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Short-time scale coupling of picoplankton community structure and single-cell heterotrophic activity in winter in coastal NW Mediterranean Sea waters

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We used flow cytometry to follow the diel variations of picoplankton community structure (PCS) and heterotrophic activity in coastal North Western Mediterranean surface waters during two successive 72 h cycles in winter 2007. Photosynthetic picoeukaryotes (pPeuk) dominated the photosynthetic fraction of the PCS during the first cycle, while *Synechococcus* (*Syn*) dominated during the second. For each group, pronounced and significant diel patterns were observed in flow cytometrically determined proxies of cell size (side scatter) and pigment content (fluorescence). *Syn* and pPeuk grew during the light period and divided at night; opposite patterns were observed in *Prochlorococcus*. The diel patterns of the overall PCS were strongly disrupted before the second cycle by a wind change event with associated rainfall and increased turbulence, suggesting that the shift observed in PCS resulted from the imbalances between growth and loss processes. During the first cycle, heterotrophic activity was higher at night than during the light period, indicating that bacterial growth was phased with the diel variations of PCS. During the second cycle, no diel patterns were observed. However, similar increasing trends in bacterial activity and small grazer abundance suggested that grazing activity was a possible source of dissolved organic matter (DOM) release that likely drove bacterial activity.

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KEYWORDS: picoplankton; heterotrophic activity; diel patterns; NW Mediterranean

INTRODUCTION

Understanding the distribution of picoplankton is one of the goals of marine microbial ecology. In addition to the picoeukaryotes (Johnson and Sieburth, 1982), two phylogenetically closely related types of photosynthetic prokaryotes: *Synechococcus* (*Syn*) (Waterbury *et al.*, 1979) and *Prochlorococcus* (*Pro*) (Chisholm *et al.*, 1988; Chisholm 1992) comprise the picophytoplankton fraction (<3 μm). The concentrations of the three groups have often been shown to peak at different periods of the year, suggesting distinct environmental controls for each of these organism types (e.g. Partensky *et al.*, 1999a). While mesotrophic regions are generally dominated by picoeukaryotes, low productive oligotrophic waters are generally occupied by large numbers of *Prochlorococcus* and to a lesser extent by *Synechococcus* (Jacquet *et al.*, 2002a), which are more common in the coastal areas (Olson *et al.*, 1990; Campbell and Vaulot, 1993; Partensky *et al.*, 1996; Partensky *et al.*, 1999a). A preference of *Prochlorococcus* for stratified over mixed waters has also been observed (Vaulot and Partensky, 1992; Lindell and Post, 1995). Although the individual geographic distributions of *Prochlorococcus* and *Synechococcus* are now well documented (Partensky *et al.*, 1999a, 1999b), less is known about the photosynthetic picoeukaryotes (pPeuk), their low numerical contribution contrasting with their dominance in the picophytoplankton biomass and production of many marine ecosystems (Li *et al.*, 1992; Li, 1994; Ishizaka *et al.*, 1997; Worden *et al.*, 2004). Yet, understanding the factors driving the picoplankton group distribution and their relative contribution to total picoplankton biomass is essential for understanding the dynamics of the ecosystem.

The distribution of the different picoplankton groups has mostly been studied on relatively large time scales with sampling frequencies ranging from once per day to 1 per month, and only a few times at a higher frequency (i.e. several samples per day). Given that events of major ecological relevance often result from transient environmental perturbation (i.e. wind stress, turbulence, high irradiance . . .), and that the microbial life history more likely operates at short time frames, it is necessary to determine the role of the short time scale in structuring the large-scale patterns in microbial communities (Seymour *et al.*, 2005).

Episodic forcing at short time scales is known to induce shifts in both phytoplankton and picoplankton community structures (PCSs) (Pannard *et al.*, 2008; Guadayol *et al.*, 2009; Thomas *et al.*, 2010), and light has often also been identified as the most important driver of diel variability. Most phytoplankton species divide at specific times of the day (Gough, 1905), and even large phytoplankton such as diatoms and dinoflagellates follow diel cycles (Swift and Durbin, 1972; Smayda, 1975). Jacquet *et al.* (Jacquet *et al.*, 1998) showed that the *Synechococcus* cell cycle was phased with the daily light cycle, possibly enforced by a "clock" controlled by genetic factors (Johnson *et al.*, 1996). Synchronization and phasing of cell growth for both *Synechococcus* and pPeuk was observed from dawn to dusk during the winter in the northwestern Mediterranean Sea and in the Alboran Sea (Jacquet *et al.*, 1998, 2002a). However, differences were reported in cultures and in natural ecosystems where the division of *Synechococcus*, *Prochlorococcus* and picoeukaryotes did not proceed at the same time (Vaulot and Marie, 1999; Jacquet *et al.*, 2001a). Whether or not such phase differences between groups are linked to the differential sensitivity of each group to light (Sommaruga *et al.*, 2005) remains unclear, but has been suggested that the *Prochlorococcus* cell cycle is tightly coupled to the irradiance levels (Jacquet *et al.*, 2001b).

In addition, the relative stability of picoplankton group cell concentrations measured on a daily or weekly scale suggests that grazing and viral lysis mortalities balance cell growth and division (Landry *et al.*, 1995). Differential grazing on *Synechococcus*, *Prochlorococcus* and pPeuk has already been described (Christaki *et al.*, 1999; Worden *et al.*, 2004) and different factors that translate into preferential grazing on some bacteria have been identified, including cell size (González *et al.*, 1990), motility (Matz and Jürgenz, 2005), surface properties (Matz and Jürgenz, 2001), phylogenetic affiliation (Jezbera *et al.*, 2005), C:N:P ratio (Shannon *et al.*, 2007), cell viability (Landry *et al.*, 1991) or membrane integrity (Massana *et al.*, 2009). Thus, a detailed knowledge of grazing is also needed to understand how microbial diel variability is associated with the intrinsic diel patterns in cell division.

Finally, tight coupling between phytoplankton and bacteria should result in bacteria also following similar circadian cycles. As a consequence of this link, a peak of bacterial activity at noon/afternoon should be expected to follow a peak of dissolved organic matter (DOM) originated either from primary production (Mague *et al.*, 1980; Fuhrman *et al.*, 1985; Herndl and Malacic, 1987; Gasol *et al.*, 1998; Pausz and Herndl, 1999) or from

circadian grazing activities (Wikner *et al.*, 1990; Atkinson *et al.*, 1992; Nagata, 2000; Jakobsen and Strom, 2004). Factors that affect the single-cell physiological status and activity level of marine bacteria, such as ultraviolet radiation, bacterivory and viral lysis are also known to often follow diel variations (Wikner *et al.*, 1990; Jeffrey *et al.*, 1996, Christaki *et al.*, 2002; Winter *et al.*, 2004). Conversely, the absence of daily coupling between phytoplankton and bacteria would imply that bacteria are not very much dependant on the DOM produced by phytoplankton on a daily basis, and instead support their growth and activity by DOM from alternative sources. Evidence for diel patterns in bacterial abundance and activity has been reported from the coastal NW Mediterranean (Gasol *et al.*, 1998; Ghiglione *et al.*, 2007), but how picophytoplankton variability is coupled with bacterial single-cell activities has not yet been analyzed.

With that objective, we followed the diel variations in picoplankton abundance using flow cytometry sampling with a high frequency (4 h intervals) during two cycles of 72 h in winter 2007 in an NW Mediterranean coastal station, the period of the year that commonly has higher chlorophyll *a* levels. Combined with flow cytometry, we used viability probes testing for bacterial activity, bacterial membrane integrity and heterotrophic nanoflagellates (HNF) abundance to determine to what extent picophytoplankton variation was coupled with heterotrophic bacterial activity. Since our sampling was disrupted by an episode of weather-induced turbulence, we were able, additionally, to describe how the diel variability and the coupling bacteria–picoalgae responded to the change.

METHOD

Sampling sites

Two diel cycles were studied during two successive 3-day periods in February–March 2007 (from 20 to 23 February 2007 and from 26 February to the 1 March 2007) at the Blanes Bay Microbial Observatory, a shallow (20 m depth) oligotrophic coastal station in the NW Mediterranean Sea, located 800 m offshore of Blanes, Catalonia, Spain (41°39.90′N, 2°48.03′E). The sampling of surface water was performed at 0.5-m depth with polycarbonate carboys at a frequency of six samplings per day (every 4 h). The samples were kept in the dark until analyses in the laboratory (<20 min from sampling). The first sampling of the two cycles (CDN01 and CDN 20, respectively) began at 10:00 a.m. Only one sample (CDN 14) could not be obtained due to sea conditions. Temperature and salinity of the waters were measured with a SAIV A/S 204 CTD probe. Irradiance measurements were obtained from the nearby station of Malgrat de Mar (Catalan Meteorological Service, www.meteo.cat), located at 5 km from the sampling station and at 4 m above the sea level. The station recorded arithmetically averaged hourly air temperature and relative humidity at 1.5 m above the ground, vector-averaged hourly wind speed and direction and global solar spectral irradiance at 2 m and accumulated rainfall at 1 m. Wave height data were collected from a scalar buoy (Datawell, Waverider) placed at 41°38′49″N, 02°48′56″E, over a depth of 74 m (XIOM Network, www.boiescat.org). Chlorophyll *a* concentration was determined from 150 mL of seawater filtered through GF/F filters (Whatman) frozen at −20°C, extracted in acetone (90% v/v) for 24 h and fluorescence was measured with a Turner Designs fluorometer following standard protocols (Yentsch and Menzel, 1963).

Picoplankton abundances

Determination of picoalgal and bacterial abundance was performed by flow cytometry using a Becton-Dickinson FACScalibur flow cytometer (Gasol and del Giorgio, 2000; Marie and Partensky, 2006) using standard operation settings: a 15-mW blue (488 nm) laser, thresholding in red or green fluorescence, depending on the specific protocol, and logarithmic acquisition. For picophytoplankton, the samples were analyzed without the addition of fixative and run at a high speed (ca. 100 μL min^{-1}) with thresholding in red fluorescence. Three main populations (*Prochlorococcus*, *Synechococcus*, picoeukaryotes) were discriminated according to their scatter and fluorescence signals (Supplementary data, Fig. S1). At this time of the year most picoeukaryotes are small and their abundances are similar to those of probe-determined Mamiellales chlorophytes (R. Massana, personal communication). For non-phototrophic bacteria, we chose to estimate the abundance following the NADS Viability protocol (see below) to avoid using fixatives. Bacterial abundances were also estimated by fixing 1.2-mL samples with a 1% paraformaldehyde + 0.05% glutaraldehyde solution and deep-freezing in liquid N_2. Afterwards the samples were unfrozen, stained with SybrGreen I at a 10× dilution and enumerated at a low speed (ca. 15 μL min^{-1}) with thresholding in green fluorescence The cells were identified in plots of side scatter (SSC) versus green fluorescence using standard conditions (e.g. Gasol and del Giorgio, 2000). At this time of the year SybrGreen-stained picophytoplankton are clearly observed in the analyses of heterotrophic bacteria and can be easily separated in red versus green fluorescence plots (Supplementary data, Fig.S1). Concentrations were obtained from weight measurement of the volume analyzed.

HNF abundances were measured following the Rose *et al.* (Rose *et al.*, 2004) protocol. From a stock solution of 1-mM Lysotracker Green (Molecular Probes), 1 μL was added to 99 μL of <0.2-μm MilliQ, and 3.8 μL of this diluted Lysotracker stock were added to 0.5 mL of the sample, generating a 75-nM Lysotracker final concentration. We analyzed the samples as in Rose *et al.* (Rose *et al.*, 2004), using a combination of SSC and green and red fluorescence plots (Supplementary data, Fig. S2). The samples were run alive at a high (ca. 100 μL min^{-1}) speed and with green fluorescence thresholding. Concentrations were obtained from weight measurement of the volume analyzed.

Bacterial single-cell activity

Measurements of the different physiological status of bacteria were done in two ways: (i) highly active prokaryotes, such as those able to reduce 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polysciences). CTC turns into a red fluorescent formazan that is detectable by epifluorescence and flow cytometry (Sherr *et al.*, 1999; Sieracki and Sieburth, 1986). Sample aliquots (0.4 mL) were amended with 5-mM CTC (from a fresh stock solution at 50 mM) immediately following collection and were incubated for 90 min in the dark at room temperature. CTC-positive (CTC+) cells were enumerated by flow cytometry using the FL2-versus-FL3 dot plot (Gasol and Arístegui, 2007). For these analyses, we used a high speed (ca. 100 μL min^{-1}) and a threshold set in red fluorescence. (ii) Cells with intact membranes were enumerated using the NADS viability protocol, based on the combination of the cell-permanent nucleic acid strain SybrGreen I (Molecular Probes, Eugene, OR) and the cell-impermeant propidium iodine (PI; Sigma Chemical Co.) fluorescent probe. We used a 10× SG1 and 10-μg mL^{-1} PI concentrations. After simultaneous addition of each stain, the samples were incubated for 20 min in the dark at room temperature and then analyzed by flow cytometry. SG1 and PI fluorescence were detected in the green (FL1) and red (FL3) cytometric channels, respectively. A dot plot of red versus green fluorescence allowed the distinction of the "live" cell cluster (i.e. cells with intact membranes and DNA present) from the "dead" cell one (i.e. with compromised membranes) (Grégori *et al.*, 2001; Falcioni *et al.*, 2008).

Data transformations and statistical analyses

To test for the significance of the periodicity of the parameters studied, we used the Fisher's Kappa statistic (Davis, 1941; Fuller 1976). For that purpose, we completed the time series with the missing CDN14 values, forecasting the CDN14 values by calculating the arithmetical average between the surrounding values CDN13 and CDN15. We then tested the null hypothesis that the values in the series were drawn from a normal distribution with variance 1 against the alternative hypothesis that the series had some periodic component. Kappa is the ratio of the maximum value of the periodogram, $I(f_i)$, and its average value. The null hypothesis is rejected if this probability is less than the significance level. All analyses were conducted in JMP 7 (SAS Institute, Inc.).

RESULTS

Background environmental variables

The two successive diel cycles were sampled in the winter of 2007 at the Blanes Bay coastal station. Water temperature was 13°C (close to the minimum of the year) and salinity close to 38.30. Both variables varied little over the period of observation (Table I). Chlorophyll *a* concentration oscillated during the cycles but was on average similar in both cycles (ca. 0.68 μg L^{-1}, Table I). From 43% (first cycle) to 51% of the chlorophyll passed through a 3-μm filter and, thus, a large percentage of the chlorophyll was in the picophytoplankton size range. The main wind direction was N/NW (340°) during the two cycles (Table II), frequently interrupted by shifts in speed and direction from North to South/SW. During the second half of the 25th of February (between the two cycles), a pronounced change from North/SW to South/SE occurred concomitantly with light rainfall (not shown) and a decrease in irradiance (Fig. 1A), rapidly followed by an increase in turbulence estimated from the changes in the average wave height, at the onset of the 26th of February (Fig. 1B), which varied from 25 to 50 cm. Compared with the first cycle, turbulence stayed at relatively higher levels during the second cycle.

Picoplankton community structure

Heterotrophic bacteria constituted the major component of the picoplankton community during the two cycles (Fig. 2). The average bacterial concentrations during the first and second cycles were 7.75 (±1.13) and 8.27 (±0.51) × 10^5 cells mL^{-1}, respectively (Table I and Fig. 2A). During the first cycle, picophytoplankton community structure appeared clearly dominated by pPeuk for which the average concentration was 1.09 (±0.40) × 10^4 cells, followed by *Synechococcus* and *Prochlorococcus* with 5.76 (±0.81) and 5.40 (±0.10) × 10^3 cells mL^{-1}, respectively (Table I and Fig. 2B). During the second cycle (week 2), a shift in the community composition followed the change in turbulence. *Synechococcus* dominated

Table I: Average values and coefficients of variation of the different environmental, PCS and activity parameters

	Average (± SD)		Coefficients of variation	
	First cycle	Second cycle	First cycle	Second cycle
Env. variables				
Chlorophyll *a* (μg L^{-1})	0.47 ± 0.02*	0.89 ± 0.03*	n.d	n.d
Temperature (°C)	13.43 ± 0.04	13.36 ± 0.01	n.d	n.d
Salinity (psu)	38.27 ± 0.03	38.30 ± 0.01	n.d	n.d
Synechococcus				
Abundance (10^3 cells mL^{-1})	5.76 ± 0.81*	15.70 ± 0.30*	14%	19%
FL2 (rel. units)	0.96 ± 0.05*	0.87 ± 0.05*	5%	6%
FL3 (rel. units)	1.39 ± 0.04*	1.32 ± 0.04*	3%	3%
SSC (rel. units)	1.16 ± 0.07*	0.86 ± 0.10*	7%	12%
Prochlorococcus				
Abundance (10^3 cells mL^{-1})	5.40 ± 0.10*	12 ± 0.17*	19%	14%
FL3 (rel. units)	0.62 ± 0.07*	0.56 ± 0.05*	12%	10%
SSC (rel. units)	0.24 ± 0.03*	0.21 ± 0.02*	14%	8%
Picoeukaryotes				
Abundance (10^4 cells mL^{-1})	1.09 ± 0.40*	1.38 ± 0.30*	40%	20%
FL3 (rel. units)	1.63 ± 0.07*	1.57 ± 0.06*	4%	4%
SSC (rel. units)	0.80 ± 0.04*	0.75 ± 0.05*	6%	6%
Heterotrophic bacteria				
Abundance (10^5 cells mL^{-1})	7.75 ± 1.13	8.27 ± 0.51	15%	6%
Live + dead cells (10^5 cells mL^{-1})	7.20 ± 0.70	7.53 ± 0.90	9%	12%
CTC+ (10^4 cells mL^{-1})	4.70 ± 0.90	5.70 ± 0.17	19%	20%
CTC+ (%)	6 ± 0.70*	6.50 ± 2.00*	11%	32%
HNA (%)	59 ± 3.00	58 ± 2.00	5%	5%
NADS-live (%)	84 ± 3.15	86 ± 5.00	10%	11%
HNF				
Abundance (10^3 cells mL^{-1})	1.14 ± 0.80	0.76 ± 0.40	68%	53%

SD, standard deviation. Coefficients of variation were calculated as (standard deviation)/(mean).
*Significantly different values between cycles (*t*-tests, $P < 0.05$).

Table II: Percentage of time in which wind blew in each direction

In %	N	NE	E	SE	S	SW	W	NW
First cycle	26.29	0.43	3.02	4.74	12.93	12.93	23.71	15.95
Weekend	30.77	2.56	7.69	4.27	14.53	9.40	18.80	11.97
Second cycle	25.41	0.66	7.26	5.61	12.87	11.22	21.12	15.94

The weather data come from the station of Malgrat de Mar (Catalan Meteorological Service, http://www.meteocat.com).

the community structure with an average concentration of 1.57 (± 0.30) × 10^4 cells mL^{-1} (representing a 172% of increase when compared with the average found during the first week), closely followed by pPeuk and *Prochlorococcus* with the average concentrations of 1.38 (± 0.30) and 1.20 (± 0.17) × 10^4 cells mL^{-1}, respectively (Table I and Fig. 2B).

Synechococcus diel patterns

As a general tendency, *Synechococcus* concentration increased during the dark period, and decreased during the light period. During the first cycle (from 20 to 23 February 2007), *Synechococcus* abundance followed a clear diel cycle with a significant 24-h periodicity (Fisher's Kappa, $P < 0.05$) (Table III, Fig. 2). The concentration increased strongly during the first part of the dark period (from 6:00 to 10:00 p.m.), followed by a plateau until dawn. After dawn, a pronounced decrease of *Synechococcus* concentration was observed until a minimum reached at dusk. In comparison with the first cycle, a less pronounced diel pattern in abundance was observed during the second diel cycle, with no significant and defined periodicity (Fig. 2, Table III). A high diel abundance variation of *Synechococcus* concentration was measured (14–19%), mostly resulting from the strong increase during the second night of observation (Fig. 2). *Synechococcus* abundance recovered a diel pattern toward the end of the second cycle, more exactly during the third light period of the second cycle (28th of February), when its concentration decreased with a pattern similar to that observed during the first week. This observation suggests that the weekend turbulence event disrupted the intrinsic diel cycles of the picophytoplankton, and that the cycles recovered after ca. 2 days to a pattern similar to that observed during the first week.

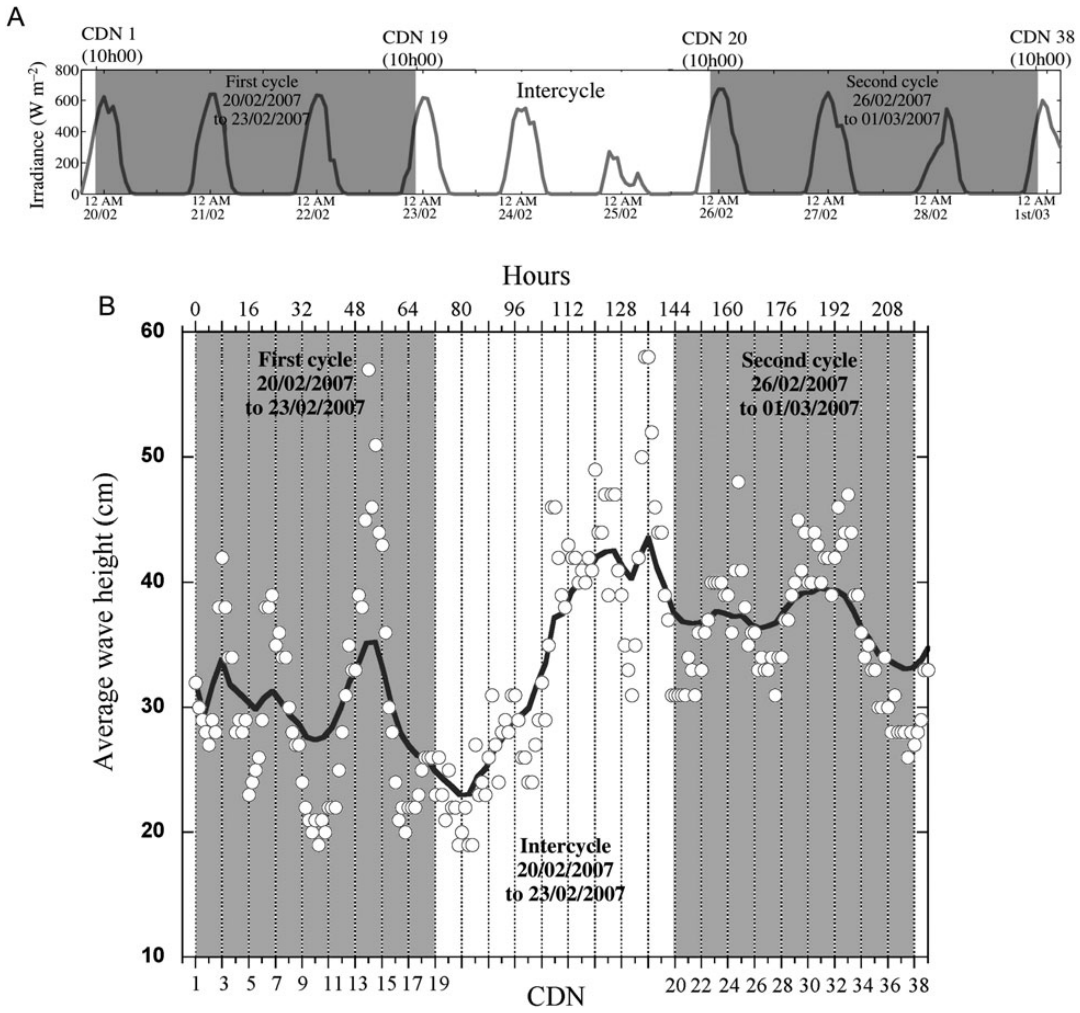

Fig. 1. (**A**) Irradiance measurements at 2 m (upper panels) during the first cycle (from the 20th to the 23rd of February) during the second cycle (from the 26th of February to the 1st of March 2007) and between the two cycles (from the 24th to the 25th of February 2007). (**B**) A smoothed average wave height measured by a scalar buoy throughout the sampling period (XIOM Network, http://www.boiescat.org). The lines were generated using a smooth fit in the software Kaleidagraph v. 3.6.2 (Synergy Software).

Cell-specific pigment content (as measured by the standardized FL2 and FL3 parameters) followed a clear diel pattern during the two cycles, with a significant periodicity of 24 h during the first cycle (Fig. 3A, Fisher's Kappa, $P < 0.05$, Table III). Contrary to the pattern observed in abundance (Fig. 2B), this trend corresponded with the accumulation of pigments during the growth process occurring during the light period of the day. The decrease of fluorescence began just after dusk and reached a minimum at noon with a stationary period until dawn. Significant differences between cycles were measured for the fluorescence parameters (Table I). A less pronounced diel pattern was observed in the SSC parameter (a surrogate of cell size) (Fig. 3B). This parameter varied opposite to abundance with a minimum around midnight, at the expected moment of cell division. Note that the average SSC of *Synechococcus* during the second cycle was significantly lower (*t*-test, $P < 0.001$) than the one measured during the first cycle with values of 0.86 (± 0.10) and 1.16 (± 0.16), respectively (Table I), indicating that possibly two different populations of *Synechococcus* were sampled during the two weeks.

Prochlorococcus diel patterns

No pronounced diel patterns of *Prochlorococcus* abundance were observed during the two cycles (Fig. 2) without any significant periodicity (Table III). During the second week, the average *Prochlorococcus* concentration was higher than the one measured the first week. The average *Prochlorococcus* abundance increased by 122% between

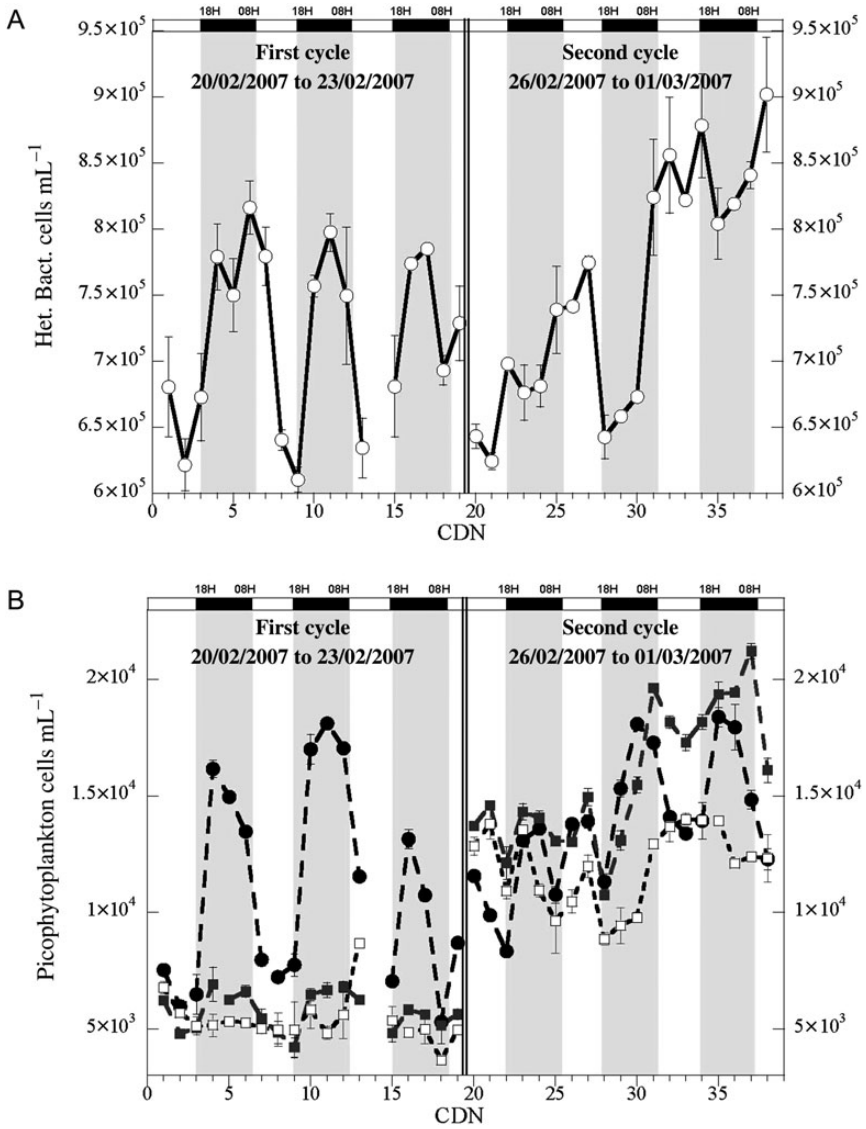

Fig. 2. Diel variations in picoplankton group abundances as measured by flow cytometry. (**A**) The Y axis of panel A corresponds to the heterotrophic bacterial concentration (open circles) and (**B**) the Y axis of panel B to the picophytoplankton abundances: closed circles for picoeukaryotes; closed squares for *Synechococcus* and open squares for *Prochlorococcus*. The gray areas correspond to the dark period (from 18:00 to 7:00 h) also indicated by solid bars on the top axis; the error bars correspond to the range of variation of the duplicate samples.

the first and the second weeks (Table I). In contrast to abundance, the FL3 parameter followed a significant 24-h periodicity (Table III). A decrease of fluorescence was observed during the day with a minimum reached at midday and increasing from midday until dawn (Fig. 3A).

pPeuk diel patterns

The concentration of picoeukaryotes followed a clear diel pattern during the first week of the experiment with a significant periodicity of 24 h (Fisher's Kappa, $P < 0.05$) (Fig. 2B, Table III). Abundance started to increase from dusk to the middle of the dark period and showed a two-step decrease. The first stage of the decrease began from the middle of the dark period to dawn, followed by a more pronounced decrease from dawn until the end of the light period (Fig. 2B). The diel variation of abundance was particularly high and calculated at 40% (Table I). During the second cycle, a less pronounced diel pattern was observed, and no significant periodicity was observed in picoeukaryote abundance (Fisher's Kappa, $P < 0.05$) (Fig. 2, Table II). While the average concentration of the second week was 26.5% higher than the first week, we measured less variability (coefficient of variation of 20%) (Table I).

Table III: Fisher's Kappa statistic testing for the significance of the diel variations

	Kappa periodicity	
	First cycle (hours) ($n = 19$)	Second cycle (hours) ($n = 19$)
Synechococcus		
Abundance	24*	Undefined
FL2	24*	Undefined
FL3	24	Undefined
SSC	24	19
Prochlorococcus		
Abundance	Undefined	Undefined
FL3	24	24*
SSC	Undefined	Undefined
Picoeukaryotes		
Abundance	24*	24
FL3	24*	24*
SSC	24*	Undefined
Heterotrophic bacteria		
Abundance	Undefined	15
Live + dead	24*	Undefined
CTC+ abundance	Undefined	nd
CTC (%)	Undefined	nd
HNA (%)	24	15
Live (%)	Undefined*	Undefined
HNF		
Abundance	24	15

"Undefined" indicates that no periodicity could be measured from the periodograms. *n* denotes the number for observations.
*Significant periodic variations ($P < 0.05$).

A pronounced diel pattern was observed during the two cycles in pPeuk pigment content (FL3 parameter, Fig. 3A) with a significant 24-h periodicity (Fisher's Kappa $P < 0.05$). This pattern was, again, opposite to the one described for abundance. Significant but weak differences were observed between the per cycle global means ($P < 0.05$). A significant diel pattern was observed in pPeuk SSC only during the first cycle with a periodicity of 24 h (Fisher's Kappa, $P < 0.05$) (Fig. 3B).

Group division rates

We estimated the *in situ* division rates for each picophytoplankton group calculating the day-to-day variations of the ratio of the minimum to the maximum light scattering (SSC) parameter, as proposed by others (Binder *et al.*, 1996; Vaulot and Marie, 1999) (Fig. 4). The amplitude of the changes in SSC varied from 1 day to the next one, and varied differently for the different groups. The highest "division rates" were estimated for *Prochlorococcus* during the first cycle, followed by *Synechococcus* and picoeukaryotes. During the second cycle, the highest $SSC_{Max:Min}$ values were measured for *Synechococcus*, followed by *Prochlorococcus* and picoeukaryotes. Considering that an $SSC_{Max:Min}$ value of 2.5 for *Prochlorococcus* in the Equatorial Pacific corresponded to 1 division per day as formulated by Vaulot and Marie (Vaulot and Marie, 1999), *Prochlorococcus* showed larger rates throughout the first cycle, reaching almost 0.5 division per day at the end of the first cycle. These rates of gross growth did not translate into an increase in cell abundances (Fig. 2).

Heterotrophic bacteria abundance and activity diel patterns

No significant differences were observed between the two per cycle average abundance values (Table I). With one of the two methods used we observed a significant cycle in the first week, with a significant periodicity measured close to 24 h (Fisher's Kappa, $P < 0.05$) (Fig. 2, Table III). Bacterial concentration increased from dusk until midnight and then decreased until dusk. Less pronounced patterns and no significant periodicity were measured during the second week for both estimations of bacterial abundance, and only a general increase was observed (Fig. 2, Table III).

The contribution of high nucleic acid (HNA) cells (%HNA) to the total bacterial abundance and those of actively respiring cells (%CTC+) followed the same pattern observed with bacterial abundance during the two cycles (Fig. 5), but the periodicity was not significant (Table III). The peaks of activity occurred around midnight during the first week (Fig. 5), with an important increase in the second night period of the second cycle, corresponding also with the increase in bacterial and *Synechococcus* abundance. %HNA was significantly positively correlated with pPeuk and bacterial abundances during the first cycle, indicating an important phasing between these parameters (Table IV). During the second cycle, the percentage of CTC+ cells (%CTC) was correlated with %HNA (Table IV), indicating that these two parameters followed similar patterns.

HNF diel patterns

During the first cycle, HNF cell concentration increased during the light periods (Fig. 6), although the periodic variations were not significant (Table III). Lower periodicity was observed during the second cycle, but after a pronounced decrease in HNF abundance observed, during the first day of the second cycle, a general trend of increase was apparent. Significant negative correlations were calculated between HNF abundances and *Synechococcus* abundance during the first week (Table IV) and a positive tendency (although no significant correlation) was observed with %CTC and %Live cells during the first and second cycles, suggesting a possible preference of HNF grazing activity for actively growing bacterial cells with intact membranes.

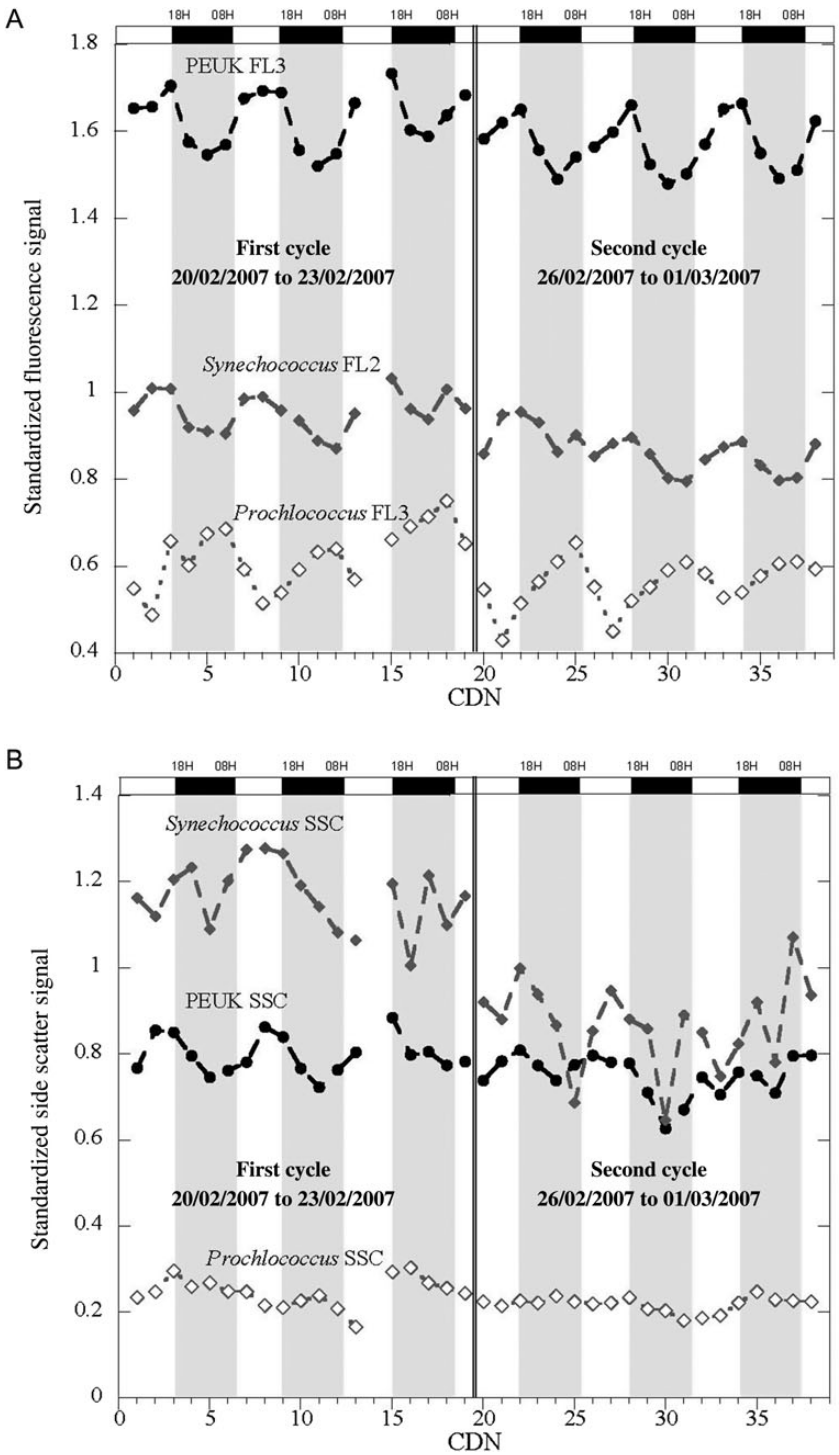

Fig. 3. (**A**) Picoeukaryotes and *Prochlorococcus* red fluorescence (from chlorophyll *a* in relative units) and *Synechococcus* phycoerythrin orange fluorescence (FL2, relative units), all standardized according to the fluorescence of Polysciences 1-μm beads. (**B**) SSC parameter for picoeukaryotes, *Synechococcus* and *Prochlorococcus*. All standardized according to the beads' SSC. The gray bars indicate the dark periods.

DISCUSSION

We focused on the diel variability of different picoplankton populations and the phasing of bacterial heterotrophic activities measured at the single-cell level. We performed the observations at the likely time of the picophytoplankton bloom, when we knew from previous research that

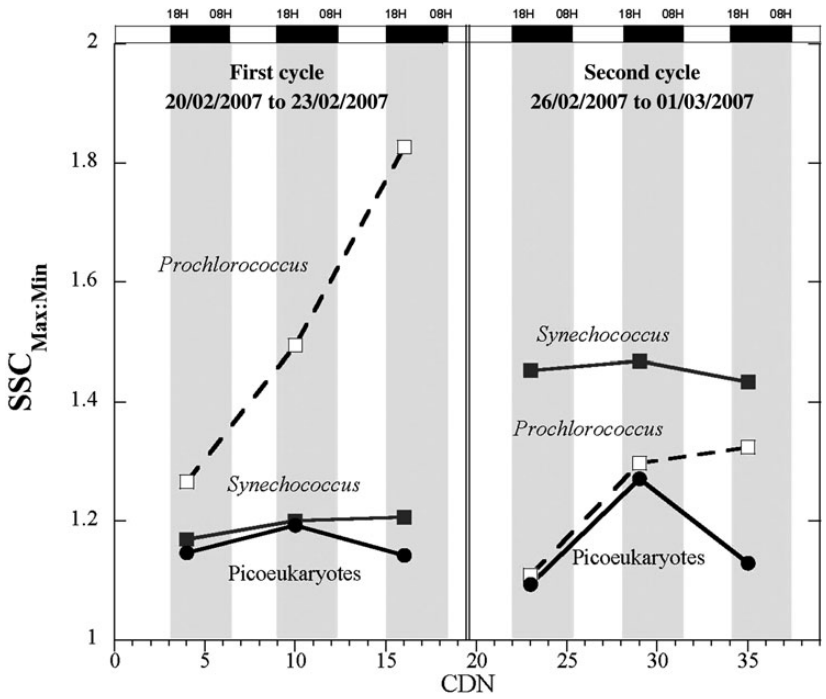

Fig. 4. The ratio of the maximum to the minimum SSC as a proxy for the *in situ* division rate of *Synechococcus*, *Prochlorococcus* and picoeukaryotes.

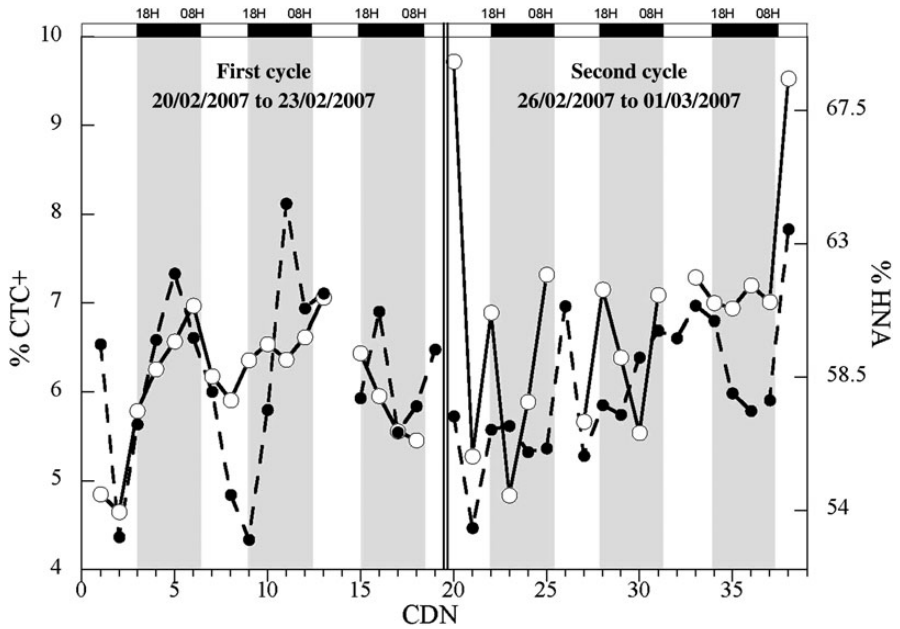

Fig. 5. Percentages of CTC-positive bacterial cells (open circles) with the scale on the left axis and of HNA (dashed black lines) with the axis on the right. The gray bars indicate the dark periods.

picoeukaryotes were at their maximal concentrations, to maximize the likelihood of observing coupled variability of picophytoplankton and bacteria. The picoeukaryotes were at ca. 2 10^4cells mL^{-1} in February 2007, which is very close to the maximum value recorded at this sampling site for the last 12 years of monthly sampling (details not shown). Indeed, during the sampled period, chlorophyll <3 μm was ca. 50% of the total chlorophyll and so the picophytoplankters were a very significant component of the phytoplankton community.

Table IV: Pearson correlation coefficients between picoplankton group abundances and heterotrophic activity parameters as measured by flow cytometry during the first cycle (A) from the 20th to 23rd of February and during the second cycle (B) from the 26th of February to 1 of March 2007

Cycle 1	Syn abund.	Pro abund.	pPEUK abund.	Bact. abund.	HNF abund	HNA%	%CTC+
A ($n = 18$)							
Syn abund.	–						
Pro abund.	0.11	–					
pPEUK abund.	**0.85**	0.05	–				
Bact. abund	**0.80**	−0.26	**0.72**	–			
Hnf abund	**−0.54**	−0.15	−0.22	−0.34	–		
%HNA	**0.72**	0.18	**0.65**	**0.61**	−0.40	–	
%CTC+	0.28	0.11	**0.54**	0.33	0.25	0.46	–
% (NADS) Live	−0.10	0.06	0.12	−0.12	0.25	−0.08	**0.50**
Cycle 2	Syn abund.	Pro abund.	pPEUK abund.	Bact. abund.	HNF abund	HNA%	%CTC
B ($n = 19$)							
Syn abund.	–						
Pro abund.	**0.60**	–					
pPEUK abund.	**0.67**	0.12	–				
Bact. abund	**0.75**	**0.45**	0.36	–			
Hnf abund	0.27	0.20	0.15	−0.14	–		
%HNA	0.39	0.18	0.30	**0.69**	0.00	–	
%CTC+	0.09	0.06	−0.14	0.37	0.40	**0.53**	–
%(NADS) Live	−0.12	−0.07	0.07	−0.51	0.10	**−0.50**	−0.28

Bold figures indicate significant correlations (Pearson's tests, $P < 0.05$). Abund., abundance; Syn, *Synechococcus*; Pro, *Prochlorococcus*; pPeuk, photosynthetic picoeukaryotes; HNF, heterotrophic nanoflagellates; %HNA, percentage of high nucleic acid content bacteria; *n*, number of observations.

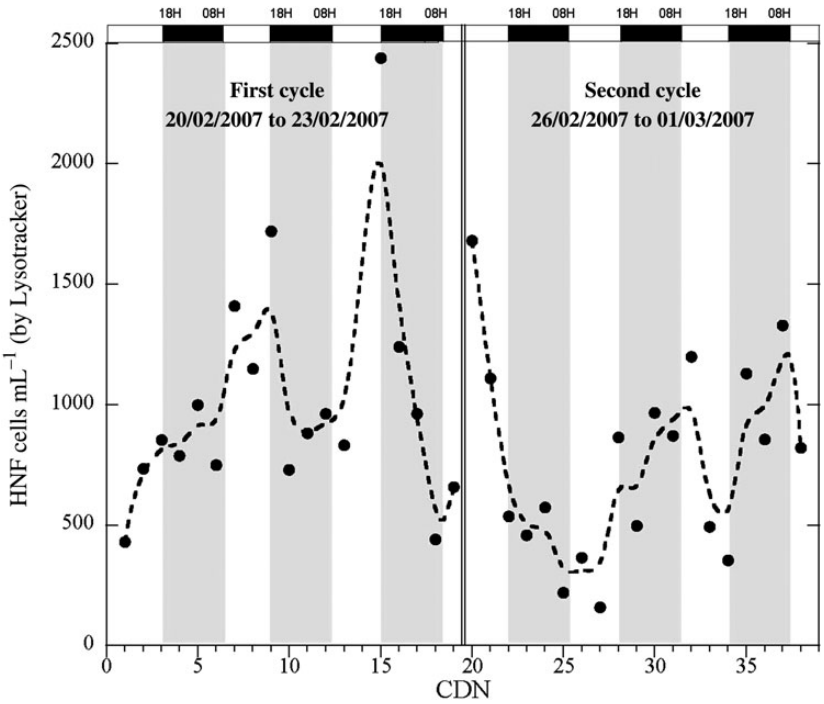

Fig. 6. The concentration of HNF after Lysotracker staining (solid black lines) during the two cycles. The lines were obtained using a smooth fit in the software Kaleidagraph v. 3.6.2 (Synergy Software). The gray bars represent the dark periods.

In brief, our results show (i) consistent diel variability of a majority of picoplankton populations, including heterotrophic bacteria and HNF, (ii) differences in the time of division and growth of different picophytoplankton groups and (iii) coupling between picophytoplankton variability and single-cell bacterial activities. We furthermore observed how a relatively small variation in weather patterns changed considerably the structure

of the microbial community and disrupted most diel cycles, which started to recover a couple of days after the disruption.

Diel patterns in picophytoplankton abundance and division

During the two cycles, diel patterns were observed in the fluorescence and scatter parameters of the different picophytoplankton groups, with a periodicity close to 24 h for the majority of the parameters studied. *Synechococcus* and picoeukaryote growth, as measured by the increase in SSC and fluorescence (FL2 and FL3), occurred during the light period, indicating that light drove the synthesis and accumulation of carbon and pigments, followed during the night by division, producing smaller cells with lower scatter (Durand and Olson, 1998). These measured diel variations are not unusual but are apparently common for *Synechococcus*, *Prochlorococcus* and picoeukaryotes communities across systems (Vaulot *et al.*, 1996; Jacquet *et al.*, 1998, 2001a,b, 2002a,b; Vaulot and Marie, 1999; Durand *et al.*, 2002; Seymour *et al.*, 2005).

However, the increase of *Prochlorococcus* FL3 fluorescence and cell size occurred during the night period instead of during the light period. A minimum of chlorophyll *a* fluorescence (FL3) was measured at midday for *Prochlorococcus* and was concomitant with the maximum irradiance measured daily. This phenomenon could be assigned to bleaching if it were not that no particular bleaching of fluorescence for *Synechococcus* and picoeukaryotes was measured during the light period of the diel cycle and that the light reaching the waters at this time of the year, in mid-winter, was not very high. Vaulot and Marie (Vaulot and Marie, 1999) measured similar patterns in the equatorial Pacific and observed only for *Prochlorococcus* fluorescence some quenching during the light period at the surface (in samples particularly exposed to high irradiance levels) and an increase of fluorescence at depth (Vaulot and Marie, 1999), suggesting that *Synechococcus* and picoeukaryotes were more protected against light damage than *Prochlorococcus*, something that can be explained by the much thicker thykaloid layer in genus *Synechococcus* and more complex photoprotective mechanisms in eukaryotes (Sommaruga *et al.*, 2005; Llabrés and Agustí, 2006). However, no decreases in *Prochlorococcus* FL3 was observed during the night, suggesting that the day light minimum was more likely related to division rather than to photochemical quenching.

Our study suggests a specific timing for *Prochlorococcus* division at least for this environment and at the time of sampling. This specific behavior could also suggest that processes other than photosynthesis were supporting *Prochlorococcus* cell growth, i.e. heterotrophy (incorporation of organic matter). It has been shown that both *Synechococcus* and *Prochlorococcus* are capable of assimilating amino acids in surface waters of the South Atlantic Subtropical front (Zubkov and Tarran, 2005) and also that *Prochlorococcus* followed pronounced diel patterns in ^{3}H-leucine and ^{35}S-methionine uptake with a minimum occurring at midday in the Atlantic Ocean (Mary *et al.*, 2008). Organic matter uptake by *Synechococcus* has been seen to follow diel patterns (Chen *et al.*, 1991; Vila-Costa *et al.*, 2006). However, we detected no significantly different uptake rates between night and day in *Synechococcus* as identified with fluorescent *in situ* hybridization probes when measured during our sampling in Blanes Bay (Ruiz-González *et al.*, 2012). *Prochlorococcus* were not tested in that study.

Disruption of the diel patterns in PCS

In comparison with the first cycle, the diel patterns of abundance during the second cycle appeared disrupted during the first and second days of observation, with a tendency toward a recovery of the diel patterns in the third day. These changes in the community structure were preceded on the 26th of February by shifts in wind direction, rainfall and turbulence conditions. However, in comparison with the weak diel periodicities observed in abundance, stronger periodicities in pigment fluorescence and SSC parameter of *Prochlorococcus*, *Synechococcus* and pPeuk were generally measured, suggesting that in spite of the shift in the community structure provoked by the turbulence and wind event, the single-cell biology was still following a fairly regular day–night pattern.

The fact that the diel patterns in abundance were generally more altered than the other parameters indicates more likely an imbalance between growth and loss processes. Different factors can explain loss processes in picophytoplankton communities, including grazing by HNFs (Dolan and Simek, 1999), and also viral lysis (Suttle and Chan, 1994). Since the diel variations in picophytoplankton community structure indicate that loss processes do not occur at a uniform rate during the day (Vaulot and Marie, 1999), that grazing activity by HNF could vary with the picophytoplankton cell cycle (Christoffersen, 1994; Dolan and Simek, 1999; Christaki *et al.*, 2002) and that diel variability of viral infection has also been demonstrated (Weinbauer *et al.*, 1995), disruption of the diel periodicity in HNF or virus abundance and activity could result in an increase in prey abundance. In our study we showed patterns of diel grazing activity on pPeuk populations with peaks at night particularly pronounced during the first cycle (Ruiz-González *et al.*, 2012). The data we present here on HNF abundance

(Fig. 6) show strong variability in HNF abundance and a decreasing trend during the first day of the second cycle directly after the wind event. This observation is likely to explain the imbalance between growth and loss rates that resulted in a general increase of all picoplankton group abundances.

Whether or not the observed changes in the community structure between weeks are linked with the sampling of different water masses over the period covering the diel cycles is difficult to ascertain and prove. No major variations of temperature or salinity were observed suggesting that we followed a relatively stable water mass. Chlorophyll *a* concentration varied little from 1 week to the next concomitantly with the shift in the picophytoplankton community structure, dominated numerically by pPeuk during the first diel cycle and by *Synechococcus* during the second. Inorganic nutrients all decreased from week to week (ammonia, nitrite and phosphate significantly, *t*-tests, $P < 0.001$, nitrate and silicate not significantly), but these could be a consequence of the organisms' growth. In the past it has been shown that the *Synechococcus* cell cycle was relatively little impacted by strong hydrological variability when compared with that of other picophytoplankton groups (Jacquet *et al*., 2002a), but the dominance of *Synechococcus* during the second cycle could also be promoted by the wind and turbulence resuspension that may have changed nutrient availability, differentially affecting the activities of the different microorganisms, as it was shown elsewhere (Cotner, 2000; Garstecki *et al*., 2002; Jacquet *et al*., 2002b). And while we observed higher division rates in *Synechococcus* during the second diel cycle when compared with other picophytoplankton groups (Fig. 4), a relatively and significantly lower light scattering value observed during the second week might also suggest that we followed two different populations, a first one with high SSC values and low division rates (as estimated from the ratio of the maximum to the minimum SSC over 1 day) during the first week, and a second with smaller cells, but with higher division rates.

Coupling between heterotroph and phototroph parameters

During the first cycle, bacterial abundance followed pronounced diel patterns, strongly phased with the relative activity measurements peaking around midnight and strongly correlated with *Synechococcus* and picoeukaryote concentrations. During the second cycle, only bacterial abundance was correlated with all the picophytoplankton groups (Pearson's tests, $P < 0.05$), and no coupling with the bacterial activity estimators was found. Despite the recognized toxicity of the CTC dye, it is also ecologically relevant for determining the highly active part of the bacterial community (Gasol and Arístegui, 2007) and has been related to bacterial production and cell growth (Choi *et al*., 1996; Lovejoy *et al*., 1996; del Giorgio *et al*., 1997; Sherr *et al*., 1999), it is reasonable to consider that bacteria were more active during the night, an idea supported by the observation of bulk and group-specific bacterial production particularly enhanced during the dark period (Ruiz-González *et al*. 2012).

The tight phasing between picophytoplankton parameters and bacterial abundance and activity found during the first cycle could indicate that the release of dissolved organic matter originating from phytoplankton growth and division processes was directly used to support heterotrophic activity (Nagata , 2000). Similarly, the pronounced diel patterns in bacterial production measured during the first cycle (Ruiz-González *et al*., 2012) and the extremely high variability associated with its diel fluctuations (calculated to be 37% during the two cycles as measured by ^{3}H-leucine incorporation) also support the idea that bacteria rapidly responded to diel changes in organic matter release from phytoplankton (Hagström *et al*., 2001), and since picophytoplankton contributed to half of the total phytoplankton biomass, to the smaller autotrophs as well. Ruiz-González *et al*. (Ruiz-González *et al*., 2012) observed that the weekend turbulence event had little effect on the bacterial community structure and activity, except for *Gammaproteobacteria* leucine uptake activity, which increased during the second cycle and followed the same increasing patterns as those measured for the different picophytoplankton groups. Some authors have hypothesized that the shift in the bacterial community structure occurring after a turbulence event was more likely driven by the change experienced by phytoplankton community structure, rather than by the physical stress alone (Pinhassi *et al*., 2004).

But were bacteria responding to the DOM excreted during production, or to DOM originating from other processes during the phytoplankton growth period? Primary production peaks generally at noon and a strong phasing between bacterial activity and phytoplankton production would suggest bacterial activity also peaking at noon. However, during the first cycle, a lag was observed since single-cell indices of activity (Fig. 5), bulk bacterial heterotrophic activity and grazing on picoeukaryotes (Ruiz-González *et al*. 2012) were seen to be higher during the night. Similarly, during the second cycle, no night peaks of CTC+ nor a night increase of bacterial production (Ruiz-González *et al*., 2012) was observed, only the same trend of increase of both parameters with HNF abundance. It is known that DOM resulting from egestion by grazing activity can represent

up to 65% of the total DOM and most of bacterial C demand (Nagata, 2000), supporting and strengthening the idea that to support their growth bacteria were likely using the dissolved organic matter released from grazing activity rather than the excreted primary production.

Concluding remarks

Improved knowledge of the diel patterns in microbial parameters and the resulting diel variability may inform us about the factors controlling the growth and loss processes of marine microbes. Not all studies of diel variability encounter the above-mentioned periodicities, and in some cases microbial abundance and activity seem to vary at random. Other than lack of sensitivity of the methods used, it is interesting to describe what environmental factors facilitate that microbial populations vary with diel periodicity in some cases and not in others. Here, we showed consistent diel periodicity of a majority of picoplankton groups and evidence of coupling between picophytoplankton variability and single-cell bacterial activities. Moreover, we showed differences in the time of division and growth of different picophytoplankton groups and observed how a relatively small variation in weather patterns could change considerably the structure of the microbial community and disrupt most of the diel cycles.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org.

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