Vertical variability and diel dynamics of picophytoplankton in the Strait of Sicily, Mediterranean Sea, in summer

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ABSTRACT: Phytoplankton pigment diversity and photoacclimation during the natural day–night cycle was investigated at a fixed location in the Strait of Sicily in July 1997 using HPLC pigment analysis on fractionated samples (<3 and >3 µm) and flow cytometry. Picophytoplankton dominated phytoplankton biomass in terms of chl *a* with an average value of 57% and was mainly represented by prokaryotes, prymnesiophytes and pelagophytes. *Prochlorococcus* and picoeukaryotes contributed equally to the picophytoplankton in terms of chl *a*, but *Prochlorococcus* were numerically more abundant and were represented by 2 ecotypes, one replacing the other according to depth. Larger phytoplankton were dominated by prymnesiophytes and diatoms. Photoacclimation was evident from changes in pigment content and strongly increased with depth. The deep chlorophyll maximum (DCM), present between 75 and 90 m, showed a diverse and rich phytoplankton community with the 2 size classes almost equally represented. Growth rates of *Prochlorococcus* and *Synechococcus*, estimated from cell cycle measurements, were 0.67 and 0.41 d^{-1}, respectively, at 75 m. Only *Prochlorococcus* was able to sustain a good growth rate of 0.43 d^{-1} at the base of the DCM (90 m) where only 0.5% of incident light was available. Light-shift experiments using onboard incubated natural seawater showed much faster kinetic coefficients for acclimation in picophytoplankton than in larger algae. In general, the data describe the dynamics of picophytoplankton and its light adaptation through the water column and in the DCM, and can be considered representative of stable summer conditions in the Mediterranean Sea.

KEY WORDS: Picophytoplankton · DCM · Pigments · Flow cytometry · Photoadaptation · Vertical variability · Diel variability

INTRODUCTION

Phytoplankton responses to light variability on a diel scale are very relevant for their dynamics as they allow the cells to acclimate to the surrounding system and to optimize their performance. Indeed, phytoplankton is highly synchronized to the light–dark cycle (Vaulot et al. 1995) and the timing of division is different in different species and groups, and even strains (Vaulot & Marie 1999). This variability is also responsible for observed variations in optical properties of seawater (DuRand et al. 2002) and is, therefore, a critical factor to consider when estimating primary production from

changes in optical properties such as beam attenuation (Siegel et al. 1989).

Picophytoplankton is an essential component of all marine ecosystems in terms of biomass, diversity and production (Veldhuis et al. 2005). It is responsible for a significant fraction of marine primary production (Li 1995) and shows elevated photophysiological plasticity when compared with larger phytoplankton (Partensky et al. 1993, Timmermans et al. 2005). Compared with the prokaryotes, picoeukaryotes have received less attention despite their significant contribution to carbon fixation (Li 1995, Worden et al. 2004) mainly because of the technical limitations to their

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identification. Recently developed molecular tools are continuously increasing the assessment of picoeukaryote diversity in terms of species and groups (Moreira & Lopez-Garcia 2002). However, very few studies report the *in situ* ecophysiological properties of picophytoplankton in general (e.g. Neveux et al. 2003, Veldhuis & Kraay 2004) and even fewer focus on the picoeukaryotes (Veldhuis et al. 2005).

We present data on pigment diversity and photoacclimation of picophytoplankton on a diel time scale, investigated at a fixed station in the Strait of Sicily, during a 51 h sampling period in summer. The aim of the study was to investigate and compare the effects of vertical and diel variability upon phytoplankton ecophysiological properties, focusing on picophytoplankton (especially picoeukaryotes) responses to variable irradiance. The responses to light variations were analysed by considering both variations with depth and with time during 2 natural day–night cycles in terms of community composition and photophysiology.

The study site, the Strait of Sicily, is an important location in the Mediterranean Sea, as it governs the exchanges between the eastern and western basins and is characterized by active mesoscale dynamics (Lermusiaux & Robinson 2001), which strongly influence the ecology of phytoplankton communities.

MATERIALS AND METHODS

Sampling. The 150 m deep sample site (36° 25.00′ N, 15° 20.03′ E) was located in a stable area of the Ionian Water away from mesoscale instabilities, which characterize the area. Hydrological conditions remained constant for the entire sampling period and were representative of the oligotrophic Mediterranean Sea in summer. The station was sampled during a 51 h period from 29 to 31 July 1997. Hydrological profiles of salinity, temperature, density, transmittance and fluorescence were obtained every 1.5 h using a CTD probe (SBE 19, Seabird Electronics) coupled with a Sea-Tech fluorometer. Underwater light profiles (PAR) were obtained from a spectroradiometer SPMR (Satlantic).

Discrete samples from each of 6 depths (5, 20, 30, 50, 75 and 90 m) were taken using a rosette sampler equipped with 24 Niskin bottles every 3 h for nutrient and pigment concentrations and flow cytometric analysis of picophytoplankton.

Dissolved inorganic nutrients. Nitrate, nitrite, silicate and phosphate concentrations were determined on fresh samples at the site on board the ship using the colorimetric procedure of Grasshof (1983) and a Technicon system.

Flow cytometry. Fresh untreated samples were analysed on board using a FACScalibur flow cytometer (Becton Dickinson) equipped with a standard laser and filter set and using 0.22 µm filtered seawater as sheath fluid. Fluorescent beads with a diameter of 0.97 µm (Polysciences) were added to each sample as internal standards, and all parameters were normalized to their values and expressed as relative units (r.u.). The red fluorescence from chlorophyll (chl) *a* and the orange fluorescence from phycoerythrin were collected through a 650 long pass filter and a 585/42 band pass filter, respectively. Two scattering parameters were also measured: FALS (Forward Angle Light Scatter), which is used as a proxy of size, and RALS (Right Angle Light Scatter), which is particularly sensitive to both size and particle refractive index. Data acquisition and analysis were performed with the CellQuest software (Becton Dickinson). Further details can be found in Casotti et al. (2000).

Three populations of picophytoplankton were identified and enumerated based upon their scattering and autofluorescence: *Prochlorococcus* spp., *Synechococcus* spp. and picoeukaryotes. In the case of *Prochlorococcus* populations with very low red fluorescence at the surface, the population was assumed to have a normal distribution and the hidden portion was extrapolated, as indicated by Casotti et al. (2003).

Tests to compare the counts in the total samples with counts in the <3 µm fractionated samples used for HPLC analyses showed that all picoeukaryotes passed through the 3 µm filter and that very few (<0.1%) and only occasionally larger cells (nanophytoplankton) were detected in the filtered samples.

Fixed (0.5% glutaraldehyde, Vaulot et al. 1989) and frozen (liquid nitrogen) samples were analysed after thawing and staining the DNA with SYBR Green I, according to the protocol of Marie et al. (1997). The proportion of cells in the different phases of the cell cycle was estimated using the ModFit software (Verity) and from these, growth rates were determined using the method of Carpenter & Chang (1988).

HPLC. For each sample, 3 l of seawater were filtered onto Nuclepore filters of 3 µm pore size and the filtrate onto Whatman GF/F filters. Filters were frozen and stored at –40°C in the dark for a maximum of 2 wk before analysis. Frozen filters were mechanically ground in 100% methanol and the extract injected into a Beckman System Gold HPLC following the procedure of Vidussi et al. (1996) using a 3 µm C_8 BDS column (100 × 4.6 mm). The mobile phase was composed of 2 solvents: (1) a 70/30 mixture of methanol/aqueous ammonium acetate, and (2) methanol. Absorbance was detected at 440 nm using a Model 168 Beckman photodiode array detector. Fluorescence was measured using an Sfm 25 Kontron spectrofluorometer with excitation at 407 nm and emission at 660 nm. Monospecific algal cultures and purified pigments from the Water

Quality Institute, International Agency for ^{14}C Determination (Denmark) were used as standards. The chemotaxonomic descriptors were alloxanthin (cryptophytes), chlorophyll *b* (chl *b*) + divinyl chlorophyll *b* (DVchl *b*) (green algae, including prasinophytes), 19′ butanoyloxyfucoxanthin (pelagophytes), fucoxanthin (diatoms), 19′ hexanoyloxyfucoxanthin (prymnesiophytes), peridinin (dinophytes) for both size classes, and zeaxanthin (prokaryotes) for the <3 µm size class.

To investigate picoeukaryote photoacclimation, intracellular chl *a* concentrations of these algae were estimated by dividing the fraction of the <3 µm chl *a* attributable to picoeukaryotes by their cell numbers obtained by flow cytometry (Brunet et al. 2006) following a 2-step procedure. Employing zeaxanthin as a marker pigment of prokaryotes, we used the mean value of 0.77 for the ratio zeaxanthin/divinyl chl *a* (DVchl *a*), as reported by Partensky et al. (1993) in a culture study of photoacclimation in a Mediterranean strain of *Prochlorococcus*. In addition, we calculated the zeaxanthin contribution of *Synechococcus* by using a value of 1.8 fg zeaxanthin per *Synechococcus* cell, following Kana et al. (1988) who investigated photophysiology of *Synechococcus* under a wide range of light intensities. This allowed us to account for the zeaxanthin attributable to the 2 cyanobacteria. The second step used a conversion factor of 0.5 for zeaxanthin/chl *a*, which allowed separation of *Synechococcus* from the picoeukaryote contribution to chl *a*. This approach is based upon a number of assumptions and represents an approximation. It is limited by the fact that it uses fixed values for the pigment ratios, which may vary with light conditions. However, the zeaxanthin/chl *a* ratio is not reported to vary considerably with changing light conditions (Kana et al. 1988, Moore et al. 1995). The limitations of the method are discussed in Brunet et al. (2006) and, despite the obvious bias introduced by extrapolating from single strains in culture to the properties of complex natural communities, the method is considered the best approximation possible and was very useful in estimating photoacclimation in picoeukaryotes. In fact, values were drawn from studies encompassing a wide range of light conditions and for *Prochlorococcus,* originated from a strain isolated in the oligotrophic Mediterranean Sea, which can be considered representative of the populations present in the study area.

Since the HPLC method used did not allow the separation of chl *b* from the DVchl *b*, the chl *b* content attributable to picoeukaryotes was assessed after eliminating the contribution of DVchl *b* from *Prochlorococcus* by using the value of 0.20 fg DVchl *b* per *Prochlorococcus* cell for low light (deep layer) and 0.10 for high light (surface layer) samples, respectively, based on values obtained from Partensky et al. (1993).

Diatoxanthin (Dt) and diadinoxanthin (Dd) were used as indicators of photoprotection as they are involved in a photodependent epoxidation–de-epoxidation cycle in chromophyte algae (Brunet et al. 2003). The ratio of the single pigments to chl *a* (i.e. Dt/chl *a* or Dd/chl *a*) indicates how much of the pigment pool is devoted to protect the cells from excess light, while the ratio of each one of them to the 2 pooled together is independent of chl *a* concentrations and is, therefore, an indicator of the activation of the photoprotection process.

From the *in situ* values of fluorescence we calculated the FRE (Fluorescence Relative Error, Estrada et al. 1996, Brunet & Lizon 2003). This parameter represents the relative deviation of the observed *in vivo* fluorescence from the value measured during the night when we assume that fluorescence is at its maximum, and was calculated as follows:

$$FRE = (Fluo_{mes} - Fluo_{estim})/Fluo_{mes} \qquad (1)$$

where $Fluo_{mes}$ is the measured fluorescence and $Fluo_{estim}$ is the estimated fluorescence, which is calculated from a linear regression between *in vivo* fluorescence and chl *a* concentrations (from HPLC and from all samples except those taken at 5 m) both measured at night ($r^2 = 0.80$, n = 25). The FRE gives an indication of the photophysiological state of the cells (Estrada et al. 1996), with negative values indicating fluorescence quenching due to high light.

Onboard incubations. The kinetic coefficients of phytoplankton photosensitive parameters in the deep chlorophyll maximum (DCM) were estimated by manipulating the natural communities incubated on board the ship. These were exposed to a shift (up or down) of light intensity in order to simulate an upwelling event from an average DCM to an average surface layer or a downwelling towards deeper waters. In addition, growth rates at 2 depths within the DCM were estimated using the cell cycle method to compare the fitness of *Prochlorococcus* and *Synechococcus* relative to light availability. Seawater (40 l) was taken at night (23:00 h local time) from the DCM at 75 m depth (1 % of the incident light at noon of the previous day) and incubated on deck in two 20 l polycarbonate containers under a constant temperature of 15ºC. The 2 containers were screened with blue plus neutral density filters (Lee Filters) to obtain either 10 % or 0.1 % of incident light. Light intensity was monitored using a 4π QSL irradiance sensor (Biospherical Instruments). Samples for HPLC pigment analysis and flow cytometry were taken every 2 h for a 24 h period starting at dawn. Variations in cell autofluorescence and concentrations of photoprotective xanthophylls normalized by chl *a* were used to calculate photoacclimation kinetic coefficients over the natural light cycle. The kinetic coeffi-

cient K (h^{-1}) was estimated only for the increasing light period (the first 6 h) according to the first-order equation:

$$X = (X0 - X\infty)\exp(-Kt) + X\infty \qquad (2)$$

where X is the photodependent parameter, $X0$ is its initial value and $X\infty$ its final value, t is time (h) and K is the first-order kinetic coefficient (h^{-1}) (e.g. Brunet et al. 2003).

RESULTS

Hydrology, biomass, phytoplankton pigment composition and diversity

The temperature profiles show thermal stratification in the first 20 m, while salinity decreased sharply between 10 and 20 m (38.2 to 37.85 psu, Fig. 1a; 25 to 19°C, Fig. 1b) due to an intrusion of Adriatic Surface Water. The vertical structure of the water column remained essentially constant during the sampling period, as indicated by the low SD values in the CTD profiles (Fig. 1a,b), with no evidence of diel periodicity. Nutrient concentrations were very low at the surface (0.5 and 0.05 μM for nitrate and phosphate) and gradually increased below 30 m to reach 3.10 and 0.35 μM, respectively, at 90 m (Fig. 1c). Light intensity in the DCM ranged from 0.5 to 2% of surface incident irradiance (E_0).

The chl a fluorescence showed a Deep Fluorescence Maximum (DFM) between 70 and 100 m, with a peak occurring at 85 m (Fig. 2a) that corresponded to a DCM with highest values of 0.40 μg l^{-1} of chl a. Chl a and DVchl a showed a relatively homogeneous distribution in the first 50 m, despite the vertical hydrological variability, and a sharp increase below 50 m (Figs. 2b & 3). DVchl a (marker of *Prochlorococcus*) accounted for 0 to
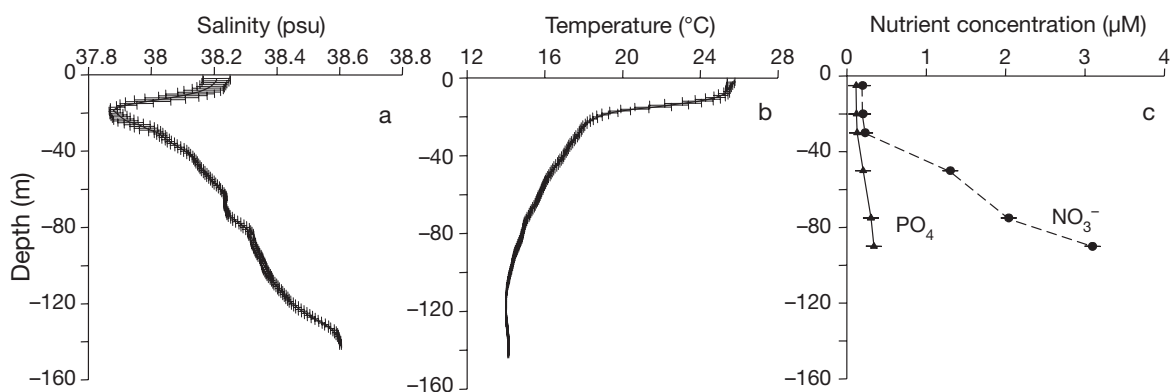

Fig. 1. Mean vertical profiles of (a) salinity (psu), (b) temperature (°C), (c) nitrate plus nitrite (dashed line) and phosphate (solid line) concentrations (μM, different depth scale). Error bars are SD, n = 34
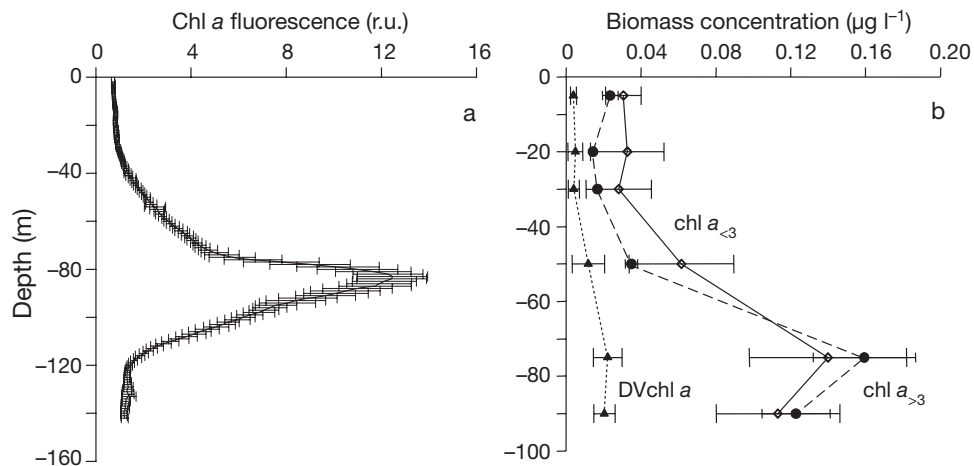

Fig. 2. Mean vertical profiles of (a) total *in vivo* fluorescence (relative units, n = 34), and (b) chl $a_{>3}$ (dashed line, μg l^{-1}, n = 17), chl $a_{<3}$ (solid line, μg l^{-1}, n = 17) and DVchl a (dotted line, μg l^{-1}, n = 17). Error bars are SD
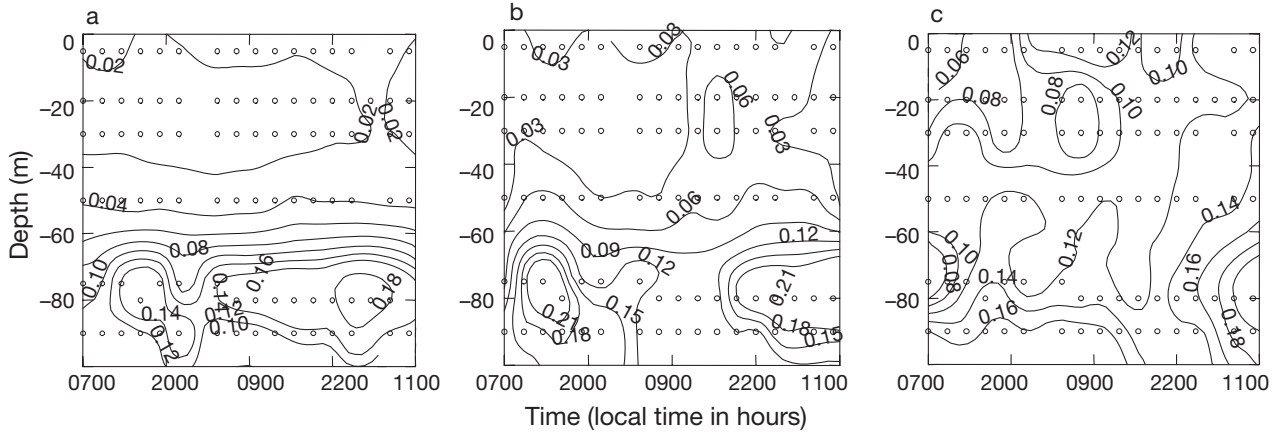
Fig. 3. Vertical distributions over time of (a) chl $a_{>3}$ (µg l^{-1}), (b) chl $a_{<3}$ + DVchl a (µg l^{-1}) and (c) DVchl a/(chl $a_{<3}$ + DVchl a)

25% of picophytoplankton chl *a*, with lower and higher values present in the surface layer and in the DCM, respectively (Fig. 3c). Picophytoplankton (<3 µm) accounted for 57% of total chl *a* on average, ranging from 40 to 90% (69% in the DCM), and within this, prokaryotes (zeaxanthin) dominated down to 50 m (26% of total chl *a*), but were gradually replaced by picoeukaryotes below 50 m (Table 1). All chemotaxonomic pigments in the picophytoplankton showed significant differences between the surface and the deeper layer (Table 1), indicating a segregation of species and adaptation. Fucoxanthin (diatoms) was the most representative pigment in the larger fraction, contributing significantly to total chl *a* both at the surface and in the DCM (Table 1). Hexanoyloxyfucoxanthin and chlc$_3$ (prymnesiophytes) were more abundant in the DCM.

Prochlorococcus cell numbers were constant in the first 20 m, but markedly increased with depth, with a peak at 50 m of 12.5×10^4 cell ml^{-1} (SD = 3.4×10^4). *Synechococcus* cell numbers showed a limited increase with depth, with a relative peak of 1.05×10^4 cell ml^{-1} (SD = 0.25×10^4 cell ml^{-1}) at 50 m (Fig. 4a). The ratio between the cell concentrations of *Prochlorococcus* and *Synechococcus* significantly increased with depth and reached its maximum value at 90 m, which is on the lower boundary of the DCM (Fig. 4b). DVchl *a* and *Prochlorococcus* cell numbers were positively correlated only when data from the surface layer and the DCM were considered separately (not shown), suggesting different photoadaptation in the 2 layers (Brunet et al. 2006).

The vertical distribution of scatter and fluorescence of *Prochlorococcus* showed a bimodal distribution in a layer between 50 to 90 m, indicating the co-existence of 2 ecotypes. Ecotype I, with lower red fluorescence and scatter, was visible from the surface down to 90 m deep and Ecotype II, with higher red fluorescence and

scatter, was present at depths from 50 m downward. The 2 types overlapped between 50 and 90 m depths.

The DVchl *a* cellular content of total *Prochlorococcus* (Ecotypes I plus II) ranged between 0.25 and 2.20 fg DVchl *a* cell^{-1}, increasing in mean value exponentially with depth (p < 0.01) (Fig. 5a) and also in variance.

To calculate the cellular content of DVchl *a* for the 2 ecotypes separately, the mean ± SD value for the surface layer of 0.55 ± 0.05 fg DVchl*a* cell^{-1} was assumed to be constant through the water column (as was apparent from the cytograms), and from this a mean value of 2.60 ± 0.91 fg DVchl *a* cell^{-1} for Ecotype II, which is the most fluorescent, was calculated.

Table 1. Average values (SD, n = 17) of the most relevant pigment ratios in the 2 size fractions (<3 µm and >3 µm) in the surface layer and the DCM; p-value is the significance level of the difference between the surface layer and the DCM (Student's *t*-test). Allo: alloxanthin (cryptophytes), But-fuco: 19′ butanoyloxyfucoxanthin (pelagophytes), chl *b*: chlorophyll *b* + divinyl chlorophyll *b* (green algae, including prasinophytes), chl c$_3$: chlorophyll c$_3$ (mainly prymnesiophytes), perid: peridinin (dinophytes), zea: zeaxanthin (prokaryotes), DVchl *a*: divinyl chl *a* (*Prochlorococcus*), fuco: fucoxanthin (diatoms), hex: 19′ hexanoyloxyfucoxanthin (prymnesiophytes)

|	Surface	DCM	p
Allo/chl $a_{<3}$	0.02 (0.02)	0.07 (0.08)	0.001
But-fuco/chl $a_{<3}$	0.35 (0.29)	0.79 (0.52)	0.001
Chl *b*/chl $a_{<3}$	0.07 (0.04)	0.17 (0.07)	0.001
Chl c$_3$/chl $a_{<3}$	0.13 (0.12)	0.38 (0.28)	0.001
Perid/chl $a_{<3}$	0.02 (0.05)	0.22 (0.23)	0.001
Zea/chl $a_{<3}$	0.89 (0.51)	0.43 (0.23)	0.001
DVchl *a*/chl $a_{<3}$	0.09 (0.04)	0.15 (0.03)	0.001
Chl c$_3$/chl $a_{>3}$	0.10 (0.10)	0.30 (0.10)	0.001
Fuco/chl $a_{>3}$	0.44 (0.74)	0.76 (0.22)	0.020
Hex-fuco/chl c$_{3<3}$	10.38 (6.34)	5.99 (4.50)	0.001
Hex-fuco/chl c$_{3>3}$	10.53 (7.40)	2.85 (1.35)	0.001
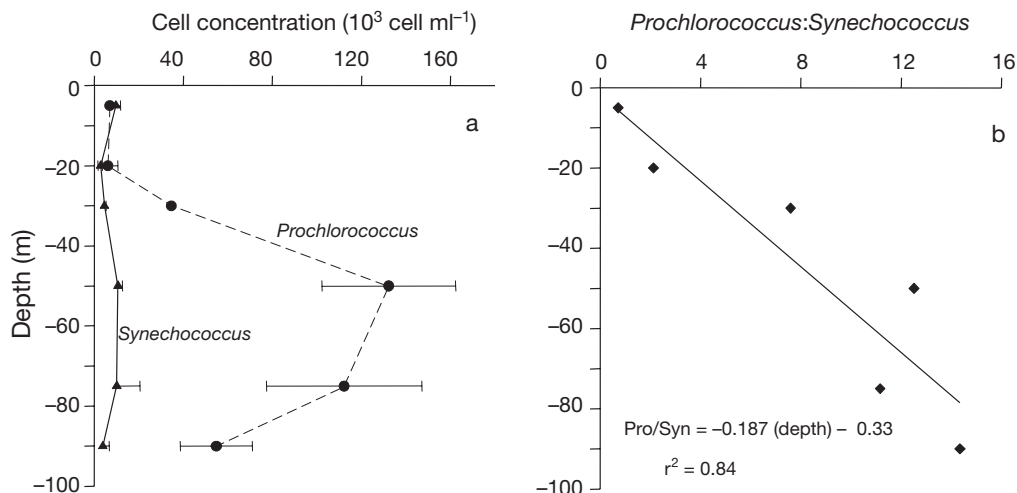
Fig. 4. Mean vertical profiles of (a) *Prochlorococcus* (dashed line) and *Synechococcus* (solid line) cell concentrations (error bars are SD, n = 17), and (b) ratio between *Prochlorococcus* and *Synechococcus* cell numbers with depth. The solid line is the linear fit of the distribution of the ratio versus depth. Equation and r^2 for the fit is reported on the plot
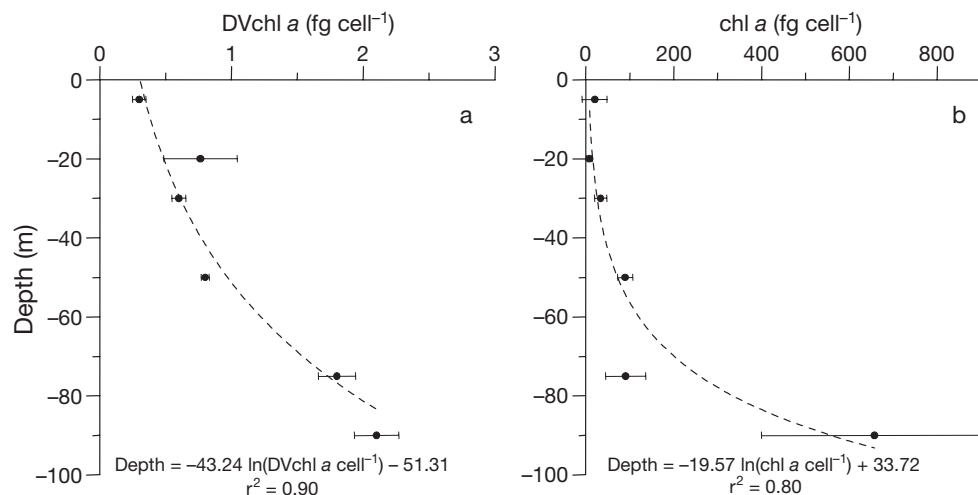

Fig. 5. Mean vertical profiles of (a) DVchl *a* cell^{-1} (fg *Prochlorococcus*$^{-1}$) and (b) chl *a* per picoeukaryote cell (fg cell^{-1}). Error bars are SD, n = 16. Equations and r^2 for the fits are reported on the plots

Average picoeukaryote concentrations were 0.6 ± 0.3 × 10^3 cell ml^{-1} and mean values of chl *a* cell^{-1} (see 'Materials and methods') ranged between 10 and 660 fg chl *a* cell^{-1}, with a significant exponential vertical increase with depth (p < 0.01) (Fig. 5b). Cellular chl *a* content of picoeukaryotes was constant in the upper layer (Student's *t*-test, p > 0.05), while values from the other depths were significantly different from one another (at least p < 0.02) except for values from 50 and 75 m (p > 0.05). Night and day values were not significantly different (p > 0.05), even for each single depth analysed separately.

Diel variations

No variation in the pigment assemblage during the time series was observed at any depth. Most of the diel variability appeared to be driven by cell division, and very little by photoacclimation, as evidenced by the negative peaks in size (FALS) and red fluorescence at dusk for all 3 picophytoplankton populations (data not shown). In the DCM, a significant diel periodicity was observed for the orange fluorescence (from phycoerythrin) of *Synechococcus* and for the red fluorescence (from chlorophyll) of Ecotype II of *Prochlorococcus*

Table 2. Sinusoidal fits of diel variations of different photodependent parameters at different depths. Dt: Diatoxanthin, (α+β)Car: α- + β-carotene, OrSyn: orange fluorescence from phycoerythrin of *Synechococcus*, RedProII: red fluorescence of Ecotype II of *Prochlorococcus* (low light adapted); n = 17. The sinusoidal regression corresponds to the following equation $Y = b + a \cdot \sin(d + c \cdot T)$, where a is the amplitude, c is the angular frequency, d is the phase and T is time (h)

Depth (m)	Equation	r^2	Periodicity (h)
20	Dt/chl $a_{<3}$ = 0.008 + 0.016 sin (−6.372 + 0.259 T)	0.94	24
20	Dt/chl $a_{>3}$ = 0.015 + 0.025 sin (−0.272 + 0.298 T)	0.96	21
75	(α+β)Car/chl $a_{<3}$ = 0.146 + 0.151 sin (−1.155 + 0.292 T)	0.86	22
75	OrSyn = 1.700 − 0.953 sin (−4.363 + 0.2888 T)	0.85	22
75	RedProII = 2.035 − 0.654 sin (−4.943 + 0.300 T)	0.72	21

(Table 2). The α and β carotene (α+βCar) to chl *a* ratio in the picofraction also showed a similar diel periodicity (Table 2), and this was also positively correlated ($p < 0.01$) to cell concentrations of *Prochlorococcus* and picoeukaryotes. The periodicity varied among the different parameters examined, ranging from 22 to 24 h, and this difference was probably caused by the time lag between samplings, which was probably too coarse for the parameters considered. Over the diel cycle, picophytoplankton chl *a* (DVchl *a* + chl $a_{<3}$) showed a significantly higher variance than the larger size class (chl $a_{>3}$, test of Fisher-Snedecor, $p < 0.01$) for every distinct depth.

The percentage of cells in the G2 phase of the cell cycle showed a peak at the end of the light period in *Prochlorococcus* and at the end of the night in *Synechococcus* (data not shown), similar to what Vaulot & Marie (1999) observed. From cell cycle measurements, *Prochlorococcus* and *Synechococcus* growth rates at 75 m were estimated to be 0.67 d^{-1} and 0.41 d^{-1}, respectively. At 90 m, the lower border of the DCM, *Prochlorococcus* still showed sustained growth rates of 0.43 d^{-1}, while the growth rate of *Synechococcus* had decreased to 0.17 d^{-1}.

Photoprotective responses

As expected, photoprotective pigments such as diadinoxanthin (Dd), diatoxanthin (Dt) and α plus β carotenes (α+βCar) had significantly higher values in the surface layer and during the light period ($p < 0.001$, Student's *t*-test) than in the deeper layer and at night (Fig. 6). Both Dt/chl *a* and (α+β)Car/chl *a* ratios were significantly higher (and their decrease with depth slower) in the larger size class (Fig. 6) ($p < 0.01$), suggesting a higher need for photoprotection than in the smaller phytoplankton. This difference was due to a higher content of photoprotective pigment per unit of chl *a*, rather than to simply more pigments, since Dt/(Dt+Dd) values and their decrease with depth were similar in the 2 size fractions (Fig. 6b,e).

Surprisingly, Dt/chl *a* and Dt/(Dt+Dd) values increased again at depth (Fig. 6a,b,d,e) in both size classes, but no oscillations with the day–night cycle were observed (contrary to the surface, Table 2) and, therefore, their involvement in photoprotective reactions could be excluded. High values of Dt could instead be due to chlororespiration, as observed by Lavaud et al. (2002) on cultures of diatoms exposed to prolonged darkness. This hypothesis is supported by the fact that only Dt increased with depth, but not Dd, and that no variation of these pigments was observed during the day–night cycle.

The Fluorescence Relative Error, which is an indicator of fluorescence quenching (see 'Materials and methods') was negatively correlated to Dt/(Dt+Dd) ($p < 0.01$, n = 50) and to the ratio between *in vivo* chl *a* fluorescence and HPLC chl *a* ($p < 0.001$). Mean values were significantly different between day and night in the surface layer ($p < 0.01$), with negative values measured during the day indicating fluorescence quenching caused by high light (Fig. 7a). The mean value of FRE was inversely correlated to the percent of incident surface light (E_0) at every depth ($p < 0.01$) (Fig. 7b). The same was true for the ratio between *in vivo* fluorescence and chl *a* concentration ($p < 0.01$) (Fig. 7c). Values from 90 m did not follow the linear relationship described on Fig. 7b & c, probably due to physiological stress caused by the very low light available at this depth, as discussed in the next section.

Onboard incubations

During the shift-up, Dt/(Dt+Dd) showed similar kinetic coefficients (K) in the 2 size classes, while Dt/chl *a* and Dd/chl *a* ratios increased more quickly in the smaller than in the larger size fraction (Table 3). During the shift-down, only picophytoplankton showed significant coefficients in the same direction as for the shift-up, except for Dd/chl *a*, which decreased more slowly, but comparably, in the 2 size classes. Kinetic coefficients for flow cytometric scatter and autofluorescence of picophytoplankton are also reported in Table 3. During the shift-up, *Synechococcus* red and orange fluorescence increased at a sustained rate, while *Prochlorococcus* red fluorescence decreased. However, the parallel decrease in DVchl *a* cell^{-1} was
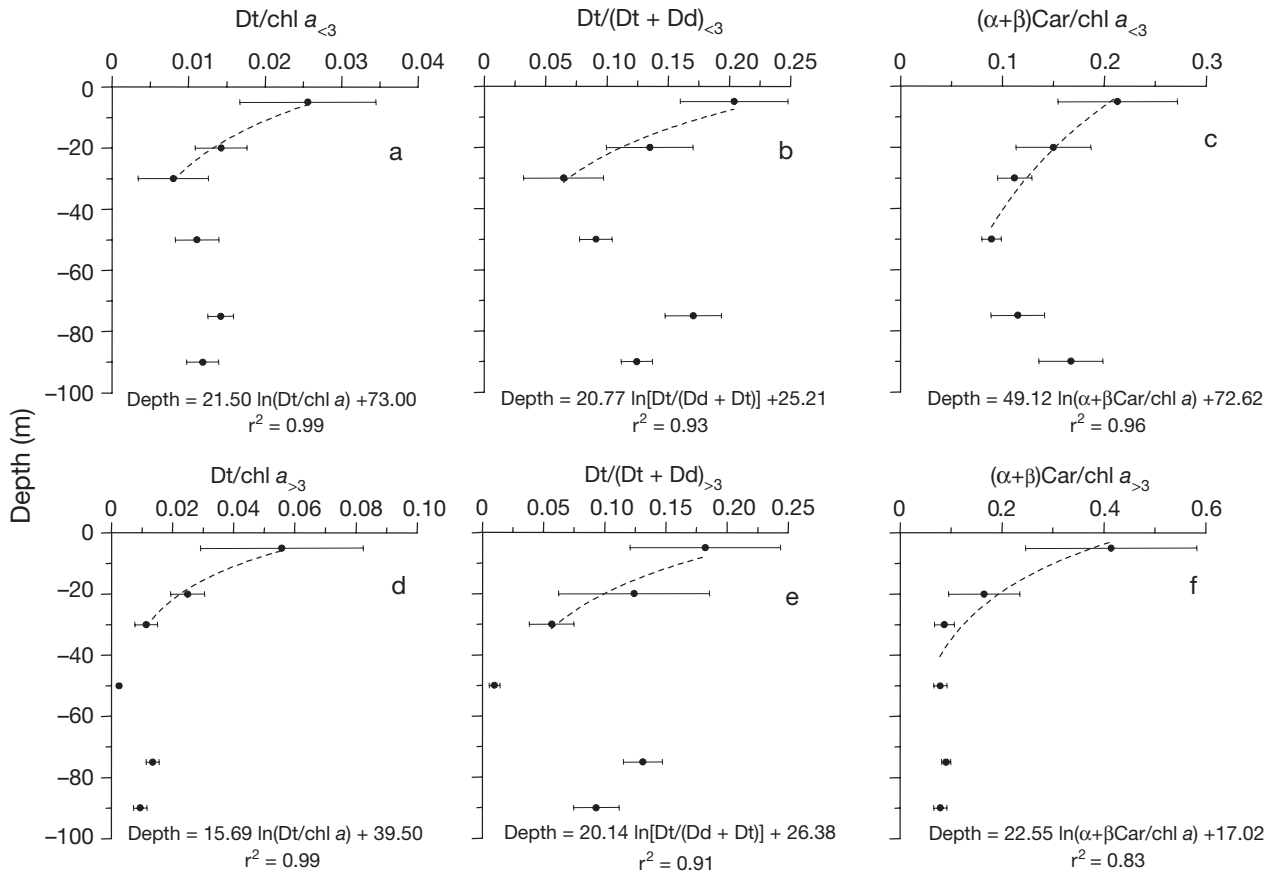
Fig. 6. Daylight mean vertical profiles of (a) Dt/chl $a_{<3}$, (b) Dt/(Dt+Dd)$_{<3}$, (c) ($\alpha+\beta$)Car/chl $a_{<3}$, (d) Dt/chl $a_{>3}$, (e) Dt/(Dt+Dd)$_{>3}$, and (f) ($\alpha+\beta$)Car/chl $a_{>3}$. Error bars are SD, n = 10. Dashed lines are exponential fits for the 0 to 30 m layer except for ($\alpha+\beta$)Car/chl a, which is 0 to 50 m layer
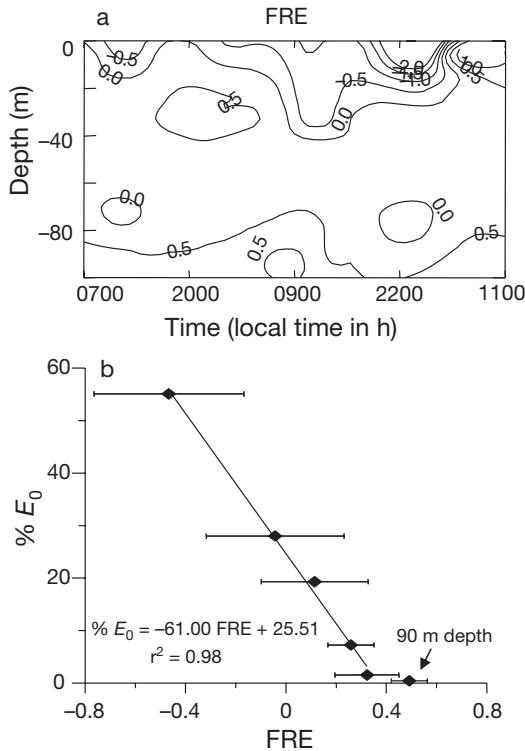

Fig. 7. (a) Vertical distribution of fluorescence relative error (FRE) over time. (b) Correlation between the percent of incident light (E_0) and the daylight mean values of FRE ($r^2 = 0.98$). (c) Correlation between the percent of incident light and the *in vivo* fluorescence versus chl a ratio ($r^2 = 0.98$). Error bars are SD, n = 10. Equations and r^2 for the fits are reported on the plots

Table 3. First-order kinetic coefficients (K, h^{-1}) and r^2 (in parentheses) for changes in pigment ratios and autofluorescence (flow cytometry on picoplankton < 3 µm or > 3 µm in size) during the light-shift experiments on natural phytoplankton from the DCM (1 % of incident light [E_0]). Data fitted are from samples collected from dawn up to the maximum of irradiance during the day. HL: high light (10 % of E_0); LL: low light (1 % of E_0); Dt: diatoxanthin; Dd: diadinoxanthin; Syn: *Synechococcus*; Pro: *Prochlorococcus*; Euk: picoeukaryotes; Eukchl *a* cell^{-1}: the content of chl *a* per picoeukaryote cell (in fg chl *a* cells^{-1}). n = 5. ns is not significant

	Dawn to HL		Dawn to LL	
	<3 µm	>3 µm	<3 µm	>3 µm
Dt/(Dt+Dd)	0.124 (0.99)	0.137 (0.99)	0.059 (0.99)	ns
Dt/chl *a*	0.291 (0.99)	0.095 (0.99)	0.360 (0.80)	ns
Dd/chl *a*	0.147 (0.99)	0.096 (0.99)	−0.354 (0.92)	−0.260 (0.99)
Syn Red	0.299 (0.98)		0.165 (0.92)	
Syn Orange	0.420 (0.94)		0.120 (0.89)	
Pro Red	−0.140 (0.92)		ns	
Euk Red	−0.110 (0.99)		ns	
Eukchl *a* cell^{-1}	−0.133 (0.99)		ns	

not significant (p > 0.05), suggesting that it was mainly due to fluorescence quenching. Red autofluorescence of picoeukaryotes and their chl *a* cell^{-1} content also decreased, with comparable kinetic coefficients between them and for red fluorescence of *Prochlorococcus*. During the shift-down only *Synechococcus* red and orange fluorescence showed significant but low values of *K*.

DISCUSSION

Biomass and diversity

The high pigment diversity of picophytoplankton in the DCM and its elevated contribution to total chl *a* indicated an elevated degree of adaptation to the quantity and quality of light available. This was not true for the larger phytoplankton, which were represented mainly by only diatoms and prymnesiophytes (fuco and Hex-fuco). We could not assess if this adaptation was realized by substitution of species or ecotypes, as in the case of *Prochlorococcus*, or by physiological acclimation. Probably only recently developed molecular tools would have provided a clear answer for all groups (Rodriguez et al. 2005). However, prymnesiophytes showed a significantly higher chl c$_3$/Hex-fuco ratio (2 chemotaxonomic pigments of this group) at the surface when compared with the DCM in both size classes (Table 1). This suggests vertical segregation of functional groups, probably related to light (chromatic) adaptation and confirms the pigment complexity and heterogeneity of this algal class as related to ecological adaptation (Moon-van der Staay et al. 2000, Zapata et al. 2004).

Variability in time in the DCM was evident from the oscillations of prokaryote autofluorescence, with peaks during the day attributed to growth and division, as confirmed by the correlation with FALS or RALS (proxies of size). This was also confirmed by the significant correlation of *Prochlorococcus* and picoeukaryote cell concentrations with (α+β)Car/chl *a*$_{<3}$, the latter showing a 22 h periodicity with a peak during the light period due to light-dependent responses.

Vertical variability within the DCM was evident when comparing properties of phytoplankton from the 2 depths above and below the fluorescence maximum, 75 and 90 m. The light available at these depths was very low, accounting for 2 % of E_0 (maximum of 20 µmol quanta m^{-2} s^{-1}) at 75 m and decreasing to 0.5 % of E_0 (maximum of 5 µmol quanta m^{-2} s^{-1}) at 90 m. Total chl *a* decreased by 20 % in this layer, with DVchl *a* decreasing by 8 %, chl *a*$_{>3}$ by 23 % and chl *a*$_{<3}$ by 20 %. Instead, the *in vivo* fluorescence increased by only 0.3 %, indicating that the DCM in terms of algal biomass was narrower than the peak in fluorescence. The discrepancy between chl *a* concentration and fluorescence may be due to the extremely low light, which increases the fluorescence per unit of chl *a* (Kiefer & Reynolds 1992). The contribution of chlorophyll degradation derivatives (phaeophytins or phaeophorbides) to total fluorescence could instead be excluded, as no change in concentration of these byproducts was detected. Therefore, light appeared to be the only factor explaining this discrepancy. This is also suggested by the lack of fit of the data from 90 m with the linear correlation between the percent of E_0 and FRE or *in vivo* fluorescence/chl *a* (Fig. 7). The measured values are 14 % higher than the expected values (from the correlation). This difference represents the degree of peculiarity in photophysiological characteristics of the phytoplankton thriving at such low light, and is confirmed by the strong increase (30 %) in chl *a* cell^{-1} for the picoeukaryotes (Fig. 5). This increase is also due to replacement of species with different capacities for chl *a* content along the water column. Although it can be surprising to see a difference between algae growing at 20 versus 5 µmol quanta m^{-2} s^{-1}, there are reports of peculiar acclimation mechanisms other than fluorescence quantum yield taking place at such very low light intensities (e.g. Quigg & Beardall 2003).

Environmental conditions at 90 m were critical for most phytoplankton, as suggested by the loss of periodicity of pigment content and autofluorescence, prob-

ably as a consequence of poor cell physiological state. For *Synechococcus*, mortality through autolysis can account for 5 to 25% of total cell numbers, with increasing values occurring with depth (Veldhuis et al. 2005).

From cell cycle estimates of the growth rates of the 2 cyanophytes, *Synechococcus* and *Prochlorococcus*, it was evident that while at 75 m both sustain reasonable growth rates, only *Prochlorococcus* is able to cope with the very low light available at 90 m and supports a growth rate of 0.43 d^{-1}, which equals one division every 1.6 d. These growth rates lie within the range of values estimated by other authors in different ecosystems (e.g. Timmermans et al. 2005 and references therein) and confirm previous speculations on the differential sensitivities of these 2 species to light and nutrient limitation, explaining their different niche occupation through the water column (Chisholm et al. 1988). These tolerances for low or high light levels were also confirmed by the incubation experiments, with *Prochlorococcus* and *Synechococcus* reacting differently in sign and kinetics to the changes in light intensities (Table 3).

In general, the data presented show that the DCM does not represent a unique and homogeneous ecophysiological entity, and call for a better understanding of its biological complexity driven by variability in time and (micro) space.

Photoacclimation

Photoprotective indicators, such as Dt/chl *a*, Dd/chl *a* and (α+β)Car/chl *a*, decreased with depth exponentially as expected in a mixed layer with low turbulence. Absolute values of these ratios were higher in the larger phytoplankton than in the picophytoplankton, and these were not due to higher pigment content in larger cells since these ratios showed similar values in the 2 size fractions below 30 m depth where no photoprotection was expected to take place. In addition, values of Dt/(Dt+Dd) were similar, indicating that larger cells were synthesizing more photoprotective pigments per unit of chl *a* than smaller cells. This strategy, involving both the direct photoprotective pigment (Dt) and its precursors (Dd and β-Car), may be attributed to larger protein-pigment antenna complexes that need to be protected inside Photosystem II. This increased need may also explain the slower rate of synthesis observed during the incubation experiments (Table 3) as compared with the smaller phytoplankton.

Picoeukaryotes were able to rapidly modify their chl *a* content in response to changing light as revealed by the decrease of 70% of chl *a* cell^{-1} during the shift-up

incubation experiment with a rate of 0.13 h^{-1}, parallel to the synthesis of Dt (0.14 h^{-1}, Table 3). This suggests a very fast synthesis of photoprotective pigments, which are also light-regulated. This photophysiological plasticity makes it a competitive factor with respect to larger phytoplankton.

Indeed, picophytoplankton showed higher sensitivity and faster reactivity to the diel changes in light than the larger phytoplankton in terms of biomass, as indicated by the higher range of values and significantly higher variance of the chl $a_{<3}$ with respect to the chl $a_{>3}$ at all depths ($p < 0.01$, Fisher-Snedecor test). Coefficients of variation (CV) of chl $a_{<3}$ ranged between 90% (in the surface layer) and 52% (at depth) whereas the CV of chl $a_{>3}$ was lower and more homogeneous through the water column (27% in the surface layer and 24% below), which probably related to the faster growth and division rates and also the faster changes in intracellular chl *a* in response to variations of light in picoeukaryotes.

The natural light–dark cycle was the main factor ruling the photophysiological properties of phytoplankton. This was reflected in the much higher variabilities of photoprotective pigment versus chl *a* and of the FRE in the surface layer (CVs of 31 and 272% on average, respectively), when compared with those in the deeper layer (16 and 51%, respectively). As a consequence of this, caution must be adopted when estimating vertical movements or physical characteristics of the upper mixed layer from light-dependent parameters (e.g. Brunet et al. 2003), especially when comparing different water masses with different algal communities. We stress the importance of using the Dt/(Dt+Dd) ratio as an estimate of the photosensitive state of phytoplankton cells in the upper layer because it is independent of cell chl *a* content and, therefore, cell size.

Throughout the water column in the hydrologically stable area of this study, the light environment, together with nutrients, selected for different ecotypes (as in the case of *Prochlorococcus*) and probably species (as in the case of picoeukaryotes), is therefore, driving the biodiversity of the ecosystem. The DVchl *a* content per cell of the 2 *Prochlorococcus* ecotypes (0.55 and 2.60 fg DVchl *a* cell^{-1}, respectively) is at the lower end of values reported (Partensky et al. 1993, Moore & Chisholm 1999), probably due to differences in strains and/or light conditions. Right-angle light scatter (RALS) (as a proxy of size) and red fluorescence were directly correlated for the low-light Ecotype II ($p < 0.001$) in the DCM, suggesting that the most important factor driving DVchl *a* variations was the growth–division process. No significant relationship was found for the high-light Ecotype I because of the scattering of the data, probably due to the overlapping of photo-

acclimation signals with the cell division cycle in the surface layer. Cell division was also responsible for the lowest values of DVchl *a* cell^{-1} measured at night until 07:00 h as well as for the highest values during the day with a peak between 16:00 and 19:00 h (local time). These results agree with previous reports from the Pacific Ocean and the northwestern Mediterranean Sea (Jacquet et al. 1998, Vaulot & Marie 1999). However, it should be noted that during this study no further increase in DVchl *a* per cell occurred at the beginning of the night period while red fluorescence of *Prochlorococcus* did, as also observed by Vaulot & Marie (1999) and Jacquet et al. (2001). This discrepancy, reported also by Neveux et al. (2003) during a diel study in the upper layer of the equatorial Pacific Ocean, was related to higher fluorescence per unit of DVchl *a* in the first part of the night and attributed to fluorescence quenching occurring during the light period.

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