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Determination of the particulate absorption of microalgae using a point-source integrating-cavity absorption meter: verification with a photometric technique, improvements for pigment bleaching, and correction for chlorophyll fluorescence

Rüdiger Röttgers, Clivia Häse, and Roland Doerffer*
Institute for Coastal Research, GKSS Research Centre Geesthacht, Max Planck Strasse 1, D-21502 Geesthacht, Germany

Abstract

The determination of the spectral light absorption coefficient of planktonic algae in seawater is crucial for many applications, including optical remote sensing. Common techniques are adversely affected by light-scattering effects, or by low particle concentrations in situ. Measurements with a point-source integrating-cavity absorption meter (PSICAM) can overcome both difficulties. In our study, we assessed the performance of a PSICAM for measuring the absorption coefficients of microalgae by a comparison with state-of-the-art techniques. The PSICAM was evaluated by comparative measurements of diluted algal cultures with a photometric setup, where the sample cuvette is placed in the center of an integrating sphere (CIS). The accuracy of both the CIS and the PSICAM technique was calibrated against a commercial spectrophotometer by measurements of dense algal cultures. The remaining sources of error for the PSICAM technique were the bleaching procedure and chlorophyll fluorescence. We propose an improved bleaching technique and introduce a correction procedure for the fluorescence effects. Applying both, the overall differences between PSICAM and CIS measurements were less than ± 1%. We further demonstrate that the accuracy of the PSICAM is at least as good as that of a commercial spectrophotometer, but its precision should be two to three orders of magnitude higher. Due to its high sensitivity and accuracy, and to the fact that sources of error can be identified and accounted for, the PSICAM has a great potential to significantly improve the determination of absorption spectra of particulate matter in natural seawater.

Light absorption in the aquatic environment can be separated in absorption by water itself, a_w, and its constituents. Conventionally these constituents are grouped into 3 fractions with different optical properties: (1) the absorption of all constituents, which pass a filter with a certain pore size (generally 0.2 μm), is called the gelbstoff absorption. This material, defined as "dissolved," consists mainly of organic matter such as humic acids and is therefore also called chromophoric dissolved organic materials (i.e., a_{CDOM}). The material that remains on the filter, defined as suspended particulate matter, can theoretically be subdivided in (2) phytoplanktonic algae, and (3) nonalgal particulate material (e.g., mineral compounds). Its absorption is called a_p, a_ϕ, and a_{NAP}, respectively. In clear waters of the open ocean a_w and a_ϕ are predominant, and a_p, a_{NAP}, and a_{CDOM} covary with phytoplankton biomass. This type of water is defined as Case 1 water (Morel and Prieur 1977). In optically more complex coastal waters (Case 2 waters), a_{CDOM} and a_{NAP} can vary independently from phytoplankton absorption due to sediment erosion and riverrine inflow of CDOM and inorganic suspended matter. The measurement of the different inherent optical properties of these substances is important for many scientific questions, e.g., for bio-optical modeling, for remote sensing applications, for the photophysiological characterization of phytoplankton, and for mapping the DOC (dissolved organic carbon) distribution and localizing origins in chemical oceanography with a_{CDOM}. However, the determination of absorption is always affected by light scattered by all kinds of particles ranging in size from molecules (Rayleigh's law) up to particles with dimensions of hundreds of micrometers. A problem with standard photometers occurs when

*Corresponding author: rroettgers@gkss.de

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the sample includes scattering constituents. In this case, the attenuation measurement comprises absorption and scattering that cannot be separated. To reduce scattering and to remove all particles, a_{CDOM} is measured after the sample is filtered, so disturbance by scattering is low. By contrast, the particulate absorption by algae is more difficult to determine, since the algal cells scatter light. One approach to determine phytoplankton absorption is to measure the attenuation of an algal suspension in a photometer cuvette in front of an integrating sphere that collects all forward scattered light (Nelson and Prézelin 1993; Kirk 1994). However, backward and sideward scattered light is lost and induces errors. To avoid these errors, the cuvette can be positioned in the center of an integrating sphere (e.g., Nelson and Prézelin 1993; Babin and Stramski 2002; Tassan and Ferrari 2003). However, this is rarely done as a special integrating sphere is needed. Both techniques are applicable for relatively dense algal cultures only. Consequently, the algae have to be concentrated for natural samples. This is typically done by retaining algae on filters and measuring the absorption in a similar photometer setup as used for the simple cuvette measurements, e.g., in front of an integrating sphere (Yentsch 1957, 1962; Trüper and Yentsch 1967; Tassan and Ferrari 1995). For this filter technique, diffuse scattering glass-fiber filters are used, amplifying the optical pathlength by multiple internal scattering. The amplification factor (β), which is the ratio of the optical to the geometric pathlength (Butler 1962), has to be determined empirically. This is mostly done by a comparison with the cuvette technique using algal cultures, which, in turn, includes the errors as described above (e.g., Maske and Haardt 1987; Mitchell and Kiefer 1988; Bricaud and Stramski 1990; Mitchell 1990; Cleveland and Weidemann 1993; Tassan and Ferrari 1998; Finkel and Irwin 2001). In another filter technique, the particulate material is concentrated on a membrane filter and transferred to a glass holder by freezing (Filter Freeze Transfer technique; Allali et al. 1995, Tassan and Allali 2002). An amplification correction is not needed because the material is embedded in frozen water and detached from the filter.

Another technique to determine the absorption of scattering media is the use of reflective cuvettes. The AC-9 of Wetlabs uses the total reflection property of a glass tube so that all light that is scattered in the forward direction is kept in the stream and finally detected. In a further reflective cuvette design (Doerffer 1979), a diffuse light input is used and both the backscattered and transmitted light is measured.

To separate the absorption by algal pigments from that by nonpigmented material, the pigments are either dissolved by organic solvents and washed out (e.g., Kishino et al. 1985) or bleached by an oxidizing reagent (Tassan and Ferrari 1995; Ferrari and Tassan 1999). Assuming this procedure does not affect the particles (e.g., the algal cells except their pigments), the remaining attenuation includes the same scattering error. This error can then be removed by subtracting the attenuation after bleaching from the total attenuation, or the attenuation of the bleached material is, however incorrectly, taken as absorption of the nonpigmented material, a_{NAP}. However, the need to concentrate the algae, the bleaching procedure, the amplification factor, and the different scattering corrections are sources for significant errors.

Most of these errors can be avoided by using an integrating sphere that can be filled with the specific water sample. A suitable size for this sphere can be selected to make it sensitive enough to be used with untreated natural water samples. A special design was proposed by Kirk (1997) as the Point Source Integrating Cavity Absorption Meter (PSICAM), basically because it has the advantage of being simpler to design than previously used ICAM types (e.g., Fry et al. 1992; Pope and Fry 1997). Since the ICAM principle is based on multiple reflection and scattering, and by this on the extension of the path length, it inherently provides the proposed reduction of scattering effects on absorption because of an already diffuse light field inside the cavity.

A PSICAM prototype was developed at the Institute for Coastal Research (GKSS Research Center). It was demonstrated that the absorption coefficient of colored solutions can be determined by this PSICAM as good as by photometry (Röttgers et al. 2005). The present paper reports on the first measurements of particulate absorption of different algal cultures conducted with the PSICAM. As the PSICAM has to be calibrated and the internal light field is not absolutely isotropic, as demanded by theory (Kirk 1997), a verification of the PSICAM measurements is necessary. To validate these PSICAM measurements, the results were compared to the theoretically best-performing technique, i.e., when using a photometric cuvette in the center of an integrating sphere. The technique to separate absorption by algal pigments from that by particles without pigments was extended by a modification of the widely used oxidative bleaching techniques. Finally, a simple technique was developed to correct the absorption determination for the influence of chlorophyll fluorescence.

Materials and procedures

Algal cultures —We compared ten different cultures from 9 different algal species. These were the diatoms (Bacillariophyceae) *Thalassiosira weissflogii*, *T. pseudonana* and *Skeletonema costatum*, the prymnesiophyte *Isochrysis galbana*, the red alga (Rhodophyceae) *Porphyridium cruentum*, the green algae (Chlorophyceae) *Chlorella sp.*, *Scenedesm*us sp. and *Nannochloropsis oculata*, and two differently colored subspecies of the cyanobacterium *Synechococcus* sp. The cultures were provided by the Alfred-Wegener-Institute for Polar and Marine Research, and by the Netherlands Institute for Ecology. The nonaxenic cultures were cultivated at room temperature in a 12h:12h light:dark rhythm at about 50 µmol photons m^{-2} s^{-1} to concentrations of > 100 µg Chl *a* L^{-1}.

Integrating sphere with a central cuvette (CIS)—Our custom-made CIS system consisted of a $BaSO_4$-coated integrating sphere (diameter: 300 mm, Labsphere), a 2-cm cuvette, a halo-

gen light source, and a spectroradiometer. The light source was a 150 W electronically stabilized halogen lamp (LQ 1700, Fiberoptic Heim). A parallel light beam with a diameter of ca. 0.7 cm was realized by a 2-mm aperture in front of the lamp opening, which was combined with a convex glass lens (f = 80 mm). After entering the integrating sphere, the light beam passes the 20 mm pathlength cylindrical quartz-glass cuvette (120 QS, Hellma) filled with the sample. The cuvette had an inner diameter of 18 mm and was fixed in the center of the sphere by a custom-made holder. The light in the sphere was collected by a small quartz-glass fiber-optic with a narrow field of view. The end of the fiber-optic pointed to the wall of the sphere, away from the cuvette, as well as away from the entrance port and from the place of the first beam reflection opposite the entrance port. The light was detected with a photodiode array spectroradiometer (Ramses ACC, TRIOS), which was connected to the other end of the fiber-optic. The lamp/detector combination allowed measurements of the spectral intensity at wavelengths between 380 and 725 nm in 2.5 nm intervals (spectral resolution 3.3 nm). A detector with a higher resolution (< 1 nm) was tested, but differences in peak position, peak form, and shape of the spectral absorption between low and high spectral resolution were negligible. The cuvette holder inside the sphere was covered with white tape and all openings, except the entrance port (diameter: 8 mm) and the port for the fiber-optic (diameter 4 mm), were closed with white filter paper (Whatman GF/F) to maximize the internal reflectivity. A cuvette filled with purified water was used as reference. The absorption of 0.22 µm filtrate of the culture medium was determined and subtracted from the total absorption of the algal culture. The same measurements were repeated using a bleached sample of the same algal culture. Some comparisons were conducted with a Cary 100 spectroradiometer (Varian) equipped with a 150-mm Spectralon-coated integrating sphere (Labsphere). The design of this sphere allows the positioning of a 10-mm cuvette in its center using a special cuvette holder (Labsphere).

PSICAM—The absorption of suspended particulates was determined with the PSICAM as described in Röttgers et al. (2005). The PSICAM consists of a cavity (diameter: 9.5 cm) made out of a highly reflective material (OP. DI. MA, Gigahertz Optik). The light was guided from the lamp to the spherical emitter, which is located in the center of the PSICAM by a fiber-optic. It was connected to the same halogen light source as used for the CIS. Also the same spectroradiometer was used for spectral detection of light. The collector of the detector measures the relative radiance intensity I and points to a position of the wall opposite to the entrance of the collector, where it avoids direct illumination from the central emitter. The "transmission" (T) of each algal culture was determined using purified water as the reference. It is calculated as the light intensity (I_s) when the cavity is filled with the sample divided by the light intensity when the cavity is filled with the reference (I_0), i.e., $T = I_s/I_0$. The transmission of the culture medium (< 0.22 µm filtrate) was determined in the same way. After the absorption coefficients were calculated from each T, the absorption of the medium was subtracted from the absorption of the algal culture. The same measurements were done with the bleached sample of the same culture and the respective < 0.22 µm-filtrate of the bleached sample. The pigmented part of the particulate absorption, a_{pabs}, was calculated as total particulate absorption, a_p, minus the particulate absorption of the bleached material, a_{bpa}. (We use a_{pabs} and a_{bpa} instead of a_ϕ and a_{NAP} to avoid confusion, since a_{bpa} is the absorption of bleached algal cells, not that of any nonalgal material, and a_{pabs} is the absorption of the bleachable pigments and other bleachable organic molecules). The critical factor for the PSICAM measurements is the determination of inner reflectivity (i.e., the reflectivity of the wall material and all other parts in the cuvette), which requires a high degree of accuracy. The reflectivity was determined before each set of measurements using solutions of nigrosine with known absorption coefficients. The reflectivity did not differ significantly (< 0.1%) among 6 determinations performed on 3 different days. The details of the PSICAM measurement are described in Leathers et al. 2000 and Röttgers et al. 2005.

It was necessary to develop two new procedures: (1) for the correction of the chlorophyll fluorescence and (2) for the correction of the absorption by the bleaching agent.

Chlorophyll fluorescence correction —To correct the CIS and PSICAM absorption spectra for the influence of light emitted by chlorophyll fluorescence, light of wavelengths > 620 nm was excluded from illuminating the sample by using a short-pass filter (600FL07, L.O.T-Oriel). The resulting intensity spectra ($I_{fl0,}$ for the reference and, $I_{flem,}$ for the sample; "water + filter" and "sample + filter" in Fig. 1), between 650 and 725 nm, were used to determine the total light emitted by fluorescence at these wavelengths, I_{fl}. I_{fl} was calculated by using the transmission, T, to correct for fluorescence light that is absorbed again by the sample, and subsequently subtracting I_{fl0} as

$$I_{fl} = I_{flem}/T - I_{fl0}. \quad (1)$$

T was determined with the standard measurement setup, i.e., without the short-pass filter. Because a part of I_{fl} is absorbed by the sample during standard measurement, the additional light collected by the detector due to fluorescence, I_{fl}' is approximately calculated by multiplying I_{fl} by T. Furthermore, the short-pass filter absorbs about 40% of the light (see Fig. 1). Thus, the fluorescence during the standard measurement, i.e., without the filter, would at least be ~1.7 times higher. Influences by the change in spectral light distribution and total intensity on the fluorescence are discussed later but implied a factor of more than 1.7. A correction factor of 2 was empirically obtained from a comparison with a spectrophotometrically determined absorption that was measured in a cuvette in front of an integrating sphere, when chlorophyll fluorescence effect will be negligible (see below). The whole calculation can be reduced to

$$I_{fl}' = (I_{flem} - I_{fl0} \times T) \times 2. \quad (2)$$

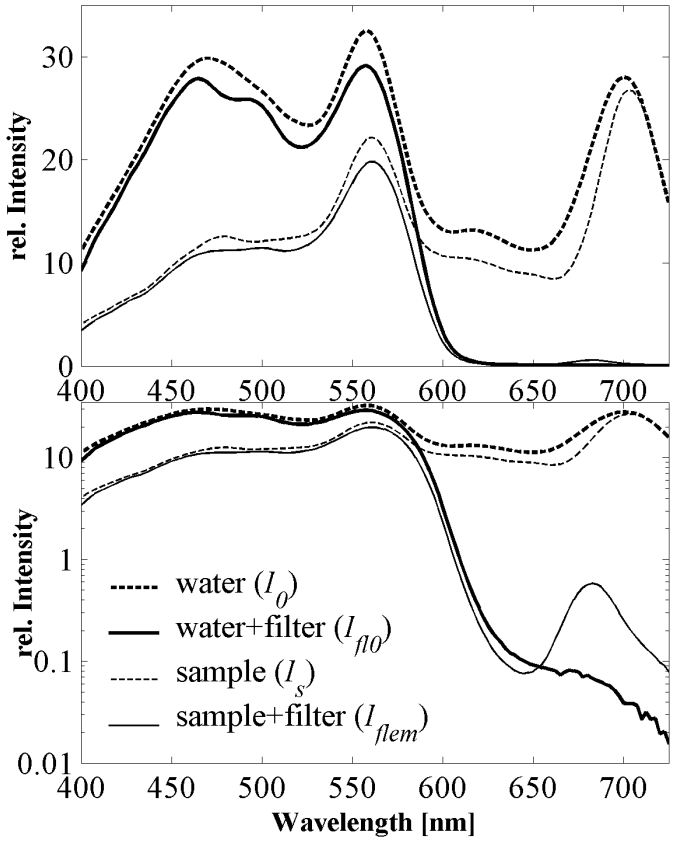

Fig. 1. A typical set of spectral intensities (in arbitrary units) for a PSICAM measurement used for determining the absorption and correcting the error related to the induced Chl *a* fluorescence. Shown are the intensities when the PSICAM cavity is filled with pure water and with the sample, without and with a < 620 nm short-pass filter, respectively. The absolute scale is shown in A to illustrate the observed transmission when the cavity is filled with the sample, and the logarithmic scale is shown in B to elucidate the fluorescence peak around 685 nm.

This light intensity (I_{fl}') was subtracted from the intensity, I_s, measured with the regular setup at wavelengths of 650 to 725 nm. The resulting corrected light intensity spectrum for the sample measurement, $I_{corr} = I_s - I_{fl}'$, was used to calculate the correct transmission ($T_{corr} = I_{corr}/I_0$) and then the fluorescence-corrected total particulate absorption, a_p (for calculation, see Röttgers et al. 2005).

Pigment bleaching—Algal pigments were bleached by the chemical oxidative bleaching procedure according to Tassan and Ferrari (1995) and Ferrari and Tassan (1999). For algal suspensions, the method was refined to remove the absorption of the NaOCl solution at wavelengths below 500 nm (see Fig. 2). Therefore, the bleaching by NaOCl was stopped after a certain time by adding small amounts of 30% H_2O_2 solution to the sample. This H_2O_2 chemically reduces the remaining NaOCl. The volume of the added H_2O_2 solution had to be reconciled with the amount of NaOCl. We used 500 μL of a concentrated NaOCl solution (6% to 12% active chlorine) added to 500 mL

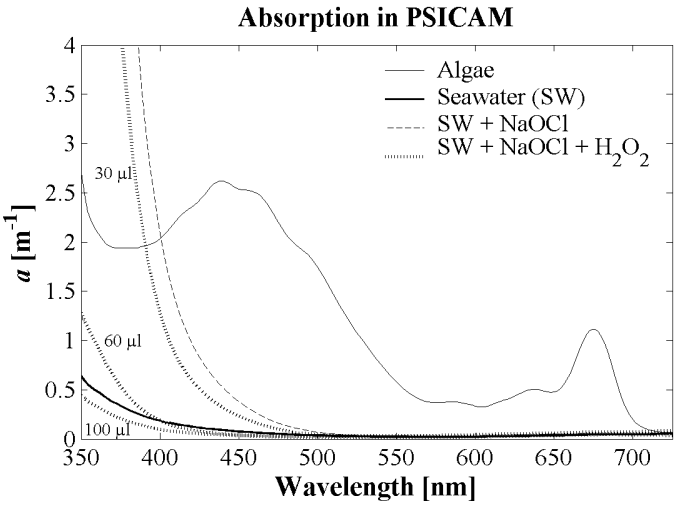

Fig. 2. Spectral absorption of filtrated seawater (SW) during the bleaching procedure when the bleaching agent (NaOCl) and different amounts of H_2O_2 were added. A typical spectrum of the algae *Isochrysis galbana* is shown for comparison, all measurements are against pure water.

of the algal suspension. Therefore, small amounts of H_2O_2 were gradually added to the bleached solution until no further decrease in absorption was observed (final amount ca. 100 μL). A surplus of H_2O_2 leads to a production of oxygen bubbles, which could affect the absorption measurement in the photometric setup (CIS) due to scattering.

Assessment

Experimental setup—Beside the PSICAM, two different devices were used for comparing the absorption measurements of suspensions of different microalgal cultures: (1) a 2-cm cuvette placed in the center of the integrating sphere of the CIS system and (2) a 1-cm cuvette placed in a commercially available photometer system equipped with an integrating sphere. The latter was used to evaluate the more sensitive and easier to manipulate CIS system.

Before each measurement, the specific algal cultures were carefully shaken. After ca. 30 min, they were decanted to remove fast sedimenting cells in order to exclude errors caused by sedimentation during the measurement procedure. For the much more sensitive PSICAM, each original test culture of each different phytoplankton species had to be diluted by 1:10 or 1:20 with the specific culture medium. The total absorption of each culture in suspension was always measured against purified water as reference. Afterwards, the sample was filtered through 0.22 μm membrane filters (GSWP, Millipore), and the absorption of the filtrate was determined in the same way. Total particulate absorption, a_p, was calculated by subtracting the absorption of the filtrate from the total absorption.

To determine the particulate absorption of the algal pigments only, a_{pabs}, the pigments were bleached using the newly developed chemical oxidation method (see below). The particulate absorption of the nonpigmented particles was deter-

mined as before and the absorption of the 0.22 µm filtrate of the bleached culture was subtracted. Each measurement was done in replicates ($n = 3$ to $n = 6$). The absorption coefficient, $a(\lambda)$, was determined from transmission T on the base of the natural logarithm.

Assessment of the CIS system—The photometric technique, which uses a cuvette in the center of an integrating sphere, was expected to provide the most reliable particulate absorption measurements for the comparison with the PSICAM. By placing the cuvette in the center of the integrating sphere, the losses due to scattering are almost eliminated, as all the scattered light remains in the sphere and is collected by the detector. However, different minor optical pathways for the measuring light beam are possible in this setup, leading to small errors in the determination of absorption. Three sources for errors had to be analyzed and procedures for the correction had to be developed:

(1) It should be noted that light reflection on the inner sphere walls of the integrating sphere results in a significant amplification of the optical pathlength in the cuvette because part of the reflected light passes through the cuvette again, leading to additional absorption. This amplification had to be determined manually for the CIS (see below) in contrast to the situation when a dual-beam spectrophotometer equipped with the same kind of integrating sphere is used. Here this amplification is automatically corrected by using the reference beam signal. The reference beam does not pass through the sample cuvette directly, but it performs exactly the same way as the sample beam after the first diffuse reflection on the sphere wall.

(2) A second source of error is the reflection on the front and back glass-window of the cuvette. In a common setup (cuvette windows perpendicular to the light beam), the part of the light beam that is reflected backwards (~5% for quartz glass) at the first two main reflection layers (1: air/glass, 2: glass/water) will leave the sphere through the entrance port. Thereby, it will not participate in the pathlength amplification. By contrast, light reflected at the back window of the cuvette and then scattered on particles in the sample will remain in the sphere and participate in the pathlength amplification, and this is not corrected by the reference beam in a spectrophotometer. Additionally, if a scattering but nonabsorbing suspension was measured against a nonscattering, nonabsorbing reference, the resulting attenuation would be negative. This is because more light is detected when a scattering suspension is filling the cuvette (Babin and Stramski 2004). To avoid this artifact, the cuvette was tilted from its position perpendicular to the light beam with a small angle of ca. 9° (see Babin and Stramski 2004). Consequently, the part of the beam reflected at the front and the back window always remains inside the sphere. The longer geometric pathlength of the cuvette (< 1%) caused by the small angle does seemingly increase the amplification, however this increase can be determined. The light reflected at the back window participates to the amplification, but the light reflected at the front window does not interact with the sample, thereby leading to an underestimation of light absorption by the sample. This effect has to be known in addition to the amplification.

The error resulting from this reflected light depends on the absolute absorption of the sample. However, within the range of transmission measured with our optically thin cultures (0.7–1), the total error will theoretically range from 5% at low absorption (high transmission) values to up to 6% at high absorption (low transmission) values (considering a reflection of 5% for quartz glass).

(3) A third source of error is related to the volume-scattering function of the particles, important in samples with high-scattering coefficients. All light, which is scattered early into backward direction, will reduce the optical pathlength, as this light can leave the cuvette before it has passed through the full geometric pathlength. Alternatively, depending on the position of the scattering event, it may increase the pathlength if it passes through the cuvette backwards again. An increase in pathlength may also be caused by multiple forward scattering at small angles. This third kind of error was not corrected because we assumed that it should be negligible at the low cell concentrations used. However, each species has a different volume-scattering function, which could be used for correction in an iterative way.

Preceding the measurements with algal cultures, two CIS specific measurements were performed: (1) the light reflected at the front window was determined by filling the cuvette with a concentrated solution of the colored stain nigrosine, which absorbs all light entering the cuvette; (2) the pathlength amplification in the CIS system was determined over a broad range of absorption by comparing different dilutions of nigrosine in purified water. The solutions were measured in a spectrophotometer (Lambda 18, Perkin Elmer) using a regular setup (i.e., without an integrating sphere) and in the CIS against purified water, respectively (considering the amount of reflected light on the front cuvette window for the CIS). The particle-free solutions should have negligible scattering by particles and the remaining Rayleigh-scattering by the water molecules should be the same for the sample and the reference. The observed amplification was constant over the measured range of absorption but showed a small wavelength-dependence; values were between 1.19 and 1.21 for the 20 mm quartz-glass cuvette used.

The performance of our custom-made CIS in determining the particulate absorption was compared to that of a commercially available system (see Materials and procedures). The cuvette in this instrument was again positioned with a small angle (ca. 9°) off the axis of the light beam. The change in absorption by pathlength amplification and by light beam reflection at the cuvette windows was determined to be only –2% for the range of the measured optical density. The theoretical 5% reduction in absorption induced by light reflected at the front window of the cuvette might partly be compensated by higher absorption due to the longer geometric path

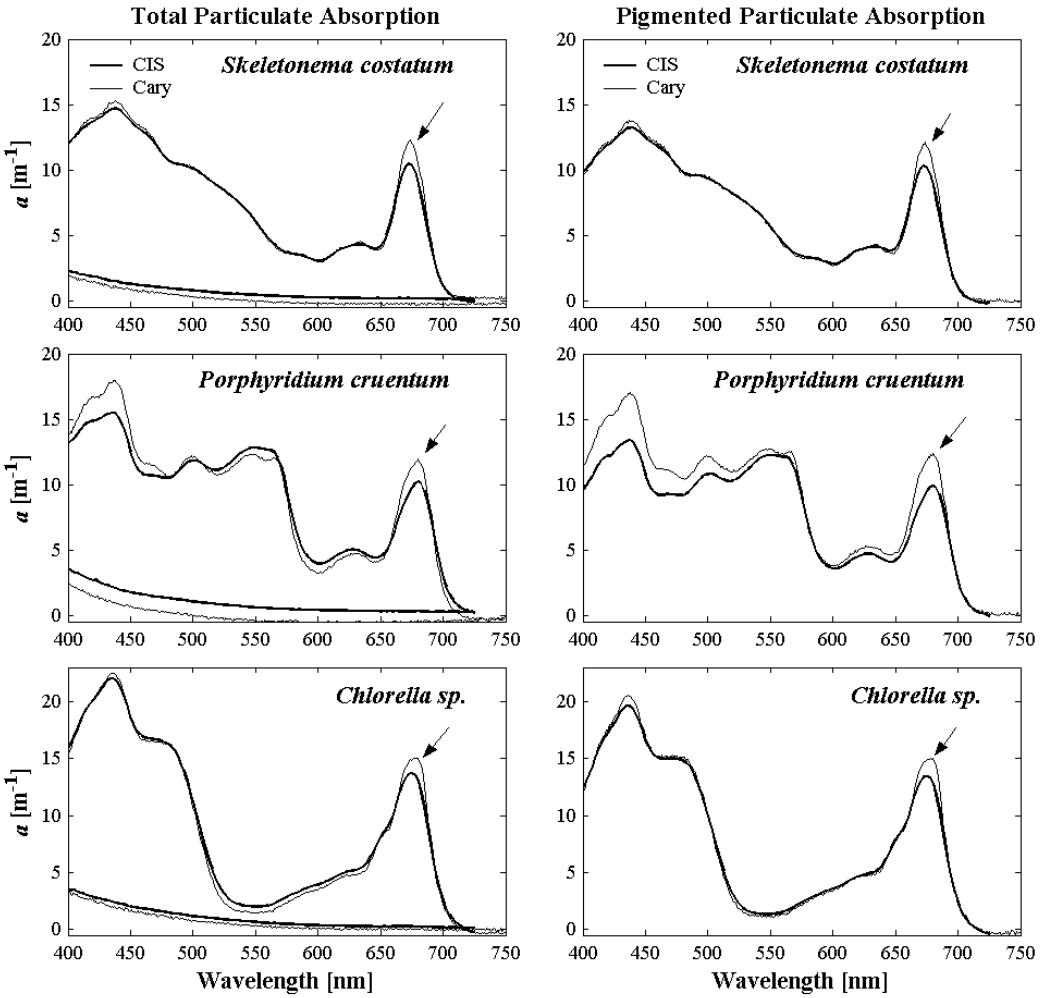

Fig. 3. Total, a_p (left panels), and pigmented, a_{pabs} (right panels), particulate absorption spectra of three different algal species. In addition, the absorption of the bleached particles, a_{bpa}, is shown together with a_p (lower spectra in left panels). The spectra were determined with the CIS system (thick lines) and a CARY 100 photometer (thin lines). An error induced by the chlorophyll fluorescence in the CIS system is visible (see arrows). Note that the a_{pabs} spectrum for *S. costatum* was null-point corrected because of the differences between a_p and a_{bpa} at 750 nm.

length within the cuvette (~ 0.6%), and by the additional absorption of that part of the light beam that is reflected at the backside window of the cuvette, thereby passing the cuvette a second time. The measured absorption was corrected for this 2% deviation. Due to the different type of absorption measurement in a photometer (wavelength scanning at low light intensity), the reflected light on the front window of the cuvette cannot be determined for an integrating sphere in a spectrophotometer.

Three different mono-specific cultures (*Chlorella* sp.*, Skeletonema costatum, Porphyridium cruentum*) were used for this comparison. Total and pigmented particulate absorption, a_p and a_{pabs}, were determined for these cultures. The left panels in Fig. 3 show a_p for the three different cultures. The overall difference between the two systems is low, except for *P. cruentum*, probably due to strong adverse scattering that leads to different scattering effects in the two measuring systems. *P. cruentum* forms large, aggregated colonies where the single cells are embedded in a protein-polysaccharide matrix, which could have a very peculiar light-scattering behavior. It basically shows that the two systems (photometer and CIS) are differently affected by scattering in the examined sample.

The difference observed in the wavelength range between 650 and 700 nm (see arrows in Fig. 3) is significant and due to the effect of Chl *a* fluorescence, which was not corrected in this comparison (see below). Marginal wavelength-dependent differences, e.g., at 440 nm or at 650 nm for *S. costatum*, are attributed to physiological differences induced by the different light conditions during the measurements, i.e., monochromatic scanning with low intensity (photometer) versus complete spectral illumination of high intensity (CIS). The same small deviations were found for a_{pabs} (Fig. 3, right panels). Small differences were observed for *S. costatum*, showing a constant offset over all wavelengths. A null-point correction

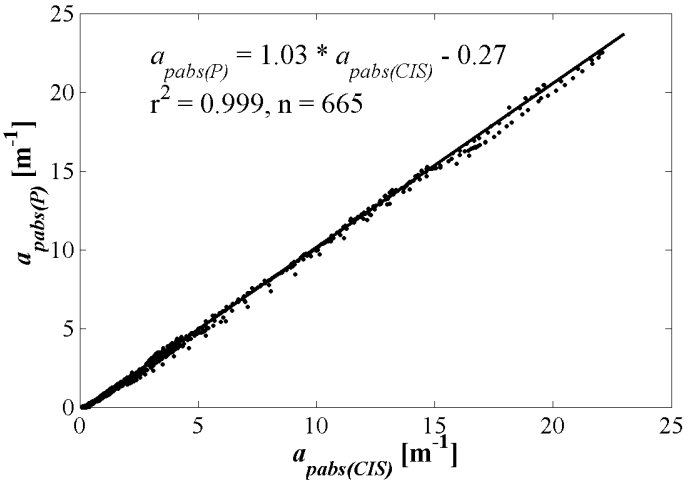

Fig. 4. Pigmented particulate absorption, a_{pabs}, determined with the CIS system plotted against a_{pabs} determined with the CARY 100 photometer. Only data of *S. costatum* and *Chlorella sp.* were used (see Fig. 3). Shown are the results of a linear regression analysis.

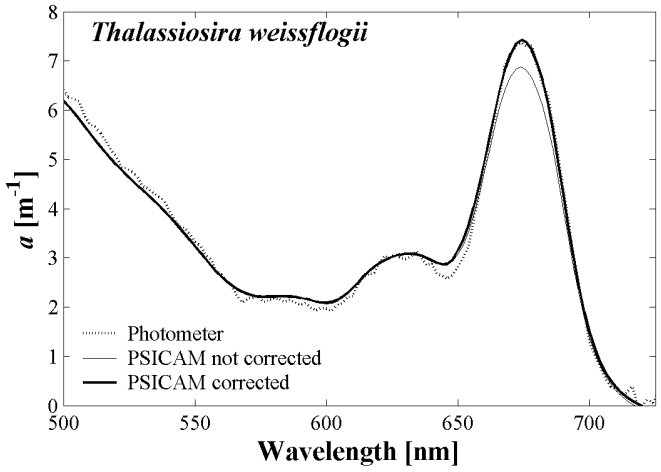

Fig. 5. Absorption spectra of a culture of *Thalassiosira weissflogii* showing the chlorophyll fluorescence effect on absorption observed with the PSICAM system and the results of the simple fluorescence correction. Shown is the absorption determined spectrophotometrically in a 1-cm cuvette placed in front of an integrating sphere (dotted line) and two spectra of the same culture determined with the PSICAM, one without (thin line) and one with the fluorescence correction (thick line). The spectrophotometrically obtained spectrum was corrected with a simple null-point correction using the absorption at 750 nm.

for the photometric measurement using the value at 750 nm would yield a better correlation.

Due to the Chl *a* fluorescence, values measured above 650 nm are not reliable for a direct correlation. For the photometer measurement, it was observed that the absorption at 750 nm is slightly negative or positive, where absorption by algal pigment should be negligible. These artifacts can be explained by different scattering properties of the algae (i.e., by different volume-scattering functions, see above for more discussion). Small deviations of a_{pabs} from the zero line can be explained by changes in scattering or background absorption induced by the bleaching procedure. Applying a null-point correction to the Cary 100-photometer data for *Chlorella sp.* and *S. costatum* reduces the mean difference between the two systems (photometer versus CIS) to less than 3%. The correlation coefficient between absorption determined with the photometer and that determined with the CIS system (Fig. 4) shows a slope slightly higher than 1 (slope: 1.03 ± 0.001, offset = –0.27 ± 0.01, n = 665, r^2 = 0.999). Considering all possible errors, the CIS system is as reliable as the commercial photometer, but due to the longer pathlength of the 20 mm cuvette, it is slightly more sensitive, and it provides the possibility to correct for most setup-related errors described above.

Correction procedure for Chl a fluorescence effects—In vivo chlorophyll fluorescence is variable as it depends on the physiological status of the algae, which to one extent is determined by the actual and the previous incident irradiance (nonphotochemical and photochemical quenching). In addition, it has to be noted that the chlorophyll fluorescence is emitted not relatively to light radiated to the sample but relatively to light absorbed by photosynthetic pigments only (i.e., not to all light absorbed by the particles), and relatively to the rate number of photons, (i.e., not relative to the light intensity). To keep the PSICAM measurements simple, we chose not to determine all these effects accurately by additional measurements or treatments. Additional information could be obtained by measuring the fluorescence excitation spectra to determine the relative contribution of the photosynthetic pigment, or by adding herbicide to decrease problems with the variable chlorophyll fluorescence. A theoretical analysis of the influence of chlorophyll fluorescence in the PSICAM, the CIS and the standard spectrophotometer shows that the effect of the induced chlorophyll fluorescence is dependent on the spectral characteristics of the illuminating light, and on the geometrical configuration of the experimental setup. When the sample is placed in front of an integrating sphere, only an insignificant part of the omni-directional fluorescence light will enter the sphere. By contrast, when the sample is placed inside the integrating sphere, all the fluorescence is collected by the detector. Absorption measurements in standard photometers are done by emitting light at different wavelengths monochromatically while the detector measures the light at all wavelengths. The consequence is that the detector does not capture only the light at the wavelength the monochromator is presently adjusted to but also the fluorescence light that is excited by this wavelength and emitted at greater wavelengths. In principle, the resulting transmission is then apparently too high, i.e., the absorption becomes too low. However, the effect of the induced fluorescence on determination of absorption of living algae in scanning spectrophotometer is supposed to be low (< 3%) as the light intensity used is low, and, hence, the fluorescence efficiency will be low (< 3%) (e.g., see Falkowski and

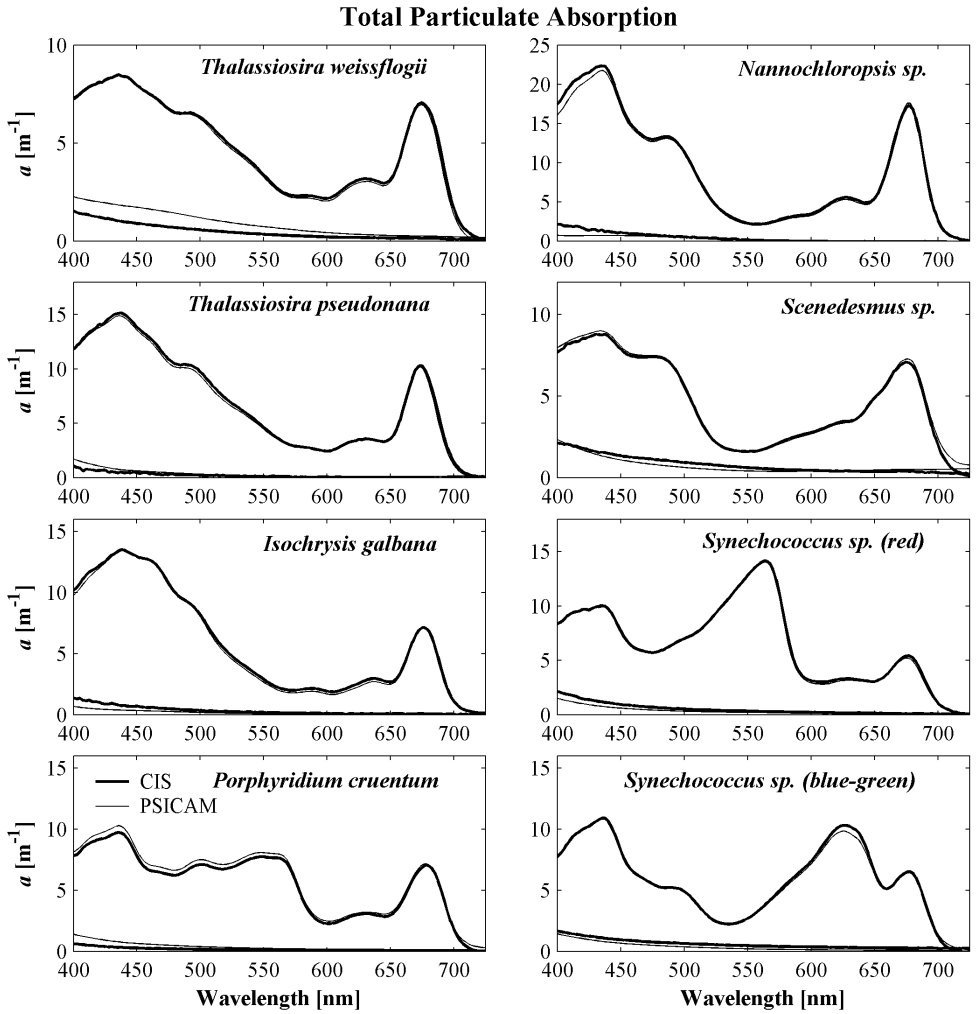

Fig. 6. Total particulate absorption, a_p, and particulate absorption of the bleached material, a_{bpd}, (lower spectra) for 8 algal species measured with the PSICAM (thin lines) and the CIS system (thick lines).

Raven 1997). It is important to note that the relative shape of the absorption spectrum is not significantly changed and that we can use this spectrum to evaluate the influence of fluorescence under other optical conditions. In the case when white light of higher intensity is used (e.g., PSICAM, CIS), the effect of the induced fluorescence becomes significant, due to the increased fluorescence efficiency (maximum variable Chl *a* fluorescence, 5% to 7%). As the light in the PSICAM and CIS setup was detected by a photodiode-array spectroradiometer, the effect of the induced fluorescence was restricted to the wavelengths of fluorescence emittance (typically 665 to 700 nm, max: ~685 nm, see Fig. 1). Using the PSICAM, the error in absorption induced by fluorescence was in the range of –5% to –15% (for one example see Fig. 5). This error was corrected by measuring the fluorescence emission spectrum of the respective algal culture in the specific setup (PSICAM, CIS) and using Eq. 2. A simple empirically determined factor of 2 was used to correct for differences in the absolute fluorescence emission between the actual light conditions during the PSICAM absorption measurement and that during the determination of the emission spectrum (Fig. 2).

Refined bleaching procedure—A prerequisite for the use of the oxidative bleaching technique in algal suspension was the compensation or removal of the substantial absorption by the NaOCl solution used (see Fig. 2). At the concentration we use for bleaching, the absorption by NaOCl leads to a significant error if not properly corrected, changing over time with the progressing oxidation process. Furthermore the absorption of the filtered water from a bleached sample, when used as a reference, leads to errors in the absorption determination. We observed that the absorption of this filtrate decreases during filtration, probably because the particles already retained on the filter get in contact with new bleaching reagents in the sample when passing through the filter cake. Compensation by subtracting the absorption of the bleached and then filtrated sample did not, however, provide reproducible results. The preferred procedure was to remove the remaining NaOCl by chemical reduction using H_2O_2. If sufficient H_2O_2 is added

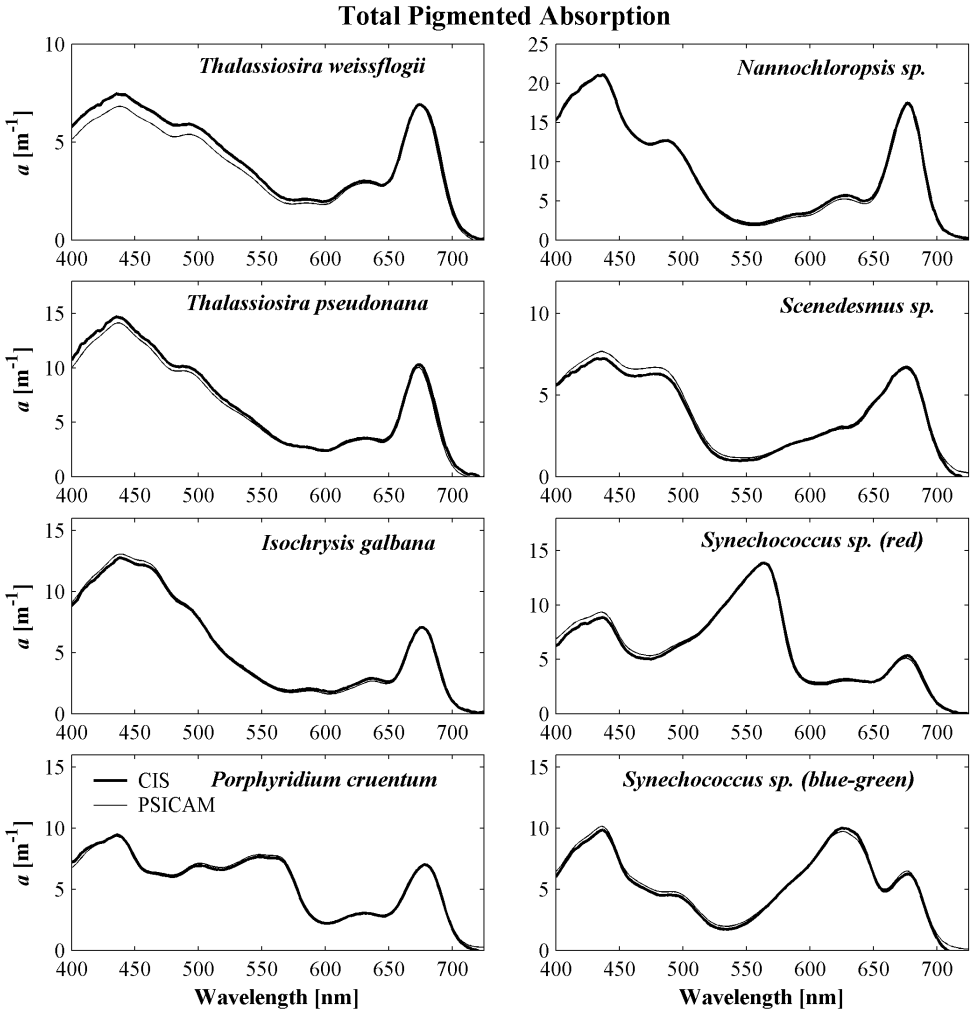

Fig. 7. Pigmented particulate absorption (total minus bleached), a_{pabs}, for 8 algal species measured with the PSICAM (thin lines) and the CIS system (thick lines).

to the bleached sample, the absorption by NaOCl disappears in a minute (Fig. 2). The remaining absorption is due to substances that are not bleached by either NaOCl or H_2O_2. This absorption is rather stable and not influenced by the filtration procedure. The remaining absorption in Fig. 2 is probably due to "Gelbstoff" absorption. The "Gelbstoff" absorption itself is affected by the bleaching procedure, whereas the absorption at 750 nm is not (Fig. 2). However bleaching by H_2O_2 of the material and the Gelbstoff fraction continues, and the time between measurements of the suspension and the filtrated reference should be as short as possible. The bleaching process was fast for the diatom species (< 10 min) and took up to 1 h for the more resistant species like chlorophytes. We assume that additional time was needed to oxidize the cell walls of the more resistant species before NaOCl could penetrate into the cells and could bleach the pigments. Further artifacts caused by the bleaching procedure can be a diminished attenuation at 750 nm, resulting from oxidation of low absorbing cell material, or from cell destruction, which would change the scattering properties of the sample. Nevertheless, this bleaching procedure allows the determination of absorption of bleached material in suspension.

PSICAM versus CIS—Total and pigmented particulate absorption, a_p and a_{pabs}, were determined with the PSICAM and the CIS system for eight cultures of different algal species (Fig. 6). Differences in a_p for all cultures were small, and the remaining deviations were not systematical (Fig. 6). Differences in absorption of the bleached algal material, a_{bpa}, between PSICAM and CIS were larger but not systematical either (Fig. 6). However, the differences in a_{pabs} between PSICAM and CIS (Fig. 7) were due to the deviations in a_{bpa}. The different sensitivity of the PSICAM and CIS required dilution of the cultures before measuring them in the PSICAM. Hence, bleaching was done afterward separately with different cell concentrations (undiluted and diluted) but with the same concentration of the bleaching reagent. This could have

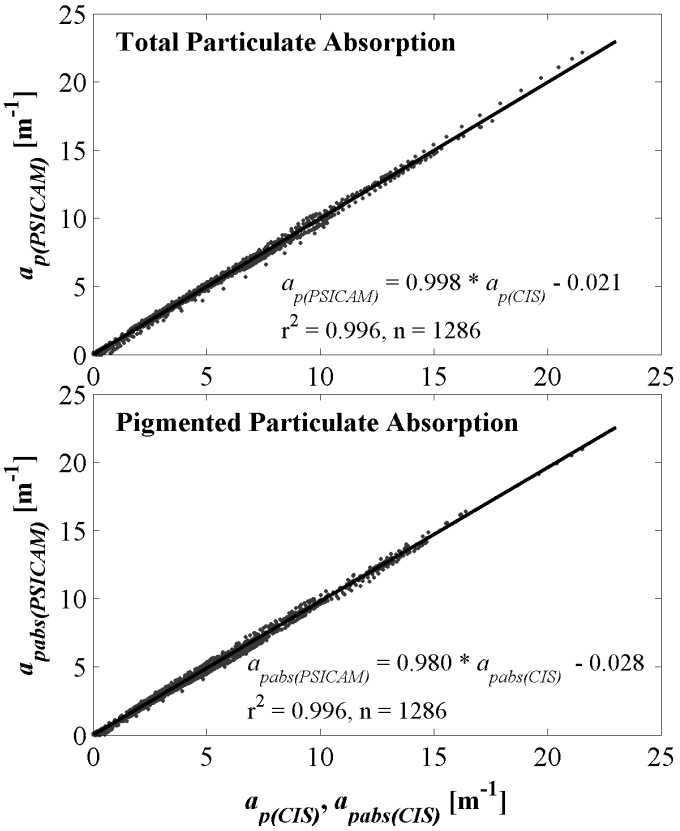

Fig. 8. Particulate absorption determined with the PSICAM plotted against absorption determined with the CIS system. Shown are the results for the total particulate absorption, a_p (upper panel), and for the pigmented particulate absorption, a_{pabs} (lower panel). Respectively shown are the fits (solid lines) and results of a linear regression analysis.

induced the differences between the two bleached absorption spectra. However, the observed absorption of the bleached material was low and negligible at longer wavelength (> 650 nm). For *Scenedesmus* sp. only, this absorption was above zero over all wavelengths for both techniques. Hence we attribute this behavior to the real absorption by the bleached material of this species. Thus, both techniques (PSICAM and CIS) did not show a significant adverse effect by scattering, as expected. No offset correction had to be applied to the data.

The correction for the Chl *a* fluorescence was applied for both measurements separately. Differences due to this correction could not be observed, despite the fact that the actual influence by fluorescence on the absorption determination was different. The fluorescence correction procedure was sufficient to receive correct absorption values.

The linear regression for all a_p data (Fig. 8) of both techniques yielded a slope of 0.998 ± 0.002 (n = 1286, r^2 = 0.996) with an offset of only –0.021 ± 0.012 m^{-1} (maximum values were as high as 20 m^{-1}). The slope is not significantly different from the 1:1 line, and the offsets not significantly different from zero.

If adverse effects by scattering would occur in both techniques, these could be compensated by subtraction of a_{bpa} from a_p, considering that the bleaching procedure does not change the scattering properties of the examined algal cells. However, the comparison between PSICAM and CIS for this a_{pabs} did show small and nonsystematic differences (Fig. 7). The linear correlation was not better (r^2 = 0.996, n = 1286) and yielded a slope of 0.980 ± 0.002 and an offset of –0.028 ± 0.012 m^{-1} (Fig. 8). The small deviation from the 1:1 line is most probably due to errors in the determination of a_{bpa}.

Discussion

The absorption determination with the PSICAM is very dependent on the calibration of the system by measuring the internal reflectivity, and a direct calculation of absorption from the measured "transmission" is not possible without a precise knowledge of this reflectivity. This makes it necessary to compare the absorption measurement of the PSICAM with state-of-the-art method using a spectrophotometric method with a cuvette of a known optical pathlength (Röttgers et al. 2005). It was necessary to conduct a comparison with particulate suspensions with a more complicated spectrophotometric method (CIS), which was evaluated with a commercially available spectrophotometer system. Nevertheless, the CIS system should provide more accurate results than the commercial photometer system because the greater diameter of the CIS integrating sphere provides a better scatter correction, the setup allows that most of the instrument-specific errors can be determined, and a cuvette with a longer optical path length can be used.

Considering the data for a_p only, no significant difference between PSICAM and CIS determinations were observed. The comparison for a_{pabs} yielded a mean difference of ca. 2% due to small errors in absorption associated with the bleaching procedure and the determination of a_{bpa}. This all indicates that adverse effects by scattering are excluded with both techniques (PSICAM and CIS) and that the rarely observed positive absorption at longer wavelengths (> 700 nm) is due to real absorption by some algal cells.

The PSICAM had been tested before, using colored solutions (Röttgers et al. 2005). In that study, it was shown that the overall difference between PSICAM and a photometric determination was less than 3%. Higher differences were observed only at very low absorption values. This can be attributed to the lower sensitivity of the photometer technique (even when using 10 cm cuvette), which induces greater relative errors.

To date, we have not determined the precision and accuracy of this prototype PSICAM. However, the PSICAM is at least as accurate as other techniques in determining absorption and its precision should be better. The PSICAM is not affected by scattering, and it has a sensitivity that is sufficient for most natural aquatic samples. The relative error will increase for natural samples with very low absorption coefficients, since the observed transmissions are in the range of 97% to 99% (pers. observations) only, whereas the error for

the prototype PSICAM is in the range of ±0.1% in transmission (Röttgers et al. 2005). For comparison, the observed transmission during the experiments described above was between 20% and 50% (see Fig. 1 for an example). However, at very low absorption coefficients, increasing the cavity diameter could increase the sensitivity of the PSICAM to some extent (Kirk 1997). Further technical developments of the PSICAM will reduce the overall error of the transmission determination.

Due to its much higher absolute sensitivity, the PSICAM will perform better than the photometric technique and will be superior to most other techniques for determining the absorption of particulate matter (but also for the CDOM absorption) of natural samples. The samples can be measured directly and quickly, without further treatments. Simple filtering techniques are used for the determination of the CDOM absorption, which is needed as a reference to calculate the absorption of the particulate matter from the total absorption of the sample, where purified water always serves as the reference. Possible errors by scattering at small particles (< 0.22 μm) are insignificant. The likewise simple, slightly improved, oxidative bleaching technique is used to determine the particulate absorption of the nonpigmented and pigmented material separately, but is still a source for a small error in the determination of the bleached and pigmented particulate absorption.

PSICAM measurements will considerably improve the determination of absorption by CDOM and by algal particles in natural waters. It is a rather simple instrument, consisting of an integrating sphere, a constant light source, and a light detector. It is proposed that, in the future, the PSICAM should become a widely used instrument in all scientific labs dealing with optical in-water measurements. The technique could also easily be applied in future in flow-through system for a continuous determination of absorption spectra along transects, and for in situ profiling systems. Furthermore, the possibility of measuring the particulate absorption of untreated water samples with a high accuracy means that already existing spectral analysis techniques can be used to analyze spectra of natural samples relatively fast (e.g., Bidigare et al. 1989). These techniques were previously limited to spectra from dense algal cultures or from concentrated algal material for which absorption was high enough to provide a good signal to noise ratio. These techniques can be used, for example, to discriminate taxonomic algal groups or algal species (e.g., Millie et al. 1997; Stæhr and Cullen 2003), to differentiate algal pigments, or to simply determine pigment concentrations, like Chl *a*, from the absorption at specific wavelengths.

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