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Assessment of a microscopic photobleaching technique for measuring the spectral absorption efficiency of individual phytoplankton cells

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Measurements of the absorptance of photosynthetic pigments in individual phytoplankton cells were made using an epifluorescence microscope equipped with a spectrograph and CCD array detector. Correction for light loss due to scattering was achieved by bleaching the cells with intense light from a mercury arc lamp, and using the bleached cells as a spectrophotometric blank. Absorption efficiency factors were calculated from knowledge of the geometrical cross-section of the cells obtained from calibrated video images acquired at the time of measurement. The single-cell efficiency factors were consistent with the average absorption efficiencies of cell suspensions measured using a spectrophotometer over most of the visible spectrum, but they were significantly lower below 420 nm. Cells of the diatom Cyclotella cryptica and the chlorophyte Chlorella salina showed clear spectral differences in spectral shape that could be related to taxonomic differences in pigment content, but absorption efficiency factors of approximately 0.4 at 675 nm were found for both species.

INTRODUCTION

The absorption efficiency factor for individual phytoplankton cells, defined as the ratio of the absorption cross-section to the geometrical cross-section, is a measure of the fraction of the light energy impinging on the cell which is absorbed (Bricaud *et al.*, 1983). It is usually deduced from measurements of cell suspensions rather than measured directly (Bricaud *et al.*, 1983; Sathyendranath *et al.*, 1987). However measurements on suspensions provide average values which mask the cell-to-cell variability which occurs within a culture or natural population. They also make it impossible to determine the absorption characteristics of individual species in a mixed natural sample. In theory, single-cell absorption measurements made using optical microscopy should avoid most of these problems. Moreover, microscopy can easily provide information on the geometrical area which the cell presents to the illuminating beam, so that absorption cross-sections and efficiency factors can be calculated. These parameters are required for optical models of phytoplankton cells and natural suspensions (Bricaud and Morel, 1986), and can also be used to validate single-cell optical measurements made by other techniques such as flow cytometry (Perry and Porter, 1989; Ackleson and Spinrad, 1988). However, in order to determine the absorptance of small pigmented particles by microscopy it is necessary to account for the reduction in light transmission through the optical system caused by scattering, which is not a trivial problem. Pioneering work in this area was carried out by Iturriaga and colleagues (Iturriaga *et al.*, 1988, 1991; Iturriaga and Siegel, 1989) who devised a method for transferring cells to a mixture of gelatine and glycerol. This mixture had a refractive index close to that of the cells, so that scattering was minimized. However, wider adoption of the Iturriaga method has been impeded by the complex sample preparation procedure and also by the length of time required to make spectral absorption measurements using a scanning monochromator and single detector. We have therefore attempted to simplify single-cell absorption measurements by introducing two modifications to current techniques. Firstly, a spectrograph and array detector were substituted for the scanning monochromator so that

measurements across the whole visible spectrum could be made in a matter of milliseconds. Secondly, the attenuance of light by individual cells was measured before and after bleaching of the photopigments in order to compensate for scattering losses. The use of bleached cells as a reference for plant cell absorption measurements was probably first suggested by Duysens (Duysens, 1956), and Doucha and Kubín developed a bleaching technique for phytoplankton which involved treatment with peracetic acid followed by exposure to a mercury arc lamp for 30 min (Doucha and Kubín, 1976). In our microscope method, the bleaching was simply carried out by exposure to the arc lamp used for epifluorescence excitation and was usually accomplished in less than 1 min. This paper presents an assessment of the feasibility of obtaining absorption cross-sections and efficiency factors for cultured phytoplankton cells using the photobleaching technique, and compares the results obtained with those from measurements made on cell suspensions using conventional spectrophotometry.

METHOD

Microscope modifications

The single-cell absorption spectrophotometer was based on an Olympus BH2 microscope fitted with an epifluorescence attachment and trinocular head. An extra tube containing relay lenses and a beamsplitter was added to the camera port to permit the attachment of a spectrometer and CCD video camera. The transmitted illumination system in the microscope stand was replaced with a fibre-optic illuminator constructed in our laboratory which incorporated an Osram Xenophot 100 W tungsten halogen lamp and blue glass filtration. The fibre-optic bundle was terminated in a condenser lens providing a reasonably collimated beam with a divergence half-angle of 6°. The optical layout of the modified instrument is illustrated in Figure 1. The field of view of the objective was brought to a 100 μm diameter image at the entrance of a spectrograph (Oriel Instruments Multispec). The image was made smaller than the 200 μm wide entrance slit of the spectrograph so that transmittance measurements were insensitive to the position of small attenuating objects in the field of view. The spectrograph was fitted with a 400 lines/mm grating and coupled to an Andor Instaspec IV cooled CCD array detector held at a temperature of 15°C. Data from the CCD detector was collected using the proprietary Andor control card and driving software. The nominal spectral range of this system extends from 300 nm to 815 nm. However, the low sensitivity of the CCD array to blue wavelengths, combined with the low blue output of the tungsten lamp, meant that signals were unacceptably noisy below 400 nm. The effective spectral resolution of the entire optical system, determined by measuring the full width half-maximum distribution of monochromatic illumination from a helium neon laser, was 1.1 nm.

Image analysis

The video output from a monochrome $^1/_2$ inch CCD camera was passed to a frame grabber and digitized at 640 × 480 pixel resolution. Geometric calibration of the digitized video image was carried out using a stage micrometer and latex beads of standard sizes. The image was then analysed using custom software written in the MATLAB image-processing language. This software calculated the area of the field of view obscured by the cell being measured. It used a simple thresholding and filling algorithm after first subtracting an image of an empty field of view to eliminate the effect of defects and dust in the relay optics and camera.

Calibration issues

The wavelength setting of the spectrograph was checked across the visible spectrum by measuring the absorption of a holmium oxide glass filter: measured absorption peak wavelengths corresponded to within ± 1 nm of the tabulated values. The radiometric linearity of the system was established using a stepped neutral density filter (Edmund Scientific), calibrated in optical densities from 0.1 to 1.0 in steps of 0.1 units: the resulting correlation coefficient was indistinguishable from unity. Positional sensitivity was checked by moving a grain of iodine-stained rice starch around the field of view: absorptance measurements were within ± 5% of the central value for all positions for which the grain was fully within the field of view.

Phytoplankton cultures

Cultures of the diatom *Cyclotella cryptica* (CCAP1070/2) and the chlorophyte *Chlorella salina* (CCAP211/25) were obtained from the Culture Collection of Algae and Protozoa at Dunstaffnage Marine Laboratory. They were grown in f/2 + Si medium made up according to the recipe in the culture collection catalogue at room temperature with uncontrolled window illumination. These species were chosen for their simple shape and lack of motility: the *Cyclotella* cells were rectangular with average dimensions of 11 μm × 8 μm, while *Chlorella* cells were spherical with a typical diameter of 6.5 μm.

Single-cell measurement procedures

For single-cell absorption efficiency measurements, a drop of the sample was held between a standard microscope slide and cover slip and the microscope set up for transmitted illumination. The measurement sequence began

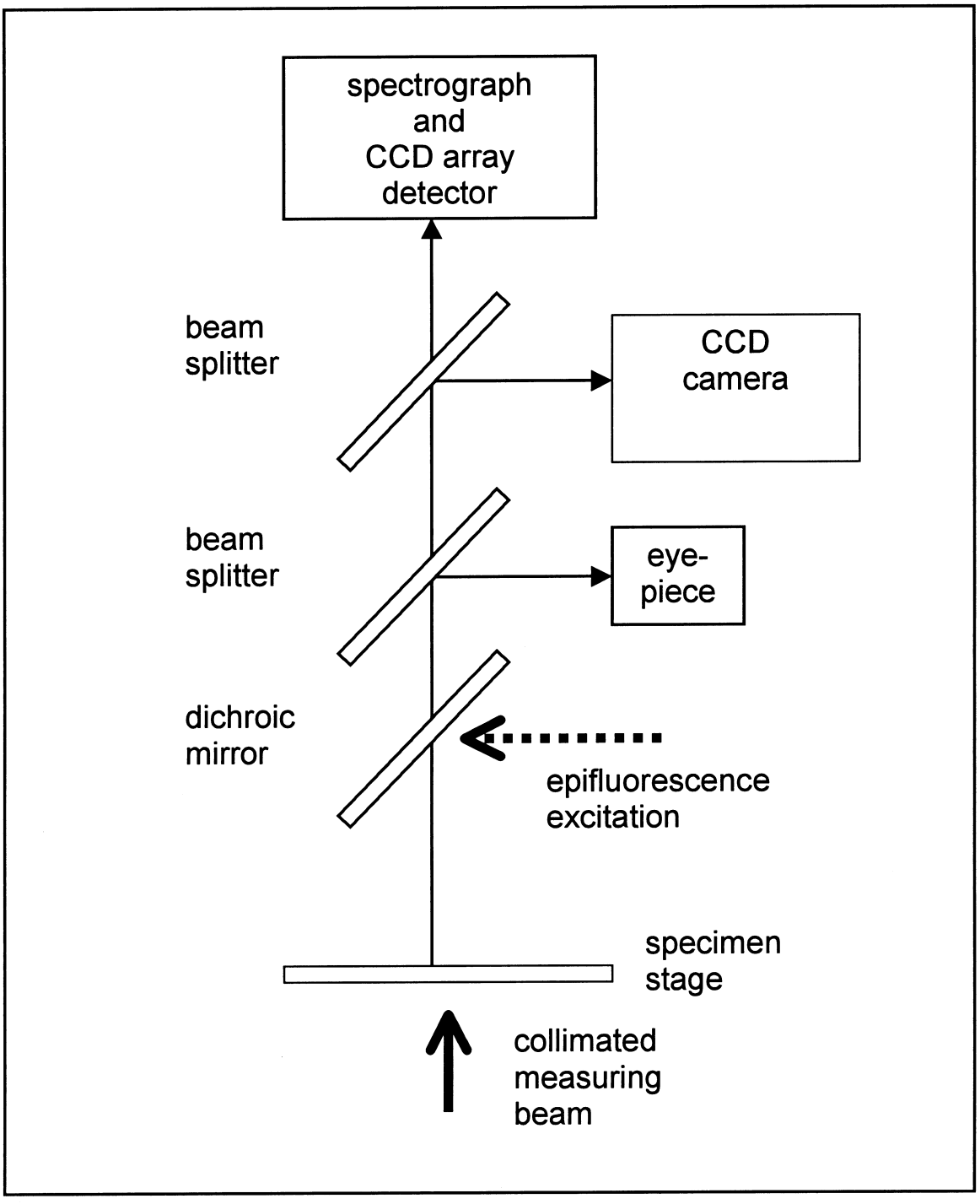

Fig. 1. Schematic optical arrangement of the modified microscope. The dichroic mirror was removed from the light path during absorptance measurements.

by finding the spectral values of irradiance produced at the spectrograph detector by an empty field, $E_0(\lambda)$, and the corresponding irradiance with a cell approximately centred in the field of view, $E_1(\lambda)$. A video image of the cells was then acquired, providing the fractional coverage of the field in terms of the ratio of cell area (A_c) to field area (A_f). The illumination of the microscope was switched to epifluorescence mode, and the decay of the

fluorescence signal from the chlorophyll antenna of photosystem 2 was monitored. It typically decayed to less than 1% of its initial value in around 20 s. Bright-field illumination was restored, the physical integrity and position of the cell were checked, and a third, $E_2(\lambda)$ measurement was made of the transmitted light with the bleached cell in the field of view. The spectral absorption efficiency, $Q_a(\lambda)$ of the cell, defined as the ratio of the absorption and geometrical cross-sections is then given by:

$$Q_a(\lambda) = \frac{E_2(\lambda) - E_1(\lambda)}{E_0(\lambda)} \div \frac{A_c}{A_f}$$

Cell suspension measurements

Measurements of the absorption coefficient $a(\lambda)$ of cell suspensions were made on samples held in a 1 cm path length cuvette using a laboratory-built single-beam spectrophotometer with 1.4 nm spectral bandwidth, using the technique proposed by Shibata *et al.* (Shibata *et al.*, 1954). A wide detector acceptance angle was obtained by placing the cuvette immediately in front of a 25 mm diameter PTFE diffusing plate, which was in turn positioned approximately 5 mm from the detecting photomultiplier. The numerical concentration of the cells in the suspension, N, and their average geometrical cross-section, g, were obtained by counting and measuring cells using the microscope in conjunction with a haemocytometer chamber. The average absorption efficiency factor, $Q_a(\lambda)$, was then obtained from the expression $Q_a(\lambda) = a/Ng$. This approach is valid only if the optical density of the suspension is less than 0.1 (van de Hulst 1981; Bricaud *et al.*, 1983). In our measurements, the maximum optical density of the cell suspensions was 0.03 for *Chlorella* and 0.05 for *Cyclotella*.

RESULTS

A series of spectra illustrating the typical reduction in cell absorption with time during photobleaching is plotted in Figure 2. There was no indication of any significant variability in the rate of bleaching of different pigments. The attenuation spectrum after bleaching shows a small but significant rise close to 400 nm. It is not clear whether this feature represents residual absorption of cellular materials which were resistant to photobleaching, or whether it is evidence for wavelength-dependent scattering by the bleached cell. During routine measurements, the progress of bleaching can be monitored more conveniently by using the spectrograph to measure the magnitude of the fluorescence peak rather than making successive absorption measurements. A fluorescence decay curve for a large pennate diatom (species not identified) measured in this way is shown in Figure 3. For illustrative purposes, the intensity of the source was greatly reduced by partially closing a variable aperture in the epifluorescence unit and adding neutral density filtration to the light path. The decay kinetics are complex, particularly in the first few seconds of exposure. This is presumably because the elimination of photochemical quenching of fluorescence from the PS2 antenna (the Kautsky effect) occurs during the early stages of bleaching. Figure 4 shows a comparison between absorption efficiency factors measured using the spectrophotometer (which gives the mean value for a suspension) and the range of values measured for 10 randomly selected cells using the microscope method. The general similarity of the results is very encouraging given the complete independence of the methodology and the notorious susceptibility of optical measurements to systematic error. The most significant discrepancy is probably in the range 400–430 nm, where measurements on the suspension gave higher absorption efficiency values. This may indicate that the feature noted in Figure 2 is residual absorption rather than wavelength-dependent scattering. Spectra published by Bricaud *et al.*, comparing the absorption of whole cells with extracted pigments show similar differences in the 400–430 nm region (Bricaud *et al.*, 1983). Figure 5 shows comparative results for single cells of *Cyclotella* and *Chlorella*. The absorption efficiency factors for these two small (nominally 10 μm) species are similar in overall magnitude, though there are obvious spectral differences. These are associated with the presence of different carotenoids and accessory chlorophylls: fucoxanthin and chlorophyll *c* for the diatom, lutein, zeaxanthin and chlorophyll *b* for the chlorophyte

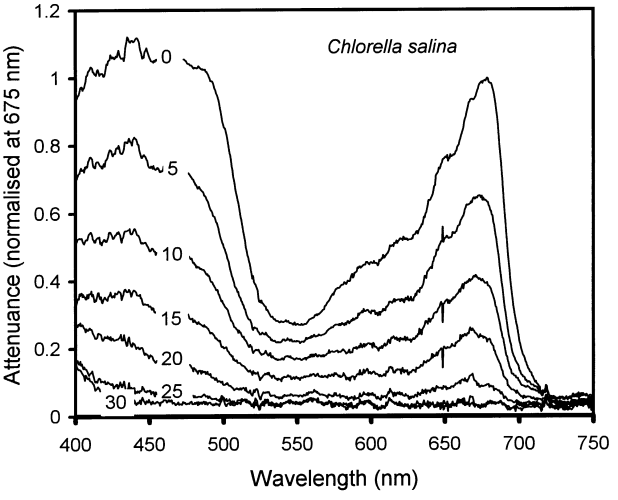

Fig. 2. Time course of photobleaching of a single diatom cell illustrated by consecutive measurements made at 5 s intervals. The graph displays the attenuation relative to a clear field of view, normalized at the red chlorophyll absorption peak of 675 nm for the initial measurement.

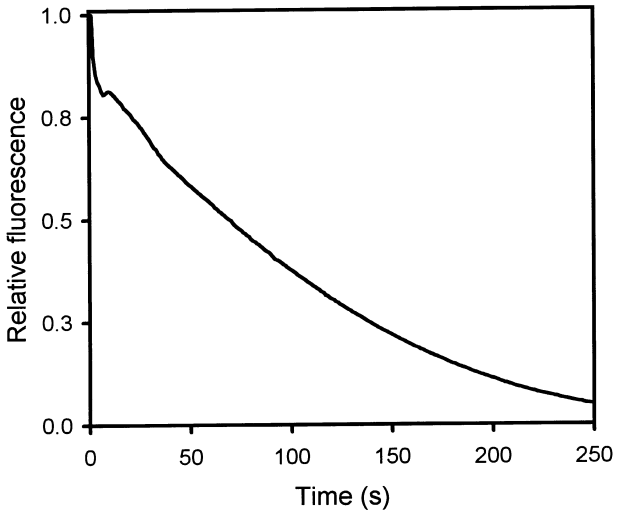

Fig. 3. Decay of the magnitude of the peak chlorophyll fluorescence signal, relative to the value measured after 1 s, for a large diatom cell exposed to very weak photobleaching irradiance.

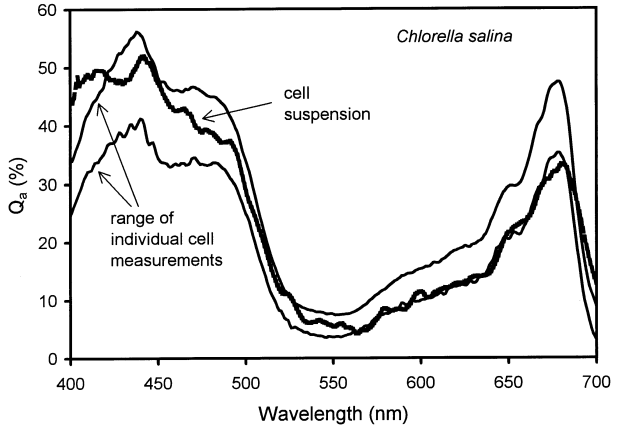

Fig. 4. Spectral absorption efficiency factors for the diatom *Cyclotella cryptica* showing the mean value for cell suspensions measured by spectrophotometry (thick line) and the range found for 10 randomly selected single cells using the photobleaching technique (thin lines).

(Prezelin and Boczar, 1986). In addition to the results illustrated, we carried out opportunistic measurements on various other phytoplankton species. Diatoms of various sizes in mixed culture gave absorption efficiencies at 675 nm values (Q_{a675}) ranging from 0.3 to 0.4 while *Prymnesium parvum*, a small flagellate, gave a mean value of 0.24. Iturriaga *et al.* made microscope measurements on single cells of the diatom *Navicula saprophila*, and found values ranging from 0.20 to 0.56 depending on the growth irradiance (Iturriaga *et al.*, 1988). Morel and Bricaud list cell suspension measurements for 17 phytoplankton species with Q_{a675} values ranging from 0.05 to 0.46 (Morel and Bricaud, 1986). All our single-cell measurements fall within this range.

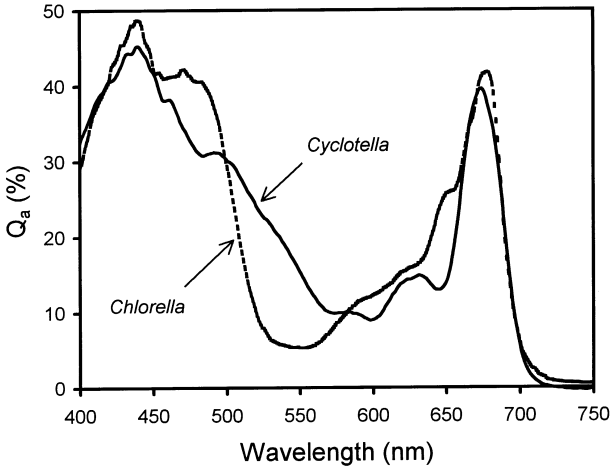

Fig. 5. Spectral absorption efficiency factors for single cells from taxonomic groups with distinctively different pigment complements.

DISCUSSION

The basic assumption underlying this work, which is that the scattering properties of the cells are not significantly altered by the bleaching process, is not strictly in accordance with optical theory. The anomalous dispersion induced by pigment absorption is known to change the cell scattering cross-section of small phytoplankton cells (Bricaud and Morel, 1986). The magnitude of this effect varies according to species, and it is usually restricted to a change of less than 10% around the main chlorophyll absorption bands. The apparent effectiveness of our scattering correction is probably due to the fact that most of the scattered light is collected by the optical system. The residual signal loss due to scattering is only around 5% of the absorption peaks (Figure 2.) so that the error attributable to anomalous dispersion is less than 1% of the absorption measurement. An additional potential error in the photobleaching technique is the attribution of absorption by resistant cellular material to scattering losses. This would create problems if whole-cell absorption measurements were used in calculations of particle refractive index. On the other hand, the fact that the absorption efficiency factor refers only to bleachable photopigments is probably advantageous for studies of the capture of photosynthetically available radiation. Finally, it must be pointed out that cells are killed by photobleaching and may physically disintegrate. For the results presented here the structural integrity of the bleached cell was checked under the microscope and subsequent measurements

were carried out as quickly as possible. In spite of these caveats, single-cell photobleaching appears to be capable of providing absorption cross-sections and efficiencies which are consistent with those obtained using cell suspensions. The technique is free of many of the ambiguities associated with population-averages measurements. It could potentially be combined with the freeze-transfer technique of Allali *et al.* (Allali *et al.*, 1995) to provide a useful means of analysing natural phytoplankton samples concentrated on membrane filters.

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