Lab 3: particle absorption and pigments **8 July 2015**

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.

SAMPLES:

- spectrophotometric measurements of acetone extracts and particles on a G/FF filter.
- ac-meter measurements of whole DRE seawater and phytoplankton cultures.

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

Station 1 – Perry Lab spectrophotometer (there is some choreography required)	
Group A	Filter in chemistry lab (location of Beer's Law tank) blank with 100 mL filtered seawater ____ mL diatom culture ____ mL diatom culture Scan filters Methanol extraction under hood in chemistry lab (location of Beer's Law tank) blank with 300 mL filtered seawater filter with higher volume filtered Rinse with filtered seawater and rescan (filters will be fragile) Spectrum of acetone extract of diatom
Group B	Filter in chemistry lab (location of Beer's Law tank) blank with 300 mL filtered seawater ____ mL green alga culture ____ mL green alga culture Scan filters Spectrum of acetone extract of green alga Filter 3 replicates DRE water for chlorophyll analysis by fluorescence of acetone extract; filter in room with Beer's Law tank.
Group C	Filter water unit on bench facing spectrophotometer C 1 blank with 100 mL filtered seawater 200 mL DRE water 300 mL DRE water 400 mL DRE water 600 mL DRE water Scan filters Methanol extraction under hood in chemistry lab (location of Beer's Law tank) blank with 300 mL filtered seawater filters with 2 higher volumes filtered Rinse with filtered seawater and rescan (filters will be fragile) Spectrum of acetone extract of DRE water

Station 2 – Mitchell Classroom	
Group A	ac-s, DRE water
Group B	ac-9, DRE water
Group C	ac-s, Session 1 – diatom culture; Session 2 – green algal culture 2

INTRODUCTION

The major absorbers in seawater are water itself, CDOM, 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) which replaced the term detrital absorption, $a_d(\lambda)$, from Kishino et al. (1985), similarly problematic because the fraction included living and detrital organic and inorganic particulate matter. 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:

$$\mathbf{a_{part}(\lambda) = a_{phyt}(\lambda) + a_{NAP}(\lambda)} \quad .(1)$$

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.

Carotenoids 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 $a_{PS}(\lambda)$ has the potential to transfer energy leading to photosynthesis:

$$\mathbf{a_{phyt}(\lambda) = a_{PS}(\lambda) + a_{PP}(\lambda)} \quad . (2)$$

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. Care should be taken when using the oxidative rather than extractive approaches because oxidized

pigments have strong absorption in the UV due to the oxidative process. This leads to overestimation of a_{NAP} and underestimation of a_{phyt} in the UV. 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 latter is typically assumed to be insignificant, which is not always true, particularly in freshwaters with high abundance of cyanobacteria):

$$a_{NAP}(\lambda) = a_{min}(\lambda) + a_d(\lambda) + a_{other}(\lambda) \quad .(3)$$

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.)

STATION 1 – spectrophotometric measurements of absorption

SCANS OF PIGMENTS IN ACETONE EXTRACTS (clear solution with no scattering)

1. Cary-50 setting 300 – 800 nm at slow scan speed.
2. Use goggles and gloves when you handle acetone.
3. 1 cm cuvette and 90% acetone as blank, stored baseline.
4. Scan sample; add drop 10% HCl and rescan.
5. Discard acetone extract in designated waste container.
6. Rinse cell WELL with water and acetone to remove acid.

CHLOROPHYLL SAMPLES FROM DRE

Filter three samples (volumes 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.

QUANTITATIVE FILTER TECHNIQUE (QFT)

1. Blanks: filter two volumes of 0.2 µm DRE filtered seawater through two different G/FF filter pads to use as filtered seawater blank – 100 mL and 300 mL.
2. Samples: filter four volumes of sample through four different G/FF filter pads. The volumes will be determined just before lab.

   Cultures (Groups A & B): volumes to be determined
   DRE water (Group C): 200, 300, 400, 600 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. We'll try to bracket those 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. Cary-50 scan 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. Save. (Note – best practice is to rotate each filter and rescan three times; we do not have time this afternoon.)
9. Scan seawater blanks. Save.
10. Scan filter pads. Save.

METHANOL EXTRACTION OF PIGMENTS (KISHINO METHOD)

1. place filters on filter rack under hood with no vacuum,
2. add about 15 mL of hot 100% methanol,
3. wait about 10 minutes, turn on vacuum, and rinse filter with filtered seawater, including under filter cup flanges,
4. carefully remove filter (it will be easy to tear) and rescan.

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 absorbance (A, unitless) measured on the spectrophotometer is:

$$a_{\mathbf{part}}(\lambda) = \frac{2.303 * 100\left(\frac{\mathrm{cm}}{\mathrm{m}}\right)}{pathlength(cm)} * A_{pad}(\lambda) - \quad (4)$$

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) and the NIR offset:

$$A_{pad}(\lambda) = (A_{sample}(\lambda) - A_{blank}(\lambda)) - A_{null}(\lambda_{ref})) \quad (5)$$

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) for a discussion on null wavelength correction for filter pad absorption. You may take the mean absorbance between 730 and 750 nm for "λ_{ref}" in A_{null} (or, you can investigate expanding or shifting the range).

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

$$Pathlength\ (cm) = \frac{Volume\ filtered\ (cm^3)}{Area\ of\ filter\ (cm^2)} \quad (6)$$

Area is calculated from the diameter of the portion of the filter that actually collects the particles (use high quality caliper).

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 was originally termed the β correction factor (Mitchell and Kiefer, 1985) and the factor was applied to the geometric pathlength term in equation (6). 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 leading to a modification of equation (6):

$$Pathlength\ (cm) = \frac{Volume\ filtered\ (cm^3)}{Area\ of\ filter\ (cm^2)*2} \quad (7)$$

Emipirical 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 :

$$A_{pad\_corr}(\lambda) = C_1 * A_{pad}(\lambda) + C_2 * A_{pad}(\lambda)^2 \quad (8)$$

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. (manuscript accepted to Applied Optics) 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 that will be recommended by NASA in the next version of ocean optics protocols. The equation to calculate absorption, while correcting for pathlength amplification is:

$$A_{pad\_corr}(\lambda) = 0.679\ A_{pad}(\lambda)^{1.2804} \quad (9)$$

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

Compare three different pathlength amplification approaches to quantify the uncertaintly that exists in current databases such as SeaBASS. Filter a sufficient volume of sample such that $0.1 < A < 0.4$ throughout the visible portion of the spectrum (this may not be possible in the low absorbing green waveband for cultures or in the NIR region).

1) Roesler: $$a_{\mathbf{part}}(\lambda) = \frac{2.303 * 100\left(\frac{\mathrm{cm}}{\mathrm{m}}\right)}{pathlength(cm)*2} * A_{pad}(\lambda)$$

2) Cleveland and Weidemann: $$a_{\mathbf{part}}(\lambda) = \frac{2.303 * 100\left(\frac{\mathrm{cm}}{\mathrm{m}}\right)}{pathlength(cm)} * (0.378 * A_{pad}(\lambda) + 0.523 * A_{pad}(\lambda)^2)$$

3) Stramski et al: $$a_{\mathbf{part}}(\lambda) = \frac{2.303 * 100\left(\frac{\mathrm{cm}}{\mathrm{m}}\right)}{pathlength(cm)} * 0.679\, A_{pad}(\lambda)^{1.2804}$$

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

$a_{\mathbf{NAP}}(\lambda)$ –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}.

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

STATION 2 – ac-meter measurements for particles, including phytoplankton 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) Run filtered seawater or medium through a and c tubes.
2) Run unfiltered DRE water or culture through a and c tubes.

Group A	ac-s, DRE water
Group B	ac-9, DRE water
Group C	ac-s, Session 1 – diatom culture; Session 2 – green algal culture 2

Measure the temperature of every sample at time of analysis.
If not measured, assume salinity of the Damariscotta River Estuary is 28.

ASSIGNMENTS – DIVIDE THE WORK. Come prepared to deliver a briefing tomorrow morning.

PHYTOPLANKTON PIGMENTS:

1) Compare the spectral shapes of the 90% acetone extracts of cultures and DRE sample. What are the major differences? (hint: first normalize the spectra to 676 nm).
2) How does the HCl acid change the spectral shape of the acetone scans? In particular, note the change in absorption at 412 nm and 676 nm; pheopigments have 54% of chlorophyll *a* absorption at 676 nm.

QFT (spectrophotometer)

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 $a_{part}(\lambda)$ using three methods for the β correction factor and consider the uncertainty in the estimated particulate absorption.
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? How does pathlength amplification impact the relationship?
6) Discuss similarities and differences in $a_{part}(\lambda)$, $a_{\mathbf{phyt}}(\lambda)$ and $a_{\mathbf{NAP}}(\lambda)$ for the DRE sample and the cultures.
7) Compare the spectral slope of $a_{\mathbf{NAP}}$ and $a_{\mathbf{CODM}}$.

AC-METER 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
5. apply the spectrally flat correction by subtracting the a(715) offset from $a(\lambda)$
6. apply the spectrally varying scattering correction presented in class. For ac-9:

$$a(\lambda) = a_{TS}(\lambda) - b(\lambda) * \frac{a_{TS}(715)}{b(715)}$$

For ac-s, one could use a wavelength further into the near infrared, e.g.:

$$a(\lambda) = a_{TS}(\lambda) - b(\lambda) * \frac{a_{TS}(730)}{b(730)}$$

where $a_{TS}(\lambda)$ indicates temperature and salinity corrected absorption observations, $b(\lambda) = c_{TS}(\lambda) - a_{TS}(\lambda)$, 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 paper by Leymarie et al. (2010) assessed the likely uncertainties associated with the different corrections.

A newer paper by Rottgers et al., 2013, suggest the following correction for ac-9:

$$a(\lambda) = a_{TS}(\lambda) - \{a_{TS}(\lambda) - a'(715)\} * (0.56\, c_p(\lambda) - a_{TS}(\lambda))/(0.56\, c_p(715) - a'(715))$$

where $a'(715)=0.212\, a_{TS}(715)^{1.135}$.
Which is based on measurements with an integrating sphere particularly useful for places where inorganic particles may contribute significantly.
Compute the scatter corrected particulate spectra using this method.

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

COMPARISONS:

1) Compare $a_{part}(\lambda)$ from the spectrophotometer with $a_{part}(\lambda)$ from the ac-meter for the cultures and the DRE sample. Are they similar? For the DRE sample, is $a_{part}(\lambda)$ from a higher or lower filter pad loading more similar to the ac-meter? How does the scattering correction use change the particulate spectra?

2) Compare $a_{phyt}(\lambda)$ from the spectrophotometer with the acetone extracts of pigments.

REFERENCES:

Babin, M., and D. Stramski,. 2002. Light absorption by aquatic particles in the near-infrared spectral region. *Limnol. Oceanogr.,* 47(3), 2002, 911–915.

Babin, M., D. Stramski, G.M. Ferrari, H. Claustre, A. Bricaud, G. Obolensky, and N. Hoepffner. 2003. Variations in the light absorption coefficients of phytoplankton, nonalgal particles, and dissolved organic matter in coastal waters around Europe. Journal of Geophysical Research Ocean 108 (C7): article number 3211 , doi: 10.1029/2001JC000882.

Cleveland, J.S., and A.D. Weidemann, 1993: Quantifying absorption by aquatic particles: A multiple scattering correction for glass-fiber filters. *Limnology and Oceanography.* 38: 1321-1327.

Culver, M.E. and M.J. Perry. 1999. Fluorescence excitation estimates of photosynthetic absorption coefficients for phytoplankton and their response to irradiance. Limnology and Oceanography 44: 24-36.

Jeffrey, S.W. and G.R. Humphrey. 1975. New spectrophotometric equations for determining chlorophylls *a*,*b*,*c1* and *c2* in higher plants, algae and natural phytoplankton. Biochem. Physiol. Pflanzen Bd. 167: 191-194.

Jeffrey, S.W., R.R.C. Mantoura, and S.W. Wright. 1997. Phytoplantkon pigments in oceanography. UNESCO Publishing. 661 pp.

Kishino, M., N. Takahashi, N. Okami, and S. Ichimura, 1985: Estimation of the spectral absorption coefficients of phytoplankton in the sea. *Bulletin of Marine Science.* 37, 634-642.

Leymarie, E., D. Doxaran, and M. Babin. 2010. Uncertainties associated to measurements of inherent optical properties in natural waters. Appl. Opt. 49(28): 5415-5436.

McKee, D., J. Piskozub, and I. Brown, "Scattering error corrections for in situ absorption and attenuation measurements,"Opt. Express 16, 19480–19492 (2008).

Mitchell, B.G. 1990. Algorithms for determining the absorption coefficient of aquatic particulates using the quantitative filter technique (QFT). *Ocean Optics X.* 137-148.

Pegau, S., J.Ronald V. Zaneveld, B. Gregg Mitchell, James L. Mueller, M. Kahru, J. Wieland and M. Stramska. 2002. Ocean Optics Protocols For Satellite Ocean Color Sensor Validation, Revision 4, Volume IV: Inherent Optical Properties: Instruments, Characterizations, Field Measurements and Data Analysis Protocols. Edited by Mueller, J. L., G.S. Fargion and C R. McClain. NASA/TM-2003-211621/Rev4-Vol.IV. Greenbelt, Maryland.

Röttgers, R., D. McKee, and S. B. Wo´zniak, 2013: Evaluation of scatter corrections for ac-9 absorption measurements in coastal waters. Methods Oceanogr. **7**, 21–39.

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 accuray in measurement of spectral particulate absorption and attenuation. Journal of Atmospheric and Oceanic Technology, 27:10, 1733-1746.