Lab 3: particle absorption and pigments  

**Laboratory Safety Issues** – Isopropyl alcohol for cleaning ac-meter cleaning; methanol for Kishino method; 90% acetone; 10% HCl. Methanol must be used under the hood. Wear goggles and gloves when handling solvents and acids. See SDS sheets for hazardous chemicals. General laboratory safety.

**Introduction**

Particulate absorption measurements provide the second part of the determination of absorption by aquatic systems, dissolved measurements being first. Suspended particulate absorption is obtained in two ways: first, by difference between paired spectrophotometric measurements of total and filtered sample in cuvettes, and second by filtration onto glass fiber filters and direct spectrophotometric analysis. This lab builds upon Lab 2 to demonstrate three ways of measuring particulate absorption spectra, with ac-meters, in cuvettes placed inside an integrating spheres, and on filters placed inside an integrating sphere. Further, this lab introduces students to the analysis of chlorophyll concentration via the extractive fluorescence technique and an approach to quantifying uncertainty in analysis.

The major absorbers in seawater are water itself, CDOM, and absorbing particles. These two components are easily separated by filtration. The total particulate absorption (designated $\alpha_{\text{part}}$ or $\alpha_p$), consists of phytoplankton (designated as $\alpha_{\text{phyt}}$, $\alpha_{\phi}$ or $\alpha_{\phi}$), other living organic particles such as viruses, bacteria, zooplankton, dead or detrital organic particles, and inorganic minerals or sediments. The particulate component is operationally separated into phytoplankton and non-algal particles (NAP, a term introduced by Babin et al. 2003 that replaced the designation $\alpha_d$, detrital absorption) via extraction with a strong polar solvent such as methanol (Kishino et al. 1985). This effectively removes the phytoplankton pigments while leaving other particles intact. While NAP consists of a large range of materials, their grouping is about more than extractive techniques, the living and dead organic and inorganic constituents within NAP all have a generally exponentially-decaying absorption coefficient which is very different from phytoplankton absorption spectra which exhibit strong features associated with distinct pigments.

Absorption coefficients are additive, hence:

$$\alpha_{\text{part}}(\lambda) = \alpha_{\text{phyt}}(\lambda) + \alpha_{\text{NAP}}(\lambda)$$

(1)

The phytoplankton absorption is determined by difference between $\alpha_{\text{part}}$ and $\alpha_{\text{NAP}}$, after computation of absorption from absorbance. This method provides the best estimate of absorption by phytoplankton pigments as they were in vivo.

Other approaches to removing the phytoplankton pigments from $\alpha_{\text{part}}$ include oxidation by bleach on the filter or H$_2$O$_2$ for particles in suspension. Care should be taken when using the oxidative rather than extractive approaches because oxidized pigments have strong absorption in the UV due to the chemical modification of the pigment molecules rather than removal that occurs with solvents. This leads to overestimation of $\alpha_{\text{NAP}}$ and underestimation of $\alpha_{\text{phyt}}$ in the UV. Phycobilipigments associated with cyanobacteria are not extractable will solvents and must be removed by hot water or phosphate buffer extraction, otherwise NAP is overestimated, $\alpha_{\text{phyt}}$ is underestimated. These extractions, when gently performed, do not disrupt the particulate
matter, including cells.

**SAMPLES**
- spectrophotometric measurements of phytoplankton cultures in suspension and DRE particles on G/FF filters;
- extractive fluorescence analysis of chlorophyll and pheophytin
- ac-meter measurements of whole DRE seawater and phytoplankton cultures.

**STUDENTS** will divide into two groups of 10 students per group. Group 1 will start in the MacAlice lecture classroom (Station 1), Group 2 will start in the Mitchell classroom (Station 2). They will switch after ~2 hours.

**STATION 1** Students will divide into two groups of five to investigate benchtop spectrophotometric analysis and extractive chlorophyll analysis.

**Part A.** Each group will begin by measuring the absorbance spectrum of a concentrated phytoplankton culture in a 1-cm cuvette placed inside the integrating sphere. Filtered seawater will be used as the reference material. We will use the Cary 300 dual beam UV-vis spectrophotometer. It is critical to use gloves during this procedure to prevent contamination of the integrating sphere.

Spectrophotometer set up (allow to warm up for 20 minutes):
- Wavelength range: 350 nm – 850 nm
- Wavelength interval: 1 nm
- Slit bandwidth: 2 nm
- Scan speed: 150 nm/min

Culture samples:
1. measure an air scan. This is done by leaving the sample holder empty but in place. Select Autozero and then Start.
2. Measure a baseline scan with the 1-cm cuvette filled ¾ full with filtered seawater in the sample holder (take care to gently place the cuvette in the holder, ensuring alignment, don’t tip or sample may spill into the sphere, damaging the sphere and the PMT detector
3. Measure a zero scan by clicking Start. This will run the reference material again correcting for the baseline. The scan should be near zero (+/- 0.001) and spectrally flat.
4. Place the culture sample into the cuvette and scan.
5. Add 1 drop of bleach to oxidize the pigments; this is one technique for determining the contribution by non-algal particles. Scan again.

Filter sample:
1. repeat the autozero air scan.
2. place the plexiglass filter holder into the sample holder. Place a blank glass fiber filter face down on the holder (this orientation ensures the light beam is incident on the sample-side of the filter. Measure this as the baseline
3. Measure a zero scan
4. Place one of the DRE sample filters on the holder and scan
5. Repeat for each of the remaining 4 filters (each student will have the opportunity to measure a filter).
6. for one filter, extract with methanol to remove the phytoplankton pigments. Scan this nap sample.
**Part B.** Each group will work in the filtration room off the main culturing facility. A whole Damariscotta River water sample collected from the dock will be filtered for spectrophotometric analysis in part B above. Each student will filter one sample but each filter will have different volumes filtered: 200 ml, 300 ml, 400 ml, 500 ml, 600 ml. In addition, prepare 3 blank filter pads with at least 200 ml of 0.2 µm-filtered seawater. Bring the filters to the students in the spectrophotometry lab.

Chlorophyll analysis. Each student will prepare triplicate sample for chlorophyll analysis. Filter 250 ml of Damariscotta River water for each triplicate. Use a pipette to ensure the most accurate volume. The largest source of error is not maintaining a well-mixed sample. Swirl three times in each direction, three time to keep mixed between measurements.

Assigned volume filtered _____________ ml. Uncertainty in filtered volume _____________ml
Place each filter into a labeled centrifuge tube. Make sure the label includes the date, the volume filtered, and your name for easy identification. We will complete the analysis next week. Place the vials into the -20C freezer.

**Station 2.** Students will divide into three groups. Each group will be assigned to either a WETLabs ac9 or acs instrument and a sample of either DRE water or one of two phytoplankton cultures (*Chaetoceros* or *Dunaliella*).

(1) Prepare the instruments by cleaning them.

(2) Perform pure water calibrations with Milli-Q water. Measure the temperature. Focus on getting good pure water calibrations: each student should run her/his own Milli-Q water cal (either a-tube or c-tube of the ac-meter, or both). Save files in your group’s folder. Note the magnitude and shape of the pure water spectra; they should be repeatable to within the instrumented resolution between calibrations (i.e. 0.005 m$^{-1}$ for absorption; 0.01 m$^{-1}$ for attenuation).

(3) Measuring the absorption and attenuation spectra of the 0.2 µm-filtered Damariscotta River Estuarine water (DRE). This will provide the best estimate of $a_{CDOM}$ for all of the samples as this water is used to dilute the cultures. Measure the temperature and salinity.

(4) Measure the assigned whole water sample. Measure the temperature and salinity.

If salinity of the Damariscotta River Estuary is not available, assume a salinity of 28.

Remember to save the files in your group’s folder:

Group A – acs with 0.2 µm-filtered DRE water and whole DRE water
Group B – ac9 with 0.2 µm-filtered DRE water and whole *Chaetoceros* culture
Group C – acs with 0.2 µm-filtered DRE water and whole *Dunaliella* culture

Instructions and code for processing data will be provided at the lab.
**DATA ANALYSIS FOR QUANTITATIVE FILTER TECHNIQUE**


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

$$a_{part}(\lambda) = \frac{2.303 \times 100 \, \frac{cm}{m}}{pathlength(cm)} \times A_{pad}(\lambda) \tag{2}$$

where 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, the factor 100 converts cm to m and the sample absorbance measured on the filter pad has been corrected for its blank (baseline):

$$A_{pad}(\lambda) = A_{sample}(\lambda) - A_{blank}(\lambda) \tag{3}$$

As discussed in class, there is much discussion regarding subtraction of the null value – essentially an offset – in the NIR to correct for scattering by suspensions in cuvettes, or by filters in the QFT method. Theoretically the integrating sphere measurements do not have scattering losses and thus any NIR signal is attributed to non-zero NIR absorption. We will explore this topic further in this lab.

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) for a discussion on null wavelength correction for filter pad absorption.

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

$$Pathlength \, (cm) = \frac{Volume \, filtered \, (cm^3)}{Area \, of \, filter \, (cm^2)} \tag{4}$$

Area is calculated from the diameter of the portion of the filter that actually collects the particles (use high quality caliper, we will use a ruler in lab, make sure you note your uncertainty in the measurement).

Effective filter diameter ______________ cm; uncertainty in diameter ______________ cm

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 when measured in transmission, rather than internally mounted integrating sphere mode). The pathlength amplification parameter was originally termed the $\beta$ correction factor (Mitchell and Kiefer, 1985) and the factor was applied to the geometric pathlength term in equation (4). A theoretical derivation of pathlength amplification (Roesler 1998) suggested that for adequately loaded filter pads (0.1<$A<$0.4) the optical pathlength was a factor of 2 greater than the geometric pathlength, when measured in
transmission mode, leading to a modification of equation (4):

\[
\text{Pathlength (cm)} = \frac{\text{Volume filtered (cm}^3\text{)}}{\text{Area of filter (cm}^2\text{)} \times 2}
\]  

(5)

Empirical estimates of pathlength amplification suggested that multiple scattering on the filter pad induced a non-linear amplification of the pathlength that depended upon the magnitude of the absorbance (e.g. Mitchell 1990; Cleveland and Weidemann 1993). These correction factors took the form of:

\[
A_{pad\_corr}(\lambda) = C_1 A_{pad}(\lambda) + C_2 A_{pad}(\lambda)^2
\]  

(6)

applied to the blank-corrected \(A_{pad}\) in equation (5) where the regression coefficients \(C_1\) and \(C_2\) varied widely across publications. \(A_{pad\_corr}\) is substituted for \(A_{pad}\) in equation (4). These previous approaches to estimating the pathlength amplification have relied on paired samples measured in a cuvette and on a filter pad, with the former representing “truth”. In reality the suspension measurements are plagued to varying degrees with scattering errors (Roesler 1998), which have yielded a lack of consensus in correction.

Recently, however, Stramski et al. (2015) compared the spectrophotometrically-measured particulate absorption for both suspended particles measured in cuvettes and filtered particles on glass fiber filters using an integrating sphere with an internally mounted sample holder (IS-mode). This configuration removes the scattering losses associated with spectrophotometric absorption and leads to more robust determination of the pathlength amplification. The IS-mode is now accepted as the preferred method for determining particulate absorption on filter pads. Although it is not yet wide spread in the field it is increasing in its implementation.

In order to provide an improved correction for pathlength amplification for measurements made in other spectrophotometric configurations (and for legacy data), the authors then computed the correction factors for filter pad samples measured in the standard transmission mode (T-mode) and the transmission-reflectance mode (T-R-mode). They additionally provided a quantitative comparison of previously published correction factors. Many of the published correction factors lead to errors approaching a factor of 2, while others were within 10%. In the absence of an IS, the T-mode approach is significantly improved using a pathlength amplification correction factor derived from IS-mode observations. This is the approach recommended by NASA in the next version of ocean optics protocols (release later this year). The equation to calculate absorption, while correcting for pathlength amplification using the internally-mounted integrating sphere mode is:

\[
A_{pad\_corr}(\lambda) = 0.323 A_{pad}(\lambda)^{1.0867}
\]  

(7)

Where \(A_{pad\_corr}(\lambda)\) is substituted for \(A_{pad}\) in the absorption equation (4) above.

\[
a_{\text{part}}(\lambda) = \frac{2.303 \times 100 \text{ cm m}^{-1}}{\text{pathlength(cm)}} \times 0.323 A_{pad}(\lambda)^{1.0867}
\]  

(8)
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. The NAP absorption is calculated as above for \(a_{part} \).

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

**Assignments – Divide the work.** Come prepared to deliver a briefing tomorrow morning.

**QFT** (internally-mounted in integrating sphere in spectrophotometer)

1) For the QFT, how variable are the blanks?
2) Do you observe a NIR offset? Is it the same for all samples?
3) What is the effect of an error in the measurement of the effective filter diameter in the computation of effective filter area in equation 5?
4) For DRE water, select a wavelength maximum (e.g., 440 nm) and a wavelength minimum (e.g., 555 nm). Plot Absorbance vs. volume filtered (for all five filter volumes). Are the plots linear? If not, any ideas why not?
5) Apply the pathlength amplification correction in equation 8. Does this change the relationship? Discuss
6) Discuss similarities and differences in \(a_{part}(\lambda)\), \(a_{phyt}(\lambda)\) and \(a_{NAP}(\lambda)\) for the DRE sample and the cultures.
7) Compare the spectral slope of \(a_{NAP}\) and \(a_{CODM}\).

**AC-meter and Internally-Mounted Cuvettes in Integrating Sphere for Particulate Absorption**

1) How did the pure water calibration for each ac-meter compare with yesterday’s calibration?
2) 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
   1. apply the spectrally flat correction by subtracting the \(a(715)\) offset from \(a(\lambda)\)
   2. apply the spectrally varying scattering correction presented in class. For ac-9:
      \[
      a(\lambda) = a_{TS}(\lambda) - b(\lambda) \times \frac{a_{TS}(715)}{b(715)}
      \]

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

   where \(a_{TS}(\lambda)\) indicates temperature and salinity corrected absorption observations, \(b(\lambda)\) =
\[ c_{TS}(\lambda) - a_{TS}(\lambda), \text{ and } b(715) = c_{TS}(715) - a_{TS}(715). \]

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

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

**COMPARISONS:**

1) Compare \( a_{\text{part}}(\lambda) \) from the spectrophotometer (both QFT and cuvette measurements) with \( a_{\text{part}}(\lambda) \) from the ac-meter for the cultures and the DRE sample. Are they similar? For the DRE sample, is \( a_{\text{part}}(\lambda) \) from a higher or lower filter pad loading more similar to the ac-meter? How does the choice of pathlength amplification impact the relationship?

**REFERENCES:**


Pegau, S., J.Ronald V. Zaneveld, B. Gregg Mitchell, James L. Mueller, M. Kahru, J. Wieland
Validation, Revision 4, Volume IV: Inherent Optical Properties: Instruments,
Characterizations, Field Measurements and Data Analysis Protocols. Edited by Mueller,
Greenbelt, Maryland.

Roesler, C.S., 1998: Theoretical and experimental approaches to improve the accuracy of
particulate absorption coefficients derived from the quantitative filter technique.
Limnology and Oceanography. 43: 1,649-1,660.

Slade, W.H. E. Boss, G. Dall'Olmo, M.R. Langner, J. Loftin, M.J. Behrenfeld, C. Roesler, and
T.K. Westberry, 2010. Underway and moored methods for improving accuracy in
measurement of spectral particulate absorption and attenuation. Journal of Atmospheric
and Oceanic Technology, 27:10, 1733-1746.

pathlength amplification in the filter-pad technique for measurements of
particulate absorption coefficient in the visible spectral region. Accepted to Applied
Optics.