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Picoplankton population dynamics in coastal waters of the northwestern Mediterranean Sea

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Abstract

High-frequency sampling of surface picoplanktonic populations was performed in Villefranche Bay (northwestern Mediterranean Sea) during the first week of July 1996. The evolution of abundance and cell parameters were monitored once per hour by flow cytometry for these populations: *Synechococcus* cyanobacteria, photosynthetic picoeukaryotes, and heterotrophic bacteria plus *Prochlorococcus*. Some parameters, such as the right-angle light scatter of Synechococcus and picoeukaryotes, or the red chlorophyll fluorescence of picoeukaryotes, exhibited a very clear 24-h diel periodicity. For other parameters, such as *Synechococcus* red chlorophyll fluorescence, it was necessary to perform a Fourier analysis to establish a major 24-h period unambiguously. This analysis also revealed that some other parameters, however, such as the cell concentration, right-angle light scatter, and red chlorophyll fluorescence of picoeukaryotes, or the red chlorophyll and orange phycoerythrin fluorescence of *Synechococcus*, had a period of 17-18 h, corresponding to the inertial frequency at this latitude. The cell cycle of *Synechococcus*, sampled twice per hour, was synchronized with the daily light cycle, allowing an estimate of their growth rate, which averaged 0.95 d^{-1} (SD = 0.16, n = 7). Its large day-to-day variability was related to the duration of the interval between the maxima of S and G2 phases that ranged from 2 to 3.5 hours. Generally, the division rate was depressed on sunny days. The loss rate of *Synechococcus* was in general lower than the division rate and appeared to follow the evolution of the latter with a l-d lag, as if grazers or viruses adapted very rapidly to changes in division rates. Over the period of study, concentrations of *Synechococcus* and other bacteria were significantly correlated (r^2 = 0.60, $p < 0.01$, n = 340), suggesting the possibility of a common controlling factor for these populations, e.g., phosphorus or grazing.

Picoplankton (i.e., the fraction of cells $<3\,\mu m$ in size) is composed of heterotrophic bacteria, two types of photosynthetic prokaryotes, *Prochlorococcus* (Chisholm et al. 1988) and *Synechococcus* (Waterbury et al. 1979), and picoeukaryotes (Johnson and Sieburth 1982). They constitute most of the plankton community in intertropical oceans and temperate oligotrophic areas and are responsible for the bulk of oceanic production (Azam et al. 1983; Li et al. 1992). Among them, the marine cyanobacterium *Synechococcus* is ubiquitous in both oligo- and mesotrophic oceanic areas, with concentrations reaching 10^5 cell ml^{-1} (Olson et al. 1990; Campbell and Vaulot 1993; Partensky et al. 1996). Picoeukaryotes are generally less abundant, although they can be large contributors to biomass and production (Campbell et al. 1994; Li 1994). They are also more heterogeneous because they include many taxa (Johnson and Sieburth 1982; Simon et al. 1994), most of which are yet to be described. Heterotrophic bacteria are generally the most abundant component of the 0.2–2-μm size fraction. Since publication of the seminal paper by Azam et al. (1983), the role of heterotrophic bacteria in the microbial food web has been largely demonstrated. This community remains poorly characterized (Giovanonni et al. 1990; Fuhrman et al. 1993), however, and most of the factors controlling its dynamics remain to be determined. Interactions between autotrophic and heterotrophic populations are probably very important for the control of oceanic productivity in oligotrophic areas (McManus and Fuhrman 1988).

Although a large set of data has been accumulated on the abundance of the different picoplankton populations throughout the world ocean, especially through large-scale programs such as JGOFS (Joint Global Ocean Flux Study), their dynamics remain poorly characterized. Their long-term evolution is driven by a variety of factors that act on different time and spatial scales. In most studies, only large scales are considered, mostly for logistical reasons. For example, cruises will typically sample at scales of 10–100 km, and time series will favor the monthly or at best the weekly scales (e.g., Karl and Lukas 1996). Short time-scales, however, and the daily scale in particular, are probably more relevant to phytoplankton dynamics. Indeed, it has been known for a very long time that phytoplankton are highly synchronized to the light : dark cycle (Gough 1905). Recent studies have shown that picoplanktonic populations are also highly synchronized and display division rates of close to once per day (e.g., Vaulot et al. 1995). It is not yet clear what kind of environmental signal (e.g., sunrise, sunset, light level threshold) is responsible for the synchronization and whether an endogenous circadian clock is implicated, as recently evidenced in cyanobacteria (Sweeney and Borgese 1989; Mori et al. 1996).

It is therefore critical to begin studying phytoplankton population dynamics at short time-scales. In doing so, we

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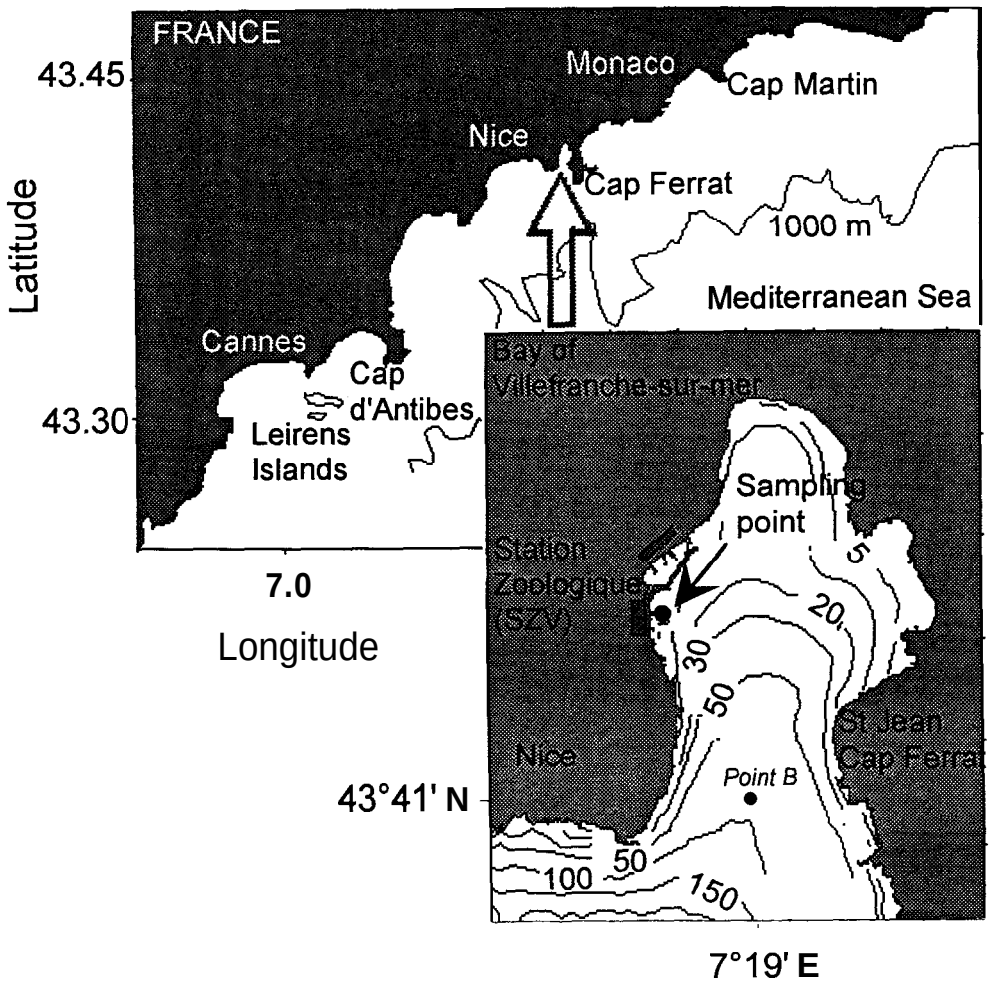

Fig. 1. Sampling site of the MEDEA workshop (July 1996). Surface seawater was collected at 2-m depth and 7.5 m from the pier of the Station Zoologique of Villefranche-sur-mer (northwestern Mediterranean Sea).

may gain a much better understanding of the factors that regulate cell growth and division, as well as those that lead to cell loss. Picoplankton appear to be particularly amenable to this type of study because their small size makes them suitable to flow cytometric analyses, permitting the very rapid recording of cell abundance as well as various cell parameters.

In the present paper, we sampled and measured the picoplankton community off Villefranche-sur-mer (northwestern Mediterranean Sea, France), by flow cytometry at frequencies on the order of once per hour for a week. Our aims were to establish which cellular parameters displayed periodic behaviors, which frequencies were the most important, how the phase and amplitude of the recorded parameters varied from day to day, and finally, how these patterns can document picoplankton population dynamics.

Materials and methods

Study site-Surface water was sampled between 1 and 8 July 1996 inside the Bay of Villefranche-sur-mer (northwestern Mediterranean Sea, 43°41 'N, 7°19'E). The sampling location was 75 m from the pier at the Station Zoologique (SZV) and 2 m below the surface (water column depth = 8 m; Fig. 1). Weather conditions were monitored during this period. Sunrise and sunset times as well as air temperature measurements were obtained every day from the Signal Station of Saint-Jean-Cap-Ferrat (Table 1).

Sampling-Seawater was collected with a peristaltic pump (Delasco model DSC 12, PCM Pompes) through 20-mm-internal-diameter polyvinylchloride (PVC) tubing about 100 m in length, running along the sea bed. The tubing was equilibrated for 2 weeks before collections were started in order to eliminate toxic effects on marine planktonic cells (see Price et al. 1986). Water was pumped continuously at the rate of about 4 liters min^{-1} into an open polypropylene IO-liter carboy (Nalgene, Bioblock) that was used as an intermediary receptacle. Four-milliliter samples were retrieved from that carboy every 30 min using an automatic device described in detail elsewhere (Jacquet et al. 1998) and stored at 4°C until processing. Briefly, a peristaltic pump (Bio-

Table 1. Meteorological conditions during the sampling period (see *Materials and methods).*

Date	Sunrise (Local time)	Sunset (Local time)	Air temperature (°C) 0600 h	1200 h	1800 h	Wind a.m./p.m.	Sky a.m./p.m.	Sea surface a.m./p.m.
30 June	0556 h	2116 h	20°C	24	22	none	partly cloudy	quiet
1 July	0557 h	2116 h	20	23	20	none/weak	sunny/cloudy	quiet
2 July	0557 h	2115 h	11	23	21	none/strong	very cloudy	quiet/rough
3 July	0558 h	2115 h	20	25	22	none/strong	sunny	quiet/rough
4 July	0558 h	2115 h	19	25	22	none	sunny	quiet
5 July	0559 h	2115 h	22	26	22	none/weak	sunny/partly cloudy	quiet
6 July	0559 h	2114 h	20	25	24	none/strong	sunny	quiet/rough
7 July	0600 h	2114 h	21	22	21	none*	partly cloudy/sunny	quiet
8 July	0601 h	2113 h	16.5	22	21	strong/weak	cloudy/very cloudy	rough/quiet

* Storm during the night.

block) retrieved water from the carboy through a 25-m-long Tygon tube (Bioblock) and delivered ±4 ml every 30 min to tubes placed on a rotating carousel; samples there were kept at 4°C for a maximum of 8 h until analysis. A preliminary experiment showed the good preservation of cell parameters (fluorescence, light scatter, and deoxyribonucleic acid [DNA] histogram) at this temperature (Jacquet et al. 1998). Fresh analysis of *Synechococcus* and picoeukaryotes (see below) was performed only on every other sample (i.e., one sample per hour), whereas determination of the abundance of heterotrophic bacteria plus *Prochlorococcus (see below)* and *Synechococcus* cell cycle analysis were performed on all samples (i.e., two samples per hour) after fixation.

Sample processing-Samples were divided into three aliquots, the first of which was analyzed fresh. The two other aliquots were fixed for 15 min with a mixture of glutaraldehyde (0.05% final concentration) and paraformaldehyde (1% final concentration), modified from the methods of Vaulot et al. (1989). The mixture of aldehydes has been shown to improve the resolution of DNA histograms for marine prokaryotes through a lower coefficient of variation (CV) of the peak of cells in the G1 phase of the cell cycle (Marie unpubl. data). The second aliquot was then immediately incubated for 5 min in the presence of potassium citrate (30 mM final concentration) and SYBR Green I (SYBR-I; 1 : 10,000 final concentration; Molecular Probes) in order to obtain bacterial counts (Marie et al. 1997). These analyses were performed continuously during the day, and efforts were made to reduce the interval between sampling and analysis for samples collected at night. The last aliquot was frozen in liquid nitrogen and stored at -80°C for delayed bacterial counts (i.e., heterotrophic bacteria plus *Prochlorococcus)* and *Synechococcus* cell cycle analysis. Back in the laboratory, this third aliquot was thawed and incubated at 37°C in presence of 0.1 g liter' of a mixture of ribonuclease (RNase) A and B (1 : 1 wt/wt; R 4875 and R 5750, Sigma Chemical) for 1 h and then stained with SYBR-I for at least 10 min (Marie et al. 1997).

Flow cytometric analysis-Samples were analyzed using a FACSort flow cytometer (Becton Dickinson). For all experiments using flow cytometry, 0.2-μm-filtered seawater was used as sheath fluid. Setup for natural fluorescence was as previously described (Partensky et al. 1996). Fluorescent beads with a diameter of 0.95 μm (Polysciences) were added into each sample, and all of the parameters were normalized to them. The following parameters were acquired on fresh samples: cell concentration, forward-angle light scatter (FALS), right-angle light scatter (RALS), and orange phycoerythrin and red chlorophyll fluorescences. On SYBR-I-stained samples, green fluorescence from the DNA–dye complex was also measured. On fresh unstained samples (Figs. 2A,B), picoeukaryotes were differentiated from *Synechococcus* cyanobacteria on the basis of the orange phycoerythrin fluorescence of the latter (Fig. 2B). *Prochlorococcus,* although present (see *Results),* had very low red chlorophyll fluorescence, typical of oligotrophic surface waters (e.g., Partensky et al. 1996), and could not be differentiated from background noise. On SYBR-I-stained samples, both picoeukaryotes and *Synechococcus* cyanobacteria could be differentiated from other bacteria because of their red chlorophyll fluorescence (Figs. 2C,D), but again *Prochlorococcus* and heterotrophic bacteria could not be separated. For *Synechococcus* cell cycle analysis, samples were run either for 6 min or until 20,000 *Synechococcus* cells were measured (Fig. 2D). Acquisition was performed at a medium rate (25-30 $\mu l\,min^{-1}$) that allowed good discrimination between the different cell cycle phases. Because of flow rate problems after 7 July involving the flow cytometer used in the field, we stopped fresh sample analysis but collected samples for one more day. Hence, one extra day of *Synechococcus* cell cycle data is available (see *Results).*

Data analysis-Data were collected in listmode files and then analyzed on a personal computer (PC) using the custom-designed freeware CYTOWIN (Vaulot 1989, available through anonymous ftp server at ftp.sb-roscoff.fr/pub/cyto). Flow cytometry cytograms (Fig. 2) were drawn using the WinMDI freeware (J. Trotter; available on the Worldwide Web through http://facs.scripps.edu/). Cell cycle analyses were performed using MultiCYCLE. This program models DNA histograms (Fig. 2D), following the algorithm of Dean and Jett (1974), as the sum of two Gaussian peaks for G1 and G2 cells and as a polynomial function broadened by Gauss variability for S cells. Initially, the fractions of cells in G1 (position of G1 peak, CV of G1 peak, ..) are esti-

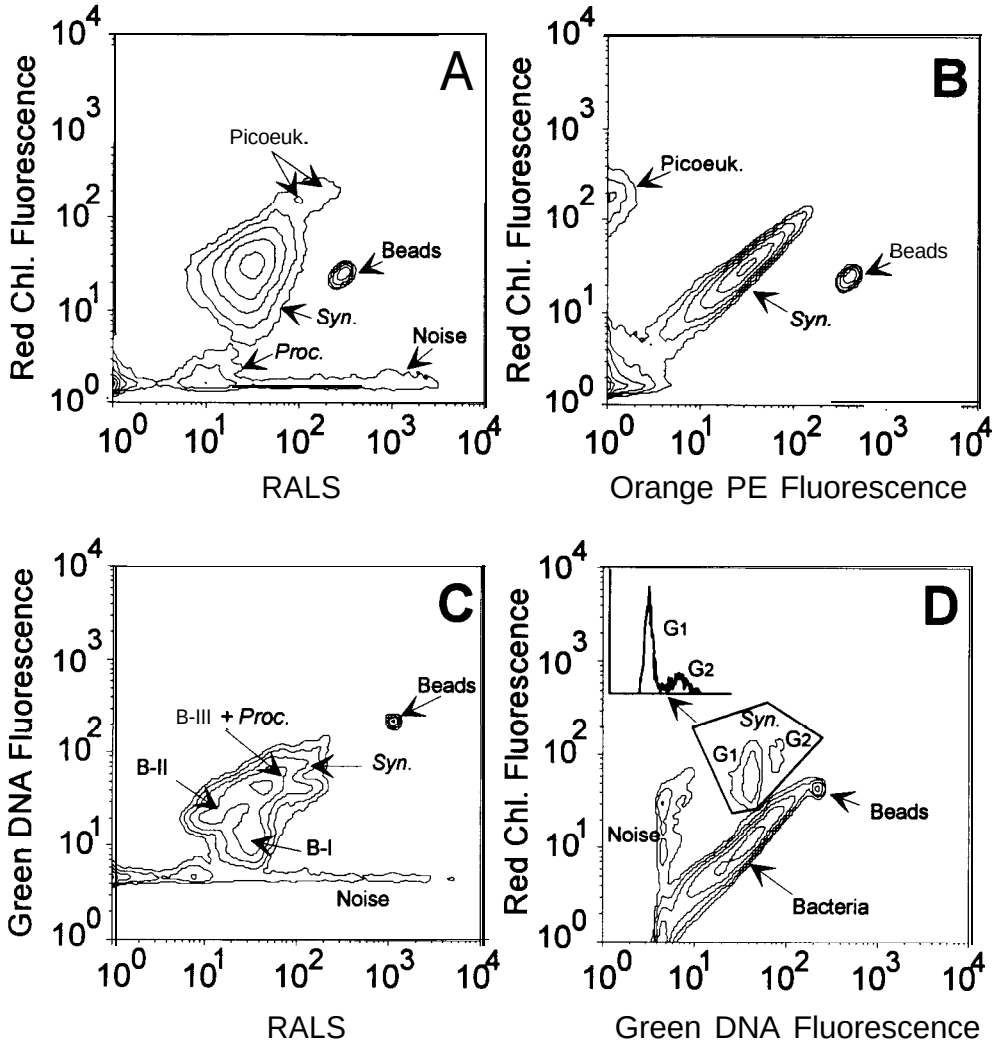

Fig. 2. Flow cytometric analysis of natural populations sampled in the Bay of Villefranche-sur-mer (2330 h, 6 July 1996). (A-B) Unstained samples. ***Prochlorococcus, Synechococcus,*** and picoeukaryotes were differentiated from each other on the basis of their right-angle light scatter (RALS) vs. chlorophyll red fluorescence (A) or phycoerythrin orange fluorescence (B). (C-D) Samples stained with the DNA dye SYBR Green I. Three bacteria populations, referred as B-I, B-II, and B-III + *Proc.,* were discriminated on the green DNA fluorescence vs. RALS cytogram (C). Picoeukaryotes do not show up because of their low relative abundance and the plot type. *Synechococcus* was well separated from other bacteria on the green DNA vs. red chlorophyll fluorescence cytogram (D). The cell cycle analysis of ***Synechococcus*** is represented as an insert on a linear scale.

mated interactively and then fitted nonlinearly using the Maquardt algorithm. Fourier analysis was performed with the Origin 4.1 software package (Microcal Software). In addition to the major frequency peak, when it existed, only significant frequency peaks were considered, i.e., those for which the ratio of their amplitude over that of the maximum frequency peak was greater than 0.5.

Division and loss rate estimations-To enable better discrimination of diel patterns, cell cycle data were smoothed with a low-pass filter, removing frequencies above 0.5 h^{-1}, using the equation: $y_i = a_6.x_{i-6} + .. + a_0.x_i + . + a_6.x_{i+6}$, with a, = 0.303, a, = 0.217, a_2 = 0.055, a, = 0.033, a, = 0.025, a, = 0.013, a_6 = 0.005, and where x, and y_i are the original and smoothed data, respectively, at time t,. The division rate was estimated from these smoothed data using the formula of Carpenter and Chang (1988):

$$\mu_{S+G2} = \frac{\sum_{i=1}^{n} \ln[1 + f_s(t_i) + f_{G2}(t_i)]}{n \times (T_S + T_{G2})} \times 24, \qquad (1)$$

where μ_{S+G2} is an estimate of the division rate (d^{-1}), n is the number of samples collected at fixed intervals during 1 d, $T_S + T_{G2}$ (h) is the sum of the duration of S and G2 phases, computed as twice the delay between the maxima of cells of each phase $[2(t_{G2max} - t_{Smax})]$ and used as the terminal event T_D, and $f_s(t_i)$ and $f_{G2}(t_i)$ are the fractions of cells in S

and *G2* phases at time t_i. Division rate was estimated from sunrise to sunrise (around 0600 h). $T_s + T_{G2}$ was not constant from day to day, and we used the real duration for each day (see Discussion).

The *Synechococcus* loss rate (g d^{-1}) was calculated from smoothed abundances (obtained with the same filter as that used for cell cycle data) and growth rate using the following formula:

$$g = \mu_{S+G2} - \ln(N_{d+1}/N_d), \quad (2)$$

where N_d and N_{d+1} are the average concentrations ($n = 3$) of the population at the beginning and the end of a 24-h sampling period, when no division occurs (i.e., around 0600 h).

Results

Synechococcus-After *Prochlorococcus (see below), Synechococcus* was the next most important autotrophic group detected by flow cytometry, with 43 X 10^3 cell ml^{-1} on average and up to 70 X 10' cell ml ' at the end of the period of study (Fig. 3A). The range in cell number during the week-long period of study varied about twofold. A weak diel pattern in the number of cells seemed to occur between 3 and 6 July, with a decrease in the first part of the day followed by an increase after midday (Fig. 3A). However, Fourier analysis of the full data set did not reveal any clear frequency peak (Table 2).

FALS and RALS, both functions of cell size and refractive index, displayed identical patterns (not shown), and only the latter will be discussed. RALS appeared to be very synchronized to the daily light cycle for *Synechococcus,* with an increase during the light period and a decrease during the dark period beginning around dawn (0600 h) and dusk (2100 h), respectively (Fig. 3B). On average, RALS increased 1.66-fold during the day. Minimum and maximum values obtained for each diel cycle were equivalent except for 6 July, when the maximum was 1.5 times lower. One notable deviation from this pattern occurred on 2 July, a very cloudy day, when RALS began to increase only 2 h after dawn.

Patterns obtained for cellular chlorophyll and phycoerythrin fluorescences of *Synechococcus* were much less apparent, although they had an obvious 24-h periodicity, as revealed by Fourier analysis (Table 2). A 17-18-h periodicity was also recorded for both parameters. For example, on 1, 2, and 5 July (Figs. 3C,D), days that were cloudy or partially cloudy, there was a clear-cut increase in fluorescence during the light period of the day. The minimum of fluorescence intensity was observed around 1400 h and the maximum between 1700 and 1900 h. During sunny days (3, 4, and 6 July), the fluorescence level increased during the night until a few hours after sunrise and then decreased. A fairly good correlation was found between phycoerythrin and chlorophyll fluorescence ($r^2 = 0.86$, $p < 0.01$, $n = 202$).

The DNA distribution of *Synechococcus* after staining by SYBR-I (Fig. 2D) was characterized by two peaks, referred to as G1 and G2 (in reference to eukaryotes) and separated by a phase of DNA replication (S phase), as previously reported by Vaulot et al. (1996). A distinct discrimination was obtained among these different phases (Fig. 4). The average CV of the G1 peak was equal to 10.5%. The analysis of the cell fractions in different phases revealed a very marked synchronization of the *Synechococcus* cell cycle. In most cases, the S phase began between 1400 and 1600 h and was most populous at 2100 h. The proportion of cells in G2 started to increase between 2000 and 2100 h, and the maximum of cells in G2 was obtained between 2200 and 2300 h (Fig. 4). On 7 and 8 July, maxima for S and G2 occurred earlier, around 1800 and 2000 h, respectively. On 1 July, two maxima for both S and G2 fractions could be observed. Minor maxima took place at 1400 and 1630 h, respectively, for S and G2, and major maxima at 2030 and 2230 h, the second of which was in agreement with what was usually observed. On 2 and 5 July, the entry of cells into S and the maximum of cells in this phase occurred about 1 h earlier than on the other days. On 8 July, an important fraction of cells was recorded in G2, i.e., about 50% more than on previous days. It is noteworthy that the maximum percentage of cells in S phase was greater during sunny days (Fig. 5B). The time measured between the maxima of cells in S and G2 phases was on average 2.5 h, but it varied between 2 and 3.5 h from day to day.

The brevity of both the S and G2 phases was a good index of the population synchrony, and we were able to estimate phase durations and growth rate using the model published by Carpenter and Chang (1988). The durations of the S and G2 phases averaged 3.3 ± 0.8 h ($n = 7$) and 1.8 ± 0.3 h ($n = 7$), respectively, but varied to a large extent from day to day (Fig. 6A). The duration of the S phase seemed somewhat related to the solar irradiance level. For example, for the two cloudy days, 1 and 2 July, the S phase duration was around 2.5 h. On 3 and 4 July, two very sunny days, the S phase duration increased, up to 4.6 h. It then decreased again on the following cloudy days (Fig. 6A). As a consequence of these changes, $t_{G2max} - t_{Smax}$ (i.e., the duration separating G2 and S maxima) was in general longer during sunny days. The daily division rate, estimated from smoothed data, was always higher than one division per day (0.95 d^{-1}, SD = 0.16, $n = 7$; Fig. 6B). The lower values were recorded on 4 and 6 July, i.e., the sunniest days of the study period, whereas the larger values were recorded on 1 and 7 July, with no clear relationship with weather conditions.

The daily loss rate, estimated from the difference between the net growth rate (that was estimated from cell abundance) and the division rate (Eq. 2), varied between 0.54 and 0.99 d^{-1}. Except for 2 and 6 July, it was always lower than the division rate (Fig. 6B).

Picoeukaryotes-The total abundance of picoeukaryotes was 2.5 X 10^3 cell ml ' on average and did not display any trend between 1 and 7 July, in contrast to *Synechococcus* (Fig. 7A). There was no significant correlation between picoeukaryote and *Synechococcus* abundances ($r^2 = 0.1$, $p > 0.1$, $n = 140$).

The diel pattern obtained for picoeukaryote RALS was not as clear as that of *Synechococcus,* although a 24-h periodicity was clearly present (Fig. 7B). Fourier analysis confirmed this pattern (Table 2).

In contrast to cyanobacteria, picoeukaryotes showed a strong daily pattern of chlorophyll fluorescence, with an in-

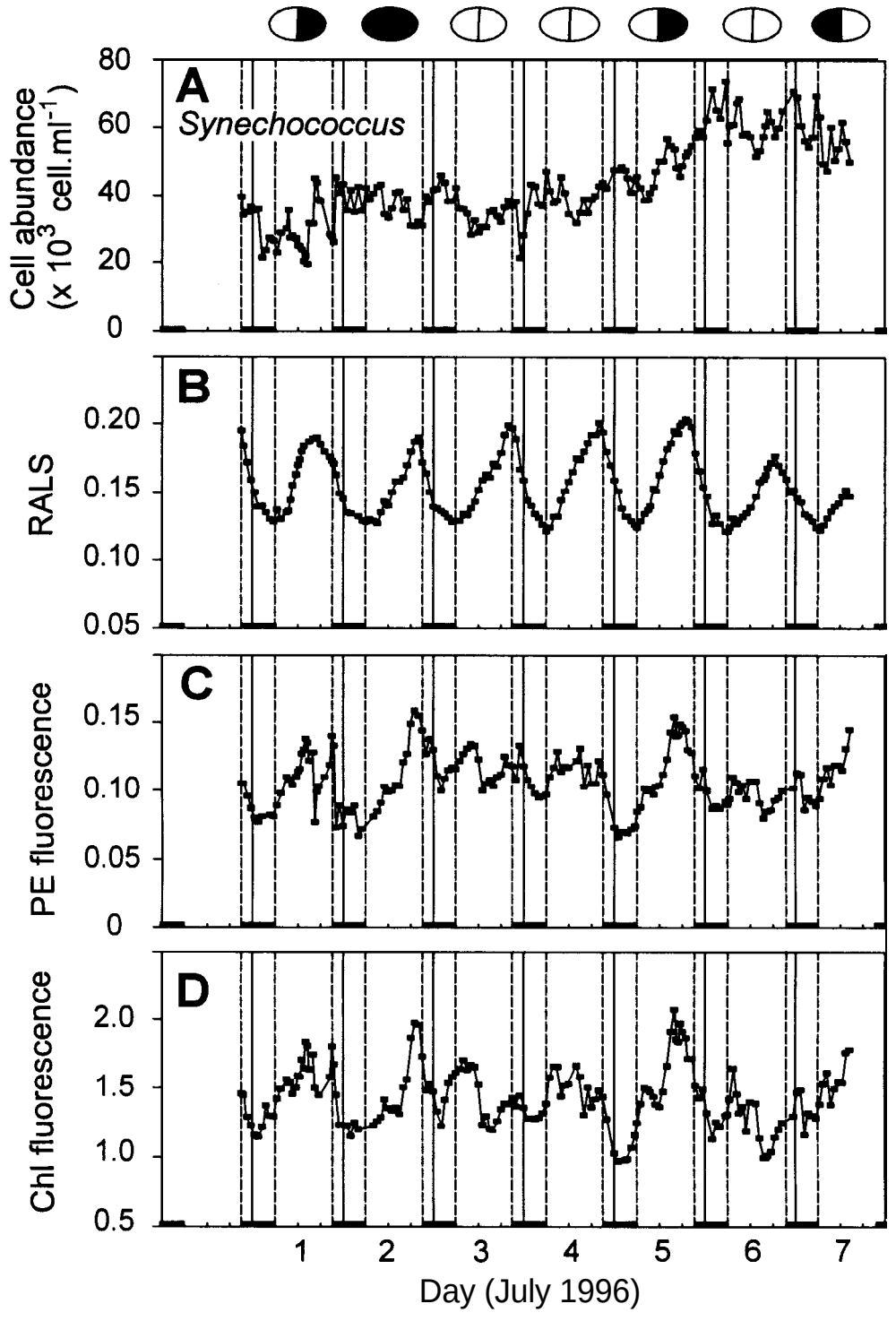

Fig. 3. Time series obtained for *Synechococcus* (fresh samples) between 1 and 7 July in the surface and coastal waters of Villefranche-sur-mer. Number of cells (A), right-angle light scatter (B), phycoerythrin (PE) fluorescence (C), and chlorophyll (Chl) fluorescence (D). These cytometric parameters were normalized to 0.95-μm diameter beads used as an internal reference. Dark bars symbolize the dark period of the day, and the relative irradiance level is symbolized by open, shaded, and solid symbols for sunny, partly cloudy, and cloudy half-days, respectively.

Table 2. Results of the Fourier analysis for each flow cytometric parameter (see ***Materials and methods***). + indicates significant frequency peak, i.e., which height is at least 50% of that of the major peak. – indicates no peak.

Population	Parameter	Periodicity: Inertial 17-18 h	Periodicity: Circadian 23-24 h
Synechococcus	Abundance	–	–
	FALS*	–	+
	RALS	–	+
	Chl	+	+
	PE	+	+
Picoeukaryotes	Abundance	+	–
	FALS	+	+
	RALS	+	+
	Chl	+	+
Bacteria	Abundance	–	–
	FALS	–	–
	FALS	–	–

* FALS, forward-angle light scatter; RALS, right-angle light scatter; Chl, chlorophyll; PE, phycoarythrin.

crease during the day and a decrease at night (Fig. 7C). On 3, 4, and 6 July (sunny days), the chlorophyll fluorescence maxima were lower than on the other days. Surprisingly, on 1 July, which was partially cloudy, very low values were recorded for chlorophyll fluorescence. As for *Synechococcus,* Fourier analysis revealed an 18-h periodicity for cell abundance, RALS, and chlorophyll fluorescence (Table 2).

Heterotrophic bacteria and Prochlorococcus-The chlorophyll fluorescence of ***Prochlorococcus*** cells, taken from fresh samples, was too dim to be differentiated from the background noise (Fig. 2A) by the FACSort flow cytometer. The concentration of ***Prochlorococcus*** was independently estimated, however, by epifluorescence microscopy, using an intensified charge coupled device (CCD) camera (Ouvemey pers. comm.), and it was found to be about 200 X 10^3 cell ml^{-1}, i.e., 4.5–5-fold larger than that of ***Synechococcus.*** This result suggested the dominance of ***Prochlorococcus*** among the autotrophic community, in agreement with what is usually observed in typical oligotrophic waters (Li et al. 1992; Campbell and Vaulot 1993) and with what Bustillos-Guzman et al. (1995) reported from divinyl-chlorophyll ***a*** measurements in summer at point B in Villefranche Bay (Fig. 1).

After staining with SYBR-I (Marie et al. 1997), heterotrophic bacteria and ***Prochlorococcus*** were detected together by flow cytometry (Fig. 2C). Abundances measured on frozen fixed samples were slightly lower than on fresh and fixed samples, but both numbers were highly correlated (r^2 = 0.76, $p < 0.01$, n = 150; $N_{frozen} = 0.93N_{fresh} + 5.5 \times 10^4$). Because frozen samples were more numerous (two samples per hour) than fresh samples, only the former are discussed. Heterotrophic bacteria and ***Prochlorococcus*** represented the major prokaryotic group, with an average concentration of 650 X 10^3 cell ml^{-1} (Fig. 8A). They ranged almost twofold during the period of study (between 4.3 X 10^5 and 8.0 X 10^5 cell ml^{-1}). Three subpopulations (B-I, B-II, and B-III, Li et al. 1995; Marie et al. 1997) could be differentiated by their respective RALS and green fluorescence (Fig. 2C). The B-III population corresponds mostly to ***Prochlorococcus*** cells (Marie et al. 1997). B-I, B-II, and B-III represented on average 28%, 28%, and 44% of the total, respectively (Fig. SD). Similar temporal trends were recorded for the three subpopulations, except on 1 July, when the B-III cells were relatively more abundant at the expense of B-I cells. An anomalous pattern was recorded on 1 July, with high values for the FALS as well as for the RALS (Figs. 8B, C). No correlation was recorded between heterotrophic bacteria plus ***Prochlorococcus*** and picoeukaryote abundances (r^2 = ***0.11,*** $p > 0.1$, n = 136), whereas a significant correlation was established between these bacteria and ***Synechococcus*** (r^2 = ***0.60,*** $p < 0.01$, n = ***340***; Fig. 9).

Discussion

Diel patterns-Numerous observations have demonstrated that many variables linked to biology display a very clear 24-h periodicity in oceanic waters. This is the case, for example, with beam attenuation (Siegel et al. 1989) or chlorophyll fluorescence (Stramska and Dickey 1992). A similar periodicity has also been demonstrated for single-cell parameters measured by flow cytometry, such as light scatter, chlorophyll fluorescence, or cell cycle (Olson et al. 1990; Vaulot et al. 1995; DuRand and Olson 1996; Vaulot and Marie in press). Quite surprisingly, many parameters measured in the present study did not display a clear periodicity. Only the RALS and the cell cycle of ***Synechococcus,*** and the RALS and chlorophyll fluorescence of picoeukaryotes, had a clear-cut 24-h period. For many of the parameters examined (cell number, RALS, and red chlorophyll fluorescence of picoeukaryotes, or the red chlorophyll and orange phycoerythrin fluorescences of ***Synechococcus),*** however, Fourier analysis revealed a peak around 17-18 h (Table 2). How can we explain this paradoxical observation? First, the sampling site was very coastal, about 75 m from shore and less than 8 m deep. It is likely subjected to strong shore currents. Therefore it is probable that we did not sample the same water mass from the beginning to the end of the study. Part of the variability we observed was likely due to the spatial variability of the picoplankton populations in Villefranche Bay and in the adjacent coastal waters. If this hypothesis holds, then we only observed strong 24-h patterns for parameters that were very homogeneous across the whole area. Clearly, this was not the case for cell abundance, which is probably very dependent on a variety of gain and loss terms (cell division, grazing, virus lysis, mixing ...). In contrast, RALS and chlorophyll are apparently more spatially homogeneous, as suggested by their very strong diel patterns. Indeed, data from the equatorial Pacific, where the same water mass was continuously sampled for 5 d and where patterns were much less variable for scatter and chlorophyll than for cell abundance (Vaulot and Marie in press), support this idea. The fact that ***Synechococcus*** chlorophyll fluorescence only exhibited a weak diel pattern when compared to RALS may be explained by the contrasting vertical distribution of the two parameters. In the equatorial Pacific mixed layer, the RALS shows only a weak vertical stratification at all times

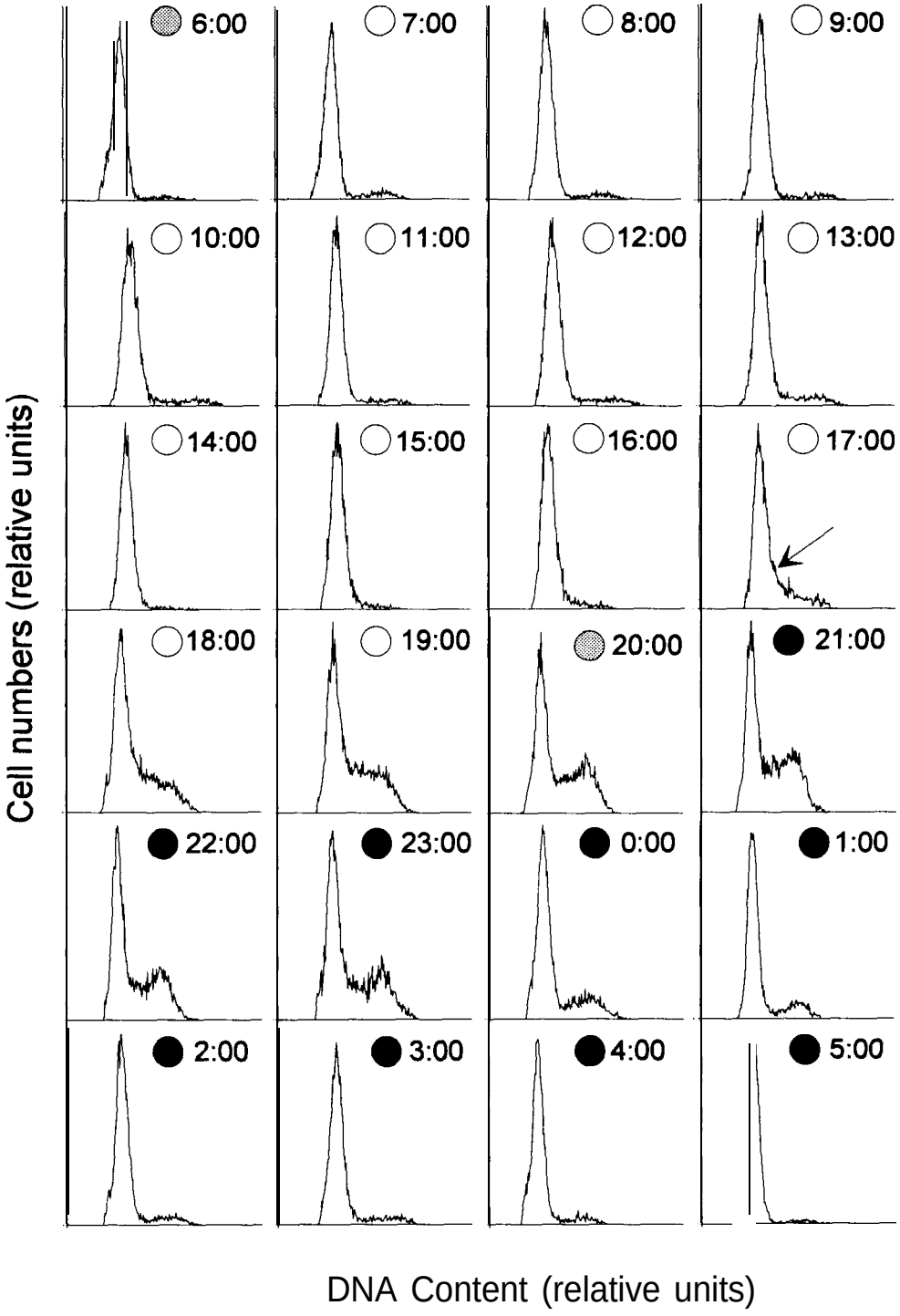

Fig. 4. Evolution of the cell cycle of *Synechococcus* during one diel cycle (5-6 July). Light, dark, and transition periods are symbolized by open, solid, and shaded circles, respectively. The arrow indicates cells entering the S phase.

of the day, in contrast to chlorophyll fluorescence, which displays very strong vertical gradients, especially in the middle of the day, due to the opposing effects of photoinhibition in surface and photoacclimation at depth. Because the situation is probably similar in Mediterranean Sea waters, water advected to our sampling point from different depths would have similar RALS but different chlorophyll fluorescence. The presence of a strong 17-18-h component in many parameters is interesting because it corresponds to the inertial period at this latitude (= 18.3 h). This reinforces the hypothesis of the advection of different water masses, because advection should display a strong component at the inertial

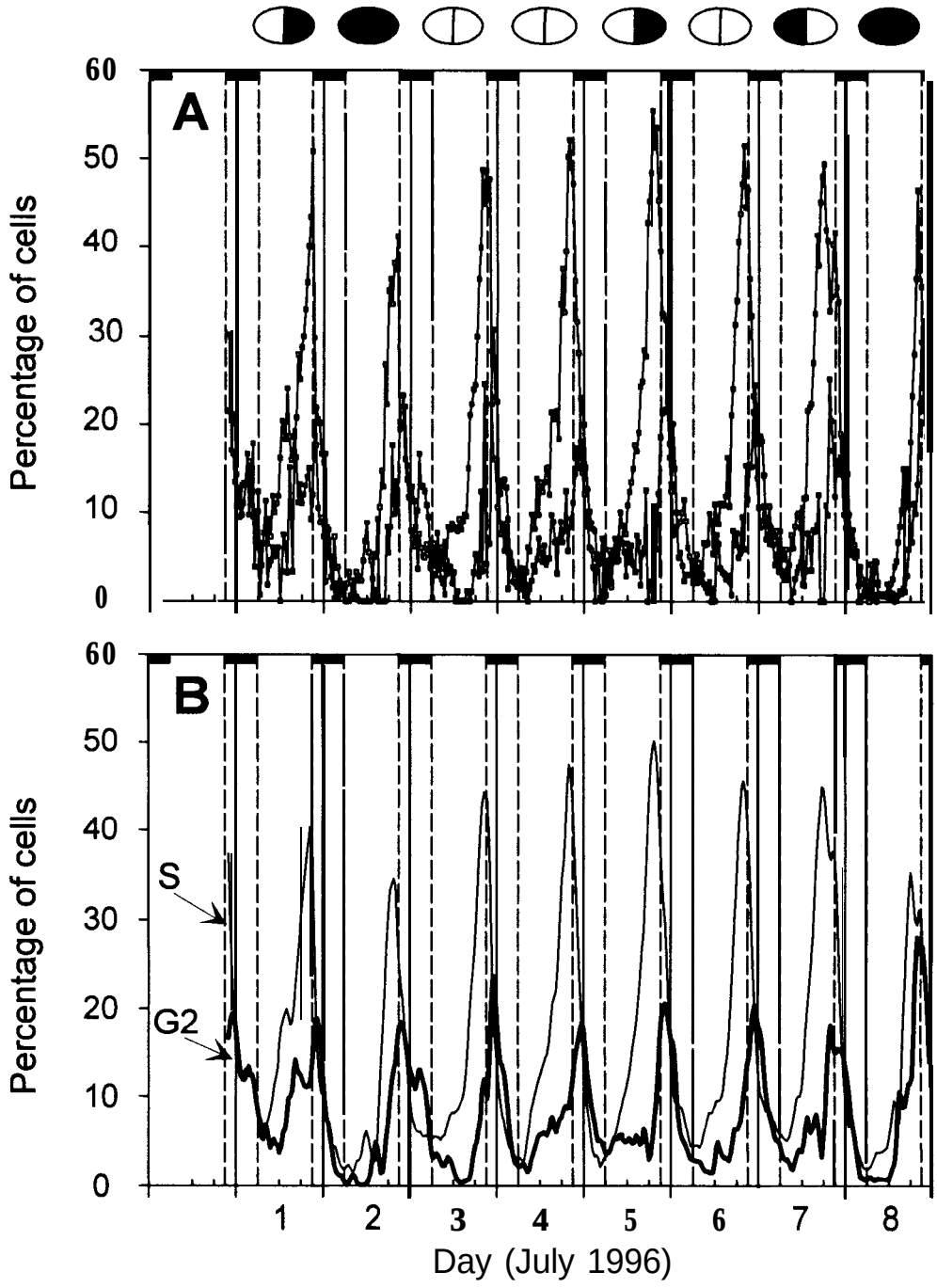

Fig. 5. Time series of the percentages of *Synechococcus* cells in the S and G2 phases of the cell cycle between 1 and 8 July. Raw (A) and smoothed (B) data. Symbols as in Fig. 3.

frequency. Population growth rates may also respond at the same time-scale, because Klein and Coste (1984) showed that wind stress would generate nutrient pulses through the thermocline at this frequency.

Synechococcus-Synechococcus abundance was in the range determined at the same site and period of the year by Ferrier-Pages and Rassoulzadegan (1994) with epifluorescence microscopy and by Vaulot et al. (1996) with flow cytometry. No clear diel pattern was observed in *Synechococcus* abundance, in contrast to what has been observed previously, e.g., in equatorial Pacific waters (DuRand and Olson 1996; Vaulot and Marie in press), most likely because of the importance of advection phenomena (see ***above).***

Minima and maxima of scatter values were recorded around dawn and dusk, respectively, as observed previously in oceanic waters (DuRand and Olson 1996; Vaulot and Marie in press). Single-cell light scatter, measured by flow cy-

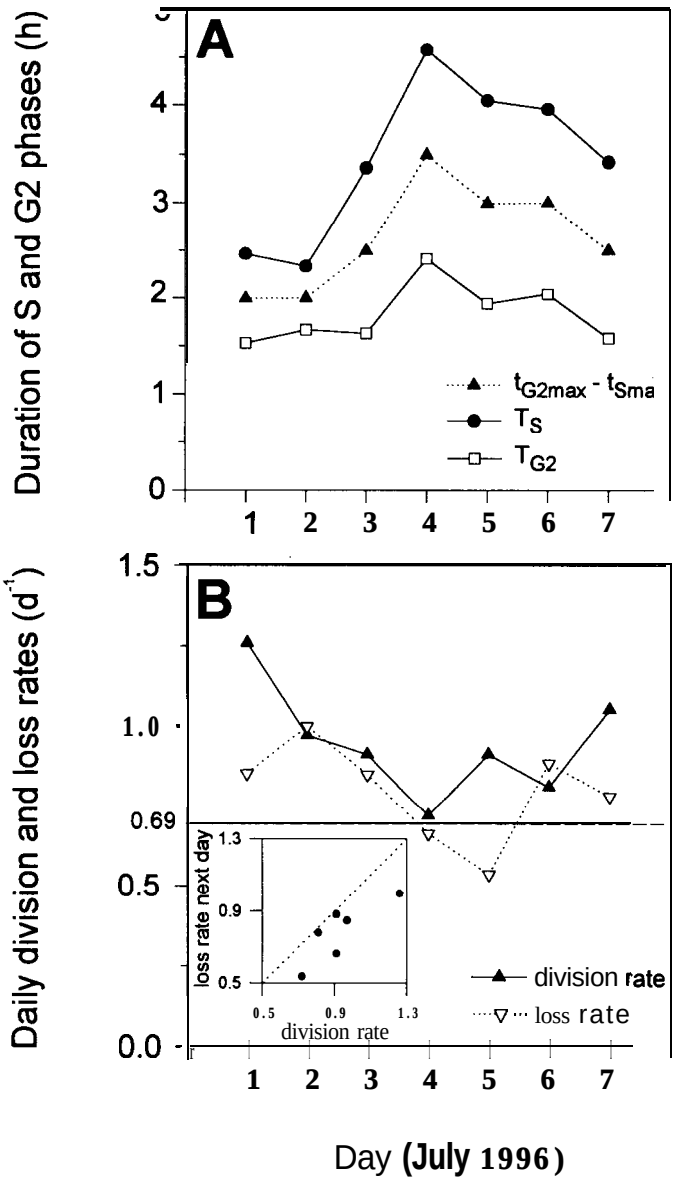

Fig. 6. (A) Duration of the *Synechococcus S* (●) and G2 (0) phases, and time difference between the S and G2 maxima ($t_{G2max} - t_{Smax}$) between 1 and 7 July. (B) *Synechococcus* division rate (shaded triangle) estimated from cell cycle and loss rate (▽) estimated from smoothed data (*see Materials and methods);* the insert shows the relationship between the division rate and the loss rate 1 d later. The hatched line corresponds to 0.69 d^{-1} or one division per day.

tometry, although it is a complex function of both cell size and refractive index (e.g., Morel 1991), has been shown to be linearly related to cell carbon content for picoplankton (DuRand and Olson 1996). The reason for the relationship between these two parameters may actually be complex, because both size and refractive index vary during the diel cycle as a function of carbon content, as shown for *Synechococcus* by Stramski et al. (1995). The pattern observed here can be interpreted most simply in terms of carbon increase when cells photosynthesize and carbon decrease when cells divide. RALS began to increase later on cloudy mornings (well observable on 2 July, Fig. 3B), hinting at a direct link between available light and carbon increase. In contrast, RALS began to decrease at the same time during sunny, cloudy, or partly cloudy days. The only exception was on 1 July, when light scatter seemed to stop increasing in the middle of the afternoon (Fig. 3). This was probably linked to the division of the first cohort, as revealed by the cell cycle analysis and discussed below (Fig. 5). Regarding *Prochlorococcus,* Binder et al. (1996) and Vaulot and Marie (in press) showed a close relationship between the growth rate and the ratio of the scatter at dusk over that at dawn; this probably reflects the requirement for cells to reach a critical carbon content or size before cell division proceeds. Surprisingly, we found a negative correlation between these parameters for *Synechococcus* ($FALS_{dusk}/FALS_{dawn} = -0.7\mu_{S+G2} + 2.77$, $r^2 = 0.86$, $p < 0.01$, $n = 6$). This paradoxical result could stem from the fact that previous studies considered the entire water column, whereas our data concern only surface waters. At the surface, the increase in carbon is probably driven by available light, and it is therefore larger on sunnier days (e.g., 3 and 4 July). In contrast, the division rate is lower on sunny days because DNA synthesis is inhibited (see below), explaining the inverse relationship. Therefore caution must be exercised when extrapolating division rate from scatter increase.

Changes in cellular fluorescence are driven by changes both in the absorption cross section (in particular due to change in chlorophyll content) and in fluorescence yield of the cells. In the equatorial Pacific, Vaulot and Marie (in press) have shown that, at the surface, chlorophyll fluorescence was strongly quenched at midday, but it presented an inverse pattern at depth. The present data set did not display such clear patterns but had more resemblance to what was observed at depth in the Pacific, i.e., an increase during the day, especially on cloudy days (1, 2, and 5 July). Such behavior is most likely due to a change in absorption cross section, as demonstrated for *Synechococcus* WH 8103 by Stramski et al. (1995). On sunny days, fluorescence began to increase in the morning, but then dropped around midday, only to recover in the afternoon. In this case, the midday drop is probably due to a change in fluorescence yield resulting from nonphotochemical quenching. A similar influence of cloud cover on chlorophyll fluorescence quenching has already been observed by Stramska and Dickey (1992). As in the case of the RALS, the night decrease in fluorescence can be attributed to cell division. As in the Pacific (Vaulot and Marie in press), phycoerythrin and chlorophyll fluorescence covaried, but the former had a weaker amplitude and showed less midday quenching (this is very clear on 2 and 5 July, when phycoerythrin fluorescence did not drop past midday, whereas that of chlorophyll did). This difference suggests that phycoerythrin might be more protected from high irradiance damage, be it direct photodamage or nonphotochemical quenching.

The cell cycle of many phytoplanktonic organisms is known to be tightly coupled to the daily light cycle (Chisholm 1981), and *Synechococcus* makes no exception, as shown either in culture (Campbell and Carpenter 1986; Binder and Chisholm 1995) or in nature (Campbell and Car-

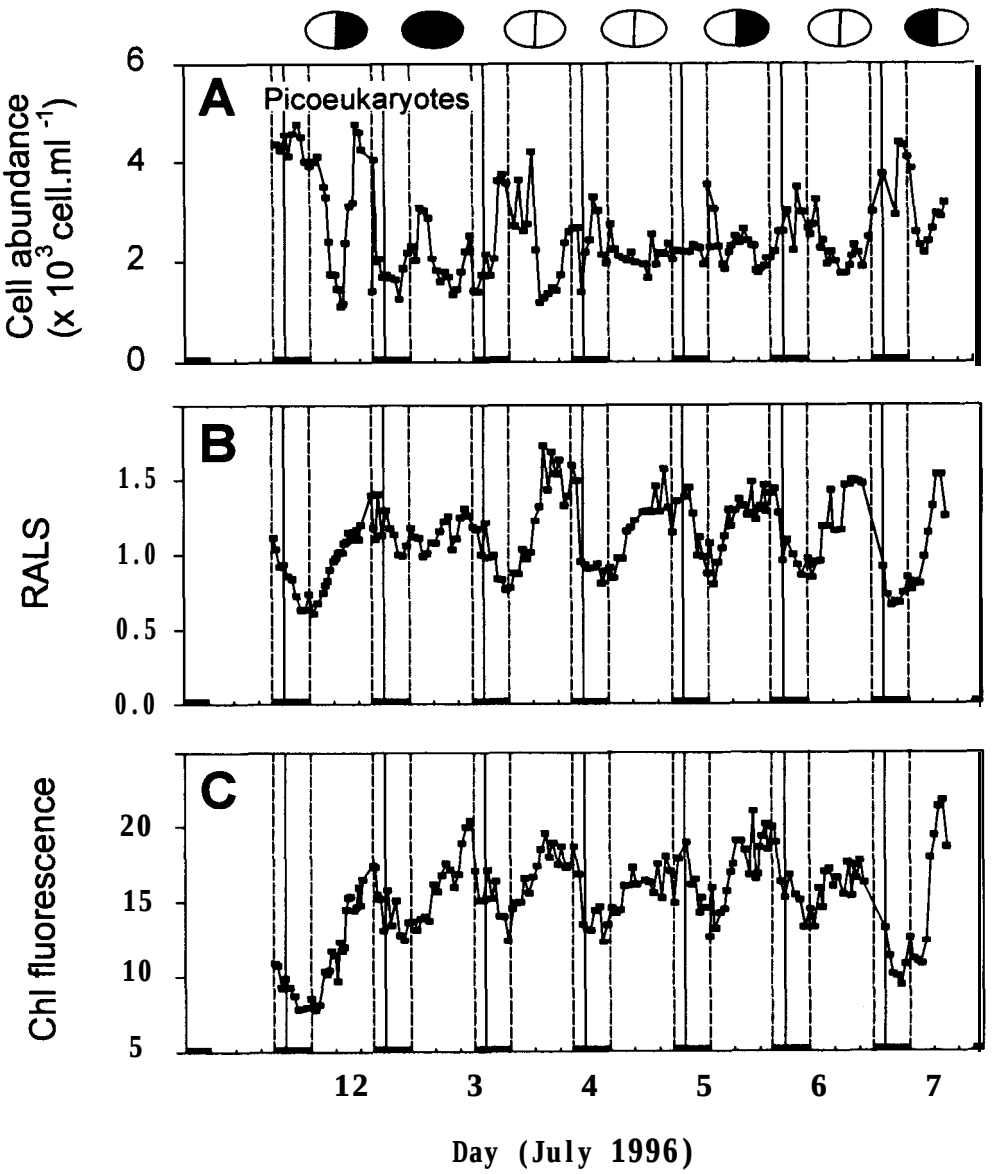

Fig. 7.Time series obtained for picoeukaryotes (fresh samples) between 1 and 7 July in the surface waters of Villefranche-sur-mer. Number of cells (A), right-angle tight scatter (B), and chlorophyll fluorescence (C). The cytometric parameters were normalized to 0.95-μm-diameter beads, used as internal reference. Symbols as in Fig. 3

penter 1986; Vaulot et al. 1996). The present data confirm the strong synchronization of the Synechococcus cell cycle and the timing of the different phases observed previously (Vaulot et al. 1996). The synchronization of Synechococcus is not as good, however, as that of *Prochlorococcus* (Vaulot et al. 1995), as revealed by the persistence of cells in S and G2 phases throughout the night and as previously observed for cyanobacteria (Campbell and Carpenter 1986; Binder and Chisholm 1995). A very interesting feature of the present data set is that it reveals day-to-day variation in cell cycling. Irradiance levels, driven by cloud cover, seem to be a key factor in that respect. On sunny days (or more precisely on days with sunny mornings), cells entered the S phase earlier (this is best seen on the unsmoothed data, Fig. 5A), and the maximum fraction of cells in S phase was higher than on cloudy days. The larger accumulation of cells in S phase was in fact due to an increase in the duration of S phase (Fig. 6A), not by an increased flux of cells through the S phase that would have translated into a higher division rate, in contrast to what was observed on these sunny days (Fig. 6B). The increased duration of S phase on sunny days might be linked to the inhibitory effects of ultraviolet (UV) wavelengths on DNA replication of phytoplankton (e.g., Buma et al. 1996; Jeffrey and Mitchell 1997), as also suggested by cell cycle data from the equatorial Pacific (Vaulot et al. 1995). In contrast, G2 duration appears to be much less affected by irradiance levels, although it follows S variation somewhat (Fig. 6A). On 8 July, although the percentage of

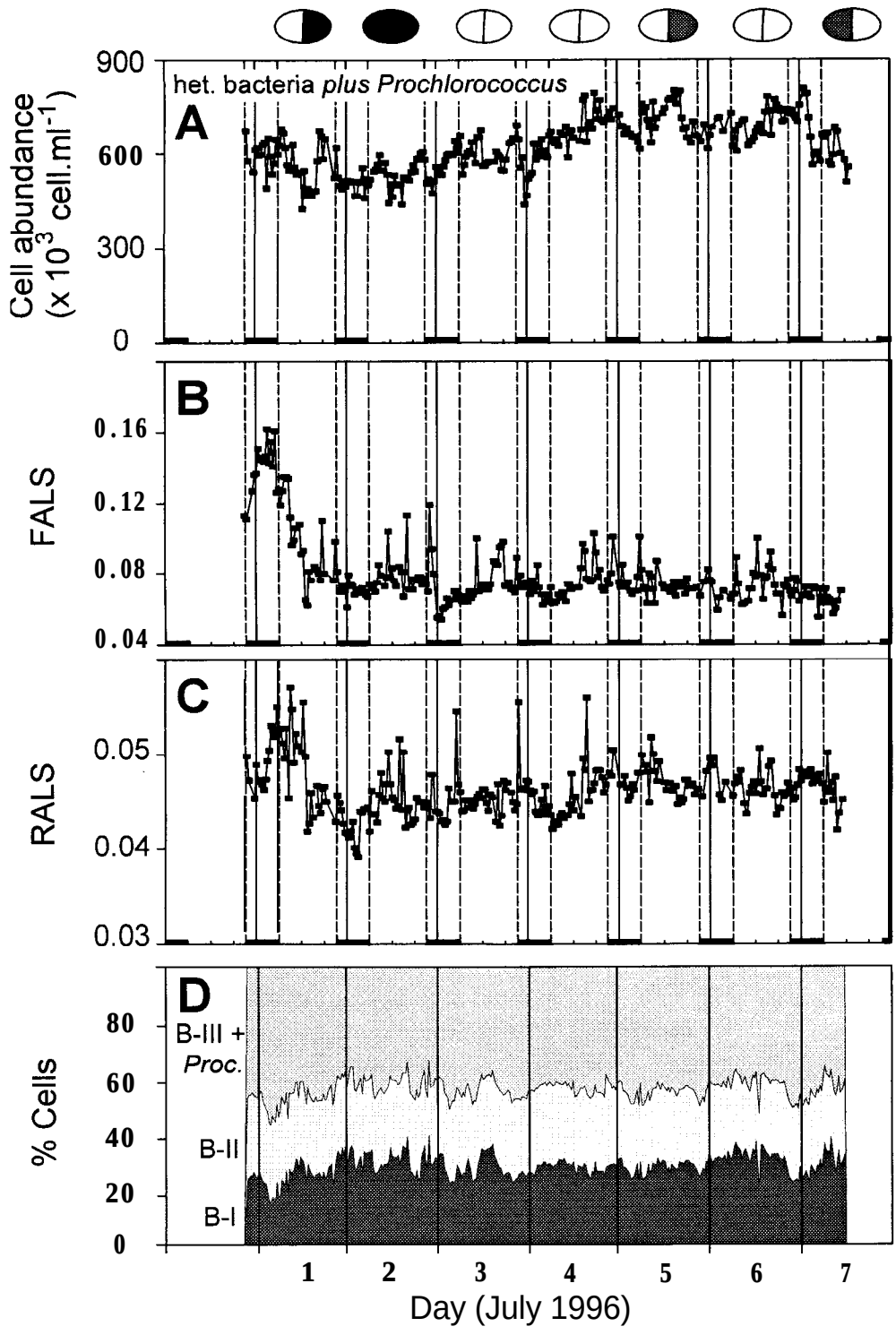

Fig. 8. Time series obtained for heterotrophic bacteria plus ***Prochlorococcus*** from fixed and frozen samples between 1 and 7 July in the surface waters of Villefranche-sur-mer. Number of cells (A), forward-angle light scatter (B), right-angle light scatter (C), and relative percentage of each subpopulation (D). Symbols as in Fig. 3.

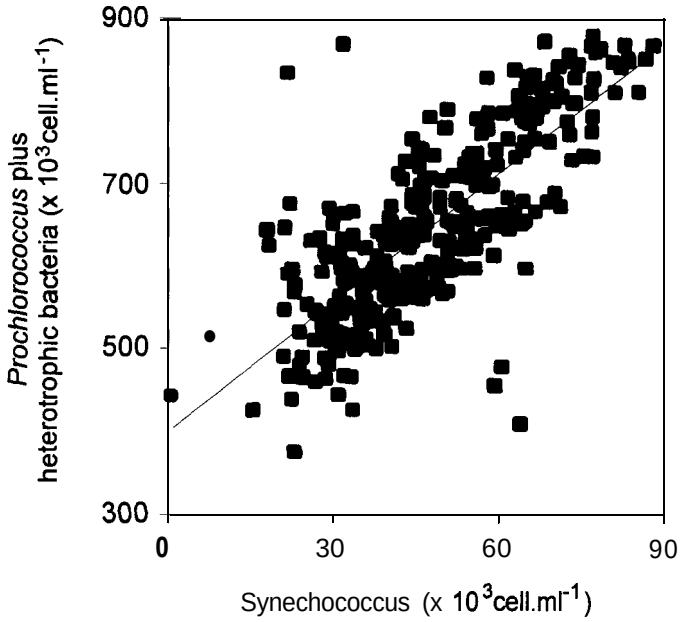

Fig. 9. Relationship between the cell concentrations of heterotrophic bacteria plus *Prochlorococcus* and *Synechococcus* (both determined on fixed and frozen samples). The solid curve corresponds to the linear fit to the data ($N_{Bact} = 7.8N_{Syn} + 3.99 \times 10^5$, $r^2 = 0.60$, $p < 0.01$, $n = 340$).

cells in S phase was low, the fraction of cells in *G2* was unusually high (Fig. 5). Stormy conditions during the previous night could be responsible for this accumulation, perhaps by disrupting cell division, as known in dinoflagellates (Berdalet 1992). Other factors, such as nutrient limitation, probably also contributed to the day-to-day cell cycle variability. In particular, Vaulot et al. (1996) demonstrated that phosphorus was controlling the Synechococcus cell cycle at the same location and time of the year in 1993, and phosphorus was also probably limiting in 1996 (Thingstad et al. 1998).

The Synechococcus division rate, estimated from cell cycle data (μ_{S+G2}), was always larger than one division per day (0.69 d^{-1}). Although Eq. 1, used to compute division rate estimates, was initially derived for a synchronized population, i.e., dividing less than once per day (Carpenter and Chang 1988), nothing in its derivation requires that the cell population divide less than once a day. The equation is in fact still valid for an asynchronous population. The only requirement is that $T_S + T_{G2}$ (and therefore $t_{G2max} - t_{Smax}$) be the same for all the cells.

When the division rate is larger than 0.69 d^{-1}, some cells have to go through two rounds of division every day. In fact, this was clearly visible on 1 July, when two cohorts went through both S and G2. Similar behavior has in fact been observed recently for *Prochlorococcus* in the Indian Ocean (Shalapyonok et al. 1998). If two cohorts of cells are going through the cell cycle, however, $T_S + T_{G2}$ might be different for each. Data from 1 July suggest that the early cohort may have a shorter $T_S + T_{G2}$. This would lead to an underestimate of the division rate if the longer $T_S + T_{G2}$ of the major cohort is used, because $T_S + T_{G2}$ appears in the denominator of Eq. 1. To get an upper bound of our error, we assumed that the first cohort has an S + G2 duration twice as short as the second one, and we applied this duration to the first cohort. On 1 July, when two cohorts were visible, this led to a 55% increase in the division rate estimate. Another problem, linked to the asynchrony resulting from cells dividing more than once per day, is that the S and G2 maxima may become blurred, leading to inaccuracy of $t_{G2max} - t_{Smax}$ estimates (ultimately, for an asynchronous population, the fraction of cells in S and G2 is constant, and $t_{G2max} - t_{Smax}$ becomes impossible to estimate), but this was clearly not the case here (Fig. 5). A third case of error in the estimate stems from the persistence of cells arrested in S + G2 phase at night that may have a much longer $T_S + T_{G2}$ than cells cycling through these phases during the day. To estimate this uncertainty, we set to zero the fraction of S + G2 cells during periods without division, i.e., when this fraction was <8%. The resulting estimated division rate could be as much as 30% lower, but the error averaged 17.5%.

The *Synechococcus* division rate varied nearly twofold during the period of study. Clearly, high irradiances in the absence of cloud cover had an inhibitory effect, because the lowest division rates were recorded on 3, 4, and 6 July, i.e., on days with cloud-free skies. It is clear from Eq. 1 that both the daily integral of the fraction of cells in S and G2 and $T_S + T_{G2}$ are responsible for the variation in μ. In the present data set, the two terms covary, i.e., both numerator and denominator are larger on sunny days. It is still $T_S + T_{G2}$, however, that has the larger effect, because μ is not constant (which would be the case if both terms balanced each other) but mirrors the variation of $T_S + T_{G2}$. In turn, the latter parameter is mostly driven by change in T_S rather than T_{G2}. These data clearly demonstrate the advantage of the cell cycle method over that of the classical mitotic index (McDuff and Chisholm 1982), which assumes a constant phase duration, clearly a hypothesis that is not fulfilled in the present case.

During the period of study, the loss rate was on average less variable as well as lower than the division rate, resulting in the observed long-term increase in *Synechococcus* cell number. Although there was no apparent direct relation between division and loss rates (Fig. 6B), if one translates the latter by 1 d to the left, the two curves now have similar trends, as if the loss rate adjusted to the division rate from the previous day (see *Fig. 6B, insert).* Although advection and mixing contribute to the loss rate (see *above),* this relation suggests that the loss rate probably involves a major biological component adjusting quite rapidly. Whether this is grazing or viral lysis, both of which have been shown recently to contribute equally to bacterioplankton loss in coastal waters (Fuhrman and Noble 1995), is still an open question.

Picoeukaryotes-Picoeukaryote abundance was more than lo-fold lower than *Synechococcus,* similar to previous observations in summer at the same station (Vaulot et al. 1996). The absence of any clear 24-h pattern in cell abundance could be due in part to their relatively low abundance, inducing statistical noise. It might also be due to the presence of two populations (Fig. 2A) that might not divide exactly

in phase, as well as being advected and mixed from different water masses. In fact, there might have been a shift on 1 July from an abundant small-sized population to a less abundant larger-sized one (Fig. 7). The clear diel pattern for RALS and chlorophyll fluorescence suggests cell growth and chlorophyll synthesis during the day. The day-to-day variability in RALS was larger than that for *Synechococcus* and seemed to be directly linked to irradiance. RALS increased more on sunny days (3-6 July). For chlorophyll, there was no clear sign of midday depression, as seen in ***Synechococcus*** here (Fig. 3D) or in picoeukaryotes in the equatorial Pacific (Vaulot and Marie in press). These two facts suggest that picoeukaryotes suffer very little from high irradiances in surface waters of the Mediterranean Sea and could even be slightly light limited and grow less on cloudy days. This absence of a deleterious effect from light could stem from the better photoprotective mechanisms available to eukaryotes, such as the xanthophyll cycle (see Demming-Adams and Adams III 1992). Although we have no direct measurement of the picoeukaryote cell cycle, both their RALS and chlorophyll fluorescence decrease (usually in the middle of the afternoon) before that of ***Synechococcus,*** suggesting that the former may divide earlier than the latter, in contrast to what was observed in the equatorial Pacific, where picoeukaryotes divided in the early part of the night about 7 h after *Synechococcus* (Vaulot and Marie in press).

Heterotrophic bacteria and Prochlorococcus-The concentrations reported here are in agreement with data previously obtained in this area (that also included *Prochlorococcus, e.g.,* Hagström et al. 1988) and more generally from pelagic waters. The presence of the three populations B-I, B-II, and B-III appears to be characteristic of relatively oligotrophic waters because in more coastal or eutrophic waters, B-III cells (in this case, they do not include *Prochlorococcus)* dominate (Li et al. 1995; Marie et al. 1997). The general increase in cell abundance over the period of study follows that of *Synechococcus (see below),* but no evolution is seen in the percentage of each of the three discriminated populations, except on 1 July, when B-III cells were relatively more abundant (this is also seen in the higher FALS and RALS observed that day, because B-III cells have higher FALS and RALS; Figs. 8B,C). As the picoeukaryote data suggest, a change in the advected water in Villefranche Bay may have occurred on that day.

Conclusion: picoplankton dynamics-In summer, conditions in the northwestern Mediterranean Sea become moderately oligotrophic, as evidenced by the relative abundance of the three picoplanktonic populations analyzed in the present study. *Synechococcus* and picoeukaryotes are one to two orders of magnitude more abundant than in extremely oligotrophic areas such as the tropical Atlantic or Pacific, whereas heterotrophic bacteria are comparable (Partensky et al. 1996; Campbell et al. 1997). Phosphorus, rather than nitrogen, is probably the major limiting factor for both photosynthetic and heterotrophic compartments (Thingstad and Rassoulzadegan 1995; Vaulot et al. 1996). Despite this limitation, *Synechococcus* populations sustain high division rates. The present work suggests indeed that, at least in surface waters, division may be controlled negatively by UV irradiance rather than positively by nutrient supply (although the latter was not quantified). Two observations made during this study point out the close coupling that exists between the different compartments of the microbial loop. First, the loss rate (due either to grazing or viral lysis) adjusts very rapidly (1 d) to variation in the *Synechococcus* division rate (Fig. 6B, insert). Second, the good correlation between the abundance of *Synechococcus* and that of *Prochlorococcus* plus heterotrophic bacteria suggests that auto- and heterotrophic bacteria are under similar controls, probably both in terms of growth, i.e., the division rate of auto- and heterotrophs may be limited by P (Thingstad et al. 1998) and inhibited by UV (Hemdl et al. 1993; Aas et al. 1996) and loss (heterotrophic bacteria, *Prochlorococcus,* and ***Synechococcus*** probably have the same predators). Heterotrophic bacteria may exploit the organic matter produced by autotrophs, that is made, in addition, more labile by surface UV action (Mopper et al. 1991). In contrast, the lack of a good correlation between bacteria and picoeukaryotes indicates that the latter are probably controlled by other factors. Indeed, high irradiances have a less visible deleterious effect on picoeukaryotes.

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