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A new method for measuring spectral absorption coefficients of marine particles

Abstract—A new method has been developed to measure the light absorption coefficients of marine particles. The procedure, adapted from one proposed earlier for microscopic observation of nanoplankton, consists of concentrating particles onto a Nuclepore filter, transferring the filtered material to a glass microscope slide using liquid nitrogen freezing, and finally measuring the particle absorption spectrum on the slide. Measurements on various phytoplanktonic species show that the transfer efficiency is 94–98% and that no alteration of absorption spectra occurs. Our new method has been tested successfully on both phytoplankton-dominated and detritus-dominated field samples. In comparison with the widely used glass-fiber filter technique, the major advantage of our procedure is to provide more accurate absorption coefficients, since the pathlength amplification effect (β factor) and its attached uncertainties are completely eliminated.

The light absorption coefficient is, together with the volume scattering function, the inherent optical property of the aquatic medium (Preisendorfer 1961) that rules radiative transfer within seawater. In oceanic (case 1) waters, particles are responsible for most of the variations in light absorption. In addition, spectral absorption coefficients of living phytoplankton are important in determining the amount of radiant energy usable for photosynthesis. These coefficients, therefore, are essential input parameters in bio-optical models developed to predict carbon fixation rates from pigment concentrations and available radiant energy (Kiefer and Mitchell 1983; Platt and Sathyendranath 1988; Morel 1991; Anderson 1993). An accurate knowledge of the absorption capacity of algal cells is also required when assessing the quantum yield for carbon fixation (another physiological parameter needed in primary production models) from the photo-

synthesis–irradiance curves measured at sea or in the laboratory.

In attempts to determine the absorption coefficients of natural phytoplanktonic populations, however, a major difficulty is encountered due to the low concentration of particulate matter in ocean water. To circumvent this difficulty, Yentsch (1957, 1962) and Trüper and Yentsch (1967) proposed to perform absorption measurements of particles retained on membrane or glass-fiber filters. More recently, Kirk (1980) and Weidemann and Bannister (1986) used different preconcentration techniques to measure absorption directly on suspensions. Because preconcentration is time-consuming and a quantitative recovery of particles is difficult to achieve, the glass-fiber filter technique has been used most extensively to measure absorption by natural phytoplankton (e.g. Lewis et al. 1985; Yentsch and Phinney 1989; Babin et al. 1993; Garver et al. 1994).

In order to interpret absorption measurements performed with this technique it is necessary to quantify the dimensionless pathlength amplification factor (β factor, Butler 1962) caused by multiple scattering within the filter and between the filter and particles. Since Kiefer and SooHoo's (1982) assumption of β constancy, many efforts have been made to evaluate β , which was found to vary within a wide range. Kishino et al. (1985) suggested that β varies with the particle type and geometrical configuration. Mitchell and Kiefer (1988) first presented an inverse relationship between the β factor and the optical density measured on the filter (OD_f), and Mitchell (1990) emphasized the fact that it could vary from one lot of filters to another. Observing a similar relationship from measurements on cultures, Bricaud and Stramski (1990) reported that β was relatively constant (~ 2) for high optical densities ($OD_f > 0.2$) and suggested filtering a suf-

ficient volume of water yielding OD_f higher than 0.2, at least within the absorption bands. At low optical densities, relationships between b and OD_f were highly variable and equivocal (indicating a wavelength dependence of β).

Mitchell (1990), however, outlined that an important part of this variability could originate from the large relative errors affecting the ratio of low OD_f to low OD_s values (where OD_s is the optical density measured on the suspension) and recommended using a quadratic relationship between OD_s and OD_f to correct for the β effect. Measurements performed by Cleveland and Weidemann (1993) on 48 phytoplankton species seem to confirm the validity of this approach because the result of their quadratic fit is close to that of Mitchell (1990). The prevalent current practice is to estimate β empirically through such polynomial expressions (Mueller and Austin 1995). Note however, that the residual variability at low optical densities influences the result of such a fitting only weakly and may therefore be overlooked.

Even if the variability in β appears to be less than previously suspected, all available data stem from empirical studies, and no theoretical modeling concerning β has been proposed so far. The complex phenomena accounting for its actual behavior remain poorly understood, and uncertainty remains on its actual values for various types of particles. In particular, no experimental determinations of β are available for detrital particles, so that empirical formulae, derived from measurements on algal cultures, are applied (perhaps wrongly) to these weakly absorbing particles. Therefore, it is desirable to develop a method for measuring the absorption coefficients of marine particles, by which the pathlength amplification effect is annihilated and uncertainties attached to its correction disappear. The method we present here is adapted from the filter-transfer-freeze (FTF) technique proposed by Hewes and Holm-Hansen (1983) for microscopic observation of nanoplankton. This method consists in transferring algal cells from filters to glass slides, freezing with dry ice (-78.5°C), and then embedding the cells in a glycerin-gel medium for cell identification and counting. As modified here for absorption measurements, this method allows optical densities to be determined without the artifactual increase in pathlength due to multiple scattering because particles are not embedded in a diffusing material (as the GF/F filter). This note describes our technique and reports results on its application to phytoplankton grown in culture and to field samples.

Samples are filtered onto a 25-mm polycarbonate (PC) Nuclepore filter under low vacuum pressure (<5 mm of Hg) to avoid cell damage. Millipore (type GTTP) filters can also be used. We recommend 0.4- μm pore size. With larger pore sizes, small cells could pass through the filter; with smaller ones, the filtration time can increase prohibitively. Filtration is stopped when a very thin layer of liquid remains on the filter. The filter is then carefully removed and quickly transferred, particle side down, onto 5 μl of Nuclepore-filtered (0.2 μm) seawater lying on a glass microscope slide. The cells are thus suspended within a thin layer of liquid underneath the filter without air

bubbles. The preparation is then frozen by placing the slide onto a metal block cooled in liquid nitrogen, so that the material, instantaneously frozen, cannot be dislodged when removing the filter. After a few seconds, when the filter is entirely frozen, the slide is removed from the metal block and the exposed surface of the filter is quickly rubbed to remove frozen water drops which might make removal of the filter difficult. Then, the filter is carefully peeled off, and a circular cover slip (with a diameter identical to that of the clearance area of the filter) is placed onto the frozen sample so as to trap the particles over the same clearance area. After the preparation has thawed, absorption measurements can be performed once water condensed on the cover slip is completely evaporated.

In our study, the absorption spectrum of particles transferred on the slide was measured with a Perkin-Elmer Lambda 19 spectrophotometer, equipped with an integrating sphere and operated in the absorbance mode. The slide was placed at the entrance of the integrating sphere, and scans were conducted between 400 and 750 nm, using the 1-nm slit. Prior to each measurement, the reference was measured on the same slide, with 5 μl of the filtrate (0.2- μm) laid between the slide and the coverslip. Absorption coefficients, $a(\lambda)$, were obtained from measured optical densities, $OD(\lambda)$, as

$$a(\lambda) = 2.3 OD(\lambda) S/V. \quad (1)$$

Here 2.3 converts from \log_{10} to \log_e , $S(\text{m}^2)$ is the area covered by particles on the slide (identical to the clearance area of the filter), and $V(\text{m}^3)$ is the filtered volume.

This technique was tested by comparing the absorption spectra for particles in suspension to those for the same material on the glass slide. Absorption coefficients of the suspensions were measured on the same spectrophotometer in 1-cm cuvettes placed at the entrance of the integrating sphere. The reference used for the baseline was the filtered (0.2 μm) sample. Algal cultures were diluted, when necessary, to keep the optical thickness below 0.3 and minimize multiple scattering (Bricaud et al. 1983). Conversely, because of the low amount of suspended particles in natural seawater, field samples had to be concentrated to make measurements on the suspension feasible. For samples collected in Mediterranean waters, 4 liters of seawater were concentrated onto a 0.4- μm Nuclepore filter under low vacuum pressure (<5 mm of Hg) and resuspended in 4 ml of filtered (0.2 μm) seawater. Samples from the equatorial Pacific were concentrated ($\sim 200\times$) by tangential filtration. Optical densities $OD(\lambda)$ were converted into absorption coefficients $a(\lambda)$ according to

$$a(\lambda) = 2.3 OD(\lambda) \times (v_f/v_i)/r. \quad (2)$$

r (m) is the optical pathlength in the cuvette, v_i is the volume of the initial sample, and v_f is the volume of the concentrated sample.

Chronologically, absorption coefficients were first measured on the suspension both for cultures and concentrated field samples; the material present in the spectrophotometric cuvette was then filtered and transferred onto the slide. This procedure ensured that measurements with

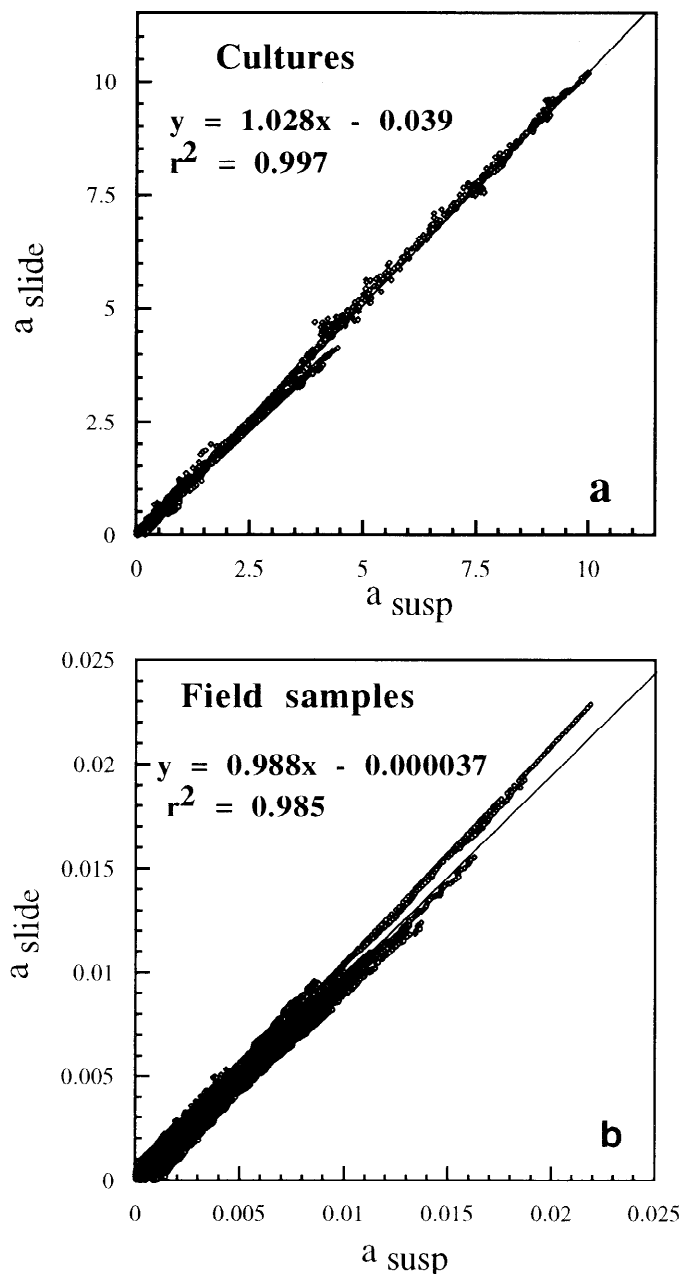


Fig. 1. Comparison between the spectral absorption coefficients (in m^{-1}) obtained on the suspensions (a_{susp}) and on the slides (a_{slide}) for phytoplanktonic cultures (six spectra, with 1-nm resolution) and field samples (35 spectra, same resolution). The equations of the regression lines and the values of the determination coefficients are indicated. This comparison was not performed directly on optical densities because, for geometrical reasons, optical densities measured on the suspensions and on the slides are not identical and their ratio varies with sample (see Eq. 1 and 2). The measurement accuracy is equivalent to $\sim 0.2 \text{ m}^{-1}$ for cultures and 2×10^{-4} – 10^{-3} m^{-1} (according to the concentration factor) for field samples.

both methods were performed on exactly the same material and eliminated uncertainties due to possible differences between culture aliquots as well as those due to incomplete resuspension of particles or selective concen-

tration for field samples. It is important to note that we used this preconcentration procedure only because absorption measurements on suspensions were needed for comparison; it is not needed during routine measurements at sea.

For cultures, as a complementary check, the efficiency of particle transfer from the filter to the slide was estimated by comparing the pigment concentrations in the material left on the filters to those in the initial samples. These concentrations were measured by spectrophotometry according to Strickland and Parsons (1968) and with the equations of Jeffrey and Humphrey (1975). Note that epifluorescence microscopy could also be used to check the absence of cells left on the filter. Both methods, however, are inappropriate for field samples, as they are unable to identify nonpigmented detrital particles. Therefore, for these field samples, testing the method was made solely by comparing the absorption spectra measured on the suspension and on the slide. This test, actually, is the most stringent and direct.

The phytoplanktonic species used to test this method were *Dunaliella tertiolecta* (Chlorophyceae), *Prorocentrum minimum* (Dinophyceae), *Emiliania huxleyi* (Prymnesiophyceae), *Anacystis marina* (Cyanophyceae), *Cryptomonas ovata* (Cryptophyceae), and *Eutreptia pertyi* (Euglenophyceae). These species were chosen to cover various taxonomic groups and to have diverse pigment composition, cell shape, and size. The first three strains were obtained from the Norwegian Institute for Water Research and the three latter from the culture collection at the University of Texas, Austin. The cultures were grown in filtered seawater, sterilized, and enriched with F/2 nutrients (Guillard and Rytner 1962). Continuous illumination was provided by "cool-white" fluorescent lamps, and scalar irradiance was $\sim 250 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. Experimental measurements were performed with cultures kept in the exponential growth phase by serial dilution with fresh medium, in order to have a negligible concentration of detrital material.

The overall agreement and the 1:1 ratio between the absorption coefficients provided by both methods for the six phytoplanktonic cultures are shown in Fig. 1a. The absorption spectra obtained for phytoplanktonic cells retained on slides, compared to those measured on suspensions, are shown as examples for three species (Fig. 2). For each species, both spectra are very similar in shape and magnitude, with features typical of the different species: Chl *a* bands around 440 and 675 nm for all species, Chl *b* (480 and 650 nm) for *D. tertiolecta*, peridinin (490 nm) for *P. minimum*, and phycocyanin (630 nm) for *A. marina*. For example, at the blue absorption maximum, absorption coefficients provided by both methods differ by no more than 7% for the six species.

The practical application of the slide method was tested on field samples collected in different waters: in coastal waters off Villefranche Bay (northwestern Mediterranean Sea) at two stations and various depths (June–September 1994), in oligotrophic waters of the equatorial Pacific during the JGOFS-France OLIPAC cruise (November 1994), and in oligotrophic waters of the northwestern

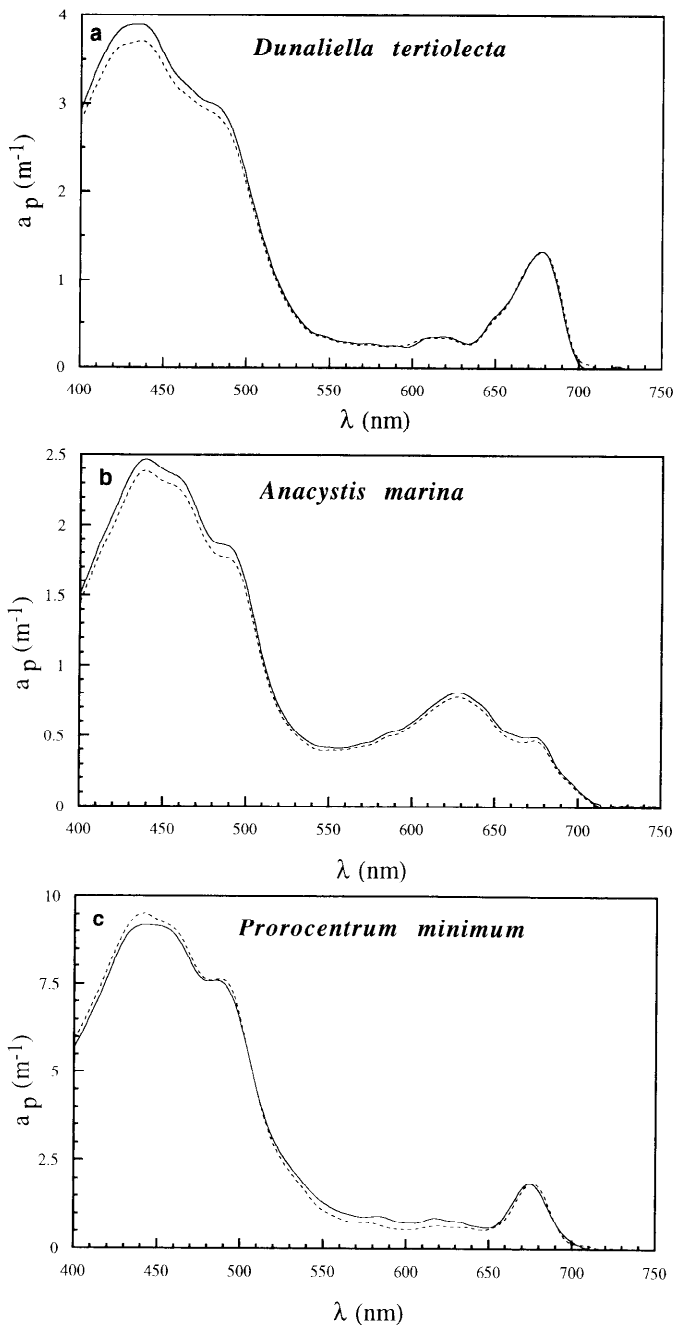


Fig. 2. Examples of absorption spectra of algal cultures measured on the suspension (solid lines) and on the glass slide (dashed lines). Spectral absorption coefficients were smoothed to eliminate instrumental noise (~ 0.001 OD) and corrected for residual scattering by subtracting the background as measured at 750 nm. Chl *a* concentrations in the suspensions are (a) 95.7 mg m^{-3} , (b) 36.1 mg m^{-3} , and (c) 189 mg m^{-3} .

Mediterranean Sea (May 1995). Samples from the first location were dominated by detrital (mineral) particles; samples from the other two locations were dominated by living algae and associated biogenous detritus.

Excellent agreement between both methods is also observed for all field samples, either dominated by detrital

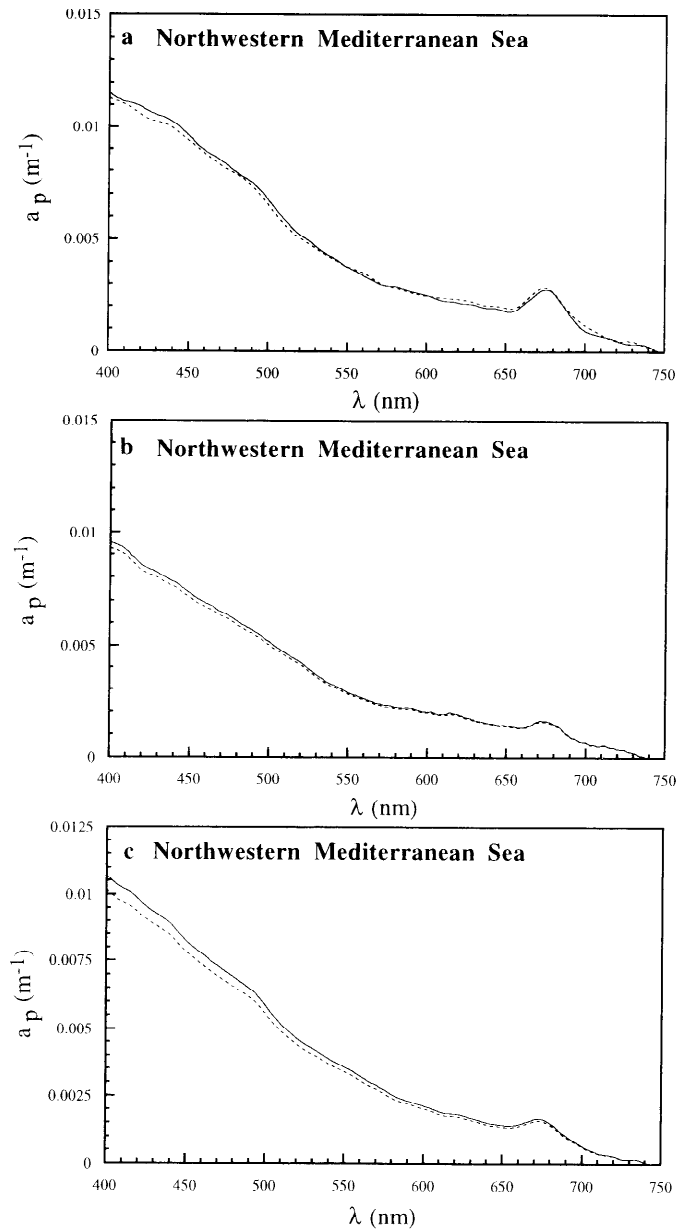


Fig. 3. As Fig. 2, but for particulate matter concentrated from field samples collected in coastal waters off Villefranche Bay ($43^{\circ}41'10''$, $7^{\circ}19'0''$ for panels a and b; $43^{\circ}39'$, $7^{\circ}17'0''$ for panel c). Samples were collected between 50 and 65 m. Chlorophyll *a* concentrations are between 0.07 and 0.10 mg m^{-3} . Sample volume is 4 liters.

particles or by living phytoplankton (Fig. 1b). The shape and magnitude of the spectra are kept when using the FTF technique (e.g. Figs. 3 and 4). At the blue absorption maximum, for instance, the maximum difference between absorption coefficients measured by both methods is 15%.

These results suggest that cell damage and loss of cellular compounds during transfer onto the slides are actually negligible. Neither spectral shifts nor absorption overestimation (as could result from a reduction of the package effect due to pigment release from burst cells) were noticed with this technique. Systematic underesti-

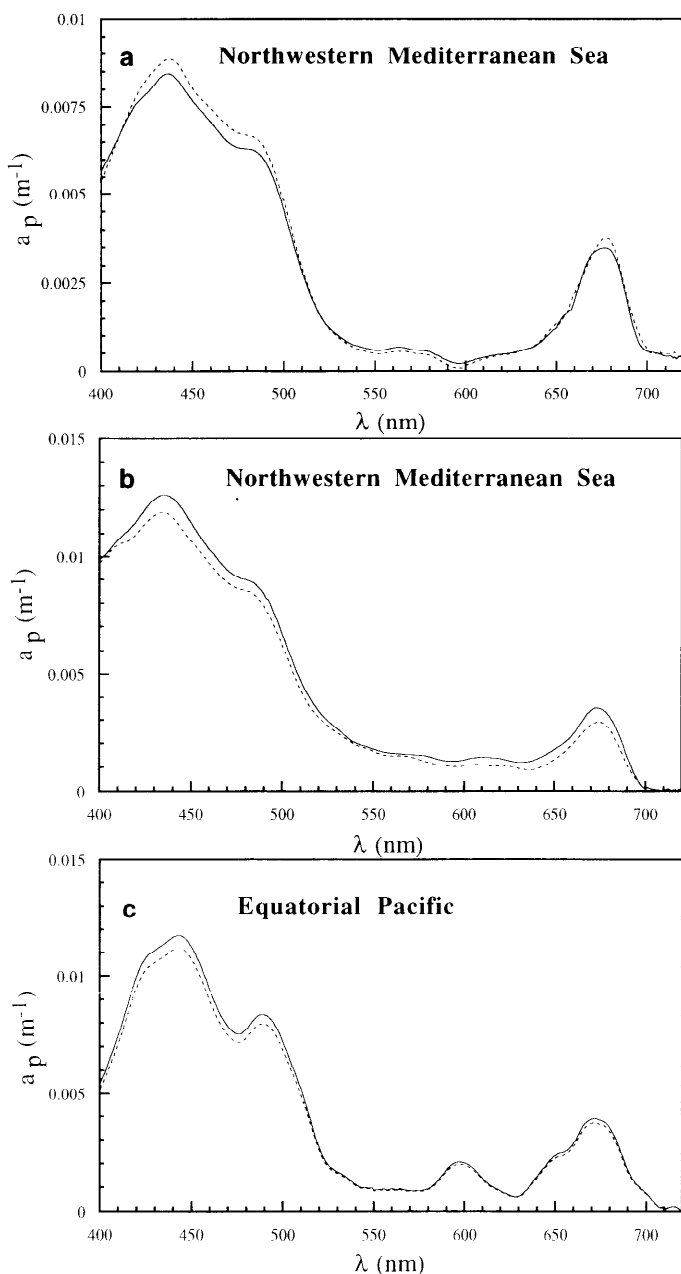


Fig. 4. As Fig. 2, but for particulate matter concentrated from field samples collected in oligotrophic waters in the NW Mediterranean Sea ($43^{\circ}25'$, $7^{\circ}51'$) and in the equatorial Pacific ($3^{\circ}S$, $150^{\circ}W$). Sample depths, Chl *a* (including divinyl Chl *a*) concentrations, and sample volumes are (a) 50 m, 0.28 mg m^{-3} , and 4 liters; (b) 30 m, 0.30 mg m^{-3} , and 5 liters; (c) 75 m, 0.18 mg m^{-3} , and 0.62 liters. Note the presence of divinyl Chl *b* bands (600 and 650 nm) on the absorption spectrum of the sample from the equatorial Pacific.

mation was not observed, which indicates that the efficiency of the particle transfer is close to 100%. In the case of cultures, this efficiency is confirmed by pigment concentration measurements, which show that 94–98% of cellular chlorophyll was transferred to the glass slides for the six species tested. Some cell damage was, nevertheless,

observed with the FTF technique by Hewes and Holm-Hansen (1983) for some particular species, such as big diatoms with large vacuoles; it might be due to the use of higher temperatures (dry ice instead of liquid nitrogen) when transferring cells. Liquid nitrogen is recommended to preserve phytoplankton for flow cytometric analyses (Vaulot et al. 1989); more importantly, microscopic examinations have shown that cells frozen in liquid nitrogen are not damaged (M. J. Chrétiennot-Dinet pers. comm.).

For most applications, once the particle absorption spectrum has been determined, it is useful to partition it into algal and nonalgal components. A procedure frequently associated with the glass-fiber filter technique is that proposed by Kishino et al. (1985), which consists of immersing the GF/F filter in methanol for pigment extraction and again measuring the residual absorption on the filter. This extractive procedure can also be applied with our method. By immersing the slide in 10 ml of methanol, pigments are extracted from algal cells. Because depigmented cells and detrital particles do not adhere to the slide (as they do with glass-fiber filters) and tend to disperse in methanol, it is then necessary to filter the methanol volume in order to collect these particles. At this stage, only Millipore filters are recommended because Nuclepore filters contain pigments that are extracted by methanol and then adsorbed by particles. Filters with $0.22\text{-}\mu\text{m}$ pore size must be used in order to collect even the smallest fragments of disrupted cells. The transfer technique can then be applied again, and the absorption spectrum of nonpigmented particles can be recorded. Another procedure might be to filter a second subsample onto a Millipore filter and to immerse the filter in methanol. This, however, doubles the sample volume, which can be problematic during routine measurements at sea. In this case also, refiltering the methanol volume would be needed because particles are not embedded in Millipore filters as they are in GF/F filters.

Measurements performed on both algal cultures and field samples using the procedure described above seem satisfactory (e.g. Fig. 5). We acknowledge that no reference method is available to validate them quantitatively because physical separation of algal and nonalgal components is not feasible within a suspension. Since it has been demonstrated, however, that no pathlength amplification occurs on the slide, we believe that these measurements are not affected by artifacts other than those of the classical method of Kishino et al. (1985). Recall that these are the following: the absorption spectrum measured after pigment extraction includes not only detritus, but also depigmented algal cells; it also includes water-soluble pigments, such as phycobilins, not (or weakly) extracted by methanol; and detrital pheopigments, as well as other chlorophylls and carotenoids present in zooplankton fecal pellets (e.g. Kleppel and Pieper 1984) are extracted by methanol and therefore erroneously included in the algal component. Several numerical decomposition methods have been proposed (Roesler et al. 1989; Morrow et al. 1989; Bricaud and Stramski 1990; Cleveland and Perry 1994) and can be used to substitute for or complement this chemical method.

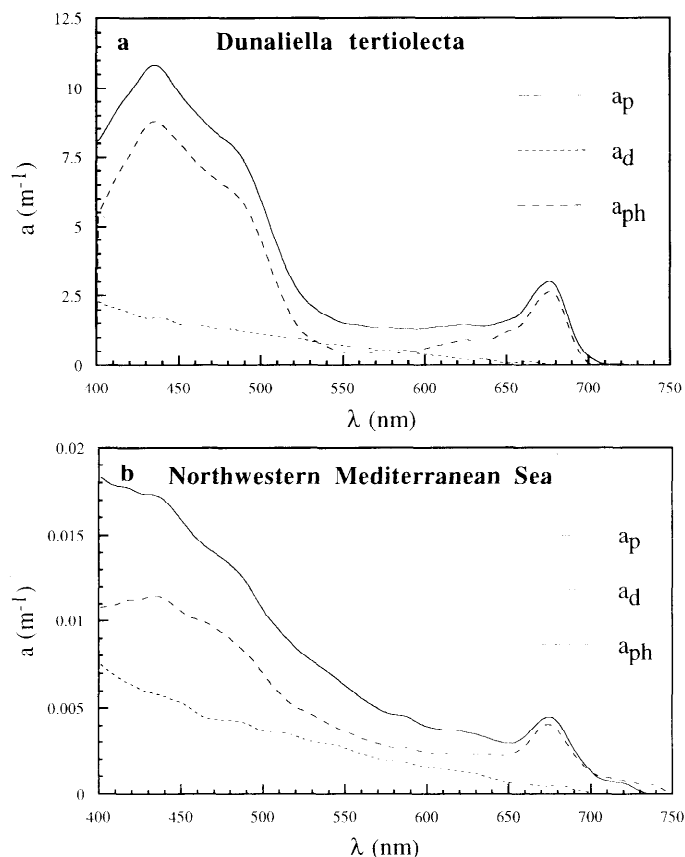


Fig. 5. Absorption coefficients before (a_p) and after (a_d) methanol treatment a_{ph} is obtained by subtracting a_d from a_p and represents the absorption coefficient of pigments extracted by methanol. [a.] Cultured sample (*Dunaliella tertiolecta*). [b.] Field sample (off Villefranche Bay, 45 m).

The reliability of the proposed method was checked by comparing the absorption spectra with those obtained on suspensions for six phytoplanktonic species grown in culture, and 35 field samples. No failure of the method was observed, provided that all the above precautions were respected. We conclude that the modified FTF technique is an efficient, convenient, and reliable method for absorption measurements on marine particles. In addition to simplicity and suitability of use at sea, its advantage, when compared to other methods and especially to the widely used glass-fiber filter technique, is to provide more accurate absorption coefficients because uncertainties linked to the pathlength amplification factor are eliminated. Use of this technique, associated with either the technique of Kishino et al. (1985) or a numerical decomposition method, should facilitate systematic measurements of algal absorption at sea and improve our knowledge of the spatial-temporal variations in both absorption properties and quantum yield for carbon fixation of natural phytoplankton.

Karima Allali
Annick Bricaud
Marcel Babin
André Morel

Laboratoire de Physique et Chimie Marines
Univ. Pierre et Marie Curie and CNRS
BP 8
F-06230 Villefranche-sur-mer, France

Patrick Chang

Unité de Biologie Cellulaire Marine
Univ. Pierre et Marie Curie and CNRS
BP 28
F-06230 Villefranche-sur-mer, France

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