

Chapter 4

Determination of spectral absorption coefficients of particles, dissolved material and phytoplankton for discrete water samples

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4.1 INTRODUCTION

The spectral absorption coefficient is one of the inherent optical properties that influence the reflectance of aquatic systems. The absorption coefficient $a(\lambda)$, in m^{-1} , at any point within a natural water body can be described in terms of the additive contribution of its components as

$$a(\lambda) = a_w(\lambda) + a_p(\lambda) + a_g(\lambda), \text{ m}^{-1}, \quad (4.1)$$

where $a_w(\lambda)$, $a_p(\lambda)$ and $a_g(\lambda)$ are the spectral absorption coefficients of water, particles, and soluble components, respectively. The spectral absorption coefficients of pure water adopted for the protocols are given in Table 1.1 (Ch. 1), and combine the results of Pope and Fry (1997), Sogandares and Fry (1997), Fry (2000) and Kou *et al.* (1993). The depth (z) dependence of the absorption coefficients is omitted for brevity. The particle absorption coefficient may be further decomposed as

$$a_p(\lambda) = a_\phi(\lambda) + a_d(\lambda), \text{ m}^{-1}, \quad (4.2)$$

where $a_\phi(\lambda)$ and $a_d(\lambda)$ are the spectral absorption coefficients of phytoplankton, and de-pigmented particles, respectively. Laboratory methods are described for determining operational estimates of these fractions. It is conceptually possible to further separate $a_d(\lambda)$ into absorption fractions due to de-pigmented organic and inorganic particles, but at present, there are no well established protocols for separately determining the absorption coefficient for inorganic particles.

To interpret aquatic spectral reflectance and better understand photochemical and photobiological processes in natural waters, it is essential to quantify the contributions of the individual constituents to the total absorption coefficients in the ultraviolet (UV) and visible region of the spectrum. The protocols presented here are based on the evolution, starting with articles by Kalle (1938) and Yentsch (1962), of methods for analyzing the absorption by soluble and particulate material in natural waters. Laboratory measurements and data analysis protocols are described for separating the total spectral absorption coefficient, $a(\lambda)$, into its components by spectrophotometric measurements of samples prepared from filtration of discrete water samples.

The spectral absorbance of the filters and filtrate from these samples, as measured in a spectrophotometer, are expressed in units of Optical Density (OD), defined as $OD(\lambda) = \text{Log}_{10}[V_o(\lambda)] - \text{Log}_{10}[V_t(\lambda)]$. $V_o(\lambda)$ is the spectrometer response for spectral flux transmitted through the reference material and $V_t(\lambda)$ is the response for spectral flux transmitted through the sample.

For the methods presented here the reference is either a properly hydrated GF/F blank filter for particle absorption, or a clean quartz glass optical cuvette filled directly from a purified water source for soluble material absorption. Note that OD is a dimensionless quantity. The use of base-10 logarithms in this context is a carryover from common practice in chemical spectroscopy and is the typical output of commercial spectrophotometers routinely used for these methods. Therefore, it is necessary to convert the OD measurements described in this chapter to the base-e representation of absorbance, *i.e.* to multiply OD by 2.303, to conform to the convention used throughout the ocean optics protocols. In general, these protocols are written assuming that the instrument that is used directly computes the optical density of the sample relative to the appropriate reference sample.

There has been considerable research to develop robust protocols that provide the most accurate estimates of absorption for various material fractions in natural waters. NASA-sponsored workshops were held at Scripps Institution of Oceanography and Bigelow Laboratory for Ocean Sciences to review absorption protocols, evaluate instrumentation, and define areas of consensus as well as areas of uncertainty that warrant further research (Mitchell *et al.* 2000).

The most widely used approach for estimating absorption by particulate matter in water samples involves analysis of the particles concentrated on filters (Yentsch, 1957). Absorption of phytoplankton suspensions determined using procedures that capture most of the forward scattered light (Shibata, 1958) can be related to the absorption measured on the filters to make quantitative corrections for the pathlength amplification effect (β) caused by the highly scattering filter medium (Duntley, 1942; Butler, 1962). The pathlength amplification parameter was symbolized as β by Kiefer and SooHoo (1982) following the nomenclature of Butler (1962). This symbol should not to be confused with the volume scattering coefficient $\beta(\lambda, \Psi)$ used in other chapters of this Technical Memorandum.

Kiefer and SooHoo (1982) reported a constant to scale the red peak of chlorophyll absorption for natural particles retained on GF/C filters to the diffuse absorption coefficients determined on suspensions by Kiefer *et al.* (1979). The diffuse absorption coefficient is double the value of the volume absorption coefficient of interest here (Preisendorfer, 1976). Mitchell and Kiefer (1984, 1988a) made direct estimates of volume absorption coefficients for phytoplankton suspensions and absorbance on glass fiber filters with the same particles to develop empirical equations that relate the amplification factor to the glass fiber sample optical density. This procedure is the basis of most laboratory methods for determining particle absorption in water samples.

Field applications of these quantitative estimates of $a_p(\lambda)$ were reported by Mitchell and Kiefer (1984, 1988b) and Bricaud and Stramski (1990). More detailed empirical results to correct for pathlength amplification were reported by Mitchell (1990) for various filter types and diverse cultures coccoid cyanobacteria, nanochlorophytes, diatoms, chrysophytes and dinoflagellates with sizes ranging from 2 μm to 20 μm . Cleveland and Weidemann (1993) and Tassan and Ferrari (1995) found that the empirical relationships of Mitchell (1990) were consistent with similar types of phytoplankton, but Moore *et al.* (1995) reported large differences in the amplification factor for *Synechococcus sp.* (WH8103) and *Prochlorococcus marinus* that were about half the size of the smallest cells studied by Mitchell (1990). Similar results were obtained by Allali *et al.* (1997) for natural populations of the Equatorial Pacific dominated by picoplankton. For samples with substantial turbidity and scattering due to inorganic matter (coastal, shelf, coccolithophore blooms), methods to correct for resulting artifacts have been described by Tassan and Ferrari (1995a, 1995b). Table 4.1 provides a summary of various published results for pathlength amplification factors.

Separation of the particle fraction into phytoplankton and other components is of considerable ecological and biogeochemical interest. Early efforts to separate absorbing components for natural particles included treatment with organic solvents, UV radiation, and potassium permanganate (references can be found in Shifrin, 1988, and Bricaud and Stramski, 1990). The most widely used chemical method is based on methanol extraction (Kishino *et al.* 1985, 1986). A recent method consists of bleaching the phytoplankton pigments by sodium hypochlorite (Tassan and Ferrari, 1995a; Ferrari and Tassan, 1999). Spectral fluorescence methods to estimate the fraction of photosynthetically active absorption, if separate total particulate absorption has been determined, have been reported by Sosik and Mitchell, (1995).

Soluble absorption observations were described by Bricaud *et al.* (1981) for diverse ocean environments, including oligotrophic and eutrophic regions. Other field reports can be found in the references listed in more recent articles (Carder *et al.*, 1989a, 1989b; Blough *et al.*, 1993; Vodacek *et al.*, 1996; Hoge *et al.*, 1993; Nelson *et al.*, 1998; D'Sa *et al.*, 1999). Spectrophotometric measurement of absorption by dissolved materials is straightforward, but has limits due to the very small signal for short pathlengths routinely employed (usually 10 cm), and to difficulties in maintaining quality control of purified water used as a reference.

This chapter defines protocols for the operational determinations of absorption coefficients for particulate and soluble matter in water samples. Methods are specified for separating particulate and soluble material by filtration, partitioning total particulate absorption into contributions by phytoplankton and de-pigmented particles (detritus), and corrections for pathlength amplification due to semi-diffuse transmittance of the filters. Recommendations are made based on widely accepted methods and processing procedures. NASA-sponsored workshops have confirmed various aspects of previously reported methods (Mitchell *et al.* 2000).

4.2 SAMPLE ACQUISITION

Water samples should be taken using Niskin (or similar) bottles at the site of, and simultaneously with, the surface in-water optical measurements, and at depth increments sufficient to resolve variability within at least the top optical depth. When possible, samples should be acquired at several depths distributed throughout the upper 300m of the water column (or in turbid water, up to seven diffuse attenuation depths for PAR irradiance, $\ln(E(0)/E(z))=7$), to provide a basis for relating the spectroscopic measurements of absorption to *in situ* profile measurements. Samples should be drawn immediately from the *in situ* sampling bottles into clean sampling bottles using clean silicon rubber or Tygon tubing or by directly filling the sample bottles from the Niskin bottle spigot. If Niskin bottles will not be sampled immediately, precautions must be taken to ensure large particles that settle are re-suspended. This can be done by transferring all water from the Niskin to a bottle or carboy larger than the total volume of the Niskin so that the entire water sample can be mixed (invert bottle numerous times to mix by turbulence), or by draining a small amount of water from the Niskin and manually inverting the entire Niskin prior to sub-sampling. Sample bottles should be kept cool (ideally near *in situ* temperatures), and dark prior to sample preparations. Preparations should be completed as soon as possible after sampling, but no later than several hours after the sample was acquired.

4.3 SPECTROPHOTOMETER CHARACTERISTICS AND CALIBRATION

A spectrophotometer used for absorption measurements following the protocols presented in this chapter must meet the following minimum performance specifications:

1. The unit's monochromator, or spectrograph, must yield a Full-Width at Half-Maximum (FWHM) bandwidth ≤ 4 nm. A larger FWHM bandwidth will not adequately resolve the red chlorophyll *a* absorption band.
2. The instrument's baseline spectrum characteristics, specified below, must be maintained over the range from 300 nm to 850 nm for measuring absorption by particles concentrated on filters, and from 250 nm to 850 nm for measuring absorption by dissolved materials.
3. For measuring absorption by particles concentrated on filters, baseline noise must be <0.01 OD, and noise <0.005 OD is strongly recommended.
4. For measuring absorption by dissolved materials, baseline noise must be <0.001 OD units, and noise <0.0005 OD is strongly recommended.
5. The instrument's baseline spectrum must be relatively flat over the wavelength range of interest and its shape and magnitude must be stable over time. Any tendencies for the spectral shape and magnitude of an instrument's baseline to drift must be well-behaved and slow enough that the rate of baseline drift may be characterized with an uncertainty less than the noise levels specified

above. It is recommended to check the instrument baseline at intervals of 1 hr to 2 hr during an extended series of measurements.

Other desirable, but not absolutely essential, spectrophotometer features are variable slit width (to allow reducing the FWHM spectral resolution, when desired), automatic baseline corrections (the adequacy of which must nevertheless be verified), and automatic spectral calibration during instrument warm-up (using mercury emission lines supplied by an internal lamp source).

The spectral accuracy of the spectrometer should be verified by scanning a holmium oxide filter, with reference to an air-to-air baseline. This spectral calibration should be repeated each time the instrument is turned on, and at the conclusion of a series of measurements. Alternatively, a spectrophotometer's spectral characteristics may be calibrated using an internal line source (*e.g.* a mercury lamp), if the instrument is so equipped, but independent checks with the holmium oxide filter are also strongly advised. A set of absorbance reference filter standards, of known *OD*, must be used to calibrate a spectrophotometer's responses over the range of *OD* associated with the samples to be measured. This calibration, together with instrument baseline spectrum determinations, should be repeated at intervals necessary to characterize (within the noise tolerances given above) any measurable drifts in the instrument baseline and/or *OD* response. Unless the investigator can confirm the stability of the instrument that is used, these calibration procedures should be repeated each time the spectrophotometer is turned on. As a minimum they should be performed at any time there is a change of lamp source, blocking filter, or other instrument setup characteristic that affects the optical response and on a regular basis during routine work.

The present version of these protocols is written assuming the use of a commercial dual-beam spectrophotometer, with the sample and reference targets illuminated by the collimated output of a grating monochromator. The protocols also apply, with minimal modifications, to measurements using a single-beam monochromator or otherwise similar optical configuration. Mitchell *et al.* (2000) report comparisons between *OD* measurements of a common set of GF/F filtered particle samples using several spectrophotometers of these types, as well as spectrophotometers based on very different optical configurations. For a diatom culture measured during the Scripps workshop, several commercial dual beam spectrophotometers estimated sample filter *OD* spectra consistently within 5 % (Figure 4.1). Some of the differently configured instruments were within 10 % of the selected reference dual-beam instrument but in some cases had limited spectral range either in the UV or the near-infrared, or both (data not shown). The largest divergence was found for a grating spectrograph instrument that illuminates the filter with diffuse white light (Figure 4.1). This unit yielded *OD* values that were significantly higher than, albeit linearly related to (with a slope of approximately 0.7), the *OD* measurements made with conventional dual beam spectrophotometers (Mitchell *et al.* 2000). This result indicates that the pathlength amplification (β factor) associated with this instrument is significantly different from previously published values (Table 4.1), which were derived using conventional dual beam spectrophotometers. An investigator using this type of spectrophotometer, or another design with yet a different optical configuration, must either compare filter *OD* spectra measurements to reference measurements on the same filters with a "conventional" spectrophotometer to derive an *OD* scaling function, or otherwise determine pathlength amplification factors for the instrument configuration, using methods discussed in Mitchell *et al.* (2000) and references cited therein.

4.4 PARTICLE ABSORPTION: SAMPLE FILTER PREPARATION AND ANALYSIS

The procedures described in this section are recommended for determining the spectral absorption coefficients of particles in discrete samples of natural waters. These laboratory measurements are complementary to methods for measuring *in situ* profiles of absorption, as described in Chapter 3 of this volume, and provide additional information on the partition of particle absorption by phytoplankton and other particles. Water samples are filtered and absorbance spectra of the filter, $OD_{fp}(\lambda)$, are estimated for the retained particles using a laboratory spectrophotometer. After measurement, the sample filters are soaked in chemical solvents to extract, or bleach, phytoplankton pigments (Kishino *et al.*, 1985; Tassan and Ferrari 1995a) then rinsed to remove the chemicals and pigments from the material retained on the filter.

The $OD_{fd}(\lambda)$ spectrum of the filter is then determined in the spectrophotometer to obtain the absorption component of the de-pigmented particles, which are sometimes referred to as detritus or tripton. Depending on the method used to de-pigment the samples, this fraction also includes bleached cells and phycobilipigments that are not extractable in methanol and also inorganic minerals that may be important absorbers in some water samples. The raw $OD_{fp}(\lambda)$ and $OD_{fd}(\lambda)$ data are used to calculate total particulate and de-pigmented absorption coefficients $a_p(\lambda)$ and $a_d(\lambda)$, respectively. The absorption coefficient of phytoplankton, $a_\phi(\lambda)$, is then calculated as the difference $a_p(\lambda) - a_d(\lambda)$.

Filtration

The Whatman GF/F™ filter (which is binder-free and combustible, with a nominal pore size of 0.7 μm) is recommended for particle absorption sampling. This type of filter is also recommended by (JGOFS 1991) for various particulate and pigment analyses. Some authors have reported that particulate material less than 0.7 μm in size will not be retained by the GF/F filter, and that this fraction may contain up to 10 % or 15 % of the phytoplankton biomass as measured by chlorophyll concentration. Chavez *et al.* (1995), however, found no statistical difference between GF/F and 0.2 μm filters for chlorophyll and productivity measurements. The absorption of particles having diameters between 0.22 μm and 0.7 μm may be selectively determined by filtering the GF/F filtrate again through a 0.22 μm Millipore cellulose acetate membrane filter, and measuring its absorbance with a spectrophotometer (Ferrari and Tassan, 1996). Note that Mitchell (1990) reported pathlength amplification factors for cellulose acetate filters that are substantially different than those for GF/F filters and also described the relative difficulty of keeping cellulose acetate filters properly hydrated.

The optical transparency of the GF/F filter relative to air decreases significantly below 380 nm but many spectrophotometers can still make optical density determinations to 300 nm with these filters. The transparency of the filter also increases with hydration; so all samples must be fully - but not excessively - hydrated for proper performance of analytical procedures and accurate optical corrections. Pre-soaking GF/F filters 1 to 2 hrs before use can lead to less variability between individual filters (Bricaud and Stramski 1990). For oceanic water samples, seawater filtered through a 0.2 μm filter should be used to hydrate the filters. Freshly filtered seawater should be used since water that is left standing in clear containers may grow considerable amounts of algae over relatively short periods of time if there are any nutrients in the filtered seawater. For fresh inland water samples, purified fresh water may be used.

Glass fiber, cellulose acetate, and other strongly diffusing filters have large scattering coefficients, which increase the optical path length of photons in the measurement beam. Filtration volume V_f should be adjusted so that the optical densities of the filter samples, relative to the blank filter satisfy the criteria that $0.05 < OD_{fp}(675) \leq 0.25$ and $OD_{fp}(440) \leq 0.4$ OD (Mitchell 1990). Optical density spectra of the sample filters should be measured as soon as possible following filtration, because pigment decomposition may occur (Stramski 1990). If filters must be stored, immediately place the unfolded filters into flat tissue containers designed for liquid nitrogen storage. Liquid nitrogen storage is recommended because alternative freezing methods were shown to have more artifacts in comparison tests (Sosik, 1999).

a. Sample Filter Preparation

- Collect water samples, and maintain them in the dark at, or near, *in situ* water temperature.
- Prepare 0.2 μm filtered seawater (FSW) in sufficient volume for hydrating sample and blank filters.
- Set up and maintain the filter apparatus in dim light to minimize photodegradation of the samples.
- For each sample, place a GF/F filter onto the filtration rig. Also prepare two blank GF/F filters by soaking them in ~25 ml of 0.2 μm filtered water while mounted on the filtration funnel (with valves closed) during the sample filtration.
- Filter the samples on GF/F filters under low vacuum (~125 mm Hg).

- Filter a sufficient volume of water V_f to yield sample optical density relative to the blank filter in the range specified above. For field samples collected in the upper 100-150 m and filtered onto 25 mm GF/F filters, V_f is typically in the range 0.5 L to 5 L, depending on the *in situ* density concentration of particles.
- Do not let the preparations run dry during filtration. Turn off the vacuum to each sample as it completes filtering. Immediately place samples on a drop of 0.2 μm FSW in the appropriate container, depending on how they will be stored.
- Record the filter and filtration funnel type, the diameter D_f of the area on the filter that contains the concentrated particles, and the volume of water filtered V_f .
- Measure the absorption spectra in a spectrophotometer, or store the filters in liquid nitrogen, as soon as possible.

b. Sample Filter Storage

- If the filter samples will be analyzed immediately, store each filter in a labeled petri dish (*e.g.* Gelman™ snap-top dishes). Ensure proper hydration of the sample by placing the GF/F filter on a small drop of 0.2 μm FSW. Store each filter sample in the dark and refrigerate it (~ 4 deg. C) until it is to be measured in the spectrophotometer.
- If the spectrophotometric measurements will be delayed more than 24 hours following sample filtration, the filter samples should be prepared for liquid nitrogen storage. Samples should be stored in containers that allow the filter to remain flat, and which are specifically designed for immersion in liquid nitrogen (*e.g.* Fisher Histoprep™ tissue capsules). One pair of blank filters should also be prepared each day for use as the reference blank for samples collected that day. Samples may be stored in liquid nitrogen for extended periods, but it is strongly recommended to analyze them as soon as possible.
- Non-pressurized liquid nitrogen dewars generally retain liquid nitrogen for 2-4 weeks. Pressurized liquid nitrogen dewars can be rented at low cost for extended cruises (4-5 weeks) so that the sample dewars may be replenished and kept full. Care must be taken at sea, and in return shipping, to ensure that the samples are properly frozen. Samples should be shipped in liquid nitrogen dry shippers, which will maintain proper temperatures for 2 to 3 weeks, if they are properly charged and in good condition.
- Air transport of liquid nitrogen dry shippers is approved under International Air Transportation Agreement (IATA 41st Edition Section A800; US Federal Aviation Administration Dangerous Good Bulletin DGAB-98-03; August 25, 1998). That approval notwithstanding, many investigators have experienced difficulties in clearing customs, and in transport of liquid nitrogen dry shippers via commercial airfreight, or as checked baggage. The investigator should contact the carriers in advance and provide the IATA approval and FAA bulletins pertaining to liquid nitrogen dry shipper transport. If the dry shipper is to be transported as checked baggage, advanced coordination with the airline is strongly recommended to avoid confiscation of samples and delays in return shipment. When samples are shipped as checked baggage or freight, the IATA memo, DOT memo, and manufacturer's certificate should be affixed to the dry shipper to minimize potential delays.
- Temporary storage of filter samples on dry ice can be considered during transport. But maximum duration of dry ice in insulated shipping boxes is several days, so the use of liquid nitrogen dry shippers is strongly recommended.

Determination of spectral optical density of sample filters

After preparation, the optical density spectrum of each sample filter is measured using a laboratory spectrophotometer. The performance characteristics and calibration requirements of the spectrophotometer used for these measurements are described above in Section 4.3.

a. Reference Blank Spectra

With a dual beam spectrophotometer, two reference filter blanks saturated with FSW are used to measure the reference spectrum, and one is left in the reference beam during sample measurements. For typical single beam instruments, generally the reference is scanned, and then samples are placed into the beam and scanned. Most modern spectrophotometers, whether single or double beam, automatically store the instrument's reference spectrum and recorded sample spectra are automatically corrected to yield $OD_{fp}(\lambda)$ relative to the reference blank filter. A new instrument reference baseline scan should be measured each time the spectrophotometer is powered up, and whenever its configuration has been changed. The baseline should also be checked regularly (every 1 hr to 2 hr) during extended periods of

analysis. Frequency of baseline verification will depend on the performance and stability of each instrument and should be determined by the investigator prior to executing routine work. Uncorrected baseline drift, and changes in sorting filters or lamp source can cause systematic measurement anomalies. Wavelength accuracy and measurement precision should also be checked during the analyses (Sect. 4.3 above).

b. Spectrophotometric Measurement Procedure

- Warm up the spectrophotometer for 30 minutes.
- Measure the initial instrument baseline and wavelength calibration.
- If using frozen samples, remove the filters from the storage container and place them in petri dishes on FSW to ensure hydration. Allow the samples to thaw for approximately 5 min and then refrigerate them in the dark until each filter is ready for analysis.
- An instrument-specific sample-mounting device is recommended to hold filters against a quartz glass mounting plate. These mounts should be secure when placed in the sample compartment and hold the sample perpendicular to the illumination beam so only the filter and the quartz plate are in the beam. Usually, these mounts must be custom fabricated specifically for each different instrument.
- Clean the quartz faceplates of the mounting device with purified water and detergent if needed. Rinse them with purified water and ethanol, and dry them thoroughly using lint-free laboratory tissues.
- Set the appropriate instrument parameters according to the manufacturer's instructions.
- Mount two pre-soaked and water saturated blank filters (one for the sample beam, and one for the reference beam).
- To test for proper filter hydration, confirm that there is a drop of FSW left on the mounting plate when the filter is lifted. With the filter on the mounting plate there should be a slight sheen on the top surface of the filter, and a very narrow (~1 mm) border of water around the edges of the filter. Be careful not to use too much water, or the sample may wash away.
- Examine the back of the filter on the mounting plate to be sure that no bubbles are trapped between the filter and the quartz glass plate on the sample holder. There should be a uniform layer of water between the filter and quartz glass mounting plate. If bubbles are present, which will be obvious, pick up the filter with forceps, and replace it on the plate with a slight dragging motion across a drop of filtered seawater. Re-inspect the back of the filter and repeat the foregoing procedure until no bubbles are present. Adjust the amount of FSW as necessary to ensure proper hydration.
- Alternative mounts that expose both sides of the filter to air may be used to avoid bubbles altogether. Sample hydration is more difficult to maintain when using this type of filter mount so the investigator must develop a satisfactory procedure to ensure proper hydration of sample and reference.
- Run the instrument baseline correction using the two blanks. For most commercial units, this baseline will be automatically used as the reference to calculate $OD_{fp}(\lambda)$. Immediately after the baseline correction is finished, and without touching the blank filters, run the two blanks as a sample scan to confirm that baseline performance is within acceptable tolerance over the spectral range of determination (Sect. 4.3 above). This spectrum should be flat spectrally. Baseline noise less than $\pm 0.005 OD$ is recommended. Save this scan for confirmation of instrument performance. If a spectrally flat baseline cannot be achieved over the spectral range of interest, the stored baseline must be subtracted from subsequent measurements of sample filter $OD_{fp}(\lambda)$.
- If using a single beam instrument, or instruments run in the single beam mode the blank is not kept in the instrument so one does not need to rehydrate the blank reference filter regularly. Most

modern single beam spectrophotometers will also automatically use the blank reference stored in memory for estimates of $OD_{fp}(\lambda)$.

- Remove the blank filter from the quartz glass sample mount in the measurement beam, and replace it with a sample filter, ensuring proper hydration of the sample (see above). Measure the sample $OD_{fp}(\lambda)$ spectrum, save it in a digital file, and record all relevant information.
- The blank reference filter will dry out over time, and must be hydrated regularly. If absorption signal deviates significantly from zero (more than 0.02 OD) in the infrared (750-800 nm), this often indicates a dry reference or sample filter. If using a quartz plate, check the reference filter after every 5-6 scans, and hydrate as needed. If the filters are mounted in air, hydrate the blank before every scan.

Sample Filter Preparation for De-pigmented Particle Absorption

After preparing an $a_p(\lambda)$ filter sample and determining its $OD_{fp}(\lambda)$ spectrum on the spectrophotometer, the sample should be processed to remove its pigments and determine $a_d(\lambda)$. The shape of the $a_d(\lambda)$ spectrum usually decreases monotonically with wavelength, following exponential form that is flatter than the shape of the soluble absorption spectrum. Since the goal is generally to get an estimate of phytoplankton absorption, if there is a residual chlorophyll *a* absorption peak in the red near 675 nm, the extraction process should be repeated to remove it. Variations of this method include use of hot or boiling methanol and varying extraction times. Use of hot methanol has risks due to flammability, and volatility. If this process is used, extra precautions must be taken.

Bleaching of the organic pigments can also be accomplished for situations with difficult to extract pigments including phycobilins or other chemically polar pigments that do not extract well in methanol. Pigment extraction in a chemical solvent, such as methanol, is a fundamentally different chemical process than bleaching the pigments using sodium hypochlorite (NaClO). Bleaching involves placing a small amount of 0.1 % active chlorine solution onto the filter, then rinsing it off with FSW. The NaClO oxidizes the pigment molecules, making their light absorption negligible. FSW rinses then remove the excess NaClO, which absorbs negligibly at wavelengths >400 nm, but absorbs strongly at shorter wavelengths. The bleaching method of pigment removal has been shown to be effective *in situations* where methanol cannot be used, as on cellulose membranes such as the 0.22 μm Millipore filter, or when phycobilins are present (Tassan and Ferrari 1995a; Mitchell *et al.* 2000). This procedure can also be adapted for use with particulate suspensions.

Neither methanol extraction, nor NaClO oxidation, provides an ideal means of separating particulate absorption into ‘algal’ and ‘detrital’ components. In each case, the action of the chemical agent is not well understood, and in many situations the two methods will yield very different results. The decision to apply either the bleaching, or methanol extraction, method will depend on the situation. For example, in inland waters where either cyanobacteria, or chlorophytes, are dominant, the bleaching technique is preferred, because of the presence of phycobilins and of extraction resistant algae (*e.g.* Porra 1990). In coastal oceanic waters, on the other hand, the methanol technique is preferred, because the results will be comparable to previously published results and there is no particular advantage to using bleach. In open-ocean samples (*e.g.* the Sargasso Sea), however, absorption by phycobilins is small, but present in some particulate absorption samples and in methanol-extracted filters (N.B. Nelson unpublished data). The methanol technique will provide results which are comparable to earlier studies, but with errors due to incomplete extraction and wavelength shifts in the phycobilin absorption bands.

a. Methanol Extraction method

- Replace the sample and blank filters on the filtration system. Treat blank filters exactly as if they were sample filters.
- Add 5 mL to 10 mL of 100 % methanol to each filter by gently pouring it down the sides of the filter funnel to minimize resuspension of the sample particles, and let stand for 1 min.

- Filter the methanol through the sample, turn off the vacuum, close the valves and add 10 - 15 mL of methanol.
- Allow the sample to stand in methanol for approximately 1 hr. Do not allow the filter to go dry during the extraction period. Time of extraction will vary depending on the filter load and phytoplankton species composition. Place aluminum foil over the filtration cups to minimize contamination during extraction.
- After extraction is complete, turn on the vacuum and draw the methanol and dissolved pigments through the filter. Rinse the sides of the filter tower twice with small amounts of methanol. Finally, rinse the sides of the filter tower three times with ~20 mL of 0.2 μm FSW. Also rinse the blanks with FSW after methanol extraction to minimize filter dehydration during spectrophotometric analysis.
- Pigment extraction is complete when the 675 nm chlorophyll *a* absorption peak is not present in the $OD_{fd}(\lambda)$ spectrum.
- Successive, short extractions of 10 minutes can sometimes improve the pigment extraction.
- Phycobilins, and some eukaryotic pigments, will not be extracted efficiently by methanol.

b. Sodium Hypochlorite oxidation method

- Prepare NaClO solution:
 - For freshwater samples: 0.1 % active chlorine in purified water (*e.g.* Milli-Q water).
 - For marine samples: 0.1 % active chlorine in purified water containing 60 gl^{-1} Na_2SO_4 , to match osmotic pressure of sample cells.
- The volume of 0.1% active chlorine solution needed to bleach pigments from a filter sample has been empirically shown to be approximately $3OD_{fp}(440)$ mL .
- Place the sample, particle side up, on the filtration system (closed valves).
- Gently pour the solution down the sides of the filter funnel.
- Let the solution act for 5 min to 10 min, adding solution as necessary to compensate for loss through the filter.
- Cover the filtration cup with aluminum foil to prevent contamination during bleaching.
- Rinse the sample by gentle filtration of 50 mL of water (either fresh water or FSW, depending on sample source).
- Complete bleaching of the pigments is indicated by the absence of a 675 nm peak, together with a concave shape near 440 nm, in the $OD_{fd}(\lambda)$ spectrum of the bleached filter. If evidence of residual pigment absorption persists, repeat the NaClO oxidation treatment, as indicated above.

Spectrophotometric Measurement of De-pigmented Optical Density Spectra

- The $OD_{fd}(\lambda)$ spectrum of the de-pigmented samples should be measured in the spectrophotometer, as described above for $OD_{fp}(\lambda)$.
- Note that methanol-extracted sample and blank filters will tend to dry out quickly if the methanol is not thoroughly rinsed from the filters prior to spectrophotometric measurements.
- NaClO oxidized sample and reference filters must be thoroughly rinsed with FSW (or fresh water for inland water samples) to extend the spectral range below 400 nm.

4.5 SOLUBLE ABSORPTION SAMPLE PREPARATION AND ANALYSIS

The measurement methods described in this section are used to determine $a_g(\lambda)$, the spectral absorption coefficient spectrum of gelbstoff, often referred to as dissolved organic matter (CDOM). Water samples are collected and particulate material is removed by filtration. The absorption of the filtrate is measured, relative to purified water, using a spectrophotometer. All equipment utilized to prepare soluble absorption samples must minimize contamination by organic, or otherwise colored, material. Samples must be protected from photo-degradation during preparation and measurements. Plastic or glass filtration apparatus may be used, provided that the units are equipped with mesh filter supports made either of stainless steel or plastic, and not with ground glass frits. Glass frits tend to become clogged over time, and may cause uneven distribution on the filter, reduce the rate of filtration and may contaminate the sample filtrate.

Membrane filters with 0.2 μm pore size (*e.g.*, Nuclepore™ polycarbonate filters) are recommended for this procedure. The membrane filters should be pre-soaked in 10% HCl, rinsed with 75-100 mL of freshly purified water, and rinsed again with a 75 – 100 mL of the sample before it is used. Tests with purified water have shown that all filters leach contamination that resembles soluble absorption (data not shown). Using polycarbonate membrane filters, an acid soak, pure water rinse and sample rinse minimizes this contamination. Still, we have found the sample preparation procedure increases the apparent absorption spectra of purified water that is prepared as though it were a sample when referenced to purified water drawn directly into the measuring cuvette from the pure water system. Therefore correction for this sample preparation blank is recommended.

Glass fiber filters should be avoided if possible because they have been shown to cause rather severe contamination of the filtrate in tests using purified water. For samples collected from very turbid waters, glass fiber filters have routinely been used as a pre-filter to minimize clogging of the final filtration with a membrane filter (Kowalczyk, 1999). In such cases the investigator must develop a procedure to rinse the glass fiber filter to ensure that the contamination from this method is minimized. Since situations requiring pre-filtration often coincide with large soluble absorption coefficients, the effects may be easily corrected but it is the responsibility of the investigator to demonstrate this. Careful assessment of the contamination of any method, and proper corrections must be carried out and reported.

Previously we recommended the use of amber-colored borosilicate glass bottles (*e.g.* Qorpak™ bottles), that screen ambient light, for sample preparation and to store laboratory prepared standard water. However, recent work (details not shown) indicate that the amber bottles may leach some colored material into the purified standard water that is prepared before cruises and used to assess the quality of purified water prepared at sea. Therefore we now recommend use of clear borosilicate Qorpak™ bottles (or equivalent) for sample preparations and for the preparation of the standard reference water. Prior to each experiment, all filtration apparatus and storage bottles should be thoroughly cleaned.

Purified water for soluble absorption measurements

Purified water freshly drawn from a water purification system, such as the Millipore Milli-Q, Millipore Alpha-Q, and Barnstead Nanopure units, or their equivalent, is strongly recommended for use at sea in preparing pure water for absorption reference, blanks and for equipment rinses specified in these protocols. Mitchell *et al.* (2000) compared the water-to-air baseline reference of purified water prepared with these three water purification systems. All three systems provided similar results in baseline tests relative to air at wavelengths between 300 nm and 900 nm, while small differences were found below 300 nm. It is also recommended to prepare a set of standard purified water samples prior to a field deployment as a reference to check daily for pure-water system degradation, *e.g.* due to poor quality feed water. Even though bottled purified water standards have been found to deteriorate slightly over time, especially from 250 nm to 325 nm, they provide invaluable quality control and an alternative source of reference water *in situations* when the purification system performance degrades dramatically.

Pre-cruise preparations

- Sample bottles (clear borosilicate Qorpak™ with polyethylene lined caps) used to collect sample filtrate or to store standard reference water need to be thoroughly cleaned in advance to remove any potential organic contaminants. Sequential soaks and rinses in dilute detergent, purified water, and 10 % HCl, followed by a final copious rinse in purified water, are recommended.
- Rinse plastic caps with 10 % HCl, twice with freshly prepared purified water (*e.g.* using a Millipore Alpha-Q system), and dry them at 70° C for 4 hr to 6 hr.
- Combust bottles with aluminum foil covers at 450° C for 4 hr to 6 hr.
- Fill clean, combusted bottles with fresh purified water drawn directly from the purification unit.
- Assemble the combusted bottles and clean caps. Store in the dark.
- These standards are used daily during cruises to evaluate the quality of purified water freshly prepared at sea.
- This carefully prepared standard water sometimes must be used as the reference material for actual sample analysis. If this is planned, the investigator should determine the optical density of the standard water preparations before and after a cruise relative to fresh purified water drawn directly into the quartz cuvettes. An assessment of the change in this water over time may indicate a need to use a time-dependent reference water correction.
- As a precaution, even if the investigator intends to have high quality purified water at sea, it is wise to determine the standard water optical density relative to freshly purified water before a cruise, and as a time-series to understand the quality of the purified water system used for reference.

Soluble Absorption Sample Preparation, Storage and Analysis

- Wash hands with soap and water to avoid contaminating the samples.
- Use 0.2 µm polycarbonate filters (*e.g.* Nuclepore or equivalent). Do not use irgalan black stained (low fluorescence background) polycarbonate filters for this preparation. Other membrane filters, or Sterivex cartridges, may also be used, but the investigator must then test for any contamination by the filter and ensure that no artifacts are introduced.
- The filtration system used should be equipped with control of vacuum for each individual filtration funnel and with a provision for direct filtration into clean bottles. An example of a suitable soluble absorption filtration assembly is illustrated in Mitchell *et al.* (2000).
- Pre-soak each filter for at least 15 min in 10 % HCl. Rinse the filter thoroughly with purified water. Mount the filter on a filtration funnel and filter ~100 mL of purified water through it into a sample bottle. Shake the bottle, and discard the water, pouring it over the inside of the cap to rinse it. Cover the filtration funnel with aluminum foil until ready to filter the sample.
- Collect ~200 mL of seawater into a clean sample bottle. For the blanks, use purified water drawn directly from the purification unit into 2 clean sample bottles.
- Filter ~75 mL of the samples and 1 blank directly into clean bottles at low vacuum (<120 mm Hg). Do not allow filters to go dry during sample rinsing. Shake the bottles, and discard the water.
- Filter ~75 mL of the samples into bottles. For the blank, filter ~75 mL of purified water. When finished, cap the bottles and store them until they are to be measured in the spectrophotometer.
- If the samples will be measured within 4 hr, store them in the dark at room temperature.
- If the samples will be measured 4 hr to 24 hr later, refrigerate them in the dark.
- Longer storage is not recommended, because artifacts of undocumented magnitude are known to occur. Several researchers have reported results from measurements of frozen samples, but no systematic evaluation of possible artifacts resulting from freezing has yet been reported.

- Warm refrigerated samples to room temperature before beginning optical density measurements. If it is practical to do so, control the samples and the reference water to equal temperatures during the spectrophotometric measurements. Absorption by water is strongly temperature dependent at red and near infrared wavelengths (Pegau and Zaneveld 1993).
- Qorpak bottles can be re-used at sea. After spectrophotometric analysis is completed, thoroughly rinse each bottle and its cap three times with purified water, pour in 20 mL of 10 % HCl acid, and close the cap. Before the bottle is reused, shake it well, discard the 10 % HCl, rinse the bottle and cap copiously with purified water, and fill the bottle with purified water, to be used later to rinse a new sample filter. Purified water should be drawn directly from the pure water system.

Determination of Optical Density of Soluble Absorption Preparations

- If samples have been refrigerated, allow them to warm to room temperature.
- Allow the spectrophotometer to warm up for 30 min. Confirm that the optical windows of the spectrophotometer are clean. If necessary, clean them with purified water and ethanol, sequentially, and dry them thoroughly with lint-free laboratory tissues.
- Verify the instrument's spectral characteristics and precision as described in Section 4.3.
- Wash hands with soap and water to avoid contamination
- Between use, 10 cm quartz window spectrophotometer cuvettes should be stored with purified water. For analysis, discard the purified water in the cuvettes, rinse inside and outside of cuvettes twice with 10 % HCl, twice with ethanol, then rinse them inside and outside using copious volumes of purified water. After the cuvettes have been cleaned, use laboratory tissues to handle them. Avoid contacting the cuvettes with bare-hands, and do not contaminate their optical windows by touching them.
- Fill both cuvettes with purified water drawn directly from the water preparation system. Use of purified water stored in containers is not recommended. However, if freshly purified water is not available at sea, the carefully prepared standard water in combusted bottles can be used as a reference, but the investigator must document its degradation over time relative to air (see above).
- Carefully dry the cuvettes. Bulk dry with paper towels, but dry the quartz optical windows with lint-free laboratory wipes only (*e.g.* Kimwipes™).
- Inspect cuvettes carefully, especially along their optical paths, to ensure that they are clean. Make sure there are no bubbles, floating dust, or contaminants on the optical windows, or in suspension. Looking through the cuvette against a black background can usually identify any problems in the samples. Repeat cleaning and drying procedures as needed to obtain a clean sample.
- Run an air-to-air baseline reference spectrum for the spectrophotometer. Record the digital air baseline. This spectrum should be spectrally flat, with noise less than $\pm 0.0005 OD$.
- Place the reference cuvette in spectrophotometer and scan $OD_{\text{rwa}}(\lambda)$, the optical density of purified water relative to air. Remove the reference cuvette and repeat the measurement for the sample cuvette. Store both spectra noting which file is for the cuvette to be used as reference in subsequent analyses, and which is to be used for samples. See Figure 4.2 for spectra of $OD_{\text{rwa}}(\lambda)$ determined during ACE-Asia.
- Compare the spectra of $OD_{\text{rwa}}(\lambda)$ determined for the reference and sample cuvettes to each other, and with a digital library of previous reference water to air optical density spectra. Ensure that the two cuvettes are well matched optically, and that both conform to tolerance of pure water relative to air. Note anomalies and plan to make any needed corrections during data processing. If anomalies are associated with poor preparation of the cuvette, repeat the preparation and run new water-to-air baseline reference scans.

- Put both reference and sample cuvettes filled with purified reference water into the spectrophotometer for a double beam unit. For a single beam unit this will be done sequentially. Run a baseline correction for purified water. After the water-to-water baseline optical density measurement is complete, record the pure water baseline as a sample, $OD_{\text{rww}}(\lambda)$. This spectrum should be spectrally flat, with magnitude less than $\pm 0.0005 OD$. Save the digital baseline spectrum. Ensure the baseline is flat and stable over time and note any anomalies. It is common for the baseline to exhibit temperature-dependent artifacts 650-800 nm. These should be minimized if possible by ensuring the purified water in the sample and reference cuvette are at the same temperature.
- If the baseline reference spectrum $OD_{\text{rww}}(\lambda)$ is not flat and stable during analysis according to specifications summarized in section 4.3, the precision of any estimate of soluble absorption may be seriously questioned. It is the investigator's responsibility to ensure satisfactory performance of the instruments and use of proper methods to ensure that the final result is reasonable. Significant deviation from the specifications in section 4.3 or improper consideration of sample preparation protocols may result in estimates of soluble absorption that are not meaningful given the small magnitude of this estimate in the visible spectral region of most interest for ocean color applications.
- Remove the sample cuvette and discard the liquid. Rinse the inside of the cuvette three times with ~5 mL to 10 mL of the next sample to be measured. A copious rinse is desired, but sample volume is often limited. Several vigorously shaken small sample rinses are recommended if the volume is extremely limited.
- Fill the sample cuvette with the purified water that has been filtered as though it were a sample and record the blank spectrum, $OD_{\text{bs}}(\lambda)$, relative to the reference cuvette filled directly from the purified water source..
- Repeat the rinsing for each subsequent sample. The first sample rinse for seawater samples is most important to eliminate all purified water, especially for seawater samples due to refractive index differences between fresh and salt water. Fill the cuvette with the next water sample.
- Prior to running each sample, dry the exterior of the sample cuvette carefully, and inspect it, as described above, to ensure a clean sample.
- Replace the sample cuvette in the spectrophotometer, and measure the $OD_s(\lambda)$ spectrum relative to freshly purified water. Store the digital data and record all necessary information.

4.6 DATA PROCESSING AND ANALYSIS

The protocols in this section should be followed to compute particle and soluble material absorption coefficients from the spectrophotometric OD measurements described above. The following discussion assumes that all measured $OD(\lambda)$ spectra, whether for samples, or reference blanks, have been corrected for the instrument baseline spectrum, either automatically, or by post-measurement calculations appropriate to a particular spectrophotometer configuration (see above in Section 4.3, and specific reference spectrum measurement checks in the protocols of Sections 4.4 and 4.5).

Computations for absorption coefficients of particles concentrated on filters, and for materials dissolved in water, differ primarily in the determination of optical pathlength and in the treatment of reference blanks.

Soluble Absorption Coefficients

For soluble absorption, the calculations are directly proportional to the sample optical density relative to the pure water reference after correction for the pure water blank and specification of a null absorption

$$a_g(\lambda) = \frac{2.303}{l} \left[[OD_s(\lambda) - OD_{bs}(\lambda)] - OD_{null} \right], \quad (4.3)$$

where l is the cuvette pathlength (usually 0.1 m), $OD_s(\lambda)$ is the optical density of the filtrate sample relative to purified water, $OD_{bs}(\lambda)$ is optical density of a purified water blank treated like a sample relative to purified water (see below), and OD_{null} is the apparent residual optical density at a long visible or near infrared wavelength where absorption by dissolved materials is assumed to be zero. Note that as long as the null wavelength region is the same for sample and blank, the sample and blank spectra can be set to zero at the null wavelength independently or after they are subtracted from each other, as indicated in Equation (4.3). Equation (4.3) assumes use of a spectrophotometer that automatically references the sample and blank optical density to freshly purified water. Most modern commercial single or double beam units will compute this optical density directly relative to the reference. The user must record both raw sample and blank optical densities relative to purified water, and assess the stability of the purified water $OD_{rww}(\lambda)$ reference by routine determinations of the purified water relative to air (*e.g.* $OD_{rwa}(\lambda)$; Figure 4.2) and also evaluate the sample preparation methods by determining the blanks routinely (*e.g.* daily when at sea; Figure 3B).

a. Filtered pure water blank spectra

There are generally small spectral effects of the filtration and preparation procedure that cause blanks prepared from purified water to have a higher $OD_{bs}(\lambda)$ at short wavelengths compared to the reference cuvette containing purified water drawn directly from the purification system. Examples of filtered blank spectra $OD_{bs}(\lambda)$ for ACE-Asia where Millipore Alpha-Q water was used as the purified water source in the reference cuvette are illustrated in Figures 4.3B and 4.3D (c.f. Mitchell *et al.* 2000). The $OD_{bs}(\lambda)$ spectrum should be determined, recorded and included with the data for each sample. It is recommended that the investigator carefully determine these blanks for each station, or at least once per day, during a field program, and evaluate the stability of this blank for quality control purposes. If the purified water system is performing well, and the preparation procedures are carefully implemented, the $OD_{bs}(\lambda)$ sample blank offsets will generally be very consistent (Figure 4.3B). In such cases, the recommended procedure is to average $OD_{bs}(\lambda)$ spectra over the entire cruise, and to then fit a smoothed exponential function over wavelength to the overall mean (the bold line in Figure 4.3B). A separate OD_{null} (see discussion below) should be determined for the averaged and smoothed $OD_{bs}(\lambda)$ spectrum before it is substituted in Equation (4.3). Because the signals are small, instrument noise is a large fraction of the signal, even for high quality spectrophotometers. Therefore subtraction of an individual blank spectrum, including its noise, is strongly discouraged as this effectively doubles the noise of an already noisy signal. Instead, it is recommended that a smoothed blank be determined from many individual blank spectra provided that the investigator can demonstrate, as in Figure 4.3B that there is consistency among the population of blank spectra that are determined. The procedure of determining blanks at least each day during routine sampling provides an important quality control on the sample preparation protocols. If the blank is found to deviate considerably from the norm, the investigator should immediately determine the cause of the discrepancy.

b. Null point corrections to soluble absorption spectra

The absorption spectrum of pure water varies strongly with temperature, especially in the wavelength region between 650 nm and 750 nm, but at other wavelengths as well (Pegau and Zaneveld, 1993). To avoid temperature related measurement artifacts, the sample and reference should be maintained at the same temperature, but in practice, this is often difficult to do. If strong temperature residuals are apparent in the spectra near 750 nm, one must inspect the data to determine an appropriate wavelength range to use as a null point. For the data in Figure 4.3, it appears that assuming a null point as the average from 590-600 nm is reasonable. This assumption may not be reasonable in turbid lake, bay and coastal waters, however, where large soluble absorption values may persist into the near IR. Selection of wavelengths for null correction must be evaluated carefully for each data set, following principles discussed at more length by Mitchell *et al.* (2000).

Particle Absorption Coefficients

To compute particle absorption $a_p(\lambda)$ in suspension from spectrophotometric $OD_{fp}(\lambda)$ measured with the particles concentrated on a GF/F filter, it is necessary to appropriately adjust the optical pathlength. This includes substituting the geometric optical pathlength of the particles in suspension, and a scaling factor, β , accounting for the increase in the optical measurement path by scattering within the filter sample. The geometric absorption pathlength l_s of the filtered material in suspension is given by

$$l_s = \frac{V_f}{A_f}, \quad (4.4)$$

where V_f is the volume of water filtered and A_f is the clearance area of the filter calculated from the diameter D_f of the part of each filter that contains the particles. D_f should be determined very carefully on numerous individual filters using AN accurate measurement tool like a caliper that is accurate to at least 0.1 mm.

Scattering of light within the GF/F filter increases the absorption pathlength. The absorption coefficient of filtered particles must be corrected for pathlength amplification and the equivalent absorption coefficient in m^{-1} in suspension is computed as

$$a_p(\lambda) = \frac{2.303A_f}{\beta V_f} \left[[OD_{fp}(\lambda) - OD_{bf}(\lambda)] - OD_{null} \right], \quad (4.5)$$

where $OD_{fp}(\lambda)$ is the measured optical density of the sample filter, $OD_{bf}(\lambda)$ is the optical density of a fully hydrated blank filter, and OD_{null} is a null wavelength residual correction from the infrared where particle absorption is minimal. See also detailed discussion of null point selection in Mitchell *et al.* (2000)

a. Particle absorption blank spectra

If a spectrophotometer with automatic reference baseline correction is used, and the reference filter blank baseline is flat over the spectral range of interest, $OD_{bf}(\lambda)$ does not need to be subtracted. Spectra of $OD_{bf}(\lambda)$ must be determined, recorded and provided with the sample data. Properly prepared blanks generally have flat spectra relative to the reference baseline filters. If the $OD_{bf}(\lambda)$ is confirmed to be flat, then it is recommended that only a null absorbance is subtracted from the $OD_{fp}(\lambda)$ to compensate for baseline offsets. Subtraction of a spectrally flat baseline that varies only due to the instrument noise increases the noise of the result. If the instrument baseline cannot be maintained within the recommendations summarized in Section 4.3, the investigator should consider using a different instrument since the errors in the methods caused by using unstable instruments are difficult to control for.

b. Null point corrections to particle absorption spectra

To correct for residual offsets in the sample filter relative to the reference, and for scattering artifacts due to particle loading, it is assumed that a null absorption wavelength in the infrared can be identified. Historically, many investigators used 750 nm as the null absorption wavelength, but recent reports indicate that this wavelength is too short for some waters. It is recommended that the null wavelength be set at 800 nm (or longer), and that the investigator must examine the spectra to evaluate residual absorption structure near the null wavelength. Rather than use a single wavelength, a mean $OD_{fp}(\lambda)$ in a 10 nm interval (*e.g.* 790 nm to 800 nm) may be used as the null value to minimize the introduction of noise in the null correction procedure. Mitchell *et al.* (2000) discuss, at more length, factors affecting the choice of an appropriate wavelength for estimating OD_{null} . In Case 2 waters, the definition of the null absorption is more difficult and the investigator may consider the benefits of the transmission-reflectance estimates of particle absorption (Tassan and Ferrari, 1995a).

c. Pathlength amplification corrections

To correct for the pathlength increases due to multiple scattering in the filter, the prevalent current practice is to estimate β empirically through either a quadratic or power function that may be expressed in the form

$$\beta = \left[C_1 + C_2 \left[OD_{fp}(\lambda) - OD_{null}(\lambda) \right] \right]^{-1}, \quad (4.6a)$$

or

$$\beta = C_0 + C_1 \left[OD_{fp}(\lambda) - OD_{null}(\lambda) \right]^{C_2}, \quad (4.6b)$$

for quadratic equation or power function fits, respectively. C_0 , C_1 and C_2 are coefficients of least squares regression fits of measured data. Recommended coefficients have been reported in the literature (Table 1). The investigator should either choose published coefficients consistent with the species composition, equipment and measurement conditions for a given data set (consider the discussion in Mitchell *et al.*

2000), or independently determine pathlength amplification factors by comparing absorption in suspension and on filters following procedures described previously (Mitchell 1990, Mitchell *et al.*, 2000).

d. De-pigmented Particle and Phytoplankton Absorption Coefficients

The de-pigmented particle absorption coefficients, $a_d(\lambda)$, may be calculated using Equation (4.5), by substituting $OD_{fd}(\lambda)$ for $OD_{fp}(\lambda)$. At present it is recommended to use the same pathlength correction factor for the de-pigmented samples as for the particle absorption sample. The validity of this operational choice of β is difficult to assess, because the de-pigmented particles are created operationally from the treatment, and the relationships between their absorption on filters compared to suspensions may differ from those derived empirically for the original particles.

The spectral absorption coefficient for phytoplankton pigments can be computed as the difference between particulate and de-pigmented estimates:

$$a_\phi(\lambda) = a_p(\lambda) - a_d(\lambda). \quad (4.7)$$

4.7 DATA REPORTING

For purposes of data reporting and archiving, the absorption coefficients will be reported in m^{-1} and computed using the equations summarized above. Uncorrected optical density spectra for the filter samples, blank filter referenced to a blank filter, pure water referenced to air, pure water referenced to pure water and soluble absorption blank spectra must be recorded and provided so alternative algorithms could be applied to the original data. The pathlength amplification factor, a description of (or reference to) the method and the procedure for assignment of the null absorption, and any blank or spectral scattering corrections for the soluble absorption calculations must be reported.

4.9 PROTOCOL STATUS AND FUTURE DIRECTIONS

Absorption spectra for particles filtered on GF/F filters

Details of various issues related to this frequently used method for estimating particle absorption for filtered samples are not significantly changed since the summary of the NASA-sponsored Workshops found in Mitchell *et al.* (2000). It is important to address a few salient issues that are routinely asked by investigators interested in implementing the method. First, most modern dual-beam spectrophotometers that have a grating before the sample and illuminate the sample with spectrally resolved light have negligible differences (a few percent) in terms of determining the raw GF/F filter $OD_{fp}(\lambda)$ of the particles relative to a properly hydrated blank filter if the protocols are carefully followed. Thus, it is not essential to determine the pathlength amplification factor, β , for each different spectrophotometer that is used as long as the investigator makes an appropriate choice of instrumentation. However, some spectrophotometers have limited spectral range, limited dynamic range, more noise and inferior stability so the investigator should evaluate the unit to be used to ensure suitability by following the recommendations in section 4.3. Second, diode-array systems that illuminate with broad-band light and then disperse the post-sample light using a spectral photodiode array may have significantly different raw OD for the filtered sample. Example OD spectra estimated for a diatom culture for various systems used at the Scripps Workshop are shown in Figure 4.1 (see also details in Mitchell *et al.* 2000). Note the Hewlett-Packard spectral diode array system has a significantly higher OD than the other instruments. An empirical relation for this offset in the range 400-700 nm is reported in Mitchell *et al.* (2000) for that specific model. The Hewlett-Packard data is reported only for wavelengths greater than 400 nm because the instrument performs poorly at short wavelengths with the glass fiber filter method. If a user chooses such optical geometry for the determination of particle absorption they should carefully assess the potential issues illustrated in Figure 4.1. We recommend that the user compare several spectrophotometers for raw optical density of properly hydrated samples relative to blank filters and ensure the unit they use does not deviate from the typical result of most systems for which amplifications factors (β) have been determined (Table 4.1). Alternatively

one must determine the pathlength amplification for the instrument of choice, a laborious and unnecessary procedure if a spectrophotometer is selected that does not cause the bias illustrated in Figure 4.1.

Absorption spectra for particles transferred to glass slides

An alternative method, developed by Allali *et al.* (1995), to estimate absorption coefficients of cultures and seawater samples is to freeze transfer the particles to transparent microscope slides, following the protocols of Hewes and Holm-Hansen (1983). The investigator must have an integrating sphere or equivalent scattered transmission accessory to implement this method. This procedure produced results comparable to the GF/F filter method in comparisons reported by Mitchell *et al.* (2000), but sufficient uncertainties remain that the GF/F method continues to be recommended for the present.

Transmission-Reflectance (T-R) Method

Tassan and Ferrari (1995a) described a modification of the light-transmission method that corrects for backscattering. This technique combines light-transmission (T) and light-reflection (R) measurements, carried out using an integrating sphere attached to a dual-beam spectrophotometer. The data analysis is performed by a theoretical model that eliminates the effect of light backscattering by the particles. At the Scripps workshop, the global error of the T-R method was comparable to the error yielded by the simpler T method for monocultures. Subsequent modifications of the T-R experimental routine (Tassan and Ferrari, 1998; Ferrari and Tassan, 1999) yielded a significant reduction of the experimental error. Tassan and Ferrari (1995) reported that for case 1 waters that have negligible inorganic particle load, the amplification factor for GF/F filters determined with the T-R methods is similar to those determined by Mitchell (1990). The T-R method is particularly suited for applications to samples containing highly scattering mineral particles that are commonly found in Case 2 waters. Despite the more complicated procedure including an instrument with an integrating sphere, this method should be considered in special circumstances, and with further development may eventually supercede the presently recommended transmission protocol.

Absorption spectra for seawater filtered through membrane filters or cartridges

For most ocean regions, the optical density of dissolved organic material, relative to purified water in a typical 10 cm pathlength cuvette, is very small in the 400-600 nm region of most interest to ocean color satellite investigations. To ensure a common frame of reference for the global data collected by diverse investigators, we recommend $OD_{\text{rwa}}(\lambda)$ spectra (250-850 nm) be determined relative to air for purified water directly introduced to properly cleaned quartz cuvettes. The purpose of such spectra is to obtain an independent reference of the quality of the purified water. $OD_{\text{rwa}}(\lambda)$ spectra for the sample cuvette used during ACE-Asia are shown in Figure 4.2. $OD_{\text{rwa}}(\lambda)$ should be determined daily for the sample and reference cuvettes used in analyses. The investigator should keep careful records of this data and assess any bias in final estimates that may be attributed to problems with the reference water. By plotting in the range of minimal absorption by water (250-600 nm; Figure 4.2A) one can assess whether or not the reference water on a ship has seriously degraded. Production of impure water by commercial systems is a relatively common problem on ships where the feed water may have serious contamination. If the purified water system fails at sea, the investigator should use the standard water prepared prior to the cruise as the reference. Spectra of $OD_{\text{rwa}}(\lambda)$ of the bottled standard water should be determined before and after a cruise for each lot of bottled standards that are prepared. This precaution is important to assist in any corrections that might be required if standard water is used as a reference, or if the purified water system degrades over time during a cruise.

There are still relatively few spectra of soluble absorption determined fresh at sea using the revised protocols recommended here. Spectra of $OD_s(\lambda)$ and $OD_{\text{bs}}(\lambda)$ collected during ACE-Asia are shown in Figure 4.3. Raw optical density, relative to Millipore Alpha-Q water are shown in 3A. We routinely find small positive offsets from 600-800 nm that we feel should be compensated by subtracting a null value. Figure 4.3B illustrates $OD_{\text{bs}}(\lambda)$ during ACE-Asia prepared as recommended in section 4.5, but plotted at 10x smaller scale as Figure 4.3A. The recommended procedure is to subtract a cruise (or global) mean of this blank (solid line in Figure 4.3B) from the raw sample OD values, and then to adjust this difference to

zero at a null reference [Equation (4.3)]. The smoothed global blank was determined by taking the mean of all blanks for each cruise we have completed since 1998, subsequently taking the mean of all cruises and lastly fitting an exponential function to the global mean after setting a null point as the average from 590-600 nm. There can be small differences in blank spectra cruise to cruise, but we do not find this to be significant relative to the overall statistics of all cruises or the variance within a single cruise. For relatively weakly absorbing samples like open oceans observed during ACE-Asia, there is negligible apparent absorption > 600 nm and there is clear evidence in 3A of uncompensated temperature effects 650-800 nm. Therefore we chose to set the null value as the mean from 590-600 nm. However, if very strong soluble absorption is present, the temperature effects 650-800 nm will be less significant, and the absorption 590-600 nm may be important. The investigator should evaluate their data to determine the best null point and report that assessment. Figure 4.3C are optical density of spectra for a 10 cm cuvette after correcting for the null value and the blank spectrum. The effort to carefully determine the purified water relative to air, and blanks during each cruise will allow different investigators to inter-compare their results better, and will ensure better quality control of data collected over time. We have also determined the time-dependent change of our standard water (data not shown), and when we use that as a reference due to the failure of our purified water system at sea, we subtract a different blank than the global fit shown in Figure 4.3B.

An alternate method for preparing samples for soluble absorption allows multiple use of Sterivex sealed filtration cartridges. Use of these cartridges has been described by D'Sa *et al.* (1999) who used the method to prepare samples delivered to a capillary light guide spectrophotometer for estimating absorption by soluble material. The procedure provides high sensitivity and can be adapted to continuous flow determinations. This new method may prove useful in various applications but has not been applied extensively at this time. Evaluation of the performance of the Sterivex cartridges for sample preparation, and of light guides for spectroscopy, warrant further research.

Constraints on the estimate of soluble and particle absorption

To constrain our water sample estimates of particle and soluble absorption we have compared them to spectral estimates of the diffuse attenuation coefficient for downwelling irradiance, $K_d(z, \lambda)$, determined using a free-fall radiometer during a Southern Ocean cruise (AMLR) and a western Pacific Ocean cruise (ACE-Asia). It is well known that accurate estimate of $K_d(z, \lambda)$ in the upper ocean is difficult. Problems include heave of the ship, foam, bubbles, shadow, tilt, sky conditions and other influences on this apparent optical property (see more detailed discussions in other chapters of these protocols). Waters *et al.* (1990) described advantages of free-fall systems and many investigators have adopted this procedure to minimize some of the problems cited above. In 2001 we deployed our Biospherical Instruments PRR 800 system at approximately 80 stations combined between our AMLR and ACE-Asia cruises. We consider this our highest quality radiometric data set because of the free-fall deployment, the spectral range from 312-710 nm and because we acquired 4-5 separate free-fall profiles at each station to improve the confidence in our final estimate. In Figure 4.4 we show estimates of the mean cosine for spectral downwelling irradiance, $\bar{\mu}_d(\lambda)$, of the upper ocean mixed layer (open symbols). Here we define $\bar{\mu}_d(\lambda)$ as the ratio $[a_w(\lambda) + a_p(\lambda) + a_g(\lambda)] / K_d$. For Figure 4.4, values for pure water are estimated from Pope and Fry (1997) for 380-700 nm, Quickenden and Irvin (1980) for 300-320 and a linear interpolation between those values for 320-380 nm as recommended by Fry (2000). If the individual components are accurate, this can be considered a reasonable estimate of the mean cosine near the ocean surface (see Mobley, 1994 for detailed discussion of the mean cosine). Theoretically the values of $\bar{\mu}_d(\lambda)$ should be less than 1.0 and for typical radiance distributions of the upper ocean, they should be in the range of 0.70-0.85 near the surface. For both AMLR and ACE-Asia all absorption data were determined fresh at sea with consistent methods between the two cruises. We found that in the region 500 nm to 650 nm there is little difference between the estimates of $\bar{\mu}_d(\lambda)$ for the Southern Ocean and the western Pacific. However, below 500 nm, the values for ACE-Asia are near 1.0 and below 400 nm they exceed 1.0. For AMLR, values approach 1.0 for wavelengths less than 350 nm.

The ratio of $a_g(\lambda) / a_t(\lambda)$ where $a_t = a_w + a_p + a_g$, is also plotted in Figure 4.4 (filled symbols). The trend clearly illustrates that the soluble component dominates at short wavelength. There are several

hypotheses that should be considered to understand the overestimates of $\bar{\mu}_d(\lambda)$ below 400 nm. These could include underestimate of $K_d(z, \lambda)$ or overestimates of any of the absorption components. A combination of these factors may prevail. The filter radiometer in the profiler has good out of band blocking, but the spectrum of surface irradiance is rapidly changing in the region <350 nm and this may cause a red shift in the effective band center of the channels, with an associated underestimate of $K_d(z, \lambda)$. There may be small particles or colloids that pass the 0.2 μm filters causing a spectrally dependent scattering error (Aas, 2000). The particle absorption we estimate is based on Mitchell (1990), which results in higher estimates compared to some other published methods (Table 1). Also, there has not been adequate attention paid to determination of the pathlength amplification factor for the region below 400 nm. It is also possible that the values for pure water absorption are too high. The very reasonable or slightly high (by about 10-15%) values for the mean cosine of downwelling irradiance shown in Figure 4.4 for 400-600 nm indicates that the absorption methods recommended here are rather robust compared to simple estimates of diffuse attenuation coefficients. Reynolds *et al.* (2001) and Stramska *et al.* (2000) have reported reasonable closure between estimates of absorption using these methods, radiometric observations and modeling.

We have used “pure water” absorption for our estimate of $a_w(\lambda)$, and salts should in fact be added, if important, in the comparison of absorption to diffuse attenuation in Figure 4.4. Our estimate of $a_g(\lambda)$ relative to purified water will include absorption by salts, if they are significant. Salts in seawater are significant absorbers at short wavelengths. Lenoble (1956; see also Shiffryn, 1988) reported values for pure salts dissolved in purified water that indicate absorption coefficients near 300 nm comparable to the sample optical density of filtered samples relative to purified water that we routinely determine at sea in this spectral region. This UV absorption (<320 nm), relative to purified water, is generally assumed to be caused by “colored dissolved organic matter” but this may be inaccurate at these short wavelengths. Therefore one must be very cautious interpreting the apparent optical density of seawater filtrates relative to purified water for wavelengths less than 320nm. We recommend that more careful research should be carried out on the methods for soluble absorption, which appears to have a potentially dominating influence on the overestimates of $\bar{\mu}_d(\lambda)$ less than 400 nm. In particular, the influence of scattering by small particles (organic or mineral) and the role of salt absorption must be more carefully assessed.

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Table 4. 1. Published coefficients for determining pathlength amplification effects. The suspension optical density, OD_{sp} , computed for a GF/F filter with $OD_{fp} = 0.2$ is provided for comparison.

Quadratic Functions	Particle Type	C_0	C_1	C_2	$OD_{sp}(0.2)$
Mitchell (1990)	Mixed Cultures	--	0.392	0.655	0.105
Cleveland & Weidemann (1993)	Mixed Cultures	--	0.378	0.523	0.097
Moore <i>et al.</i> (1995)	<i>Prochlorococcus marinus</i>	--	0.291	0.051	0.060
Moore <i>et al.</i> (1995)	<i>Thalassiosira weissflogii</i>	--	0.299	0.746	0.090
Moore <i>et al.</i> (1995)	<i>Synechococcus</i> WH8103	--	0.304	0.450	0.080
Tassan & Ferrari (1995)	<i>Scenedesmus obliquus</i>	--	0.406	0.519	0.102
Nelson <i>et al.</i> (1998)	<i>Dunaliella tertiolecta</i>	--	0.437	0.022	0.088
Nelson <i>et al.</i> (1998)	<i>Phaeodactylum tricorutum</i>	--	0.294	0.587	0.082
Nelson <i>et al.</i> (1998)	<i>Synechococcus</i> WH7803	--	0.277	0.000	0.055
Power Functions					
Mitchell and Kiefer (1988a)	<i>Dunaliella tertiolecta</i>	1.3	0.540	-0.467	0.082
Bricaud and Stramski (1990)	Field samples; <i>D. tertiolecta</i> Cultures of Mitchell & Kiefer (1988a)	0.0	1.630	-0.220	0.086
Kahru and Mitchell (1998)	Mitchell (1990) data	0.0	1.220	-0.254	0.109
Constant					
Roesler (1998)	Assume $\beta = 2.0$	--	--	--	0.100

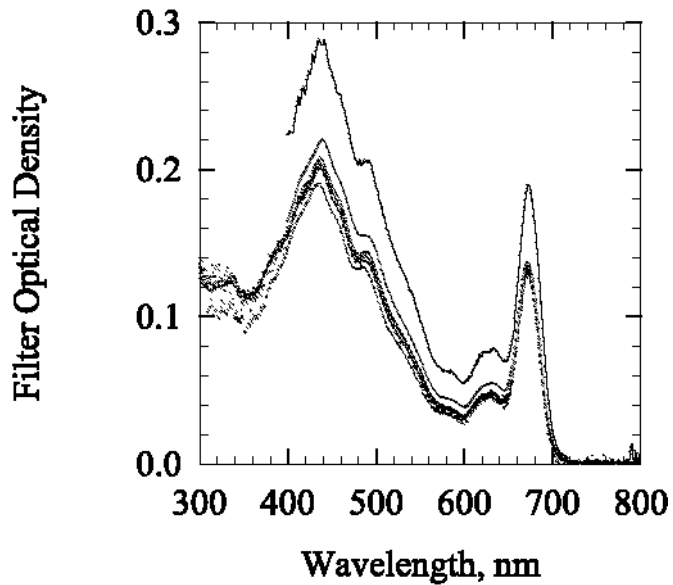


Figure 4.1: Optical density for various spectrophotometers for a diatom culture filtered onto GF/F filters. The average from 790-800 nm was used for a null value and the same volume was used for all samples. The data from the Hewlett Packard diode array system is higher than the other spectrophotometers as discussed in detail in Mitchell *et al.* (2000). Below 400 nm, the Hewlett Packard unit was too noisy for the glass fiber filter method.

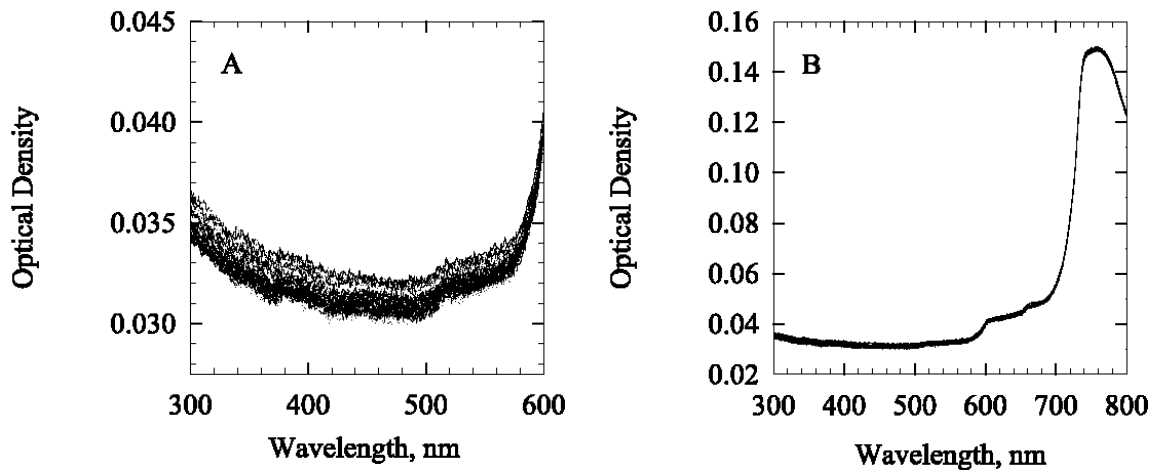


Figure 4.2: Optical density for fresh Millipore Alpha-Q water in the sample cuvette referenced to air in a dual beam spectrophotometer, $OD_{rwa}(\lambda)$, determined during the ACE-Asia experiment. **A).** $OD_{rwa}(\lambda)$ plotted for the spectral range 300 nm to 600 nm. **B).** Plotted for the spectral range 300 nm to 800 nm.

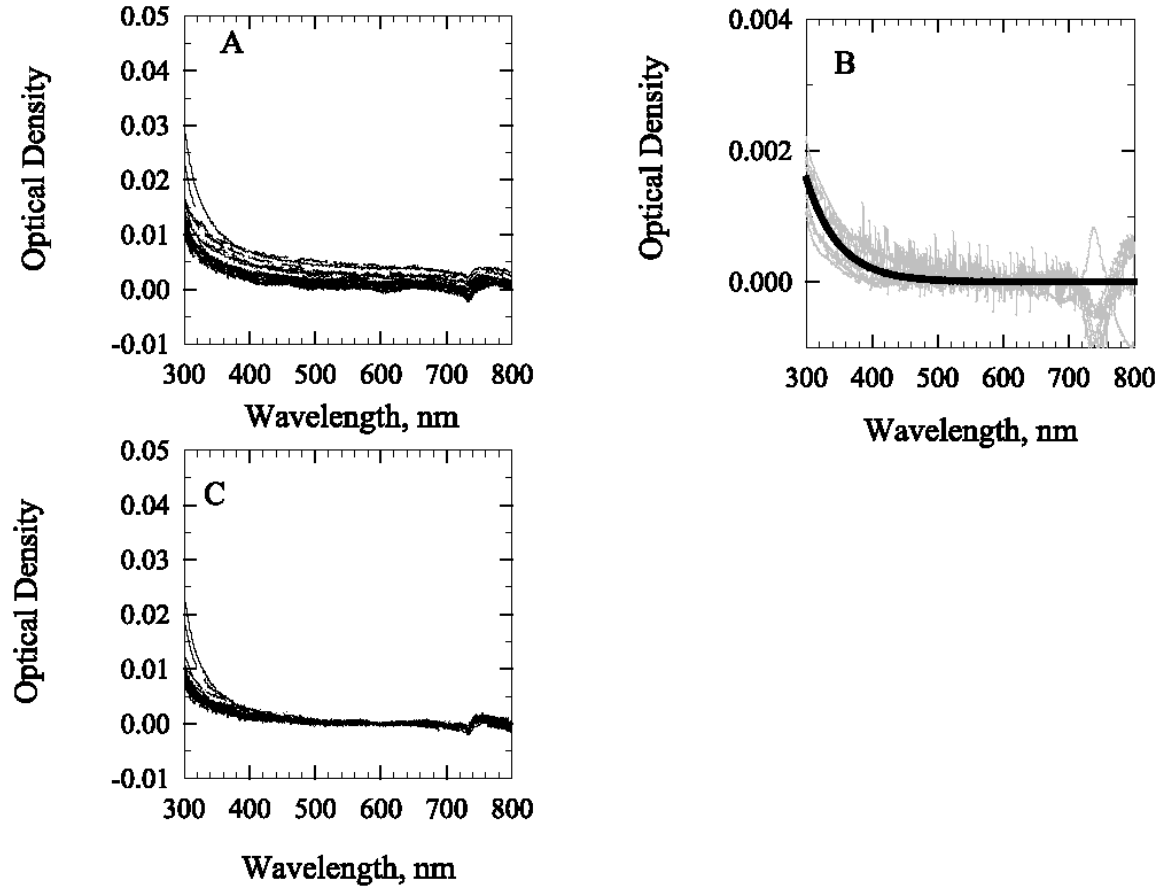


Figure 4.3: Typical results for soluble absorption determined during the ACE-Asia cruise (March – April 2001) in the western Pacific according to the protocols recommended here. **A**). Raw optical density, $OD_s(\lambda)$, for samples relative to Millipore Alpha-Q water. **B**). Blank optical density spectra, $OD_{bs}(\lambda)$ (after null offset, gray) compared to a global value (solid line). The global blank is determined by fitting an exponential function to the mean blank for more than 15 cruises from 1998-2001 where the mean for each cruise was determined as the mean of all individual blanks for each cruise. A fitted curve to a cruise or global mean for $OD_{bs}(\lambda)$ is recommended for correction of the soluble sample blank because individual spectra (gray) have significant instrument noise. Note the scale for 3B is approximately 10x smaller than the scale in 3A. **C**). Estimates of sample optical density spectra after subtraction of the null value (average of raw values 590-600nm) and after subtraction of a global blank according to Equation (4.3). Temperature effects are evident 650-800 nm in the individual spectra.

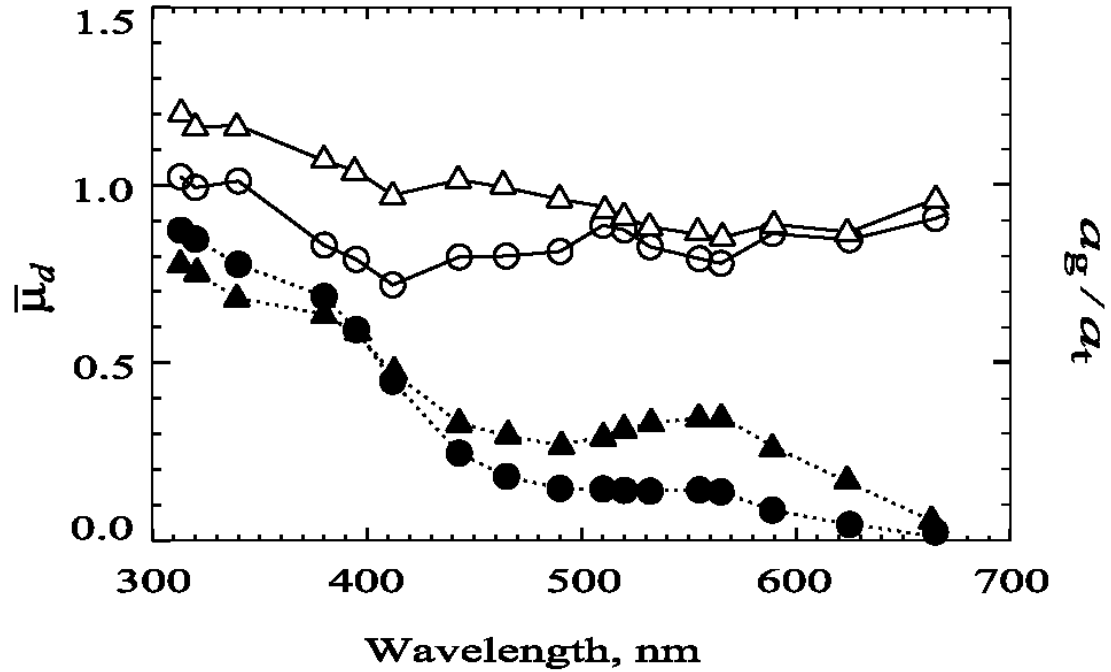


Figure 4.4: Median values for $\bar{\mu}_d(\lambda)$, the mean cosine for downwelling irradiance (open symbols, see text for definition) determined for the upper mixed layer. Values are plotted at each wavelength of the PRR 800 reflectance radiometer deployed during 2001 cruises to the Southern Ocean (AMLR) and the Western Pacific (ACE-Asia). The ratio of $a_g(\lambda)/a_t(\lambda)$ for the same data set are shown in solid symbols and plotted to the same scale. For both sets of spectra, AMLR data are circles and ACE-Asia data are triangles.