Chapter 5: Spectrophotometric Measurements of Particulate Absorption Using Filter Pads

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5.1 General Considerations

Prior to the quantitative filter pad approach to assess absorption spectrophotometrically, particulate absorption was measured in cuvettes. These measurements clearly demonstrated the impact of scattering by the suspended particles on the estimation of absorption, because scattered photons are generally not collected by the detector and their contribution is assigned to absorbance. In the extreme, the derived coefficient more resembled beam attenuation rather than absorption. An additional consequence of particle scattering is that it redirects photons through the suspension of absorbing particles, increasing the absolute path length of those photons over that which would have been observed in a non-scattering environment, thereby increasing the probability of absorption. This latter effect is termed path length amplification, the ratio of the mean optical path length to the geometric path length (Butler 1962); it leads to overestimated absorption coefficients and, in the extreme, to flatted absorption peaks (Duysens 1956). Modeling suggests that path length amplification is minimized by maintaining dilute suspensions where only single scattering occurs over the geometric path (van de Hulst 1957). In most natural samples, however, the suspended particles are significantly dilute as to require long geometric path cuvettes, which are more susceptible to scattering losses. Filtering particles onto glass fiber filters solved a number of technical issues inherent in the suspension approach but yielded others.

Advantages: Filtering large sample volumes onto glass fiber filters (e.g., Whatman® glass microfiber filters, grade GF/F with nominal pore size 0.7 μm) solved the issue of the dilute medium and low signal to noise ratios by concentrating particles. This yielded a higher optical density in the spectrophotometer by increasing the geometric path length. Additionally, the filtration removed the solute from the measurement resulting in separation of the particulate from dissolved fractions of the total absorption. Finally, the extraction of pigments from the filter leaving the non-extractable cellular material and inorganic particles provided a means for estimating the contribution to absorption by the phytoplankton pigments as they were *in vivo*, i.e. as they were packaged (Kishino et al. 1985; Sosik and Mitchell 1990).

Disadvantages: Glass fiber filters are highly scattering. Depending upon the configuration of the spectrophotometer, this yielded significant losses of the incident light from the detector and if not corrected lead to an estimate of particle absorption that included significant scattering by the filter pad. Additionally, the highly scattering nature of the filter particles increased the optical path length of the photons significantly over the geometric path length, increasing the likelihood of absorption. As was true for suspensions that do not satisfy the measurement criteria of single-scattering regime, the path length amplification factor on filters is even more significant and leads to a non-negligible overestimation of the absorption coefficient. Finally, the filter pads have their own optical properties that vary slightly from filter to filter. Because the optical properties of the filter pad are typically much larger than those of the natural particles, variations between filters that are not removed by blank filter subtraction provide additional non-negligible contributions to the computed sample absorption coefficient and increasing the uncertainty in the derived values.

Prior to the implementation of integrating spheres for filter pads (Section 5.7), particulate absorption was assessed on filter pads configured in the transmittance mode (alternatively referred to as transmission mode) on the spectrophotometer (Fig. 5.1a). Note that the use of the term "transmittance mode" in this context of filter pad measurement configuration is not to be confused with the transmittance output of the spectrophotometer. Actually, the filter pad measurements are typically made using an absorbance output of the spectrophotometer, rather than transmittance output (see Section 5.3.1 below). In this configuration the transmittance through the filter pad with particles is measured relative to the transmittance through a blank filter (Kiefer and SooHoo 1982).

A large portion of the incident light is not detected and although this is mostly corrected for by the reference through a blank filter, the difference in scattered loss for a blank filter is not the same as for a filter with embedded particles. This error in uncorrected scattering loss is generally lumped into the path length amplification factor, β, the

so-called “beta correction factor”, which theoretically should only correct for the increases in the optical path length compared to the geometric path length through the absorbing particles.

Consensus: As spectrophotometric technology has improved, including the implementation of integrating spheres, the disadvantages of the filter pad approach are declining and the uncertainty in filter pad absorption approaches are improving. In the following sections, the three basic configurations for determining particulate absorption from filter pads are described and configuration-specific protocols are outlined (Fig. 5.1). It is now recognized that the internally-mounted integrating sphere approach (IS-mode) is superior to either the transmittance mode (T-mode) or the transmittance-reflectance mode (T-R-mode) in terms of accuracy, precision, labor and sample handling. However, the vast historical data sets were primarily collected using the T-mode and not every research group has access to the more expensive integrating sphere accessories. Thus, it is critical to continue providing protocols that maximize the quality of data collected while providing clear methods for identifying the sources of errors and quantifying the uncertainties that do exist.

5.2 Sample Collection and Handling

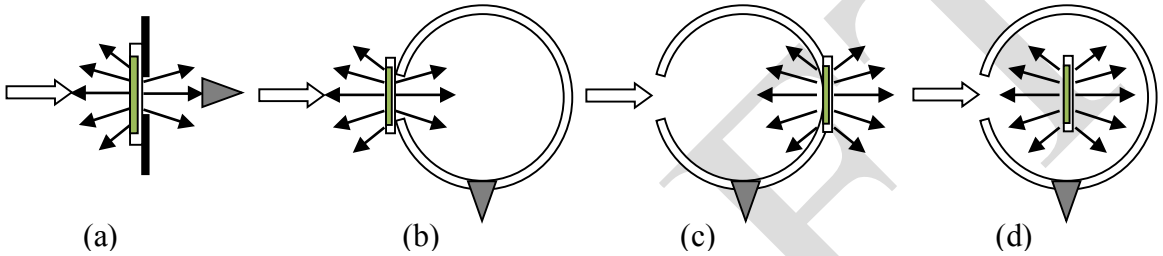

Figure 5.1. Spectrophotometric configurations for determining filter pad optical density: (a) transmittance mode (T-mode), (b and c) transmittance and reflectance mode measured with an integrating sphere with externally mounted samples (TR-mode); (d) internally mounted sample in integrating sphere (IS-mode). Open arrow indicates incident beam, black arrows indicate beams scattered from filter, grey cone indicates detector for the generalized model.

Water samples are collected by clean Niskin bottles (with non-reactive internal tubing). One large and uncorrectable source of error in the measurement is the preferential settling of particles with time as subsamples are collected from the Niskin bottles. Thus, each bottle should be transferred in its entirety to a large volume carboy protected from light and heat during subsampling. Particles are to be kept in suspension while subsampling by careful but vigorous swirling of the carboy. Swirling three times clockwise, followed by three times counter clockwise, followed by three times clockwise effectively resuspends sinking particles. The reversal of swirling direction is critical as it provides the chaotic mixing motion that is necessary to avoid a non-uniform distribution of particles due to centrifugal forces that results from uniform swirling. This resuspension method is also necessary in the sample bottle prior to measuring out the filtration volume. Sample bottles should never be shaken.

Place a set of filters into the filtration manifold. A sample volume sufficient to obtain an optical density value of 0.1 to 0.4 in the wavelength range of interest is required, for example 400 nm to 700 nm, recognizing that that there are regions of minimal absorption that may have optical density values <0.1 when peak absorption is within the range. This may require two or more filters to be prepared for a single sample in order to maintain the optical density range for both the UV and visible portions of the spectrum. Until experience provides the intuition for filter pad loading, multiple filter volumes should be prepared. Vacuum pressure should not exceed 5 mmHg or 0.1 psi in order to minimize cell breakage. As the final volume of water goes through the filter, the valve should be turned off to prevent air from being drawn through the filter, leading to cell breakage.

Prepare 3 - 5 blank filters along with sample filters, filtering a like volume of pure water (such as MilliQ®) or 0.2 μm-filtered seawater (FSW) through each. Filter fibers compress as more water is filtered through them thus they will have different scattering properties with a 50 ml filter volume versus 1000 ml (Roesler 1998). If samples are to be stored from a cruise, collect at least 5 blank filters from each batch used and keep track of which samples and blanks are from each lot to ensure that analyses are processed within a single lot number.

Filters should be removed from filter cups for immediate spectrophotometric scanning or immediate freezing and storage (Sosik 1999). Notch the edge of each filter to provide a means for identifying the orientation in the spectrophotometer, which is especially important for replicate scans of the sample filter in different orientations as well for repositioning the sample filter in the same orientations for scans after the methanol extraction treatment (see

below). If filters are going to be scanned immediately, place them in a petri dish, which has been prepared with a bed of very moist Kimwipes® (or like tissues that don't shed particles; use a compatible water, filtered seawater or purified water, to moisten tissue while maintaining sample isotonic balance). Put the lid on the petri dish to maintain moisture and wrap in foil to prevent exposure to light. Filters change their optical properties as they dry (Roesler 1998), likely due to enhanced scattering by air pockets (Fig. 5.2). Additional changes may occur to some samples containing phytoplankton species that are susceptible to pigment degradation on filters over short temporal scales during a spectral scan (Stramski 1990). When making replicate scans on a filter, it is essential to remoisten between the scans.

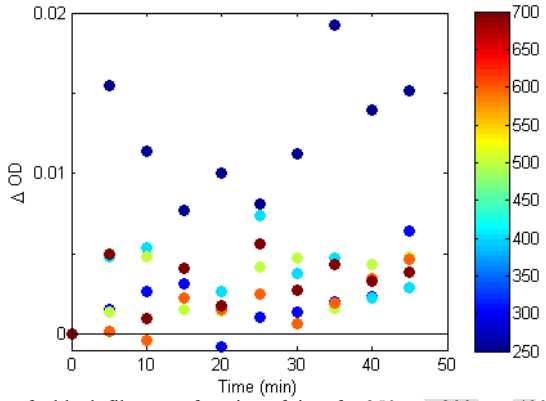

Figure 5.2. Increase in optical density of a blank filter as a function of time for 250 nm, 300 nm, 400 nm, 500 nm, 600 nm and 700 nm (colorbar). Filter was left in spectrophotometer and scanned every 5 minutes as it dried. Measurements were corrected for initial values.

If filters are going to be stored for later analysis they should be flash frozen with liquid nitrogen either by placing them unfolded in individual labeled Tissue-Teks® (such as the plastic disposable capsules manufactured by Sakura Finetek; Fig. 5.3) and placing them directly into a liquid nitrogen dewar, or by freezing them on a spatula that has been sitting in liquid nitrogen. Once flash frozen, the filters can be quickly placed in a -80° C freezer until analysis. The flash freezing prevents the differential freezing of particulate and dissolved molecules and best preserves the optical properties of the particles.

Figure 5.3. Example of plastic capsules for storing individual sample filters in liquid nitrogen.

Immediately before measurement, frozen filters, which have lost their original water content but maintained the original salts, are remoistened by placing filter on top of a drop of MilliQ® or other pure water or filtered seawater on a glass slide or in a petri dish. In this case the MilliQ® water plus the original salts will create isotonic balance. The filter should absorb most but not all of the water within a matter of seconds. Scan immediately.

The first scan of the particles provides the measurement for computing the particulate absorption coefficient, $a_p(\lambda)$. Non-algal particle absorption coefficient, $a_{NAP}(\lambda)$, is computed from the measurement on the same sample filters following pigment extraction. This is done by returning each sample filter to the filtration manifold and gently extracting with a small volume (e.g., 10 ml) of methanol (this is the reason to carefully notch the filter so that it doesn't impact the filtering portion). In order to minimally impact the particles on the filter, the methanol is slowly sprayed down the side of the filter cup with a squirt bottle. Gently filter the methanol through the filter pad, taking care not to draw air through. Add another small volume of methanol and let sit for approximately 15 minutes to fully extract remaining pigments. Gently rinse filter with 15 ml of filtered seawater (or MilliQ® for freshwater samples), applied in the same fashion, and filter through. This treatment is also performed on a set of blank filters, which are then used in the baseline, zero and blank scans for the extracted sample filters. This method involving the methanol treatment of filter pads was originally proposed by Kishino et al. (1985) and it is here recommended for routine use. We note,

however, that other approaches for experimentally partitioning the total particulate absorption into phytoplankton and non-algal components have been also proposed, for example treatment of sample with a highly oxidizing agent such as sodium hypochlorite (Ferrari and Tassan, 1999). This method does not remove the pigments from the sample; the oxidized pigments remain on the filter and their absorption shifts to the short-wavelength portion of the visible spectrum and UV making assessment in that wavelength region inaccurate.

5.3 Computing Absorption from Absorbance

5.3.1 Absorbance

Commercially available spectrophotometers typically allow selection of output in either absorbance or transmittance. The output of absorbance is referred to by the community of optical oceanographers as optical density, *OD* (c.f., although the International Union of Pure and Applied Chemistry (IUPAC) recommended against this term in the Compendium of Chemical Terminology). The relationship between these two outputs is such that $OD = \log_{10}(1/T)$, where T is transmittance. It is important to emphasize that transmittance T in this definition may have different interpretations depending on the geometry of measurement. In particular, if only the radiant power that is directly transmitted through the sample, Φ_t, is measured at the detector, then $T = \Phi_t/\Phi_o$ where Φ_o is the power of collimated beam incident on the sample. Such geometry of measurement is required by an *ideal beam attenuation meter*. In contrast, if both Φ_t and total scattered power in all directions, Φ_B, are measured at the detector, then $T = (\Phi_t + \Phi_B)/\Phi_o$. Such geometry of measurement would yield an *ideal absorption meter* (see Chapter 2).

In practice, it is difficult to perfectly satisfy the geometrical requirement of an ideal absorption meter because it is difficult to ensure that the total scattered power Φ_B is measured at the detector. When a certain portion of scattered power is not detected, the absorption coefficient is overestimated owing to the so-called scattering error (see Eq. 2.6 and related text in Section 2.2). For the spectrophotometric filter-pad technique, the issue of imperfect geometry and associated scattering error is most pronounced in the T-mode configuration (Fig. 5.1a). In contrast, the IS-mode with sample mounted inside an integrating sphere (Fig. 5.1d) approaches an ideal geometry of absorption measurement.

The absorption coefficient is defined as $a = -(1/L)\ \ln[(\Phi_t + \Phi_B)/\Phi_o]$ (see Section 2.1), while the optical density output from the spectrophotometer is provided as $OD = \log_{10}[\Phi_o/(\Phi_t + \Phi_B)]$. This gives rise the relationship between spectral optical density measurements and the spectral absorption coefficients, $a(\lambda)$ in units of m^{-1}:

$$a(\lambda) = \ln(10)\ OD(\lambda)/L \qquad (5.1)$$

where ln(10) converts the common base 10 logarithm ($\log_{10}$) to the natural logarithm that has the number e as its base ($\ln \equiv \log_e$) and L is the geometric path length of the sample expressed in units of (m).

5.3.2 The geometric path length

Geometric path lengths for cuvette measurements are given by the width of the cuvette, equivalent to the geometric path through the sample. For filter pad measurements the geometric path length, L, is computed from the volume filtered, V (m^3), and the effective area of the filter, A (m^2), measured as the area over which particles are collected onto the filter:

$$L = (V/A) \qquad (5.2)$$

which yields the height of a column of the sample projected onto the filter pad. In practice, V is typically measured in units of cm^3 (or mL) and A in cm^2 or mm^2, so conversions to m^3 and m^2 are required, respectively. The conversion is 100 cm m^{-1}, for volume and area measurements in mL and cm^2, respectively.

5.3.3 Optical path length and path length amplification

The assumption in the expression for absorption (Eqs. 5.1 and 5.2) is that the geometric path length (i.e., V/A) is equal to the optical path length (the actual average distance that a photon travels through the sample). However, comparisons between particulate samples measured on particle suspension in cuvette placed inside an integrating sphere (which is close to an ideal absorption measurement) and those measured on filter pads indicates that there is an amplification of the mean photon path through the filter compared to geometric path caused by the highly scattering nature of the filter pad. The increased optical path length relative to the geometric path length allows for increased probability for absorption by particles collected on the filter and therefore overestimation of the absorption coefficient. The correction factor for path length amplification is the so-called beta correction, β. Recent routine implementation

of the center-mounted integrating sphere configuration for both filter pads and suspended cuvette samples have clearly identified that the lack of consensus in published β correction factors is due to errors in measurements used to derive those correction factors, e.g., measurements of the suspended particulates made without an integrating sphere (Stramski et al., 2015).

The consensus for obtaining the absorption coefficient of particles, $a_p(\lambda)$, or non-algal component of particulate absorption, $a_{NAP}(\lambda)$, from filter-pad measurements, which are corrected for path length amplification, implements the following relationships:

$$a_x(\lambda) = \ln(10)\, OD_s(\lambda) / (V/A) \tag{5.3}$$

where units for the volume filtered V are (m^3) and the effective area of the filter A (m^2), and subscript x represents either the p or NAP components. The optical density $OD_s(\lambda)$ represents the absorbance by particles, which is corrected for path length amplification factor, β. In other words, $OD_s(\lambda)$ can be interpreted as the optical density of the same particles as collected on the filter pad, which would be measured in suspension over the path length V/A under single-scattering regime *without* the effect of path length amplification. The $OD_s(\lambda)$ values are calculated from a predetermined relationship involving the optical density of particles measured on the filter, $OD_f(\lambda)$:

$$OD_s = \mathbf{f}(OD_f) \tag{5.4}$$

where the function **f** essentially quantifies the path length amplification factor β. Note that β can be calculated from Eq. (5.4) as a ratio OD_f / OD_s. Generally, this ratio can vary as a function of OD_f (particle load on the filter) as the function **f** can be nonlinear. In practice, however, in routine processing of filter-pad measurements there is no need to calculate β or use explicit values of β because the right-hand side of Eq. (5.4) is simply substituted for OD_s in Eq. (5.3). The OD_f values in Eq. (5.4) represent the optical density of particles on the filter after all necessary corrections for baselines were made (i.e., blank filter baseline and instrument baseline or drift). Note also that the light wavelength argument, λ, is omitted from Eq. (5.4) because this relationship is typically determined by combining data covering a broad spectral range, usually the entire visible part of the spectrum. Therefore, Eq. (5.4) is applicable to any wavelength within the spectral range for which the relationship was determined.

The determination of Eq. (5.4) requires special laboratory experiments, and many such dedicated experiments have been conducted in the past. As a result of these experiments different functional forms were proposed, for example a second-order polynomial or power function. Recently, Stramski et al. (2015) examined the path length amplification relationships, $OD_s = \mathbf{f}(OD_f)$, with diverse samples for all configurations of filter pad spectrophotometry (transmittance T, transmittance-reflectance T-R, and inside-sphere IS), and compared their derived relationships to previously published results, with a few showing close agreement. Importantly, in these experiments the OD_s was measured on particle suspensions within the integrating sphere to provide a very close estimate of the true reference absorption coefficient. The methodology of measurements of particle suspensions placed inside the integrating sphere is described elsewhere (Babin and Stramski 2002, 2004; Stramski et al. 2007). As a result of the study by Stramski et al. (2015) we recommend the following relationships to correct for the path length amplification of the filter pad technique:

T-mode: $$OD_s = 0.679\, (OD_f)^{1.2804} \tag{5.5}$$

T-R-mode: $$OD_s = 0.719\, (OD_f)^{1.2287} \tag{5.6}$$

IS-mode: $$OD_s = 0.323\, (OD_f)^{1.0867} \tag{5.7}$$

The right-hand side of these equations should be substituted for OD_s in Eq. (5.3) in final calculations of $a_p(\lambda)$ or $a_{NAP}(\lambda)$.

5.3.4 Quantifying Uncertainty in the Filter Pad Absorption Coefficients

A full model for filter pad absorption uncertainty is achieved by arithmetically propagating the uncertainty quantified for each methodological step (JCGM 2008). In general, an experimental measurement equation for the quantity to be determined or measured, y (referred to as the measurand), can be written as

$$y = \mathbf{f}\,(x_1, x_2, \ldots x_n) \tag{5.8}$$

where the function **f** is defined by the physics of the measurement problem and $x_1, x_2, \ldots x_n$ are the experimentally determined input variables to which the measurand y is related. The variables $x_1, x_2, \ldots x_n$ have uncertainties associated

with them, which give rise to an uncertainty in the estimate of measurand y. In addition, the variables $x_1, x_2, \ldots x_n$ may themselves have measurement equations representing separate determinations.

In our case of determinations of the particulate absorption coefficient from the filter pad technique the measurand at any light wavelength λ is a_p or a_{NAP} (in what follows in this section we omit the argument λ and use the symbol a for a_p or a_{NAP} for brevity). By combining Eq. (5.3) with one of Eqs. (5.5), (5.6), or (5.7) which characterizes the path length amplification correction for one of the filter pad modes (i.e., T, T-R, or IS-mode), we obtain the experimental measurement equation for a:

$$a = \ln(10)\ \alpha\ (OD_f)^{\gamma}\ \frac{A}{V} \tag{5.9}$$

This equation has five input variables x_i which are OD_f, α, γ, A, and V. Note that although the fixed values of α and γ are used for each filter pad mode in the calculations of the measurand a, these quantities should be considered as variables in the context of uncertainty analysis because the assumed fixed values of α and γ just represent the statistical estimates established as the best option in a statistical sense for path length amplification correction. In addition, note that the variable OD_f can be written in terms of experimental measurement equation:

$$OD_f = (OD_{fs} - OD_{infs}) - (OD_{fb} - OD_{infb}) \tag{5.10}$$

where the input variables are: OD_{fs} is the best estimate of optical density measured on the sample filter, OD_{fb} is the best estimate of optical density measured for the blank filter, and OD_{infs} and OD_{infb} are the best estimates of optical density representing the instrument baselines (typically the air vs. air measurements in dual beam spectrophotometer) which are applicable to the sample filter and blank filter scans, respectively. OD_{infs} and OD_{infb} may or may not be the same depending on the sequence of specific measurements during the period of measurements. The best estimate of OD_{fs} can be obtained by repeating the scans on the sample filter, for example for different filter orientations (Fig. 5.4a), as well as by taking measurements on replicate sample filters (Fig. 5.4b), if available. Arithmetic propagation in a best-case scenario yields small uncertainties (Fig. 5.4c). The best estimate of OD_{fb} can be obtained by averaging measurements taken on multiple blank filters (Fig. 5.5), including repetitive scans for a given filter or, if allowed by the design of experiment, by making measurements of OD_{fb} for a given blank filter that is subsequently used to collect a sample for the OD_{fb} measurements. The magnitude of variability in OD_{fb} is highly dependent upon spectrophotometric configuration.

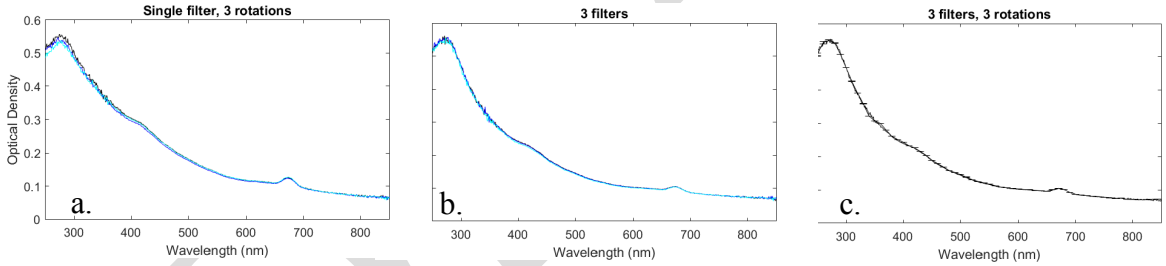

Figure 5.4. Example of best case scenario for sample uncertainty as quantified by (a) three replicate scans of a single filter with three rotations within the beam, (b) scans of three replicate sample filters, and (c) arithmetically propagated uncertainty shown by error bars (every 10 nm for clarity).

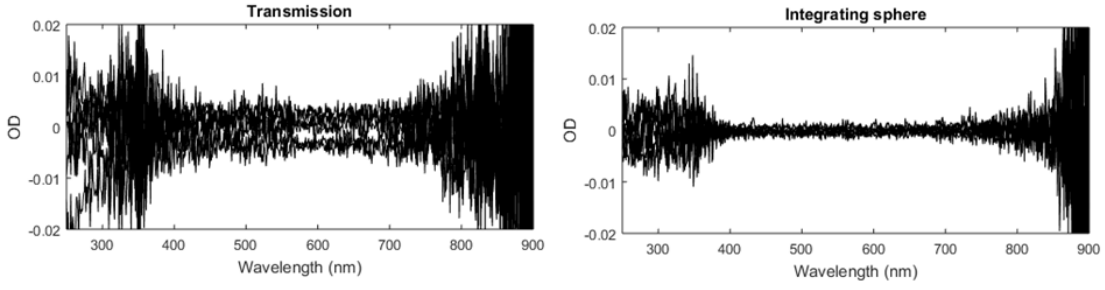

Figure 5.5. Spectrophotometric scans (optical density spectra) for a set of five blank filter pads that have been baseline corrected (a single or average blank filter pad signature removed) as measured in transmittance mode (*left*) or center-mounted integrating sphere mode (*right*).

The equation for the combined (total) standard uncertainty, $u_c(a)$, in the absorption coefficient, a, can be expressed as:

$$u_c(a) = \sqrt{\sum_{i=1}^{n} \left(\frac{\partial a}{\partial x_i}\right)^2 u^2(x_i)} =$$

$$\sqrt{\left(\frac{\partial a}{\partial OD_f}\right)^2 u^2(OD_f) + \left(\frac{\partial a}{\partial \alpha}\right)^2 u^2(\alpha) + \left(\frac{\partial a}{\partial \gamma}\right)^2 u^2(\gamma) + \left(\frac{\partial a}{\partial A}\right)^2 u^2(A) + \left(\frac{\partial a}{\partial V}\right)^2 u^2(V)} \quad (5.11)$$

where the partial derivatives are referred to as sensitivity coefficients which relate the change in the measurand a with respect to the input variable x_i, and the quantities $u(x_i)$ are the uncertainties assigned to individual variables x_i that are used to calculate a. As explained in relation to Eq. (5.9) the variables x_i are OD_f, α, γ, A, and V, so in this case $n = 5$. Note that the uncertainty associated with the path length amplification is represented by the two terms that are associated with α and γ. Equation (5.11) is applicable when there are no correlations between variables x_i which is a reasonable assumption for our experimental problem.

This equation indicates that in order to estimate the total standard uncertainty, $u_c(a)$, it is necessary to determine both the sensitivity coefficients with respect to each individual variable x_i and the uncertainty of each variable x_i. Note also that the variable OD_f involved in Eq. (5.11) is itself described by the experimental measurement Eq. (5.10). Therefore, this variable has its own combined standard uncertainty, $u_c(OD_f)$, which can be expressed by an uncertainty equation that is analogous to Eq. (5.11) in which the variables x_i are OD_{fs}, OD_{fb}, OD_{infs}, and OD_{infb}. These variables are also subject to uncertainties which need to, and can, be quantified. For example, the uncertainties in the instrument baselines are associated with the inherent random noise of the instrument for the air vs. air scan, and possibly also a temporal drift in these baselines during the period of measurements. These uncertainties will necessarily vary from instrument to instrument and should be determined and reported. The uncertainties in OD_{fs} and OD_{fb} can be estimated from repetitive scans for a given sample or blank filter and measurements taken on multiple sample or blank filters.

Rigorous quantification of total uncertainty, $u_c(a)$, for the experimental problem at hand is very difficult, if not impossible, because of the lack of complete information required to rigorously evaluate each term involved in Eq. (5.11). In general, the uncertainties $u(x_i)$ of individual variables x_i could be estimated from the experimental standard deviation, $s(x_i)$, determined from a series of N measurements of variable x_i according to:

$$u(x_i) = \sqrt{s^2(\overline{x}_i)} = \sqrt{\frac{s^2(x_i)}{N}} \quad (5.12)$$

where $\overline{x}_i$ is the estimated average value of x_i and $s^2(\overline{x}_i)$ is the experimental variance of the mean. Whereas such estimation appears relatively straightforward for some x_i variables, such as A and V (e.g., Fig. 5.6), this task is more difficult and would require special, generally highly laborious, experiments for other variables, OD_f, α, and γ, involved in Eq. (5.11). The additional complexity in the evaluation of $u_c(a)$ from Eq. (5.11) results from the fact that the sensitivity coefficients, $\partial a / \partial x_i$, with respect to any specific input variable x_i depend on the magnitude of other input variables used in Eq. (5.9). It thus appears that a simpler more pragmatic approach for approximate estimation of the total uncertainty $u_c(a)$ for the filter pad technique is to conduct dedicated experiments on many diverse samples, in which the measurand a obtained from the filter pad measurements is simply compared with reference measurements taken on the same samples in suspension with a technique that provides the measurand a in the closest possible agreement with the true particulate absorption coefficient (for example, PSICAM method or particle suspension inside the integrating sphere of the spectrophotometer). Under the assumption that the reference measurements are subject to much smaller uncertainty than the filter pad measurements, the differences between the two measurements are largely attributable to the uncertainty of the filter pad measurements. The drawbacks of this approach involves the uncertainty of the reference measurements (which, however, may be easier to quantify than for filter pad technique) and the inability to resolve the influences of individual input variables x_i on the total uncertainty $u_c(a)$ of the filter pad technique. Some experiments aimed at addressing these uncertainty issues have been recently undertaken by the NASA PACE Science Team but more work in this area will be required to rigorously quantify both the total uncertainty $u_c(a)$ and the individual terms of Eq. (5.11) for the filter pad technique.

For illustrative purposes of the conceptual framework of the uncertainty analysis based upon Eq. (5.11), the contribution of a single variable, x_i to $u_c(a)$, assuming that other input variables make no contribution to $u_c(a)$, can be

considered for V, filtration volume as x_i. Eq. (5.11) simplifies to (assuming that the uncertainty terms associated with OD_f, α, γ, and A are all null):

$$u_c(a) = u_V(a) = \frac{\partial a}{\partial V} u(V) \quad (5.13)$$

where

$$\frac{\partial a}{\partial V} = -\ln(10)\ \alpha\ (OD_f)^{\gamma} A\ V^{-2}$$

which indicates that the sensitivity coefficient is inversely proportional to the squared volume. The uncertainty of volume, $u(V)$, from Eq. (5.12) is obtained by assuming a reasonable value for the standard deviation in V, $s(V)$. This is determined by the graduated cylinder used and each person's ability to measure volume accurately. The former is determined by the graduated cylinder (resolution to one half the distance between marked intervals) and uncertainty is reduced by selecting a graduated cylinder with a volume closest to but larger than the filter volume. The latter is difficult to quantify but effort should be made to assess the user's random uncertainty under standard measuring conditions (obviously greater on a ship in rough seas compared to in the laboratory) by calculating the standard deviation for a reasonable number of measurements, N. To see the impact of filter volume uncertainty, the sensitivity coefficient $\partial a/\partial V$ is calculated by assuming reasonable values used in the filter pad measurements for all components (Fig. 5.6). For example, two samples with equal filter volumes but different measured optical density spectra, within the recommended 0.1 to 0.4 range, will have the same uncertainty spectrum associated with $u(V)$, but it will represent a larger proportional uncertainty for the low optical density sample (Fig. 5.6A, black and blue curves, respectively). Similarly, two identical optical density spectra will have very different absolute uncertainties if one results from filtering 100 mL sample and the other from 500 mL sample (Fig. 5.6B, black and green curves, respectively), even though the proportional uncertainty is the same.

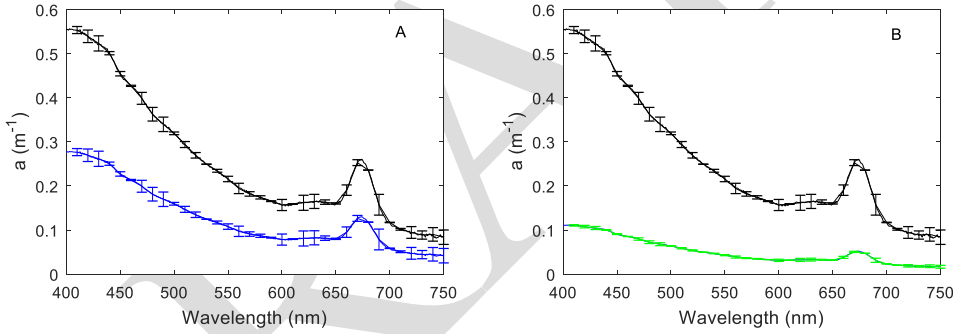

Figure 5.6. Absorption spectra with propagated uncertainty spectra are expressed as error bars for (A) two particulate optical density spectra for which the optical density magnitudes vary but the filter volumes are the same, (B) for two identical optical density spectra for which only the filter volumes vary (100 mL and 500 mL).

In practice, some of the uncertainty terms can be determined for a given laboratory setting with robust protocol standards. Some may be easier to analyze (like V and A), while others like α and γ (which both contribute to the uncertainty associated with path length amplification) may be harder to quantify because the assumptions underlying their uncertainty are still somewhat speculative in the absence of focused experimentation. However, assessing the level of uncertainty in as many of the variables as possible, and quantifying the contribution of each, provides guidance to improving laboratory practices and reducing uncertainty in spectrophotometric technique.

5.3.5 Partitioning Particulate Absorption into Contributions by Phytoplankton and Non-Algal Particulates (NAP)

Once the optical density spectra for particulate and non-algal particulate contributions have been converted to their respective absorption values using Eqs. (5.3), and (5.5), (5.6), or (5.7) depending on the measurement configuration (T-mode, TR-mode, or IS-mode, respectively) the spectral absorption coefficients for phytoplankton, $a_{ph}(\lambda)$, are calculated by difference:

$$a_{ph}(\lambda) = a_p(\lambda) - a_{NAP}(\lambda) \quad (5.14)$$

The partitioning of the particulate absorption into phytoplankton and non-algal particles is understood to be an operational definition based upon pigment extraction (Fig. 5.7). The phytoplankton component is better described as the "absorption by methanol-extractable phytoplankton pigments *in vivo*". It necessarily does not include other phytoplankton cellular material such as cell walls, membranes, etc., which instead are included in the "non-algal particle" fraction. Note that this operational definition of non-algal particulate component includes all kinds of non-algal particles such as organic detritus, mineral particles, mixed organic-inorganic particles, and heterotrophic organisms.

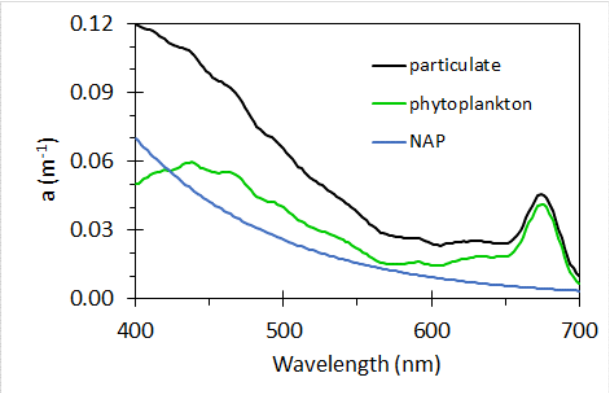

Figure 5.7 Example of particulate absorption spectrum measured on a filter pad (black), the absorption by NAP, measured after methanol extraction (blue), and the phytoplankton absorption determined by difference (green).

5.4 Measurement of Filter Pad Absorption in Transmittance Mode (T-Mode)

While likely the least accurate of the spectrophotometric modes for determining particulate absorption on filter pads, the transmittance mode (T-mode) has the longest legacy. That it does not require expensive accessories such as integrating spheres suggests it may continue to be the most utilized configuration mode. For these reasons, it is critical to understand the uncertainties encountered in this approach and strategies for both minimizing uncertainties and correcting for those that remain. Presently the largest uncertainties are those due to scattering losses to the detector that are not accounted for by blank filter correction and scattering impacts on path length amplification. By employing paired analyses with an internally-mounted integrating sphere (IS-mode), both uncertainties can be quantified.

5.4.1 Spectrophotometer configuration in T-Mode

The baseline, blank and sample filter pads will all be placed against the detector side of the sample chamber. The moisture of the filter pad will provide the cohesive properties necessary to hold the filter in place. To protect the spectrophotometer from filter moisture, it is recommended that a thin Plexiglas slide, with a central opening that exceeds the size and shape of opening of the spectrophotometer aperture, be secured to the spectrophotometer for placement of the filters (Fig. 5.8).

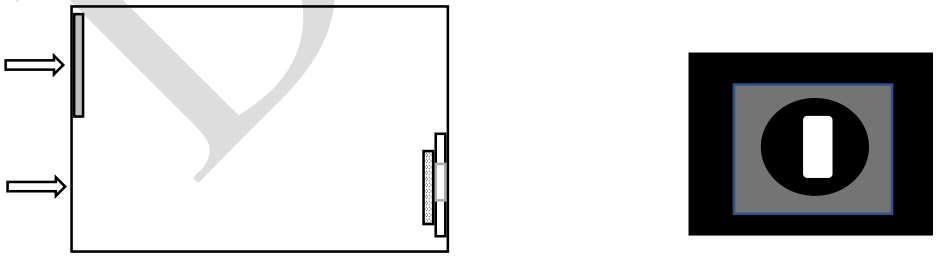

Figure 5.8. *Left diagram* Top view of sampling chamber in dual beam spectrophotometer. Arrows indicate incoming beams for reference (top) and sample (bottom). Neutral density filter (grey) placed on entrance of reference beam. Filter holder with aperture (white) and glass fiber filter (dotted) on exit port of sample beam. *Right diagram* Front view of exit port (white) of sample beam showing spectrophotometer wall (black), glass or Plexiglas filter holder with round aperture (grey). The filter holder aperture is larger than the exit port but smaller than the glass fiber filters.

The spectral optical density, *OD*, of a blank filter pad in transmittance mode is between 2.2 (T = 0.6%) and 2.5 (T = 0.3%) from 350 nm to 850 nm, and increases exponentially into the UV to a value of approximately 4.1 (T = 0.008%) at 200 nm when corrected for air baseline (Fig. 5.9). These values are meant to be illustrative, the specific values will vary slightly between instruments. Thus, in the visible waveband blank glass fiber filters transmit less than 0.6% of the incident beam to the detector, making for a very low signal to noise ratio. The situation can be vastly improved by balancing the amount of light energy that passes through the sample and reference beams. This is achieved by placing a quartz neutral density (ND) filter against the reference port entering the sample compartment (Fig. 5.8, left diagram). Quartz is preferred over glass because of its superior transmittance in the UV portion of the spectrum and the reduced likelihood of being scratched. In this configuration, there is a comparable amount of light energy passing to the detector from both the sample and reference beam, which minimizes the instrumental noise (and in many models, allows the gain to be increased). Fig. 5.9 shows the optical density scan for blank filters relative to air, 0.5 and 2.0 neutral density filters. The 2.0 ND is optimal; the resulting blank filter optical density ranges from 0.3 to 0.4 in the visible, which is equivalent to 50% and 40% transmittance, respectively, a vast improvement in signal to noise. Again, the exact choice of the ND filter may vary between instruments depending upon geometry and how the T-mode is configured.

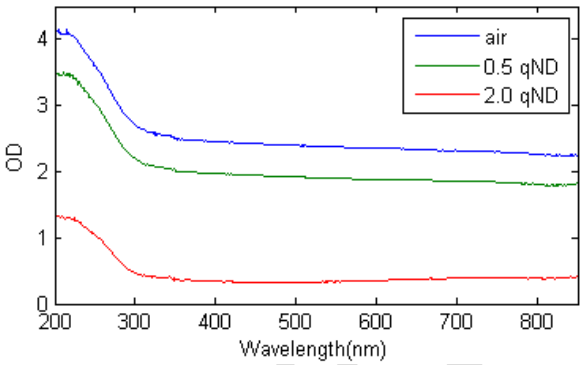

Figure 5.9. Optical density spectra for blank filter pad with air baseline correction applied (blue). Placing a neutral density filter of optical density 0.5 (green) and 2.0 (red) on the entrance port of the reference beam reduces the overall optical density signal of the blank filter pad in dual beam mode by balancing the energy in the sample and reference beams, thereby increasing signal to noise.

5.4.2 Dual beam versus single beam spectrophotometry in T-Mode

Dual beam spectrophotometers are preferred to single beam spectrophotometers because the dual beam automatically corrects for short-term variations in lamp energy that occur both within a single scan and between scans.

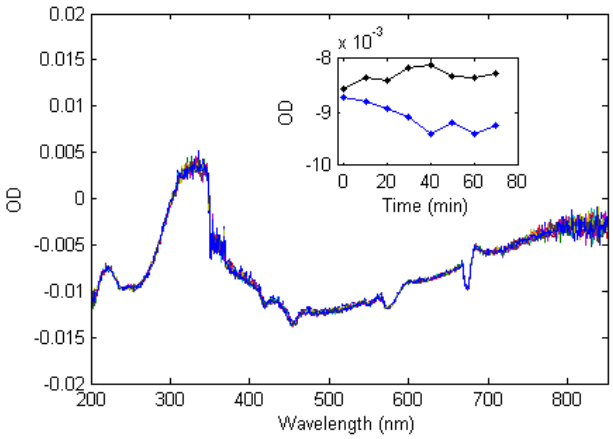

Figure 5.10. Example of time series of spectrophotometric air scans collected over approximately 70 minutes every 10 minutes after instrument is turned on. The inset shows the time series of air OD measured at 275 nm (black) and 400 nm (blue) representing the two lamps, deuterium and tungsten, respectively.

Instruments should be allowed to warm up for at least 60 minutes as the spectrum of lamp energy changes during warm up. The warm up time can be assessed by running air scans every ten minutes from the time the instrument is

turned on until subsequent scans approach differences in optical density < 0.0005 (the target noise value; Fig. 5.10). Establish the warm up time for your instrument; it may change as the lamps age.

5.4.3 Instrument performance, instrument settings and spectrophotometric noise in T-Mode

Instrument performance tests should be performed prior to every measurement session. These include tests for wavelength accuracy, absorbance calibration and instrument noise. Maintaining a record of these tests allows the user to identify misalignment, lamp degradation and detector failures.

The recommended wavelength range for filter pad measurements is 250 nm – 850 nm. The lower (UV) end of the spectrum will be noisy because of the strong absorption by the glass fiber filters and the relatively weak light energy of the instrument; the upper red end of the spectrum will be noisy because of the generally weak light/detector sensitivity and strong scattering by the filter. The crossover wavelength between the deuterium and tungsten lamps is between 300 nm and 350 nm and should be consistently maintained. The wavelength resolution, scan speed and integration times are recommended to be 1 nm; 120 to 300 nm per minute; 0.1 to 0.2 s, respectively. A slit band width (SBW) of 2 nm is recommended. Smaller SBW lead to reduced light energy and lower signal to noise while larger SBWs reduce the resolution of spectral variations associated with distinct pigments.

Spectrophotometric noise varies between manufacturers, between instruments, and over time. This is best assessed by collecting multiple air scans and computing the standard deviation spectrum over the entire wavelength range. The target value is approximately 0.0005 optical density units and is the lowest level of uncertainty.

5.4.4 Baseline, zero and blank scans in T-Mode

Baseline scans are necessary to remove the instrument signal which encompasses the variations due to lamp energy spectrum, spectral sensitivity of the detector and the optical signature of the glass fiber filter. There are two approaches to performing baseline scans: air baselines and filter pad baselines. The end product particulate absorption will be the same but the differences are in what appears on the screen as samples are processed.

The air baseline approach involves collecting a single air scan as a baseline which is automatically removed from subsequent blank and sample scans. The average spectrum of a set of 3 - 5 blank filter pads scans are then subtracted from each sample scan to remove the optical signature of the filter pad. The standard deviation spectrum of the blank scans is used to compute absorption uncertainty using Eq. (5.6). What appears on the screen during measurement is a spectrum that is the sum of the filter pad and the sample optical properties. To maintain the proper sample loading on the filter (optical density between 0.1 and 0.4), the contribution by the blank filter has to be mentally removed. Thus, if the optical density of the blank filter is 0.25 in the UV (as is typical for T-mode) and the sample filter optical density is 0.6, the sample optical density is 0.35, still less than the maximal 0.4 optical density target (Fig. 5.11).

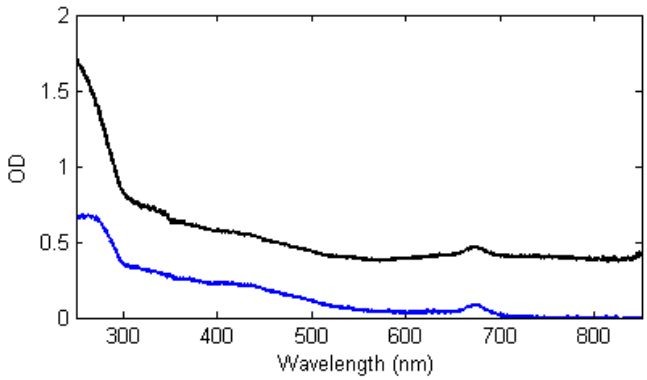

Figure 5.11. Example of a particulate optical density scan run in T-mode with air as a baseline (black) or with a blank filter as a baseline (blue). Note the sample optical density range is between 0.1 and 0.4 (the target range) for the visible portion of the spectrum but begins to exceed this optimal range in the UV portion of the spectrum.

The filter pad baseline approach involves collecting a single scan of a prepared blank filter pad as the baseline which is automatically removed from subsequent blank and sample filter scans. The average spectrum of the blank filter scans should be spectrally flat about zero (Fig. 5.4). The advantage of this approach is that the scans that appear on the screen show the optical density of the sample material and instantly allow the user to determine if the proper

loading has been achieved throughout the spectral range. It also provides an instant assessment of the blank filter spectral variations.

5.4.5 Sample analysis for T-Mode

The sequence of sample scans includes the assessment of a baseline, a series of blank filter pads, and the initial scan of the set of sample filter pads. The initial scan of each sample provides the assessment of particulate optical density. After pigment extraction (Section 5.2), the sample filters are scanned again to provide assessment of the non-extractable particulate contribution to optical density, also known as non-algal particles (Fig. 5.12).

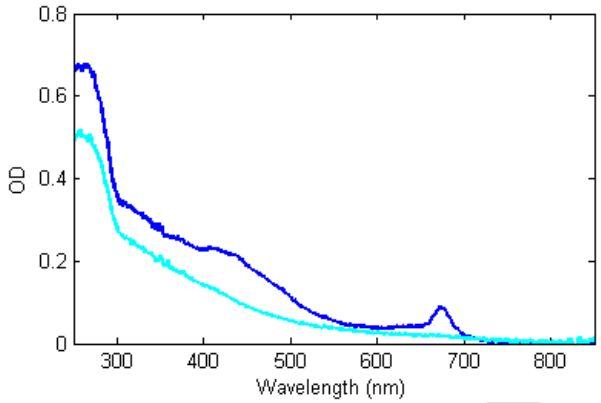

Figure 5.12. Example of a particulate (blue) and extracted non-algal particle (cyan) optical density scans measured in T-mode with a blank filter as a baseline. Note the magnitude and shape of the optical density spectra in the near IR (~700nm -850 nm) is zero and spectrally flat within the uncertainty of T-mode blank filter readings.

The operating protocol of the spectrophotometer with regards to spectral bandwidth, spectral sampling, and scan rates are the same as described earlier in this section. The recommendations regarding maintaining filter hydration between measurements should also be followed. A typical sequence of making measurements is as follows:

1. The baseline scan is initiated with the quartz neutral density filter placed securely to the light source side of the reference port and a moist blank filter placed securely to the detector side of the sample port (diagram). This is the baseline scan. For most instruments, this scan is stored internally and automatically subtracted from subsequent samples scans.

2. Without opening the sample compartment, a second scan is immediately collected. This scan will have the baseline scan automatically removed. Because no changes have been made this scan should be spectrally flat about zero; this is the zero scan. If there is some spectral dependence to this scan or noise of a level exceeding 0.001 the blank filter pad should be replaced with a different moist pad and the baseline and zero scan repeated.

3. A series of 3 – 5 blank filters scans are collected relative to the baseline scan; these are the blank scans. These should likewise exhibit no spectral dependence and with signals of order 0.001 throughout the spectrum. The largest variations are likely found in the UV and far-red portions of the spectra (Fig. 5.6). If all the blank filter scans are similar to each other but very different from zero, the baseline filter pad is anomalous and one of the blank pads should be rerun in baseline mode. Repeat the zero and blank scans as above.

4. Sample filters are scanned similarly relative to the baseline scan. A notch at the edge of the filter is used to align the position of the filter on the holder. This provides a mechanism for placing the filter in the same orientation after pigment extraction. Remoistening and rotating the filter 90^{o} for a second scan provides a measure of within sample variability. These measurements provide the optical density signature for computing the spectral particulate absorption coefficients, $a_p(\lambda)$, via Eqs. (5.3) and (5.5).

5. After pigment extraction (Section 5.2), each sample filter is rescanned to assess the optical density signature of the non-algal particles for computing absorption properties, $a_{NAP}(\lambda)$, via Eqs. (5.3) and (5.5).

6. Blank filters are scanned throughout the measurement period to assess any drift in the instrument relative to the initial baseline. If the blank filter spectra vary relative to their initial scans in spectral shape (flat) and magnitude (+/- 0.001 maximally), a new series of baseline, zero and blank scans should be run.

5.4.6 Data processing for T-Mode

General processing of data and calculation of the absorption coefficient is similar to the general guidelines described in Section 5.3. An important variation unique to the T-mode is that the so-called "null point" correction, in which subtraction of a spectrally-constant value from the NIR spectral region is used to account for large scattering losses. Experience with healthy phytoplankton cultures suggests that there is negligible absorption in the NIR, thus when non-negligible absorption is measured in the NIR using the filter pad technique in T-mode, it is assumed that it is due to scattering losses by the filter pad. The assumption is that these scattering losses are spectrally invariant due to the large size of the scattering fibers of the filter relative to the wavelength of light (and confirmed by their white scattering appearance). Thus, the average absorption value computed in the NIR region is subtracted from the entire spectrum, resulting in a shift down (in the case of positive NIR signal) or shift up (in the case of a negative NIR signal). Problems arise when the sample is composed of particulates other than healthy phytoplankton. In this case the NIR null point correction likely removes some contribution to absorption by these non-algal particles and leads to a larger measurement uncertainty. However, in the absence of other supporting measurements, the null point correction provides the lowest error estimate for T-mode absorption measurements. The data processing procedure is as follows:

1. Instrument drift is quantified from the blank filter pad measurements made at different times throughout the measurement period. If required, all filter baselines and sample spectra are corrected for any observed drift.

2. The average and standard deviation optical density spectra from all blank filter pad scans are computed. If the air baseline approach is used, the average is subtracted from each measured spectrum. If the blank filter baseline approach is used, no additional subtraction is necessary (as the average of the blank filters relative to a blank filter baseline should be zero within 0.001 optical density units).

3. Replicate measurements of baseline-corrected sample filter optical density, $OD_f(\lambda)$, obtained on the same sample filter are averaged.

4. The blank-corrected and averaged $OD_f(\lambda)$ of the sample can be smoothed, for example with a moving average. The choice of smoothing window width and number of iterations is determined based on characteristics of the sample spectra (i.e., presence or absence of sharp peaks, behavior of instrument noise).

5. The particle absorption coefficient, $a_p(\lambda)$, or non-algal particle absorption coefficient, $a_{NAP}(\lambda)$,for each sample are calculated from $OD_f(\lambda)$ using the known filtration volume (V in m^3) and the measured interception area of filtration (A in m^2) as:

$$a_x(\lambda) = \ln(10)\ 0.679\ [OD_f(\lambda)]^{1.2804} / (V/A) \qquad (5.15)$$

which utilizes a beta-correction modeled as a power function for the relationship between OD_s and OD_f (Eqs. 5.3 and 5.5).

6. For the particulate and non-algal particle absorption spectra, the null point correction can be applied by subtracting the respective average absorption values in the NIR (e.g. over the range 800 nm – 850 nm).

7. The phytoplankton absorption spectrum, $a_{ph}(\lambda)$, is computed from the particulate and non-algal particle absorption spectra by difference using Eq. (5.14).

The largest uncertainty in the T-mode approach is associated with the unknown level of absorption in the NIR which cannot be quantified because of the unknown quantity of scattered loss by the filter with imbedded particles compared to the blank filter. The IS-mode provides quantitation of the NIR absorption, and, in the presence of any particulate material other than healthy phytoplankton cultures, there is measurable NIR absorption coefficients, which clearly violates the assumption for the null point correction in step 6. It also provides evidence that the scattering corrections approaches for reflecting tube absorption meters that require the null point correction at red wavelengths are also in error (Chapter 2).

5.5 Measurement of Filter Pad Absorption in T-Mode Using Fiber Optics

5.5.1 General Considerations for Fiber Optic T-Mode

Particulate absorption measurements using the filter pad method have been assessed on a fiber-optic based spectrophotometer (Beltz et al. 2006; Miller et al. 2011; Naik and D'Sa 2012). The portable fiber-optic based system consists of a single beam optical path with a light source, a filter holder and a fiber-optic spectrometer all connected

serially using optical fibers (Fig. 5.13a), The filter holder consists of a filter fixture for the GF/F filter and a holder (Fig. 5.13b; QFT1-89575, WPI). In its basic configuration, the output of a high intensity light source (Fig. 5.13c; e.g., D_2H consisting of a deuterium and halogen lamp; WPI Inc.) is coupled via an optical fiber with a core diameter of 600 μm to the filter holder. A combination of 600 μm input fiber and a fused silica lens collimates the input light into an approximately parallel beam of 5 mm diameter (Belz et al. 2006). The collimated light beam incident perpendicular to GF/F filter (blank, particulate or extracted) is transmitted or scattered through the filter and is collected by a second collimating lens behind the filter and coupled into an exit 600 μm fiber that is then connected to a photodiode array spectrometer (Fig. 5.13d; e.g., Tidas, J&M Analytische Messung Regeltechnik GmbH) that is optimized for the spectral range of 195 - 725 nm. The Tidas spectrometer connects via a RS-232 to USB adapter to a Windows based computer with vendor supplied Spectralys software that is used to acquire, display and analyze the spectral data.

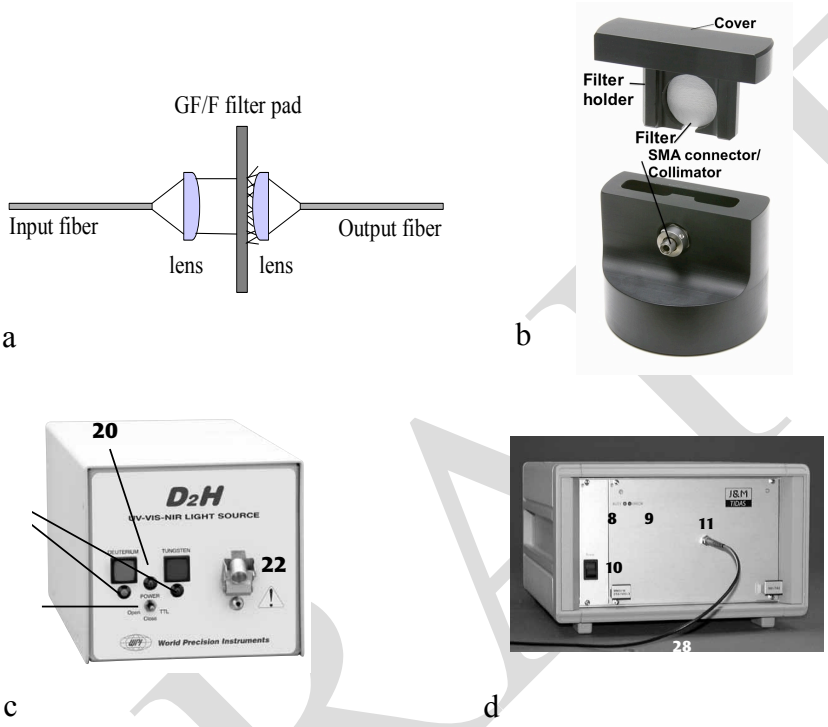

Figure 5.13. (a) Schematic of a portable fiber-optic based filter holder for measuring particle absorption using the QFT method. The input fiber connects to the light source (e.g., D_2H; b) while the output fiber connects to the spectrometer (e.g., Tidas; c). (d) The filter holder (WPI) with the GF/F filter fixture that is inserted into the holder.

Advantages: The fiber optic based spectrophotometer for filter pad measurements is small and portable and relatively inexpensive in comparison to the laboratory based spectrophotometers (e.g., Perkin-Elmer Lambda-850). It can be easily setup on a ship during field campaigns and absorption measurements of suspended particles obtained following seawater sampling onboard the ship. The use of a photodiode array-based spectrometer also allows for greater sensitivity in the absorbance measurements by increasing the integration time of the detector.

Disadvantages: Results of a comparison study of particle absorption on a filter indicated small differences (~5%) in $OD_f(\lambda)$ between the fiber-optic based system and a high performance spectrophotometer (e.g., Perkin Elmer Lambda 850; Miller et al. 2011; Naik and D'Sa 2012).

5.5.2 Sample analysis for Fiber Optic T-Mode

1. The spectrophotometer (lamps and the spectrometer) should be allowed to warm for one hour before running the samples.
2. The Spectralys software is started.
3. The steps for blank and sample GF/F filter preparation (Section 5.2) should be followed.
4. A blank GF/F filter is placed in the fiber optic filter holder.

5. Integration time is adjusted on the spectrometer to optimize the light transmission through the blank hydrated filter to obtain peak intensity of ~70% and then select absorbance mode.

6. A 'dark spectrum' is obtained with the shutter closed.

7. The shutter switch is moved to 'open' mode and 'reference spectrum' is obtained.

8. The blank filter is replaced with a filter that contains all particles and then non-algal particles following methanol extraction.

9. A 'sample spectrum' is obtained and repeated after rotating the filter 90°.

10. The spectra are export and saved as an ascii file.

5.5.3 Data processing for Fiber Optic T-Mode

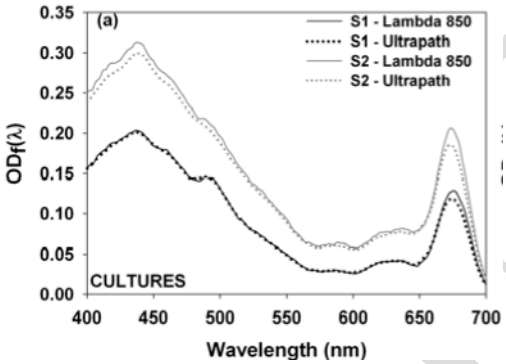

Figure 5.14. Comparison of spectral shape of optical density ($OD_f(\lambda)$) of particles on GF/F filter measured with the fiber-optic based system and a Perkin-Elmer Lambda 850 spectrophotometer with an integrating sphere (Naik and D'Sa 2012).

1. A null correction should be applied by subtracting the average of spectrally flat region between 712-722 nm.

2. Optical density $OD_f(\lambda)$ for representative culture samples measured on the fiber optic based system and the Lambda 850 showed overall very good agreement with strong linear relationship observed between $OD_f(\lambda)$ at chlorophyll absorption peaks (443 and 676 nm) and also over the entire visible domain from 400 to 700 nm (Fig. 5.14). These results suggested that the beta correction factor derived for the Perkin Elmer Lambda 850 with an integrating sphere could be applied to the fiber optic based QFT system. The equation for beta correction for path length amplification effect caused by multiple scattering in the glass-fiber filter applicable to the fiber-optic based absorbance measurements is given as (Naik and D'Sa 2012):

$$OD_s(\lambda) = 0.405[OD_f(\lambda)] + 0.475[OD_f(\lambda)]^2 \quad (5.16)$$

where $OD_s(\lambda)$ is the corrected optical density of particulate matter. The absorption coefficient of the particulate matter, $a_p(\lambda)$ or $a_{NAP}(\lambda)$, is then calculated from Eq. (5.3) using $OD_s(\lambda)$ values from Eq. (5.16). The phytoplankton absorption coefficient, $a_{ph}(\lambda)$, is calculated from Eq. (5.14).

5.6 Measurement of Filter Pad Absorption in Transmittance and Reflectance Mode (T-R Mode)

5.6.1 General Considerations for T-R Mode

Tassan and Ferrari (1995) described a modification of the light-transmittance method that corrects for differences in backscattering between the sample and reference filter and, thus, accounts for backscatter differences between different sample filters. This technique combines light-transmittance (T) and light-reflectance (R) measurements carried out using an integrating sphere attached to a dual-beam spectrophotometer. In contrast to T mode, the T-R mode enables a measurement of a large fraction of both forward-scattered light (T-mode) and backward-scattered light (R-mode), which largely circumvents (or minimizes) the issues associated with scattering error (owing to undetected portion of scattered power for T-mode alone) and null-point correction. The T-R data analysis is performed by a theoretical model that eliminates the effect of differences in light backscattering by the particles and different filters.

Modifications of the T-R experimental routine (Tassan and Ferrari 1998; Ferrari and Tassan 1999) yielded a significant reduction of the experimental error. Absolute errors are typically lower for the T-R method than for the T method (Tassan and Ferrari 2002, Röttgers and Gehnke 2012). Tassan and Ferrari (1995) reported that for Case 1 waters that have negligible inorganic particle load, the amplification factor for GF/F filters determined with the T-R method is similar to that determined by Mitchell (1990). Similar results were obtained for Case 2 waters (Tassan et al. 2000). The T-R method is particularly suited for applications to samples containing highly scattering mineral particles that are commonly found in Case 2 waters. When sample measurements of T and R are made with a good spectrophotometer and integrating sphere, the scattering errors are greatly reduced, usually to the point that a null-point correction becomes unnecessary (Tassan and Ferrari 2003, Röttgers and Gehnke 2012). The method should be considered when a good spectrophotometer equipped with an integrating sphere is available, but the sphere does not allow placing a sample inside it and using a superior IS-mode of measurement (see Section 5.7).

The most recent procedure that includes some modifications of the Tassan and Ferrari (1995) routine is described by Tassan and Ferrari (2002). Here, the method is described in a simplified way.

5.6.2 Sample analysis for T-R Mode

Common procedures for sample preparation, handling, and spectrophotometric measurements as described above should be followed. Some minor differences may be related to the necessity for the use of the integrating sphere in the T-R mode. The properties of the integrating sphere are of lower importance, but usually a sphere with a larger diameter provides better performance. A sphere operating with a double-beam spectrophotometer has typically four ports, two entrance ports and two exit ports, one set for the sample beam and the other set for the reference beam. The exit ports are normally closed with white reflective plates (typically calibrated reflectance standards), and black light traps are usually placed behind these plates. Alignment of the two light beams should be checked with respect to the beam position being in the center of these reflective plates. The baseline is recorded with both entrance ports void and both exit ports closed with reflectance standards.

All spectral measurements can be done using the absorbance (*OD*) output of spectrophotometer and the following procedure is based on *OD* measurements. A hydrated blank filter is measured as a reference. When using 25 mm GF/F filters, these, when wet, can be placed directly onto the integrating sphere ports. Some extra support for the filters can be arranged to avoid salt water coming in direct contact with the outside of the integrating sphere. Using glass plates behind the filter as a support is not recommended. Each filter is first measured when placed at the sample beam entrance port, when the reference beam entrance port is void and the exit ports are closed with the reflectance standards. This results in the respective *OD* of the transmittance mode for the sample filter and the reference filter, OD_{fs}^{T} and OD_{fr}^{T}, respectively. Secondly, the same filter is measured (after another hydration if necessary) in reflectance mode when placed (sample side facing the light beam) at the sample beam exit port. The reflectance standard at this port is removed and the space behind the filter serves as a black light trap (otherwise support the filter with a black material). These measurements result in the respective *OD* of the reflectance mode for the sample filter and the reference filter, OD_{fs}^{R} and OD_{fr}^{R}, respectively

5.6.3 Data processing for T-R Mode

According to the equations provided by Tassan and Ferrari (2002) and omitting the wavelength argument λ for brevity, the *OD* values are converted to specific transmittances and reflectances using $OD^{T} = log_{10}(1/T)$ and $OD^{R} = log_{10}(1/R)$. This results in absolute transmittances and reflectances of the sample and reference filter, T_{fs}, T_{fr}, R_{fs} , and R_{fr}. The ratios of the two transmittance and two reflectance measurements gives the *T* and *R* spectra of the sample, i.e., $T_f = T_{fs}/T_{fr}$ and $R_f = R_{fs}/R_{fr}$, respectively. T_f is used to calculate OD_{fs}^{T}, the optical density of the sample in the transmittance mode. The full set of T and R measurements is used to calculate the absorptance of the sample as

$$A_f = \frac{1-T_f+R_{fr}(T_f-R_f)}{1+R_{fr}T_f\tau} \tag{5.17}$$

where τ compensates for the fact that the reflected light inside the filter is diffuse and is no longer a collimated beam. Its effect on A_f is low (<3%). This factor was determined empirically and can be calculated for each sample filter as:

$$\tau(\lambda) = 1.15 - 0.17\ \mathrm{OD}_{\mathrm{fs}}^{\mathrm{T}^*}(\lambda) \tag{5.18}$$

where

$$OD_{fs}^{T\,*}(\lambda) = OD_{fs}^{T}(\lambda) - 0.5\, OD_{fs}^{T}(750) \quad (5.19)$$

The formulation for τ is valid for $0.02 < OD_{fs}^{T\,*} < 0.7$.

The absorptance of the sample is defined as the fraction of incident power that is lost from the beam owing to absorption (Mobley, 1994), so in this case we have $A_f = \Phi_a/\Phi_o$ where Φ_a is the absorbed power. In addition, by virtue of energy conservation $\Phi_o = \Phi_a + \Phi_t + \Phi_B$, we obtain $1 - A_f = (\Phi_t + \Phi_B)/\Phi_o$. As a result, the final values of the optical density of the sample measured in T-R mode can be calculated from A_f as (see Section 5.3.1):

$$OD_f = \log_{10}[1/(1 - A_f)] \quad (5.20)$$

The particle absorption coefficient, $a_p(\lambda)$, or non-algal particle absorption coefficient, $a_{NAP}(\lambda)$, are calculated from $OD_f(\lambda)$ using the known filtration volume (V in m^3) and the measured interception area of filtration (A in m^2) as:

$$a_x(\lambda) = \ln(10)\ 0.719\ [OD_f(\lambda)]^{1.2287} / (V/A) \quad (5.21)$$

which utilizes a beta-correction modeled as a power function for the relationship between OD_s and OD_f (Eqs. 5.3 and 5.6). The phytoplankton absorption coefficient, $a_{ph}(\lambda)$, is calculated from Eq. (5.14).

5.7 Measurement of Filter Pad Absorption Inside an Integrating Sphere (IS-Mode)

5.7.1 General considerations for IS-Mode

Historically, the most common implementation of the filter pad technique involves measuring the transmittance (T) of a sample filter relative to a blank reference filter (Mitchell et al. 2003). This method suffers from a poor geometry as a large fraction of light scattered by the filter is not detected by the spectrophotometer, resulting in unknown errors in the spectral determination of filter optical density, $OD_f(\lambda)$, and ultimately in the particulate absorption coefficient, $a_p(\lambda)$. An alternative approach, referred to as the transmittance-reflectance (T-R) method, employs multiple scans of the sample and reference filters placed at the entrance (transmittance) and exit (reflectance) ports of an integrating sphere (Tassan and Ferrari 1995, 1998). The underlying assumptions of the T-R method are based on the law of energy conversation, but uncertainties arise as these assumptions are not necessarily fully satisfied with the actual measurement configuration. The need for multiple scans at different filter positions also increases uncertainties and makes the method more laborious to implement.

To circumvent these limitations, we recommend an improved refinement of the filter pad technique in which the sample or reference filter is placed inside an integrating sphere during measurement. This inside sphere (IS-mode) technique ensures the detection of nearly all photons scattered by the sample, resulting in improved accuracy and precision of absorption measurements (Maske and Haardt 1987; Babin and Stramski 2002, 2004; Stramski et al. 2004, 2007; Röttgers and Gehnke 2012; Stramski et al. 2015). Because this method does not require multiple scans of the same filter in different optical configurations, the effort is no more laborious than the traditional T-mode.

5.7.2 Sample preparation for IS-Mode

Sample collection and filtering follow the same guidelines recommended for the general filter pad technique (Section 5.2). Filter volumes are adjusted to target an optical density of the sample filter between 0.1 and 0.4 (after corrections for instrument baseline and blank filter baseline, see below). For some spectral regions, especially the UV, multiple filtrations of the sample with different volumes may be needed to satisfy these criteria.

5.7.3 Spectrophotometer configuration for IS-Mode

A suitable dual-beam spectrophotometer equipped with an integrating sphere (e.g., 15 cm diameter sphere) is required to implement the IS method. The instrument performance with regards to wavelength accuracy and absorbance calibration should be verified as described in Section 5.4.3.

Some integrating sphere manufacturers provide a fixture for mounting of samples within the beam inside the sphere, which can be adapted to positioning of sample filters. Alternatively, a custom mounting system can be fabricated. The mounting system should center the sample filter perpendicular to the illumination beam, and be secured in a way that ensures reproducible positioning of a filter at the same location. Ideally, the mounting mechanism should be constructed such that only the filter itself interacts with the beam (i.e., no filter supporting structure within the

illuminated portion). All materials within the sphere should be made of Spectralon or coated with a similar highly reflective material.

Through the use of various apertures placed with the light path between the source and entrance port of the integrating sphere, the size and shape of the sample beam is adjusted to provide a beam illuminating the center of the filtered area. Beam size should be sufficiently large to cover a representative portion of the filter (e.g., 3 mm wide x 6 mm high). The size of the reference beam is adjusted accordingly to provide a similar amount of light energy associated with the sample and reference beams propagating in air.

5.7.4 Sample analysis for IS-Mode

The operating protocol of the spectrophotometer with regards to spectral bandwidth, spectral sampling, and scan rates are the same as described earlier in Section 5.4.3. The recommendations regarding maintaining sample filter hydration between measurements should also be followed. A typical sequence of making measurements is as follows:

1. After a suitable warm-up period, the spectrophotometer is initially autozeroed by scanning air-vs-air with the empty mounting mechanism placed within the sphere; for most instruments, this scan is automatically stored in memory and subtracted from subsequent scans. The scan is then repeated and the data saved to a data file to provide an actual measure of instrument baseline. This air-vs-air instrument baseline should be performed and saved at regular intervals to check for instrument drift throughout the course of sample measurements.
2. Hydrated blank filters (minimum of 3 to 5) drawn from the same batch as the sample filters are positioned on the mounting mechanism and placed within the center of the integrating sphere for measurement. These blank filters are scanned (relative to air in the reference beam) and these spectra of optical density (absorbance values) are saved to data files for determination of the average blank-filter baseline. These can be run initially before beginning analysis of samples, or spaced intermittently between sample filters. This protocol assumes a typical scenario when it is impractical to measure individual blank-filter baselines for each specific sample filter, such as when sample filters are collected and frozen on the ship for post-cruise analysis in the lab. However, in some lab experiments with limited number of samples it is possible to measure the filter baseline and then immediately collect sample on the same filter for subsequent measurement of the sample filter. In this case there is no need for the determination of average filter baseline for the purpose of its application to multiple sample filters.
3. After ensuring an appropriate level of hydration and no excess moisture of the sample filter (frozen filters have to be first remoistened by placing them on a drop of water, see Section 5.2), the sample filter is positioned on the mounting mechanism and placed within the center of the integrating sphere for measurement. It is useful to notch or mark an edge of the filter before initial measurement to ensure reproducible positioning of the filter for subsequent scans (e.g., for replicate scans and after pigment extraction).
4. The sample filter is scanned (relative to air in the reference beam) and the measured optical density values (absorbance values) are saved to a data file. Following the initial measurement, the filter is repositioned in a different orientation (e.g., 90° rotation) and measured a second time to check reproducibility and homogeneity of sample distribution on the filter. The replicate scans of sample filter provide the optical density data that are used to calculate the particulate absorption coefficient, $a_p(\lambda)$.
5. Following measurement, sample filters can be extracted in solvent to remove phytoplankton pigments (Section 5.2 and 5.3.5) and re-measured in the spectrophotometer to estimate the non-algal particulate absorption coefficient, $a_{NAP}(\lambda)$.

5.7.5 Data processing for IS-Mode

General processing of data and calculation of the absorption coefficient is similar to the guidelines described in Section 5.3. In contrast to T-mode, the so-called "null point" correction, in which subtraction of a spectrally constant value of particulate absorption from the NIR spectral region is used to account for scattering losses, is not applied. Experience suggests that scattering losses with the IS technique are small enough to be considered negligible, and the application of a null point correction can mask true particulate absorption in the NIR.

The general sequence of data processing is:

1. Instrument drift is checked from the air-vs-air measurements (i.e., instrument baselines) made at different times throughout the measurement period. If needed, all blank filter baselines and sample spectra are corrected for

any observed drift of the instrument using appropriate instrument baselines.

2. Measurements from all blank filters are averaged to create the final values of optical density for the filter baseline (relative to air). Importantly, the *OD* values of blank filters mounted inside the integrating sphere as measured relative to air in the reference beam are close to zero (typically within ±0.04 in the spectral region between 300 and 850 nm).
3. The spectrum of the final filter baseline is subtracted from each spectrum of sample filter optical density.
4. Replicate measurements of baseline-corrected sample filter optical density, $OD_f(\lambda)$, obtained on the same sample filter are averaged.
5. The blank-corrected and averaged $OD_f(\lambda)$ of the sample can be smoothed, for example with a moving average. The choice of smoothing window width and number of iterations is determined based on characteristics of the sample spectra (i.e., presence or absence of sharp peaks, behavior of instrument noise).
6. The particle absorption coefficient, $a_p(\lambda)$, or non-algal component, $a_{NAP}(\lambda)$, of each sample is calculated from $OD_f(\lambda)$ using the known filtration volume (V in m^3) and the measured interception area of filtration (A in m^2) as:

$$a_x(\lambda) = \ln(10)\ 0.323\ [OD_f(\lambda)]^{1.0867} / (V/A) \qquad (5.22)$$

which utilizes a beta-correction modeled as a power function for the relationship between OD_s and OD_f (Eqs. 5.3 and 5.7). The phytoplankton absorption coefficient, $a_{ph}(\lambda)$, is calculated from Eq. (5.14).

As mentioned previously, no null point scattering correction is applied to the calculated $a_p(\lambda)$. If a corresponding absorption spectrum of the particles after pigment extraction (a_{NAP}) is measured, this spectrum is adjusted with an offset in the near-infrared so that the average value of $a_{NAP}(\lambda)$ equals the average value of $a_p(\lambda)$ in the NIR spectral range (e.g., 800 - 820 nm). When using the IS technique this adjustment of $a_{NAP}(\lambda)$ is usually very small which supports the common assumption that phytoplankton pigments do not absorb in this spectral region.

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