

Diel patterns in optical properties of the chlorophyte *Nannochloris* sp.: Relating individual-cell to bulk measurements

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Abstract

To investigate how cell growth and division affect the optical properties of phytoplankton, cultures of the chlorophyte *Nannochloris* were sampled over a diel cycle to measure cell size and concentration, light-beam attenuation and absorption, flow cytometric forward light scattering and chlorophyll fluorescence, and carbon content. Refractive index was calculated using the anomalous diffraction approximation. At six different light levels, ranging from 60 to 1,500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, cell division was tightly phased to the light:dark cycle, occurring soon after dark. There were pronounced diel patterns, with minima near dawn and maxima near dusk, in cell size, cell-specific beam attenuation and absorption, flow cytometric forward light scatter, and carbon content. The diel variations in attenuation cross section were primarily caused by changes in cell size due to growth and division, although there was some influence from changes in refractive index. Because the major constituents of many phytoplankton communities are small eukaryotic cells similar to *Nannochloris* in cell size and division pattern, the results presented here have important consequences for the interpretation of diel variations in optical properties observed in the ocean.

There is increasing interest in using optical measurements, such as ocean color and beam attenuation, to provide convenient estimates of ocean biomass and primary productivity on large scales. Algorithms to interpret remote optical measurements in terms of biogeochemical processes are based on the fact that the color of the ocean is largely determined by the optical properties of the suspended particles, particularly the phytoplankton and their products (Lewis and Cullen 1991). Understanding the variability in optical properties among the particles in the ocean can lead to a better interpretation of these bulk measurements and thus better estimates of larger-scale production.

Because phytoplankton growth is dependent on light, one of the most important scales of variation is over the daily cycle of light and dark (LD). Many researchers have observed diel variations in bio-optical properties in the ocean (Dickey et al. 1990; Hamilton et al. 1990; Stramski and Dickey 1992; Gardner et al. 1995), and diel variations in beam attenuation have been used to estimate primary production by assuming a constant carbon-specific beam attenuation (Siegel et al. 1989; Cullen et al. 1992; Walsh et al.

1995). However, there is evidence of diel variation in the carbon-specific beam attenuation (Ackleson et al. 1993; Stramski and Reynolds 1993; Stramski et al. 1995), and the effect of this variation in estimates of productivity has been explored (Cullen and Lewis 1995). To more completely interpret such bulk measurements made at sea, the diel variations in optical properties of phytoplankton cells must be better understood. Stramski and Reynolds (1993) investigated the diel variations in optical properties of the diatom *Thalassiosira pseudonana* and found that variations in refractive index were equal to or more important than variations in cell size in affecting changes in optical cross sections. The measured carbon-specific beam attenuation varied over the diel cycle (up to almost 30%). A recent study of *Synechococcus* indicated that, for a slowly growing culture, optical cross sections were minimum near dawn or mid-morning and maximum near dusk (Stramski et al. 1995). The mean carbon-specific beam attenuation showed substantial diel variability, changing by up to twofold over the cycle, and was different for *Synechococcus* ($2.48 \text{ m}^2 (\text{g C})^{-1}$ at 660 nm) than for *T. pseudonana* ($3.81 \text{ m}^2 (\text{g C})^{-1}$ at 660 nm). Stramski et al. (1995) suggested that there may be considerable variability in the carbon-specific beam attenuation, which should be considered when estimating productivity from diel variations in beam attenuation.

In the experiments reported here on diel variations in optical properties of a phytoplankton, we chose the chlorophyte *Nannochloris* because it is similar in size and shape (2–3 μm in diameter, coccoid) to open-ocean ultraphytoplankton and has division patterns typical of many eukaryotic phytoplankton, i.e., cell division occurs during the night (Chisholm 1981). We measured cell concentration, beam attenuation and absorption, cell volume, and carbon content and calculated optical cross sections and refractive index. We also measured forward light scatter (FLS) and chlorophyll fluorescence of these cells using flow cytometry, a method that can measure the optical properties of individual phytoplankton cells. Thus, the results of this study will aid in

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Table 1. Summary of laboratory experiments on *Nannochloris*.

Experiment	Light level ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$)	Type of experiment	Measurements
Nan250 (Jul 92)	250	Replicate batch, carboys in incubator	Cell size Concentration Beam attenuation Absorption Carbon Forward light scatter
Nan500 (Aug 92)	500	Replicate batch, carboys in incubator	Cell size Concentration Beam attenuation Absorption Carbon Forward light scatter
Nan1500 (Aug 94)	1,500	Replicate batch, flasks in natural light	Cell size Concentration Beam attenuation Absorption Carbon Forward light scatter
Nan60 (May 93)	60	Cyclostat, in incubator	Cell size Concentration Forward light scatter
Nan120 (May 93)	120	Cyclostat, in incubator	Cell size Concentration Forward light scatter
Nan330 (May 93)	330 (up from 120 previous day)	Cyclostat, in incubator	Cell size Concentration Forward light scatter

interpreting flow cytometric measurements of phytoplankton populations in the field and in understanding the impact of particular populations on the bulk optical properties of seawater (DuRand 1995; DuRand and Olson 1996).

Materials and methods

Batch cultures—Three experiments were performed in which the chlorophyte *Nannochloris* sp. was grown in *f/2* medium in replicate batch cultures at 22°C (Table 1). The *f/2* medium was autoclaved, then sterile-filtered through a 0.22- μm Millipore filter (142- μm diameter). For two experiments, designated Nan250 and Nan500, the cultures were grown in 10-liter carboys in an incubator on a 14:10 LD cycle at photosynthetically available radiation (PAR) of 250 and 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, respectively (Cool-White fluorescent lights, measured with a Biospherical Instruments QSL-100 4π sensor). The carboys were mixed with a magnetic stir bar and bubbled with moisturized, filtered air. Samples were obtained through a sterile sampling port (forced out by air pressure). A third experiment (Nan1500) was performed outdoors under natural sunlight with an average integrated light level of 1,500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a maximum of 2,200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (14 h daylight on a mostly sunny day in August) (Fig. 1). The light levels were measured with a Biospherical Instruments QSR-240 2π sensor in the air. The cultures were in the shadow of the building from dawn (~0600 h) until ~0800 h, after which they were in full sunlight. The cells were grown in Fernbach flasks set

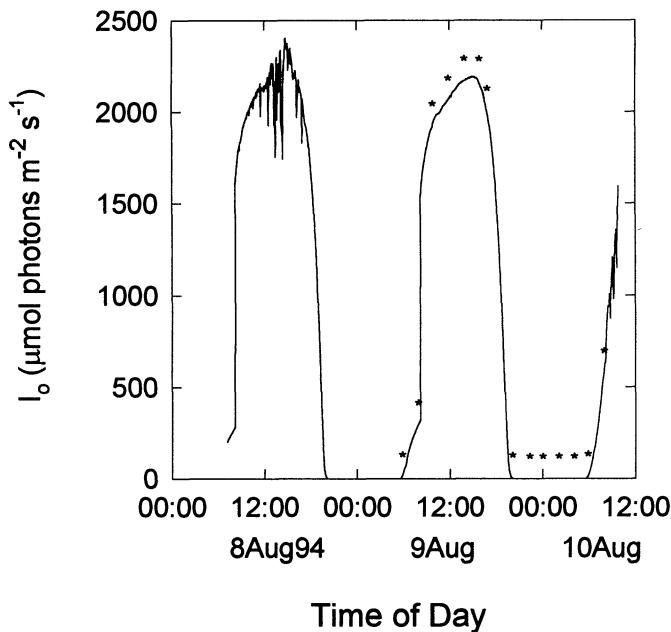


Fig. 1. I_o (scalar PAR, in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) measured outdoors near the experimental setup for Nan1500 for the days the cultures were in natural light. Sampling times (9–10 August) are marked with asterisks.

in a clear Plexiglas water bath with the temperature controlled by a recirculating water bath. The flasks were stirred throughout the experiment, and samples were taken by transporting the flasks to a laminar flow hood, then pouring an aliquot. The cultures were first grown in an incubator at 500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, then placed outdoors for 1 d before sampling began.

For each of the three experiments, the exponential-phase cultures were sampled, beginning at dawn, every 2 h for 24 h, and a number of measurements were made (*see below*). All measurements were completed within 2 h except where noted. During the nighttime samplings, all samples were kept in dim light during analysis.

Cyclostat cultures—An additional set of experiments was performed using two cyclostats (nutrient-replete continuous cultures on a 14:10 LD cycle), in which samples were collected automatically every hour with a fraction collector (Table 1). The samples were refrigerated at 4°C for up to 12 h, then analyzed on a Coulter Multisizer; 2-ml aliquots were preserved and frozen for later analysis on the flow cytometer (*see below for description of analysis*). Light levels for the two cyclostats were 60 and 120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ for the first 24-h sampling, then a second 24-h sampling was started after the light level was increased to 330 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ for the cyclostat that was previously at 120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Flow cytometry—Triplicate samples were analyzed on an EPICS 753 flow cytometer modified to analyze 50-ml seawater samples at 5–10 ml min^{-1} (Olson et al. 1991, 1993). The culture was diluted 500-fold with 0.22 μm filtered seawater (FSW). A previous study indicated that optical properties of phytoplankton cultures can be affected within 1 h of dilution (Ackleson et al. 1988b). However, our dilutions were performed immediately before samples were run, and similar dilutions were made before other measurements (Coulter cell size and beam attenuation), so any change would be expected to influence all the measurements. Fluorescent microspheres (3.79 μm , Polysciences) were added to each sample as an internal standard. The concentration of beads was calibrated on the flow cytometer to calculate cell concentrations (Olson et al. 1993). For each particle, FLS (3–19° at 488 nm), side scatter (73–107°), red fluorescence (chlorophyll, 660–700 nm), and orange fluorescence (530–630 nm, used to differentiate beads from cells) data were collected. The resulting “list-mode” files were analyzed using CytoPC software (D. Vaultot, Station Biologique, Roscoff, France) to obtain numbers of cells and beads analyzed and the mean values for each parameter. The FLS data were converted from bead units to forward scattering cross section ($\sigma_{b,3-19^\circ}$, in μm^2) as in DuRand and Olson (1996). For Nan500 and Nan1500, the samples were analyzed live; for Nan250 and the cyclostat experiments (Nan60, Nan120, and Nan330), the samples were fixed with 0.1% glutaraldehyde and stored in liquid nitrogen until analysis (Vaultot et al. 1989; Olson et al. 1993). A comparison of live and frozen samples for Nan500 showed little difference in forward scattering cross section (average, 5%). However, side light scat-

ter did change significantly and thus was not used (data not shown).

Beam attenuation—Beam attenuation was measured with a SeaTech transmissometer (25-cm path length and 665-nm wavelength) enclosed by a polyvinyl chloride (PVC) sleeve (11-cm inner diameter) with a sampling port. Both a white and a flat black PVC sleeve were tested, and no significant differences were found (data not shown). First, 1.6 liters of FSW was added to the sleeve, and a voltage reading was made. Then, culture was added, progressively, to total 8, 12, and 16 ml, and the voltage was recorded after each addition. For each measurement, the culture was added to the FSW, the mixture was stirred, and any bubbles were cleared from the transmissometer windows before the reading was taken. This stepwise addition was repeated twice for each culture. To analyze the data, voltages were converted to beam attenuation (c , in m^{-1}), the preceding FSW blank reading was subtracted from each culture measurement, and the value was normalized to the concentration of cells in the transmissometer sleeve (calculated from the measured volume of the culture addition and the flow cytometric cell concentrations). The samples in the transmissometer sleeve were determined to be optically thin (because the sequential addition series did not depart from linearity), so that multiple photon-particle interactions were negligible (Bricaud et al. 1983).

Cell size—Cell size was measured using a Coulter Multisizer equipped with a 30- μm orifice (except in Nan250, in which a 50- μm orifice was used). Samples were diluted with FSW to keep the coincidence rate below 5%. At each sampling point, duplicate or triplicate samples were mixed and measured. The samples were analyzed immediately upon sampling, except for the cyclostat experiments (Nan60, Nan120, and Nan330), which were refrigerated for up to 12 h before analysis. There was no difference in cell size between replicate test samples run on the Multisizer immediately after collection or after 11 h of refrigeration. For all samples, the 256-channel data of cell-diameter distributions were used to calculate the geometric projected area of the mean cell (\bar{G}), thus including the effects of polydispersion, as in Stramski and Reynolds (1993):

$$\bar{G} = \frac{\pi}{4} \int_{D_{\min}}^{D_{\max}} D^2 F(D) dD \times \left[\int_{D_{\min}}^{D_{\max}} F(D) dD \right]^{-1}.$$

The diameter of the mean cell, determined on the basis of the distribution of cross sections is then $\bar{D} = [(4/\pi)\bar{G}]^{1/2}$ (Stramski and Reynolds 1993). The volume of the mean cell was calculated as

$$\bar{V} = \frac{\pi}{6} \int_{D_{\min}}^{D_{\max}} D^3 F(D) dD \times \left[\int_{D_{\min}}^{D_{\max}} F(D) dD \right]^{-1},$$

as in Reynolds et al. (1997).

Carbon—Replicate samples (25 ml) were filtered onto precombusted GF/F filters, frozen, and later dried overnight at 60°C and analyzed on a Perkin-Elmer 2400 CHN analyzer with acetanilide as the standard. Replicate blanks consisting of a wet filter were made at each sampling point. The blanks

were subtracted from the samples to obtain the carbon content of the known volume of culture (and thus number of cells).

Absorption—Absorption was measured in a 1-cm cuvette using a Perkin-Elmer Lambda 18 spectrophotometer with a 60-mm integrating sphere. The samples were preserved with 0.1% glutaraldehyde and stored in liquid nitrogen before analysis. Test samples of fresh versus preserved and frozen samples of *Nannochloris* were virtually identical over the spectra and identical at 665 nm, the wavelength of the beam transmissometer. Two spectra were collected for each sample and averaged. Sterile-filtered *f/2* medium or FSW was used in the reference cuvette and subtracted as the blank. The value at 750 nm was subtracted from the absorption values across the spectra because absorption by phytoplankton is negligible at that wavelength and any signal is due to scattering losses, which are assumed to be spectrally flat.

Calculations of optical properties—Optical properties were calculated assuming that the particles were homogeneous and spherical. The scattering coefficient (b) was calculated from the measured attenuation (c) and absorption (a) coefficients ($b = c - a$; at 665 nm). The cross sections for attenuation (σ_c), absorption (σ_a), and scattering (σ_b) are the relevant coefficients normalized to the cell concentration (e.g., $\sigma_c = c/(N/V)$, where N/V is the number of cells per volume). Cross sections are in units of length squared. Using the anomalous diffraction approximation (Van de Hulst 1957 as described in Morel and Bricaud 1986), a dimensionless efficiency factor for attenuation (Q_c , and similarly for absorption and scattering) was calculated: $Q_c = \sigma_c/\bar{G}$. The imaginary part of the refractive index, n'' , where $n' = (\rho'/4)\{[2\pi(D/2)]/(\lambda/n_w)\}$, with ρ' = optical thickness of absorption, λ = wavelength of light, and n_w (the refractive index of seawater) = 1.34, was then calculated after determining ρ' through iterations from

$$Q_a(\rho') = 1 + 2 \frac{\exp(-\rho')}{\rho'} + 2 \frac{\exp(-\rho') - 1}{\rho'^2}$$

(Morel and Bricaud 1986; Bricaud and Morel 1986). Once ρ' (and thus n'') was determined, the real part of the refractive index was calculated through iterations from

$$Q_c(\rho) = 2 - 4 \exp(-\rho \tan \zeta) \left[\frac{\cos \zeta}{\rho} \sin(\rho - \zeta) + \left(\frac{\cos \zeta}{\rho} \right)^2 \cos(\rho - 2\zeta) \right] + 4 \left(\frac{\cos \zeta}{\rho} \right)^2 \cos 2\zeta$$

where ρ is the phase lag and $\tan \zeta = \frac{1}{2} (\rho'/\rho)$ (Morel and Bricaud 1986; Stramski et al. 1988). Because there are multiple solutions for Q_c , the results were restricted to those reasonable for phytoplankton cells (the first solution with $n > 1.02$ was accepted; all calculated n values were between 1.04 and 1.07).

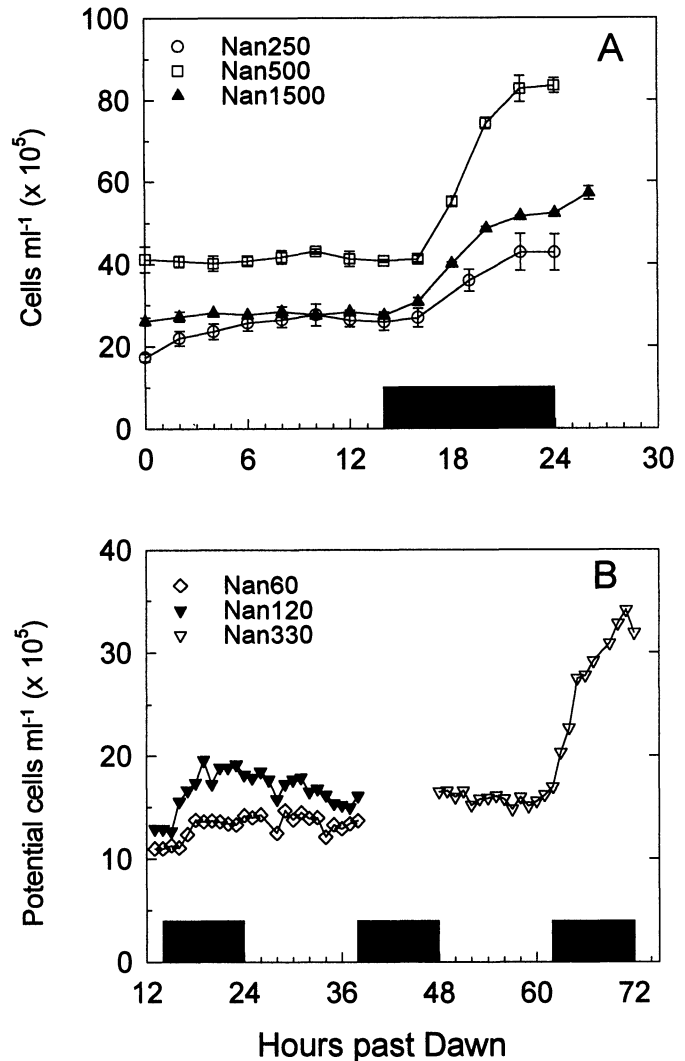


Fig. 2. Cell concentration changes as measured by flow cytometry in batch culture (A) and cyclostat (B) experiments. Black bars indicate periods of darkness. In A, each point represents the average value for replicate cultures, and error bars show standard errors. In B, the potential cell concentration is shown (measured cell concentration with the dilution rate of the cyclostat taken into account: potential cells ml⁻¹ = measured cells ml⁻¹ + hourly dilution rate × number of hours elapsed × measured cells ml⁻¹). Note that Nan120 and Nan330 were the same culture with only the light level changed for the second day.

Results and discussion

Measurements of cell properties—All experiments showed tight phasing of cell division to the LD cycle (Fig. 2). In each case, the major burst of division started 2 h after darkness and continued for 4–6 h (although for Nan250, there was some cell division occurring during the early part of the preceding light period). Even though Nan1500 was performed outdoors in natural light, and thus the cells were exposed to a higher light level and a true dawn and dusk (Fig. 1), the timing of the cell division for this experiment was similar to that of the others. The fraction of cells divid-

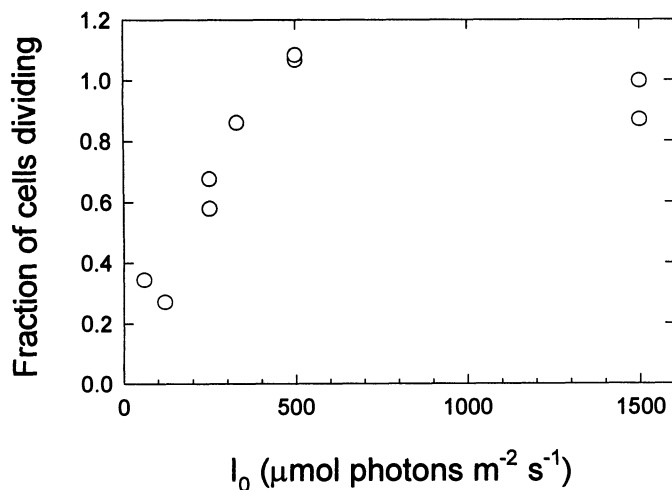


Fig. 3. Fraction of cells dividing at night, determined from changes in cell concentration during the night, for cultures grown at different light levels (I_0 = scalar PAR, in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; LD = 14:10).

ing during the division burst ranged from 0.3 to 1.1, which corresponds to specific growth rates of 0.2 to 0.7 d^{-1} (Fig. 3).

In each experiment, the mean cell volume of *Nannochloris* was minimum near dawn and maximum near dusk, as evident from the time series of \bar{V} (Fig. 4) and the cell-diameter distributions at selected times (Fig. 5). It should be noted that the mean cell size for the dawn time points (when the cells were smallest) is an overestimate because the smallest cells were in the region affected by instrument noise and were not included. This effect was most obvious in Nan250, in which a larger orifice (50 μm) was used on the Coulter Multisizer. Flow cytometric measurements of mean forward scattering cross section (Fig. 6) showed patterns similar to those of mean cell volume. Side light scattering (also from flow cytometric measurements) showed similar patterns and was internally consistent within each experiment, but comparisons between experiments were not possible because the signal changed significantly with preservation (data not shown). Carbon content per cell also increased during the day and decreased at night (Fig. 7). All these results are consistent with individual cells photosynthesizing to add carbon and grow in size during the day, then dividing in the dark to produce smaller cells with less carbon and lower scattering per cell.

The calculated values of the real part of the refractive index (n) ranged from 1.04 to 1.066 (Fig. 8A), which is within the range expected for phytoplankton cells (Aas 1981 as referred to by Morel and Bricaud 1986; Bricaud et al. 1988). There appears to be a small diel pattern in n , with n decreasing during the night. The slope of a linear regression fitted to the values of n over time during the night was significantly less than zero for all the experiments (t -test, $P < 0.05$). The real part of the refractive index increased significantly during the day for Nan500 (t -test, $P < 0.0025$). Although n also increased during the daytime for Nan250 and Nan 1500, the increases were not significant, perhaps because most of the increase occurred between the first two

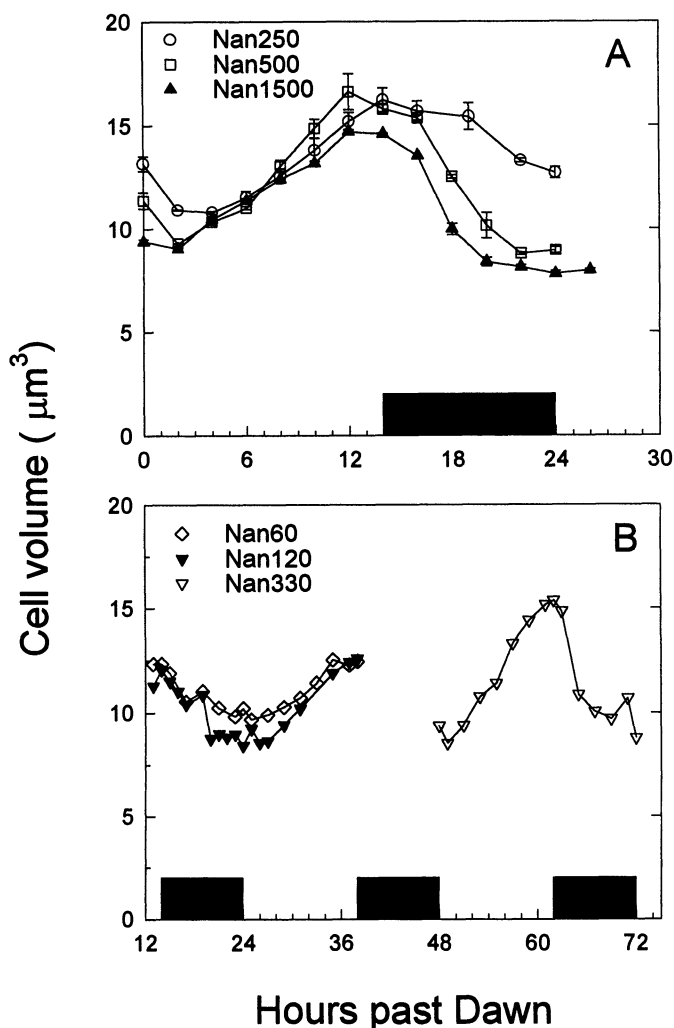


Fig. 4. Diel variations in mean cell volume (\bar{V}) from Coulter electronic particle sizer in batch culture (A) and cyclostat (B) experiments. For batch cultures (A), average ($n = 2$) and standard error bars are shown.

points for Nan1500 and because the first point for Nan250 was lost.

Changes in refractive index most likely reflect the changing composition of the cells, due to the different refractive indices of internal components and the cell water content, as they grow and divide (Aas 1981 as referred to by Morel and Bricaud 1986). The intracellular carbon concentration (C_i) shows a pattern similar to that of n , the real part of the refractive index (Fig. 8B). For Nan250 and Nan500, both n and C_i clearly decrease at night, when the cells are dividing. This may reflect either uptake of water (swelling) or loss of carbon through respiration. Markager et al. (1992) observed a faster rate of dark respiration for the first 2 h in the dark for a community of phytoplankton in a eutrophic lake. In studies of *Isochrysis galbana*, Carder et al. (1972) suggested that increases in refractive index were caused by increases in the surface area-to-volume ratio of the cells, because the cell wall was expected to be one of the most refractive parts. However, if this were the case, we would expect an increase

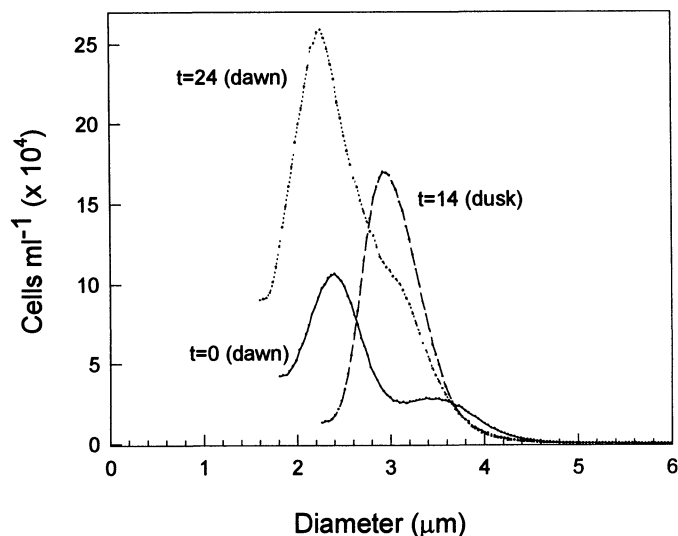


Fig. 5. Cell size distributions for Nan500 at first dawn ($t = 0$), dusk ($t = 14$), and second dawn ($t = 24$) from Coulter Multisizer data.

in n with cell division instead of the observed decrease, because the newly divided (smaller) cells would have a higher surface area-to-volume ratio. It is interesting to note that the highest n (close to 1.06) and C_i values and the smallest changes in both properties during the diel cycle are for the natural light experiment (Nan1500). This could be due to the higher light level or the fact that there was a true dawn and dusk for that experiment. Additional experiments are required to further investigate these effects.

The calculated value of the imaginary part of the refractive index, n' , generally ranging from 0.002 to 0.004 (Fig. 8C), was similar to that determined by others for phytoplankton cells ($n' = 0.004$ at 660 nm for *T. pseudonana*; Stramski and Reynolds 1993). For all three experiments, the changes seen were relatively small, and there was no consistent diel pattern.

Both the attenuation cross section (σ_t) and the scattering cross section (σ_s) at 665 nm increased during the day to reach a maximum at dusk, then decreased at night (Fig. 9A,B). These patterns were similar to those seen in mean cell volume (Fig. 4), FLS per cell (Fig. 6), and carbon per cell (Fig. 7). The absorption cross section (σ_a ; Fig. 9C) was always small compared with the attenuation cross section; it tended to increase during the day and decrease at night. The trends in Q_a were similar to those of σ_a , with an average value of 0.12 (data not shown). The chlorophyll fluorescence per cell, measured with the flow cytometer, showed similar patterns to σ_a (Fig. 10); chlorophyll fluorescence per cell and σ_a were strongly correlated for the batch culture experiments ($r^2 = 0.89$, $n = 39$). Chlorophyll fluorescence normalized to cell volume ranged from lowest at the highest light level to highest at the lowest light level in the following order: Nan1500, -500, -250, -330, -120, and -60 (data not shown).

The real part of the refractive index (at 665 nm) and intracellular carbon concentration were strongly correlated ($n = 36$, $r^2 = 0.75$; Fig. 11). The slope of the relationship (1.17×10^{-4} , which is a 0.117 increase in n for every 1 g cm^{-3}

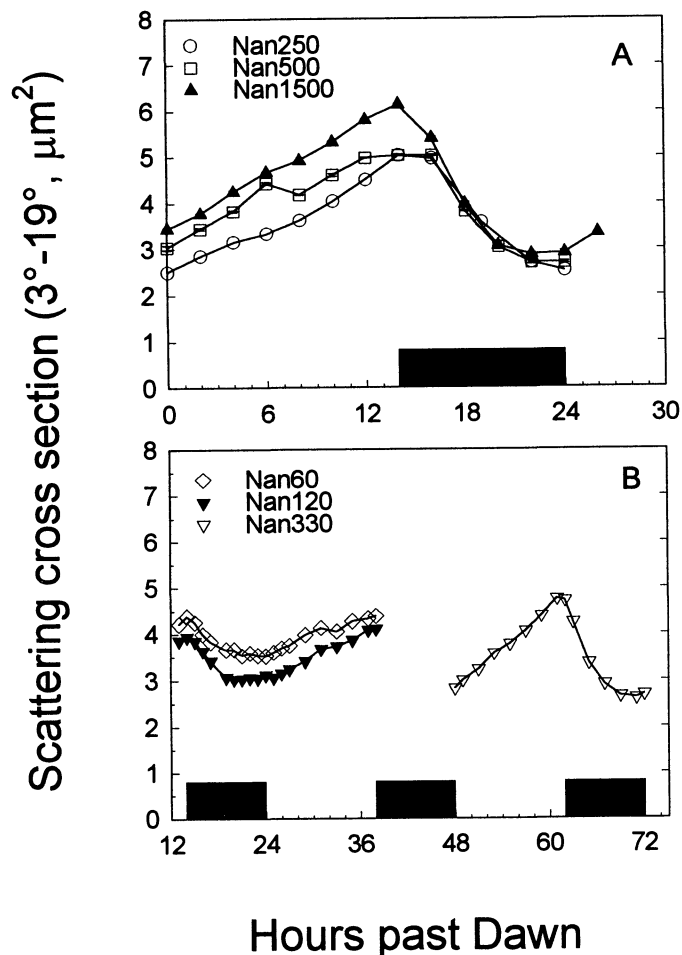


Fig. 6. Diel variations in forward scattering cross section ($3\text{--}19^\circ$, in μm^2) for batch culture (A) and cyclostat (B) experiments. Standard error bars plotted in A are smaller than the symbols.

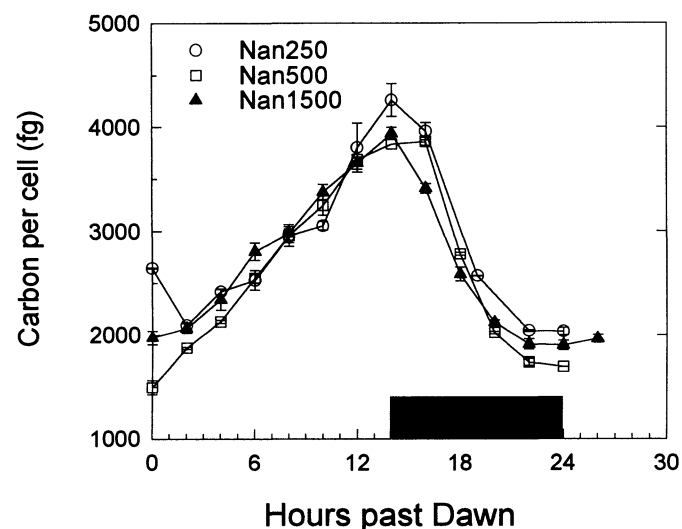


Fig. 7. Diel variations in carbon per cell for Nan250, Nan500, and Nan1500. Average ($n = 2$) and standard error bars are shown.

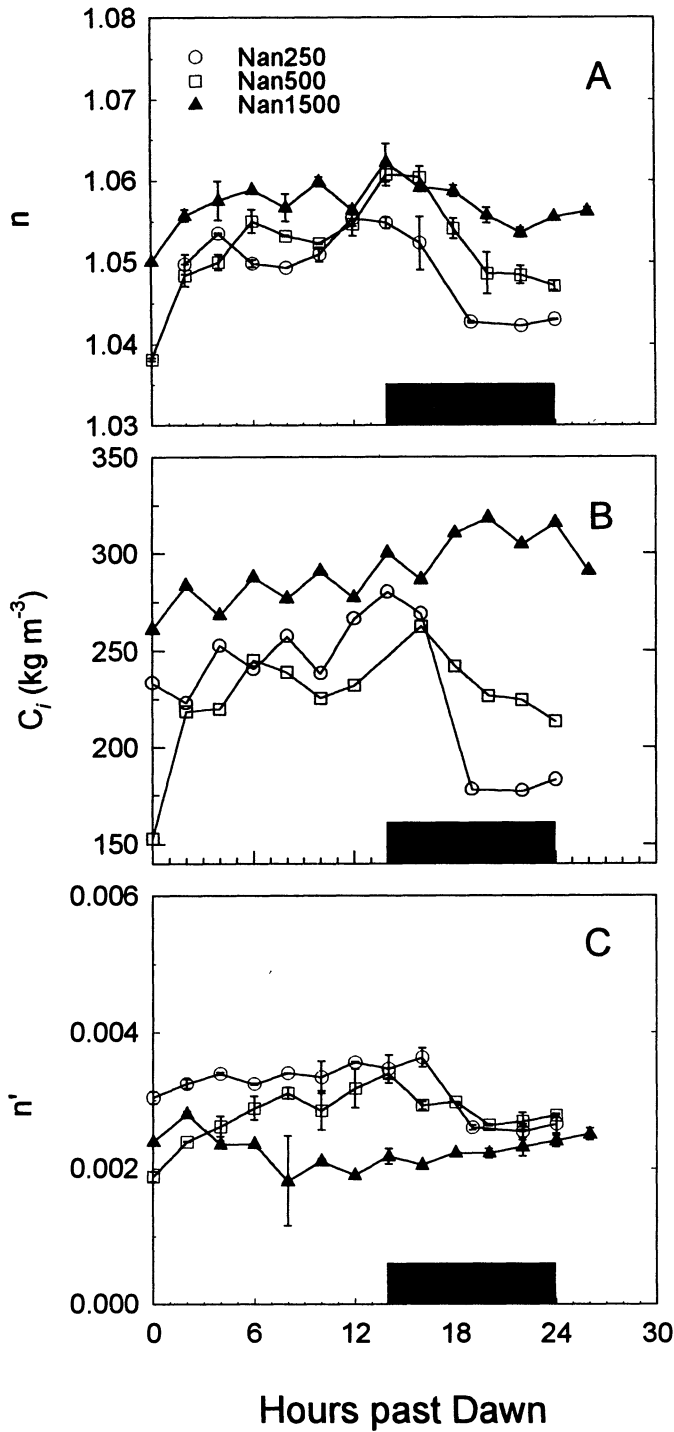


Fig. 8. Time series of n , the calculated real part of the refractive index (A), C_i , the intracellular carbon concentration (B), and n' , the calculated imaginary part of the refractive index (C) of the *Nanochloris* cells for Nan250, Nan500, and Nan1500. All calculations were done at 665 nm.

increase in C_i) was lower than the slope calculated by Morel and Ahn (1990) based on the contributions of the components of a cell (2.33×10^{-4} at 415 nm). It was also lower than that measured by Stramski and Reynolds (1993) for *T.*

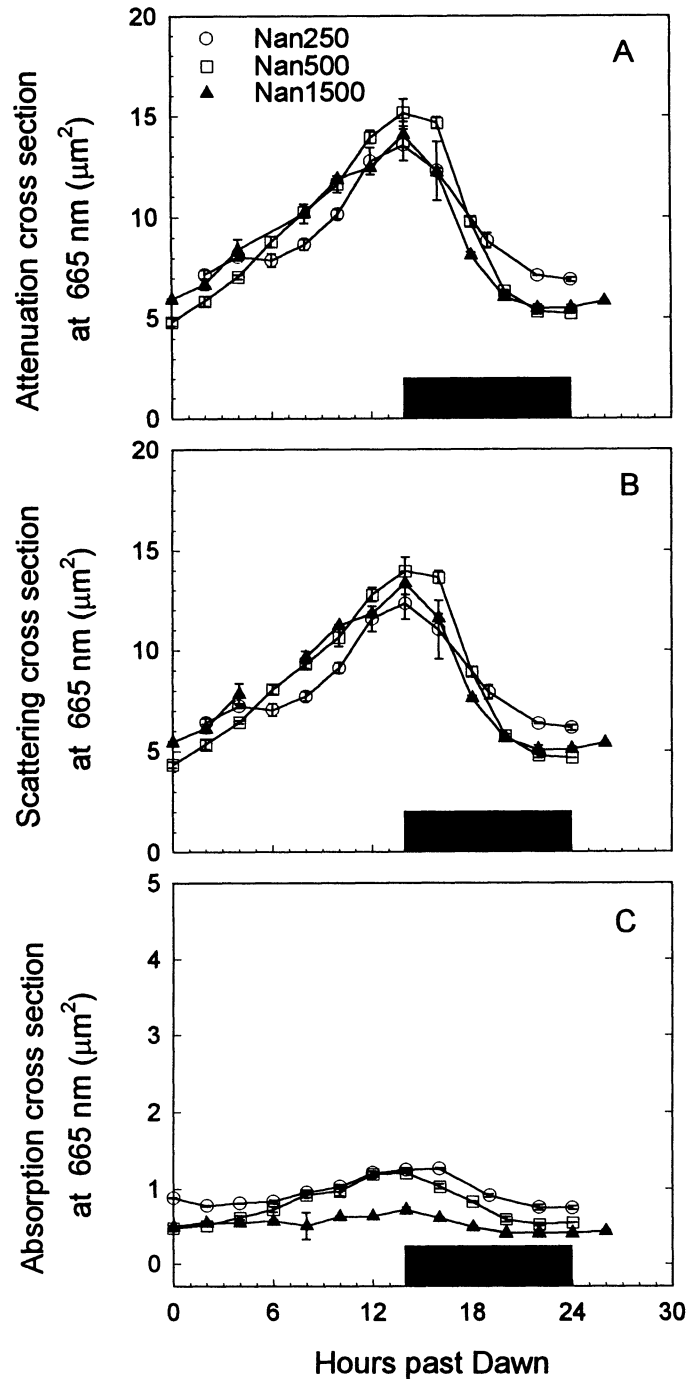


Fig. 9. Diel variations in the attenuation (σ_t) (A), scattering (σ_b) (B), and absorption cross sections (σ_a) (C) at a wavelength of 665 nm for Nan250, Nan500, and Nan1500. The attenuation and absorption cross sections are from bulk measurements of attenuation and absorption normalized to the cell concentration. The scattering cross section was obtained by subtraction. Note the different y axis scaling for C.

pseudonana (1.75×10^{-4} at 660 nm), Stramski et al. (1995) for *Synechococcus* (1.65×10^{-4} at 443 nm and 1.74×10^{-4} at 660), and Stramski and Morel (1990) for *Synechocystis* (2.43×10^{-4} at 440 nm). Thus, compared with previous cells

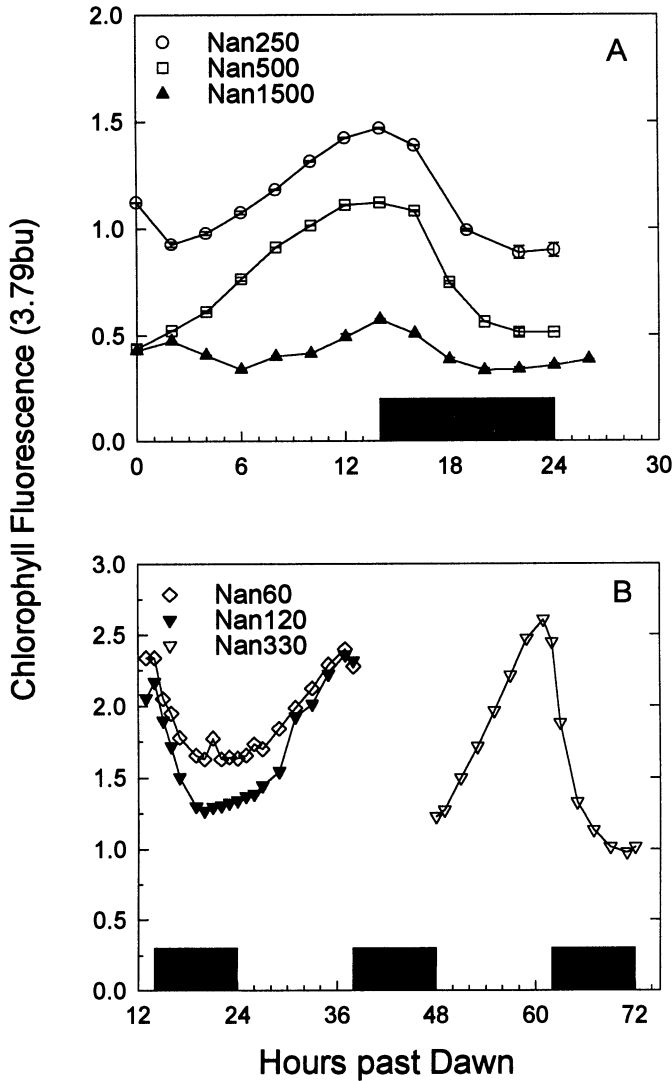


Fig. 10. Chlorophyll fluorescence per cell from flow cytometric analysis for batch culture (A) and cyclostat (B) experiments as a function of time. Values are expressed relative to a standard (3.79- μm) bead.

tested, *Nannochloris* appears to have a smaller increase in n for the same change in intracellular carbon concentration.

There was a strong correlation between the mean flow cytometric forward scattering cross section (at 488 nm) and the total scattering cross section (σ_b , at 665 nm) determined by subtraction of the measured σ_c and σ_a values when data from the three batch culture experiments were grouped ($r^2 = 0.82$; Fig. 12). The strong agreement of these independent assessments of scattering cross sections is consistent with Mie theory for cells in certain ranges of size and refractive index (i.e., for diameters of about 1–5 μm and n values of 1.04–1.07, which includes cells in these experiments). This is evidence that flow cytometry can provide a quantitative link between bulk optical properties and those of individual cells. This link would be expected to be valid only when the optical properties of the water are dominated by cells in these size and refractive index ranges (for particles outside

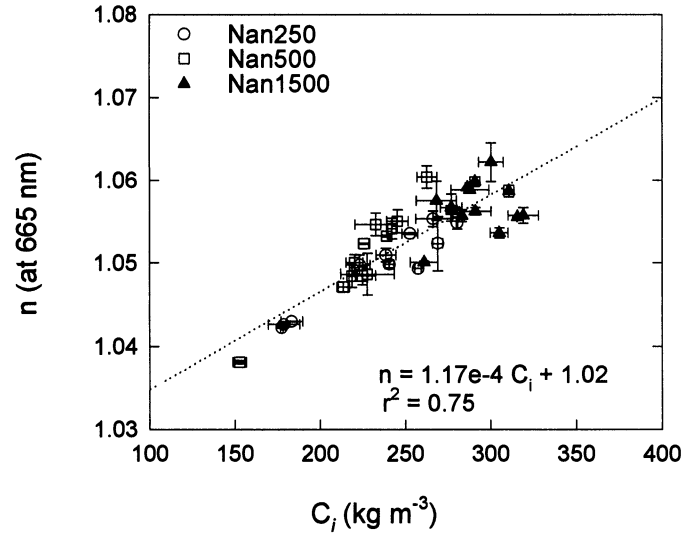


Fig. 11. Relationship between the real part of the refractive index (n , at 665 nm) and intracellular carbon concentration (C_i , in kg m^{-3}) for Nan250, Nan500, and Nan1500.

these ranges, the oscillatory nature of the size–scattering relationship makes the situation much more complex), but previous observations suggest that this situation is not uncommon in the ocean. We have observed in the equatorial Pacific (DuRand and Olson 1996) and the Sargasso Sea (DuRand 1995) that flow cytometric forward light scattering of *Prochlorococcus*, *Synechococcus*, and small eukaryotic phytoplankton exhibits diel variations similar to those reported here for cultures of *Nannochloris*. When the contributions of the different groups of phytoplankton to bulk water beam attenuation are calculated, it is most often cells similar in size to *Nannochloris* that make the greatest contributions.

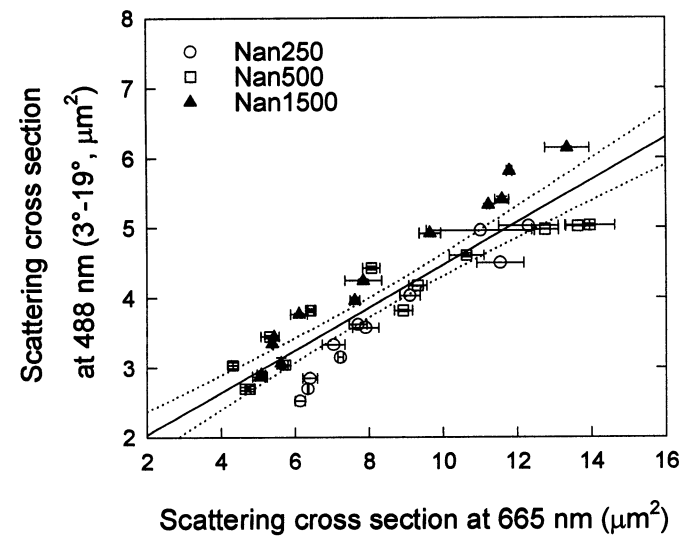


Fig. 12. Relationship between flow cytometric forward (3–19°, at 488 nm) and total scattering cross section (calculated from measured attenuation and absorption at 665 nm). Linear regression ($r^2 = 0.82$, $y = 0.30x + 1.4$) and 95% confidence intervals are shown.

Estimation of cell volume and cell division—If cell division is phased to the diel cycle, as it appears to be for many phytoplankton groups (Chisholm 1981), it should be possible to estimate division rates from changes in mean cell volume. If, for example, all the cells in a population divide during the night (when photosynthesis is not a factor), then the average cell volume would be expected to double during the day and then halve upon division at night. In general, the relative changes in mean cell volume will be a minimum estimate of the fraction of cells dividing, because both asynchrony and cell division during the day will tend to reduce the observed excursions in mean cell volume. In numerous intensive sampling experiments in oceanic waters, we have observed diel variations in flow cytometric FLS (from which cell volume can be estimated) for different phytoplankton groups similar to those seen in these laboratory experiments (DuRand 1995; DuRand and Olson 1996). Field observations of phytoplankton groups show steadily increasing FLS over the day with a single peak near dusk, indicating that the bulk of the cells are phased and dividing at night.

The relationship between cell size and FLS changes is nonlinear; for cells in the size range of *Nannochloris*, FLS should vary with diameter to the second or third power (i.e., with area or volume) depending on the assumed refractive index and angles of collection (results of Mie theory calculations performed using the program of Ackleson and Spinrad 1988). It should be emphasized that small changes in the refractive index can considerably change the relationship between FLS and cell size. Over the small ranges of size in these laboratory experiments (close to twofold), a linear relationship between volume and FLS described the data well for *Nannochloris*. Even with the changes in refractive index observed in these experiments, there was a strong correlation between forward scattering cross section and cell volume.

For each *Nannochloris* experiment, a linear regression was obtained between forward scattering cross section ($\sigma_{b,3-19^\circ}$) and cell volume (Fig. 13). An analysis of covariance was performed, and the hypothesis that all slopes (β) were equal was rejected ($P < 0.0005$). A multiple comparison testing of the slopes (Tukey test) revealed that they came from two separate populations, where $\beta_{60} = \beta_{120} = \beta_{330} = \beta_{500} \neq \beta_{1500}$. Nan1500, the natural light experiment, is different from the others, probably because the refractive index was higher (Fig. 8A). (We cannot conclude how β_{250} is related to the slopes of the other populations. This may be the result of overestimates of cell volume at the time points that are outliers on Fig. 13 because the Coulter volume measurements in the Nan250 experiment were made with a larger orifice than for the other experiments and because some cells were below the detection limit).

For each of the two distinguishable groups of data, we found well-correlated relationships (for Nan1500: volume = $2.22 \times \sigma_{b,3-19^\circ} - 1.30$, $n = 27$, $r^2 = 0.97$; for Nan60, Nan120, Nan330, and Nan500 combined: volume = $2.83 \times \sigma_{b,3-19^\circ} - 0.79$, $n = 77$, $r^2 = 0.75$), despite the changes in refractive index observed over the diel cycle. However, even if the refractive index does not change significantly over the diel cycle, different refractive indices (e.g., for different types of cells or different amounts of nutrient limitation) will

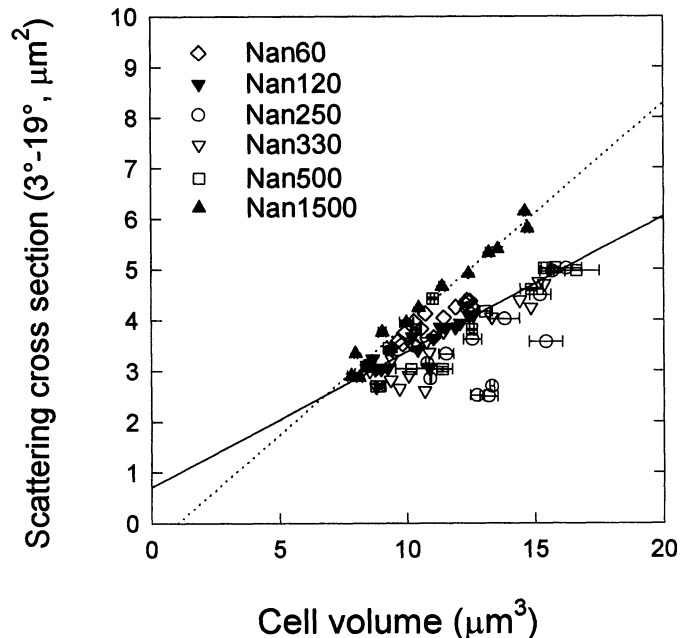


Fig. 13. Relationship between forward scattering cross section (3–19°, in μm^2) and cell volume (in μm^3) for Nan60, Nan120, Nan250, Nan330, Nan500, and Nan1500. The dotted line is a linear regression on the Nan1500 data only ($r^2 = 0.987$, $y = 0.44x - 0.44$). The solid line is a linear regression on the combined data for Nan60, Nan120, Nan330, and Nan500 ($r^2 = 0.75$, $y = 0.27x + 0.71$).

change the relationship between volume and $\sigma_{b,3-19^\circ}$. Despite this caveat, relationships such as these have been used with some success to estimate mean cell volume from flow cytometric mean forward scatter of phytoplankton populations in the field and thus to make estimates of the number of dividing cells (DuRand 1995). Such estimates would be improved if refractive index as well as volume were estimated. This has been done from flow cytometric measurements of side light scatter in conjunction with FLS (Ackleson and Spinrad 1988; Ackleson et al. 1988a). We are investigating this approach.

Because nearly all cell division occurred during the night, decreases in FLS or volume (which are strongly correlated, Fig. 13) during the night should provide good estimates of growth rates. For these experiments, growth rates calculated from the decrease in FLS or volume during the night, $\mu = \ln(\text{max}/\text{min})$, are well correlated with the growth rates calculated from the cell concentration changes during the nighttime division burst, $\mu = \ln(N_{\text{after}}/N_{\text{before}})$. The value of r^2 is 0.88 for the linear regression (with zero intercept) for the FLS calculation ($\beta = 0.93$) and 0.78 for the volume calculation ($\beta = 0.81$, $n = 9$, $P < 0.001$ for each regression). Thus, diel changes in cell forward light scattering or cell volume provide reasonable estimates of the growth rate of *Nannochloris* cultures determined from the nighttime division burst, which would be expected to be a minimum estimate of the specific growth rate (and equal to it if the cells are phased and dividing only at night).

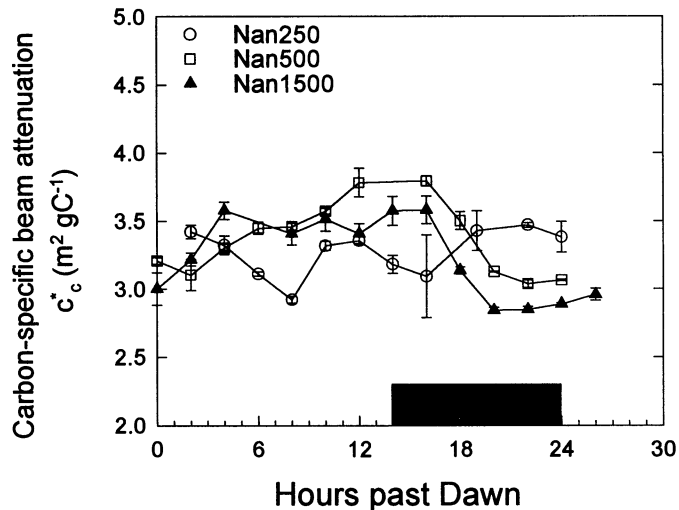


Fig. 14. Time series of the carbon-specific beam attenuation (c_c^* , in $\text{m}^2 (\text{g C}^{-1})$) for Nan250, Nan500, and Nan1500.

Carbon-specific beam attenuation—The carbon-specific beam attenuation (c_c^* , Fig. 14) varied by up to 25% over the course of the measurements. Both Nan500 and Nan1500 tended to increase during the day and decrease rapidly concurrently with cell division at the beginning of the night; Nan250 showed no distinct pattern. This is important in relation to estimates of carbon production from diel variations in beam attenuation at sea (Siegel et al. 1989; Cullen et al. 1992; Walsh et al. 1995). In these cases, a constant c_c^* was applied to represent all phytoplankton at all times of the day. If, indeed, c_c^* changes during the day, then these conclusions may not be valid. The increase from dawn to dusk is up to 25% for *Nannochloris* (reported here) and about 30% for slowly growing *Synechococcus* (Stramski et al. 1995) but has no consistent pattern from dawn to dusk for *T. pseudonana* (although it had up to 30% variation over a 3-d sampling period; Stramski and Reynolds 1993). Our average c_c^* for *Nannochloris* of $3.38 \text{ m}^2 (\text{g C}^{-1})$ ($n = 49$, $\text{SD} = 0.34$) is similar to that of Stramski and Reynolds for *T. pseudonana*, 3.81. Stramski et al. found *Synechococcus* to have a lower value of $2.48 \text{ m}^2 \text{ g}^{-1} \text{ C}$. We also found a lower c_c^* value for *Synechococcus*, 1.52 ($n = 8$ and $\text{SD} = 0.26$ for four strains, unpubl. data). It therefore appears that there can be significant differences in the carbon-specific beam attenuation both with time of day and among different species. Thus, it may be possible to improve estimates of production by using values for c_c^* that reflect these diel changes for different groups of phytoplankton (DuRand and Olson 1996). Without accounting for the composition of the phytoplankton community and the diel variations in c_c^* , accurate estimates of primary production from diel variations in optical measurements cannot be made. By using a variable c_c^* in a physiological-optical model, Cullen and Lewis (1995) found the resulting production estimates to be consistent with other measurements in the equatorial Pacific. They state that, in a “fairly arbitrary” way, a c_c^* value that increased by 30% during the early part of the day was chosen. This is similar to the changes that we saw from dawn

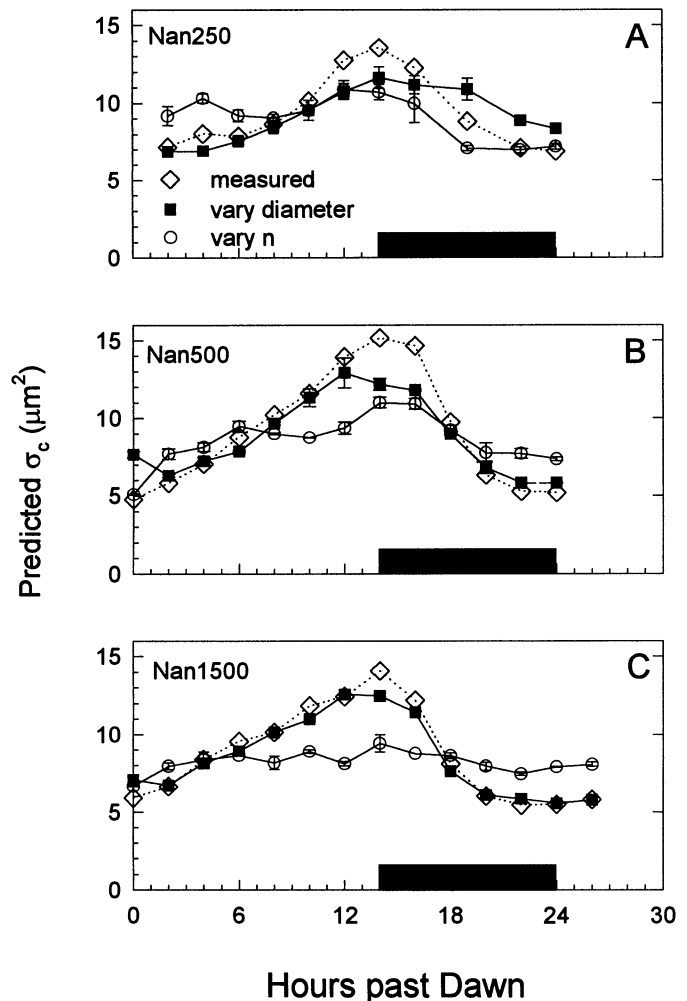


Fig. 15. Results of the sensitivity analysis of attenuation cross section (σ_c) to varying diameter at a constant real part of the refractive index, n , and varying refractive index at a constant diameter compared with the measured σ_c for Nan250 (A), Nan500 (B), and Nan1500 (C).

to dusk for *Nannochloris*. However, their initial starting value of $2 \text{ m}^2 (\text{g C}^{-1})$ was considerably lower than that determined in this work ($3.38 \text{ m}^2 (\text{g C}^{-1})$).

Contributions of diameter and refractive index to σ_c —Previous studies on *T. pseudonana* and *Synechococcus* indicated that changes in refractive index could be even more important than changes in cell size in determining σ_c , depending on the time of day (Stramski and Reynolds 1993; Stramski et al. 1995). To determine the relative effects of the two variables on *Nannochloris*, we performed a sensitivity analysis to estimate the contributions of changes in diameter and in the real part of the refractive index to the attenuation cross section (σ_c ; Fig. 15). Using the data from each carboy in each diel experiment, the mean cell diameter was held constant at its average value, and Q_c and σ_c were calculated to determine the effect of changing n on σ_c (n' , which had only a small effect, was assumed to be zero for

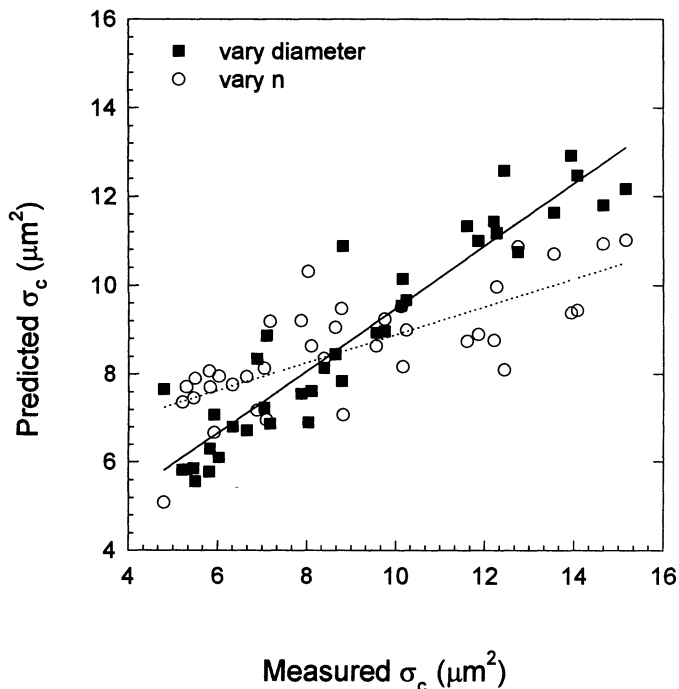


Fig. 16. Relationship between the measured σ_c and predicted σ_c determined by varying diameter (\bar{D}) or varying refractive index (n). The linear regression for varying \bar{D} is $y = 0.70x + 2.45$ (solid line, $r^2 = 0.88$) and for varying n is $y = 0.31x + 5.75$ (dotted line, $r^2 = 0.57$).

these calculations). Similarly, n (calculated as described in *Materials and methods*) was held constant (at its average value) and Q_c and σ_c were calculated to determine the effect of changing diameter on σ_c . It should be noted that inaccuracies in σ_c due to measurement errors or effects of non-phytoplankton attenuation in the cultures will affect the calculated refractive index, because n is originally calculated from diameter and σ_c . Thus, n is not independently determined; measurement errors in σ_c will spuriously improve the correlation between n and σ_c . In addition, any overestimates in diameter at the dawn time points (due to the smallest cells being lost in the noise, *see above*) would cause a smaller increase in diameter over the diel cycle, which would lead to an underestimate of the effect of variations in diameter. Even with these artifacts, it is apparent that the observed variations in diameter through the diel cycle had a greater effect on the variation in σ_c than did those of refractive index (Fig. 16). We quantified the relationship between measured σ_c and predicted σ_c by linear regression, which attributes 88% of the variations in measured σ_c to variations in predicted σ_c from varying diameter and 57% to variations in predicted σ_c from varying n . The artifacts noted above most likely have falsely increased the percentage attributed to varying n (and contributed to our accounting for >100% of the variations in σ_c).

Conclusions

Cell division of *Nannochloris* was tightly phased to the LD cycle, occurring soon after dark for light levels ranging

from 60 to 1,500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Cell size and cell-specific attenuation, absorption, flow cytometric light scatter, and carbon were all minimum near dawn and maximum near dusk. Diel variations in cell size due to growth and division were the primary cause of changes in the attenuation cross section, although variations in refractive index also contributed. Because eukaryotic cells similar in size to *Nannochloris* are significant components of many phytoplankton communities, these results have important consequences for interpreting diel variations in oceanic optical properties. For example, the relationship between flow cytometric FLS and beam attenuation calculated in this work can be applied to field measurements made using flow cytometry (within certain ranges of cell size and refractive index) to estimate the contributions of particular phytoplankton populations to this bulk optical property (DuRand and Olson 1996). In addition, because the variations in FLS largely reflect cell growth processes, it should be possible to estimate in situ growth rates of different phytoplankton groups from diel measurements of FLS. The accuracy of such estimates, however, depends on simultaneous estimates of refractive index.

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