**Lab 3: particle absorption and pigments**   **10 July 2013**

LABORATORY SAFETY ISSUES – Isopropyl alcohol for cleaning ac-x; methanol for Kishino method; 90% acetone for extract of pigments; 10% HCl.   ONLY use methanol under the hood. Wear goggles and gloves when handling solvents and acids. See MSDS sheets for hazardous chemicals. General laboratory safety.

INTRODUCTION
The major absorbers in seawater are water itself, chromophoric or color-absorbing dissolved organic matter, and absorbing particles. Total particulate absorption is designated as $a_{part}(\lambda)$. Phytoplankton are one of the dominant types of absorbing particles, due to their photosynthetic and photoprotective pigments; phytoplankton pigment absorption is designated as $a_{phyt}(\lambda)$. The difference between as $a_{part}(\lambda)$ and $a_{phyt}(\lambda)$ is designated as $a_{NAP}(\lambda)$, a term introduced by Babin et al. (2003). a_{NAP} represents the absorption associated with compounds (chemicals) that cannot be extracted by a polar solvent such as methanol. While the term NAP refers to absorption by Non-Algal Particles, that term is technically incorrect because water-soluble pigments such as the phycobilins are not removed from the filter pad by methanol. NAP should, therefore, be considered as absorption of non-methanol extractable materials and may include phycobilin proteins, cytochromes, absorbing minerals, and non-pigmented components of phytoplankton frustules, etc.
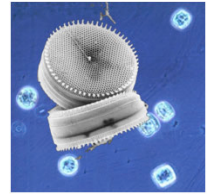
Absorption coefficients are additive, hence:

$$a_{part}(\lambda) = a_{phyt}(\lambda) + a_{NAP}(\lambda)$$
.

Phytoplankton absorption arises primarily from absorption by pigments. Chlorophyll *a* is one of the dominant pigments, and is present in all oxygen-evolving photosynthesizers (with the exception of *Prochlorococcus*, which possesses the divinyl version of chlorophyll *a*). Chlorophyll *a* is the most widely used index of phytoplankton biomass, whether rightly or wrongly so. Most marine eukaryotic phytoplankton contain chlorophyll *c* (although Prasinophytes contain chlorophyll *b* instead) and carotenoids.
Caotenoids can be photosynthetic or photoprotective. Phytoplankton absorption can be correspondingly decomposed into absorption by photosynthetic (PS) and photoprotective (PP) pigments. This is done by using a fluorescence excitation technique, which is based on excitation of chlorophyll *a* (Culver and Perry, 1999); only photosynthetic pigments transfer energy to chlorophyll *a*, which then fluoresces. This distinction is important for absorption based models or assessments of primary productivity, because only $a_{PS}(\lambda)$ leads to photosynthesis:

$$a_{phyt}(\lambda) = a_{PS}(\lambda) + a_{PP}(\lambda)$$
.

The absorption of NAP particles can be separated from that of pigments by extracting the pigments on a filter pad with a strong polar solvent (methanol extraction method of Kishino et al., 1985) or with oxidation by bleach on the filter or H_2O_2 for particles in suspension. NAP particles are composed of suspended inorganic mineral particles (min); organic particles including cell material that absorbs but is not methanol extractable, detrital material and non-phytoplanktonic living organisms (d); and non-methanol extractable pigments such as

phycobilins (the later is typically assumed to be insignificant, which is not always true, particularly in freshwaters with high abundance of cyanobacteria):

$$a_{NAP}(\lambda) = a_{min}(\lambda) + a_d(\lambda) + a_{other}(\lambda)$$.

In practice, is difficult to physically separate the above subcomponents, and we will not attempt to do so. (N.B.: in older literature, the term detrital absorption, a_d, was commonly used in place of non-algal particles, a_{NAP}, and should not be confused with the a_d term above which does include living and non-living detrital organic matter.)

STATIONS AND SAMPLES:
- spectrophotometric measurements of acetone extracts of pigments and of particles retained on a G/FF filter (from whole DRE seawater and a phytoplankton culture)
- ac-s/ac-9 measurements of whole DRE seawater and a phytoplankton culture

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

	1st session	2nd session
Group 1	Station 1 – spec in MJP Lab: acetone extract of spinach; absorption of particles from DRE	Station 2 – Mitchell Classroom: DRE water; ac-s
Group 2	Station 1 – spec in MJP Lab: acetone extract of DRE water; absorption of particles from DRE	Station 2 – Mitchell Classroom: culture; ac-s
Group 3	Station 1 – spec in MJP Lab: acetone extract of culture; absorption of culture	Station 2 – Mitchell Classroom: DRE water; ac-9
Group 4	Station 2 – Mitchell Classroom: DRE water; ac-s	Station 1 – spec in MJP Lab: acetone extract of spinach; absorption of particles from DRE
Group 5	Station 2 – Mitchell Classroom: culture; ac-s	Station 1 – spec in MJP Lab: acetone extract of DRE water; absorption of particles from DRE
Group 6	Station 2 – Mitchell Classroom: DRE water; ac-9	Station 1 – spec in MJP Lab: acetone extract of culture; absorption of culture

STATION 1 – spectrophotometric measurements of absorption

SCANS OF PIGMENTS IN ACETONE EXTRACTS

Extracts of phytoplankton pigments in 90% acetone have been prepared from spinach (with chlorophyll *b*), water samples from the Damariscotta River Estuary, and a culture.
1. Use goggles when you handle acetone.
2. Run Cary-50 calibration protocol.
3. Set scan limits to 300 – 800 nm at medium scan speed.
4. Use 1 cm cuvette and 90% acetone as blank; store baseline.
5. Transfer an aliquot of extract to 1 cm cuvette and run scan; store file.
6. Add drop 10% HCl and rescan.
7. Discard acetone extract in designated waste container.
8. Rinse cell WELL with water and acetone to remove acid.

QUANTITATIVE FILTER TECHNIQUE (QFT)

1. Blanks: filter two volumes of filtered seawater through two different G/FF filter pads to use as filtered seawater blank – 100 mL and 300 mL.
2. Samples: filter 2 volumes of sample through two different G/FF filter pads. The volumes for DRE may be adjusted; culture volumes will be determined based on cell density):
   > Groups 1 & 4: DRE water, 100 mL and 300 mL
   > Groups 2 & 5: DRE water, 300 mL and 500 mL
   > Groups 3 & 6: culture, ---- mL and --- mL

   In principal, the target absorbance values on the spectrophotometer are ~ 0.1 – 0.2 for the red peak and 0.4 – 0.5 for the blue peak. In practice, expect some discussion and trial and error to get the desired absorbance values.
3. Place all filter pads immediately on moist Kimwipes in a Petri dish in dim light; note and record position of filters. Hold filter with forceps by the clean edge only (don't scrape filtered material off the pad).
4. Milli-Q blank: moisten a G/FF filter pad with Milli-Q water.
5. Measure the diameter of the filtered area with the calipers.
6. Run Cary-50 calibration protocol and review settings; set scan limits to 300 –800 nm and medium scan speed.
7. Blank Cary 50 spectrophotometer in air and save air scan as baseline.
8. Scan Milli-Q-blank. Rotate position and repeat for total of three scans.
9. Scan seawater blanks and samples. Rotate each filter and rescan three times.
10. Select the filter with greater volume and one seawater blank for Kishino methanol extraction (under hood):
    – place filters on filter rack under hood with no vacuum,
    – add about 15 mL of hot 100% methanol,
    – wait about 10 minutes, turn on vacuum, and rinse filter with filtered seawater, including under filter cup flanges,
    – carefully remove filter (it will be easy to tear) and rescan.

CHLOROPHYLL SAMPLES

Filter three samples (volume as directed) on G/FF filters for chlorophyll *a* and place in tube with 5 mL of 90% acetone; record tube number; store in freezer for 48 hours. You'll run these samples on Friday.

DATA ANALYSIS FOR QUANTITATIVE FILTER TECHNIQUE
(QFT, *sensu* Mitchell, 1990, Pegau et al., 2002, and Roesler, 1998):

The basic equation for calculating *a*, the absorption coefficient (m^{-1}), from ambsorbance (A) measured on the spectrophotometer is:

$$a_{part}(\lambda) = \frac{2.303 * 100}{pathlength * \beta} * \{A_{pad}(\lambda) - [A_{blank}(\lambda) - A_{null}("\lambda")]\}$$

Absorbance is multiplied by 2.303, as you did for the Tuesday lab to convert from log base$_{10}$ to natural log, to compute the absorption coefficient.

A null value – essentially an offset – may need to be applied if the filters vary considerably in water content (hydration) or manufacturing. See Pegau et al. (2002) for an extensive discussion of "issues" with selecting a null wavelength where theoretically absorption is zero or negligible. Also see Babin and Stramski (2002). You may take the mean Absorbance between 730 and 750 nm for "λ" in A$_{null}$ (or, you can investigate expanding or shifting the range).

The optical pathlength of particles on the filter is equivalent to the pathlength, as if the particles were in suspension:

$$pathlength = \frac{Volume\ filtered\ (cm^3)}{Area\ of\ the\ filter\ (cm^2)}$$

Area is calculated from the diameter of the portion of the filter that actually collects the particles (use high quality caliper). The factor of 100 convert cm to m.

As well as collecting particles, the glass fiber filter also provides a highly diffusing environment for the spectrophotometric measurement, and increases the effective or geometric pathlength that photons travel between the source and detector. The consequence is higher photon absorption (on the order of a factor of two). The pathlength amplification parameter is termed the β correction factor (Mitchell, 1990). Try both methods for equivalence of the β correction: 1) At high pad loadings the β correction factor approaches 2.0, for theoretical reasons discussed in Roesler (1998). 2) Cleveland and Weidemann (1993), equations below, determined an absorbance dependency of the path amplification that they found to be wavelength independent; this relationship replaces the β correction factor in the first equation, where:

$$A_{\beta\text{-corrected}}(\lambda) = 0.378\ \{A_{pad}(\lambda) - [A_{blank}(\lambda) - A_{null}("\lambda")]\} + 0.523\ \{A_{pad}(\lambda) - [A_{blank}(\lambda) - A_{null}("\lambda")]\}^2$$

$a_{part}(\lambda) = $ 1) Roesler: $\dfrac{2.303 * 100\ (cm/m)}{pathlength\ (cm) * 2}$ or 2) Cleveland: $\dfrac{2.303 * 100\ (cm/m}{pathlength\ (cm)} * A_{\beta\text{-corrected}}(\lambda)$

$a_{part}(\lambda)$ – is measured by first collecting particles from seawater on a G/FF (i.e., fine glass fiber filter).

$a_{NAP}(\lambda)$ –is measured by removing all methanol-extractable pigments with hot methanol (Kishino et al., 1985); the residual absorption is due to non-pigmented organics, minerals, and non-methanol extractable absorbing organic material.

$a_{phyt}(\lambda)$ –is measured by difference between $a_{part}(\lambda)$ and $a_{NAP}(\lambda)$.

STATION 2 – ac-s/ac-9 measurements for particles and cells

INTRODUCTION
The particulate absorption coefficient can be resolved from measurements of total absorption and filtered absorption, measured with an ac-9 and ac-s, by

(1) taking two profiles with a single meter, first without a filter and the second with a filter at the intake. The difference between total and filtered absorption is the particulate absorption:

$$a_{part}(\lambda) = a_{Total}(\lambda) - a_{CDOM}(\lambda) \ .$$

(2) with two meters, one with a filter on its intake port. A single profile is performed and the two sets of observations are rectified for differences in flow rates (see Roesler and Boss 2007) and then the difference is used to compute $a_{part}(\lambda)$.

A third implementation of this approach, applied to a single instrument in-flow through mode involves an automated filter switch into position at short intervals (see Slade et al. 2011). N.B.: the ac-instruments do not collect all the scattered light. Hence a correction that relies, in the least, on the simultaneous measurements performed with the c-side, needs to be implemented.

MEASUREMENTS
Clean the sensor windows and tubes prior to measurements with lens paper and ethanol.

1) Focus on getting good cals with Milli-q water, both a and c tubes.
2) Run filtered seawater or medium through a and c tubes.
3) Run unfiltered DRE water or culture through a and c tubes.

Groups 1, 4	ac-s	DRE water
Groups 2, 5	ac-s	culture
Groups 3, 6	ac-9	DRE water

Measure the temperature of every sample at time of analysis.
Salinity of the Damariscotta River Estuary was 28 on 2 July.

ASSIGNMENTS – DIVIDE THE WORK AND CONQUER! COME PREPARED TO DELIVER A BRIEFING TOMORROW MORNING.

QFT:

1. For the QFT, how variable are the blanks? Are the filtered seawater blanks the same as the Milli-Q blanks?
2. Does the choice of null wavelength(s) matter? Try using an average Absorbance for 720 – 750 nm.
3. What is the effect of an error in the measurement of the filter diameter?
   $(A = \pi r^2)$.
4. Calculate $a_{part}(\lambda)$ sample using two methods for the β correction factor and consider why they (dis)agree:

   $\beta = 2.00$, with uncertainty calculation (Roesler, 1998);

   β with an Absorbance-dependence (Cleveland and Weidemann, 1993); see

   equation above for $A_{\beta\text{-corrected}}(\lambda)$.
5. For DRE water, select a wavelength maximum (e.g., 440 nm) and a wavelength minimum (e.g., 555 nm). Plot Absorbance vs. volume filtered (four volumes). Are the plots linear? If not, any ideas why not?
6. Calculate $a_{phyt}(\lambda)$ and $a_{NAP}(\lambda)$ for the DRE sample and the culture:
   – for DRE, compare $a_{part}(\lambda)$ with $a_{phyt}(\lambda)$; how do they differ?
   – for DRE, compare spectrum of NAP with that of CDOM; are their spectral slopes similar?
   – for culture, compare $a_{part}(\lambda)$ with $a_{phyt}(\lambda)$; how do they differ?
   – compare spectra of $a_{phyt}(\lambda)$ for DRE and culture; how do they differ?

AC-METER FOR PARTICULATE ABSORPTION
(Roesler, 1998; Twardowski et al., 1999; Slade et al. 2010)

7. How did the pure water calibration for each ac-meter compare with yesterday's calibration?
8. Compute **a** and **c** for each set of observations – whole DRE sample and filtered sample; repeat for culture and cell-free medium. You can use provided Excel templates or MATLAB code. Apply the following scattering corrections to the absorption scans
   – apply the spectrally flat correction by subtracting the a(715) offset from **a**(λ)

   – apply the spectrally varying scattering correction presented in class. For ac-9:
   $a(\lambda) = a_{TS}(\lambda) - b(\lambda) * \dfrac{a_{TS}(715)}{b(715)}$

   For ac-s, one could use a wavelength further into the near infrared, e.g.:
   $a(\lambda) = a_{TS}(\lambda) - b(\lambda) * \dfrac{a_{TS}(730)}{b(730)}$

   where $a_{TS}(\lambda)$ indicates temperature and salinity corrected absorption observations, $b(\lambda) =$

$c_{TS}(\lambda) - a_{TS}(\lambda)$, and $b(715) = c_{TS}(715) - a_{TS}(715)$.

N.B.: When backscattering measurements are available, there exists a correction for the ac-9 that uses those values, e.g., McKee et al., 2008. A recent paper by Leymarie et al. (2010) assessed the likely uncertainties associated with the different corrections.

9. For each scattering correction, compute $a_{part}(\lambda)$ from the difference between unfiltered and filtered observations: $a_{Total}(\lambda) - a_{CDOM}$.

COMPARISONS:

10. Compare $a_{part}(\lambda)$ from the spectrophotometer with $a_{part}(\lambda)$ from the ac-9 or ac-s for the culture. Are they similar? Is $a_{part}(\lambda)$ from one filter pad volume closer?

11. Compare $a_{part}(\lambda)$ from the spectrophotometer with $a_{part}(\lambda)$ from the ac-9 or acs- for the culture. Are they similar? Is $a_{part}(\lambda)$ from one filter pad volume closer?

PHYTOPLANKTON PIGMENTS:

12. Compare the spectral shapes of the 90% acetone extract of culture and Damariscotta River Estuary sample. What are the major differences? (hint: first normalize the spectra to 676 nm).

13. Compare the spectral shape of the 90% acetone extract of the culture and the Damariscotta River Estuary sample with that of the corresponding $a_{phyt}(\lambda)$ by normalizing both spectra to the red peak. How similar and different are they? Why are they different?

14. How does acid change the spectral shape of the acetone scans? Note the change in absorption at 412 nm and 676 nm. N.B.: pheopigments have 54% of chlorophyll *a* absorption at 676 nm.

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