Lecture 30: Pigments and their Proxies

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11 August 2021

Outline

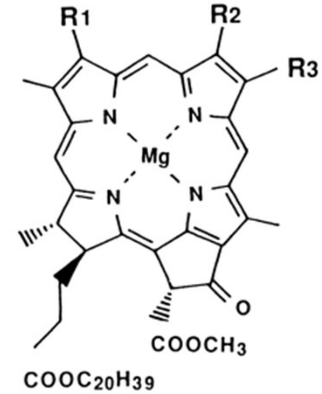
- Classifying Pigment
- Diagnostic pigments and taxonomy
- Quantifying Pigments with HPLC
- Estimating Pigments
  - absorption deconvolution
  - single channel fluorescence
  - multichannel fluorescence

Classifying pigments

- What is a pigment?
- Definition:
  - Compound that absorbs light and imparts color
  - Nearly insoluble in water (but we know this from lab)
  - In contrast to dyes, which are soluble and chemically bond to a substrate, imparting color to it
- Biological roles
  - light harvesting for photosynthesis (PS – photosynthetic)
  - light protection under high light (PP – photoprotective)

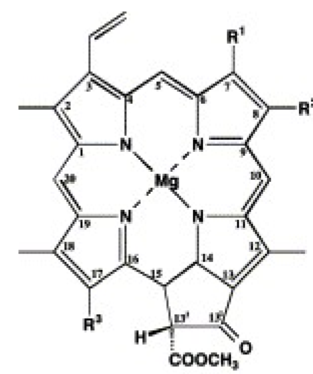
Major Types – Chlorophylls

- Chlorophyll *a* – dominant and ubiquitous across phytoplankton
- Chlorophyll *b* or *c* – they don't have both, accessory pigments, blue peaks are greener, red peaks are more orange,
- Divinyl chl *a* and *b* are found in *Prochlorococcus* (diagnostic pigments)



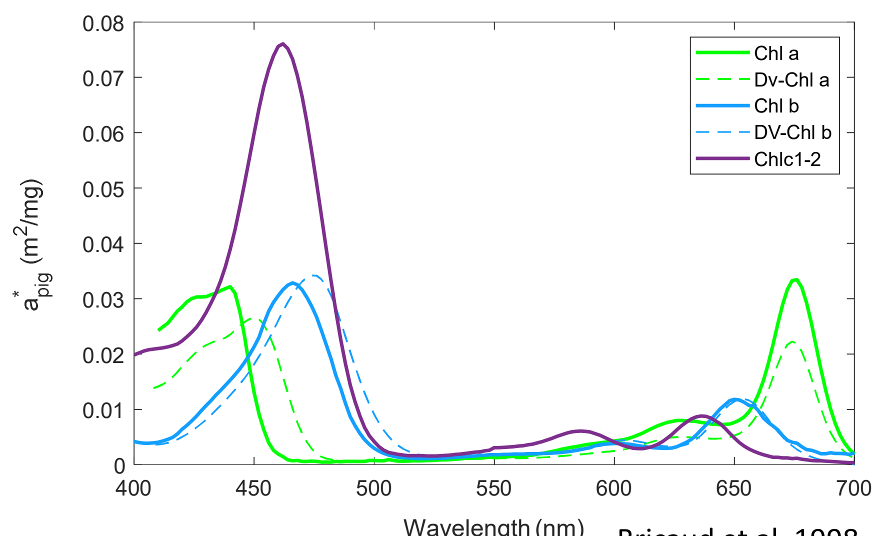
	R1	R2	R3
Chlorophyll *d*	CHO	CH_3	CH_2CH_3
Chlorophyll *a*	$CHCH_2$	CH_3	CH_2CH_3
Chlorophyll *b*	$CHCH_2$	CHO	CH_2CH_3
Divinyl Chlorophyll *a*	$CHCH_2$	CH_3	$CHCH_2$
Divinyl Chlorophyll *b*	$CHCH_2$	CHO	$CHCH_2$

Hu et al., 1998. PNAS 95 (22) 13319-13323; https://doi.org/10.1073/pnas.95.22.13319



Pigment	R[1]	R[2]	R[3]
Chl c_1	CH_3	CH_2CH_3	acrylic acid
Chl c_2	CH_3	$CH{=}CH_2$	acrylic acid
Chl c_3	$COOCH_3$	$CH{=}CH_2$	acrylic acid

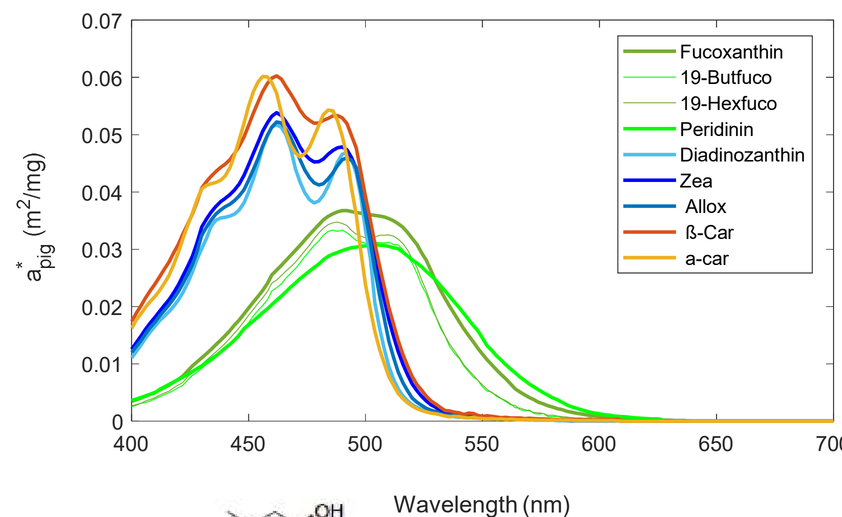
Helfrick et al. 2003. Biochim. Biophys., Acta https://doi.org/10.1016/S0005-2728(03)00081-1

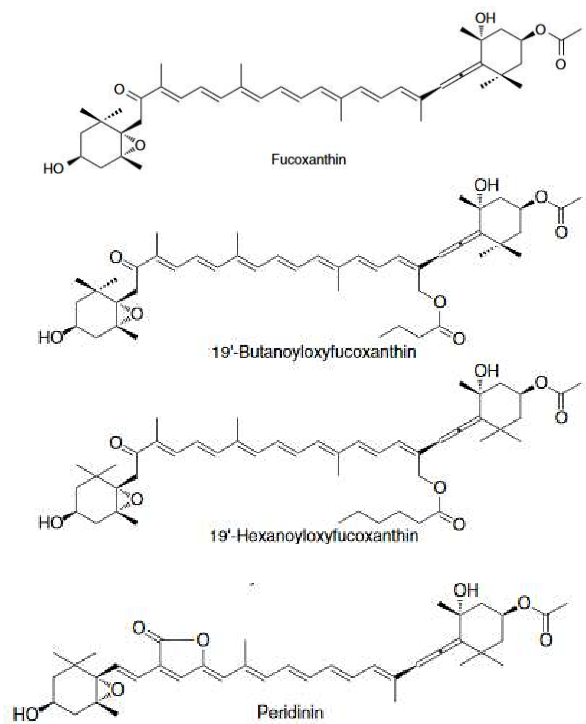


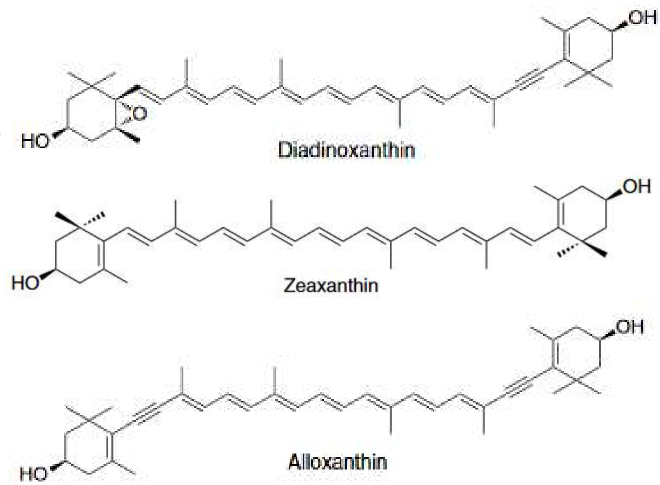
Bricaud et al. 1998

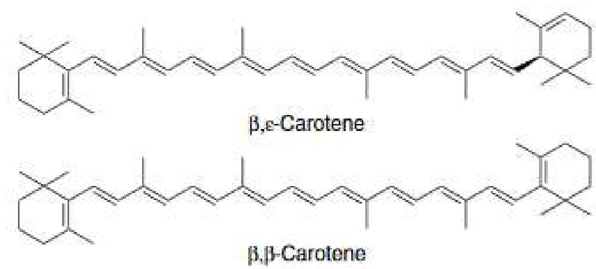
Major Types - Carotenoids

- Carotenoids
  - Fill in the blue- green bands

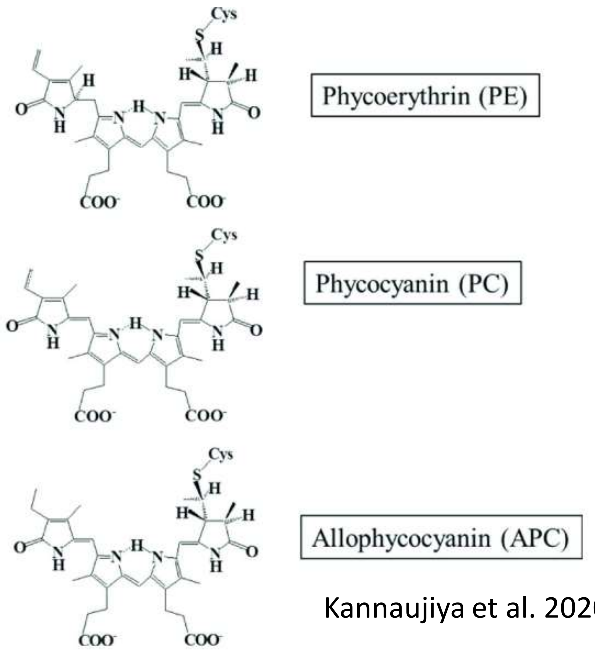


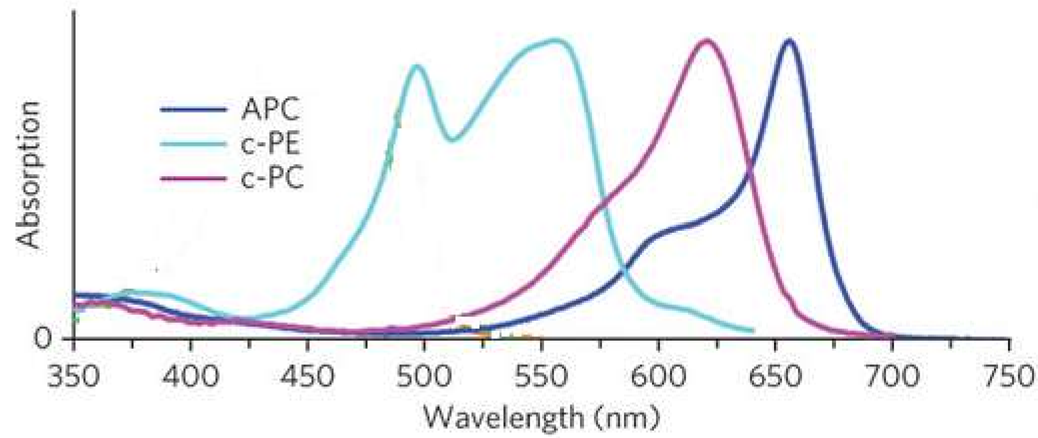






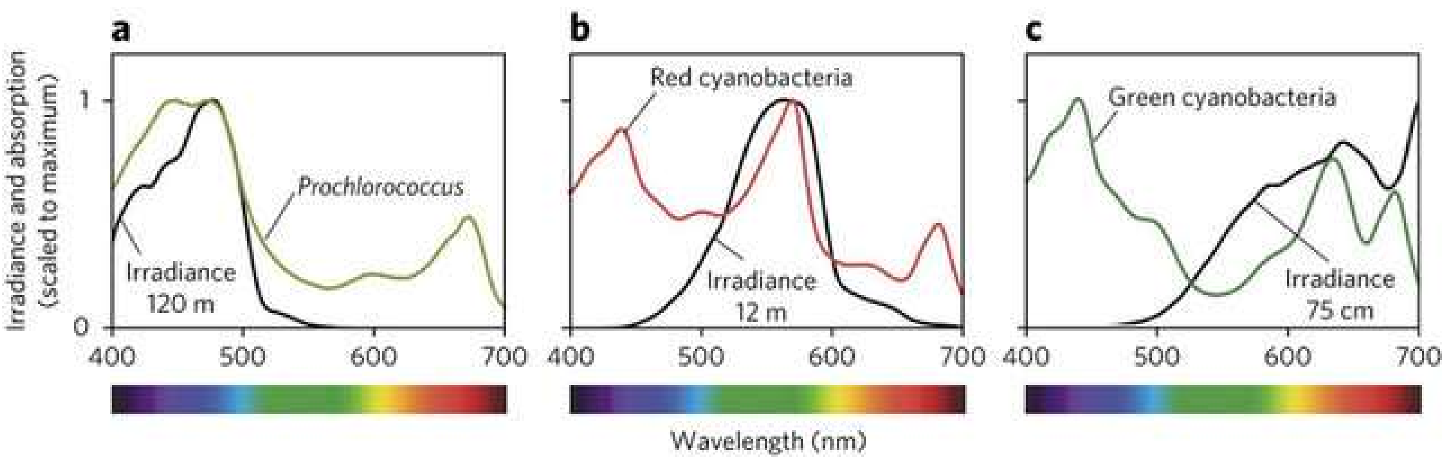
Major Types - Phycobilins





Kannaujiya et al. 2020

Croce & van Amerongen 2014



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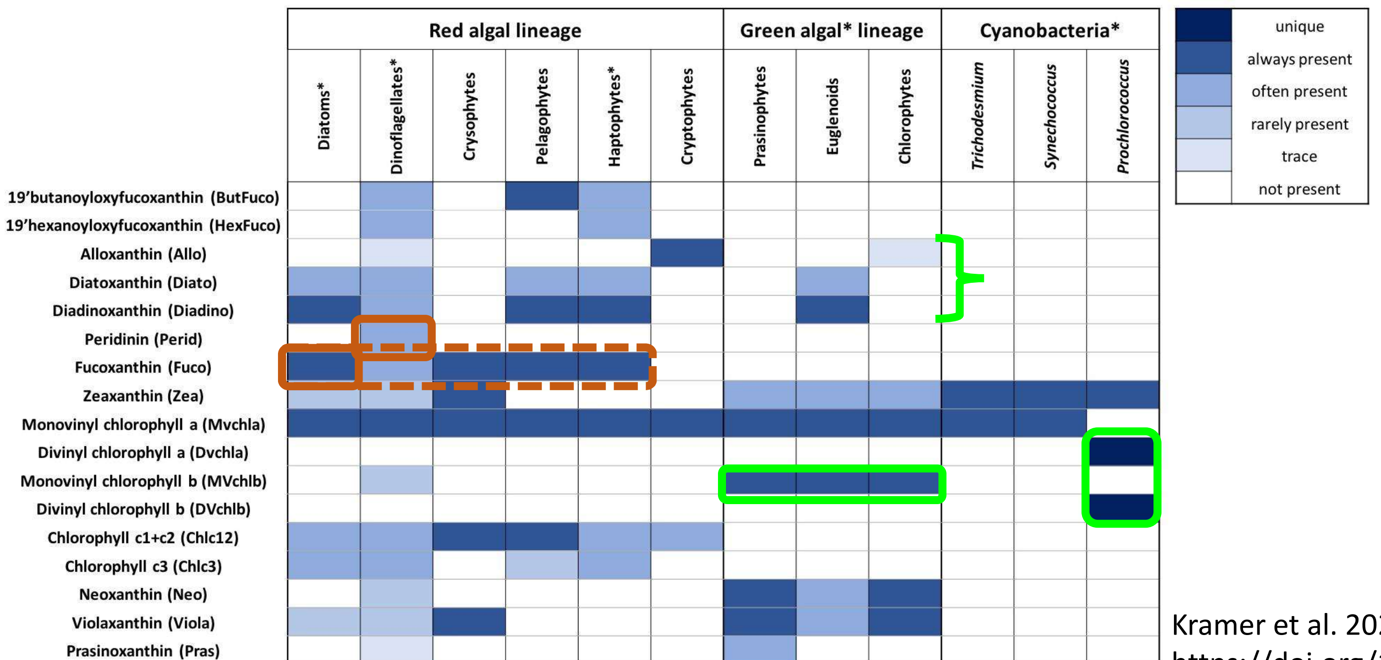
Diagnostic Pigments and Phytoplankton Taxonomy

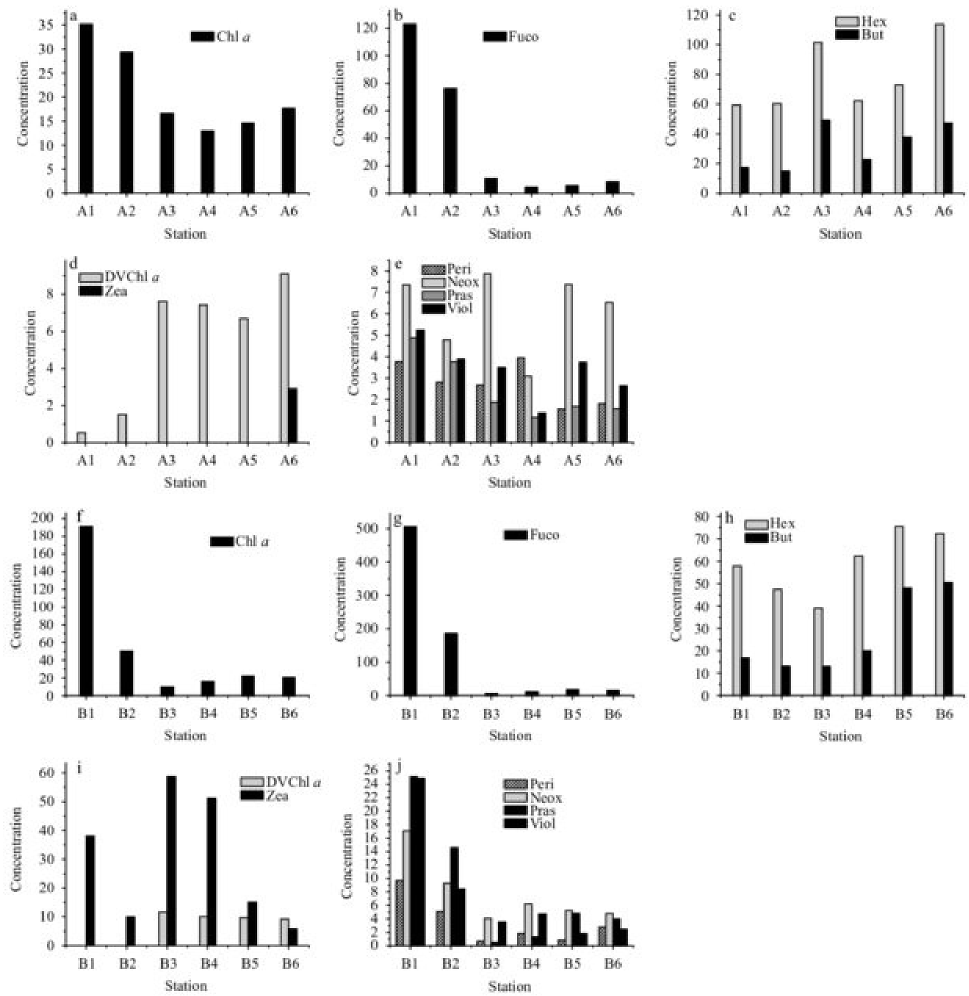
- Some pigments or combinations of pigments are characteristic of specific phytoplankton taxa
- For every example there are notable exceptions



Kramer et al. 2020 Front. Mar. Sci.
https://doi.org/10.3389/fmars.2020.00215

Statistical Approach – CHEMTAX[1]

- Step 1 – Measure
  - pigment composition
  - pigment concentration



[1]Mackey et al., 1996

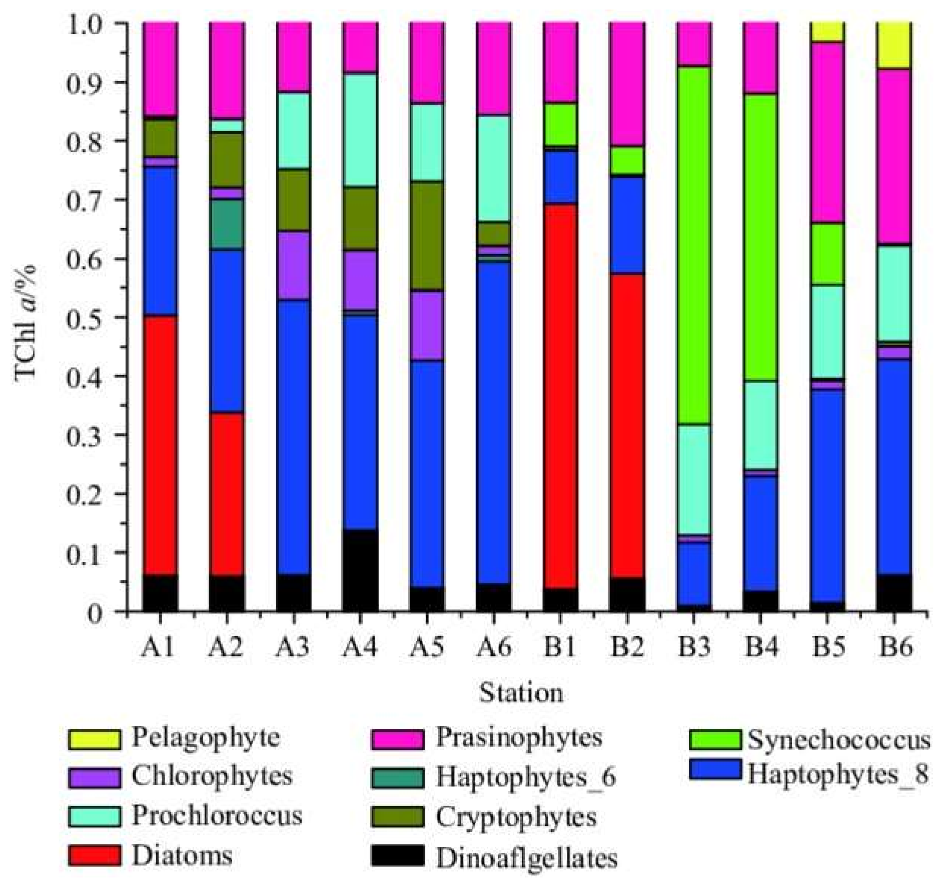
Gao et al. 2018
DOI: 10.1007/s13131-018-1342-x

Statistical Approach - CHEMTAX

- Step 2 – Know something about your ecosystem
  - Tune the pigment ratio for the taxonomic groups you are likely to encounter
    - e.g., how much of the measured fucoxanthin is partitioned into diatoms or the other groups?
  - Most people who use CHEMTAX don’t do this critical step
  - GIGO

Statistical Approach - CHEMTAX

- Step 3 – Model
  - Phytoplankton composition from (1)measured pigment composition and (2)input taxonomically-based pigment ratios



Gao et al. 2018
DOI: 10.1007/s13131-018-1342-x

Diagnostic Pigments and Size

- In the Mediterranean Sea pigments are generally associated with specific groups and those groups have characteristic cell sizes
  - $BP_{pico} = \frac{Zea+Chl_b}{DP}$ $< 2\mu m$ cells
  - $BP_{nano} = \frac{Allox+19\prime Hexfuco+19' Butfuco}{DP}$ $2\mu m - 20\mu m$ cells
  - $BP_{micro} = \frac{Fuco+Perid}{DP}$ $> 20\mu m$ cells
- For this scenario, pigments *may* be used to identify these size classes and their associated taxa

Table 1. Taxonomic Pigments Used in This Study and Their Significance in Term of Size Class

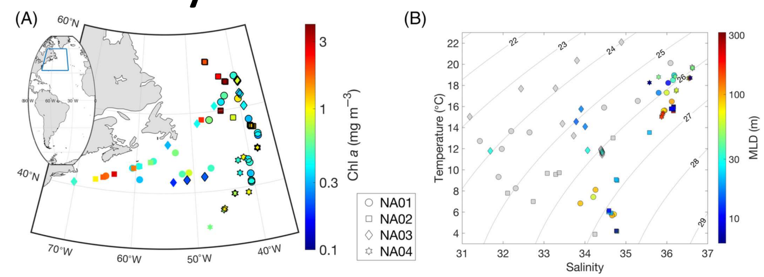
Pigments	Abbreviations	Taxonomic Significance	Size µm	References[a]
Zeaxanthin	Zea	cyanobacteria and prochlorophytes	< 2	1, 2, 5
Divinyl-chlorophyll *a*	Dv-chl *a*	prochlorophytes	< 2	3, 4, 5
Chlorophyll *b*+Divinyl-chlorophyll *b*	Tchl *b*	green flagellates and prochlorophytes	< 2	6, 7, 8, 9
19' hexanoyloxyfucoxanthin	19'-HF	chromophytes nanoflagellates	2-20	10, 11, 12, 13, 14
19' butanoyloxyfucoxanthin	19'-BF	chromophytes nanoflagellates	2-20	10, 13, 14, 15,16
Alloxanthin	Allo	cryptophytes	2-20	14, 17
Fucoxanthin	Fuco	diatoms	> 20	10, 11, 13, 18
Peridinin	Peri	dinoflagellates	> 20	18, 19, 20

[a]References are 1, *Gieskes et al.* [1988]; 2, *Guillard et al.* [1985]; 3, *Goericke and Repeta* [1992]; 4, *Gieskes and Kraay* [1983a]; 5, *Chisholm et al.* [1988]; 6, *Partensky et al.* [1993]; 7, *Moore et al.* [1995]; 8, *Jeffrey* [1976]; 9, *Simon et al.* [1994]; 10, *Bjørnland and Liaaen-Jensen* [1989]; 11, *Hooks et al.* [1988]; 12, *Arpin et al.* [1976]; 13, *Wright and Jeffrey* [1987]; 14, *Jeffrey and Vesk* [1997]; 15, *Andersen et al.* [1993]; 16, *Bjørnland et al.* [1989]; 17, *Gieskes and Kraay* [1983b]; 18, *Kimor et al.* [1987]; 19, *Johansen et al.* [1974]; and 20, *Jeffrey et al.* 1975.

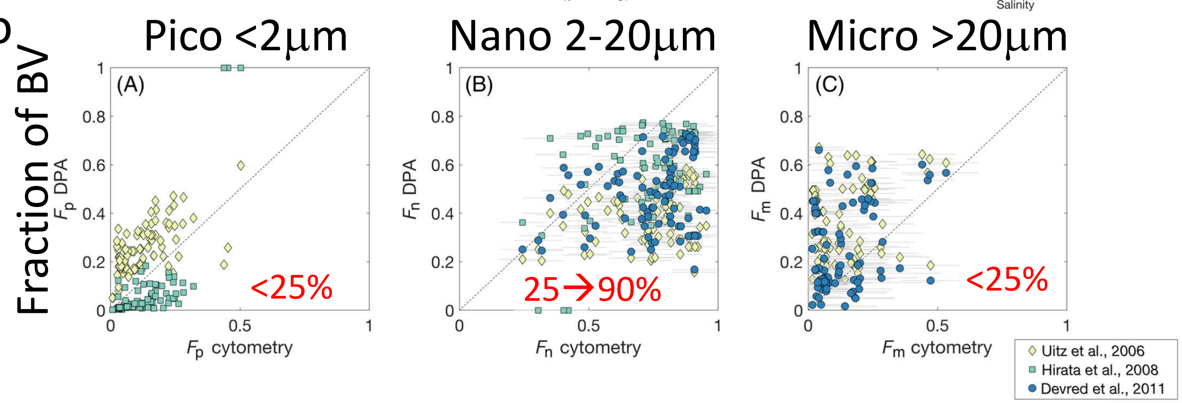
Vidussi et al. 2001. J Geophys Res 106
DOI: 10.1029/1999JC000308

Diagnostic Pigments and Size

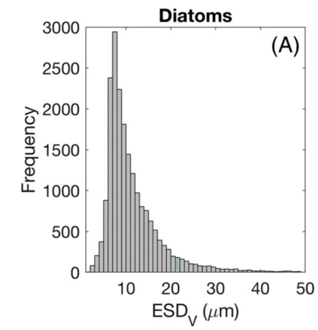
- How does it work in other locations?
- Chase et al (2020) tested 3 ‘tuned’ DP models on a data set from the North Atlantic and validated with cytometric classifications (broad optical range)



- Partitioning biovolume into size bins
  - Models bracket pico
  - Underestimate nano
  - Overestimate micro
- Partitioning chl



Diagnostic Pigments and Size



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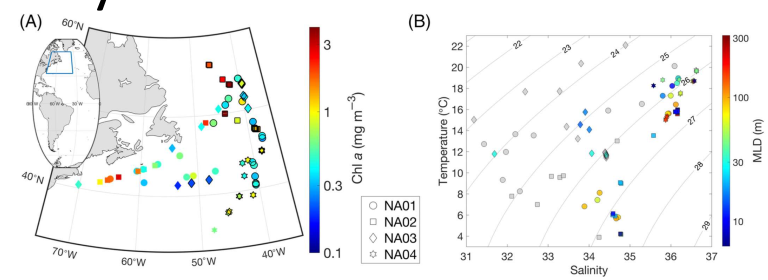
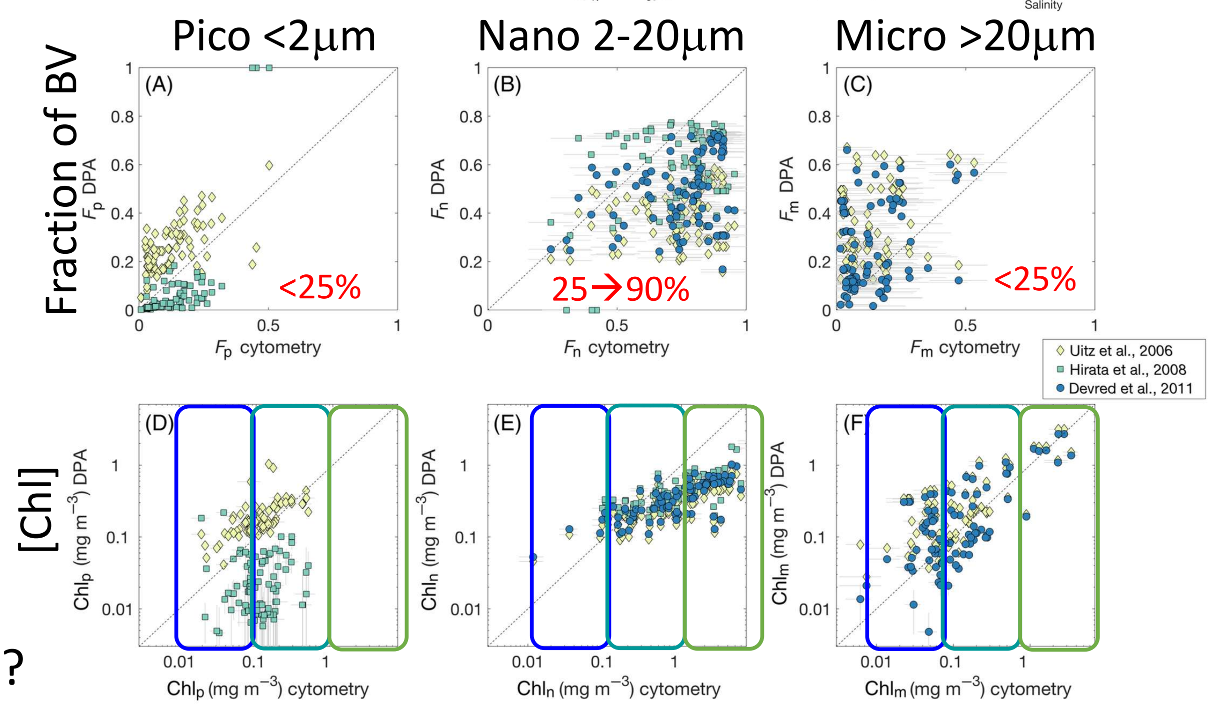
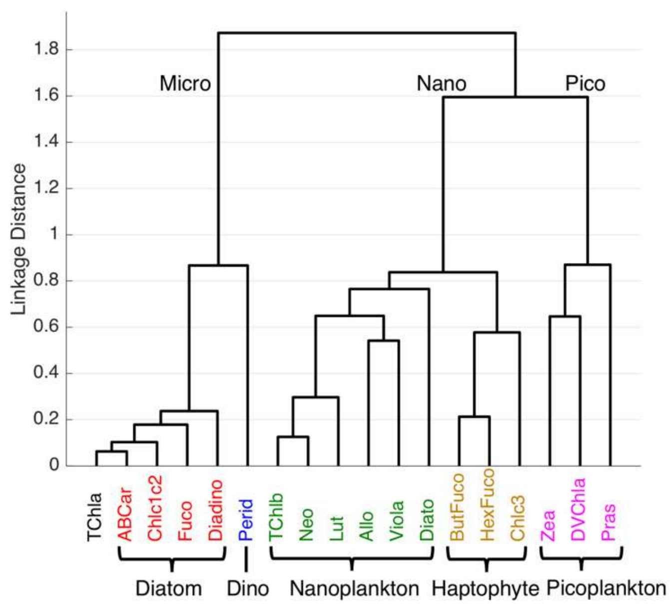


- Partitioning biovolume into size bins
  - Models bracket pico
  - Underestimate nano
  - Overestimate micro
- Partitioning chl
  - 0.01 – 0.1 mg/m3
    - All overestimated
  - 0.1 – 1 mg/m3
    - Micro overestimated
  - 1 – 10 mg/m3
    - Nano underestimated
- Where do models go wrong?

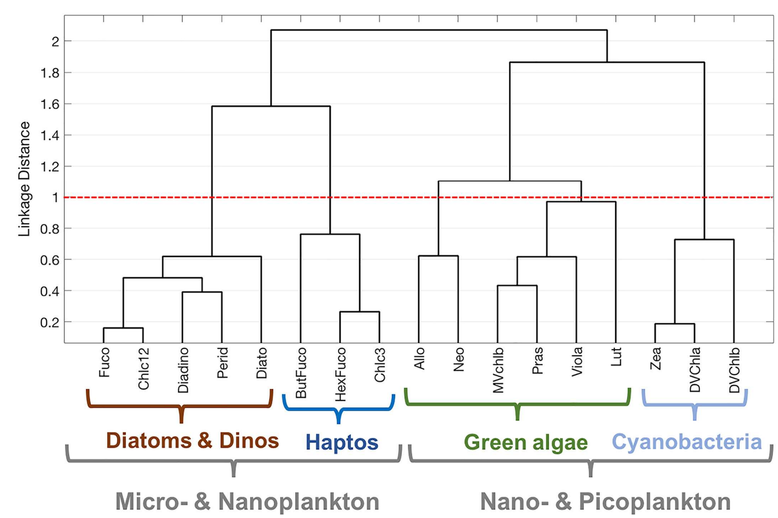


Hierarchical cluster analysis

- Hierarchical cluster analysis → 3 pigment clusters linkage distances larger than 1.5 → 3 phytoplankton size classes (upwelling system off California)
- Similar results for a global data set (but regional analyses revealed a range of ecological patterns)



Catlett and Siegel 2017
https://doi.org/10.1002/2017JC013195



Kramer and Siegel 2019
https://doi.org/10.1029/2019JC015604

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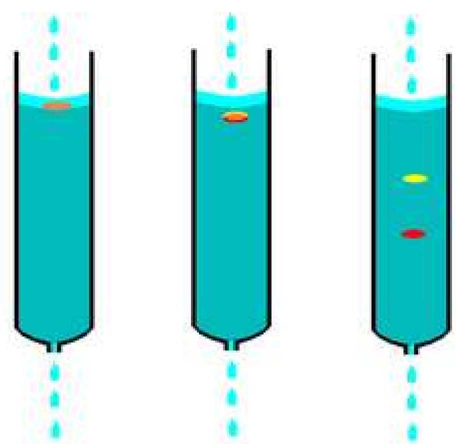
High Performance[1] Liquid Chromatography

[1] formerly known as high pressure liquid chromatography

Basic mode of operation

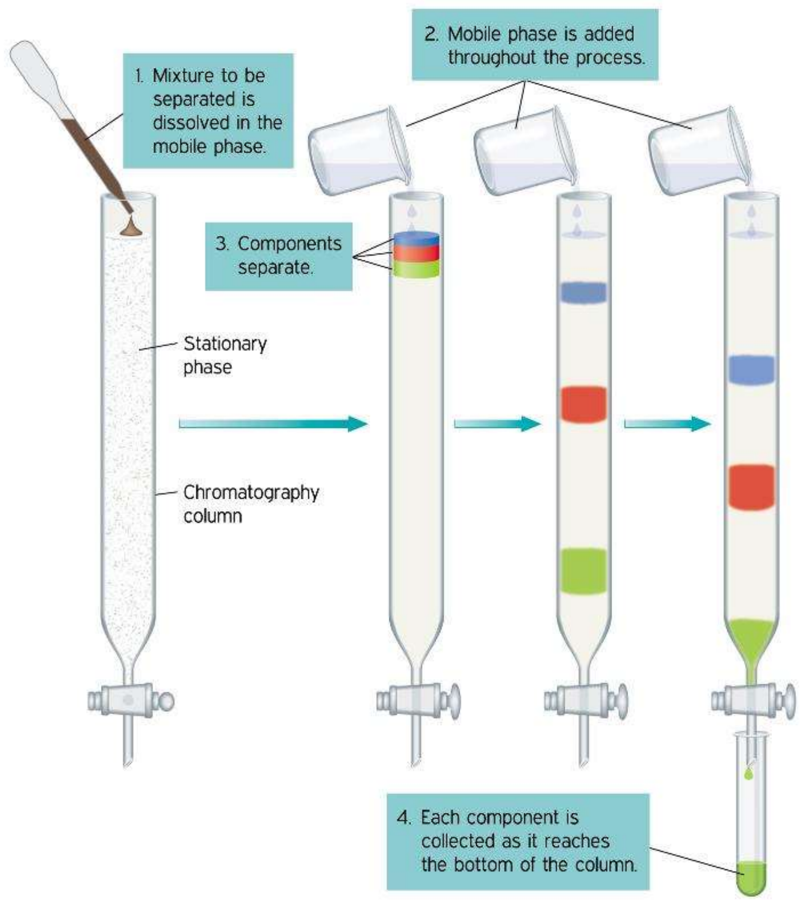
- **Column Chromatography** - technique used to separate chemical mixtures into individual compounds
- Chemical mixture (e.g., extracted phytoplankton pigments)
  - Carried by the liquid mobile phase (solvent gradient)
  - Through the solid stationary phase (silica resin)
  - Individual compounds (i.e., pigments) in mixture travel at different rates due to differential adhesion to the silica

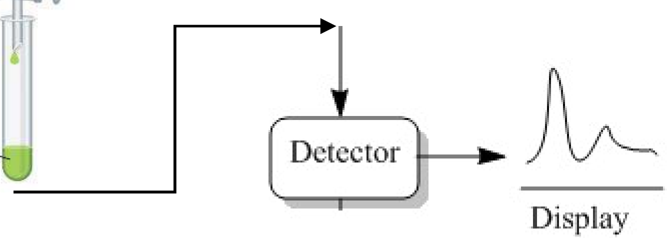


https://chemdictionary.org/column-chromatography/

Basic mode of operation

- Each compound is collected an analyzed for identification
- Pigments have strong and unique absorption features
- As each compound exits the column the absorption properties are measured
  - Absorption → [concentration]
  - Absorption spectrum → identification





https://chemdictionary.org/column-chromatography/

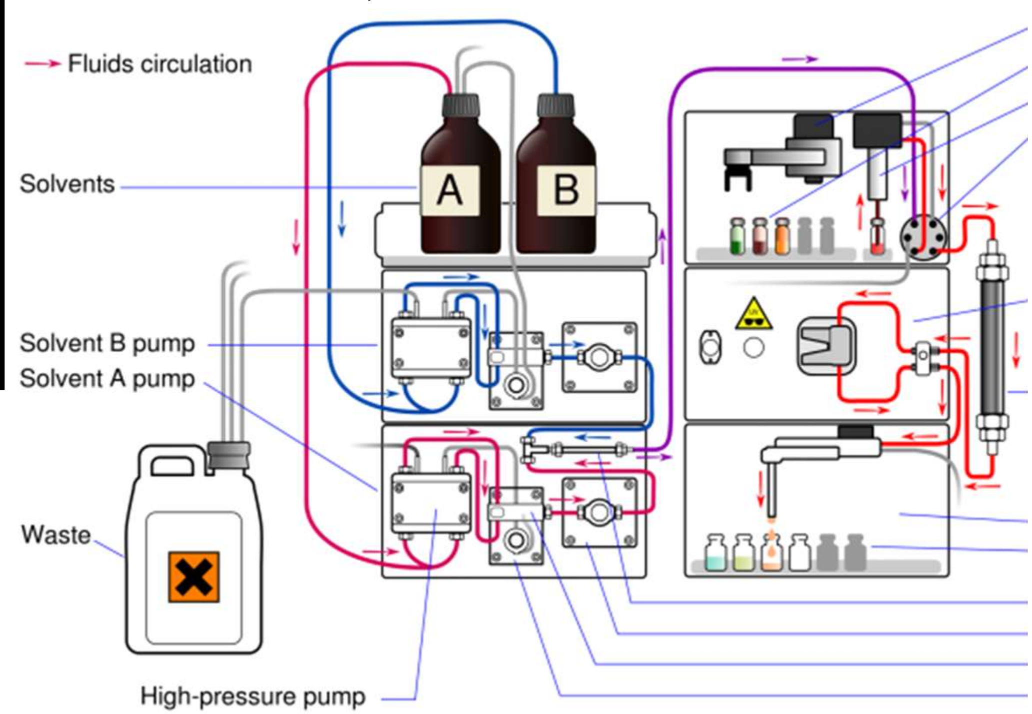
Basic mode of operation

- High Performance (Pressure) Liquid Chromatography
  - 400 atmosphere pressure applied to injection → faster
  - Much smaller packing of column → better separation
  - Great method for separating pigment mixtures into individual pigments

High Performance Liquid Chromatography

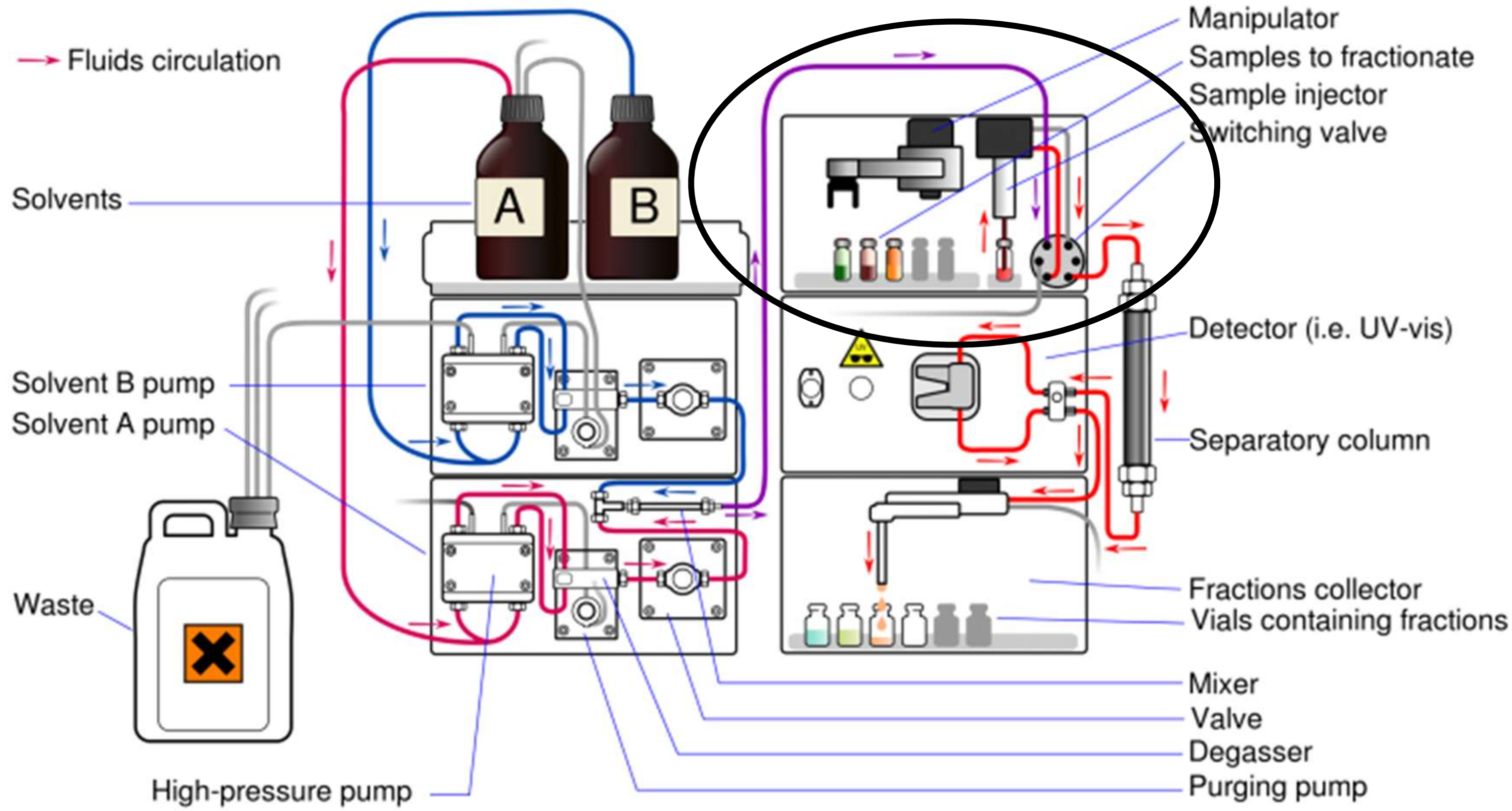
- ***Step 1: Solvent reservoir***
  - Gradient of two solvents
    - A 70% methanol: 30% 28 mM tetrabutyl ammonium acetate
    - B 100% methanol

Step	*Time*	A [%]	B [%]
Start	0	95	5
2	22	5	95
3	29	5	95
4	31	95	5
End	36	95	5



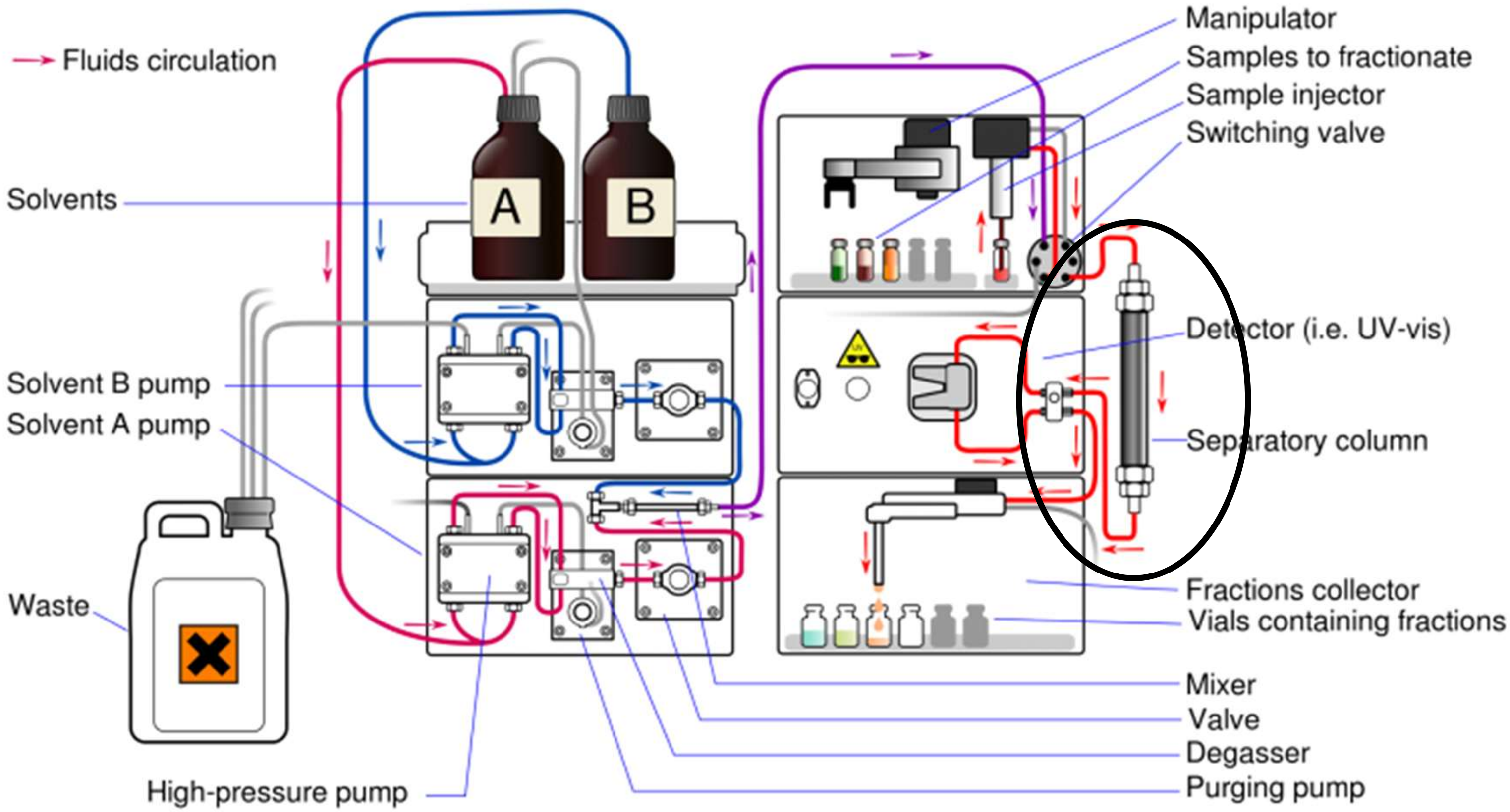
High Performance Liquid Chromatography

- ***Step 2: Injection of sample***



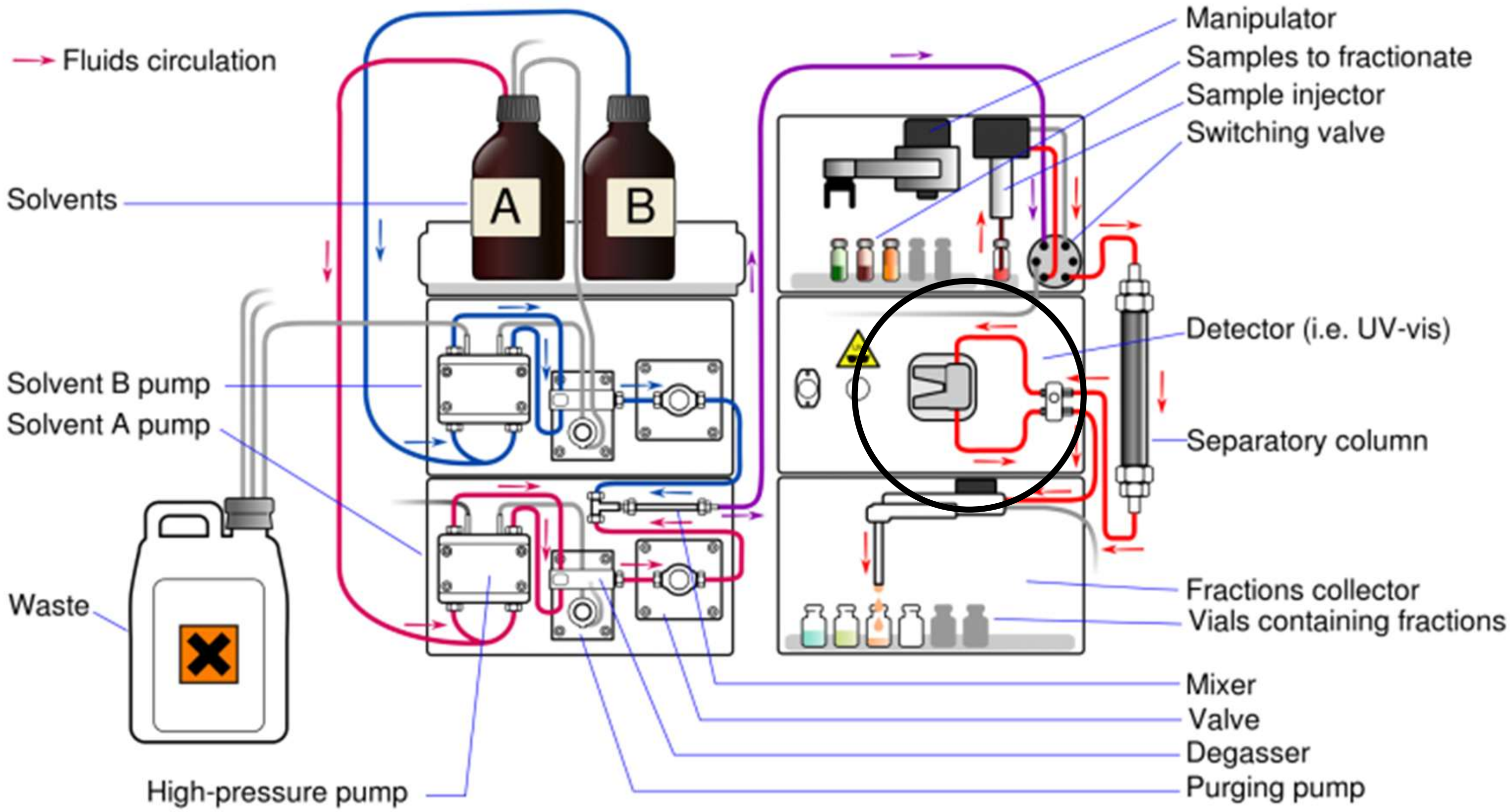
High Performance Liquid Chromatography

- ***Step 3: Retention time (0-36 minutes)***



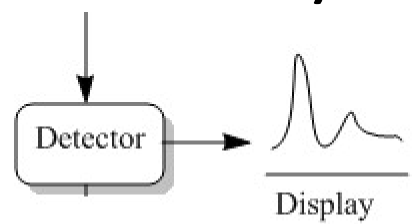
High Performance Liquid Chromatography

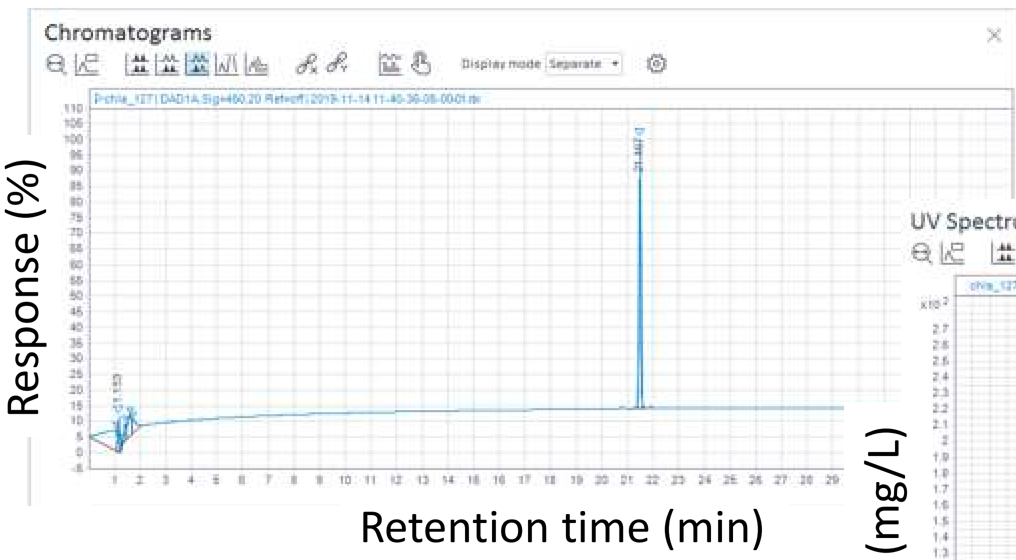
- ***Step 4: Detection***

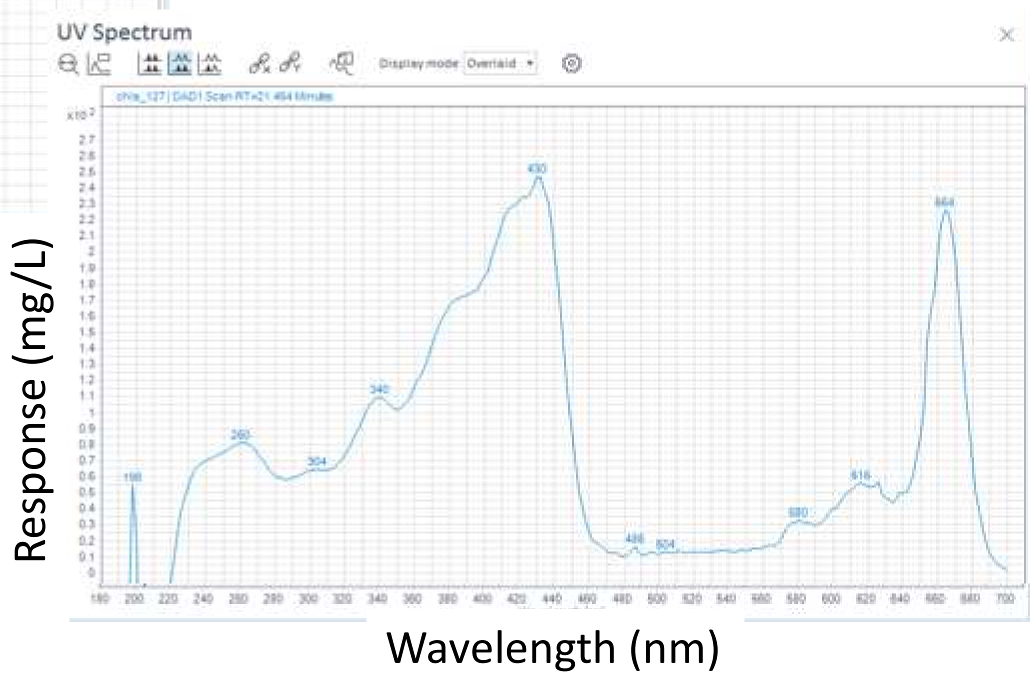


High Performance Liquid Chromatography

- ***Step 4: Detection***
  - **Signal (absorption) as a function of (retention time)**
  - **Associated absorption spectrum**

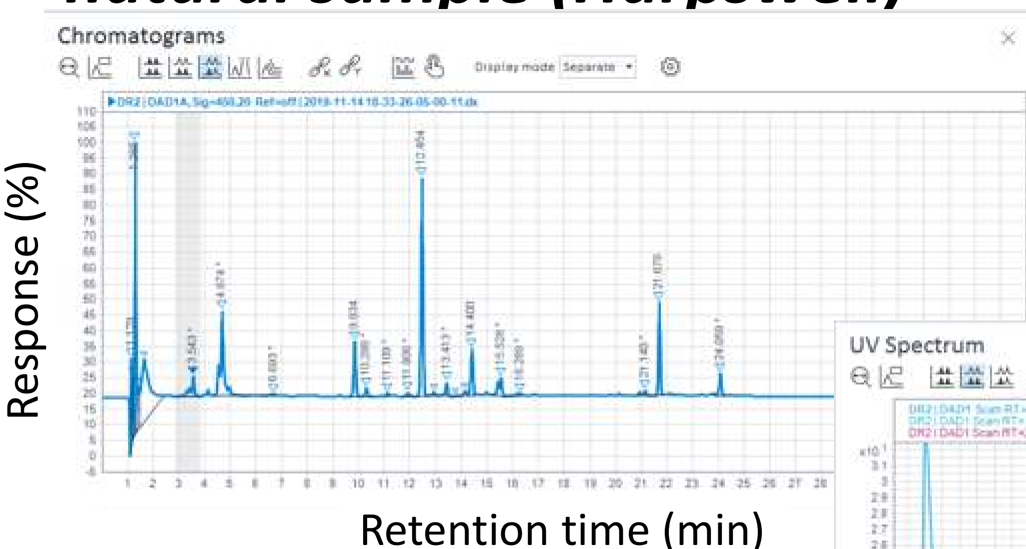


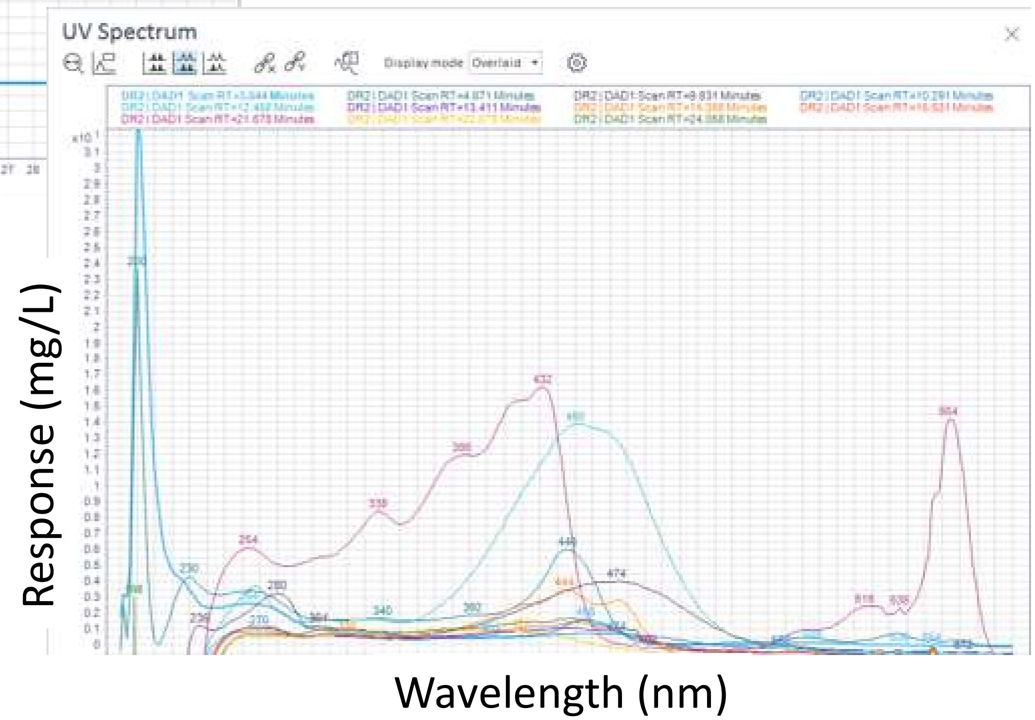




High Performance Liquid Chromatography

- ***Step 5: Displaying the peaks versus retention time for natural sample (Harpswell)***





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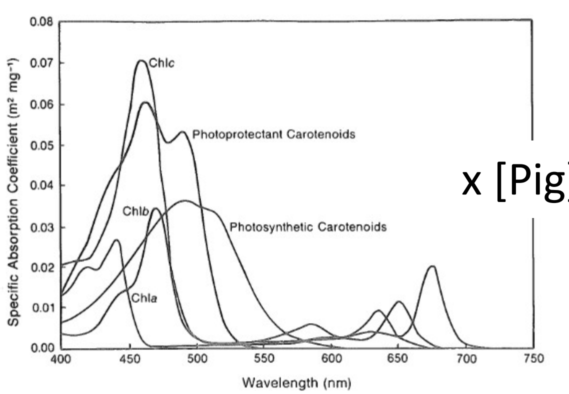
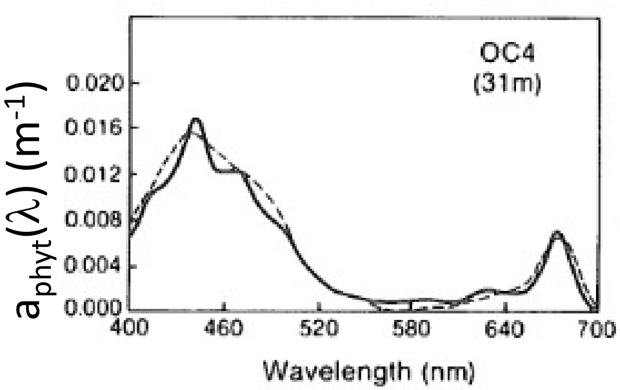
Estimating Pigments from Absorption

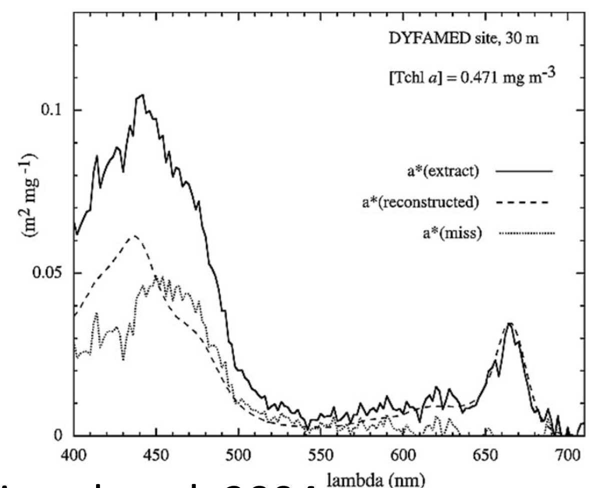
- Pigments to absorption – Forward model
  - $\sum_{n=1}^{N}[Pig]_n \times a^{*}_{pig_n}(\lambda) = a_{phyt}(\lambda)$
  - account for variations between *in vitro* and *in vivo* absorption properties
  - (e.g., Bidigare et al. 1990; Hoepffner and Sathyendranath 1991; Bricaud et al. 2004)



Bidigare et al. 1990

x [Pig] =

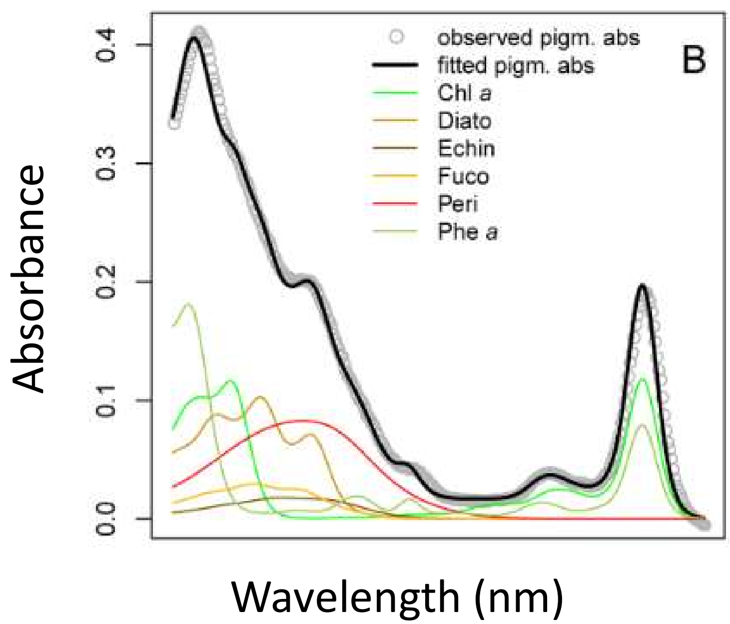




Bricaud et al. 2004

Estimating Pigments from Absorption

- Pigments from absorption – Inverse model
  - $a_{phyt}(\lambda) = \sum_{n=1}^{N} [Pig]_n \times a_n^*(\lambda)$ → pigment-based classification
  - Solve for $[Pig]_n$ by multiple linear regression
  - (Thrane et al. 2015)



Estimating Pigments from Absorption

- Pigments from absorption – Inverse model
  - $a_{phyt}(\lambda) = \sum_{n=1}^{N} g_n \times G_n(\lambda)$
  - Solve for magnitude of gaussians, g_n, by multiple linear regression
  - Correlate Gaussian magnitudes to HPLC pigments→
  - (Chase et al. 2017)

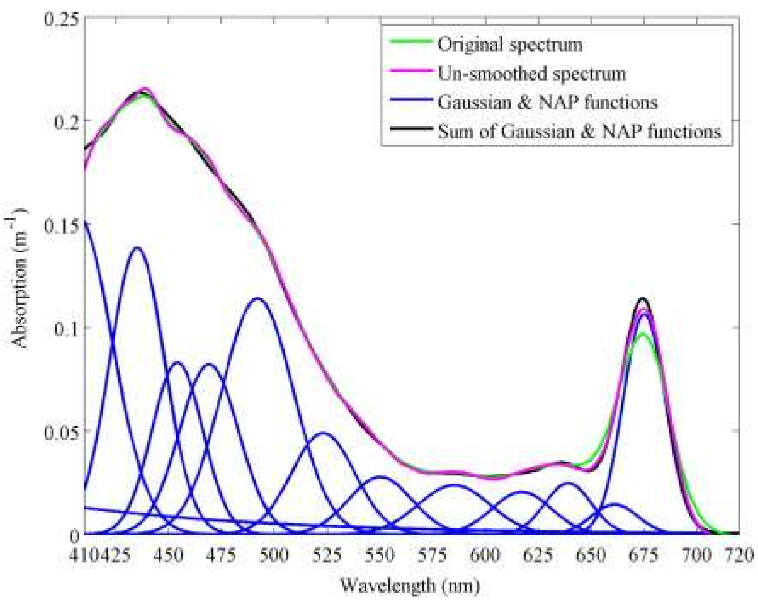


Table 2. Correlations between HPLC pigment concentrations and $a_{gaus}(\lambda_i)$ at ten different pigment absorption wavelengths. Correlation values are Spearman's rank correlation coefficient (non-parametric; denoted ρ). A and B are coefficients determined using Eq. (4) (Section 2.4).

Wavelength (nm)	Pigment(s)	ρ	A	B	e_{median} (%)
435	TChl a	0.868	0.031	0.578	35
617	TChl a	0.834	0.003	0.758	36
675	TChl a	0.899	0.014	0.798	30
454	0.03(TChl b) + 0.07(Chl c)	0.845	0.028	0.414	57
469	TChl b	0.783	0.066	0.533	52
661	TChl b	0.747	0.018	0.668	40
585	Chl c	0.846	0.014	0.582	53
639	Chl c	0.894	0.012	0.641	41
492	PPC	0.606	0.046	0.650	51
523	PSC	0.855	0.013	0.588	49

PPC = α-carotene + β-carotene + zeaxanthin + alloxanthin + diadinoxanthin.

PSC = 19′-hexanoyloxyfucoxanthin + fucoxanthin + 19′-butanoyloxyfucoxanthin + peridinin.

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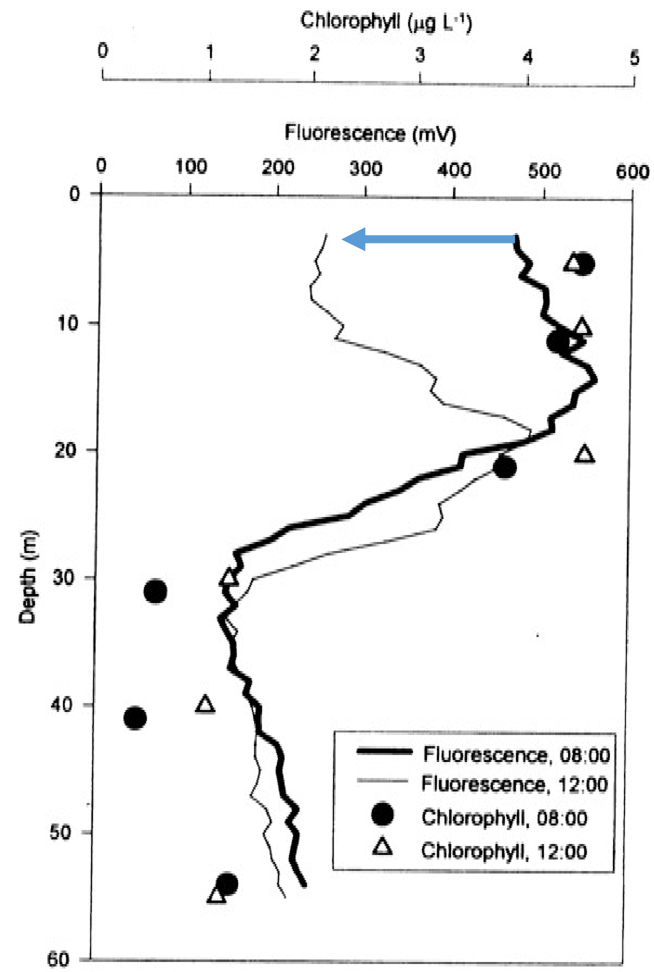
Estimating Pigments from Fluorescence

- We spent a lecture and lab on using fluorescence to estimate chlorophyll concentration
- You demonstrated the difference in calibration between identical samples that were protected from ambient light versus exposed to ambient light (F/chl ↓as E↑)
- You may have observed a subsurface fluorescence maximum at your sampling stations that was not supported by the extracted chlorophyll values
- High light quenches fluorescence (and photosynthesis) → Non-photochemical quenching (NPQ)
- There are a variety of strategies for dealing with NPQ in *in situ* data sets

Non-Photochemical Quenching (NPQ)

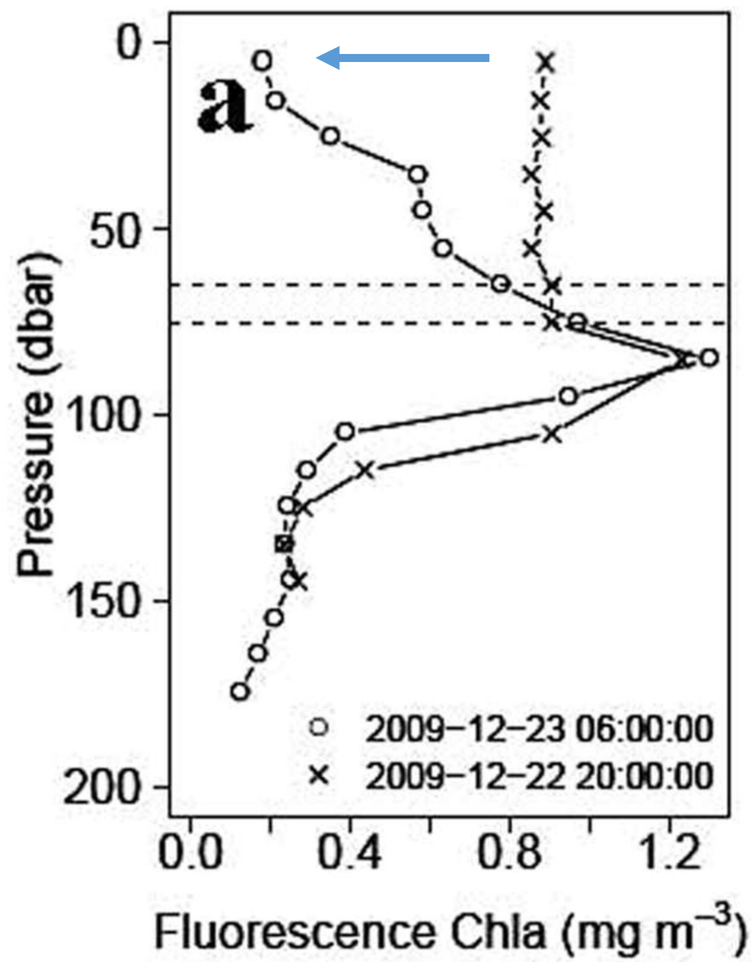
- Chl profile constant over 4-hr interval
- Surface in situ F_{chl} decreases at surface from morning to noon
- Non-photochemical quenching, NPQ



Falkowski and Raven 1997

Non-photochemical quenching

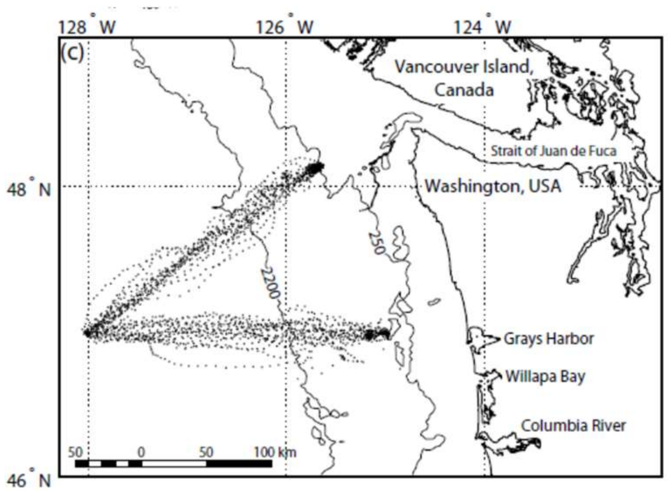
- On a profiling float



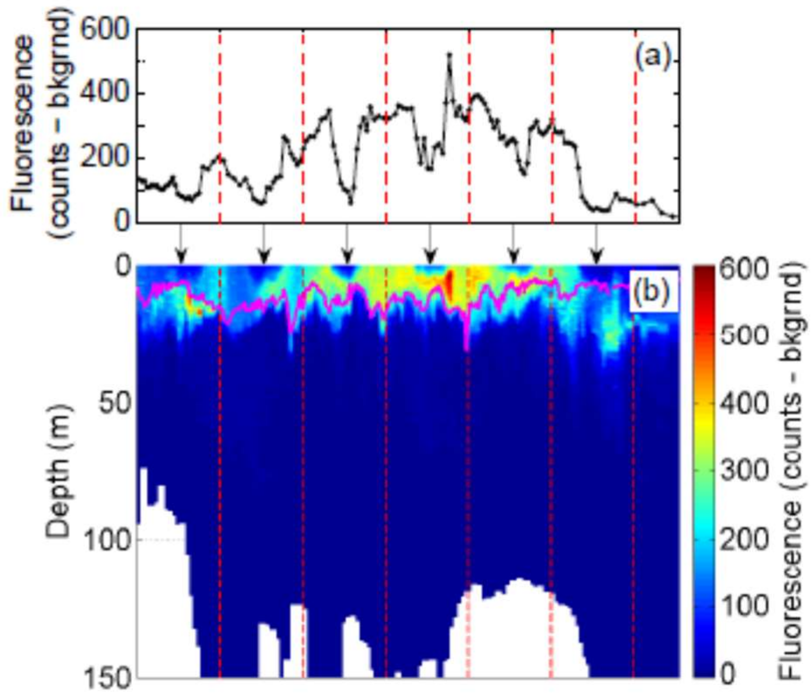
Xing et al. 2012

Non-photochemical quenching

- Glider



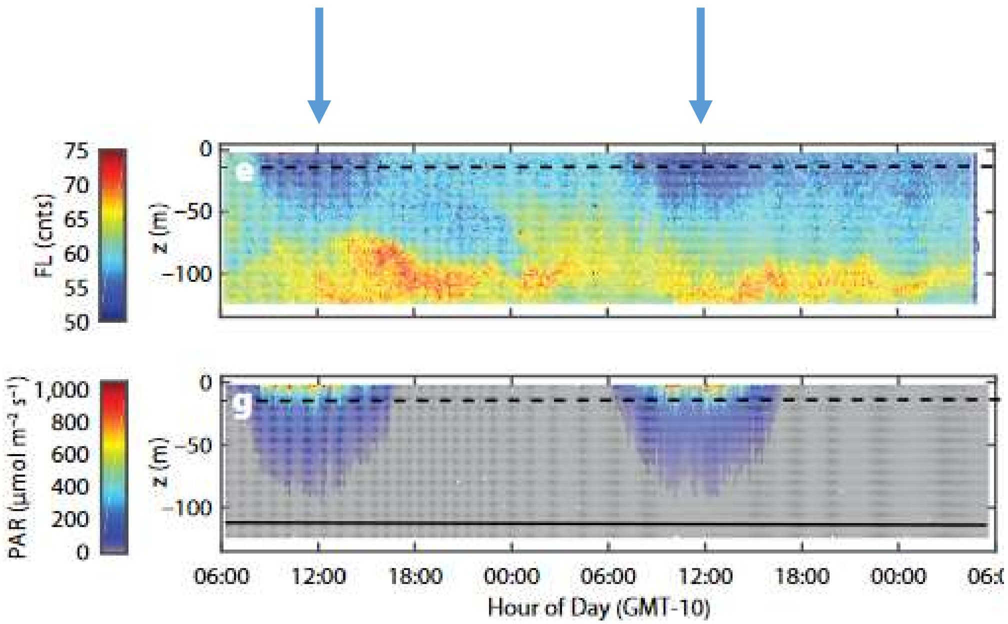
midnight

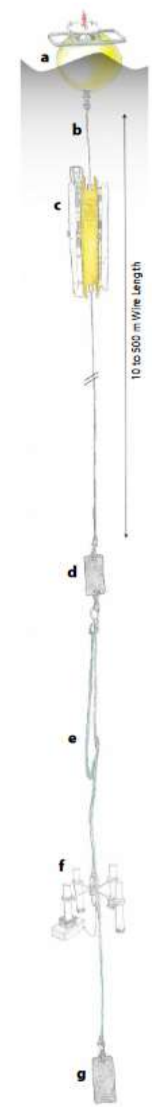


Sackman et al. 2008

Non-photochemical quenching

- Wirewalker



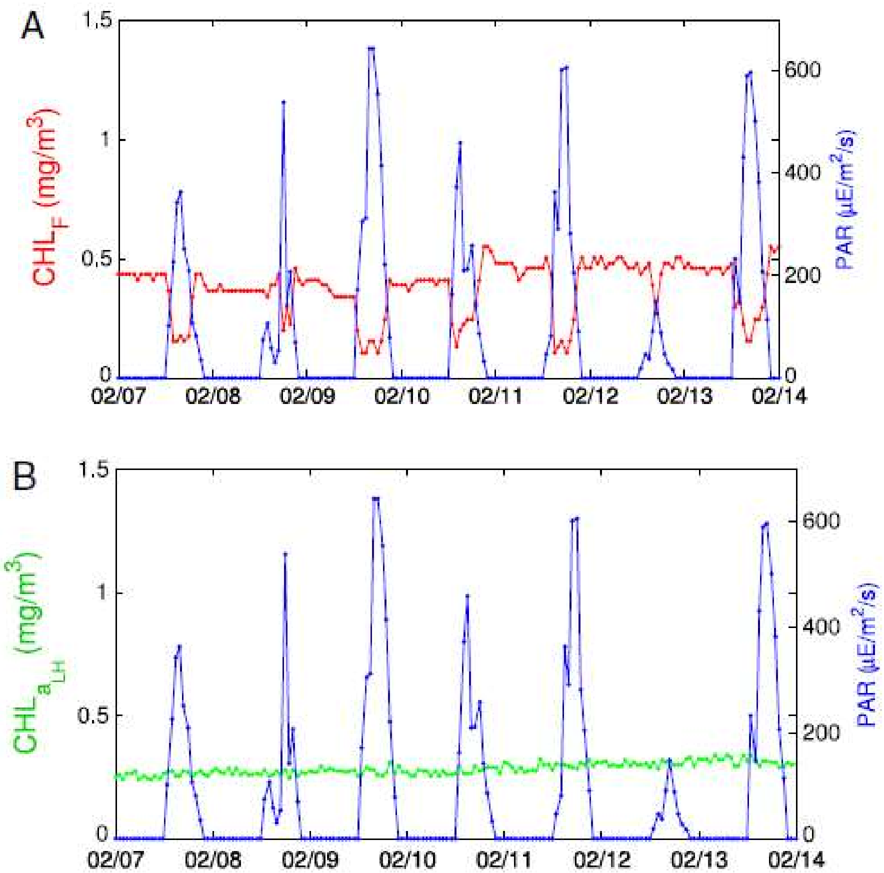


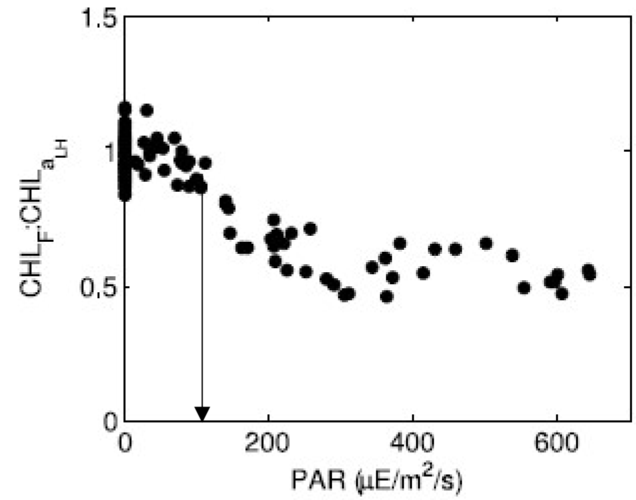
Omand et al. 2017

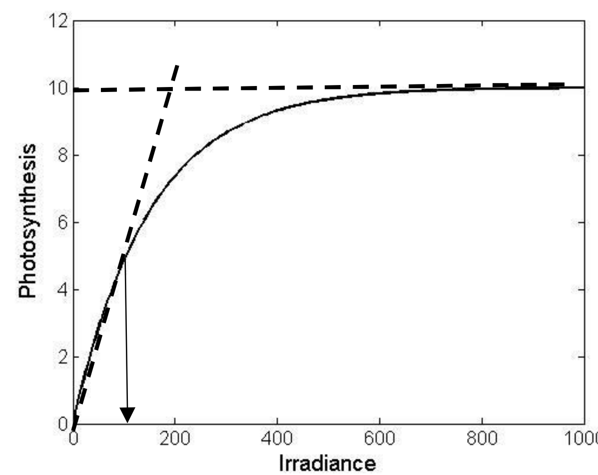
Non-photochemical quenching

- Mooring (LOBO)

The irradiance at which F_{chl} is quenched is a good proxy for E_k, the half saturation constant for photosynthesis





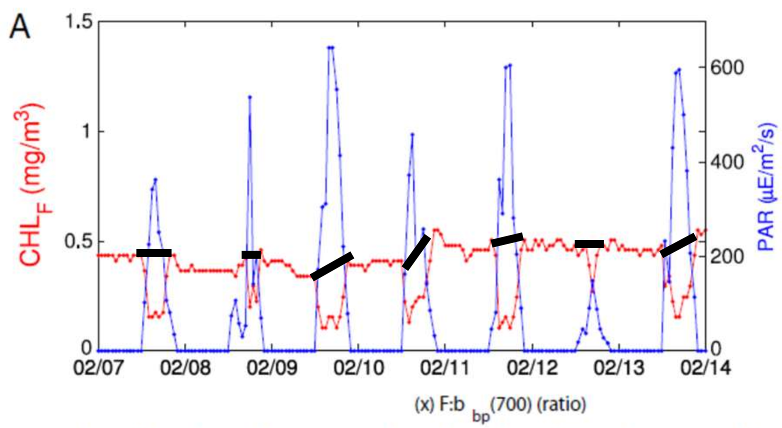


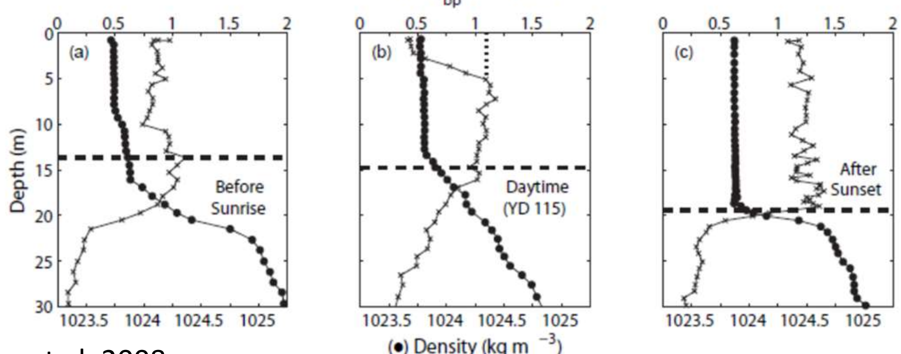
Roesler and Barnard 2013

NPQ – corrections

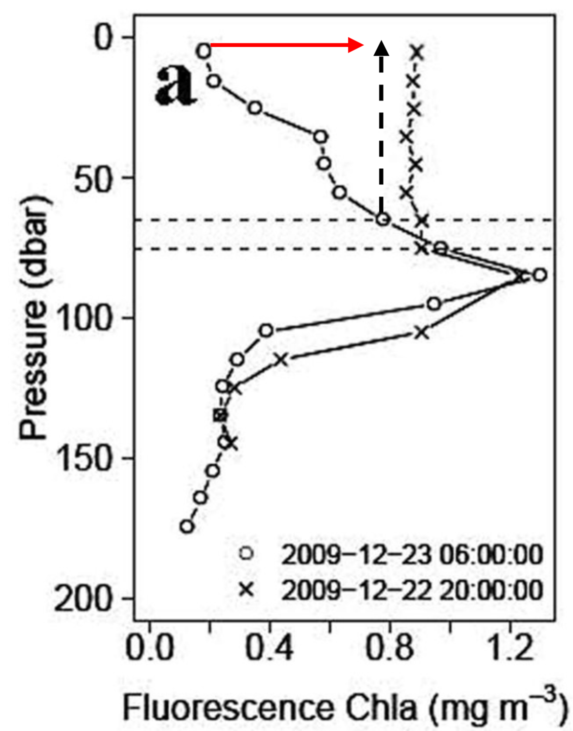
- mooring – extrapolate over nighttime observations
- Profiler – extrapolate over the mixed layer
- Glider – use F:bb ratio from nighttime observations

Roesler and Barnard 2013



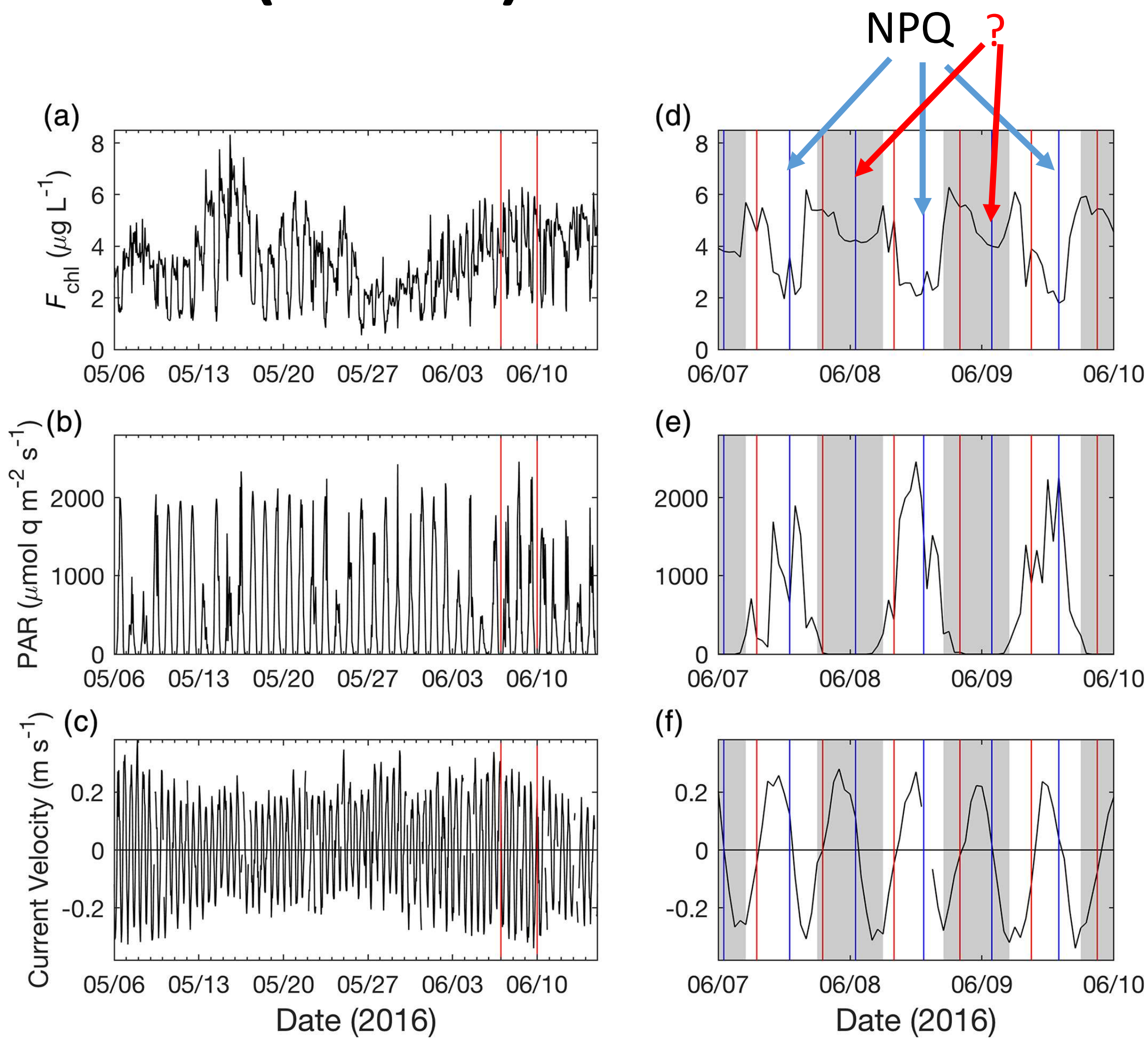


Sackman et al. 2008



Xing et al. 2012

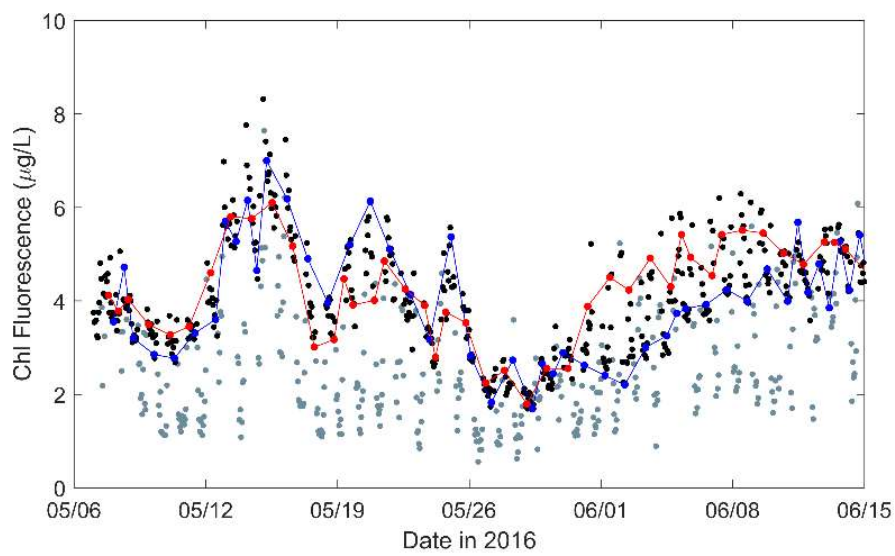
Diel (NPQ) and Tidal variability



Carberry et al. 2019

Diel (NPQ) and Tidal variability

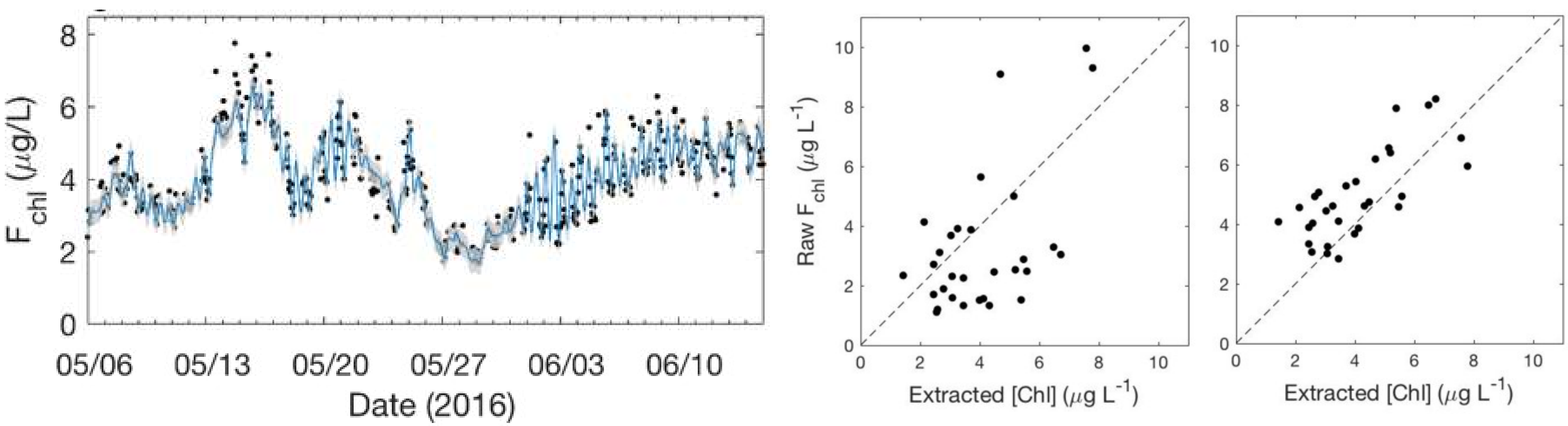
- Identified NPQed observations
- Identified high and low tide conditions
- Identified unquenched high and low tide observations



Carberry et al. 2019

Diel (NPQ) and Tidal variability

- Fit hourly tidal sinusoid to unquenched high and low tide observations
- Encapsulates most non-quenched observations
- Validated with weekly discrete [Chl] observations



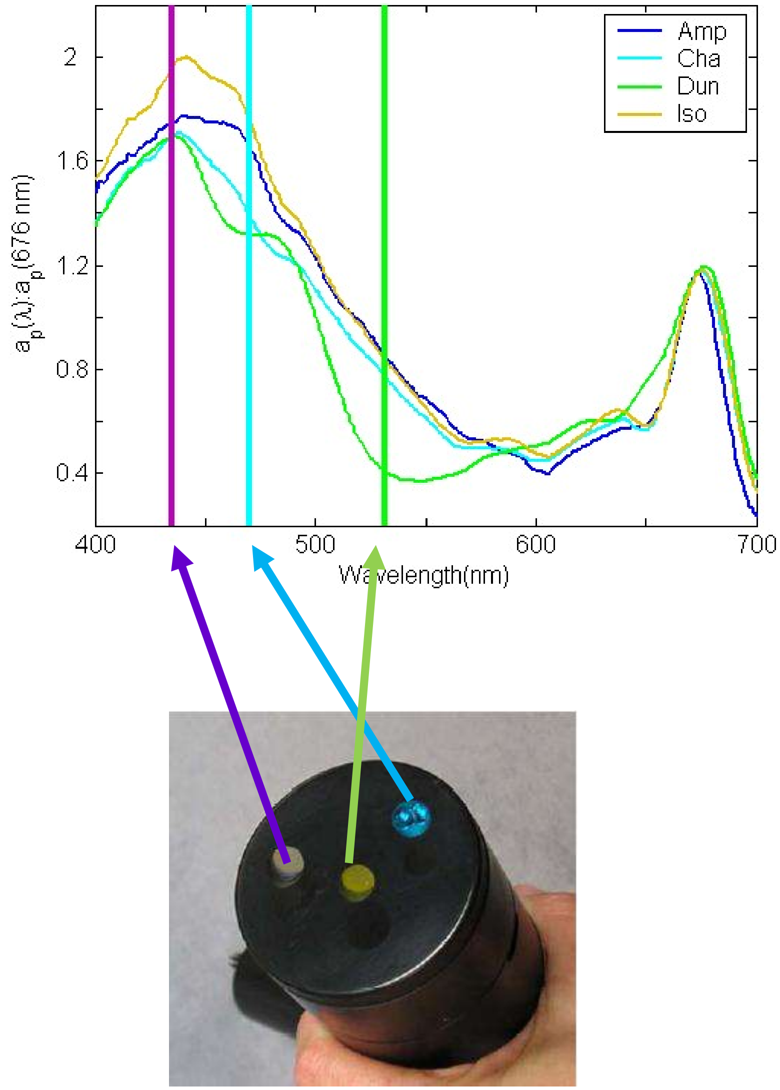
Carberry et al. 2019

Outline

- Classifying Pigment
- Diagnostic pigments and taxonomy
- Quantifying Pigments with HPLC
- Estimating Pigments
  - absorption deconvolution
  - Single channel fluorescence
  - Multichannel fluorescence

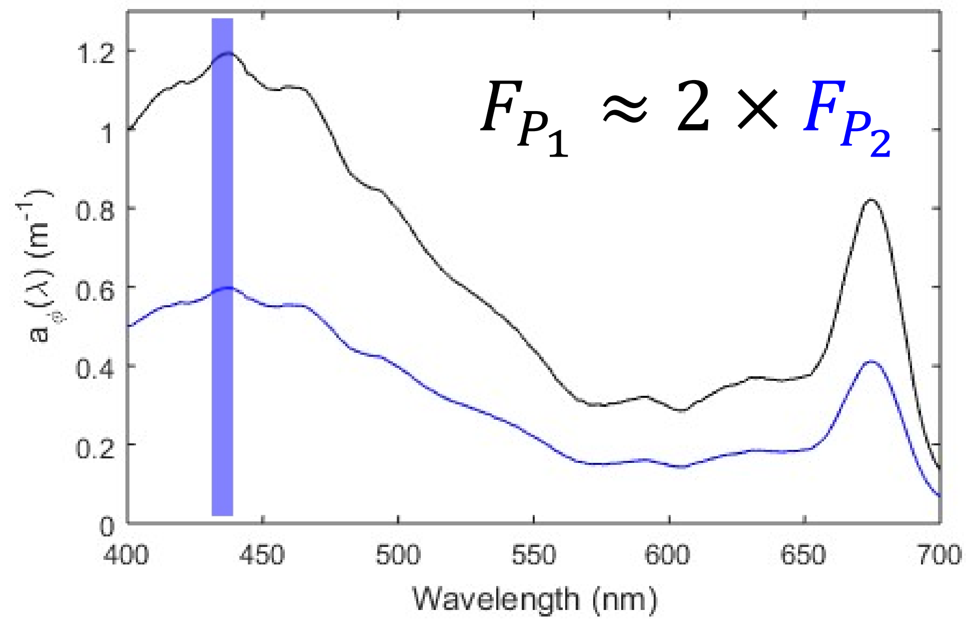
Multi-excitation chlorophyll fluorescence

- Not a new idea (e.g. Yentsch and Phinney 1985; Poryvkina et al. 1994; Seppälä et al. 1998; MacIntyre et al 2010)
- Multichannel fluorometers enable *in situ* observations
- Based upon probing different spectral regions (pigment bands)
- → pigment-based taxonomic differences
- Pigment-based PFGs
- e.g., WETLabs 3X1M (3 excitation LEDs, 1 fluorescence emission detector (695 nm), new model F3



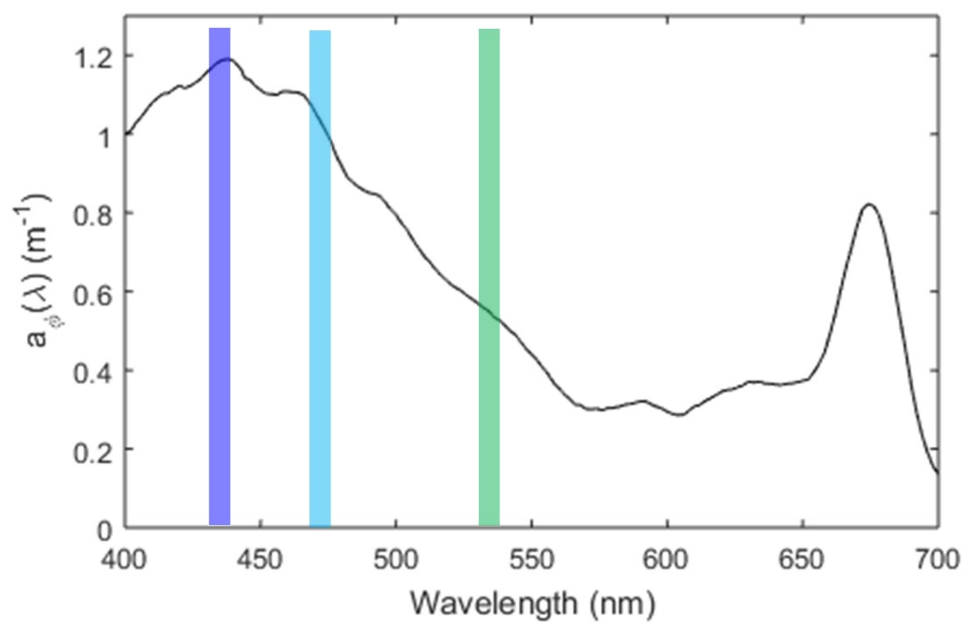
A quick quiz part 1

- Given two phytoplankton populations, P_1 and P_2
- Excite with 440 nm LED
- What is the relative measured fluorescence response?



A quick quiz part 2

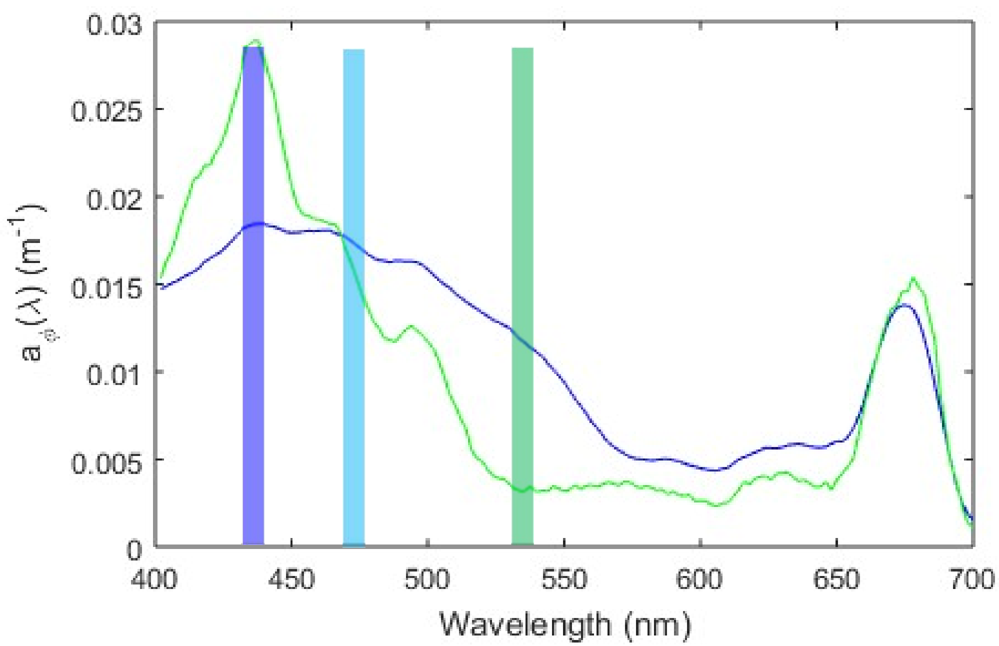
- Given one phytoplankton population
- Excite with 440 nm, 470 nm, 532 nm LEDs (assume same constant quantum flux)
- What is the relative measured fluorescence response?



$$F_{Ex_1} > F_{Ex_2} \gg F_{Ex_3}$$
$$F_{Ex_1} \approx 2 \times F_{Ex_3}$$

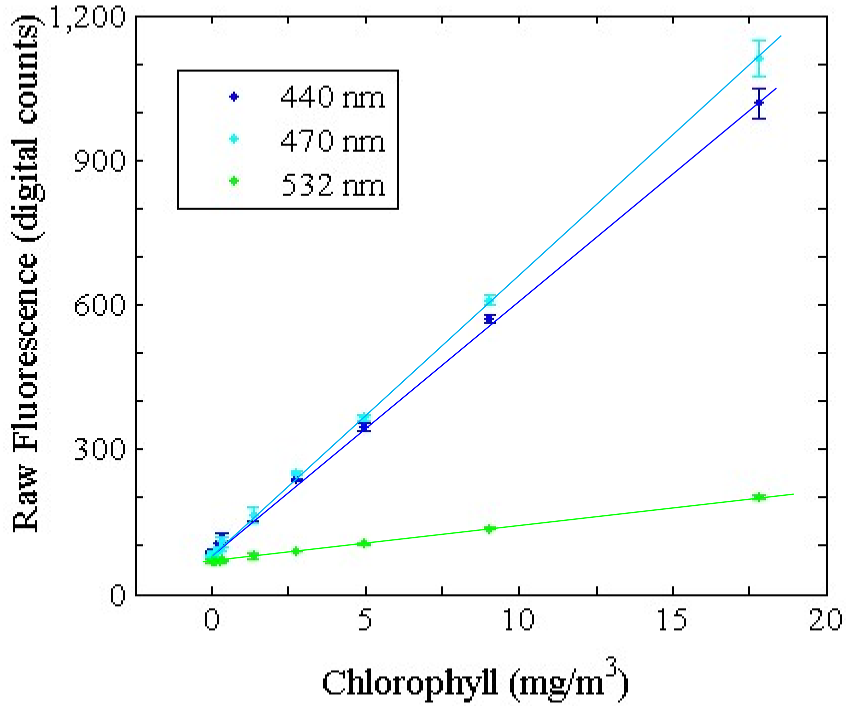
A quick quiz part 3

- Given two phytoplankton populations, P_1 and P_2
- Excite with 440 nm, 470 nm, 532 nm LEDs (assume same constant quantum flux)
- What is the measured relative fluorescence response at each λ_{ex}?



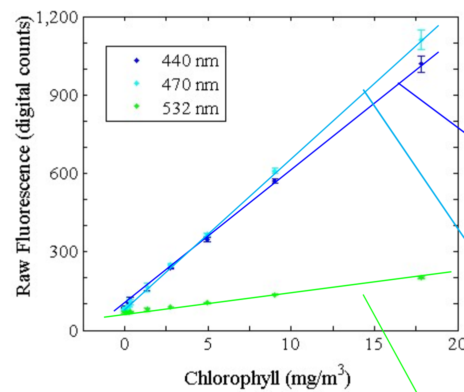
There is a lot of information in just 3 channels!

Calibrate the 3-channel fluorometer in the same manner

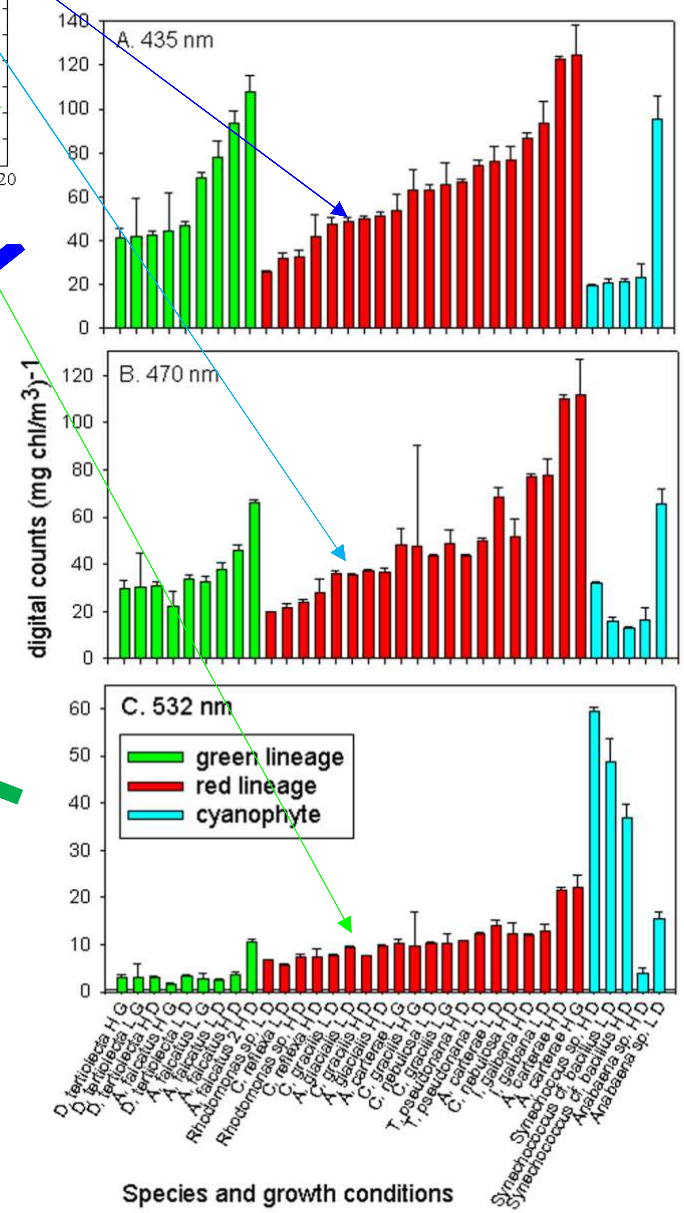


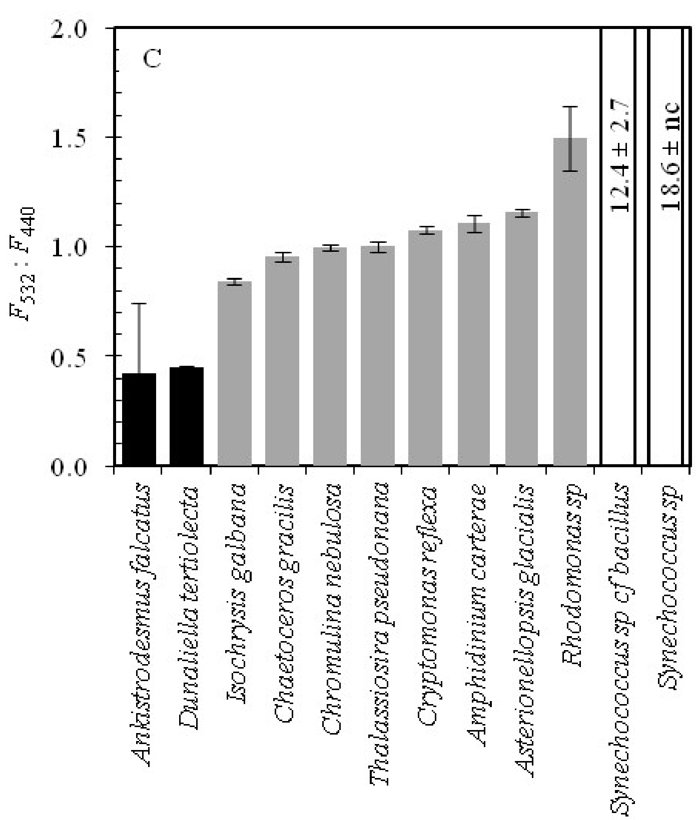
- 19 cultures
- H, L growth irradiance
- Exponential and stationary phase
- Dilution series for each culture
- Obtain slope for each experiment

3-channel fluorometer calibration

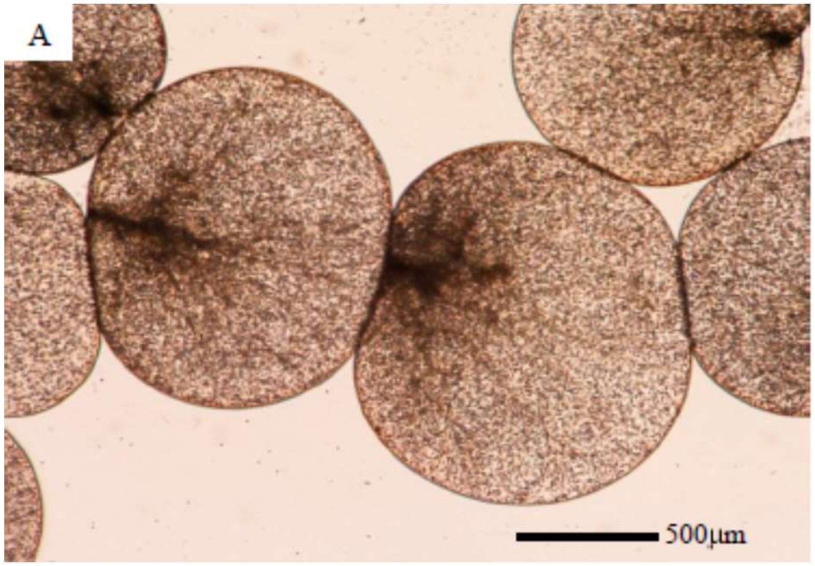


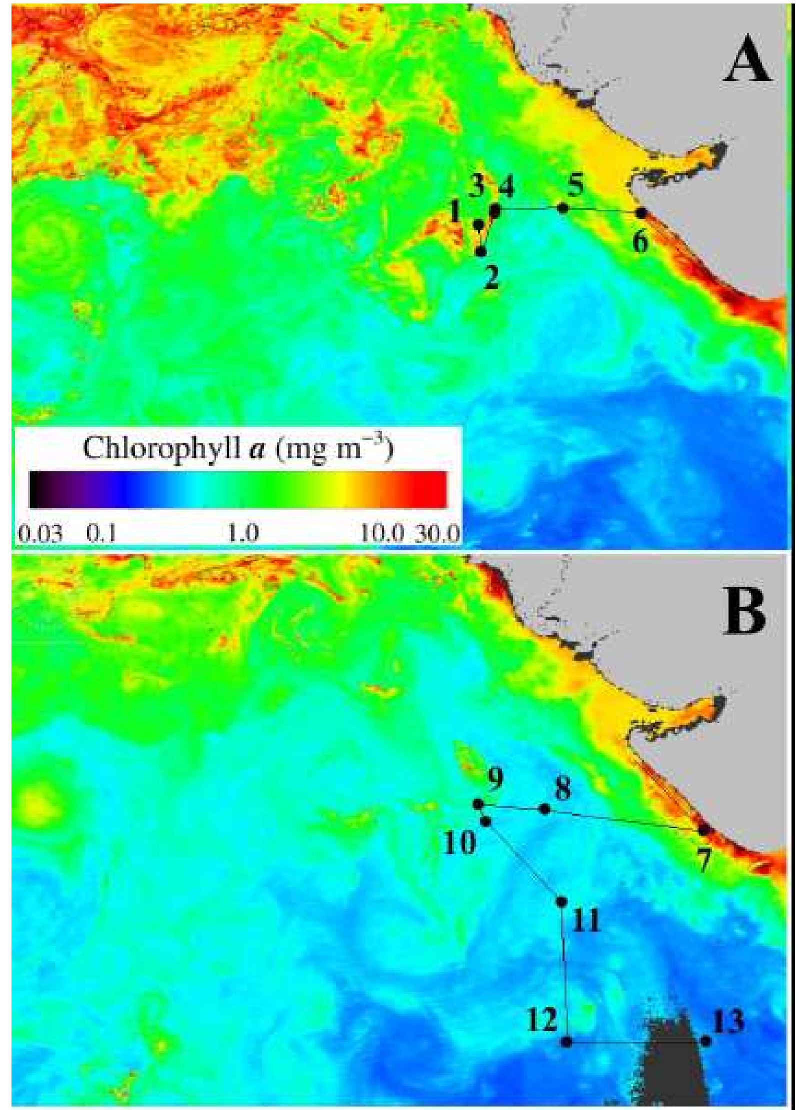
- Plot cal slopes for each λ, each species
- Calculate Fluorescence ratios





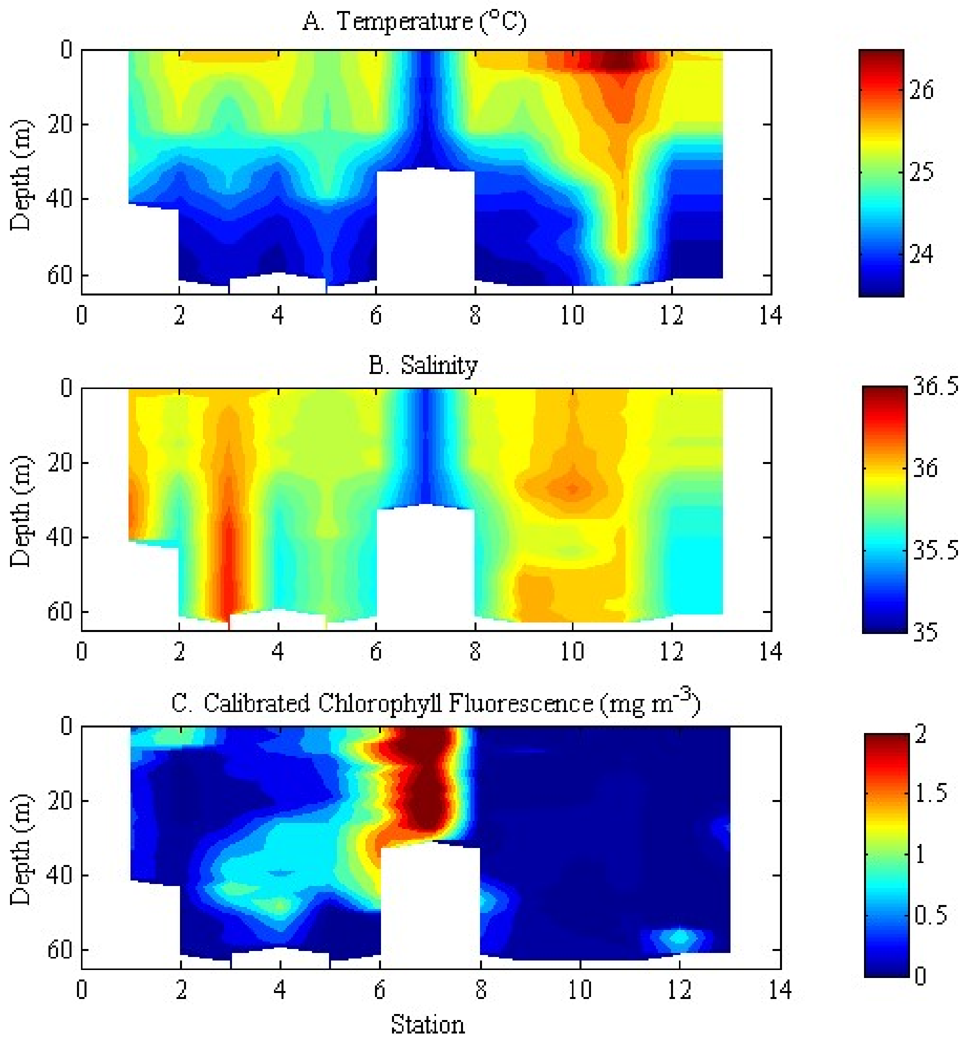
Application: The Arabian Sea

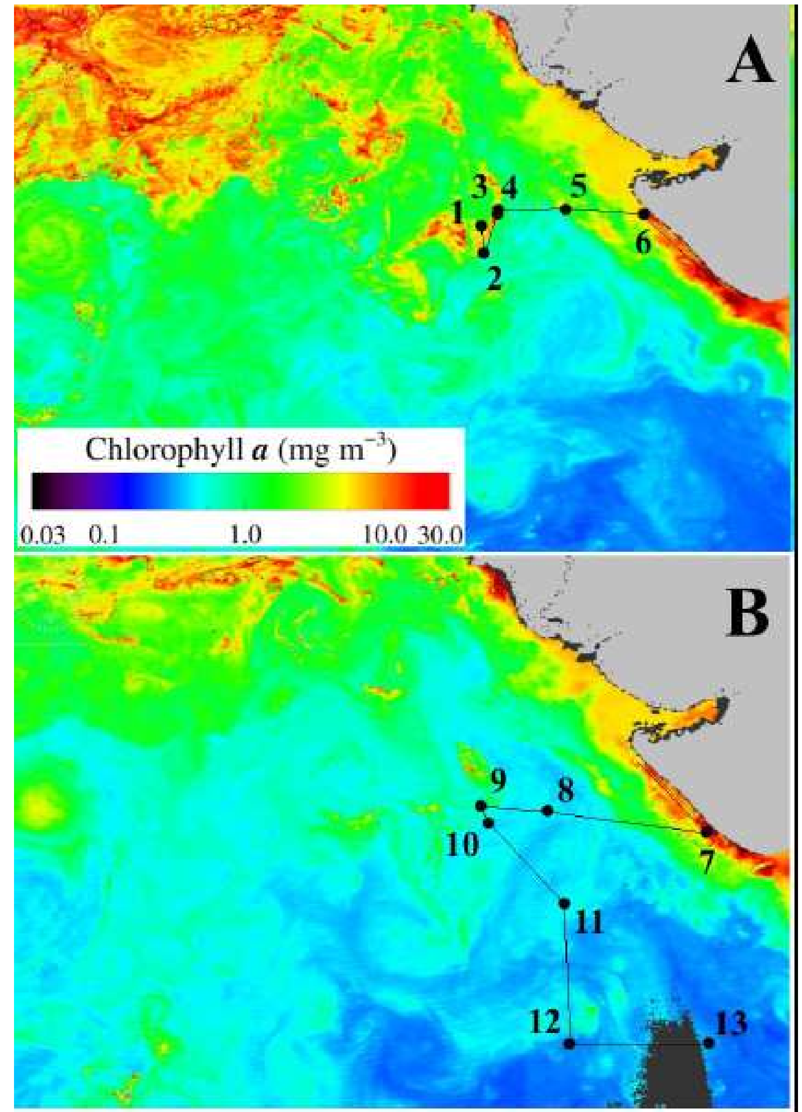




Thibodeau et al. 2014

Application: The Arabian Sea

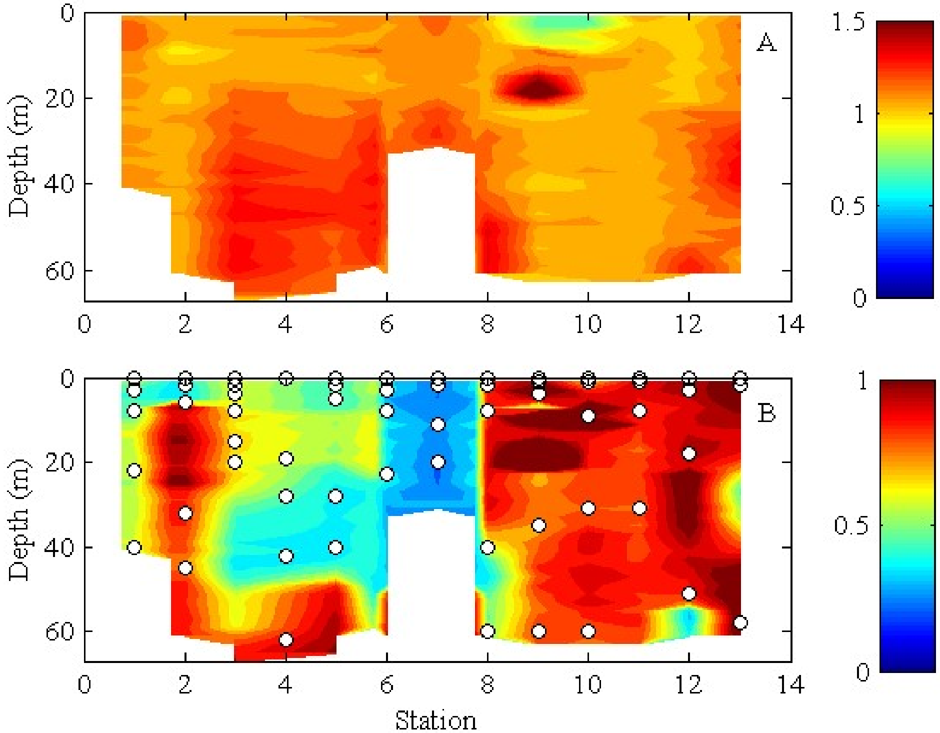




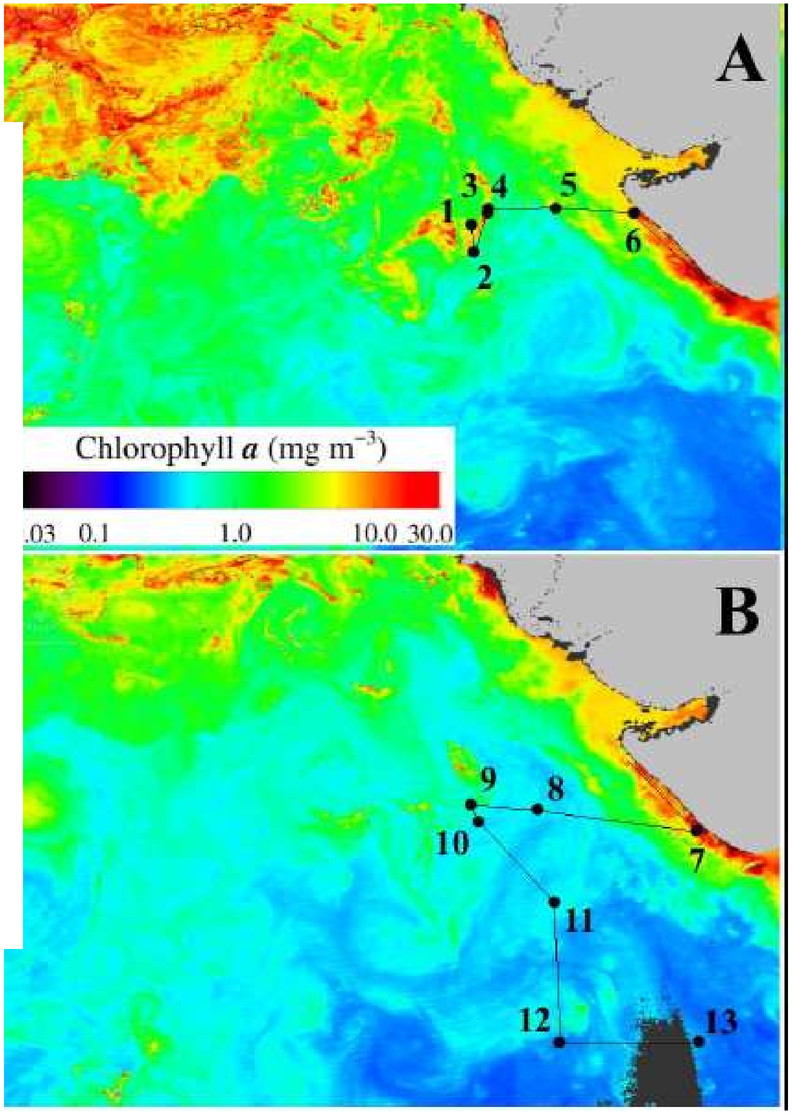
Thibodeau et al. 2014

Application: The Arabian Sea

- F470:F440



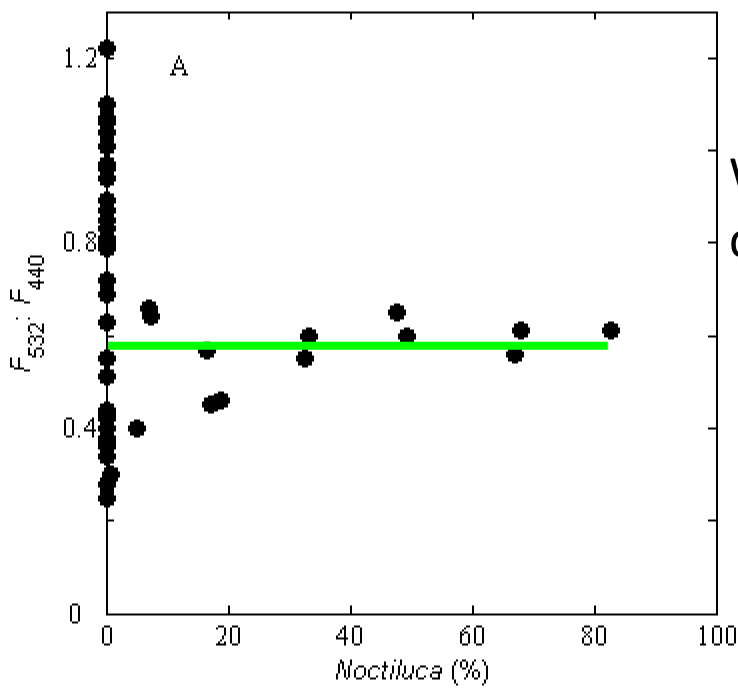
- F532:F440



Thibodeau et al. 2014

Application: The Arabian Sea

- Fluorescence ratios
- Microscopy



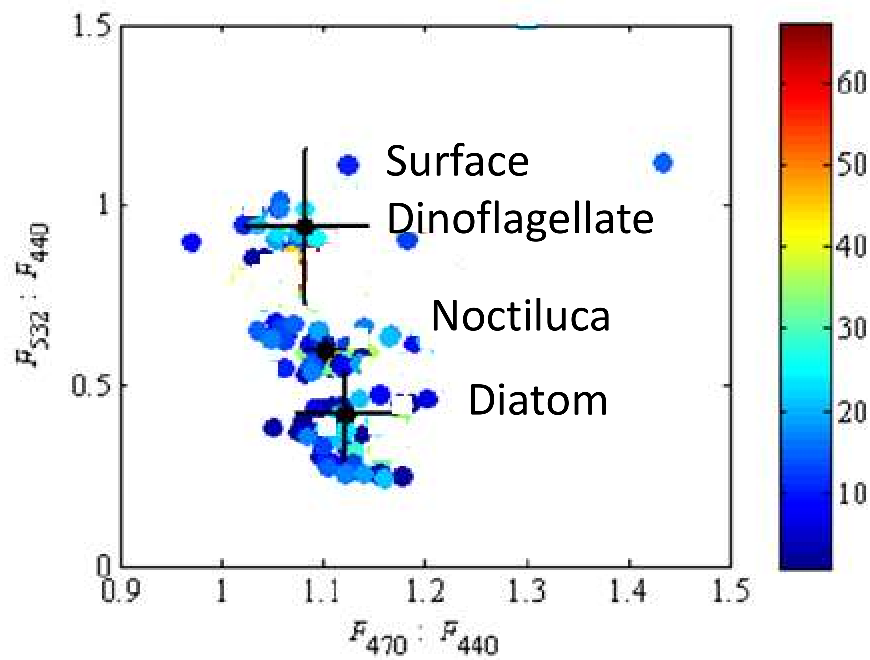
When *Noctiluca* >30% of population, it dominates the F532:F440 fluorescence ratio

Thibodeau et al. 2014

Application: The Arabian Sea

- Fluorescence ratios (for 90% dominance)
- Plot in ratio/ratio space with surface obs (<20m)
- Observe data three clusters associated with three plankton groups

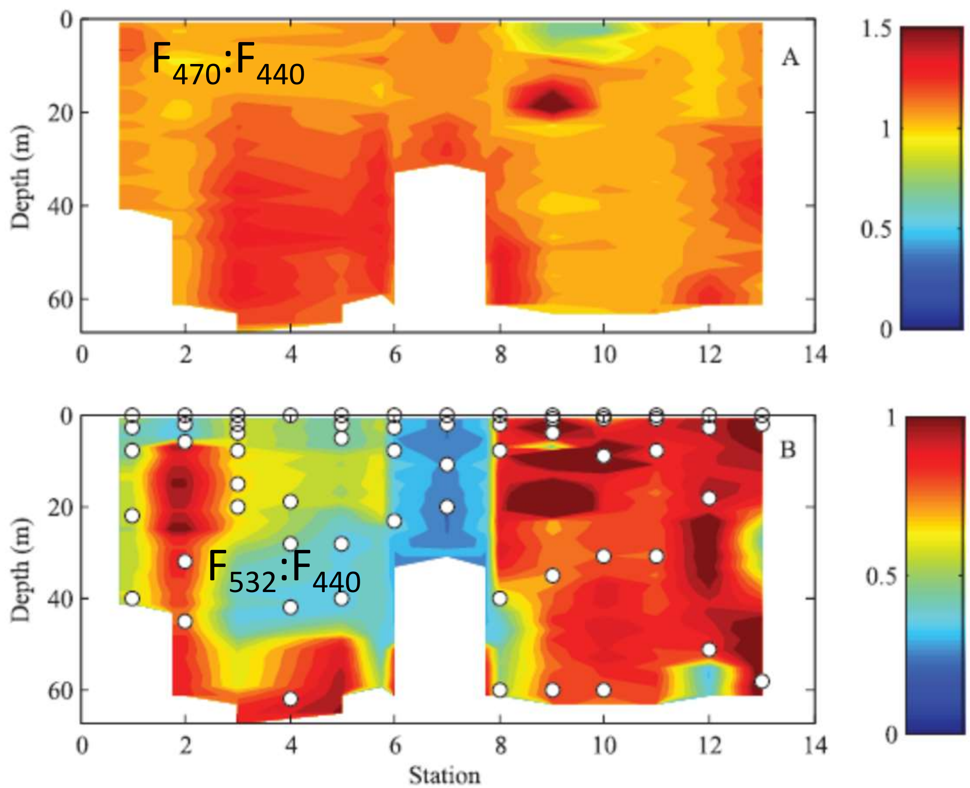
Phytoplankton group	$F_{532}:F_{440}$	$F_{470}:F_{440}$
N. miliaris	0.60 +/- 0.03	1.10 +/- 0.02
Diatom	0.42 +/- 0.13	1.12 +/- 0.05
Dinoflagellate	0.94 +/- 0.22	1.08 +/- 0.06

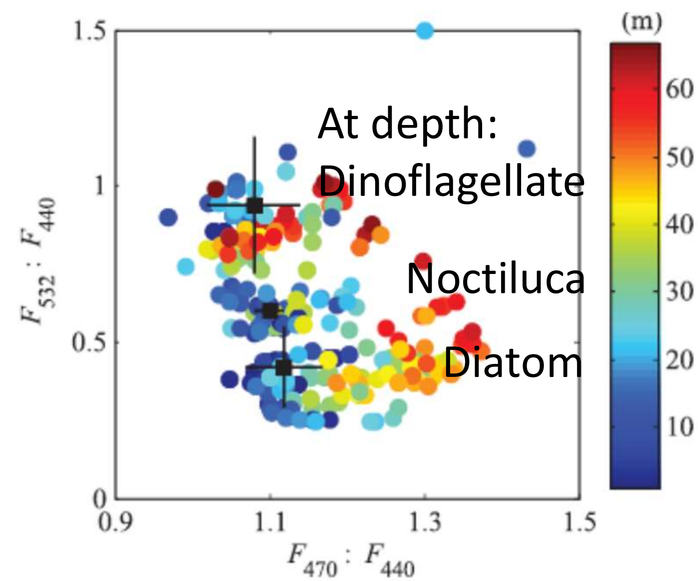


Thibodeau et al. 2014

F_{470}:F_{440} and F_{532}:F_{440} statistically distinct between pigment groups

- Along a transect in the Arabian Sea

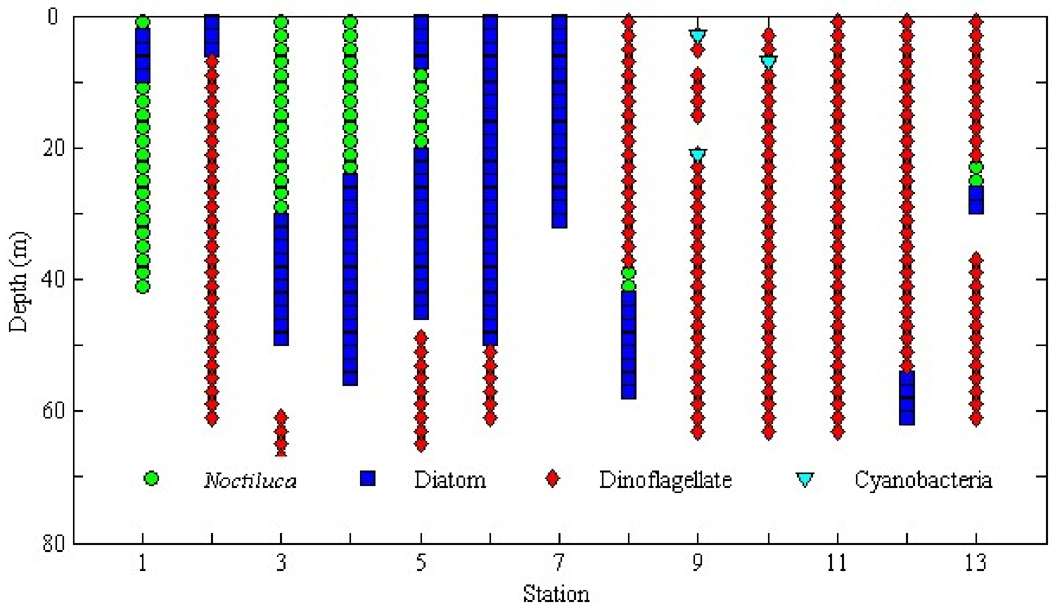




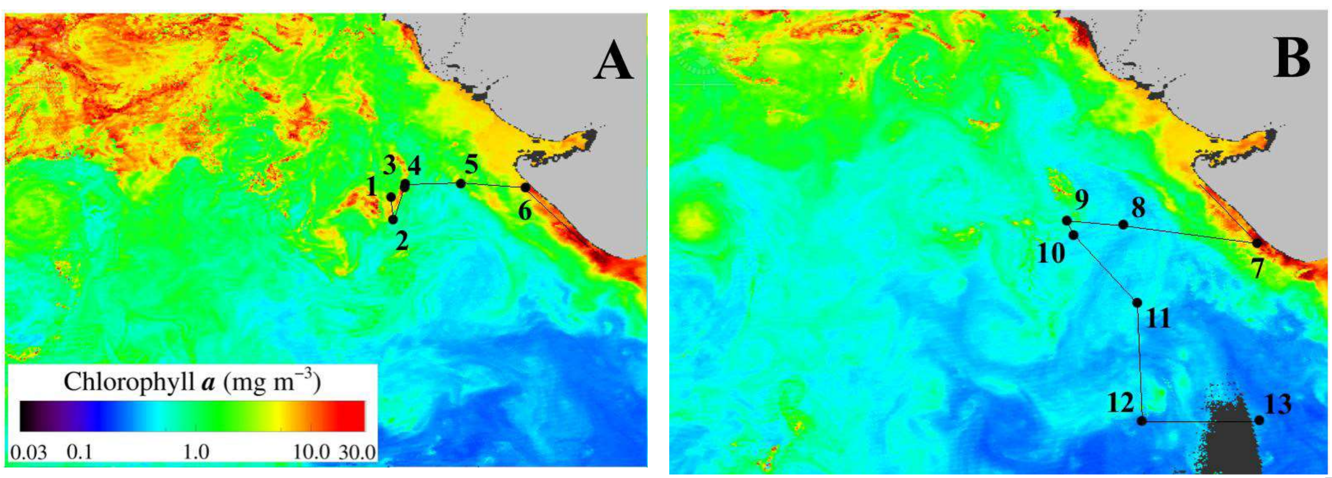
- Data points cluster
  - Diatoms
  - Dinoflagellates
  - *Noctiluca*

Thibodeau et al., 2014 Limnol Ocean

Application: The Arabian Sea

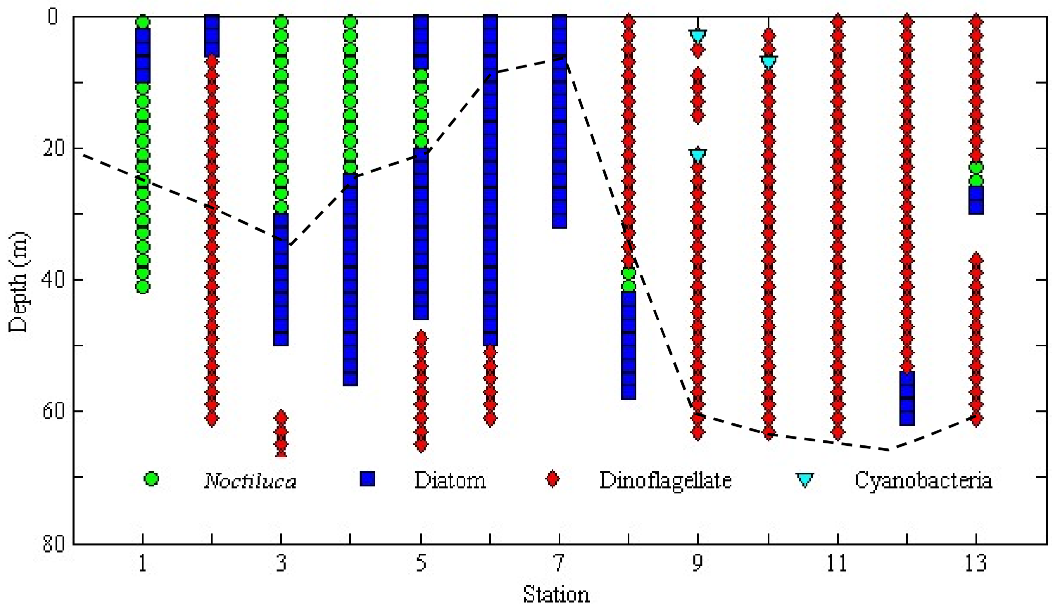


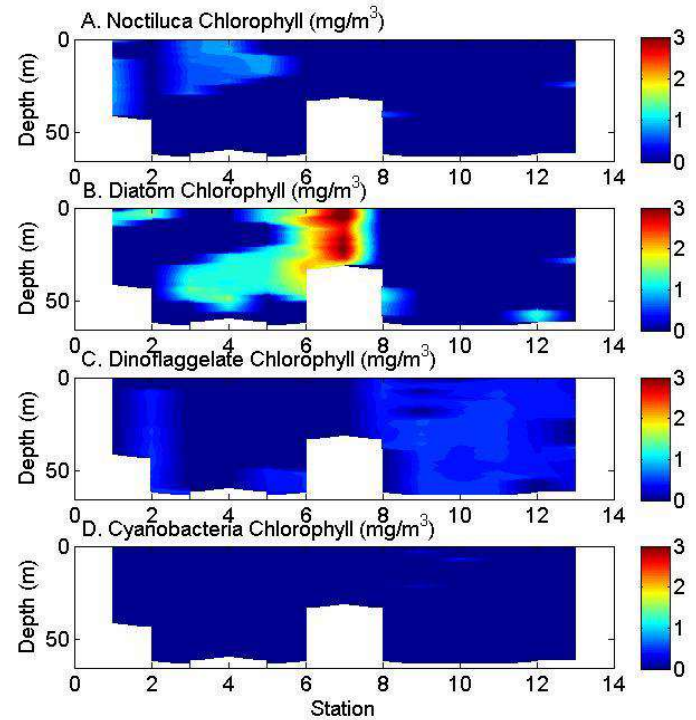
- Assign algal group to each depth bin based upon paired fluorescence ratios



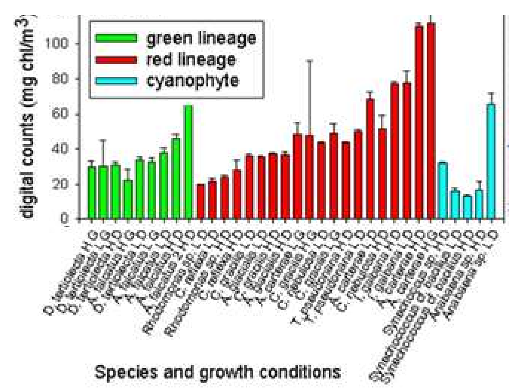
Thibodeau et al. 2014

Application: The Arabian Sea





- Apply group-specific calibration slope to Fchl observations for more accurate chlorophyll estimate
- Exponentially scale over optical depths for more accurate satellite validation



Werdell et al. 2014

Pigments

- ‘Easy’ to measure if you are good in the lab
- Enticing products to work with because of their relationships to phytoplankton classification, photosynthesis, absorption spectra
- Take care in the interpretation