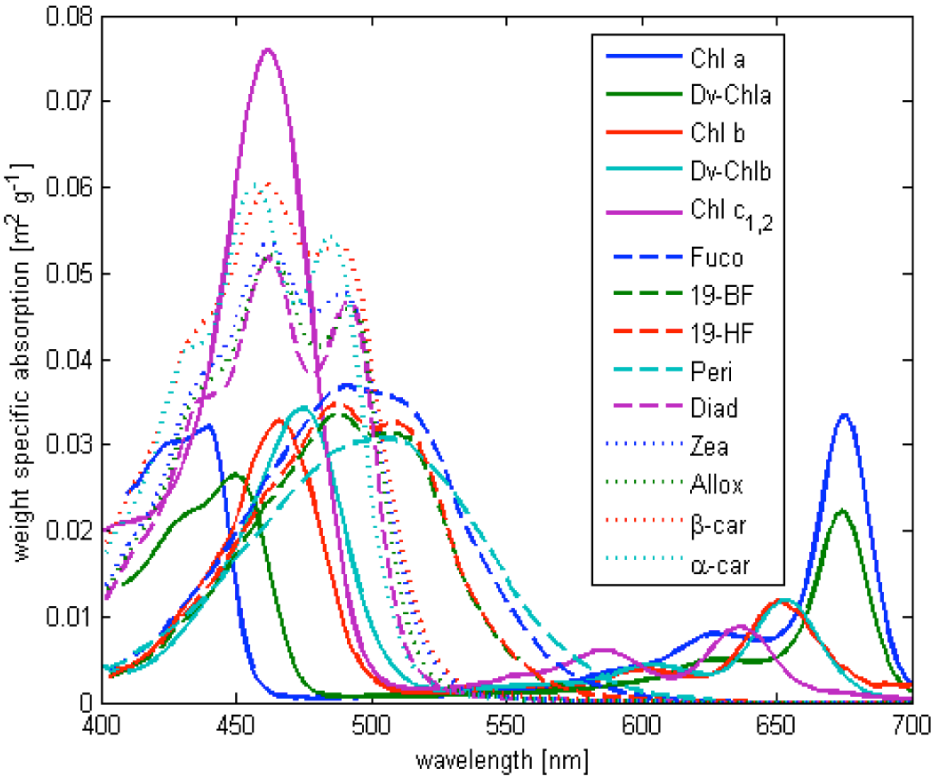
Pheophytin
lost Mg^{++}; peak shifts to ~415

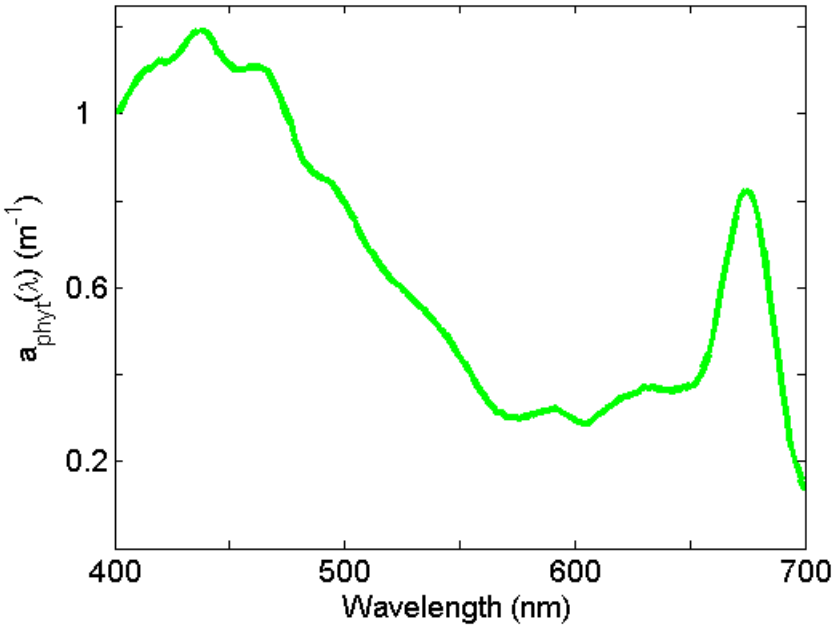
Pheophorbide
lost Mg^{++} and phytol tail

Pure chlorophyll a: absorption and fluorescence peaks will vary, depending on environment – protein complex & pH in membrane or polarity of solvent

www.ch.ic.ac.uk/local/projects/ steer/cloroads.gif

All phytoplankton have chlorophyll a (*Prochlorococcus* – divinyl chl a) plus other pigments – some photosynthetic and photoprotective.





Composite absorption
– overlapping absorption by different pigments

Chlorophyll molecule is attached to a binding protein.

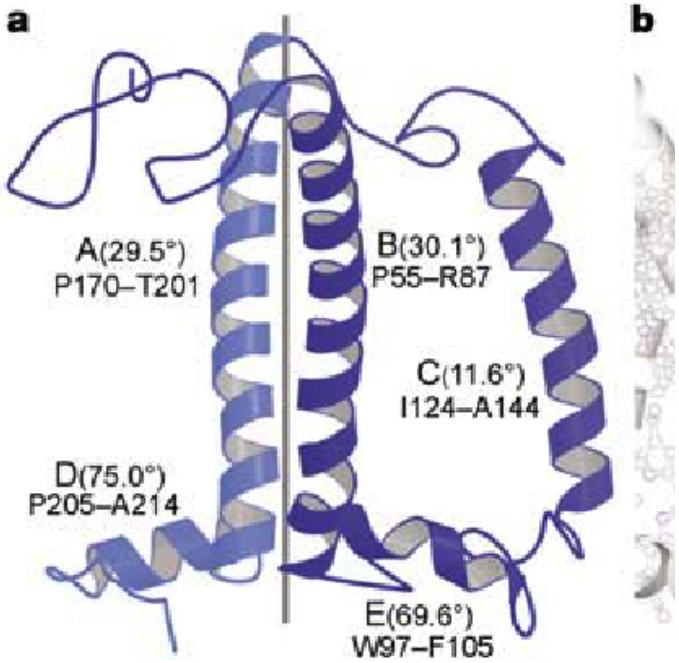
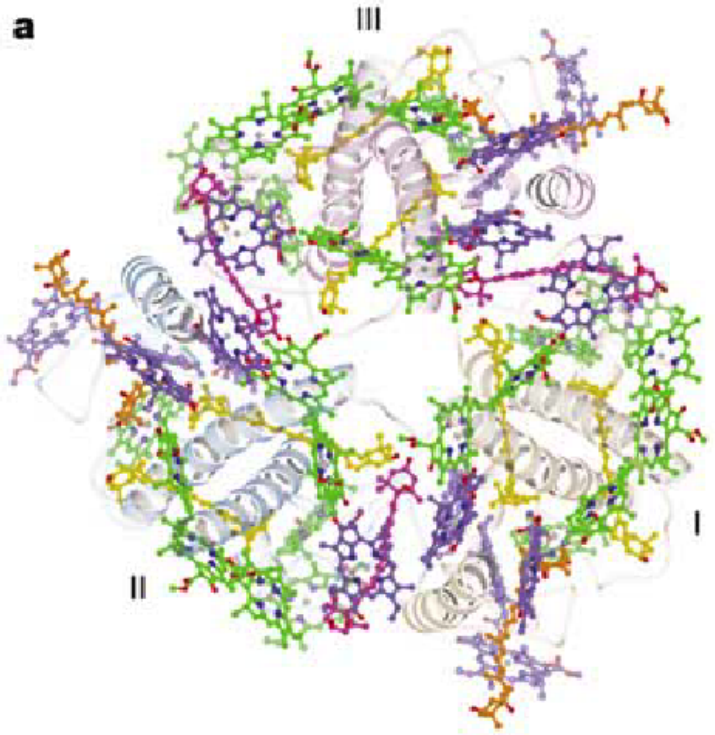


Figure 3 Secondary structure of monomeric LHC-II

protein backbone of monomeric LHC-II protein complex, from electron density mapping

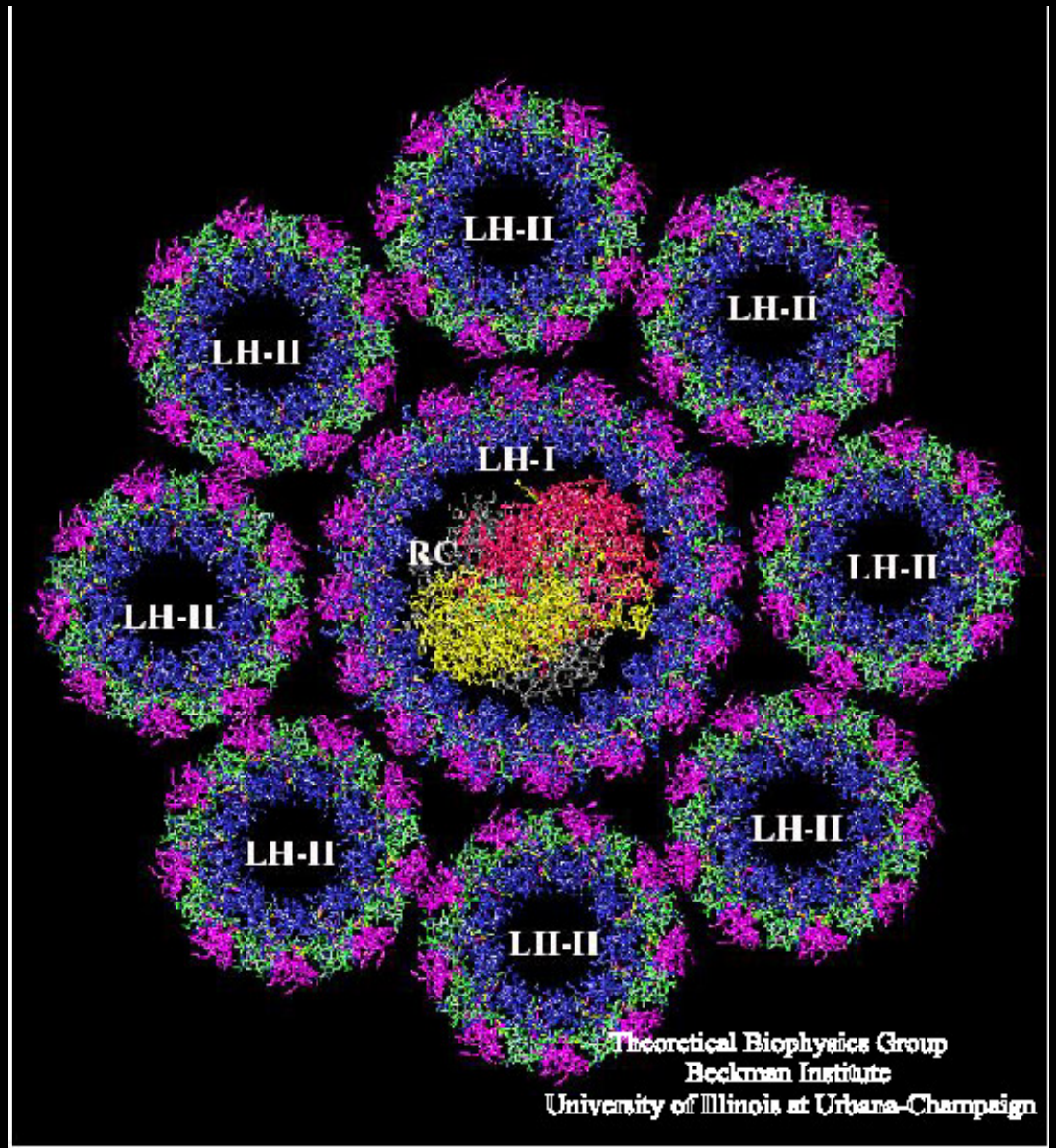
Trimeric complexes of Chl and binding protein.



3 monomers = 1 trimer
green: chl *a*; blue: chl *b*
yellow/orange: P carotenoids
magenta: PP carotenoids

Lui et al., 2004, Nature 428: 287ff for spinach LHC-II)

Many light harvesting trimers around reaction center (PS II)
to form a light harvesting complex.



Lui et al., 2004

Light harvesting complexes and other functional complexes are located in thylakoid membrane.

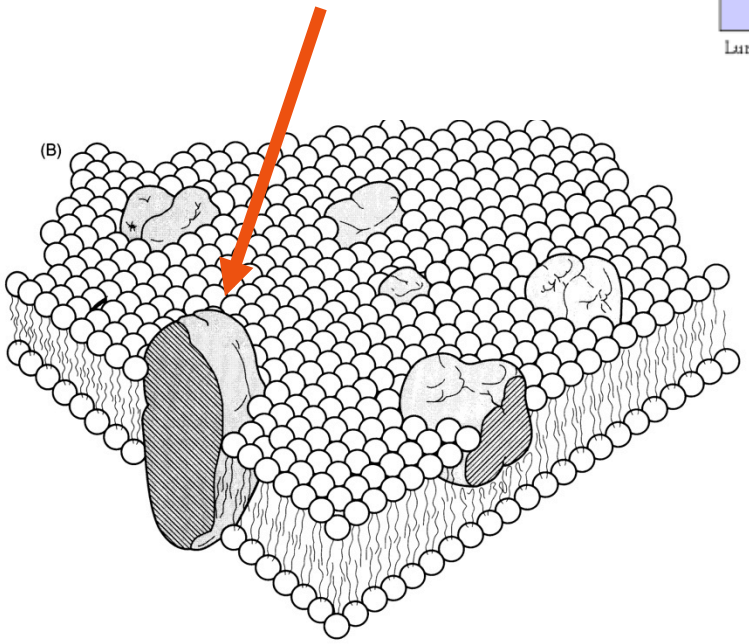
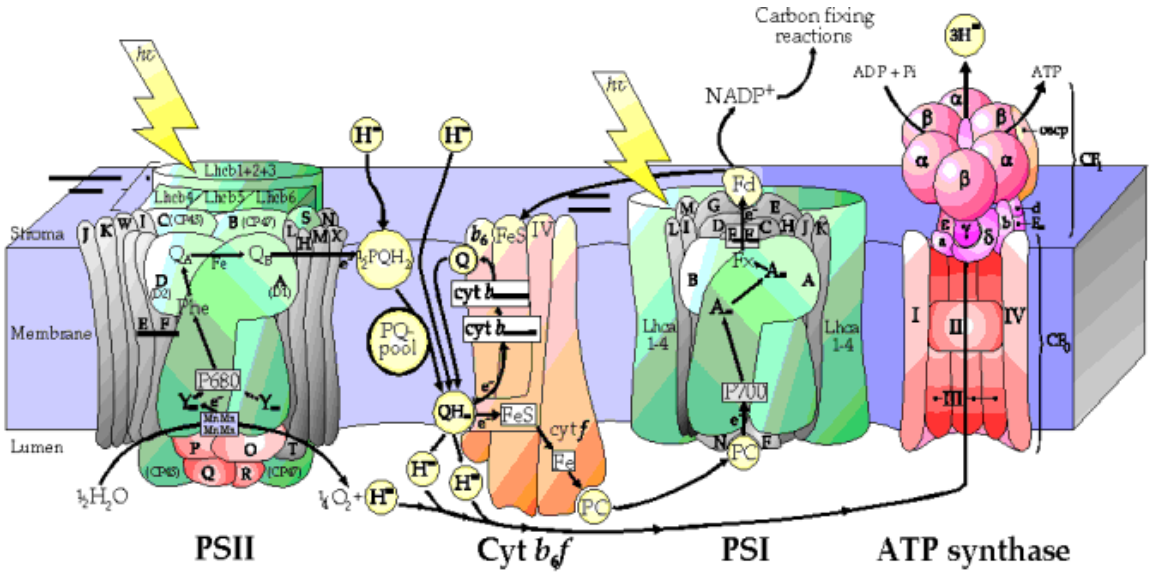
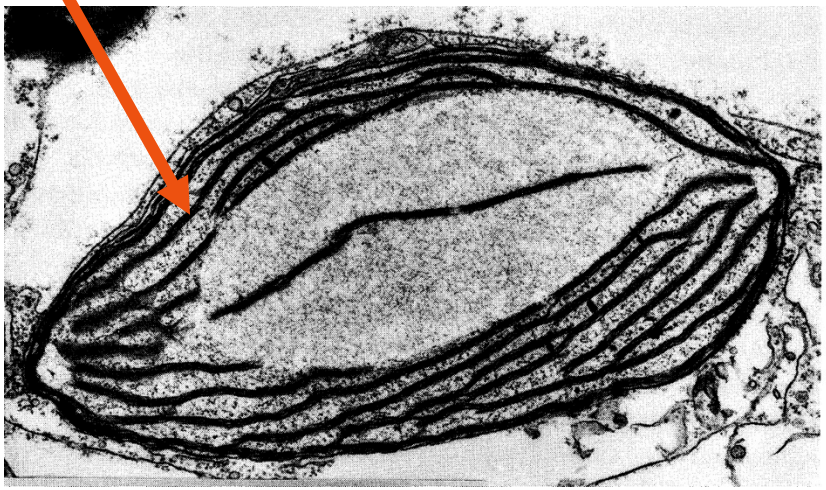


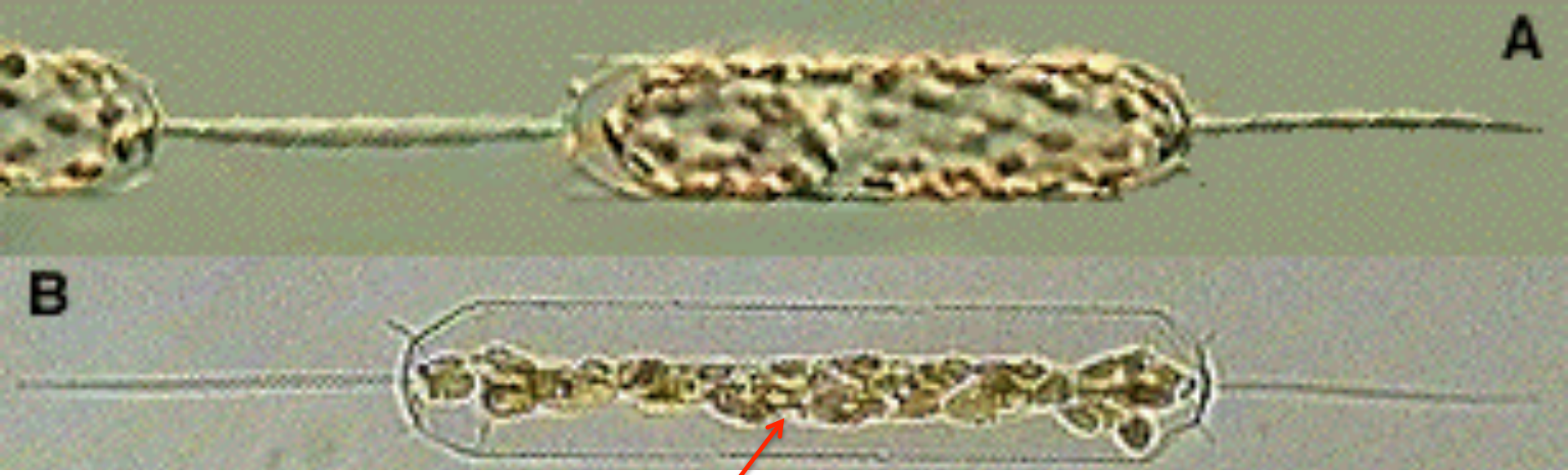
Figure 1.2 (A) Structure of two of the most important lipids that make up thylakoid membranes: monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG). In the formation of membranes, the polar sugar groups face the aqueous phases, while opposing nonpolar alkyl groups are oriented toward each other to form a lipid bilayer. The width of the bilayer is approximately 4 nm. (B) A schematic diagram of a thylakoid membrane (modified from Singer, Nicolson 1972). Thylakoid membranes are largely composed of MGDG and DGDG with other polyunsaturated fatty acids. Proteins are oriented within the membrane in a nonrandom fashion. Some proteins span the membrane, whereas others may only partially protrude. The proteins will have specific "sidedness," with some functional groups facing the lumen and others facing the stroma.



http://www.bio.ic.ac.uk/research/barber/index.html

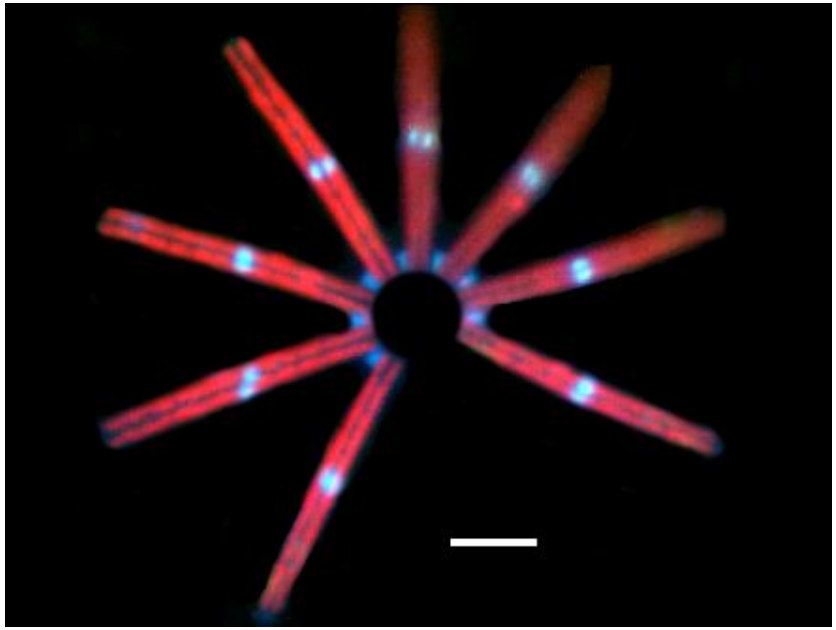
Thylakoid membranes in chloroplast

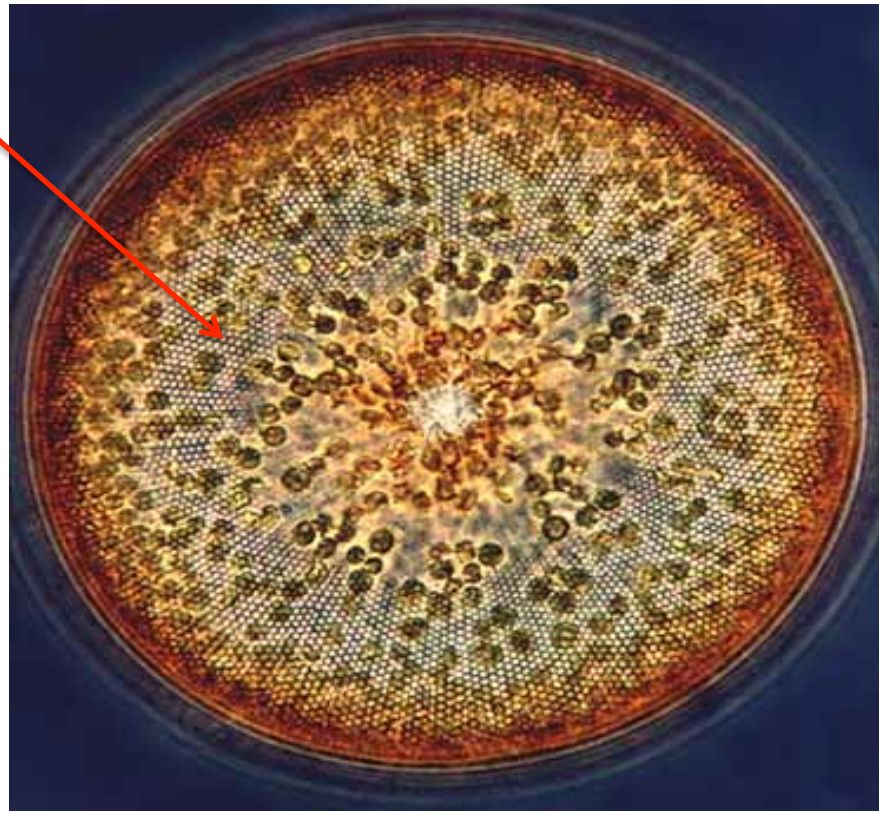




Diatom chloroplasts

In vivo chlorophyll fluorescence





Proxies or surrogates

Keep at the top of your thinking – **what is a phytoplankton?** particle? species? molecule – chlorophyll or carbon? … or what?

Potential surrogates or proxies for phytoplankton:

Pigment based:

* chlorophyll fluorescence
* extracted chlorophyll, other pigments (HPLC)
* absorption coefficients

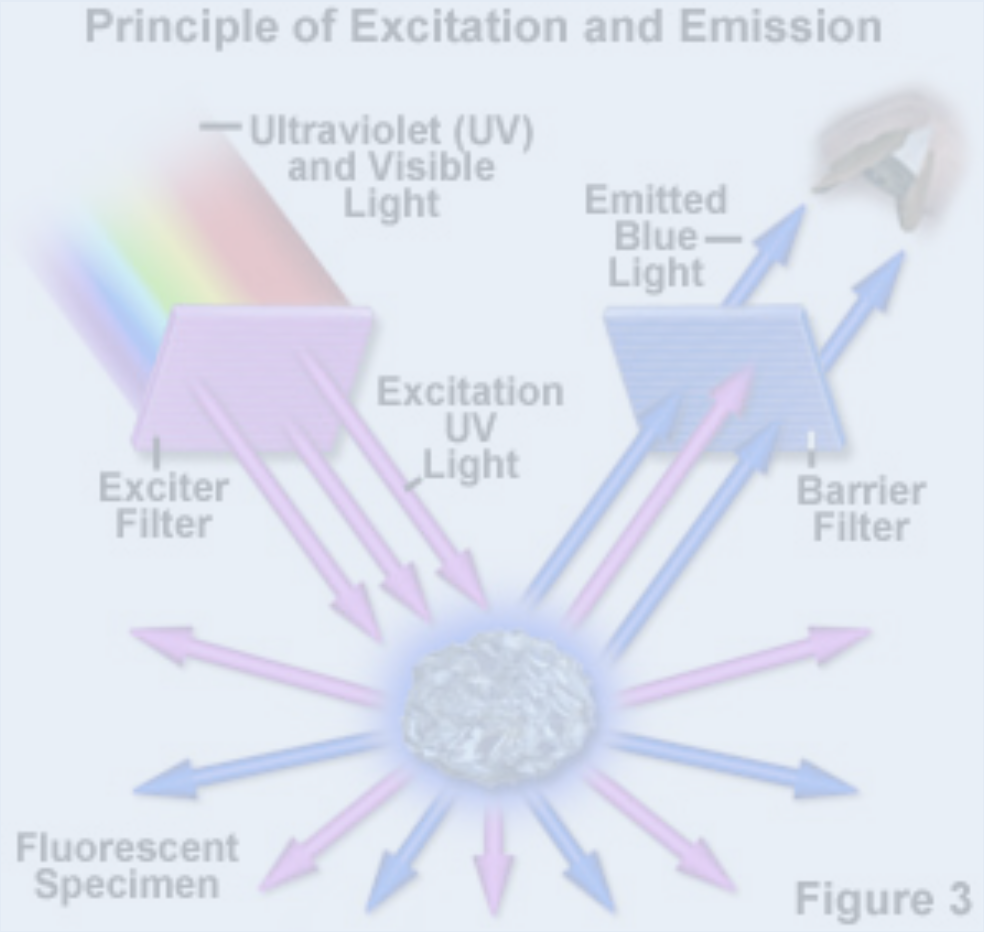
Particle based:

* beam c or backscatter
* particle size distribution

What else ?

2) What is fluorescence?

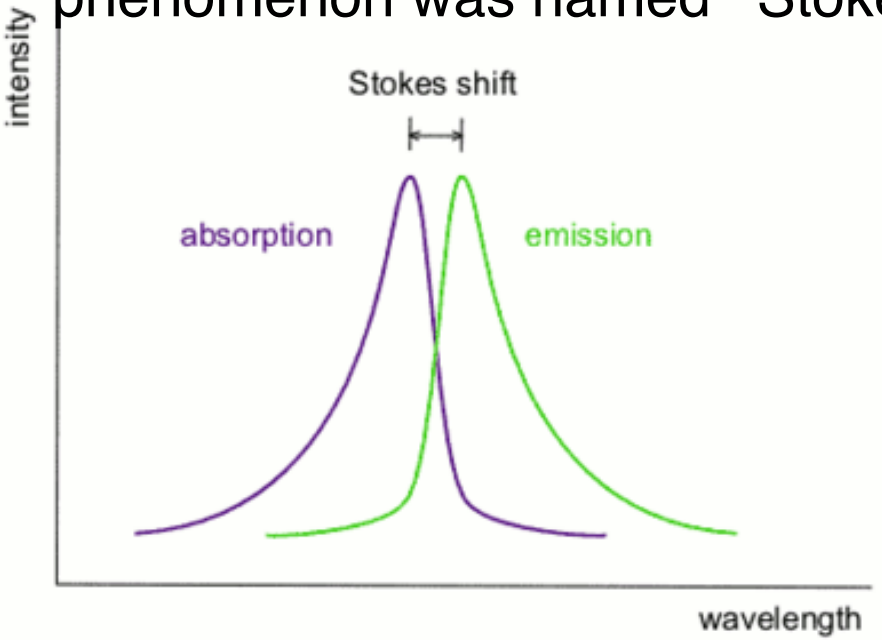
What is the relationship with absorption?



Sir George Gabriel Stokes

"I am almost inclined to coin a word and call the appearance fluorescence, from fluor-spar, as the analogous term opalescence is derived from the name of a mineral." –Phil. Trans. 479 (1852)

In 1852 Stokes described fluorescence, as exhibited by fluorspar and uranium glass. **He noted emission of visible light when he exposed them to UV light.** This phenomenon was named 'Stokes shift'.

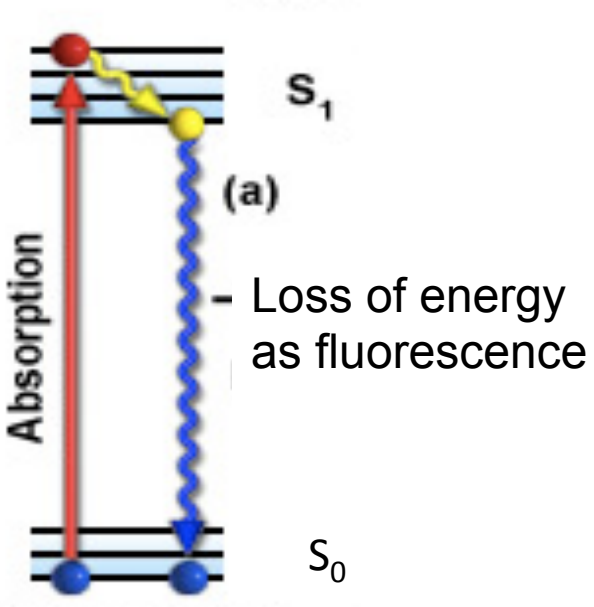


A fraction of energy absorbed at **shorter wavelength** (higher frequency, higher energy) is re-emitted as a photon at **longer wavelength** (lower frequency, lower energy).

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

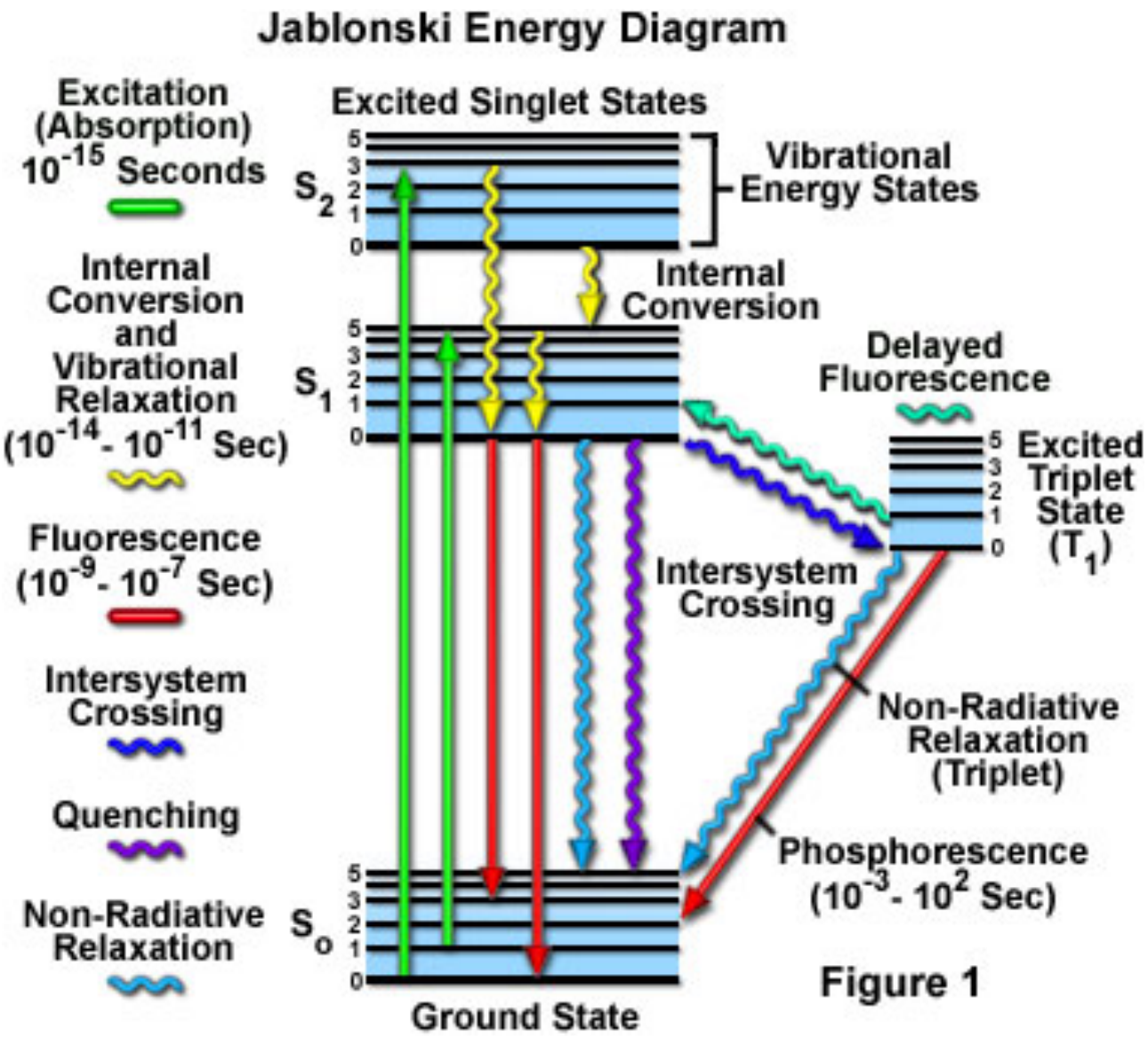
Fluorescence: A fraction of energy absorbed at a shorter wavelength (higher frequency, higher energy) is re-emitted as a photon at a longer wavelength (lower frequency, lower energy).

Energy (as a photon) can be absorbed **IF and ONLY IF** the energy of the photon ($E = h\nu = hc/\lambda$) is equal to <u>Δ energy</u> between an electron in the ground electronic state (S_0) and in a higher electronic state (S_n).



<u>Absorption</u> is an "electronic transition" ($O(10^{-15}$ s)), leading to an excited state. The excited electron returns to ground state by vibrational loss of energy (radiation-less decay). Certain molecules can lose some energy through photon loss, e.g., fluorescence. Note: from lowest electronic state of S_1 (not S_n). Other processes on next slide, but we'll stick to F.

Fluorescence is only ONE potential pathway for dissipation of absorbed energy;
That is one challenge for 'calibration' of chlorophyll *a* fluorescence.

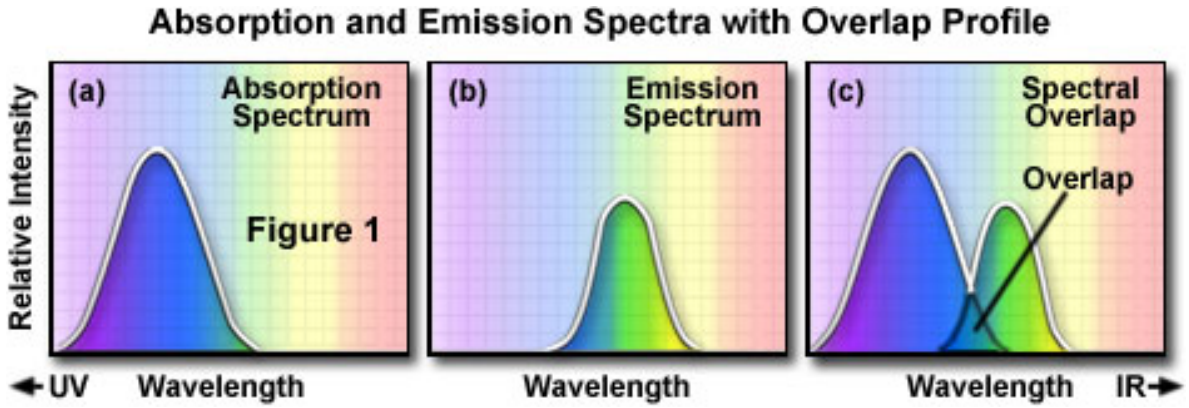


Neat applet: http://www.micro.magnet.fsu.edu/primer/java/jablonski/lightandcolor/

http://www.micro.magnet.fsu.edu/optics/timeline/people/jablonski.html

Summary: **fluorescence emission**

1. always from lowest vibrational state of S_1
2. red shifted – Stokes shift (higher λ, lower E)
3. mirror image of absorption



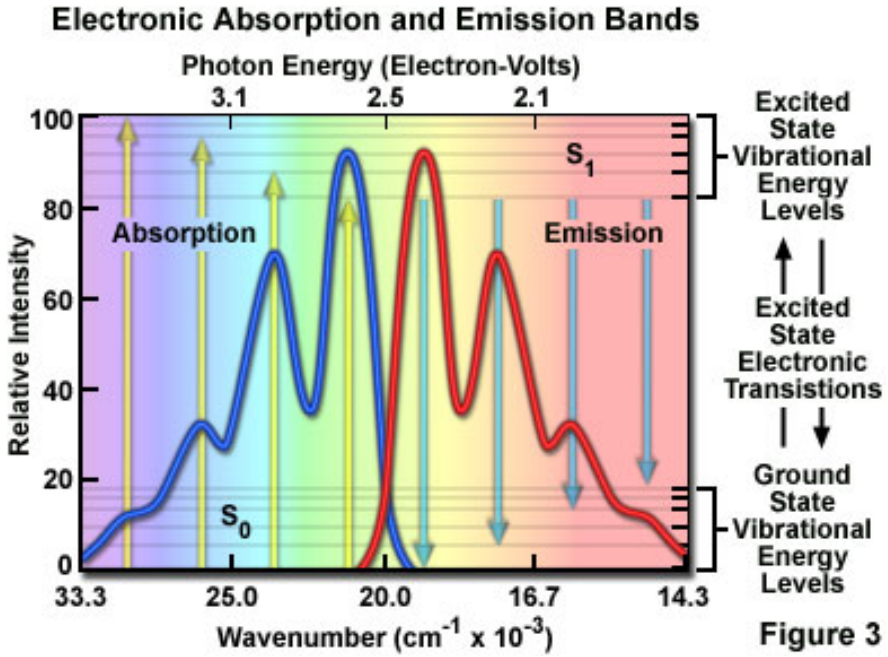
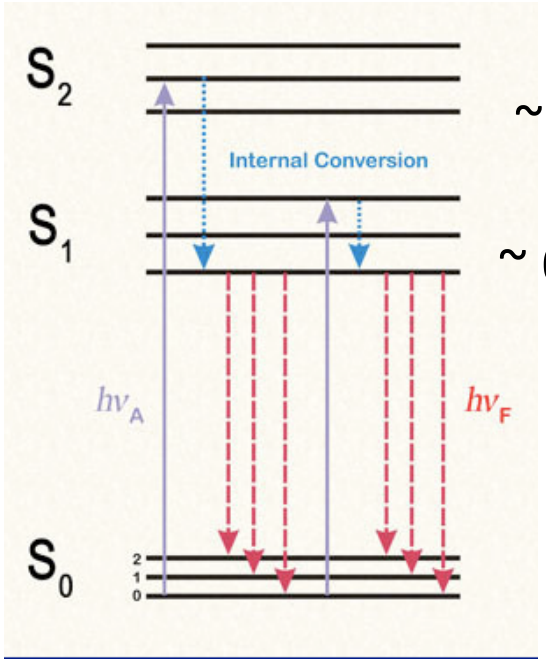


Figure 3

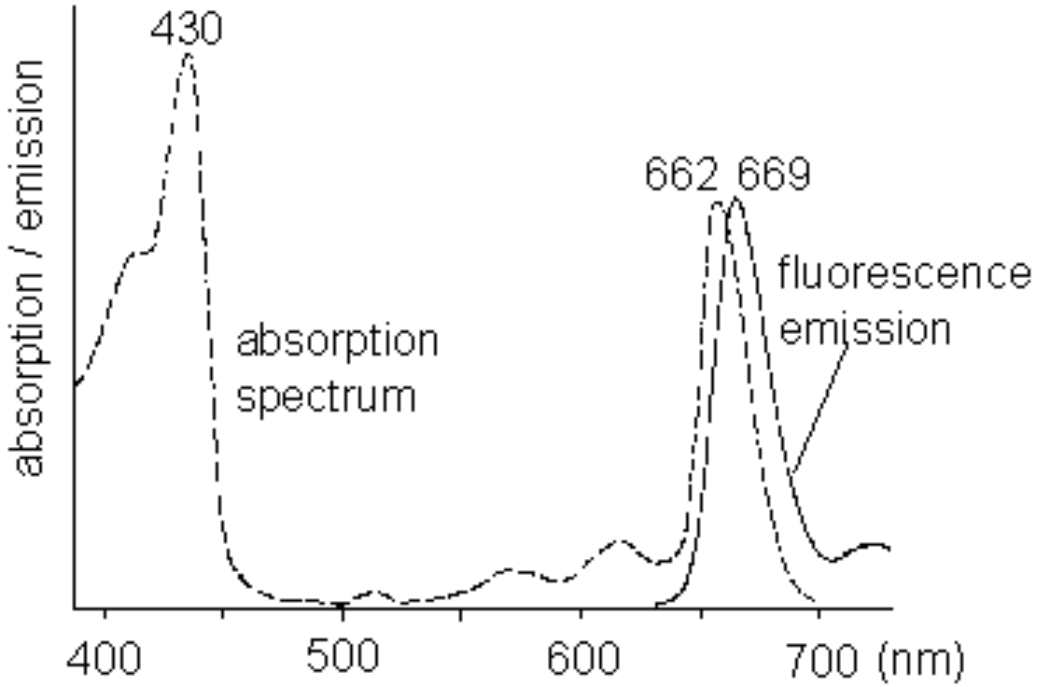
Chlorophyll *a*

– is a single pigment molecule with <u>two</u> primary absorption bands: blue Soret band (S2) and red Q band (S1); fluorescence emission and Stokes' shift is only from Q band (that is why chl. fluorescence is red, not blue).



~ 436 nm

~ 676 nm

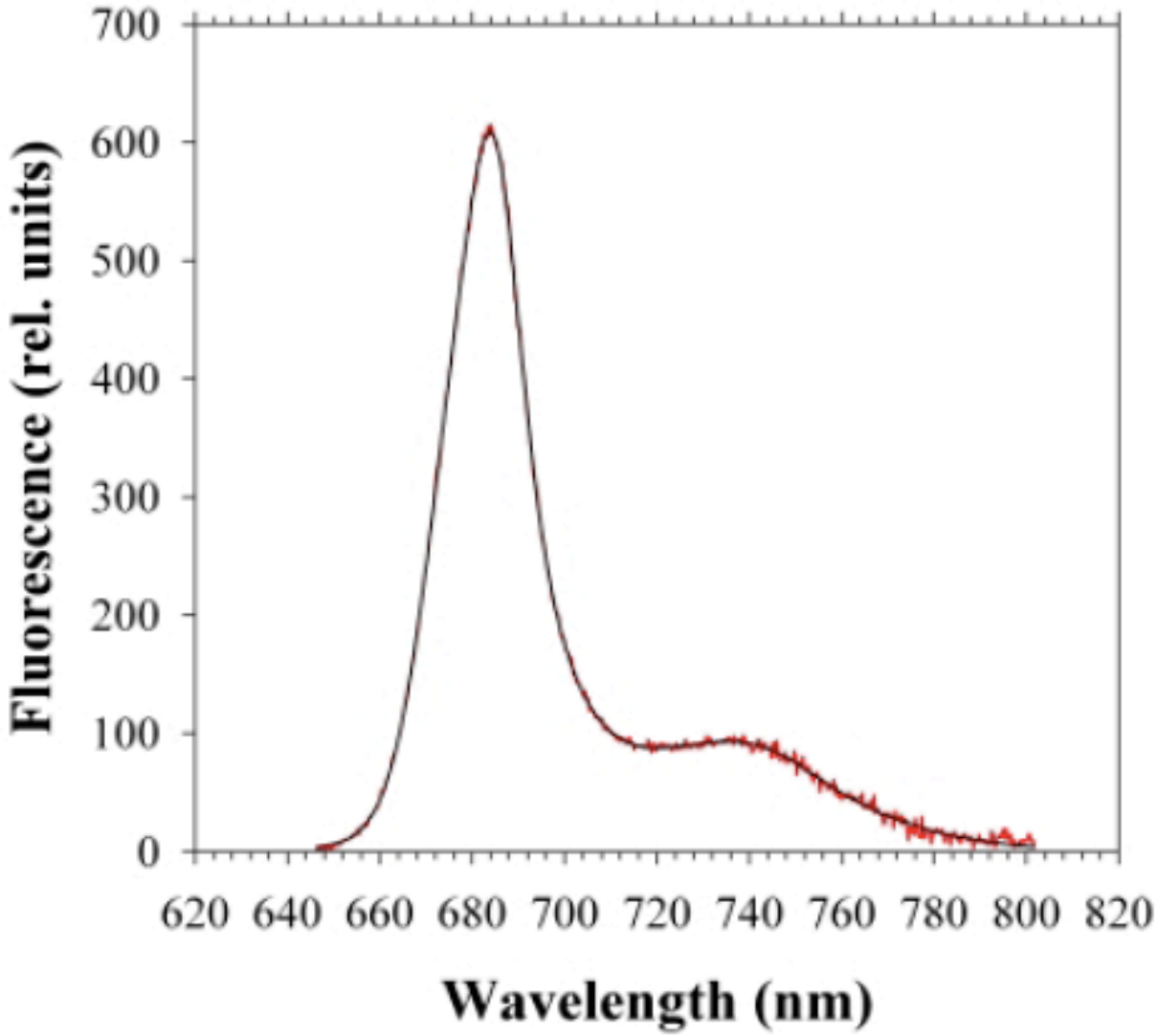


Absorption *in vivo (living cell)*

Absorption in vitro (pigment in a solvent)

In a living cell, **ONLY** chlorophyll *a* fluoresces.
In solvent extract, chlorophyll *b* also fluoresces.

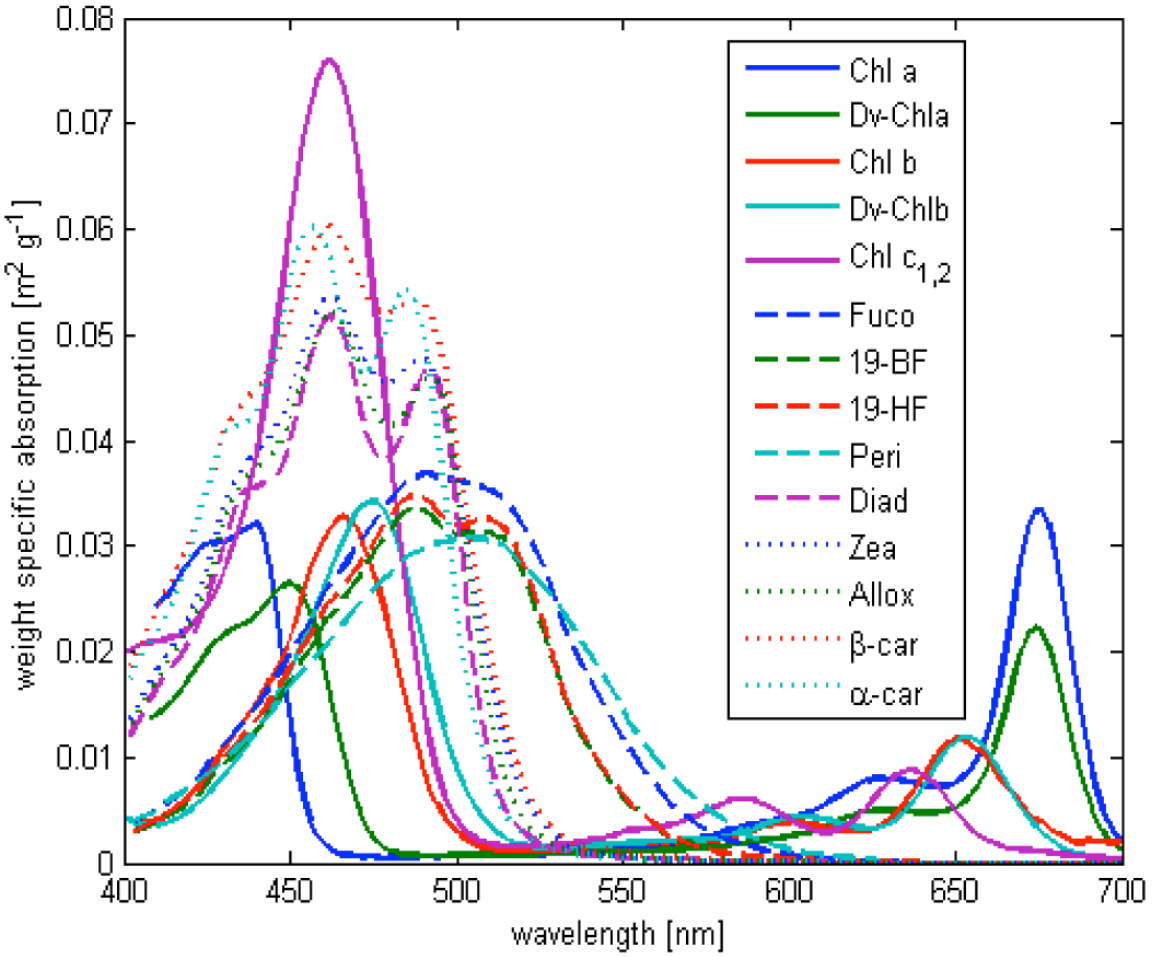
Blow up of emission spectrum



In living cell, chlorophyll *a* absorbs at 436 nm.

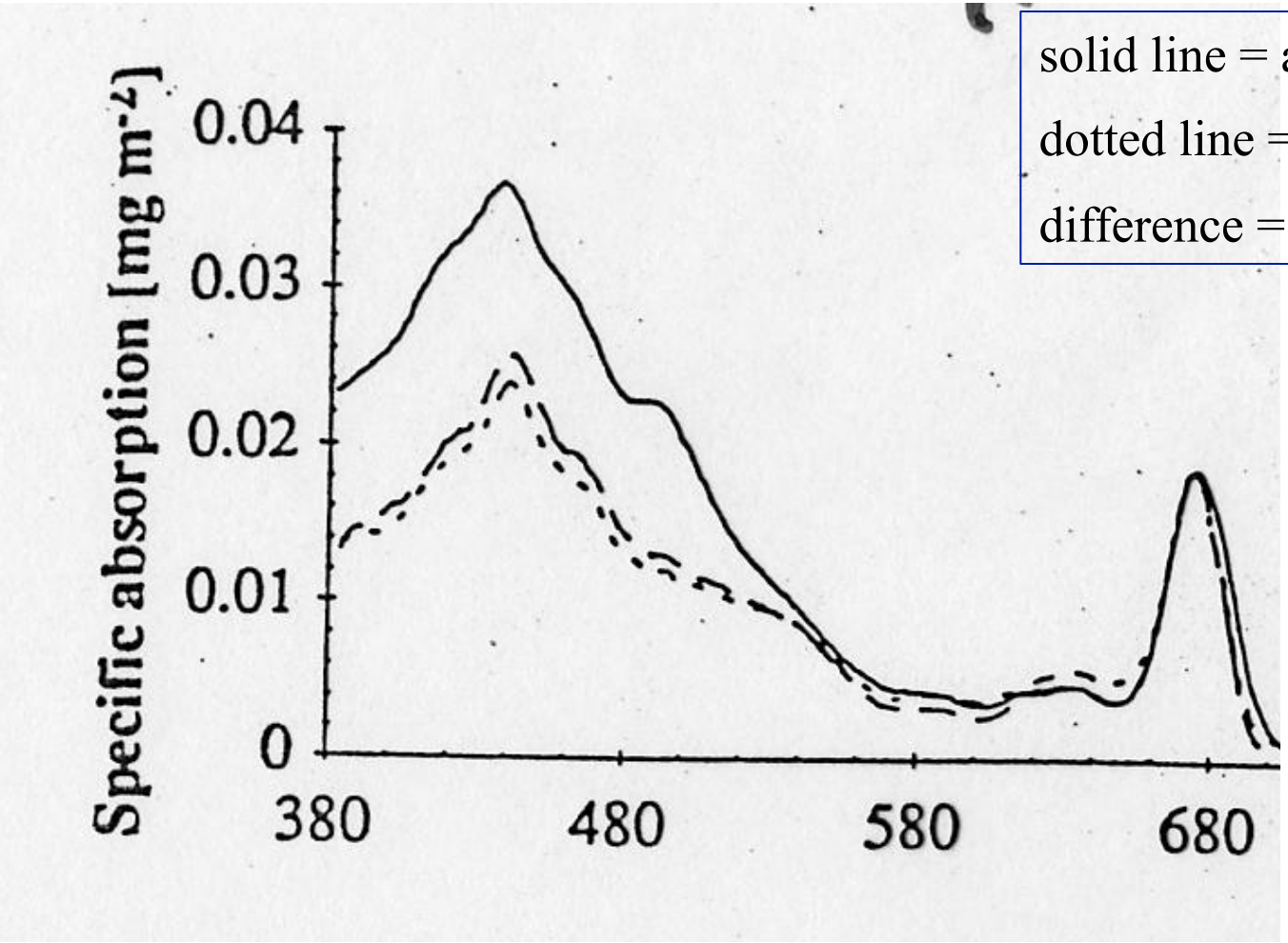
What wavelength is used for excitation in ocean fluorometers?

www



Hint energy transfer from accessory pigment to chlorophyll *a* Q band; therefore, fluorescence efficiency will be a function of accessory pigment concentration.

Absorption by photoprotective and photosynthetic pigments



Chlorophyll *a* *(also fluoresce in ocean – CDOM & phycoerythrin).*

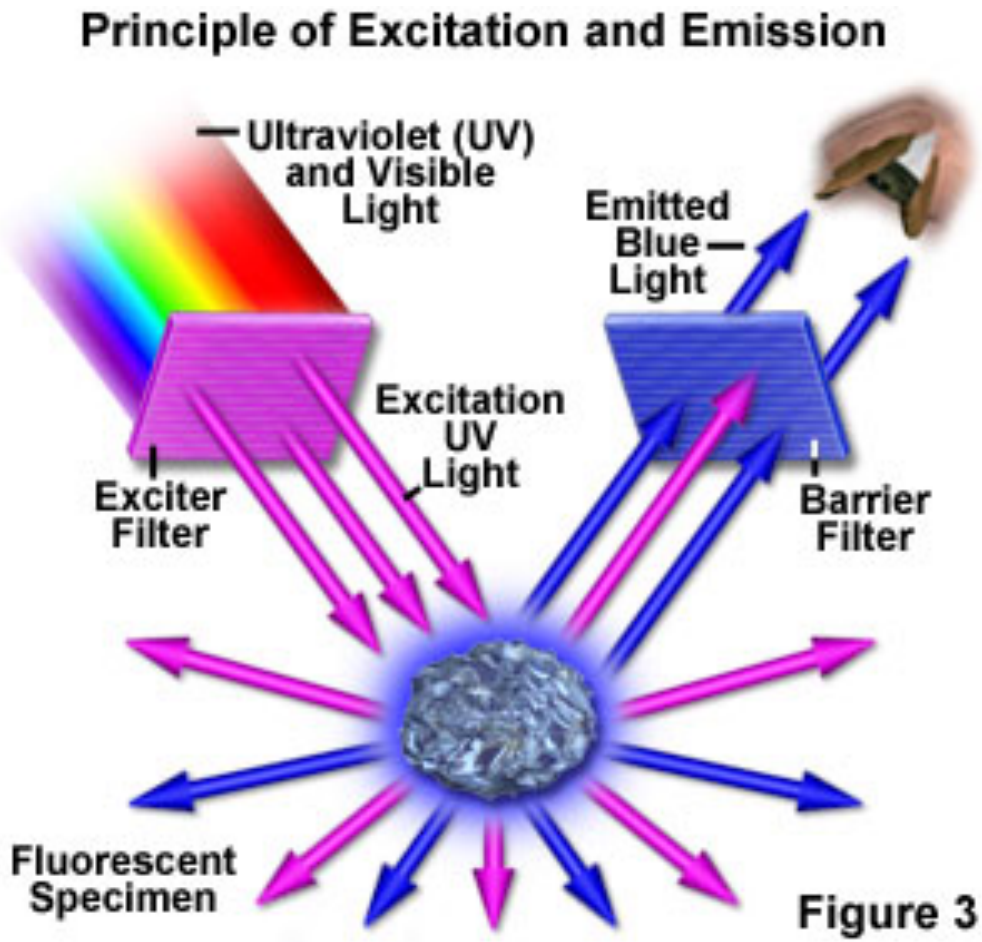
– is a single pigment molecule with two primary absorption bands: blue Soret band (S2) and red Q band (S1); fluorescence emission and Stokes' shift is only from Q band.

Technical notes:

1) Two absorption bands of chlorophyll provides a great technical advantage

– allows better **separation of excitation (blue) and emission (red) light.**

2) excite and detect fluorescence orthogonally to reduce stray light (slide 15 – filters are NOT 100% efficient; this works because fluorescence is isotropic.



http://micro.magnet.fsu.edu/primer/lightandcolor/fluorointroduction.html

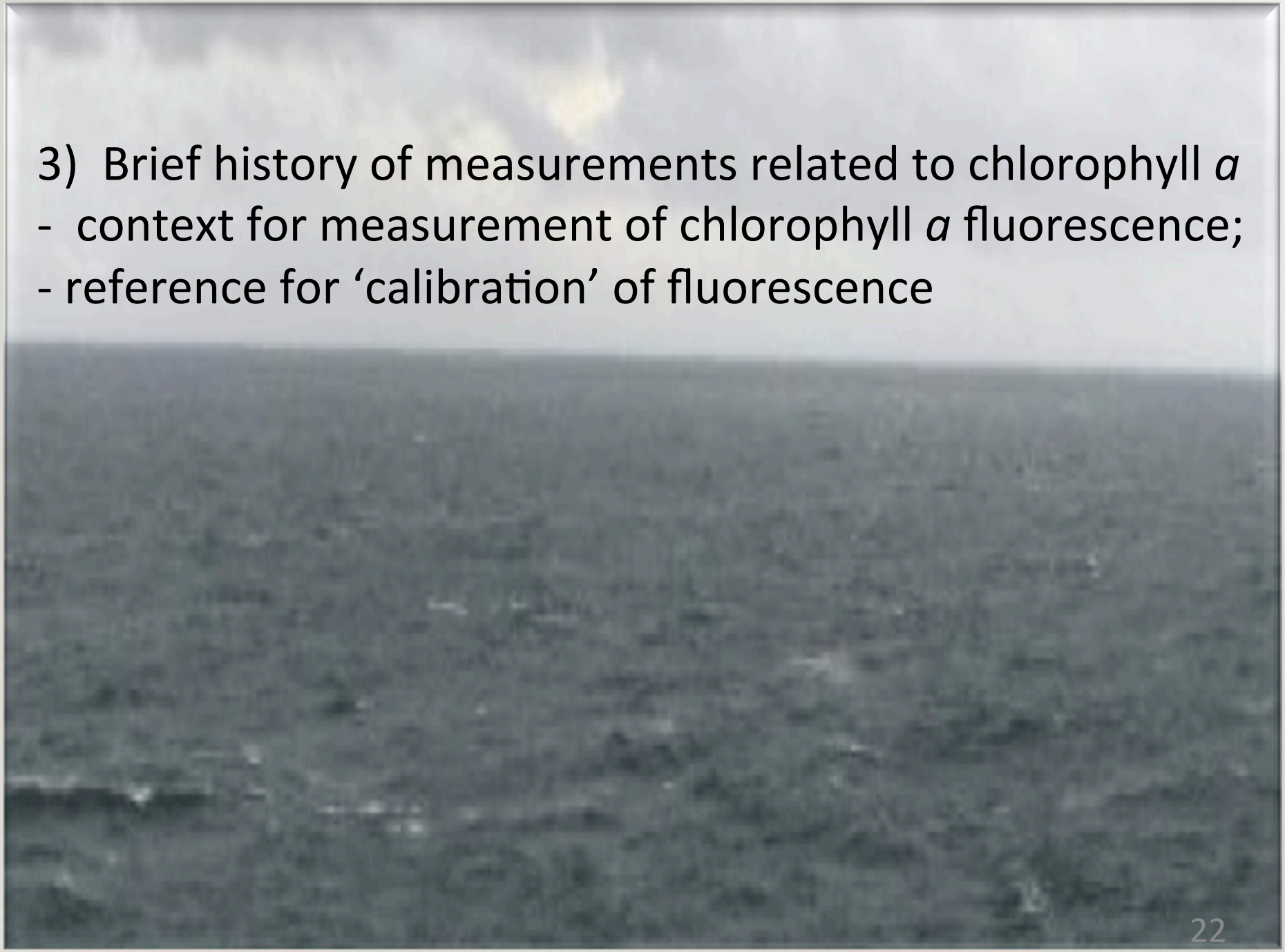
Two types of fluorescence measurements

1) **active – artificial light source for E(λ)**

- static: use for profiles of chlorophyll fluorescence; moorings; mobile platforms

— time resolved (true tr is ~ femo/picos for chemistry, like hole burning in CDOM; but could consider pump & probe, variable F)

2) **passive** – sun is light source



3) Brief history of measurements related to chlorophyll *a*
- context for measurement of chlorophyll *a* fluorescence;
- reference for 'calibration' of fluorescence

in vivo	color of the water, by eye (and by smell)
'in vivo'	HPPU and Munsell color chart, by eye, of filtered samples
in vitro	spectrophometry of solvent extracts (filtered)
in vitro	fluorescence of extracts; simple filter fluorometer & more λ
in vitro	HPLC of extracts
in vivo	chlorophyll fluorescence profiles by pump (fluorometer on deck), then *in situ* fluorometry with sensor on CTD
in vivo	epifluorescence microscopy (typically not quantitative)
in vivo	flow cytometry on bench; now a few FCM *in situ*
in vitro	spectrophometry of filter pads (a_676)
in vivo	spectrophometry of water samples with ac9/acs (a_676), profiles and underway
in vivo	pump and probe fluorescence for physiology (profiles and bench top)
in vivo	ocean color remote sensing reflectance
in vivo	ocean color remote sensing fluorescence line height

Color by eye – here by camera:
Rhizosolenia patch photographed from the deck of the oceanographic research vessel, R.V. Thompson, on 25 August, 1995 near 2 N, 132 W during Equatorial Pacific JGOFS.

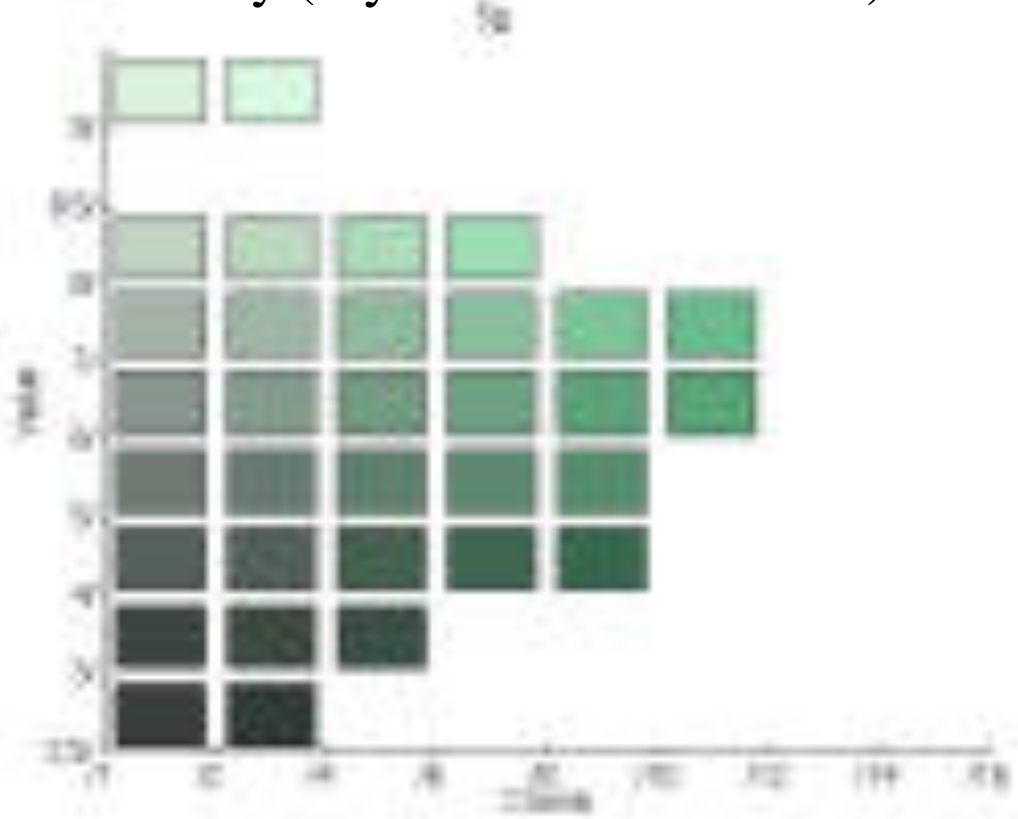
http://po.gso.uri.edu/color/yoder2.html

Harvey Plant Pigment Unit (HPPU) - up to ~ 1950

– standardized color on filters (Munsell chart,

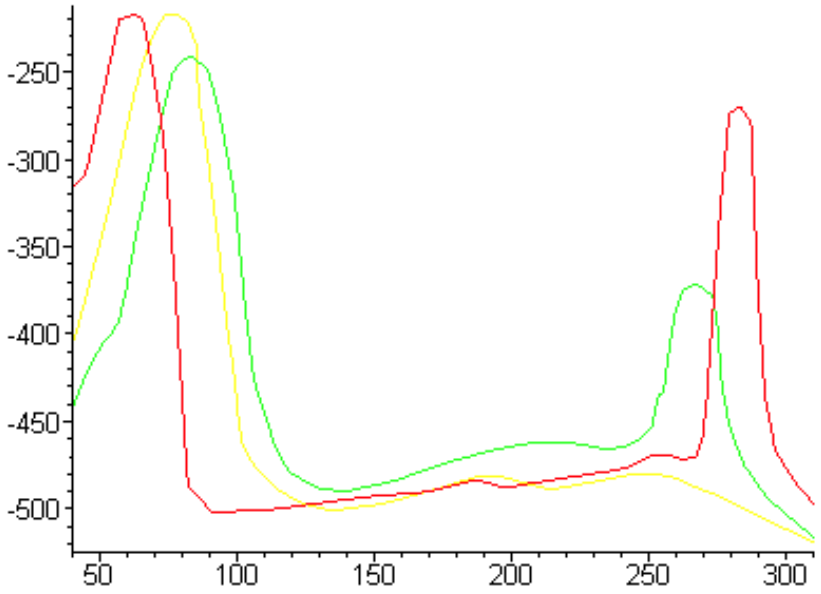
which is still used for soils and tobacco).

Harvey (Plymouth Lab ~ 1930's) calibrated HPPU to phosphorus.



Not bad: http://en.wikipedia.org/wiki/Munsell_color_system

Spectrophotometry of extracts of multiple pigments in solvent;
therefore had to try to mathematically separate pigments.
trichromatic eq. to separate pigments.
Introduced in ~ 1950s when the old Beckman DU spectrophotometer became widespread, and was used into the 1960s.
(Values are in NOAA's NODC data base. Use caution.)



$$OD_{664} = \varepsilon_{664,a}\, a\, L + \varepsilon_{664,b}\, b\, L + \varepsilon_{664,c}\, c\, L$$

$$OD_{647} = \varepsilon_{647,a}\, a\, L + \varepsilon_{647,b}\, b\, L + \varepsilon_{647,c}\, c\, L$$

$$OD_{630} = \varepsilon_{630,a}\, a\, L + \varepsilon_{630,b}\, b\, L + \varepsilon_{630,c}\, c\, L$$

Fluorescence of acetone extracts of filtered water – protocol from dock. ~1960's. Benchtop filter fluorometer. Reasonably fast. Cheap. Still widely used, and used for calibration of *in situ* fluorometers.

chl *a* = K (Fm/ Fm-1) x (Fo - Fa) x (v/V)

pheo *a* = K (Fm/ Fm-1) x [(Fm x Fa - Fo)] x (v/V)

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

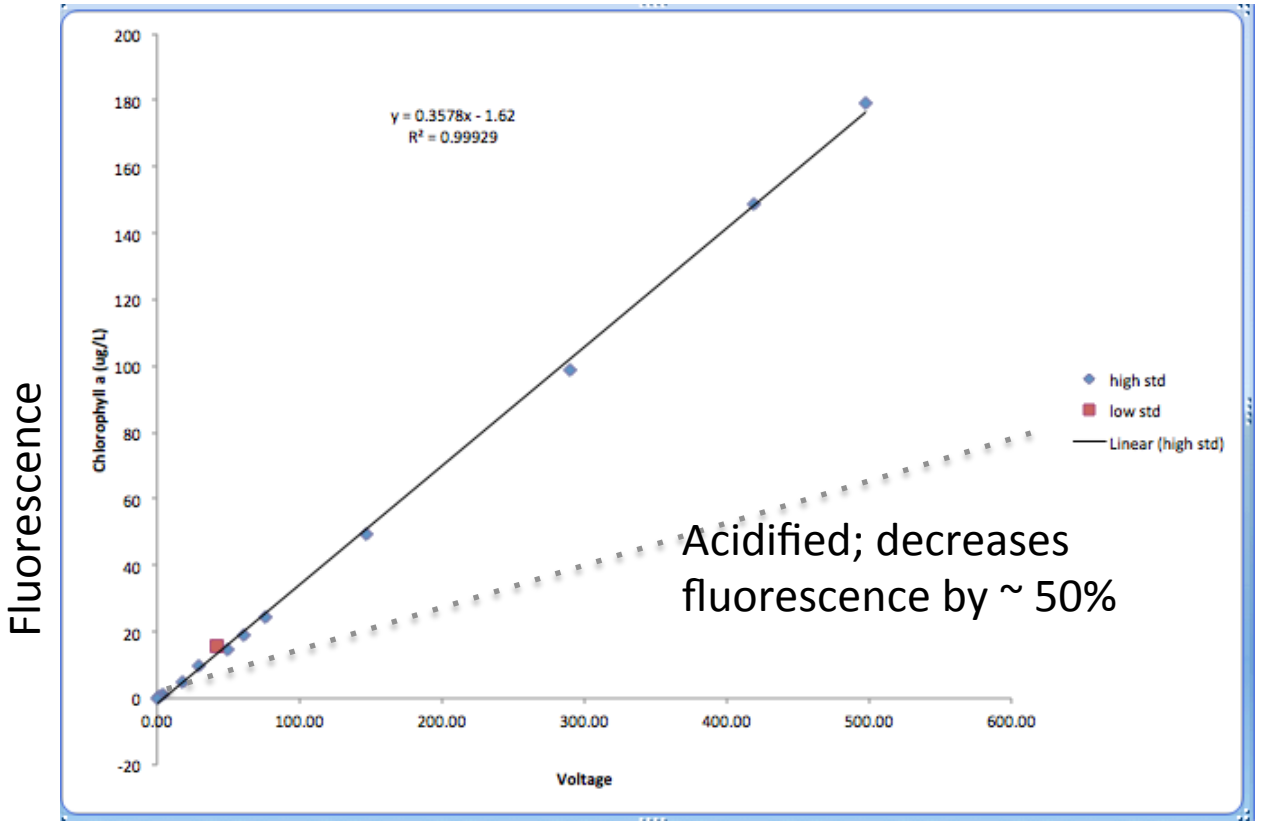
v = 90% acetone extract volume (L)

V = volume filtered (L)

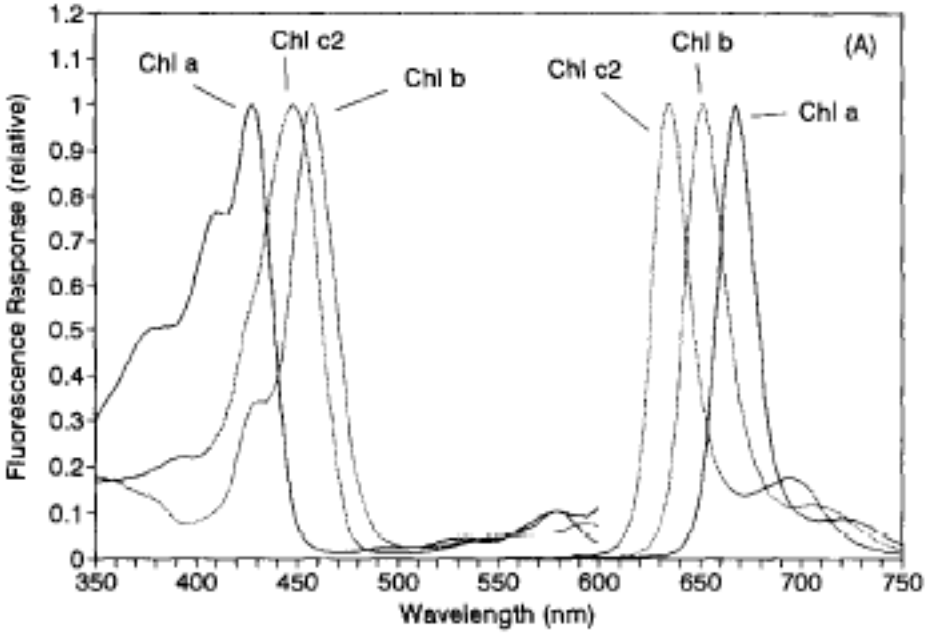
Standardization of chlorophyll fluorescence with commercial extracts of pure chlorophyll *a*.

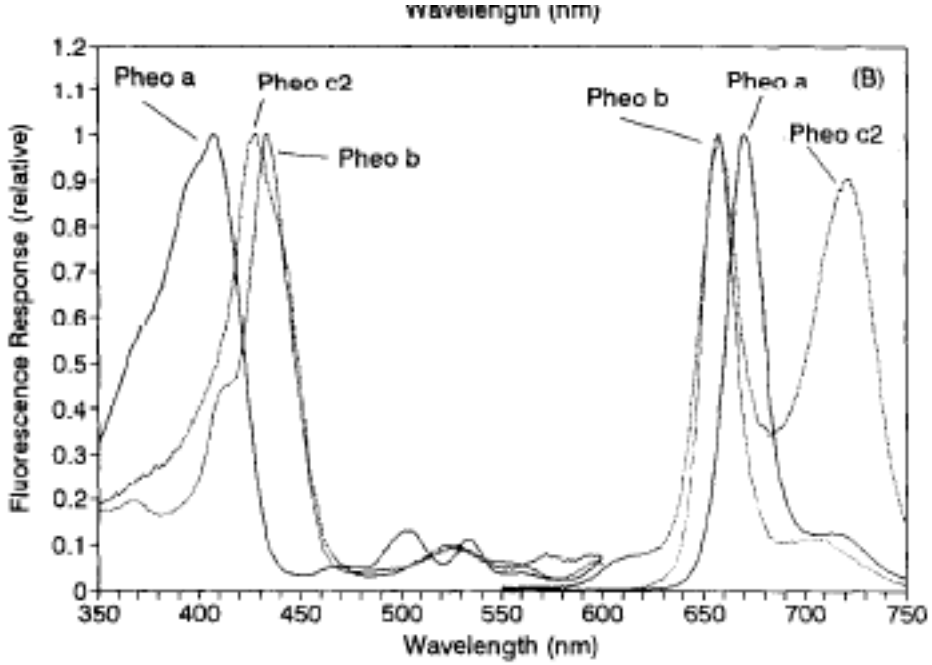
Calibrate with primary standard 1-2 times/year.

Calibrate with solid secondary standard with each use.



Problem: Chlorophyll *b* is fluorescent in vitro.
Leads to underestimation of chlorophyll *a* and overestimation of pheopigment (degraded pigment, often by product of grazing).

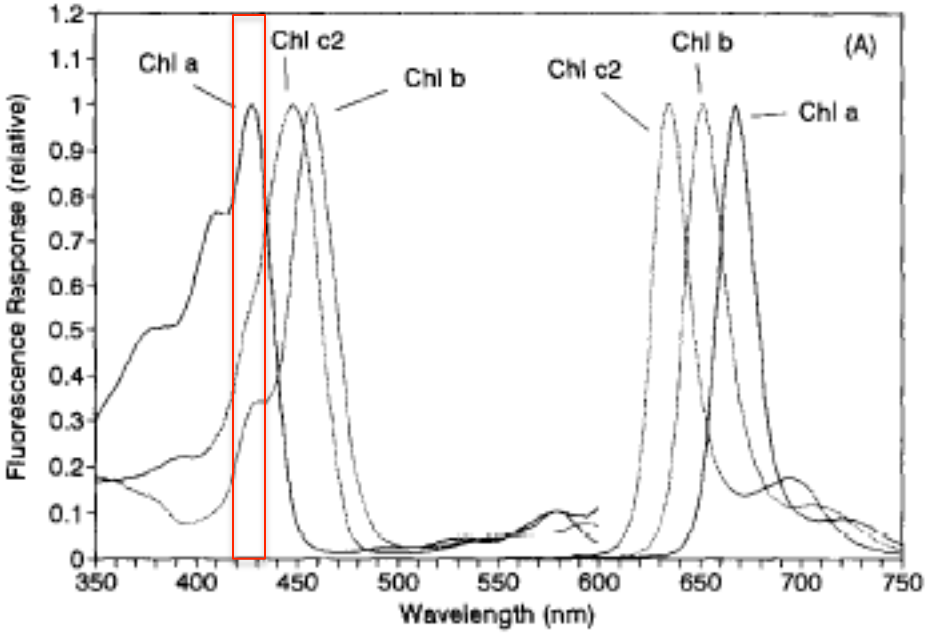


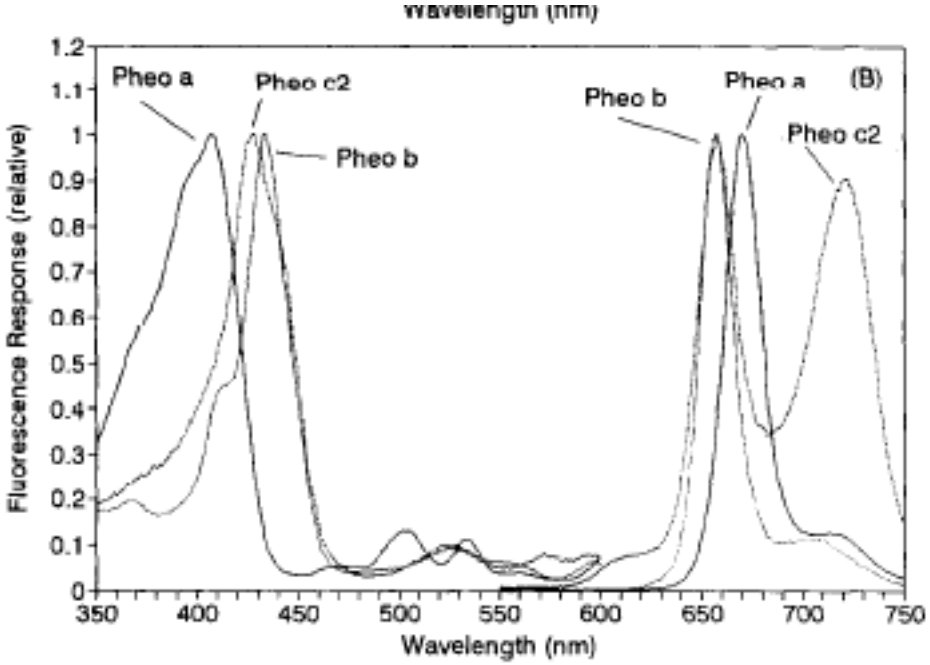


Before acid (Fo)
Combination of chl a, chl b, pheo a and pheo b.

After acid (Fa)
Only pheo a and b.

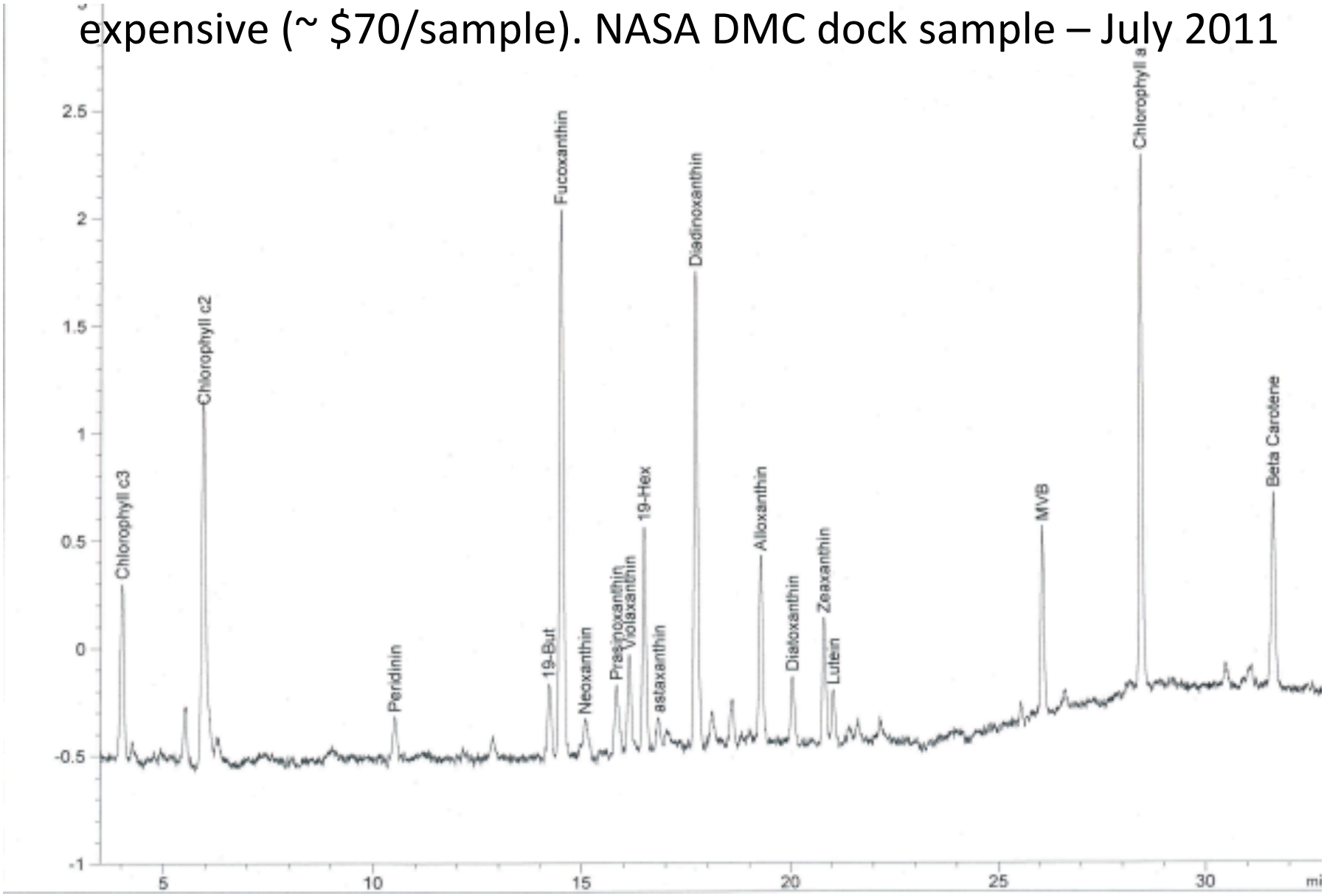
Problem: Chlorophyll *b* is fluorescent in vitro.
Welschmeyer method – use a narrow filter that excites only chlorophyll a (and not chlorophyll *b)*.
No acidification. Don't get pheopigment. Filter set sold by Turner Designs.



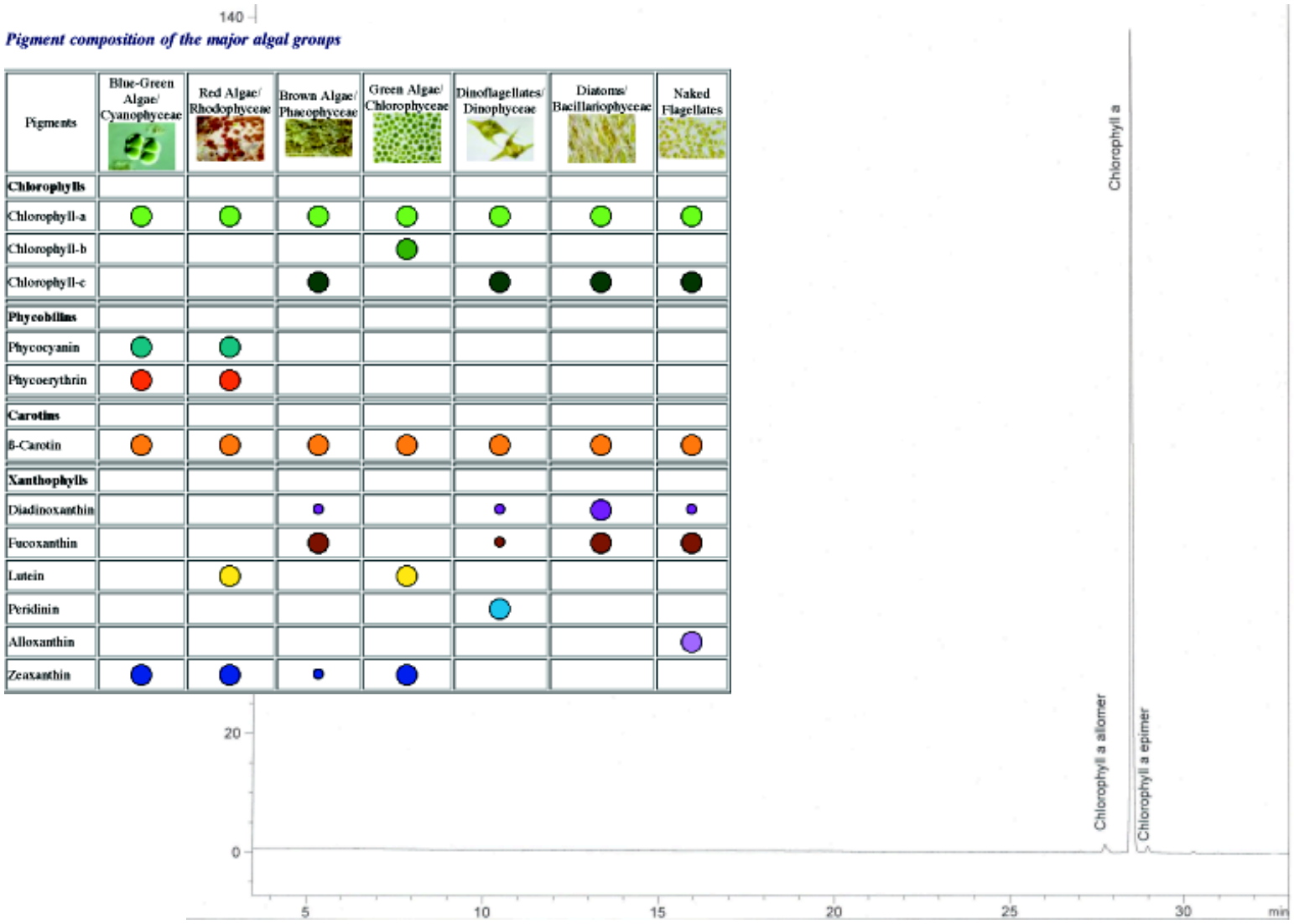


No acidification – narrow filters that measure chlorophyll a (mostly)

HPLC – separation of pigments, removes ambiguity. Slow & expensive (~ $70/sample). NASA DMC dock sample – July 2011



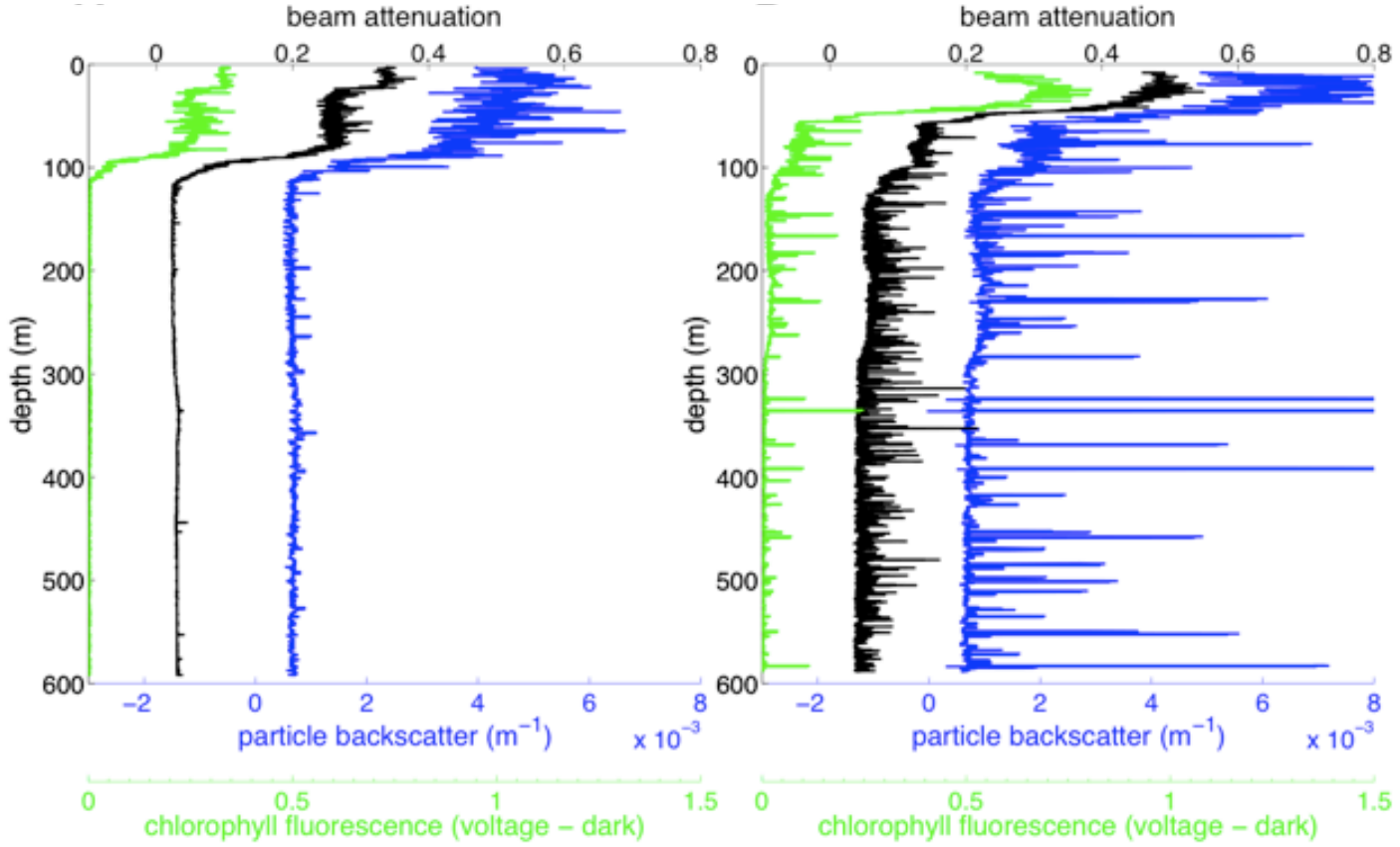
HPLC standard –chlorophyll *a*. Time for elution under defined conditions.



Chlorophyll fluorescence profiles by pump ~ late 1960's (with fluorometer on deck), then *in situ* fluorometry with sensor on CTD, mooring, glider, float, etc.

Challenge is to calibrate it, especially for autonomous ops.

5 May [Chl F, b_{bp}, c_p] 10 May



Three week experiment, > 1000 chlorophyll samples

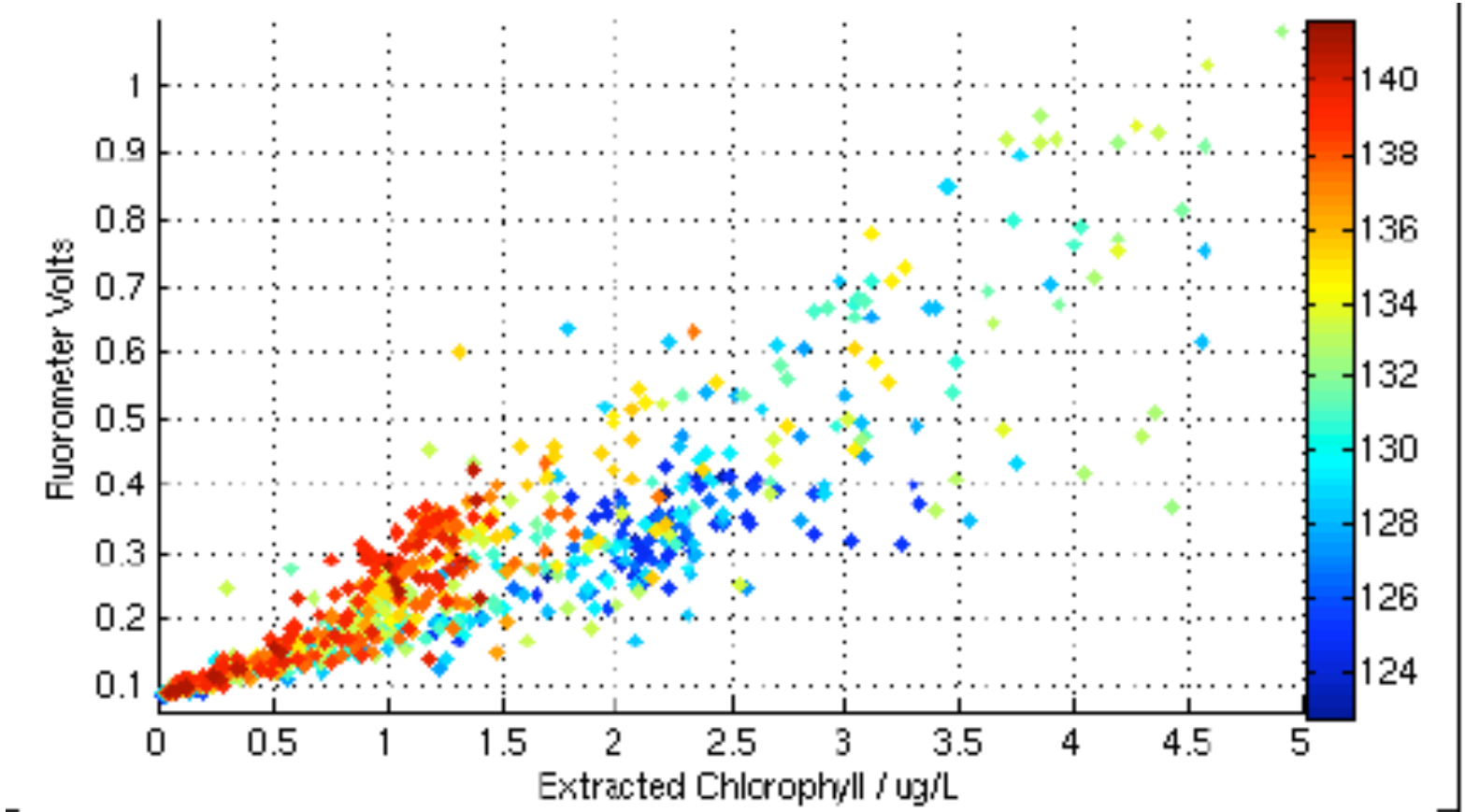
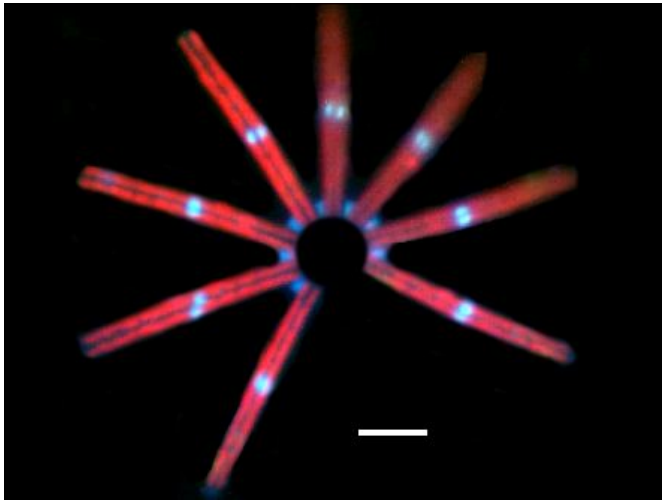
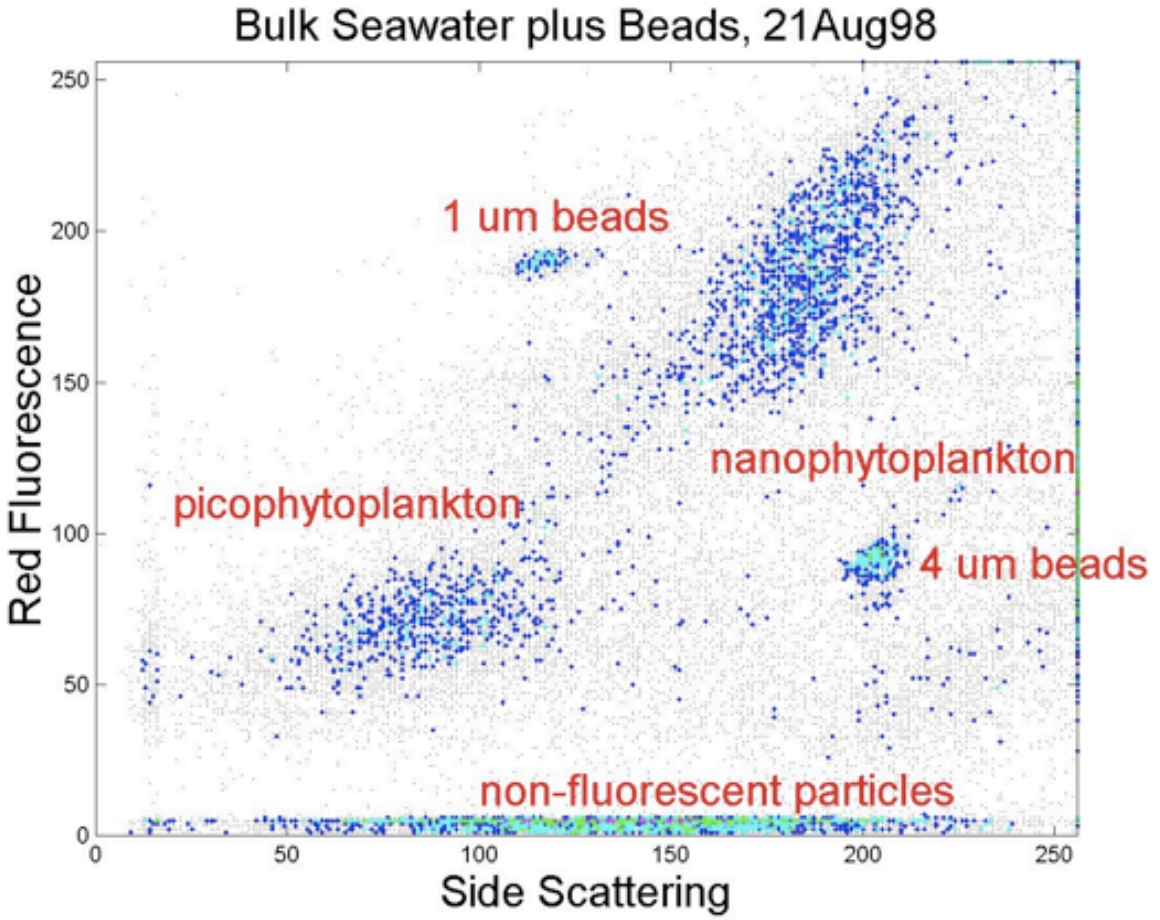


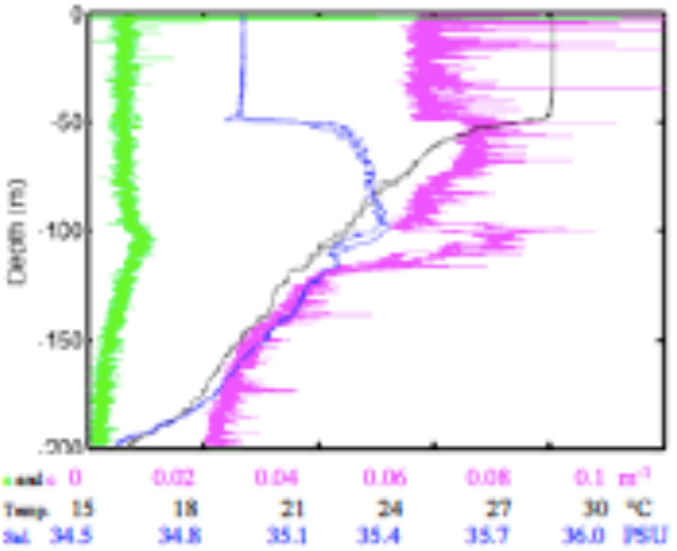
Fig. 2. Scatter plot of raw fluorometer output against extracted chlorophyll for all *Knorr* bottle samples. Color is yearday of 2008.

Epifluorescence microscopy, and flow cytometry

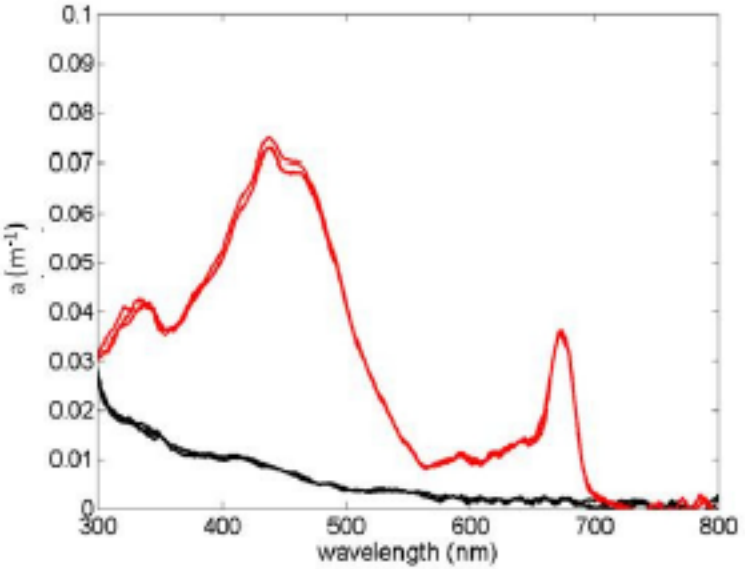




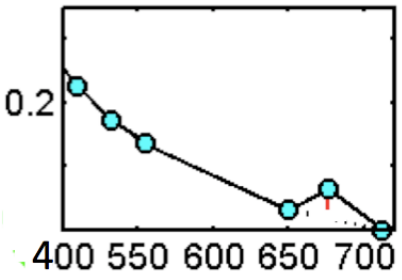
Filter pad absorption and ac-x absorption at 676



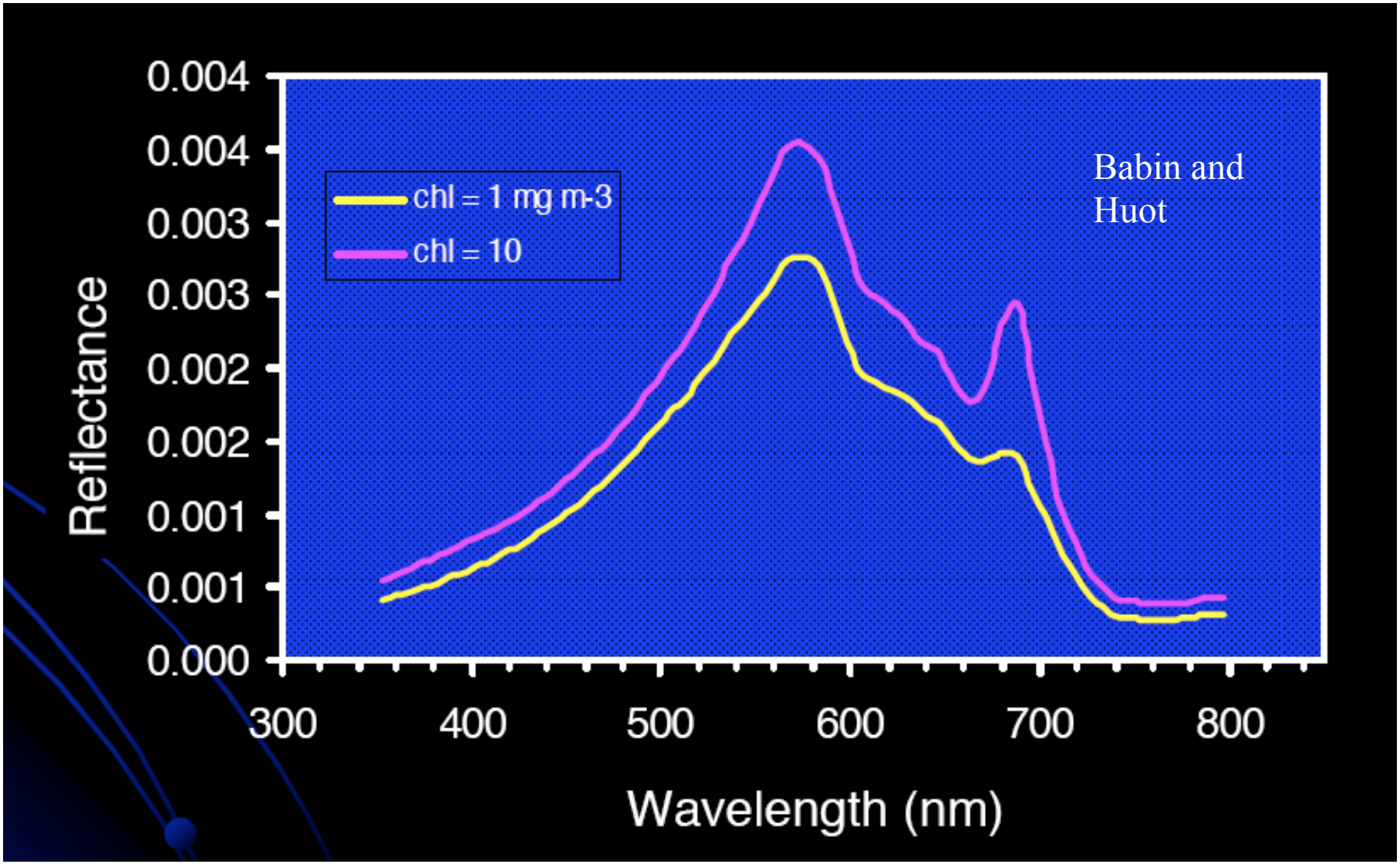
Profile from HOTS, August 2004)



a_phyt (676) is a good estimator of chlorophyll concentration in cell (Roesler)



Solar stimulated fluorescence



pump and probe fluorescence for physiology (bench top and profiles)

Sheri's data

4) General principles of fluorometric measurement and types of oceanographic fluorometers

These vary with manufacturers (bench top and *in situ*)

- excitation/emission wavelength
- light source, filters
- geometry
- temporal resolution and average
- calibration for fluorometers
  - With cells? With fluorescent standard?
- drift – electronics, filter degradation
- temperature effects – electronics, fluorescence quenching

5) Interpretation of data, and challenges therein, and potential workarounds

_ fluorescence quenching due to sun and innate diel rhythms

_ pigment packaging – cell size and photoadaptation

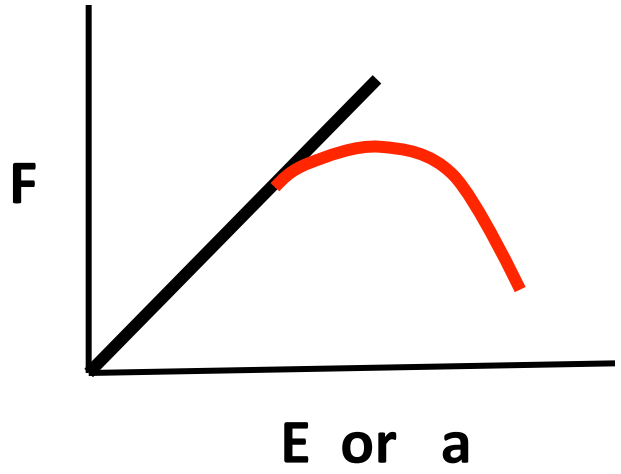
_ variable ratio of photosynthetic pigments

_ nutrient limitation

Really can NOT calibrate a chlorophyll fluorometer, except with many discrete samples.

But you CAN still get useful information

$$F(\lambda) = a(\lambda) * E(\lambda) * \Phi_f$$



F = fluorescence emission
measure as photons or energy – difficult to get absolute measurement, so typically measured as relative fluorescence, in digital counts or analogue detector in volts)

a = absorption coefficient; 'related' to **C**, concentration (and a*)

E = energy of excitation light

λ = wavelength

Φ_f = quantum yield of fluorescence = $\frac{\text{moles photons emitted}}{\text{moles absorbed}}$

$$F = a(\lambda) * E(\lambda) * \Phi_f$$

Three things to note in the fluorescence equation:

1. a = absorption coefficient (not chlorophyll concentration; in acetone extract 'a' ~~ chl concentration, but not in live cells).

2. there is a λ for both absorption and E, excitation energy

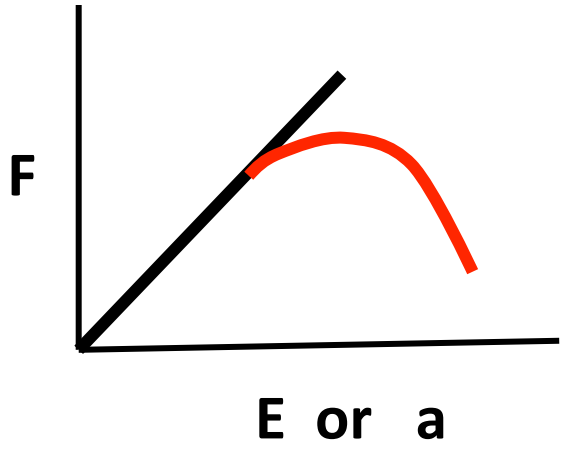
3. quantum yield of fluorescence, Φ_f , varies:
   - in solution (*in vitro)*, F is a function of solvent and temperature
   - in living cell (*in vivo),* F is a function of physiology

$$F = a(\lambda) * E(\lambda) * \Phi_f$$

1) a = absorption coefficient

in vitro (e.g., in acetone extract: a ~ chl conc., hence F ~ conc; Turner Designs 10-AU calibration protocol.

in vivo (living cells): F ~ a …… with other caveats, such as constant Φ_f



a vs. F (flow cytometry)

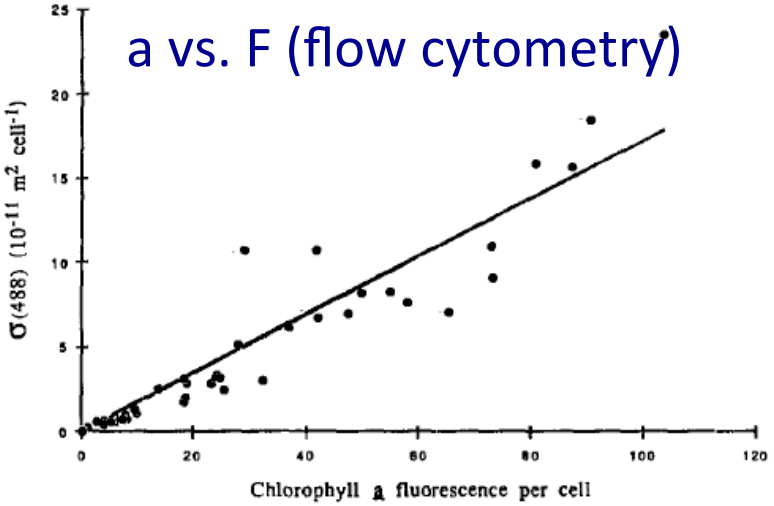


Fig. 5. Regression of geometric mean of Chl *a* fluorescence per cell (converted to linear units) measured in the flow cytometer vs. $\sigma(488)$ for all species and growth irradiances listed in Table 1; $\sigma(488) = 0.173 \times 10^{-11} \times$ Chl *a* fluorescence per cell; $r^2 = 0.93$.

Technical note: fluorescence is linear function of concentration at low concentrations; re-absorption occurs at higher concentrations.

Perry & Porter 1989

a* changes due to pigment packaging – i.e., size and photo-adaption.
Therefore, fluorescence-to-chlorophyll must too.

F/Chl was a function of cell size in San Francisco Bay, due to greater pigment packaging in larger cells.

Sizes were separated w/ screens
- netplankton (>22 µm)
- nanoplankton (5–22 µm)
- ultraplankton (<5 µm)

F / Chl was linearly related within size class, but was significantly different between sizes: ultraplankton = 2 * nanoplank. = 2 * netplank.

Data from San Francisco Bay;
Alpine and Cloern (1985). J Plankton Research 7: 318.

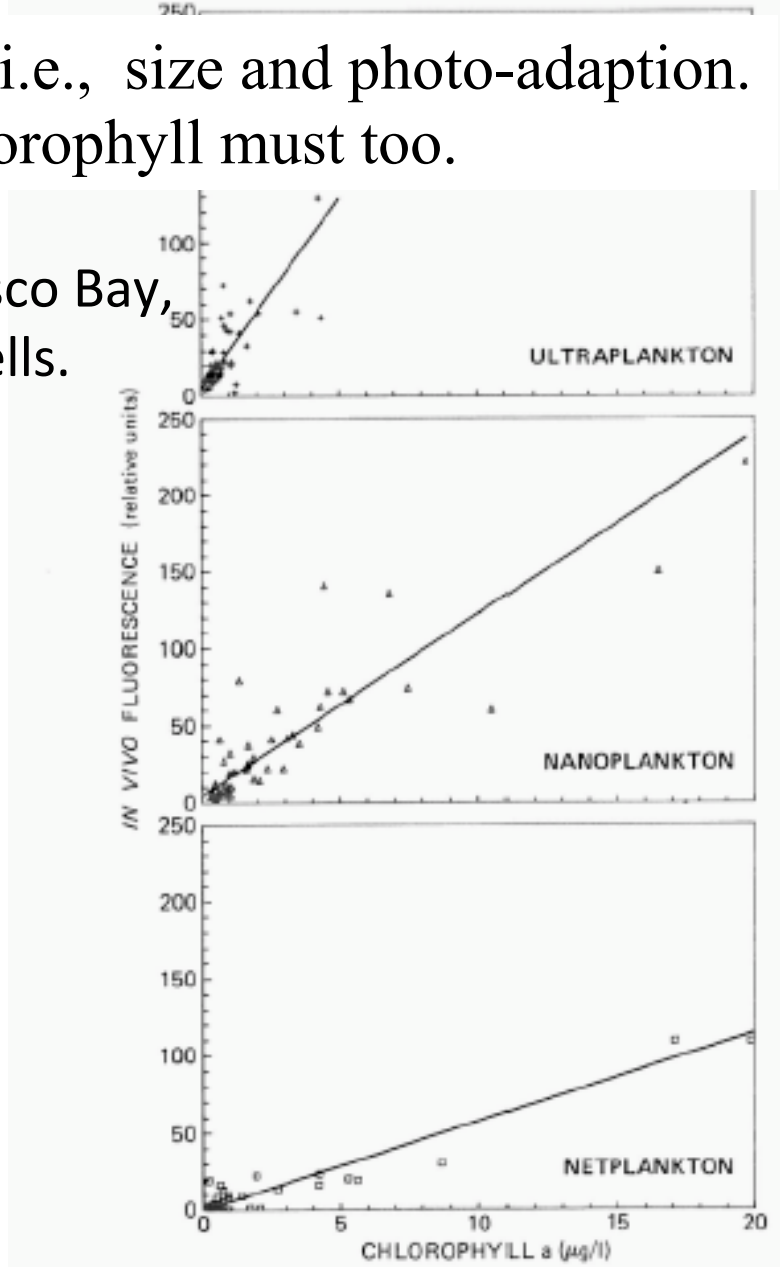
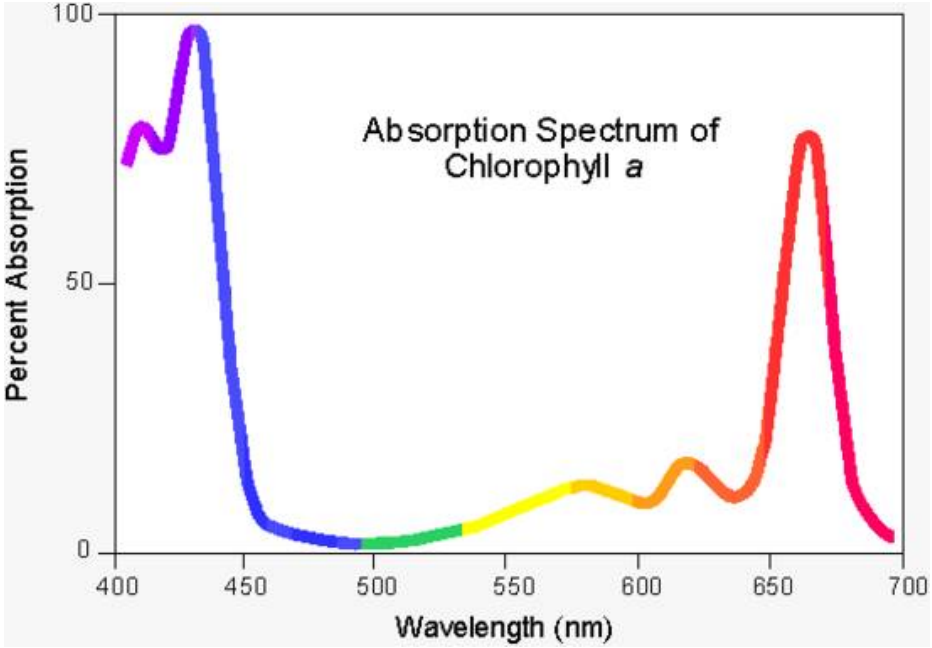


Fig. 2. *In vivo* fluorescence plotted against chlorophyll *a* for each size class. Data are pooled sites in San Francisco Bay over a year long period. Regression lines are shown for each size

$$F = a(\lambda) * E(\lambda) * \Phi_f$$

2) λ– dependence for both absorption and E, excitation energy:

there must be a match between wavelengths of phytoplankton absorption spectrum and lamp excitation spectrum.



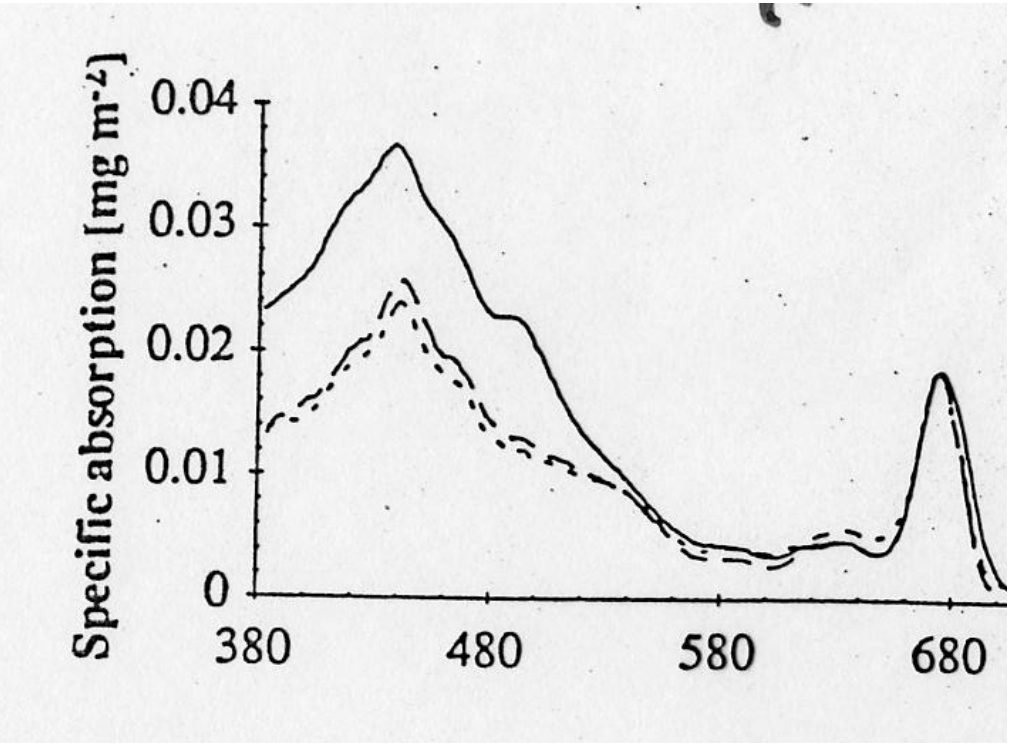
What is the excitation spectrum of a typical *in situ* fluorometer?

(Models do differ in λ; also, calibration issues associated with changes in E and/or λ over time)

$$F = a(\lambda) * E(\lambda) * \Phi_f$$

λ– dependence for both absorption and E, excitation energy:

phytoplankton absorption at 470 nm can be separated into absorption by photosynthetic pigments(a_ps) and photoprotective pigments (a_pp). Only photosynthetic pigments are capable of transferring energy to chlorophyll Q-band, resulting in fluorescence. Here, fluorescence is proportional to a_ps.



solid line = a_phyt

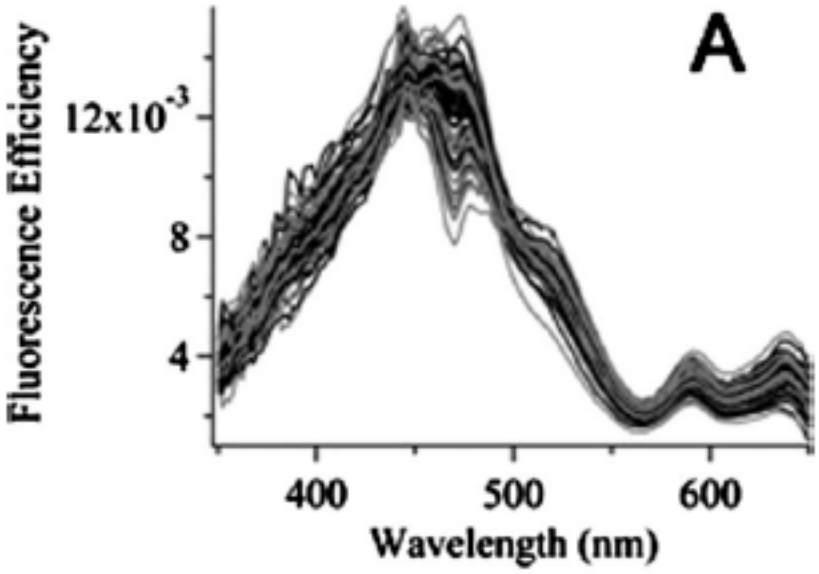
dotted line = a_ps

difference = a_pp

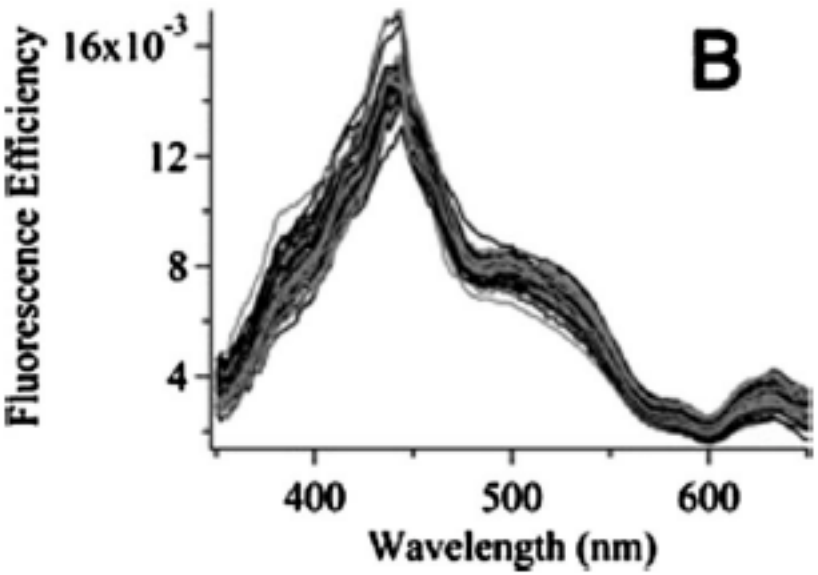
$$F = a(\lambda) * E(\lambda) * \Phi_f$$

λ− dependence for both absorption and E, excitation energy:

Is the ratio between chlorophyll *a* and accessory pigments constant?



Emiliania huxleyi cells



Thalassiosira pseudonana

Richardson et al. 2010. Rev. Sci. Instrum. 81, 013103

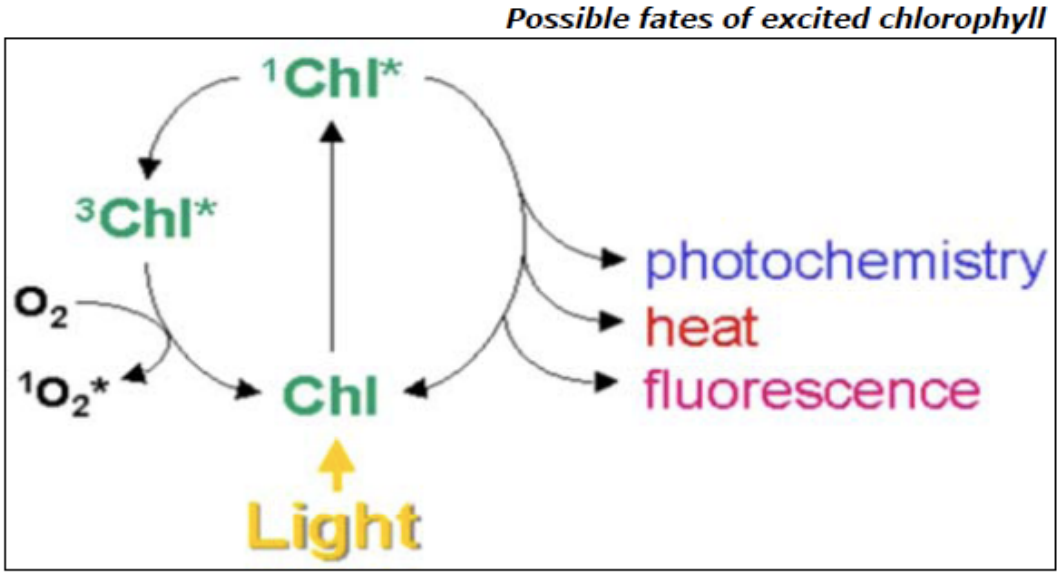
Single-cell excitation spectra (O(50-100 individual cells))

$$F = a(\lambda) * E(\lambda) * \Phi_f$$

3) quantum yield of fluorescence varies spatially & temporally (Φ_f = moles photon fluoresced/ moles photon absorbed):

– in solution (*in vitro)*, F is a function of environment (solvent, temperature, pH, ionic strength); $\Phi_f \sim 0.33$ for Chl *a* acetone extract

– in living cell (*in vivo),* F is a function of photosynthetic physiology, and is influenced by light (photo-quecching and photo-damage) and nutrient limitation; typically Φ_f is <0.03.



http://biologie.univ-mrs.fr/upload/p222/1_fluorescence.pdf

Fluorescence quenching a challenge

(sometimes have to look at night time data only, or try correction)

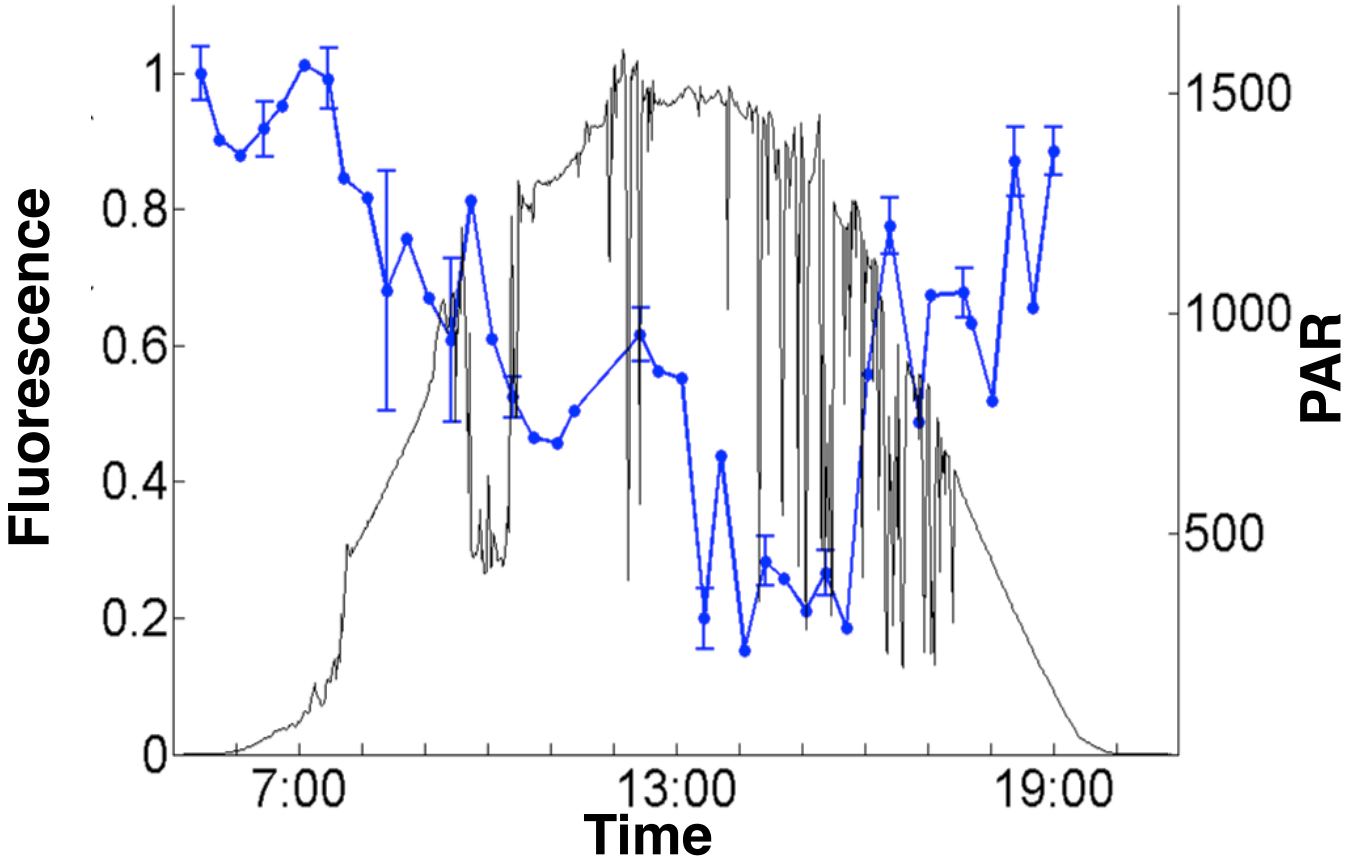
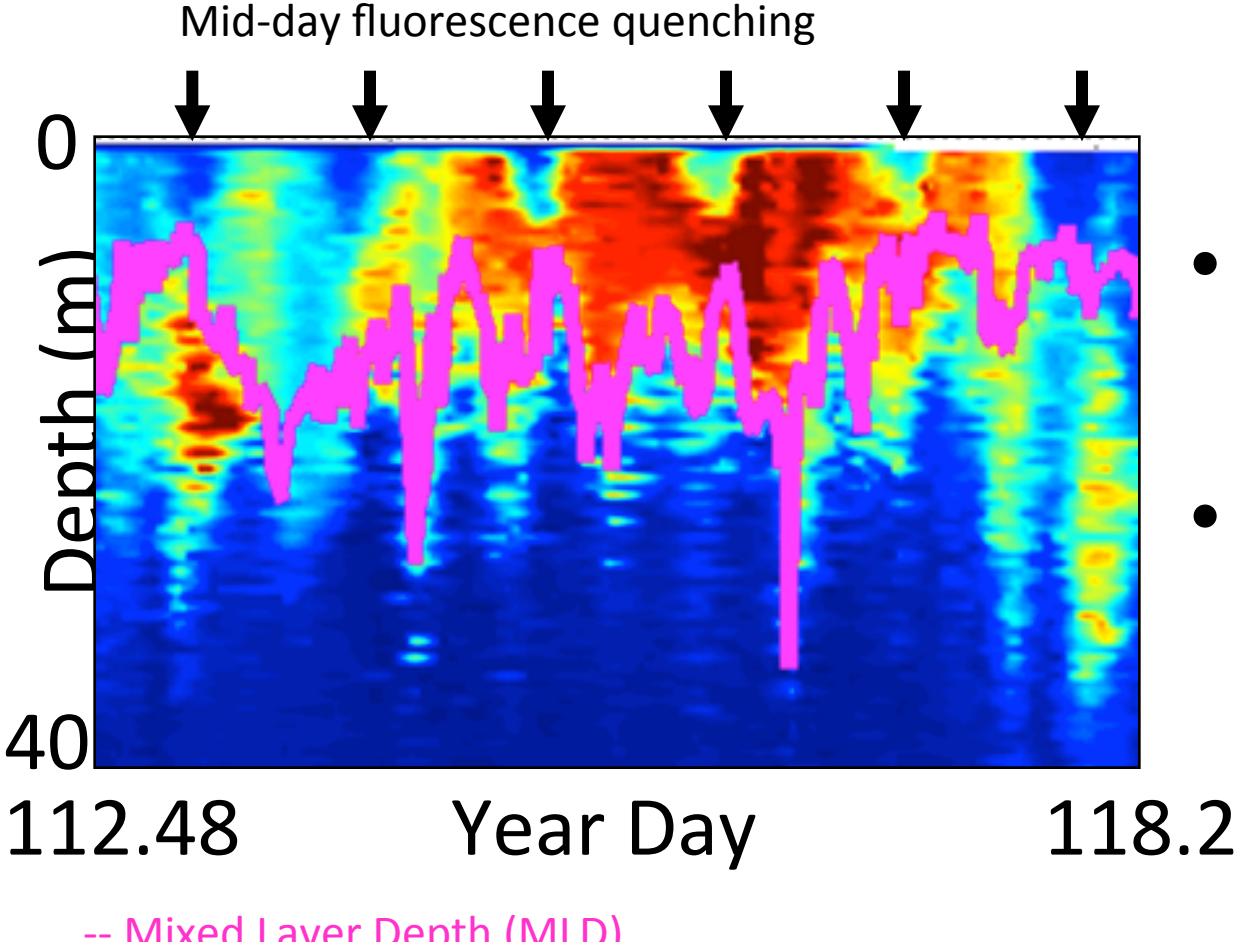


Figure 2: Damariscotta River *in situ* chlorophyll a fluorescence and PAR (µmol photons/s/m^2) vs. time.

Drzewianowski 2008 – MS thesis

Another example of mid-day fluorescence quenching



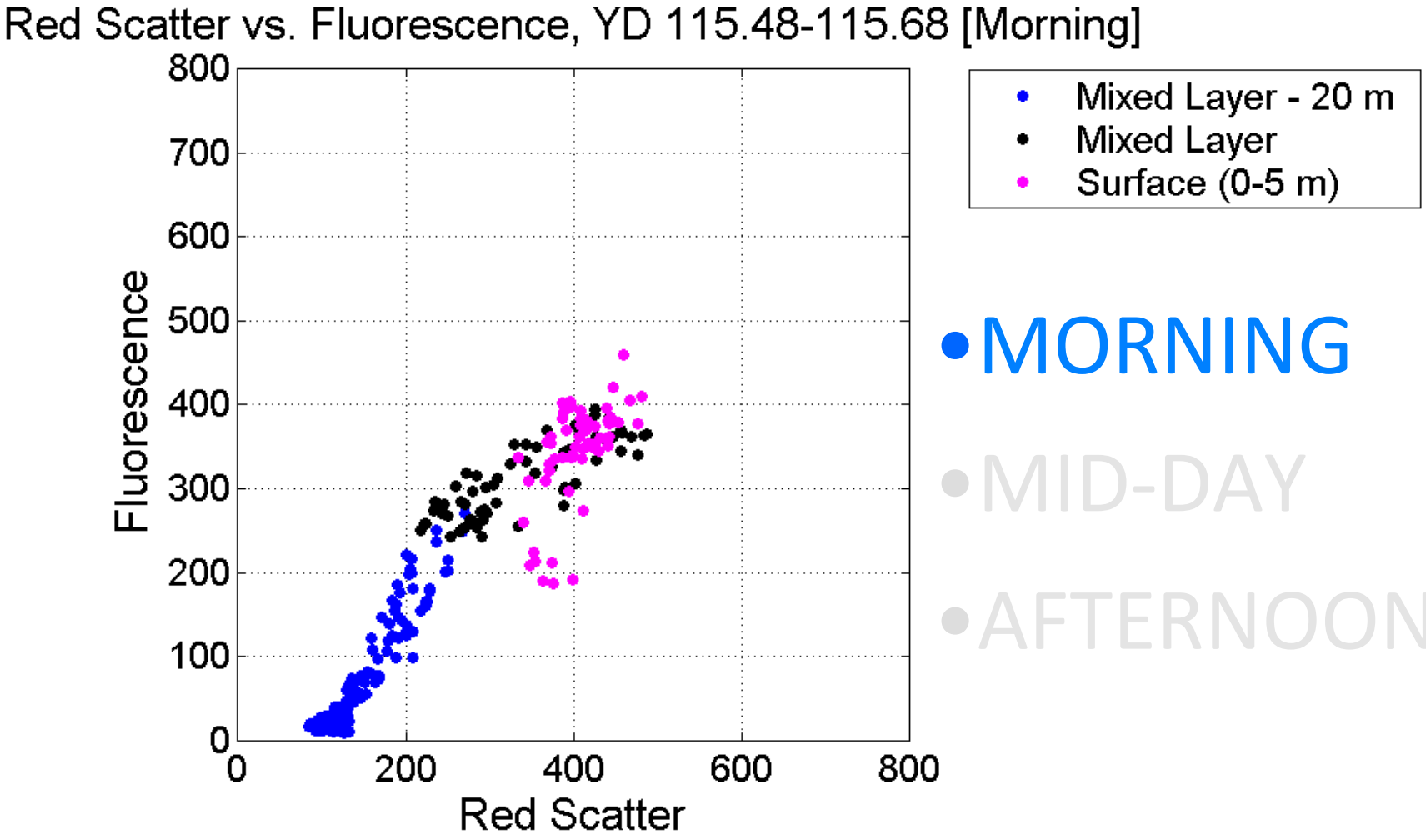
- Quenching observed to 11m
- Fluoresence quenched up to 80% at surface

-- Mixed Layer Depth (MLD)

So maybe for biomass, should we concentrate on night-time measurements *in vivo* fluorescence measurements?

Sackmann 2007 PhD.

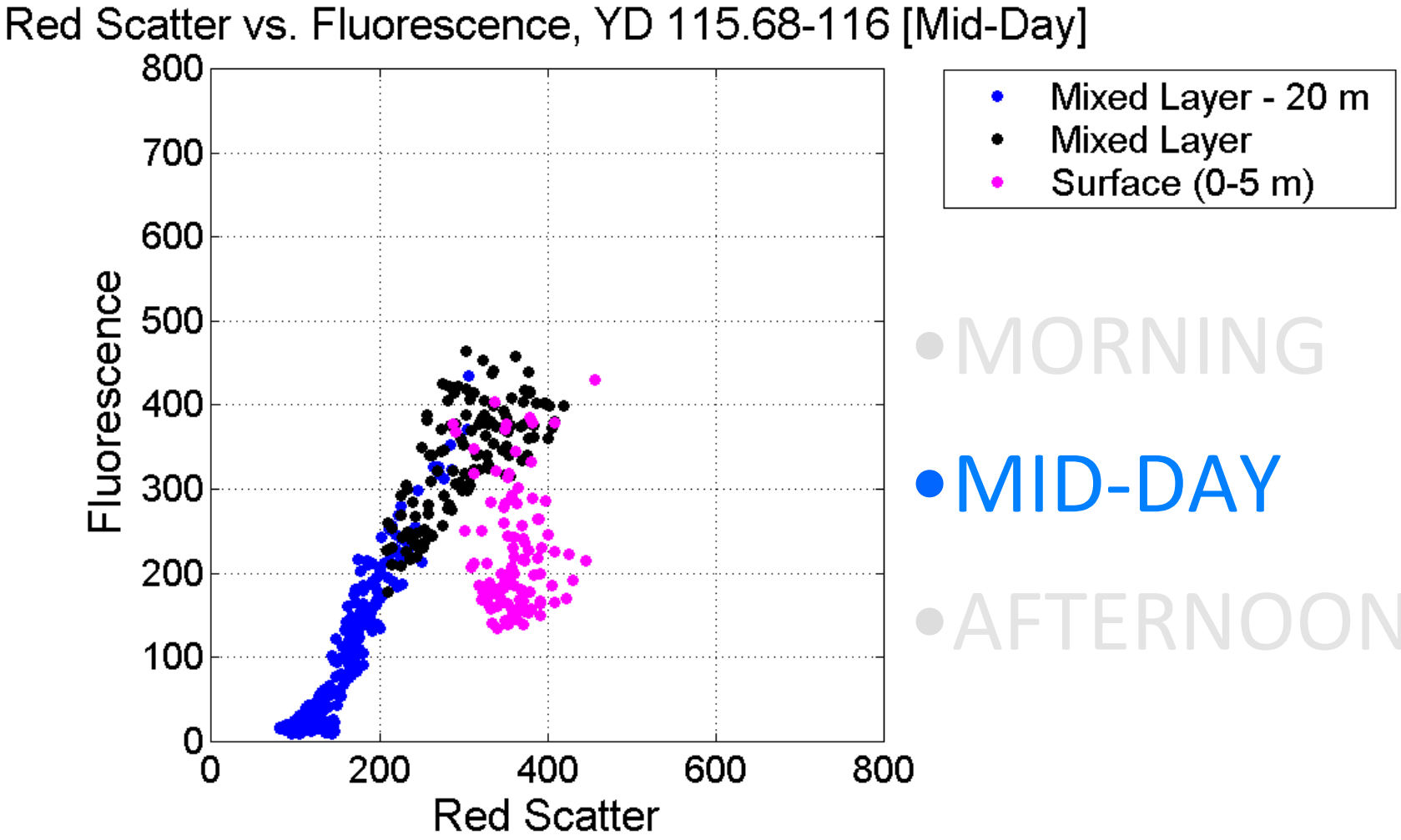
Mid-day fluorescence quenching



- MORNING
- MID-DAY
- AFTERNOON

Sackmann et al., unpub.

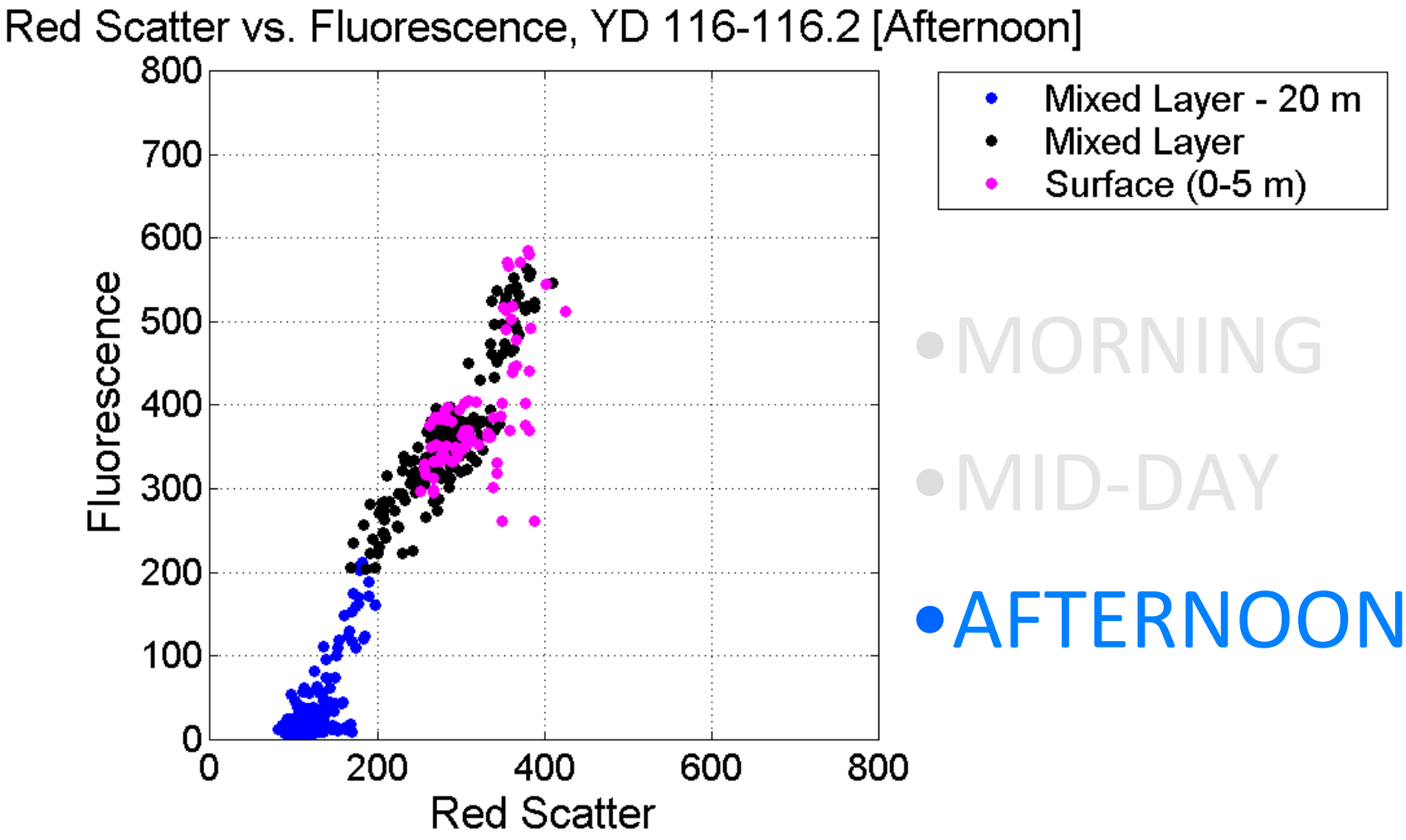
Mid-day fluorescence quenching



- MORNING
- **MID-DAY**
- AFTERNOON

Sackmann et al., unpub.

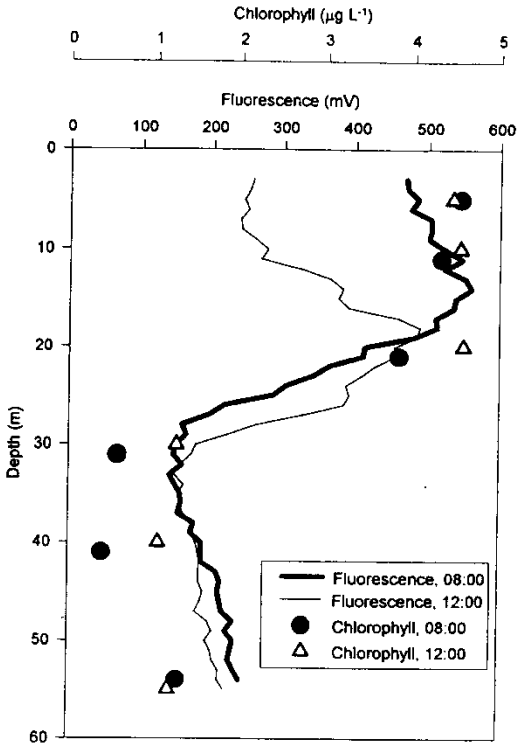
Mid-day fluorescence quenching



- MORNING
- MID-DAY
- AFTERNOON

Sackmann et al., unpub.

Fluorescence measurements on a ship can be well calibrated, because you can collect frequent water samples.



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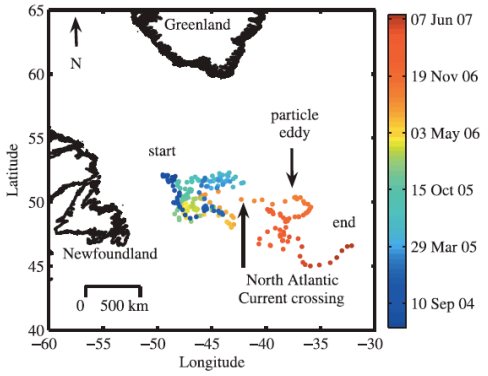
From Falkowski and Raven 1997
Chlorophyll fluorescence and extracted concentration of chlorophyll early AM vs. noon.

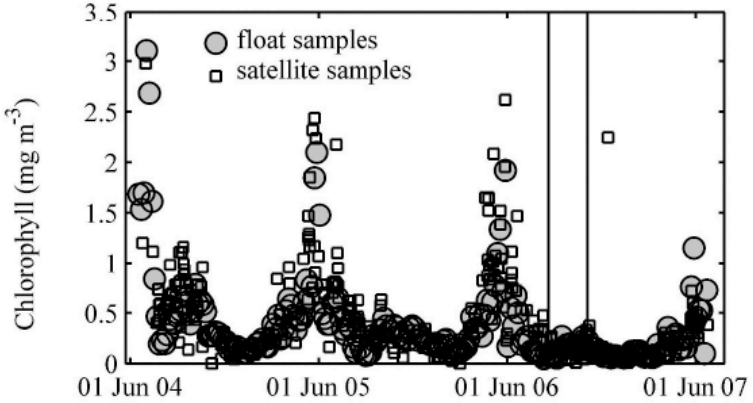
This profile shows the effect of day-time fluorescence quenching on mid-day fluorescence profile. Symbols are extracted chlorophyll from bottle samples.

Fluorescence to chlorophyll more difficult on remote, autonomous platforms

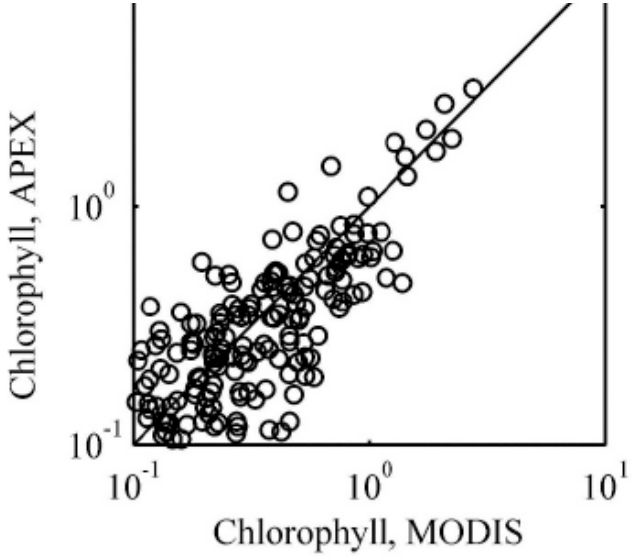
Boss et al. (2008) Limnol. Oceanogr.

Observations of pigment and particle distributions in the western North Atlantic from an autonomous float and ocean color satellite

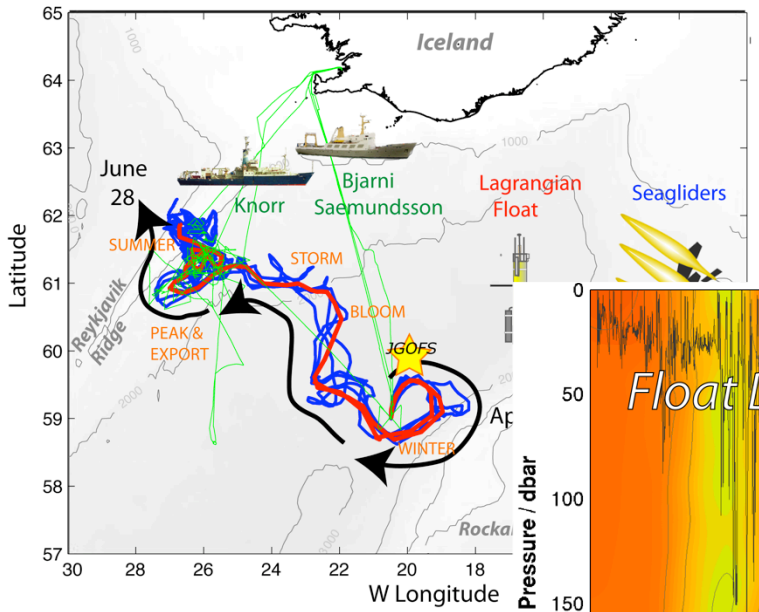


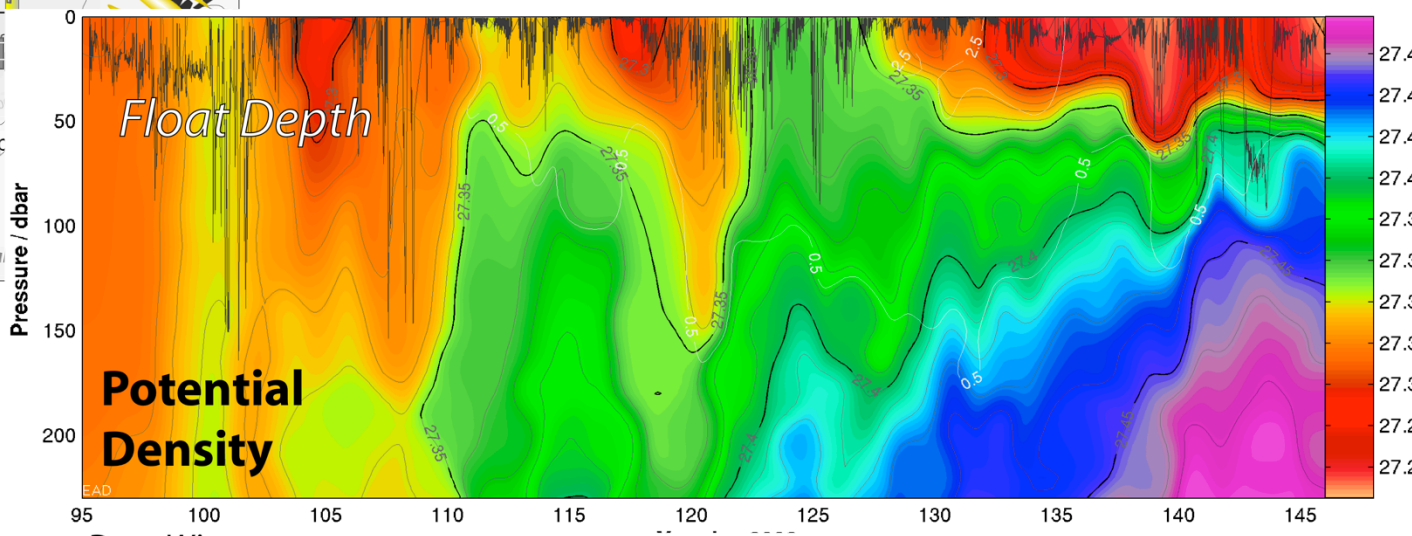


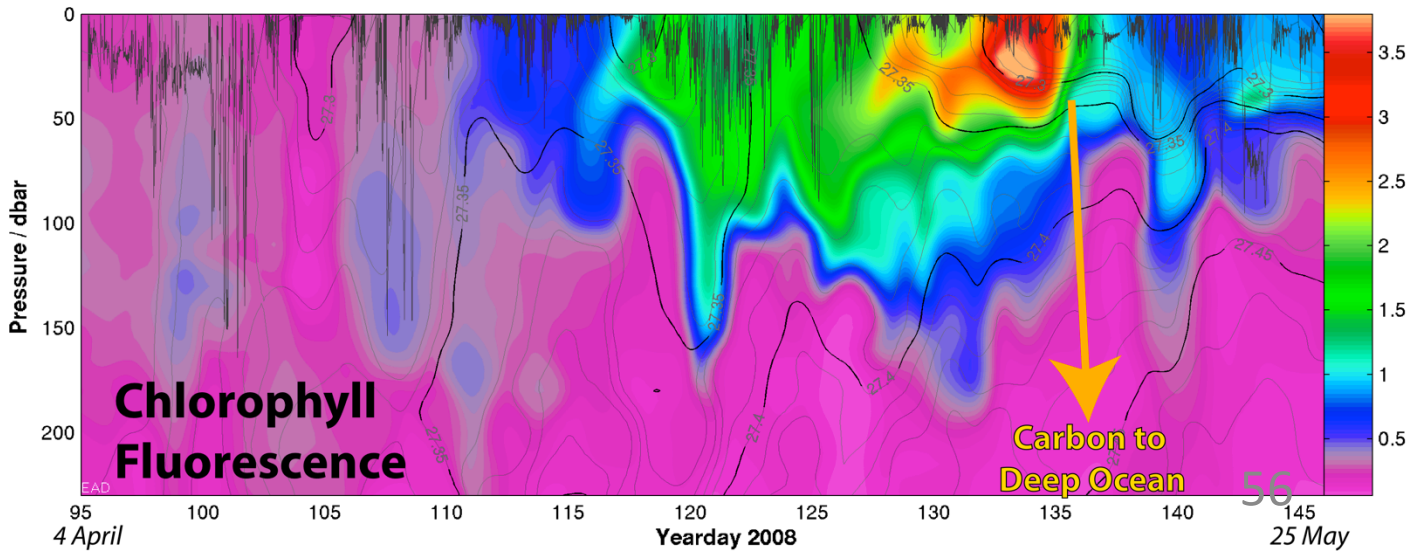
Time series and comparison of chlorophyll concentration as measured by the float and satellite ocean color sensors.



Timing of a bloom from a float – evolution of float patch from early April to late May.







Alkire et al., sub.