Ocean Optics Protocols For Satellite Ocean Color Sensor Validation, Revision 2

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Chapter 12

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

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12.1 INTRODUCTION

The spectral absorption coefficient is one of the inherent optical properties that influence the reflectance of aquatic systems. Therefore, knowledge of total absorption and its component parts is required to improve our understanding of variability in spectral reflectance estimated with ocean color sensors. Protocols for estimating the volume absorption coefficients for particles, phytoplankton, photosynthetic pigments, de-pigmented particles and soluble material are described. Methods are based on prior literature and results from NASA-sponsored workshops on methodological protocols. Issues, assumptions, and alternative methods are reviewed and recommendations for future research are made. Workshop participants are listed in Table 1.

The absorption coefficient at any point within a natural water body is an inherent property that can be described in terms of the additive contribution of its components:

$$a(\lambda) = a_w(\lambda) + a_p(\lambda) + a_g(\lambda)$$

where w, p, and g refer to water, particles, and soluble components. The particle component may be further decomposed:

$$a_p(\lambda) = a_\phi(\lambda) + a_d(\lambda) + a_i(\lambda)$$

where ϕ, d and i refer to phytoplankton, de-pigmented and inorganic components. Depth (z) dependence of the coefficients are omitted for brevity. Absorption methods are described for operational estimates of these fractions.

Scripps Institution of Oceanography Workshop		
Name	Affiliation	Instrument Brought
Lore Ayoub	Lehigh University	
Annick Bricaud	Laboratoire de Physique et Chimie Marines	
Joan Cleveland	Office of Naval Research	HP Diode Array
Giovanni Ferrari	CEC JRC, Institute for Remote Sensing Applications	
Piotr Flatau	University of California San Diego	
Jim Ivey	Naval Research Lab - Stennis Space Center	
Mati Kahru	University of California San Diego	
Motoaki Kishino	Photosynthesis Research Laboratory	
Helmut Maske	CICESE/Ecologia	Elyptica
Greg Mitchell	University of California San Diego	Varian Cary 1 with integrating sphere
Tiffany Moisan	University of California at San Diego	Perkin Elmer λ6 with integrating sphere
Lisa Moore	MIT School of Engineering	
John Morrow	Biospherical Instruments	
Norm Nelson	Bermuda Biological Station for Research	
Jennifer Patch	University of South Florida	Custom built spectral radiometer
Scott Pegau	Oregon State University	AC-9
Rick Reynolds	University of California San Diego	
Heidi Sosik	Woods Hole Oceanographic Institute	Perkin Elmer λ18 with integrating
Dariusz Stramski	University of California San Diego	Kontron Uvikon 860 with integrating sphere
Stelvio Tassan	CEC JRC, Institute for Remote Sensing Applications	
Tony Vodacek	Rochester Institute of Technology	
AlanWeidemann	Naval Research Lab - Stennis Space Center	
John Wieland	University of California at San Diego	
Ron Zaneveld	Oregon State University	
Bigelow Absorption Workshop		
Name	Affiliation	
Rick Gould	Naval Research Lab - Stennis Space Center	ASD Field Spec
Jim Hopkins	Bigelow Laboratory	
Phil Hovey	NOAA, NESDIS	HP Diode Array
Dave Phinney	Bigelow Laboratory	Bausch & Lomb Dual Beam Spec
Charles Trees	San Diego State University/CHORS	Perkin Elmer Lambda 3b
Sara Woodman	Bigelow Laboratory	

Table 1. NASA-sponsored workshops participants

To interpret aquatic spectral reflectance and better understand photochemical and photobiological processes in natural waters, it is essential to quantify the contributions of the total absorption coefficients in the ultraviolet (UV) and visible region of the spectrum. The material presented here is based on the evolution of methods starting with articles by Kalle (1938) and Yentsch (1962) on the absorption by soluble and particulate material in the oceans. Laboratory measurements and data analysis protocols are described for separating the total spectral absorption coefficient, $a(\lambda)$, into its components by spectrophotometric measurements on processed samples that have been prepared from filtration of discrete water samples. All filtration methods define operational fractions with assumptions depending on the methodology employed. There has been considerable research to develop robust protocols that provide the most accurate quantitative estimates of various fractions. 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. A summary of participants and the instrumentation that was evaluated is provided in Table 1. Recommendations provided here are based on results from the workshops and published literature. In general, recommendations are provided for protocols that have gained broad acceptance by diverse researchers. We also discuss alternative methods, assumptions for various methods, and issues that lead to uncertainties that should be the focus of future research.

12.2 BACKGROUND

The most widely used approach to estimate absorption by particulate matter 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). Kiefer and SooHoo (1982) reported a constant to scale the red peak of chlorophyll absorption for GF/C filter measurements of natural particles to the diffuse absorption coefficients determined on suspensions by Kiefer et al. (1979).
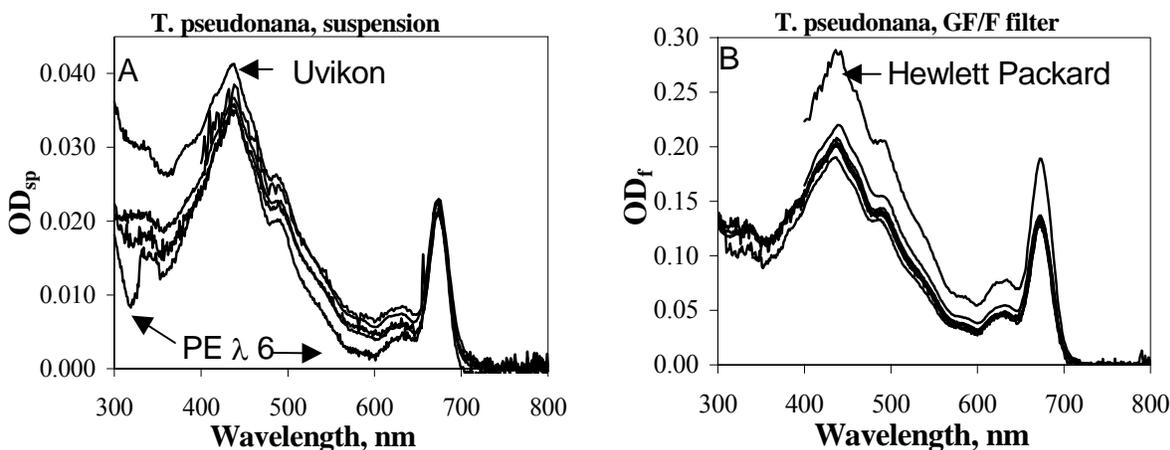

Figure 1. Instrument comparison of optical density (OD) spectra for a culture studied during the Scripps workshop. **A.** $OD_{sp}(\lambda)$ 300-800 nm for suspensions of *Thalasiosira pseudonana* cultures determined with different spectrophotometers. All units had integrating sphere attachments. Performance in the visible is in good agreement for all instruments except the Perkin Elmer Lambda 6, which had baseline stability problems during the workshop. UV region absorbance is in poor agreement for unknown reasons. The HP unit was not capable of performing below 400 nm. **B.** $OD_f(\lambda)$ 300-800 nm for *T. pseudonana* cultures on Whatman GF/F™ glass fiber filters determined with various optical geometries. The mean value from 790-800 nm was subtracted as a null value for all spectra. $OD_f(\lambda)$ in the UV agreed better between instruments compared to $OD_{sp}(\lambda)$ determined with integrating sphere systems.

The diffuse absorption coefficient is double the value of the volume absorption coefficient of interest here. 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 methods for determining particle absorption on water samples. Example data for one culture studied at the Scripps workshop are shown in Figure 1.

Field applications of these quantitative estimates of a_p were reported by Mitchell and Kiefer (1988b) and Bricaud and Stramski (1990). More detailed empirical results to correct for path length amplification were reported by Mitchell (1990) for various filter types and diverse cultures ranging from 2 μm coccoid cyanobacteria to 10-20 μm diatoms, chrysophytes and dinoflagellates. Cleveland and Weidemann (1993) and Tassan and Ferrari (1995) found 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; see also Table 2). Similar results were obtained by Allali et al. (1997) for natural populations of the Equatorial Pacific dominated by picoplankton. For samples with substantial turbidity due to inorganic matter (coastal, shelf, coccolithophore blooms), artifacts in the estimates can be caused by the large scattering load of the inorganic material; methods to correct for this have been described by Tassan and Ferrari (1995a, 1995b).

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, 1995; 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). Estimating the quantitative absorption of soluble material is rather direct, but has limits due to the very small signal for short pathlengths routinely employed (usually 10 cm). A difficult issue is quality control of reference water and specification of an appropriate blank. A summary of average blanks from several cruises is shown in Figure 2.
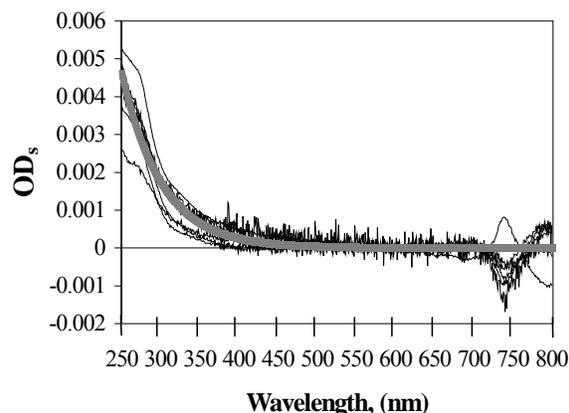

Figure 2. $OD_s(\lambda)$ 250-800 nm spectra for a 0.1m optical path of blank samples prepared by filtering purified water as though it were a sample. Measurements were determined with either Varian Cary 1 or Cary 100 in dual beam mode referenced to purified water. Mean blanks for individual cruises (typically 15-30 total blanks per cruise) are shown as thin lines; the bold line is an exponential fit to the mean for all cruises, where individual cruises were weighted by the relative number of blanks determined. Data collected by Scripps Photobiology Group. For one cruise the instrument baseline noise was $> \pm 0.0005$ which was out of specification for these units. Accurate estimates of $a_g(\lambda)$ require instrumentation with very small baseline noise.

We report recommendations for the determination of absorption coefficients for particulate and soluble matter as defined by operational definitions specified by preparations of water samples. Results of the methods, including separation of particulate and soluble material by filtration methods, partitioning of total particulate absorption into phytoplankton and de-pigmented (detritus), and corrections for various artifacts including scattering and pathlength amplification, will depend on the protocols used for preparation and the methods used for data processing. Recommendations are made based on widely accepted methods and processing procedures. NASA-sponsored workshops have confirmed various aspects of previously reported methods. Significant uncertainty still exists regarding the ideal methods for achieving the most accurate results for various absorption components. Therefore a review of important uncertainties, alternative methods, and recommendations for further research are also included in the Workshop Summary section of this report.

12.3 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, 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 Tygon tubing. If Niskin bottles will not be sampled immediately, precautions must be taken to ensure large particles that settle are resuspended. 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 vertical profile was acquired.

12.4 PARTICLE ABSORPTION SAMPLE PREPARATION ANALYSIS

For quantitative estimates of particle absorption coefficients, water samples are filtered and absorbance spectra of the filter $\left(OD_f\right)$ are estimated for the retained particles using a laboratory spectrophotometer. After measurement, the sample filters are extracted or bleached to remove phytoplankton pigments (Kishino et al., 1985; Tassan and Ferrari 1995a) and the OD_f of the filter is determined again in the spectrophotometer. This yields the de-pigmented absorption component sometimes referred to as detritus or tripton. Depending on the method, this fraction also includes bleached cells, and phycobilipigments which are not extractable in methanol. The raw OD data are used to calculate total particulate and de-pigmented absorption coefficients $\left(a_p \text{ and } a_d\right)$. The absorption coefficient of phytoplankton $\left(a_\phi\right)$ is then estimated as the difference $a_p - a_d$.

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 10-15% 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. Vacuum pressures below ~5 inches of mercury, i.e., ~120 mm Hg, are recommended to reduce the chances of particle breakage. The absorption of the particle fraction with 0.22-0.7 μm diameter can be selectively determined by measuring the GF/F filtrate deposited on a 0.22 μm Millipore cellulose acetate membrane filter (Ferrari and Tassan, 1996).

Filtration volume should be adjusted to keep the samples in the optical density range that is ideal for the pathlength amplification corrections. Glass fiber, cellulose acetate, and other strongly diffusing filters have large scattering coefficients, which increases the optical path length of photons in the measurement beam. Mitchell (1990) studied the effects of variable optical density on the performance of algorithms and recommended that optical densities be in the range 0.05-0.4 for best

performance of empirical algorithms. The optical transparency of the GF/F filter relative to air decreases significantly below 380 nm but many spectrophotometers can still make determinations to 300 nm with these filters. Optical density spectra of the sample filters should be measured as soon as possible, because pigment decomposition may occur (Stramski 1990). If filters must be stored, 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). The transparency of the filter also depends on hydration, so all samples must be fully - but not excessively - hydrated for proper performance of analytical procedures and accurate optical corrections.

Sample Filter Preparation

- Collect water samples, and maintain in the dark at or near *in situ* water temperature.
- 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 0.2 μm filtered water while mounted on the filtration funnel (with valves closed) during the sample filtration.
- Filter samples on GF/F filters under low vacuum (~5 in. Hg) in dim light.
- Filter sufficient volume for proper performance of correction algorithms. For typical field samples collected in the upper 100-150 m appropriate filtration volumes are typically in the range 0.5 – 5 L, depending on abundance of particles. A reasonable volume to filter (Vf, liters) can be estimated if Chl *a* concentration (Chl, μg/l) is known ($Vf = 0.4 \, [Chl]^{-0.7}$).
- 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 filtered water in the appropriate container depending on how they will be stored.
- Record the filter and filtration funnel type, diameter of the area on the filter with the concentrated particles, and volume filtered.
- Run absorption spectra as soon as possible.

Sample Filter Storage

- If 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 filtered seawater. Store dark and refrigerated (~4 deg. C) until analysis.

- If samples will be run more than 24 hours after collection sample filters should be prepared for liquid nitrogen storage. Storage should be done in containers that allow the filter to remain flat, and are specifically designed for liquid nitrogen (e.g. Fisher Histoprep™ tissue capsules). One pair of blank filters for each sample date should be prepared for the subsequent analysis. Samples on liquid nitrogen may be stored for extended periods but analysis as soon as possible is always preferred.
- Non-pressurized liquid nitrogen sample 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) to keep the sample dewars 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 can last 2-3 weeks if properly charged and in good condition.
- Air transport of liquid nitrogen dry shippers is approved by International Air Transportation Agreement (IATA 41st Edition Section A800; US Federal Aviation Administration Dangerous Good Bulletin DGAB-98-03; August 25, 1998) but many issues have been reported in clearing customs, or 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 bulletin of liquid nitrogen dry shipper information. 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. 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 on dry ice can be considered during transport. But maximum duration of dry ice in insulated shipping boxes is several days, so liquid nitrogen dry shippers are recommended.

Sample Filter Preparation for De-pigmented Particle Absorption

After preparing an a_p sample filter and determining OD_f on the spectrophotometer using the standard procedures, the sample should be processed to prepare a de-pigmented sample for determination of a_d.

Methanol Extraction method

- Place the sample and blank filters back onto the filtration system. Treat blank filters exactly as if they were sample filters.
- Add 5-10 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. Let stand for 1 minute.
- Filter methanol through, close valves, and add 10-15 ml methanol. Allow sample to stand in methanol for approximately 1 hour. Do not allow the filter to go dry during the extraction period. Time of extraction will vary depending on filter load and species composition on the filter. Place aluminum foil over the filtration cups during extraction to minimize contamination.
- After extraction, turn on the vacuum and draw the methanol through the filter. Rinse the sides of the filter tower twice with small amounts of methanol. Last, rinse the sides of the filter tower three times with ~20 ml of 0.2 μm filtered seawater. Also rinse blanks with filtered seawater after methanol extraction to minimize filter dehydration problems in analysis.
- If the 675 nm absorption peak of the sample is absent, samples can be considered fully extracted.
- Successive short extractions of 10 minutes can sometimes improve the pigment extraction.
- Phycobilins and some eukaryotic pigments will not be extracted by methanol.

Sodium Hypochlorite oxidation method

- *Prepare NaClO solution:*
  For freshwater samples: 0.1% active chlorine in Milli-Q water.
  For marine samples: 0.1% active chlorine in Milli-Q water containing 60 g l^{-1} Na$_2$SO$_4$, to match osmotic pressure of sample cells.
- Determine the solution amount needed to bleach the sample by the empirical expression:

ml of solution (0.1% active chlorine) = 3 OD_f (440).

- 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 5-10 min time, adding solution to compensate loss through the filter.
- Cover cup with aluminum foil to prevent contamination.
- Rinse the sample by gentle filtration of 50 ml of water (either fresh or salt, depending on sample source).
- Disappearance of the 675 nm peak in the bleached sample, and evidence of concave shape of the OD spectrum about 440 nm, can be considered evidence of complete pigment bleaching.
- If residual pigment absorption remains, repeat NaClO oxidation treatment, as indicated above.
- Treat blank filters in the same way.

Determination of spectral optical density of sample filters

After preparation, the sample filters are scanned in a spectrophotometer. The following procedure is written generically for a dual beam spectrophotometer. Single beam units and a variety of custom-built instruments may be used provided the investigator will invest the extra effort for carefully characterizing the baseline and spectral performance of the instrumentation. Regardless of instrument configuration, care must be taken with respect to maintaining proper knowledge of instrumentation baseline, noise, spectral range, and other characteristics. Due to both large attenuance of sample filters and instrument limitations including dynamic range and spectral performance, there is a wide range of performance between different instruments. Careful selection of instrumentation by the investigator is very important for achieving satisfactory results. Performance of a wide variety of commercial and custom units have been compared and results are discussed in the Workshop Summary section below. In general, research-quality dual beam spectrophotometers had superior performance to various alternative instruments.

With a dual beam spectrophotometer, two reference filter blanks saturated with filtered seawater are used to measure the reference spectrum, and one is left in the reference beam during sample measurements. Most modern spectrophotometers automatically store the

instrument's reference spectrum and recorded sample spectra are automatically corrected. A new instrument reference baseline scan should be measured each time the spectrophotometer is powered and when its configuration has been changed. The baseline should be checked regularly (every 1-2 hours) during extended periods of analysis. Frequency of baseline verification will depend on the performance and stability of individual instrument and should be determined by the investigator prior to executing routine work. Baseline drift, and changes in sorting filters or lamp source can be causes of sample anomalies. Wavelength accuracy should be checked during the analyses (see Spectrophotometer considerations section below).

Analysis Procedure

- Warm up spectrophotometer 30 minutes.
- If using frozen samples, remove filters from container and place in petri dishes on filtered water to ensure hydration. Allow to thaw at least ~5 min; store refrigerated until 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. In general, these mounts must be fabricated specifically for each different instrument.
- Clean the quartz faceplates of the mounting device with purified water and detergent if needed. Rinse with purified water and ethanol. Dry thoroughly with lint-free laboratory tissues.
- Set the appropriate instrument parameters.
- Mount two pre-soaked and water saturated blank filters (one for sample beam, one for reference beam).
- To test for proper filter hydration, confirm that there is a drop of FSW left on the mounting plate when filter is lifted. With 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 on the quartz plate. Be careful not to have too much water, as the sample may wash away.
- No bubbles should be between the filter and the glass on the sample holder. Test by examining the back of the filter on the mounting plate. There should be a uniform layer of water between the filter and quartz mounting plate. Bubbles will be obvious. If they are present, pick up the filter with forceps, and place back on the plate with a slight dragging of filter across a filtered water drop. Check again. Repeat until no bubbles are present. Adjust amount of filtered water as necessary.
- Mounts that allow both sides of the filter to be in air are an alternative to eliminate the issue of bubbles, but sample hydration is more difficult to maintain.
- Run instrument baseline correction using the two blanks. For most commercial units, this baseline will be automatically subtracted from subsequent scans. Immediately after baseline correction is finished and without touching the filters, run the two blanks as a sample scan to confirm baseline performance within acceptable tolerance over the spectral range of determination. This spectrum should be flat spectrally with baseline noise less than ± 0.005 OD. Save this scan for reference and 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 estimates of sample filter $OD_f(\lambda)$. If using a single beam instrument, or instruments run in the single beam mode, it is possible to run a baseline with a blank filter against air; in this case the sample filter will then be run against air, which will avoid the necessity of rehydrating the blank reference filter regularly.
- Remove the blank filter from the quartz glass sample mount, and replace it with a sample filter ensuring proper hydration of sample. Run sample spectrum, save the 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 (> $\pm$ 0.02 absorbance) from zero 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 mounted in air, hydrate blank every scan.
- After determination of particle optical density, samples should be de-pigmented and de-pigmented optical density should be determined as described above.
- Note that methanol-extracted samples and blank filters tend to dry out quickly if the methanol is not thoroughly rinsed from the filters prior to analysis. NaClO oxidized

sample and reference filters should be thoroughly rinsed to extend the spectral range below 400 nm.

12.5 SOLUBLE ABSORPTION SAMPLE PREPARATION AND ANALYSIS

This technique measures the spectral absorption by soluble material $\left(a_g\right)$ dissolved in seawater. Seawater 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 should minimize contamination by organic or colored material and should protect samples from photo-degradation. Plastic or glass filtration apparatus may be used but the units should have mesh filter supports (e.g. stainless steel or plastic) and not ground glass frits. Glass frits tend to clog over time and change particle retention efficiencies of the units. Amber-colored borosilicate glass bottles are preferred for filtrate collection because they screen ambient light. Prior to each experiment, all filtration and storage bottles should be thoroughly cleaned. For preparations, 0.2 μm membrane filters, (e.g., Nuclepore™ polycarbonate) are recommended. Glass fiber filters are not recommended because of large contamination when tested with purified water. Membrane filters should be pre-soaked in 10% HCl and rinsed copiously with pure water and a small aliquot of sample before preparation. An alternate method for preparing samples 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 light guides for spectroscopy warrant further research but are not discussed further in this report.

Preparation procedures for soluble samples can introduce artifacts so a careful evaluation of the blank is essential. Spectral blanks for soluble absorption must be determined and subtracted from sample spectra. Blanks determined on numerous cruises are presented in Figure 2. For the blanks in Figure 2, the reference water was delivered directly to a pre-cleaned 10 cm quartz cuvette and the baseline was determined from 250-800 nm with Varian Cary 1 or Cary 100 spectrophotometers. The blank was prepared by filtering freshly purified water (Alpha-Q, Milli-Q or Nanopure) with the identical protocol used to prepare samples. The mean value of raw optical density from 590-600 nm was subtracted from the full spectrum as a null absorption correction.

Despite careful consideration of clean techniques, pre-rinse of filters, and sample handling, the purified water blank exhibits significant deviation from the reference below 450 nm. For all cruises, the instrument noise of samples was less than ± 0.0005 optical density units, except for one cruise where the instrument performance was out of specification for baseline noise. Temperature differences between the blank and reference samples lead to the spectral anomalies between 650-800 nm and must be carefully considered in the selection of a null point for soluble absorption determinations. Both reference and sample cells must be maintained at the same temperature if accurate estimates from 650-850 nm are required. For weakly absorbing oceanic samples soluble absorption can be negligible greater than 600 nm and thus 600 nm may be considered a null point. For more strongly absorbing samples the null point should be set at a longer wavelengths and careful consideration of temperature effects in specifying the null point should be made (See Figure 2). These issues are discussed in more detail in the Absorption Workshop Summary below.

Pre-cruise preparations

- Sample bottles (Qorpak™) used to collect sample filtrate need to be thoroughly cleaned in advance to remove any potential organic contaminants. Sequential soaks and rinses in mild detergent, purified water, 10% HCl with final copious rinse in purified water is recommended.
- Rinse plastic caps with 10% HCl, twice with Alpha-Q, then dry at 70°C for 4-6 hrs.
- Combust bottles with aluminum foil covers at 450°C for 4-6 hours.
- Fill clean, combusted bottles with fresh Alpha-Q (directly from tap).
- Assemble the combusted bottles and clean caps. Store in the dark.
- These standards are used during cruises to

evaluate the quality of freshly prepared purified water.

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 be used, but the investigator must then test for any contamination by the filter and ensure that no artifacts are introduced by the filter type.
- Filtration systems with individual control of vacuum for each sample and direct filtration to clean bottles should be used. See Figure 3 for a diagram of a custom-made soluble absorption filtration assembly that achieves these goals.
- Pre-soak filters for at least 15 minutes in 10% HCl. Rinse filters thoroughly with purified water. Mount filters on funnel and filter ~100 mls of purified water through filter into sample bottles. Shake bottles, rinse inside of caps with bottle rinse, discard water (pour discard over inside of caps to rinse them). Cover filtration cups with aluminum foil until ready to filter sample.
- Collect ~200 ml of seawater sample. For the blank, use purified water drawn directly into 2 clean sample bottle.
- Filter ~75 ml of sample and 1 blank directly into clean bottles at low vacuum (<5 in. Hg). Shake bottles, discard water. Do not allow filters to go dry during sample rinsing.
- Filter ~ 75 ml of sample into bottles. For the blank, filter ~75 ml of purified water. When finished, cap bottles and store until analysis.
- For samples that will be run within 4 hours, store in the dark at room temperature.
- For samples that will be run 4-24 hours later, refrigerate samples in the dark.
- Longer storage is not recommended, as there are artifacts of undocumented magnitude. Several researchers have reported results on frozen samples but no systematic evaluation of freezing artifacts has been reported.
- For refrigerated samples, warm to room temperature before analysis.
- The Qorpak bottles can be re-used at sea. After analysis, thoroughly rinse bottles (and caps) 3x with fresh purified water and store

with 20 ml of 10% HCl. Before re-use, shake well, discard the 10% HCl, and rinse copiously with fresh purified water. Fill the bottle with purified water to be used as the rinse for sample filters.
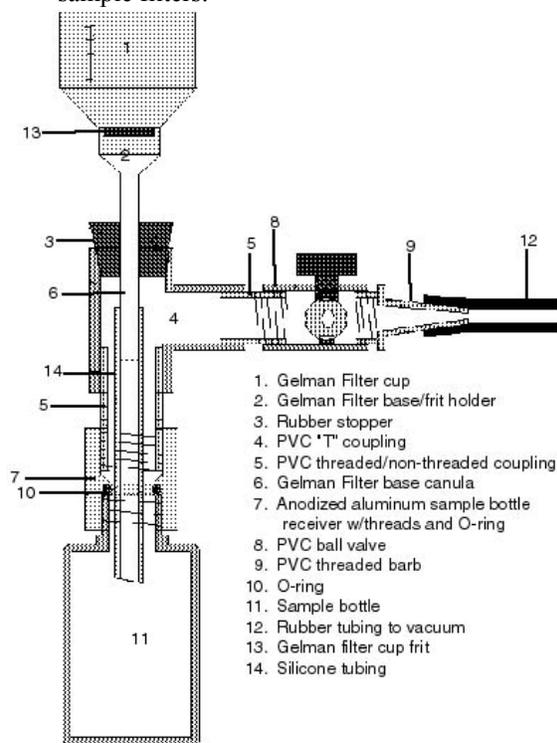
1. Gelman Filter cup
2. Gelman Filter base/frit holder
3. Rubber stopper
4. PVC 'T' coupling
5. PVC threaded/non-threaded coupling
6. Gelman Filter base canula
7. Anodized aluminum sample bottle receiver w/threads and O-ring
8. PVC ball valve
9. PVC threaded barb
10. O-ring
11. Sample bottle
12. Rubber tubing to vacuum
13. Gelman filter cup frit
14. Silicone tubing

Figure 3. Diagram of filtration apparatus designed to collect clean filtrate directly to a clean sample bottle for determination of soluble absorption coefficients.

Determination of Optical Density of Soluble Absorption Preparations

- If samples have been refrigerated, allow them to warm to room temperature.
- Allow spectrophotometer to warm up for 30 min. Confirm that the optical windows of the spectrophotometer are clean. If necessary, clean with purified water followed by ethanol and dry thoroughly with lint-free laboratory tissues.
- 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 2x with 10% HCl, 2x with ethanol, then rinse inside and outside copiously with purified water. After cleaning, use laboratory tissues when handling the cuvettes,

avoid contact with bare-hands. In particular do not contaminate the optical windows of the cuvette.

- Fill both cuvettes with purified water directly from water preparation system. Use of water stored in containers is not recommended. If purified water is not available at sea, the carefully prepared water in combusted bottles can be used as a reference, but the investigator must document its degradation over time relative to air.
- 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 including visual inspection along the optical path to ensure 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 for a clean sample.
- Run an air vs. air baseline for the spectrophotometer. Record the digital air baseline. This spectrum should be spectrally flat, with noise less than ± 0.0005.
- Place one cuvette in spectrophotometer and scan relative to air. Remove and repeat for the second cuvette. Store both spectra noting which file is for the cuvette to be used as reference in subsequent analyses, and which to be used for samples.
- Compare the two pure water vs. air spectra with each other and with a digital library of previous air-water 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 from poor preparation of the cuvette, repeat the preparation and run new air-water scans.
- Put both cuvettes in the spectrophotometer. Run baseline correction for purified water in both beams. After baseline is complete record the pure water baseline as a sample. This spectrum should be spectrally flat, with magnitude less than ± 0.0007. Save the digital baseline spectrum. Ensure the baseline is flat and note any anomalies. If baseline is not flat, the spectrum must be used for data processing.
- Remove sample cuvette and discard liquid. Rinse inside of cuvette 3x with ~5-10 ml of next sample. A copious rinse is desired but sample is often limited. Several vigorously shaken small sample rinses are recommended if volume is extremely limited. The first sample rinse is most important to eliminate all purified water, especially for seawater samples due to refractive index differences between fresh and salt water. Fill cuvette with next sample.
- Dry sample cuvette carefully and inspect as described above to ensure clean sample.
- Place sample cuvette back into spectrophotometer, and run spectrum. Store digital data and record all necessary information.

12.6 DATA PROCESSING

Spectral absorption coefficients in units of m^{-1} are calculated from optical density spectra $OD(\lambda)$ measured using the protocols described above. Subscripts *b, d, f, r, s, sp* and *null* used below are defined as referring to *OD* for the blank, de-pigmented particles, filter, reference baseline, soluble material, suspension of particles, and null point, respectively. Raw data collected as described above must be processed to provide the absorption coefficients of interest. For simplicity in the following discussion, it is assumed that the baseline reference spectrum $(OD_r(\lambda))$ for either soluble or particulate samples has been automatically compensated in the raw data record through instrument software. If not, it must be corrected in data processing and its omission in the formal equations does not imply that it is not necessary to evaluate this reference baseline and correct for it as required.

For soluble absorption, the calculations are directly proportional to the sample optical density relative to the pure water reference

$$a_g(\lambda) = \frac{2.303}{l}\left[OD_s(\lambda) - OD_b(\lambda)\right], \qquad (12.1)$$

where l is the cuvette pathlength (usually 0.1m) OD_s is the soluble preparation absorbance relative to freshly purified water and OD_b is the blank for the preparation. When double beam spectrophotometers are used that produce flat baseline spectra and automatically correct for the pure water reference, the $OD_r(\lambda)$ need not be subtracted since its value is simply zero plus instrumentation noise. If a flat reference spectrum and its noise is subtracted from the sample spectrum, noise is introduced to the result with no change in the basic

spectrum.

There are generally small spectral effects of the filtration and preparation procedure that cause blanks prepared from purified water to have a higher $OD_b(\lambda)$ at short wavelengths compared to purified water drawn directly from the sample system (Figure 2). The value of $OD_b(\lambda)$ for the blank must be subtracted from the raw soluble sample optical density. The $OD_b(\lambda)$ should be determined, recorded and included with the data. It is recommended that the investigator carefully determine these blanks for a cruise or field program for each station, and carry out an evaluation of the stability of this blank for quality control purposes. If the purified water system is performing well and the preparation procedures carefully implemented, the blanks are generally very consistent. In this case it is best to determine a mean spectrum from numerous determinations (e.g. all blanks for a cruise), and then fit a smoothed exponential function to the overall mean. Figure 2 illustrates these results for blanks, prepared from Millipore Alpha-Q, Milli-Q, or Banstead Nanopure water, and determined immediately after preparation for numerous cruises. The OD for the soluble sample and the blank should be set to zero at the selected null point, and then the blank subtracted from the sample. Temperature and salinity effects from 650-750 nm (Pegau et al., 1995) make choice of a null wavelength problematic since it is often not feasible to keep samples and references at identical temperatures. Influence of these effects are evident from ~620-800 nm in the blank spectra shown in Figure 2. The choice of a null point wavelength is discussed in the Workshop Summary section.

For particle absorption determinations, a correction for increased optical pathlength due to scattering by the filter medium is required (Kiefer and SooHoo, 1982; Mitchell and Kiefer 1984, 1988a; Mitchell 1990). The geometric absorption pathlength l_s of the filtered material in suspension is given by

$$l_s = \frac{V}{A}, \qquad (12.2)$$

where V is the volume of water filtered and A is the clearance area of the filter determined by measuring the diameter of the portion of the filter with concentrated particles. Scattering of light within the GF/F filter increases the absorption pathlength. 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 $\beta(\theta)$ used in other chapters of this Technical Memorandum. 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

$$a_p(\lambda) = \frac{2.303A}{\beta V}\left[OD_f(\lambda) - OD_{null}\right]. \qquad (12.3)$$

$OD_f(\lambda)$ is the absorbance of the sample filter measured relative to a fully hydrated blank filter. As with the analysis for soluble absorption, if a spectrophotometer with automatic reference baseline correction is used, and the reference filter blank baseline is flat over the spectral range of interest, $OD_r(\lambda)$ does not need to be subtracted. Spectra of $OD_b(\lambda)$ 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 $OD_b(\lambda)$ is confirmed to be flat, then it is recommended that only a null absorbance is subtracted from the $OD_f(\lambda)$ to compensate for baseline offsets. The investigator must ensure that $OD_b(\lambda)$ is flat if it is not to be subtracted from the raw $OD_f(\lambda)$. 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 (OD_{null}) in the infrared can be defined. Previously, investigators have used 750 nm as the null absorption wavelength, but numerous reports indicate this wavelength is too short for many waters. It is recommended that the null wavelength be set at 800 nm (or longer) and the investigator must examine the spectra to evaluate residual absorption structure near the null wavelength. Rather than use a single wavelength (which may be influenced by the instrument noise), a mean OD_f over 10 nm (e.g. 790-800 nm) can be used as the null value to minimize the introduction of noise in the null procedure. This issue is discussed in more detail in the Workshop Summary section.

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

$$\beta = \left[C_1 + C_2 \left[OD_f(\lambda) - OD_{null}(\lambda) \right] \right]^{-1}, \qquad (12.4a)$$

or

$$\beta = C_0 + C_1 \left[OD_f(\lambda) - OD_{null}(\lambda) \right]^{C_2}, \qquad (12.4b)$$

Table 2. Published coefficients for determining pathlength amplification effects. The OD_{sp} derived from Equation 4 for a GF/F filter with OD_f = 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 et al. (1995)	*Prochlorococcus marinus*	--	0.291	0.051	0.060
Moore et al. (1995)	*Thalassiosira weissflogii*	--	0.299	0.746	0.090
Moore et al. (1995)	*Synechococcus WH8103*	--	0.304	0.450	0.080
Tassan & Ferrari (1995)	*Scenedesmus obliquus*	--	0.406	0.519	0.102
Nelson et al. (1998)	*Dunaliella tertiolecta*	--	0.437	0.022	0.088
Nelson et al. (1998)	*Phaeodactylum tricornutum*	--	0.294	0.587	0.082
Nelson et al. (1998)	*Synechococcus* WH7803	--	0.277	0.000	0.055
Power Functions					
Mitchell and Kiefer (1988a)	*Dunaliella tertiolecta*	1.3	0.540	-0.467	0.082
Bricaud and Stramski (1990)	Field samples; *D. tertiolecta* 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 β = 2.0	--	--	--	0.100

for quadratic equation or power function fits, respectively. C_1 and C_2 are coefficients of least squares regression fits of measured data. Several recommended coefficients for C_1 and C_2 have been reported in the literature (Table 2).

The best overall performance of the GF/F algorithm evaluated by Mitchell (1990) was achieved when filtered sample density yields $OD_p(675)$ between 0.05 and 0.25 with blue absorption ≤ 0.4. The volume of water filtered for particle absorption measurements should therefore be adjusted accordingly. The various coefficients in Table 2 will predict absorption coefficients that vary by a factor of ~2 but the overall results indicate a much narrower range.   For all the coefficients shown in Table 2, the mean OD_{sp} = 0.087 when OD_f =0.2; the 95% confidence range is 0.078-0.096. This range is comparable to the estimated precision of the method discussed by Mitchell (1990) where the errors of the method were estimated to be of the order ± 15% for an experiment done carefully with a single filter lot, and perhaps 20-30% considering all aggregated errors of different filter lots, instrumentation, volume filtered, raw optical density, and particle type. The best fit to the data in Mitchell (1990)

results in estimates of OD of suspensions approximately 20-30% higher than values based on the power function coefficients of Mitchell and Kiefer (1988a) determined from a limited study of *D. tertiolecta* (e.g. 28% difference in Table 2 example).   Note that results of Bricaud and Stramski (1990) agree within 5% of the results for the Mitchell and Kiefer (1988a) equation because both fits were based on the same *D. tertiolecta* data set.   The smallest estimates of OD_{sp} shown in Table 2 correspond to β experiments done for small phytoplankton (*Prochlorococcus, Synechococcus*). There is considerable uncertainty still regarding the influence of particle type, refractive index and size on the β values. The best estimate for β may depend on particle types within a sample that are not known *a priori*. Therefore it is important to report the absorption coefficient estimates, the correction procedure, and the raw data to allow future revisions in the estimates from the original data.   Ancillary data including particle size distributions, inorganic sediment mass, flow cytometry and HPLC pigments would be useful to evaluate the ideal correction factors for OD_f . 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). These issues are discussed further in the Workshop Summary section.

For de-pigmented absorption coefficients, $a_d(\lambda)$, one may use a calculation analogous to Equation 3, but where the filter optical density of de-pigmented particles corrected for the reference baseline, is used for OD_f. Generally the same pathlength correction for the de-pigmented samples is applied. The validity of this operational choice of β is difficult to assess because the de-pigmented particles are created operationally from the treatment and therefore true empirical relationships between their absorption on filters compared to suspensions have not be performed. The spectral absorption coefficient for phytoplankton pigments can be computed as the difference between particulate and de-pigmented estimates:

$$a_\phi(\lambda) = a_p(\lambda) - a_d(\lambda) \qquad (12.5)$$

12.7 ESTIMATION OF PHOTOSYNTHETICALLY ACTIVE ABSORPTION

Methods have been proposed for the estimation of photosynthetically active absorption. Total phytoplankton absorption is attributed primarily to the sum of photosynthetic and photoprotective pigments (e.g. $a_\phi = a_{ps} + a_{pp}$) Bidigare et al. (1987) reported a method for reconstruction of the phytoplankton absorption from HPLC-determined pigment concentrations and estimates of the mass-specific spectral extinction coefficients of different pigments. HPLC pigment reconstruction was compared to estimates of phytoplankton absorption determined with glass fiber filters for samples from the Sargasso Sea. HPLC reconstruction methods are useful for improving our understanding of the relative contribution of phytoplankton to total absorption, as well as the relative importance of photosynthetic and photoprotective pigments for the phytoplankton fraction. However, issues remain due to the lack of knowledge about the true *in vivo* extinction coefficients of individual pigments, and the uncertainty in the degree of packaging of a polydisperse natural particle assemblage. Estimates of spectral mass extinction coefficients for these methods are provided in Bidigare et al., (1990). The method is useful if pigment-packaging effects are negligible. For this

assumption to hold, both small cells and low pigment per cell volume are required. Compared to directly measured *in vivo* absorption spectra of phytoplankton cultures, the HPLC reconstruction generally over predicts the true value, and results in spectral shapes that are different from *in vivo* estimates (Sosik and Mitchell, 1991; Moisan and Mitchell, 1999). An improvement of Bidigare's method has been proposed by Babin et al. (1996) and applied by Allali et al. (1997) to natural populations of the Equatorial Pacific. It consists of partitioning the measured absorption spectrum into its photosynthetic and non-photosynthetic components using the HPLC information, and thus takes into account the actual degree of packaging for the population.

Fluorescence excitation spectra for chlorophyll *a* have been shown to be a good proxy of the spectral shape of the photosynthetic action spectrum for many phytoplankton types (Neori et al., 1988). Thus, they can represent the relative absorption spectrum of photosynthetically active pigments for some phytoplankton types. Sosik and Mitchell (1995) have proposed a normalization of the fluorescence excitation spectrum to the red peak of absorption for oceanic particulates to estimate absorption by photosynthetically active pigments, $a_{ps}(\lambda)$. They found $a_{ps}(\lambda) \le a_\phi(\lambda)$, where the difference is attributable to photo-protective pigments in the phytoplankton. Issues related to scaling the relative fluorescence spectrum for cultures to the chlorophyll *a* absorption at the red peak have been discussed further by Johnsen et al., (1994) and Moisan and Mitchell (1999). The method assumes that pigments are equally distributed between the two photosystems (the fluorescence of PSI is undetectable at ambient temperature). This assumption is not valid for phycobiliprotein absorption (Neori et al., 1988) and some other phytoplankton groups (Allali, 1997; M. Babin, unpublished data).

12.8 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 reference, pure water reference, and soluble absorption blank spectra must be recorded and provided so alternative algorithms could be applied to the original data. The pathlength amplification factor and a description of (or reference to) the method and the

procedure for assignment of the null absorption must be reported.

12.9 SUMMARY OF WORKSHOP RESULTS

Two workshops were sponsored by NASA to evaluate different spectrophotometers, previously published literature, and new methods for absorption analyses. The participants in the workshops at Scripps Institution of Oceanography and Bigelow Laboratory for Ocean Sciences are listed in Table 1. Several questions were addressed including comparison of different spectrophotometer systems, evaluation of published methods for determination of β, sample preservation, null absorption wavelength subtraction, de-pigmentation, and other issues. Many of these issues could benefit from further research on advanced protocols.

Spectrophotometer considerations

Various spectrophotometer options exist ranging from commercially available research-quality systems that have scattered transmission and reflectance options to low-cost analytical units, to custom built instruments that may be very simple or quite sophisticated. With appropriate training and knowledge, high quality results may be achieved with diverse spectrophotometers. The investigator must weigh the merits of the flexibility of custom systems with the ease of operation of certain robust analytical or research-quality units. Various instruments that were evaluated during the NASA workshops are listed in Table 1. In terms of baseline noise and stability, spectral precision and range, and ability to measure both filters and suspensions, the top performing spectrophotometers - not surprisingly - were the more expensive commercial research-quality systems. Still, many intermediate cost analytical units performed well throughout the spectral range. However, numerous instruments exhibited problems with baseline noise, spectral range, spectral accuracy and stability. Often several of these problems were found for an instrument. To carry out appropriate work on particle suspensions requires either a high quality scattered transmission accessory or an integrating sphere. For the workshops, the analyses on suspensions were limited to the moderate to expensive commercial systems that had integrating sphere accessories.

Verification of the spectral and quantitative accuracy of the optical density estimates must be carried out by investigators. Commercially available research quality analytical dual beam UV-visible spectrophotometers are recommended for the absorption determinations described here. Many commercial instruments use lines in the mercury lamp to ensure spectral calibration during start up procedures. Still, it is possible for some instruments to develop spectral anomalies during operations including baseline drift, or mechanical mis-alignment of the grating, etc. Therefore it is recommended that investigators have a holmium oxide filter as an independent reference of instrument spectral performance. Periodic checks should be determined by scanning the filter relative to an air-air baseline. Any spectral anomalies found must be corrected. Numerous spectrograph devices and non-commercial spectrophotometers do not have automatic spectral performance adjustment. For such instruments, careful determination of the spectral performance using a holmium oxide filter should be done. If the unit exhibits instabilities, the spectra must be repeated regularly. Since all raw optical density determinations described here are carried out relative to a reference (e.g. blank filter, purified water) proper treatment of the baseline and sample spectra should provide accurate optical density results. To ensure accurate estimates of optical density, absorbing reference standards should be run regularly relative to air with the analytical equipment. Absorption reference filters for different optical densities, and holmium oxide filters can be purchased from manufacturers or scientific optics supply companies to carry out these performance tests.

At the Scripps workshop, several research-quality instruments provided the best overall performance. The Perkin Elmer Lambda 18 was the most consistent in the spectral range from 300-800 nm for all sample types and measurement geometries and is used in comparison to other instruments listed in Table 1. At the Bigelow workshop, the moderately priced Perkin Elmer Lambda 3 was used to compare various units. Scripps workshop results for one culture are shown in Figure 1 as an example of the type of results attained. In general, with proper consideration of instrumental baselines and care in sample preparations, all spectrophotometers that could determine the optical density of suspensions agreed well in the visible within routine analytical error (Figure 1a). This was also true for determinations of cultures or natural particles concentrated on GF/F filters both using standard optics in commercial grating monochromator systems, or

collecting the energy with a scattered transmission accessory or integrating sphere (Figure 1B). When OD_f from 400-700 nm for the traditional dual beam commercial instruments were regressed against each other the slopes of the regression usually were between 0.95 – 1.05 implying minimal difference caused by different instruments. In Figure 4B the slopes for the Cary and the Kontron, relative to the Perkin Elmer Lambda 18 were 0.96 and 0.98, respectively. At the Bigelow workshop, the ASD fiber optics system had a slope of 1.15 relative to the Perkin Elmer Lambda 3.

Optical densities in transmittance mode for GF/F filters were determined at the Scripps workshop for diverse optical geometries including integrating spheres (PE Lambda 18), diffuse transmittance accessory (UVIKON Shibata method), standard optics of pre-sample grating commercial spectrophotometers (Cary 1, Perkin Elmer Lambda 6), diode array with integrating sphere illumination and collection (HP), and sample illumination from broad band light source with spectral dispersion using a post-sample diode array spectrograph (USF). Table 3 summarizes the regression slopes of these diverse optical geometries relative to the Perkin Elmer Lambda 18 integrating sphere estimates. Both the Cary and the Perkin Elmer Lambda 6 with samples placed in the beam of the standard optics path in the sample compartment, as well as the Kontron configured with a proper scattered transmission accessory (Shibata, 1958) resulted in raw OD_f values that were within 5% of the PE Lambda 18 integrating sphere results. These differences for replicate filters prepared individually for each instrument are within the routine methodological uncertainty of replicate filters run on the same instrument. Results in Table 3 indicate that many spectrophotometers, with extremely different optical geometries, can determine raw OD values that are equivalent within methodological uncertainty of the preparations. Results in Table 3 are consistent with the results of Mitchell (1990) that raw OD_f for a filter measured either with standard optics or an integrating sphere were not significantly different.

Large differences among instruments were observed, however, in the baseline noise, spectral range, and transition across instrumentation optical-mechanical wavelengths (e.g. lamp or sorting filter wavelength change positions). These latter performance issues are very important with respect to achieving high quality results, and the investigator should carefully assess the performance of

Table 3 Example of OD_f regression slopes between instruments for replicate GFF filtered samples of *T. weissflogii* analyzed with various spectrophotometers at the Scripps Workshop. Comparisons are relative to OD_f determined with the Perkin Elmer Lambda 18 integrating sphere. Extremely different optical geometries are represented. Data are 400-700 nm after null correction. Other comparisons for different cultures yielded similar results.

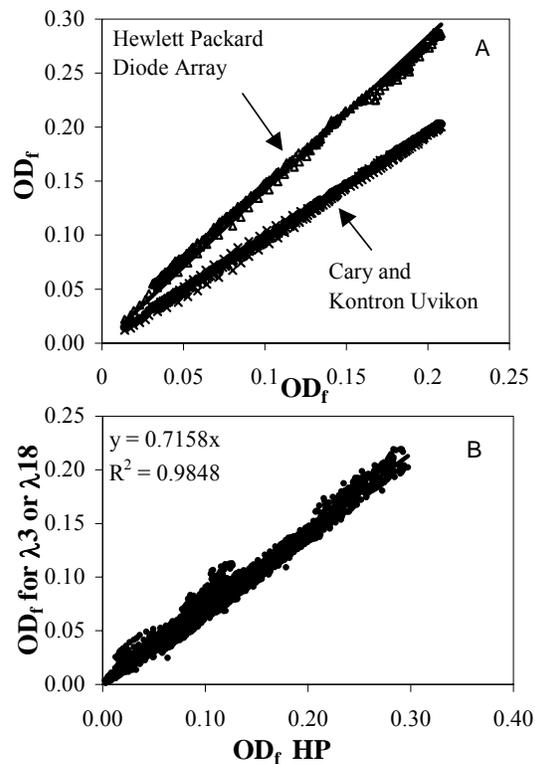| Instrument	Slope	Optical Geometry
Cary	0.98	Standard Optics
Lambda 6	0.95	Standard Optics
UVIKON	0.96	Shibata Optics
USF	1.07	Fiber Diode
HP	1.42	Sphere Diode
Elyptica	0.91	Single Beam

Figure 4. **A.** Linear regression of OD_f values between 400-700 nm for Perkin Elmer Lambda 18, Varian Cary 1 and Kontron. **B.** Merged regression analysis of HP Diode Array unit for various comparisons by SDSU CHORS and Scripps Photobiology Group. Data are for OD_f from 400-700 nm after setting a null point as the mean 790-800 (SIO) or 750 nm (SDSU).

various instruments before choosing the best unit for the work to be achieved. An important generality is that many spectrophotometers, with or without scattered transmission or integrating spheres, provide $OD_f(\lambda)$ within ± 5% for GF/F filters. The investigator must carefully evaluate raw OD_f of a candidate instrument for various filter loading and particle types relative to other units to ensure that the raw data is comparable. One cannot assume all instruments provide a raw OD_f that are equivalent. For example, the ASD unit at the Bigelow Workshop estimated OD_f values 15% higher than the Perkin Elmer Lambda 3.

The Hewlett Packard diode array unit has been confirmed to have raw OD_f that is considerably larger than values determined with more traditional units. The optical design of the Hewlett Packard spectrophotometer requires illumination of the sample in a diffuse flux source from an integrating sphere attachment to achieve results with diffusing materials such as particles on filters. This illumination geometry is significantly different from the collimated beam illumination of pre-sample grating monochromator systems. This difference results in observed optical densities of the diode array system being 35-45% higher than estimates on the same filter samples determined by various dual beam grating monochromator references or single beam units (Figure 4a). The results from comparisons on three different dates with diverse cultures are shown in Figure 4b. We excluded high optical density samples because the relationship became slightly non-linear at $OD_f > 0.4$. For the Perkin Elmer Lambda 18 and Lambda 3 used as reference instruments for the Scripps and Bigelow workshops, respectively, the Hewlett Packard unit had a consistent offset that is easily predicted with a linear regression for $OD_f < 0.35$. These results for cultures agree well with previous work comparing many natural field samples (Cleveland, pers. Comm). Based on the regression in Figure 4b we recommend that the investigator multiply OD_f for the Hewlett Packard diode array system by 0.72, after setting the null point in the infrared, to convert to the comparable OD_f for the traditional dual beam scanning monochromator systems. Alternatively, estimates of β can be determined by the investigator if appropriate determinations of suspensions can be done to compare to filtered samples. Another major limitation of the Hewlett Packard diode array unit was that it had a very noisy baseline for glass fiber filters, and could not achieve good results with

these filters below 400 nm or above 750 nm. Noise from 700-750 nm made estimation of the null absorption wavelength more problematic. The advantage of this type of system is that it records a spectrum very rapidly. The sacrifice of data quality for speed is not recommended.

Instrumentation issues imply that an investigator carefully choose an instrument that can perform the analyses of interest. Appropriate, rigorous evaluation should be carried out prior to selection. It is recommended that investigators chose a high quality spectrophotometer that can effectively record raw optical density spectra for filters from 300-850 nm and for soluble samples from 250-850 nm. Systems with variable slit widths are preferred and spectra should be run at a 4 nm bandwidth or smaller. Bandwidth larger than 4 nm will smear the red absorption peak of chlorophyll in $a_p(\lambda)$ determinations. Baseline performance recommendations below are specified for a 4 nm bandwidth. Baseline noise for the glass fiber filters should stay within ± 0.01 over the full spectral range, but performance better than ± 0.005 is strongly recommended. The units should maintain baseline flatness over time with minimal drift in offset. For soluble absorption, the baseline noise over the full spectral range for 10 cm quartz cuvettes with purified water should be less than ± 0.001 but performance better than ± 0.0005 is strongly recommended. For either preparation, baseline anomalies caused by lamp or sorting filter changes, or other instrumentation effects must be corrected. Automatic baseline corrections for many commercial units do an adequate job. Still, the investigator must carefully evaluate the baseline of all measurements and correct for any residual artifacts. Based on numerous comparisons, the Varian Cary 1 (now called Cary 100) has proven to be a high performing instrument that is moderately priced. Selection of a unit with comparable performance or better is strongly recommended.

Effective Pathlength Corrections

The methods to determine the pathlength amplification factor based on transmission spectrophotometry of filters and suspensions has been described in detail elsewhere (Mitchell and Kiefer, 1988a; Bricaud and Stramski, 1990; Mitchell, 1990). The recommended approach is to determine the suspension optical density (OD_{sp}) on relatively high transmittance (optically thin) samples to minimize multiple scattering errors (Bricaud et al., 1983; Mitchell and Kiefer, 1988a).

In the following discussion, it is assumed that $OD_p(\lambda)$ has been corrected for the baseline reference and nulled in the infrared. The culture of interest should be filtered at multiple concentrations on different filter preparations to achieve a range of $OD_p(\lambda)$. Scaling of the estimated absorbance of the optically thin suspension $\left(OD_{sp}\right)$ is made by multiplying the geometric pathlength for different volumes (l_s) (Equation 12.2) after determining the effective filtration area of the filter $\left(l_s OD_{sp}\right)$. The investigator should not measure very high suspension absorbance to match high filter loading for estimates of β as this will cause possible errors in the suspension estimates due to multiple scattering effects (cf. Lohrenz, 1999). For natural populations, measurements on suspensions are in general not feasible due to low particle concentration so pre-concentration is required with possible artifacts such as particle loss, aggregation, etc. A possible alternative, introduced by Allali et al. (1997), is to compare absorption spectra measured on filters to those measured on glass slides (modified FTF technique, see later).

Several ad hoc comparisons of methods have been accomplished by various co-authors on this report, as well as more formal comparisons at the Scripps and Bigelow absorption workshops. In general, the previous results reported by Mitchell and Kiefer (1984; 1988a) that β exhibits variability due to changes in sample OD have been confirmed (e.g. Bricaud and Stramski, 1990; Cleveland and Weidemann, 1993; Tassan and Ferrari, 1995a; Moore et al., 1995). An example plot of OD_{sp} vs. OD_f for *T. pseudonana* for the spectral range 400-700 nm is shown in Figure 5a. Two different volumes of culture were filtered on replicate GF/F filters and the filter OD_f were determined with various spectrophotometers at the Scripps workshop. Published fits corresponding to variable β are also indicated as numbered lines. The Hewlett Packard diode array data fall well outside the data for other instruments. Also, the results for *P. marinus* (reported by Moore et al., 1995) were confirmed at the Scripps workshop (Figure 5b). This result indicating β depends on particle type is not well understood and warrants further research.
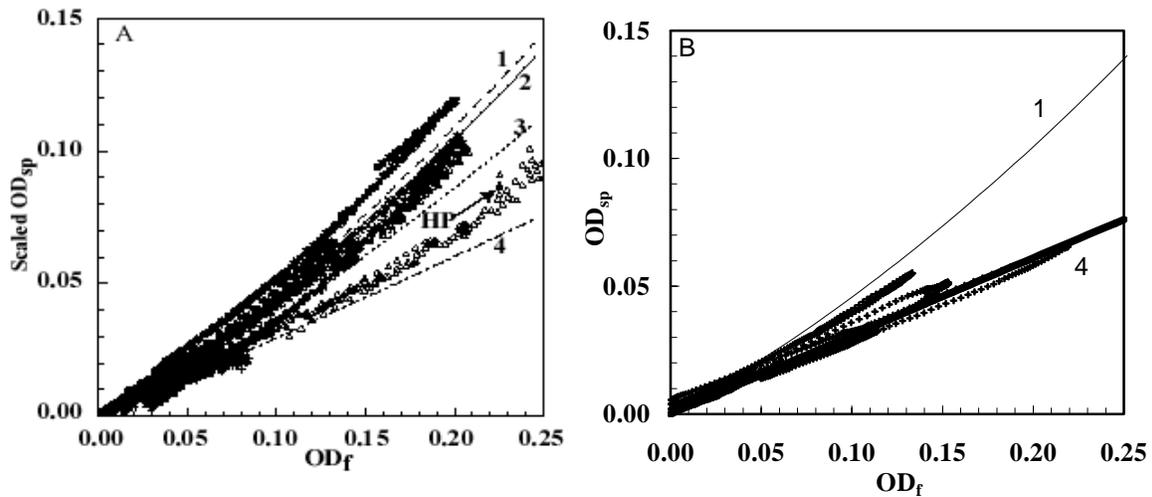

Figure 5. Following the procedures of Mitchell (1990) scaled optical density of suspensions are plotted against GF/F filter optical density to estimate the pathlength amplification factor, β. **A**. Optical density of the suspension scaled to geometric pathlength $\left(l_s OD_{sp}\right)$ for *T. pseudonana* plotted against OD_f for instruments with integrating spheres used at the Scripps Absorption Workshop. Multiple filtration volumes were used to achieve significant range for the raw OD_f values. Numbered lines correspond to published coefficients (1=Mitchell, 1990; 2=Kahru and Mitchell, 1998; 3=Bricaud and Stramski, 1990; 4=Moore et al., 1995). All instruments at the Scripps workshop that could determine OD on both suspensions and filters, except the Hewlett Packard Diode Array unit, produced results similar to those originally reported by Mitchell (1990) for this small (5-6 μm) diatom. Data for the HP system are indicated (see also Figure 4). **B**. OD comparisons for suspensions and filters for *Prochlorococcus marinus* using the Perkin Elmer Lambda 18 at the Scripps workshop compared to the previously reported relationships for various cultures (line symbols same as panel A). The earlier differences noted by Moore et al. (1995) for *P. marinus* were confirmed during the Scripps workshop. The differences are not understood at this time.

An alternative method based on β being a constant equal to 2.0 is discussed by Roesler (1998). The assumption is that the glass fiber filter method estimates the diffuse absorption of a sample, which is exactly 2 times the volume absorption coefficient (cf. Preisendorfer, 1976). True measurements of the diffuse absorption coefficient of phytoplankton culture suspensions are reported by Kiefer et al., (1979) and it is evident that the chlorophyll-specific absorption coefficients at the red peak of those determinations are approximately 2 times those routinely reported for the volume absorption coefficient (e.g. Mitchell and Kiefer, 1988a; Johnsen et al., 1994; Sosik and Mitchell, 1991; Moisan and Mitchell, 1999). While it would be useful if β could be quantified by a simple constant, the theory of Duntley (1942) and empirical results on the optical dependency of β were confirmed for dyes in diffuse preparations (Butler, 1962) and for phytoplankton on glass fiber and cellulose acetate filters by Mitchell and Kiefer (1988a). This dependency has been found by numerous researchers as summarized in Table 2. The typical measurements in standard spectrophotometers illuminate the sample with a collimated beam, which becomes diffuse as it is transmitted through the filter. However, even the emerging beam is not fully diffuse and this can be demonstrated easily by visualizing a point source of light through a single fully hydrated glass fiber filter. While the point source becomes highly diffused, it is still visible as a distinct source, indicating that the illumination beam is not fully diffuse. Thus, the glass fiber filter does not achieve a measurement of diffuse absorption required to satisfy the optical geometry discussed by Preisendorfer (1976) so the simplification that β = 2.0 does not appear justified on theoretical grounds. However, 2.0 is a reasonable approximation in many cases since according to the Mitchell (1990) relationship, β equals 2.35 and 1.5 when $OD_f(\lambda)$ is equal to 0.05 or 0.4 respectively, the range for optimal algorithm performance. Table 2 shows that for $OD_f(\lambda)$ equal to 0.2, the resulting estimate for the suspension using β = 2.0 is in the middle of various methods recommended in the literature.

Absorption spectra for particles transferred to glass slides

At the Scripps workshop, the method of Allali et al. (1995) was used to estimate absorption coefficients of cultures and seawater samples by freeze transfer of the particles to transparent microscope slides following the protocols of Hewes and Holm-Hansen (1983). This transfer allows determination of OD_f in a non-diffusing preparation to avoid the pathlength amplification. For the filter support, a polysulfone Gelman unit was used (due to clogging, fritted glass supports should be avoided, especially for natural samples). Nuclepore™ 0.2 μm polycarbonate filters were used (0.4 μm filters are also adequate and more convenient for use at sea). Samples were filtered under low vacuum pressure (<5 in. Hg).

Immediately at the end of filtration, the filter was removed from the filtration unit and transferred, particle side down, onto a glass microscope slide (with or without a drop of water). No fixative was used in the preparations at the workshop. The preparation was then frozen by laying the slide (filter on the upper side) on a metal block cooled in liquid nitrogen (it is convenient to use a small-size Dewar container). The temperature of the metal block must be low enough for the filter to become almost immediately "white" with frost. After 10-15 seconds, the slide was removed from the block and the filter was carefully peeled off (when properly frozen, there is some resistance to peeling) and examined by eye to check the efficiency of the transfer. Then a circular cover slip of the same size as the clearance area of the filter was placed on the transferred particles. For most experiments, the Nuclepore™ filter was then put into 90% acetone for chlorophyll extraction to quantify the transfer efficiency of the chlorophyll containing particles. The FTF procedure produced results comparable to the GFF filter method. Performance for a diatom culture was not as good as other cultures, but the results were still quite reasonable (Figure 6a). Other comparisons for cultures of phytoplankton and natural samples are shown in Allali et al., (1995). Diatoms or other larger cells may become crushed by the slide and cover slip preparation in which case release of pigments could lead to reduction of pigment package effects. At the Scripps workshop, results for smaller cells including *Synechococcus*, *Prochlorococcus* and *Emiliania* were better than the results for the diatom. The natural samples concentrated from Scripps Pier also resulted in excellent comparison between the freeze transfer and suspension estimates of absorption (Figure 6b) for wavelengths longer than 350 nm. The reason for larger discrepancies below 375 nm for this preparation is not known.

The transfer of particles from the filter to the slide is a critical step for FTF sample preparation. Visual examination of the filter and slide
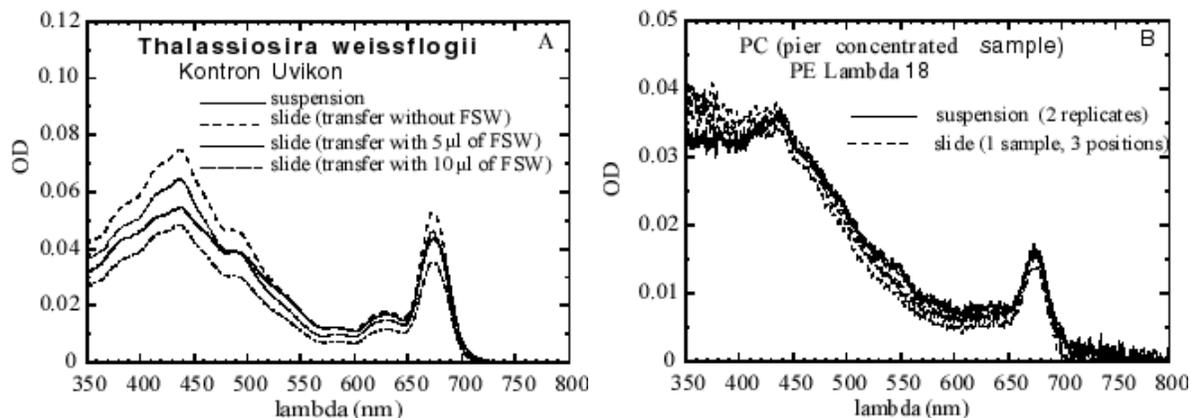
Figure 6. Examples of *OD* determined on a diatom culture and seawater particles transferred to glass slides using the Allali et al. (1995) method and suspensions of the same material determined (using the Perkin Elmer Lambda 18 spectrophotometer or the Kontron Uvikon) at the SIO Absorption Workshop. For these and other cultures (data not shown), the FTF method provides estimates of $a_p(\lambda)$ comparable to those measured on suspensions.

preparation is essential as it gives a quick, albeit qualitative, indication of the success of this operation. The transfer generally appears to be easier and more successful without addition of water to the slide before freezing, except for some species such as diatoms. The additional extraction of the filter and determination of the fraction of total chlorophyll not transferred must be performed for quantitative work. For most of the investigated samples, the technique has given satisfactory results, and the overall uncertainty is similar to that expected for measurements with the GF/F technique because of uncertainty in β. The modified FTF technique is not significantly more time-consuming than the GF/F technique and can provide results that agree well with determinations on suspensions. While filtering on 0.2 μm polycarbonate filters takes longer than GF/F filters, there is no need to reach high optical densities so sample volumes can be reduced. An important limitation remains that methanol or bleach de-pigmentation is feasible but not easily achieved (See Allali et al., 1995) so that it may be more practical to exploit numerical decomposition methods (e.g. Morrow et al., 1989; Bricaud and Stramski, 1990). A more practical issue is that successful determinations of the transmittance of the diffusing particles on the slide require an integrating sphere or scattered transmission accessory for the spectrophotometer. This adds an additional burden of expense and analytical complexity that may make this procedure less amenable to routine applications. Also, as pointed out by Sosik (1999), some artifacts of freezing,

particularly in the UV, occur immediately upon freezing. Sufficient issues remain with the FTF method so that the GF/F method is still recommended at this time for most routine work.

Purified water for soluble absorption

At the Scripps workshop, the performance of purified water preparation systems were compared. The units (Millipore Milli-Q and Alpha-Q, and Barnstead Nanopure) all provided similar results in tests relative to air for the spectral range 300-900 nm. Below 300 nm there were small differences (data not shown). These units, or equivalent systems, should be capable of delivering purified water required as a reference for soluble absorption measurements and are recommended as standard equipment for field programs. In many field programs, however, the available feed water is of such an inferior quality that the systems can become overwhelmed and their performance significantly diminished. The experience of the co-authors is that this is a major limitation with respect to knowledge of the pure water baseline. Therefore it is recommended that a set of standard purified water samples be prepared prior to a field deployment and analyzed daily in reference to purified water prepared in the field. Procedures for preparing this standard water are provided in the protocols section. The standard prepared water has been found to deteriorate over time, especially from 250-325 nm, but the magnitude of the observed increase in optical density relative to freshly purified water is much smaller than the magnitude

of the potentially dramatic degradation in the output of pure water systems. Therefore, routine determination of the reference standard can assist in quality control of, or serve as an alternative source to, low quality output from a purified water system. Given the issues regarding frequent failure of pure water systems during fieldwork, the investigator must routinely record a spectrum of the purified reference water used for analysis relative to air. This procedure provides an absolute check for the quality of the purified water and could serve as the basis for making corrections. Further work on the UV absorption of pure water should be carried out to extend our knowledge of pure water in the visible and infrared reported by Pope and Fry (1997). See also a recent recommendation for UV-Visible absorption coefficients for pure water (Fry, 2000).

Null point normalization of particle absorption measurements

The accurate determination of particulate light absorption requires some procedure to correct measured absorption coefficients for errors, which arise from scattering losses within the measurement system. Both soluble and particulate absorption methods require adjustment of the spectrum at a null point. The most common approaches begin by identifying a spectral region where sample absorption can be assumed negligible, which allows an initial assessment of the scattering error for a limited portion of the spectrum. For measurements

of absorption by aquatic particles, this "null point" wavelength is usually taken from a spectral region in the near-infrared typically 750 nm to 800 nm for the correction of spectrophotometric measurements of water sample preparations.

Differences in light absorption and scattering properties among individual filters used for sample filtration are one source of variability. The optical properties of blank GF/F filters can vary significantly between individual filters (up to 0.05 OD), presumably as a result of differences inherent in the manufacturing process. While differences in blank OD are noted, the spectrum of two blank filters relative to each other is essentially flat. Different glass fiber filter types have different transmittances (Mitchell and Kiefer, 1988a) and there are variations between manufacturing lots of the same filter type (Mitchell, 1990). The relative degree of water saturation between baseline and sample filters may also lead to differences in the measured $OD_f(\lambda)$ of sample filters so proper hydration of samples is an essential part of the protocol. Pre-soaking filters in filtered seawater 1-2 hrs before use can lead to less variability between individual filters (Bricaud and Stramski 1990). Blanks used as references must be adequately soaked and remain hydrated during analyses.

The choice of a null point in the infrared originates primarily from the assumption that absorption by phytoplankton cells is negligible in this wavelength region. For *T. pseudonana* raw OD values measured at 750 nm relative to a
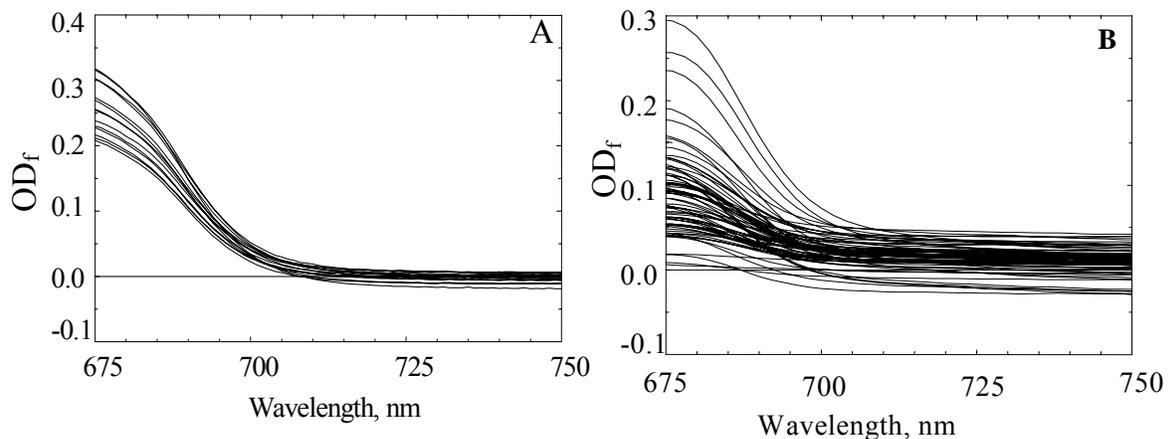

Figure 7. A. Raw OD_f 675-750 for a culture of *T. pseudonana*. **B**. Raw OD_f 675-750 for seawater samples. The mean value $OD_f(750)$ for *T. pseudonana* is not significantly different from 0.0, but the mean $OD_f(750)$ for seawater samples is significantly greater than 0.0 (p < 0.001). This suggests the possibility of true absorption at 750 nm that may result in an absolute error in routine application of a null value and/or significant scattering with unknown spectral dependency. Scattering error may have spectral dependency leading to either positive or negative errors depending on the particle types and the spectral range of interest. Routine selection of a null wavelength in the infrared contributes error that is not well quantified.

pre-soaked and hydrated filter varied from -0.019 to 0.01. The mean value of -0.0016 (15 measurements) is not significantly different from zero (Figure 7a). These results suggest that phytoplankton have negligible absorption in the near infrared, and introduce minimal scattering error when collected on GF/F filters for absorption measurements. However, there are examples, especially in the presence of suspended sediment loads, that significant absorption for field samples is still present at 750 nm. Minerals may exhibit significant absorption in the near infrared (e.g. Bukata et al. 1995). Figure 7b illustrates examples of optical density spectra for natural particle assemblages from Case 1 waters of the California Current collected on glass fiber filters. In contrast to the culture, the field data indicate that the measured optical density signal in the infrared is frequently positive, with mean values significantly greater than zero implying that scattering by the particles on the filter contribute to this non-zero raw optical density. The magnitude of $OD(750)$ is not trivial. Despite the careful baseline treatment and efforts to minimize filter-to-filter variability in these measurements, this rather systematic tendency towards positive values of OD in the infrared for field samples is difficult to interpret if only transmittance measurements are determined because it is not possible to distinguish true absorption from scattering error. The transmittance-reflectance method can improve on this uncertainty (Tassan and Ferrari, 1995a).

The error in estimating absorbance from OD using a near-IR null point will depend on the relative magnitude of absorption to scattering at the null point. Subtracting out true absorption introduces an absolute error equal to the true absorption at the null wavelength. Spectral dependence of scattering can introduce positive or negative bias at shorter wavelengths depending on the size of the particles and their refractive index. For particles greater than several microns, the spectral dependence of scattered losses will be nearly flat, so small errors may be expected. If the scattering is dominated by small particles, the errors can be greater, and their relative magnitude difficult to assess *a priori* without detailed knowledge of the size and refractive index. Since natural particle size distributions and refractive indices can vary substantially, these factors can introduce errors of uncertain magnitude. Still, at least for Case 1 waters and many Case 2 waters, the errors from using a null absorption correction will be smaller than if no null absorption is used.

For soluble absorption, temperature differences between the reference water and sample can lead to strong spectral absorption features (Pegau and Zaneveld, 1993). These absorption bands are strongest in the range 650 - 750nm but appear to have harmonics both shorter and longer so that choice of a null point is an important consideration. To avoid temperature effects, sample and blank should be maintained at the same temperature. It is often difficult in practice to ensure temperature equivalence in which case care must be taken regarding the wavelength for setting a null point for soluble absorption analysis. If strong temperature residuals are in the spectra, one must inspect the data to determine an appropriate wavelength range to use as a null point. In many clear open ocean waters, the $OD_s(\lambda)$ values greater than 600 nm are typically not significantly different from the baseline, so it is feasible to utilize a shorter wavelength null point in these situations (Mitchell et al., 1998). However, more turbid lake, bay and coastal waters have large soluble absorption into the near IR. Figure 2 illustrates that the magnitude of the temperature effects for pure water blanks determined for various cruises is typically ± 0.001 OD for a 0.1 m pathlength. While this may seem to be a small analytical uncertainty, it corresponds to a_g ± 0.02 – similar to the magnitude of pure water 400-500 nm, or phytoplankton absorption at the red peak for chlorophyll of 1 μg/L. Situations where the reference was both colder and warmer than the sample are shown in Figure 2. It is evident that for these samples, the region near 600 nm is a preferred null wavelength compared to any choice between 600-800 nm. When soluble absorption values are large, the relative effects of temperature are smaller, and one may be able to choose a null point greater than 600 nm. The performance of some spectrophotometers diminishes at longer wavelengths in the infrared, especially for particulate samples. The investigator must carefully inspect baseline, blank and sample spectra to determine an appropriate wavelength for null assignment. The final choice will introduce some uncertainty and error in the derived absorption coefficients, which leads to the requirements of reporting raw data for $OD_s(\lambda)$, blanks, and purified water vs. air.

De-Pigmented Particle Absorption

Material collected on glass-fiber filters includes phytoplankton and other particles, including bacteria, microzooplankton, organic

detritus (e.g. dead organisms, phytodetritus, and marine snow), and inorganic particles (sand, dust, coccoliths, etc.). Separation of the total particulate absorption coefficient as measured on glass-fiber filters $\left(a_p\right)$ into phytoplankton $\left(a_\phi\right)$ and non-phytoplankton or "detrital" $\left(a_d\right)$ components is an important pre-condition for using these absorption data to validate ocean color satellite products, including pigment biomass indexes and primary productivity. Early efforts to separate absorbing components in natural samples included treatment with organic solvents, UV radiation, and potassium permanganate (references can be found in Shifrin, 1988, and Bricaud and Stramski, 1990).

Methods to partition a_p into its components can be grouped by methodology. Chemical techniques extract or bleach the more labile pigments on the filter, leaving refractory absorbing material behind. The treated filter is scanned again to retrieve a_d, which is then subtracted from a_p to yield a_ϕ (e.g., Kishino et al., 1985, Tassan and Ferrari 1995a). Statistical techniques to decompose total particulate absorption spectra into these two components have been proposed (e.g., Morrow et al., 1989, Bricaud and Stramski 1990, Cleveland and Perry 1994). Mathematical methods are not truly independent since they are typically validated using the results of chemical separation methods. Microspectrophotometric observations of individual particles to estimate each component directly (e.g., Iturriaga et al., 1988; Iturriaga and Siegel 1989), are time consuming and therefore not amenable to routine estimates, but of great value in understanding the details of particle absorption within a sample. Reconstruction of spectra from the concentration of HPLC-determined phytoplankton pigments (e.g., Bidigare et al., 1990) can be used but this method does not directly result in an estimate of non-phytoplankton (detrital) absorption. At the Scripps workshop, an intercomparison of the most commonly used chemical partitioning methods, were evaluated to assess differences and to provide recommendations for common procedures.

Chemical methods are the most widely used as they have the advantages of requiring no specialized equipment (e.g. microspectrophotometer) or assumptions about the spectral nature of component absorption (as is the case in some mathematical methods or HPLC reconstruction). It must be stressed at this point that definitions resulting from partitioning of the total particulate absorption coefficient using chemical or mathematical techniques are purely operational, as

any extraction or bleaching technique does not purely select for (or against) phytoplankton pigments. Any non-phytoplankton pigments extracted or bleached in a chemical method would thus result in an overestimation of a_ϕ, while any phytoplankton pigments left on the filter after treatment would result in an overestimation of a_d. Mathematical methods also involve various assumptions leading to un-quantified uncertainties. The a_d spectrum generally has a monotonically increasing absorption with decreasing wavelength usually with a slight exponential form that is flatter than soluble absorption. Since the goal is generally to get an estimate of phytoplankton absorption, if there is a residual chl a absorption peak in the red near 675 nm the extraction process should be repeated until the peak disappears. 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. 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.

Extractive methods such as methanol are fundamentally different in action from sodium hypochlorite (NaClO) used to bleach, rather than extract, phytoplankton pigments. Bleaching involves placing a small amount of 0.1% active chlorine solution onto the filter, then rinsing with water. The NaClO oxidizes the pigment molecules, making their light absorption negligible. Water rinses then remove the excess NaClO, whose absorption is negligible above 400 nm but increases steeply below that wavelength. This method was found to be effective in situations where methanol cannot be used, as on cellulose membranes such as the 0.22 micron Millipore filter, or on phycobilins. Also this procedure can be adapted for use with particulate suspensions.

Several chemical methods for extracting pigments from marine particles collected on glass-fiber filters were compared. Test samples included pure cultures of *Thalassiosira weissflogii* (a diatom), *Dunaliella tertiolecta* (a chlorophyte), *Synechococcus* strain WH7805 (a cyanobacterium), and an offshore sample with mixed population including large diatoms. Hot and cold absolute methanol treatments had similar results for extraction times ranging from 1 to 30 minutes. Methanol and methanol + water treatments failed to extract phycobilins from WH7805 (Figure 8). Bleach (NaClO) treatments succeeded in rapidly

removing phycobilin and other pigment absorption but in some cultures and field samples an artifact resembling 'detritus' absorption was also produced in the wavelength range below 400 nm. Independent studies conducted outside the Scripps workshop were consistent with these results.
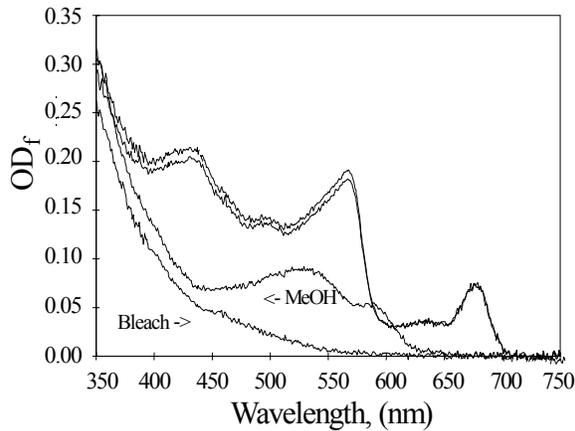

Figure 8. $OD_f(\lambda)$ spectra 350-750 determined at the Scripps Workshop for *Synechococcus sp.* compared to methanol extraction and NaClO oxidation de-pigmentation. OD_f values were set to zero at 750 nm. Phycobiliproteins of the cyanobacterium do not extract in methanol. Adequate rinsing of the NaClO bleach allows extension of this method below 400 nm.

Neither methanol extraction nor NaClO oxidation provide ideal means of separating particulate absorption into 'algal' and 'detrital' components. In both cases the action of the chemical agents is not well understood, and in many cases is quite different. The decision to apply either the bleaching or methanol extraction method will depend on the situation. For example, for inland waters where either cyanobacteria or chlorophytes are dominant, the bleaching technique will be preferable because of the presence of phycobilins and of extraction resistant algae (e.g. Porra 1990). In coastal oceanic waters the methanol technique will be preferable because 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) absorption by phycobilins is small but present in some particulate absorption samples and in methanol-extracted filters (N.B. Nelson unpubl. 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.

Modifications of the bleaching procedure based on the results at the Scripps workshop and

subsequent work at CEC JRC Ispra (Ferrari and Tassan, 1999) and Bermuda Biological Station has permitted better control of the treatment. In the wavelength range from 400 to 750 nm the agreement between pigment absorption spectra obtained by methanol extraction and NaClO bleaching is generally good. With some phytoplankton types bleaching yields a detritus-like absorption in the 350-400 nm interval higher than that obtained by methanol extraction. This is likely an artifact caused by NaClO-induced reactions, but could also be due to incomplete rinsing of the residual sodium hypochlorite. NaClO bleaching is effective with a very large variety of phytoplankton types (in fact no resistant type has been found so far), including the water-soluble pigments of the cyanobacteria that are poorly extracted by methanol.

All techniques include uncertainties and assumptions not considered in the present studies. For example, resuspension and redistribution of particles from filters when solutions are added may have some effect on the absorption of the sample. Also, changes in the size or shape of the particles on the filter may be induced by the chemical treatments, changing their scattering properties and possibly changing the package effects and β. Finally, it is well known that these techniques do not merely remove the absorption by the primary phytoplankton chlorophylls, carotenoids (and phycobilins in the case of the NaClO technique), but they may also remove absorption by other pigments such as flavins, cytochromes, breakdown products (e.g. phaeophytins and phaeophorbides) and animal pigments. These considerations should be taken into account when interpreting results of chemical separation methods.

Transmission-Reflectance (T-R) Method

Backscattering of light by particles represents an error source for absorption measurements carried out by the routine light-transmission technique (T), leading to an overestimate of the true sample absorption. These errors are partially compensated for OD_f determined in T-mode by subtracting the OD_{null} at a wavelength assumed to have negligible absorption. But as discussed above such assumptions regarding a null point choice may lead to errors of uncertain magnitude. The backscattering loss, and its spectral dependence depends on the particle size distribution as well as on the type of material (through the refraction index). For medium-to-large phytoplankton cells (≥ 3 μm) spectral dependence of scattering is small

300 – 800 nm, but is more significant for small cells (prochlorophytes, heterotrophic bacteria), fine organic detritus and inorganic suspended sediment. Large backscattering is frequently observed with algal species containing inorganic material (e.g. coccolithophores).
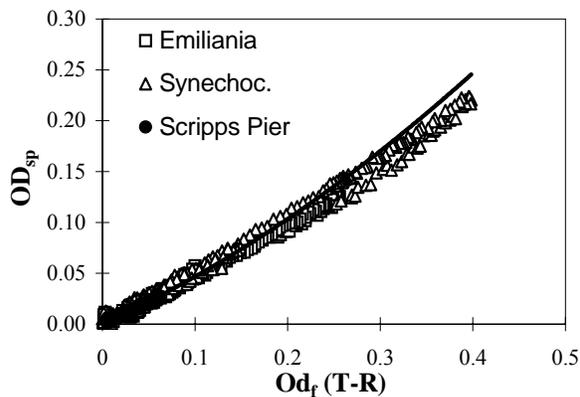

Figure 9. Comparison of the absorbance 400-700 nm for suspensions and filter pads using the T-R method of Tassan and Ferrari (1995) for cultures and concentrated pier water during the SIO Absorption Workshop. Note the coherence of the data compared to Figure 5A. Consideration of both transmittance and reflectance in development of corrections for glass fiber filters can improve methods, especially when particle scattering introduces large errors associated with assignment of a near-IR null point.

A modification of the current light-transmission method that corrects for backscattering was described by Tassan and Ferrari (1995). This technique combines light-transmission (T) and light-reflection (R) measurements, carried out using an integrating sphere attached to the dual-beam spectrophotometer. The data analysis is performed by a theoretical model that eliminates the effect of light backscattering by the particles. The conversion of the optical density of the sample filter measured by the T-R method, into the equivalent optical density of the particle suspension is obtained by means of an empirical correlation that is determined comparing the scattering-free pigment optical densities of particles in suspension and retained on the filter. Because of the risk of NaClO-induced artifacts absorbing below 400 nm, only the $OD(\lambda)$ portion above 440 nm is analyzed for the determination of the correlation (Tassan and Ferrari, 1998). At the Scripps workshop, the global error of the T-R method was comparable to the error yielded by the simpler T method. Subsequent modifications of the

T-R experimental routine (Tassan and Ferrari, 1998; Ferrari and Tassan, 1999) yielded a significant reduction of the experimental error. The T-R method is particularly suited for applications to samples containing highly scattering particles that are commonly found in Case 2 waters. Figure 9 illustrates the results for a β relationship using Scripps Pier water with a considerable amount of light-scattering detritus. The T-R method, being more complicated than the T method, is affected by a larger number of error sources that must be considered. Also, since the method requires a high quality spectrophotometer with integrating sphere attachment that is more costly and difficult to maintain during many field campaigns, this method does not yet have the widespread use of the simple T method. Still, to the extent feasible, more investigators should evaluate the advantages of the T-R methods.

12.9 CONCLUSIONS

Spectral absorption and backscattering govern the reflectance of the ocean. In principle, it is trivial to determine absorption coefficients at hyperspectral resolution on water samples with accuracies of 20-30% after appropriate preparation and attention to optical analysis and data processing. Such data is strongly recommended in support of ocean color science to allow a better understanding of ocean reflectance, and for various photochemical and photobiological applications. Standard protocols for determining the absorption by different fractions in seawater are described. Laboratory comparisons of different instruments and procedures at two NASA-sponsored workshops have led to several important conclusions regarding the methods and a set of simple analytical protocols that should ensure consistent data quality if properly implemented.

The demonstration that raw $OD_f(\lambda)$ of replicate samples for many instruments with diverse optical geometries were within 5% (Table 3) implies that investigators can determine raw data that is essentially equivalent using very different configurations. However, some instruments provide significantly different results (e.g. the HP diode array) and the investigator must evaluate raw data of any instrument prior to quantitative work. A trivial linear transform can convert raw OD_f determined with the HP diode array system to the equivalent optical density of standard spectrophotometers (Figure 4b). More uncertainty is associated with application of the pathlength

amplification factor (β) since it may be dependent on particle type that is not known *a priori*. At this time, it is not feasible to accomplish a full error analysis for natural particle assemblages because most studies of β have been carried out on cultures that may not adequately represent the diversity of all particle types of interest. Corrections for β published in the literature generally agree within 20-30%, with the most significant difference reported for *Prochlorococcus* (Table 2). The workshops confirmed previously reported *OD*-dependence of β, as well as the divergence of β for very small phytoplankton (*Prochlorococcus*) compared to large phytoplankton. The cause of the difference in β for *Prochlorococcus* is still not understood, and may be related to errors in spectral scattering that are not compensated via the null point normalization, differences in the interaction between scattering and absorption within the glass fiber filter or other unresolved issues with the methods.

Soluble absorption estimates are rather simple, but there are serious practical considerations related to creation of appropriate reference water in the field, baseline stability and noise, and assignment of a null value. Long-term storage of samples remains an issue that has been given relatively little attention and no systematic studies have been done on artifacts caused by storage for soluble absorption. To achieve satisfactory results, we recommend that the investigator use a high quality commercial spectrophotometer that achieves specific performance criteria outlined above for the particle and soluble baseline noise, stability, spectral bandwidth, and spectral range. These criteria are trivial to test during instrument demonstrations provided by manufacturers and should be given very high priority in selecting the best unit for the work. All water sample preparations should be analyzed as soon as possible due to artifacts caused by long-term storage.

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