Diel changes in bulk and single-cell bacterial heterotrophic activity in winter surface waters of the northwestern Mediterranean Sea

Clara Ruiz-González, Thomas Lefort, Ramon Massana, Rafel Simó, and Josep M. Gasol*

Institut de Ciències del Mar–Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Abstract

Two diel cycle studies were conducted to determine the effect of day–night light changes on winter bacterial activity in the coastal Mediterranean (Blanes Bay Microbial Observatory). Bacterial abundances, bacterial heterotrophic activity, and flagellate grazing counts were determined at 4-h intervals during two 3-d periods separated by 2 d. Twice a day, the single-cell activity of major bacterial groups was further analyzed by applying microautoradiography combined with catalyzed reporter deposition–fluorescence in situ hybridization. During the first cycle, all the measured parameters (including the number of active cells in most groups) varied synchronously, with higher values at night and lower during the day. An episode of strong winds between the two studied periods disrupted this periodicity at the onset of the second cycle. The bulk incorporation of ³H-leucine recovered the diel pattern after 2 d, mostly driven by the activity of Gammaproteobacteria. Among the possibilities considered, the observed nighttime increases in the grazing activity of heterotrophic nanoflagellates appeared to partially drive the activity of all bacterial taxa through potential periodic release of dissolved organic matter.

Day–night variations of biological parameters in the ocean are originally a consequence of the relation between sunlight and marine organisms. Because the different components of the microbial food web are continuously interacting, any synchronization of life cycles and behaviors, e.g., grazing or excretion, will ultimately be reflected in marine bacterial activity, with implications for extrapolation of carbon cycling from hourly to higher scales.

Both photosynthetic release and excretion through grazing activities are thought to be major sources of dissolved organic matter (DOM) for marine bacterial use in oceanic environments (Nagata 2000). Because photosynthetic organisms have to deal with diurnal variations in light availability, and grazing activities are often synchronized with circadian cycles (Wikner et al. 1990), the rates of DOM supply for marine bacteria may also follow diel patterns. Thus, depending on the origin (and quality) of the DOM that bacteria use, and how fast they respond to changes in substrate supply, different diel bacterial activity trends will be detected. If bacteria are closely coupled to photosynthetic production of DOM, we should expect activity cycles showing peaks around noon and afternoon (Fuhrman et al. 1985; Herndl and Malacic 1987; Gasol et al. 1998). On the contrary, if bacterioplankton depend on DOM released by grazers (Nagata 2000) or on allochthonous organic carbon, they will be synchronized to the rhythms of these supplies, if any.

The diel patterns of bacterioplankton activity in the upper layers of the ocean, however, might also be influenced by other factors varying at the daily scale, such as ultraviolet radiation (UVR; Herndl et al. 1993; Jeffrey et al. 1996), bacterivory (Wikner et al. 1990), or viral lysis (Winter et al. 2004). Such short-term variations seem to occur more intensively in oligotrophic environments, where substrate supplies are low and we may expect tightly coupled covariation between bacteria and DOM production (Gasol et al. 1998). In coastal areas or more eutrophic waters, though, where DOM supply may be independent from circadian cycles and/or many different substrates are available, we might find inconsistent or unclear diel cycles (Riemann and Søndergaard 1984; Gasol et al. 1998).

Thus far, such diurnal variability of bacterial activity has been addressed mainly at the community level, and very few studies have considered this issue from a single-cell or group-specific point of view. Different phylogenetic groups are well known to show distinct seasonal patterns in their activities (Alonso-Sáez and Gasol 2007; Vila-Costa et al. 2007), yet very little is known about how the different bacterial groups behave throughout a diel cycle. Among them, Cyanobacteria have been more carefully studied, showing obvious diurnal rhythms in the uptake of organic substrates (Chen et al. 1991; Mary et al. 2008*a*). On the contrary, Pernthaler and Pernthaler (2005) did not find clear diel cycles in cell proliferation when focusing on three bacterial taxa.

Considering that the different phylogenetic groups may differ in their preferences for organic substrates or phytoplankton species (Pinhassi et al. 2004; Alonso-Sáez and Gasol 2007), and given that there are bacteria with differential sensitivities to sunlight or photoheterotrophic capacities (Béjà et al. 2000; Kolber et al. 2000; Alonso-Sáez et al. 2006), we expected that populations within the bacterial assemblages would exhibit distinct amplitudes and rhythms in their diurnal fluctuations.

For that purpose, we studied the short-term variability of bacterial activity in five bacterial taxa from coastal northwest Mediterranean waters during two consecutive 72-h cycles, along with bulk bacterial heterotrophic activity measured as both ³H-leucine and ³H-thymidine incorporation. Microautoradiography combined with catalyzed reported deposition–fluorescence in situ hybridization

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* Corresponding author: pepgasol@icm.csic.es

(MAR–CARD–FISH) allowed the analysis of diurnal variations at the single-cell level. In order to correctly interpret the observed patterns, several light and dark incubations for bacterial activity and microscopic observations of grazing activities were also performed. We expected to find variable behaviors of different bacterial groups depending on the light levels or DOM supplies, which would provide insight into the daily rhythms of particular bacterial taxa.

Methods

Sampling and basic parameters—The study was carried out in the Blanes Bay Microbial Observatory, a shallow (20-m depth) oligotrophic coastal station in the northwest Mediterranean Sea, located 800 m offshore of Blanes, Catalonia, Spain (41°39.90′N, 2°48.03′E). Experiments were performed in two blocks, on 20–23 February (first cycle) and on 26 February–01 March (second cycle) 2007. Surface-water samples (0.5 m depth) were collected with polycarbonate carboys every 4 h during these two 72-h periods, and transported to the lab under dim light. Incubations were started less than 30 min after water collection. The sample of the third day at noon could not be collected because of rough sea conditions. The temperature and salinity of the sampled waters were obtained with a SAIV A-S 204 conductivity–temperature–depth probe at every sampling time. Irradiance measurements during the sampling period were obtained from the nearby station of Malgrat de Mar (Catalan Meteorological Service, www.meteo.cat), located 5 km from the sampling station and at 4 m above sea level. The station recorded arithmetically averaged hourly air temperature and relative humidity at 1.5 m above ground, vector-averaged hourly wind speed and direction and global irradiance at 2 m, and accumulated rainfall at 1 m. Wave height data were collected from a scalar buoy (DATAWELL, Waverider) placed at 41°38.82′N, 02°48.93′E over a depth of 74 m (XIOM Network, www.boiescat.org).

Nutrients and chlorophyll *a* concentrations were determined only once at the beginning of each cycle. Samples for nutrient concentration were filtered through 0.2-μm–pore size polycarbonate filters (47-mm diameter, Supor-200, Gelman Sciences). Dissolved inorganic nitrogen (NO_3^-, NO_2^-, NH_4^+) and phosphate (PO_4^{3-}) were measured spectrophotometrically with an Alliance Evolution II autoanalyzer following standard procedures (Grasshoff et al. 1983). Chlorophyll *a* concentration was determined from triplicate 150-mL samples filtered through GF/F filters (Whatman) extracted in acetone (90% v:v), and fluorescence was measured with a Turner Designs fluorometer.

Abundance of prokaryotes and eukaryotic picophytoplankton—*Prochlorococcus*, *Synechococcus*, and photosynthetic picoeukaryote (PPeuk) abundances were enumerated by flow cytometry in unstained samples and distinguished by their size and pigment properties following common procedures (Olson et al. 1993). Heterotrophic prokaryotes were also quantified by flow cytometry after staining with SybrGreen I (Gasol and del Giorgio 2000).

Bacterial heterotrophic activity—Bacterial activity was estimated every 4 h from both radioactive ³H-leucine and ³H-thymidine incorporation. For leucine we used the ³H-leucine assimilation method described by Kirchman et al. (1985) with the modifications of Smith and Azam (1992). Briefly, four aliquots (1.2-mL) and two trichloroacetic acid (TCA)-killed controls were incubated with radiolabeled leucine (40 nmol L⁻¹; final concentration, 160 Ci mmol⁻¹) for about 2 h in the dark at in situ temperature. The incorporation was then stopped by adding 120 μL of cold TCA 50% to the samples, which were stored at −20°C until processing by the centrifugation method of Smith and Azam (1992). Bacterial activity was also measured as ³H-thymidine incorporation following Fuhrman and Azam (1980) with the modifications of Smith and Azam (1992). Samples were incubated with 10 nmol L⁻¹ ³H-thymidine (final concentration) and processed like the ³H-leucine samples.

Furthermore, every day at 13:00 h six additional aliquots amended with ³H-leucine were incubated under an artificial light source (∼ 100 μmol photons m⁻² s⁻¹ of visible light only) parallel to standard dark incubations for comparison of both light and dark conditions.

Grazing activity of heterotrophic nanoflagellates (HNFs) on PPeuks—Samples of 30 mL fixed with glutaraldehyde (2% final concentration) and stained with 4,6-diamidino-2-phenylindole (DAPI) to a final concentration of 5 mg mL⁻¹ were filtered through a 0.6-μm polycarbonate black filter (Poretics). Only samples from the first cycle were analyzed. Colorless flagellates < 5 μm in size (HNFs) were enumerated with an Olympus BX61 epifluorescence microscope by UV excitation (to detect the protists) and blue light excitation (to check for the absence of chloroplasts). At this excitation, the presence of ingested pigmented prey (small PPeuks) was easily observed inside the cells. The HNFs with ingested algae were distinguished from phototrophic forms by the lack of conspicuous plastids and/or the general morphology of both prey and predator.

MAR–CARD–FISH—Twice a day (GMT 13:00 h and 01:00 h) 30-mL water samples were incubated with added radioactive ³H-leucine (160 Ci mmol⁻¹; 0.5 nmol L⁻¹ final concentration) for 4 h in the dark at in situ temperature. Controls killed with paraformaldehyde (PFA) were also run simultaneously with all live incubations. After 4 h the live samples were fixed overnight with PFA (1% final concentration) at 4°C in the dark.

For the analysis of the single-cell bacterial activity, we followed the protocol described in Alonso-Sáez and Gasol (2007). Aliquots of 10 mL were gently filtered through 0.2-μm polycarbonate filters (GTTP, Millipore), rinsed with milliQ water, air dried, and stored at −20°C until processing. Hybridization of the filters was done following the CARD–FISH protocol. Several horseradish peroxidase probes were used to characterize the composition of the bacterial community in the water samples: Eub338-II-III for most Eubacteria (Daims et al. 1999), Gam42a for most Gammaproteobacteria (Manz et al. 1992), CF319 for many clades belonging to the Bacteroidetes group (Manz et al.

1996), Ros537 for the *Roseobacter* clade (Eilers et al. 2001), SAR11-441R for the SAR11 cluster (Morris et al. 2002), and Syn405 for the cyanobacterial genus *Synechococcus* (West et al. 2001). The Eub antisense probe Non338 (Wallner et al. 1993) was used as a negative control. All probes were purchased from biomers.net.

Cell walls were first permeabilized with lysozyme (37°C, 1 h) and achromopeptidase (37°C, 0.5 h) before the hybridization. Hybridizations were carried out on sections of the filters at 35°C overnight, and specific hybridization conditions were established by addition of formamide to the hybridization buffers (45% formamide for the SAR11 probe, 20% for Non338, 60% for Syn405, and 55% for the rest of the probes). Smaller pieces from each hybridized section were cut and stained (DAPI, 1 μg mL^{-1}) to estimate the relative abundance of each group before applying the microautoradiography. Between 500 and 800 DAPI-positive cells were counted manually within a minimum of 10 fields under an epifluorescence microscope.

For microautoradiography, the filter sections were glued onto slides and embedded in 46°C melted photographic emulsion (KODAK NTB-2) containing 0.1% agarose in a dark room. The slides were placed face up on an ice-cold metal bar for about 5 min for the emulsion to solidify, and then stored inside black boxes at 4°C until development. The optimal exposure time was determined for all the samples and resulted in an average of 8 d. Slides were developed by placing them into the developer (KODAK D19) for 3 min followed by fixation with KODAK Tmax fixer for 3 min and 5 min of washing with tap water. Slides were then dried in a dessicator overnight, stained with DAPI (1 μg mL^{-1}), and counted manually by epifluorescence microscopy.

Statistical analyses—The Shapiro-Wilk *W*-test for normality of data and Levene's test for homogeneity of variance were applied prior to analysis, and either one-way ANOVA or the nonparametric Kruskal-Wallis test were used to analyze statistically significant ($p < 0.05$) differences in the measured variables between day and night samples or between cycles, and post hoc analyses (Tukey's honestly significant difference test) for comparison among different sampling times. Correlations between variables were calculated using Pearson's correlation coefficient. These statistical analyses were performed using JMP software (SAS Institute). For calculating uncertainties on ratios (i.e., bacterial-specific activities), error propagation was taken into account. Standard errors were calculated using the formula for the propagation of error as described by Bevington and Robinson (2003).

Results

Background information—Sampling was done at the coldest period of the year (average temperature $\sim$ 13.4°C), and temperature did not vary significantly ($p > 0.05$) between the two weeks. The water column was totally mixed (data not shown) as is typical for this area in winter pre-bloom conditions (Estrada et al. 1985). Chlorophyll *a* concentration was estimated for only the first sampling

Table 1. Averaged measurements of temperature, salinity, leucine incorporation rates (LIR), and cell concentration of heterotrophic bacteria (Het. bact.), *Synechococcus* (Syn.), *Prochlorococcus* (Prochl.), and PPeuk measured at each sampling cycle. Total chlorophyll *a* (Chl *a*) and nutrient concentration were analyzed only at the beginning of each week. Values represent mean ± SE. Asterisks (*) indicate significant differences between both cycles ($p < 0.05$).

	First cycle	Second cycle
Temperature (°C)	13.43±0.02	13.36±0.01
Chl *a* (μg L^{-1})	0.47±0.02*	0.89±0.03*
Salinity	38.3±0.01	38.3±0.01
PO$_4$ (μmol L^{-1})	0.17	0.16
NH$_4$ (μmol L^{-1})	1.75	1.79
NO$_2$ (μmol L^{-1})	0.36	0.18
NO$_3$ (μmol L^{-1})	1.47	1.24
LIR (pmol leucine L^{-1} h^{-1})	171±18	129±9
Het. bact. (10^5 mL^{-1})	7.2±0.2	7.5±0.2
Syn. (10^4 mL^{-1})	0.6±0.02*	1.6±0.1*
Prochl. (10^4 mL^{-1})	0.5±0.02*	1.2±0.04*
PPeuk (10^4 mL^{-1})	1.1±0.1*	1.4±0.1*

time of each cycle, and it almost doubled (from 0.47 to 0.89 μg L^{-1}) between the first and the second cycle (Table 1). Whereas the average number of heterotrophic bacteria did not significantly change between both cycles, averaged *Synechococcus*, *Prochlorococcus,* and PPeuk abundances significantly ($p < 0.05$) increased from the first to the second week (Table 1), in concordance with the higher chlorophyll *a* concentrations found in the second week. During the unsampled days between the two cycles, wind direction changed (shifting from south and southwest to east) causing increased sea turbulence and wave action (Fig. 1B), with simultaneous rainfall and low-light conditions (Fig. 1A).

Diel variations in bulk bacterial activity—Both ^{3}H-leucine and ^{3}H-thymidine incorporation rates were measured every 4 h during two periods of 72 h in order to assess the effect of daily light changes on bacterial heterotrophic activity. During the first cycle (20–23 February), bacterial activity showed a marked diel pattern, with higher ^{3}H-leucine and ^{3}H-thymidine incorporation values at night and lower values during the day (Fig. 2A). Throughout the 72 h there were extremely large variations in bacterial activity among sampling times. ^{3}H-leucine incorporation increased from 53.8 to 314.2 pmol L^{-1} h^{-1} between 13:00 h on 20 February and 05:00 h on 21 February, meaning a $\sim$ sixfold increase in less than 24 h. On 21 and 22 February the peaks reached 269.8 and 243.9 pmol L^{-1} h^{-1}, respectively, and they appeared to be displaced in time with respect to the first day, because they both were reached at 21:00 h instead of at 05:00 h. Minimum values were around 50 and 75 pmol ^{3}H-leucine L^{-1} h^{-1}. ^{3}H-thymidine incorporation was also higher at night than during the day, and it very closely paralleled the ^{3}H-leucine incorporation pattern. Maximum ^{3}H-thymidine incorporation values ranged from 80.4 pmol L^{-1} h^{-1} on 21 February to 59.4 pmol L^{-1} h^{-1}
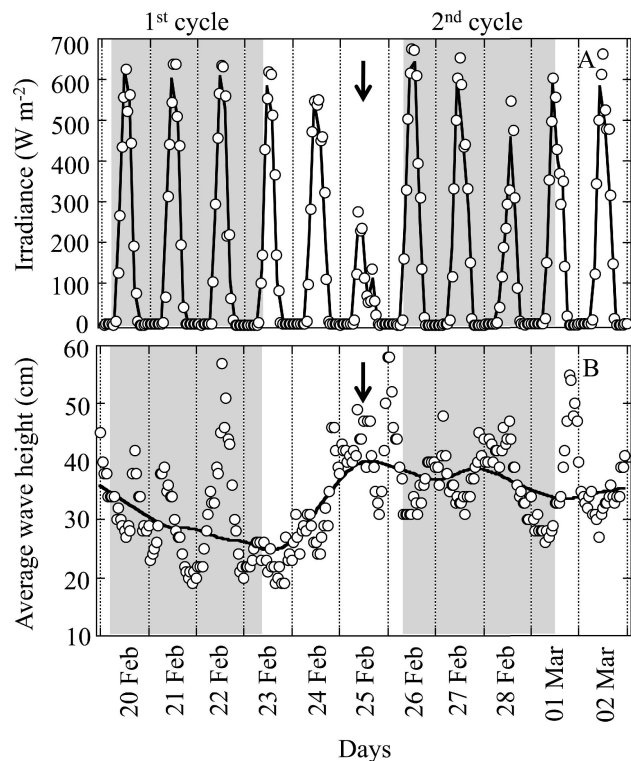
Fig. 1. (A) Irradiance measurements during the sampling period obtained from the station of Malgrat de Mar (Catalan Meteorological Service [SMC]); (B) mean wave height measured by a scalar buoy throughout the sampling period (XIOM Network, www.boiescat.org). Grey areas behind show each 72-h cycle. Arrows indicate an episode of increased wave height and reduced irradiance just before the beginning of the second cycle. The line is a best-fit smooth curve through the center of the data calculated using the locally weighted least-squared error method (Kaleidagraph version 4.1.1, Synergy Software).

Fig. 2. Diel changes in bulk ^{3}H-leucine and ^{3}H-thymidine bacterial incorporation rates during (A) the first and (B) the second cycles. The values are averages and standard errors of four replicate measurements. Arrows indicate when incubations for MAR–CARD–FISH analyses were taken. Solid bars on top axis represent dark periods.

on 22 February. Minimum values were obtained during the daytime at around 5 and 20 pmol L^{-1} h^{-1} (Fig. 2A).

During the second week (26 February–01 March) the diel cycles of bacterial activity seemed to have been disrupted after the 2 unsampled days (Fig. 2B). No diurnal pattern was found at the beginning of the week; however, in the course of the last 2 d, ^{3}H-leucine incorporation started to recover the pattern again, showing two maximum peaks at 01:00 h on 28 February and 01 March (175.0 and 203.9 pmol L^{-1} h^{-1}, respectively). There was a general increasing trend in ^{3}H-leucine incorporation throughout the second 72 h.

During the second week we did not observe any consistent diel pattern for ^{3}H-thymidine incorporation. Values were variable and ranged from 6.1 pmol L^{-1} h^{-1} at the first sampling point to 60.8 pmol L^{-1} h^{-1} at 01:00 h on 28 February. Unfortunately, we missed 24 h of incorporation, so the data set is not complete.

The cycle in bacterial abundances was less pronounced (T. Lefort unpubl.) and, as a result, bacterial-specific activities (Fig. 3) followed the same cycles as those of bulk bacterial activity. During the second week, however, bacterial-specific activity did not present any clear cycle,
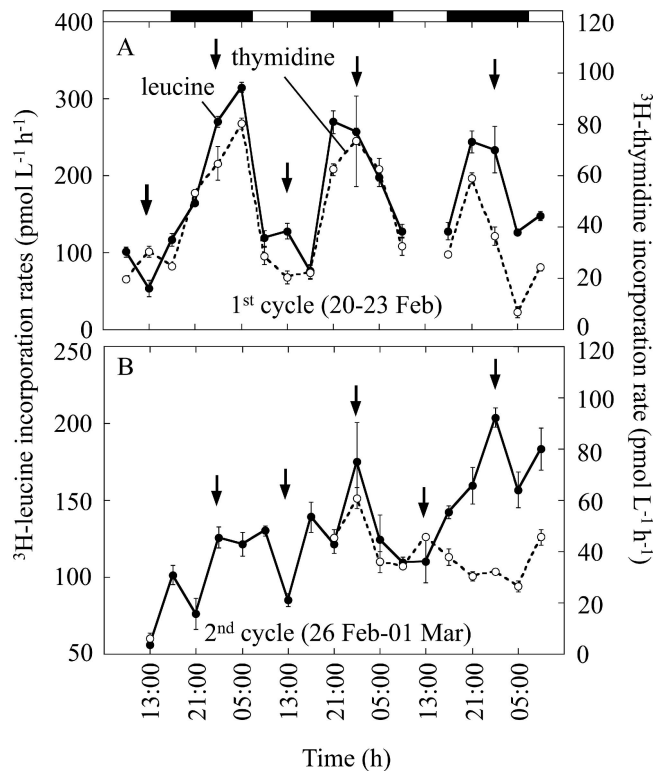
although towards the end of the week we could detect two night peaks of ^{3}H-leucine–specific incorporation rates, in a way similar to that for bulk incorporation (Fig. 3B).

On 20, 21, 26, and 28 February (days 1–4 as presented in Fig. 4) six subsamples from the noon sampling were amended with ^{3}H-leucine and ^{3}H-thymidine and incubated under an artificial visible-light source in parallel to standard dark incubations in order to explore the effects of light on bacterial activity measurements. ^{3}H-leucine incorporation was higher by 50–135% (always significant at $p < 0.05$) when incubated in the light. The effect of light on ^{3}H-thymidine incorporation was more variable; it caused a significant increase (twofold to fourfold) on days 2 and 3 and no significant differences between treatments on days 1 and 4.

Diel variability of the grazing activity of HNFs on PPeuks—Among all the preys available for HNF grazing, picoeukaryotes (mostly 1-μm *Micromonas*-like cells) were the group presenting more biomass (ca. 45% of picoplankton biomass, details not shown), comparable to that of heterotrophic bacteria. This is a recurrent seasonal feature of coastal Mediterranean stations, and for that reason we quantified during the first cycle both HNF abundance and the ingested PPeuks inside them every 4 h (Fig. 5). We found a clear pattern of higher grazing activity at night and nearly no ingestion during the day. This activity seemed to
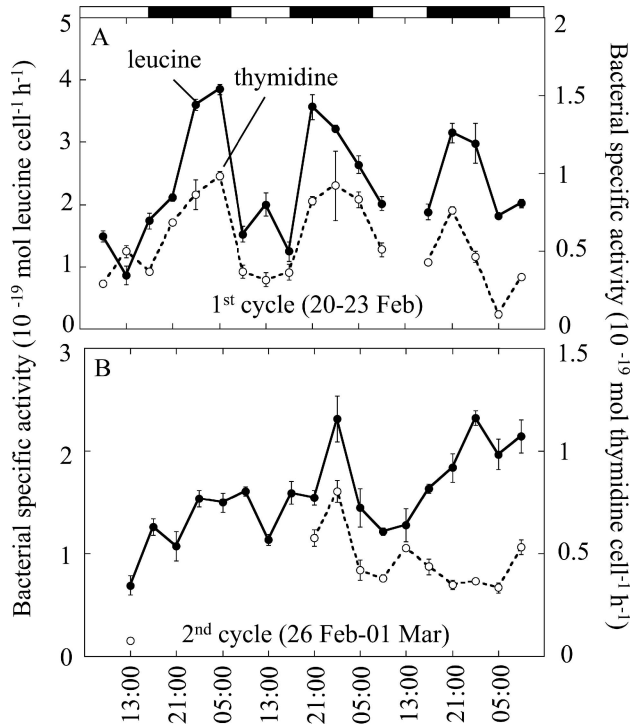
Fig. 3. Diel changes in ^{3}H-leucine and ^{3}H-thymidine bacterial specific activities during (A) the first and (B) the second cycles. The values were obtained by dividing bulk bacterial production by the concentration of bacteria at each sampling time. Solid bars on top axis represent dark periods.

be explained by the availability of prey, as shown by the significant correlation between PPeuk abundances (Fig. 5B) and ingested cells ($r = 0.73$, $p < 0.001$, $n = 18$). Interestingly, this relationship was stronger during the second 24-h period ($r = 0.99$, $p < 0.0001$, $n = 6$) compared to the other 2 d ($r = 0.88$ and $r = 0.82$ for the first and third day, respectively, $p < 0.05$, $n = 6$ each), and by the end of the sampling period it seemed that the HNFs, which had increased their numbers, had caused a significant decrease in PPeuk abundances. Unfortunately, we did not determine

the grazing activity during the second cycle, and HNF cells were only quantified in six different sampling points (Fig. 5C). However, it seemed that they were relatively less abundant during this cycle, and PPeuks, instead, appeared to restore a diel pattern in their abundances from the second day onwards, showing greater numbers at night than during the day (Fig. 5C).

Diurnal variations in community composition and single-cell activities—The composition of the bacterial community during both cycles was analyzed by CARD–FISH for day (13:00 h) and night (01:00 h) samples (Table 2). The fraction of DAPI-stained cells hybridized with as Eubacteria ranged from 81% to 88% during the first week and from 78% to 88% during the second week. Hybridization with specific probes showed that the bacterial community was mainly dominated by the SAR11 clade of the Alphaproteobacteria, which accounted for 31–42% of the total DAPI counts. Also Bacteroidetes comprised an important fraction of the bacterial community, with percentages ranging from 14% to 24%. The rest of the groups (Gammaproteobacteria, *Roseobacter,* and *Synechococcus*) were always below 11%. No significant differences in the average relative abundances of groups were found between the two cycles except for *Roseobacter,* which showed significantly higher numbers in cycle 1 than in cycle 2 (8% vs. 5% of DAPI counts, respectively, $p < 0.05$).

The relative abundances of the studied groups remained constant throughout the first week; only Gammaproteobacteria showed significantly higher numbers at 01:00 h on 21 February compared to the rest of the sampling times (Table 2, first cycle). On the contrary, the percentages of bacterial groups during the second week were more variable (Table 2, second cycle). Although *Roseobacter* and SAR11 did not show changes, total bacteria occurred at slightly lower percentages at 13:00 h on 27 February with respect to some of the sampling times. Conversely, Gammaproteobacteria and Bacteroidetes had significantly higher contributions to total abundance at 13:00 h on 28 February, but remained more or less constant during the rest of the cycle.
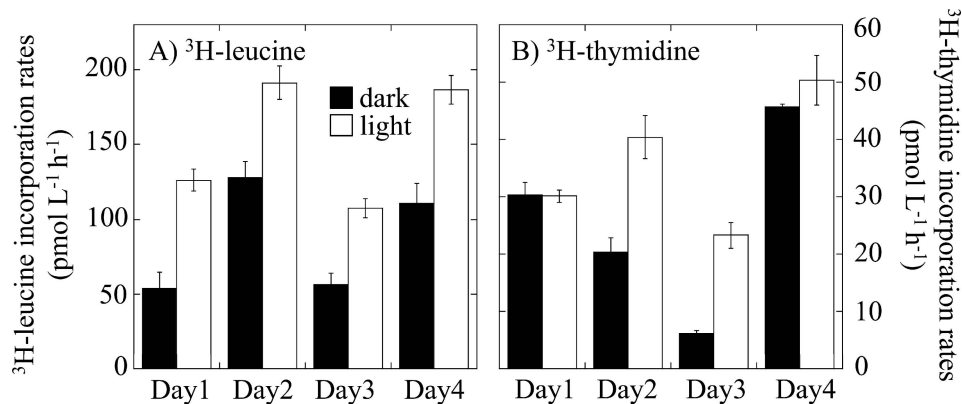

Fig. 4. Bacterial bulk activity measured as (A) ^{3}H-leucine and (B) ^{3}H-thymidine incorporation both in the dark and under an artificial light source at 13:00 h on 20 and 21 February (first cycle, days 1 and 2) and on 26 and 28 February (second cycle, days 3 and 4). Values are averages ± standard errors of four replicates.
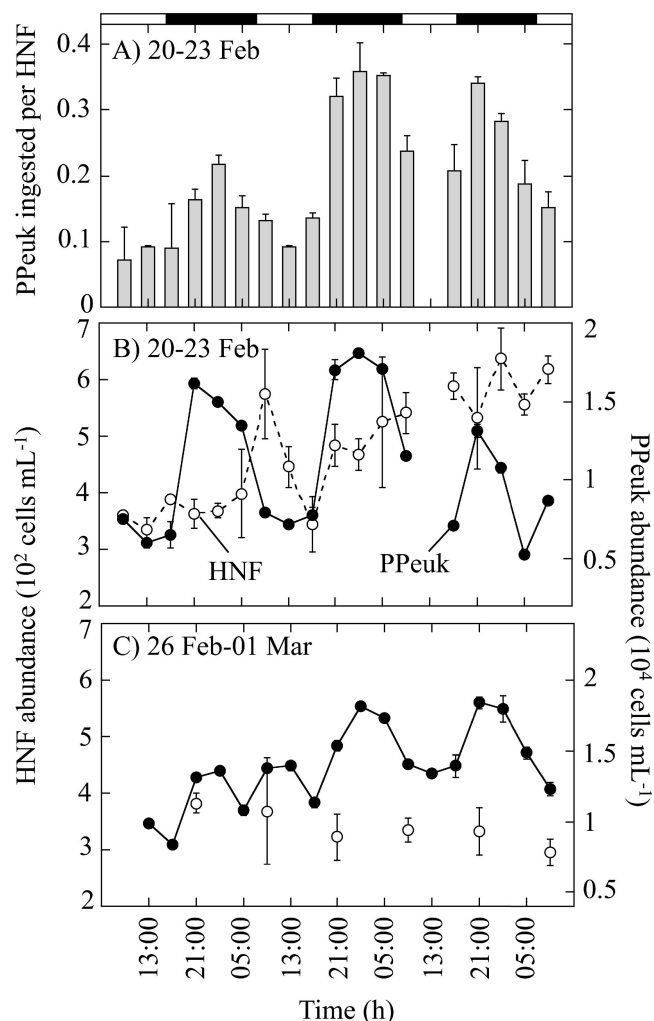
Fig. 5.   (A) Diel variations in the grazing activity of HNFs on PPeuks throughout the first cycle (average ± range of variation between two transects), and evolution with time of HNF (average ± range of variation between two transects) and PPeuk (average ± range of variation of duplicate samples) abundances during (B) the first and (C) the second cycle. Solid bars on top axis represent dark periods. HNFs during the second cycle were only counted in six different occasions.

The diurnal variations in single-cell activity of the different phylogenetic groups of bacteria were investigated in the two cycles using the MAR–CARD–FISH technique. On average, 28% (19–34%) of total Eubacteria were labeled (active) in ^{3}H-leucine uptake during the first week, and 20% (14–27%) during the second week. In general, most of the studied groups showed no significant differences in averaged percentages of active cells between the two cycles. Only members of Gammaproteobacteria presented on average many more active cells during the first cycle (39–87%, mean 65%) than during the second cycle (14–53%, mean 27%), even if the number of labeled cells tended to increase throughout the sampling period.

Considering the 2 weeks together, the average proportions of active cells were 6% (3–12%) for Bacteroidetes,

23% (13–41%) for SAR11, 82% (68–93%) for *Roseobacter*, and 7% (5–11%) for *Synechococcus*.

In the first week, the majority of the bacterial groups showed an obvious and similar diurnal pattern of activity, with greater percentages of active cells at night than during the day (Fig. 6). These changes between day and night were significant ($p < 0.05$) in all cases. Instead, no clear diurnal pattern was registered during the second cycle: just Gammaproteobacteria seemed to start to recover the same diel trend from the second night onwards (Fig. 7C).

With regard to variations in single-cell activity throughout the day in the first cycle (Fig. 6), we found that the percentage of labeled Eubacteria increased at night and decreased during the day by an average factor of ∼ 1.5 (Fig. 6A). When we focused on specific bacterial groups, a similar response was generally found. Most groups showed a stronger increase in the percentage of active bacteria from 13:00 h on 20 February to 01:00 h on 21 February (the first night) than during the second night. This was in agreement with the lower rates of ^{3}H-leucine incorporation registered at 01:00 h on 22 February compared to the first peak at 05:00 h on 21 February (Fig. 2A). Gammaproteobacteria, *Roseobacter*, and SAR11 showed night : day increases of 80%, 32%, and 93% during the first night, and 40%, 20%, and 55% during the second night, respectively. This night stimulation of the number of active cells was more similar between both nights for Bacteroidetes (108% and 91% increase, respectively), and *Synechococcus* did also show this nocturnal activation of their heterotrophic activities, although conversely to the rest of the groups, its stimulation was greater during the second night (57%) than during the first one (24%).

During the second cycle (26 February–01 March) we could not find any consistent diel pattern, but there was a general increase in the number of active cells towards the end of the sampling period (Fig. 7). Only Bacteroidetes, which remained constant during the whole cycle, did not show this significant increase in activity (Fig. 7E). Members of the Gammaproteobacteria cluster instead exhibited some diel pattern with higher percentages of active cells at night than during the last sampled day (from 18% at 13:00 h on 28 February to 53% at 01:00 h on 01 March, Fig. 7C). Interestingly, whereas bulk ^{3}H-leucine incorporation did not significantly correlate with prokaryote abundances (as measured by flow cytometry) when considering the two cycles together, it did correlate nicely with the number of MAR-active Eubacteria ($r = 0.83$, $p < 0.005$, $n = 10$).

Figure 8 shows the percentage contribution of each group to the total bacteria active in ^{3}H-leucine uptake plotted against their relative contribution to total Eubacteria abundance in all the samples. Data points on, or near, the 1 : 1 line indicate groups that were participating in substrate uptake proportionally to their abundance share in situ. In both cycles, *Roseobacter* and to a less extent Gammaproteobacteria were overrepresented in ^{3}H-leucine uptake compared to their abundance. On the contrary, SAR11 were closer to the 1 : 1 line and members of the Bacteroidetes cluster were always underrepresented in the uptake. In general, the upwards displacement observed in the right panel was due to the significant nocturnal

Table 2. Bacterial assemblage structure described as percentage of hybridized cells with specific probes by CARD–FISH in five samples of the first cycle and five samples of the second cycle. Eub338-II-III (Eubacteria), CF319a (Bacteroidetes), Gam42a (Gammaproteobacteria), Ros537 (*Roseobacter*), SAR11-441R (SAR11 clade), and Syn405 (*Synechococcus*). SAR11 and *Roseobacter* are subgroups of Alphaproteobacteria. Values are expressed as percentage of the total DAPI counts ($\pm$ standard error of 10–40 fields). Letters refer to results with a post hoc Tukey's test ($p < 0.05$) of the comparison between times. Different letters indicate significant differences between the different sampling times.

Date	Time	Fraction (%) of total DAPI counts detected with CARD-FISH probe					
		Eub338-II-III	CF319a	Gam42a	Ros537	Sar11-441R	Syn405
First cycle							
20 Feb	13:00	83±1[a]	17±2[a]	5±1[a]	6±1[a]	42±2[a]	<1[a]
21 Feb	01:00	85±2[a]	24±2[a]	8±1[b]	10±1[a]	42±2[a]	1.0±0.3[a]
22 Feb	13:00	84±1[a]	21±1[a]	4±1[a]	7±1[a]	40±2[a]	<1[a]
22 Feb	01:00	88±2[a]	21±3[a]	6±1[ab]	10±1[a]	38±4[a]	<1[a]
23 Feb	01:00	81±3[a]	17±2[a]	5±1[a]	8±1[a]	34±3[a]	<1[a]
	Average±SE	84±1	20±1	6±1	8±1	39±2	<1
Second cycle							
27 Feb	01:00	85±1[a]	16±1[a]	3±1[a]	4±1[a]	31±2[a]	2.0±0.6[a]
27 Feb	13:00	78±1[b]	15±1[a]	4±1[a]	5±1[a]	36±2[a]	1.1±0.3[a]
28 Feb	01:00	83±2[ab]	18±2[ab]	4±1[a]	6±1[a]	36±2[a]	1.5±0.4[a]
28 Feb	13:00	88±1[a]	22±1[b]	10±1[b]	4±1[a]	34±2[a]	1.6±0.7[a]
01 Mar	01:00	83±2[ab]	14±1[a]	6±1[a]	5±1[a]	37±3[a]	<1[a]
	Average±SE	83±2	17±1	5±1	5±0.4	35±1	1.3±0.2

increases in the fraction of active cells of most groups during the first cycle. Because all groups showed this behavior, the whole picture did not change significantly between day and night.

Discussion

The heterotrophic activity of marine bacteria in surface waters is driven by a complex framework of biological and physicochemical processes that are expected to undergo day–night variations as a consequence of the relation between sunlight and marine biota. In the course of the two cycles studied in February 2007, we first found that ^{3}H-leucine and ^{3}H-thymidine incorporation rates showed pronounced diurnal patterns with maximum values at night and lower values during the day, but this trend seemed to be disrupted after the unsampled days between both cycles. During those 2 d, there was a rainfall episode and a change in the wind direction from south and southwest to east, which seemed to alter the phased trend found during the first week. In this area, east winds are typically accompanied by relatively high waves and promote sediment resuspension (Ferré et al. 2005) that might modify the quality of DOM and inorganic nutrients available for marine organisms and thus might alter diel patterns. Episodic resuspension events have been shown to affect planktonic activities in both laboratory and field studies (Cotner 2000; Garstecki et al. 2002), although no specific information is available on how this may affect the bacterial diel periodicity. In our study we found that towards the end of the second cycle, after relaxation of the east wind episode, some parameters appeared to recover the day–night pattern, suggesting that these microbial populations are resilient to this kind of physical perturbations.

Water mass characteristics during both cycles were usual for that time of the year in the northwest Mediterranean, with cold temperatures, totally mixed water column, and late winter pre-bloom conditions (Estrada et al. 1985). Our physical data showed no great changes in temperature or salinity over time, indicating that we were sampling a reasonably stable and coherent patch of water during each week (details not shown). Nevertheless, we cannot discard the possibility that the East winds could have pushed offshore waters into the coast that might explain the higher abundances of picophytoplankters found during the second week.

Difference in average bacterial activity between the two 3-d cycles—It is remarkable that the average bacterial activity had decreased after the unsampled days. This minimum in bacterial activity coincided with a minimum in abundance at the beginning of the second cycle (T. Lefort unpubl.). If sediment resuspension occurred in between cycles (and not the entrance of a different water mass), we might expect an increase in bacterial activity (Cotner 2000). However, a decrease in activity might also occur if either the resuspended material was more recalcitrant, or if a notable fraction of free-living bacteria attached to suspended particles (Chróst and Riemann 1994) and were under-sampled with the small volumes used for bacterial activity assays and flow cytometry analyses. Unfortunately, because we did not specifically test this, we cannot give an accurate explanation for this difference between cycles.

Diel changes in bulk and specific bacterial activity—Marked diel cycles in bulk bacterial activity were detected during the first cycle but not during the second one. From 20 to 23 February bulk bacterial activity showed much higher ^{3}H-leucine and ^{3}H-thymidine incorporation rates at
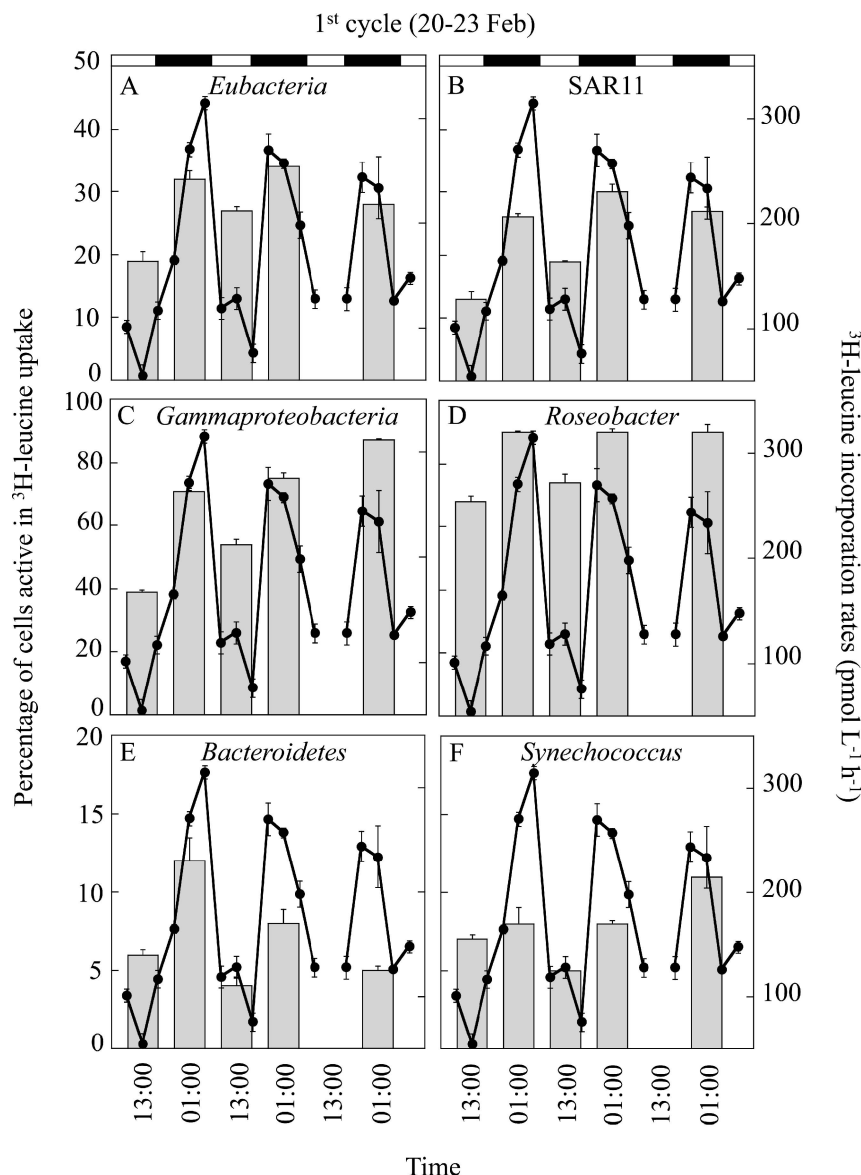
1st cycle (20-23 Feb)

Fig. 6. Percentage of positively hybridized cells taking up ^{3}H-leucine as measured by MAR–CARD–FISH during the first cycle (20–23 February) in both 13:00 h and 01:00 h samples. Columns represent means of duplicate samples and error bars indicate the range of variation between them. The dotted line behind shows bulk ^{3}H-leucine incorporation rates for comparison as displayed in Fig. 2A. Solid bars on top axis represent dark periods. 13:00 h sampling time is missing in the third day because of bad weather conditions.

night than during the day. Likewise, specific bacterial activity was also greater at night, meaning that the nocturnal increase in total ^{3}H-leucine and ^{3}H-thymidine incorporation was not just because there were more bacteria; on average, each cell was incorporating more leucine (and thus grew faster) throughout the night. Such diel fluctuations were surprisingly higher than the range reported for seasonal variation in bacterial activity within that year: the greatest change found during the first 24 h in ^{3}H-leucine incorporation rate was 260.4 pmol L^{-1} h^{-1} (from 13:00 h on 20 February to 05:00 h on 21 February) whereas the maximum variation recorded for the whole of 2007 (daytime sampling only) was found to be

188.6 pmol L^{-1} h^{-1} between March and October (I. Lekunberri unpubl.). Some authors (Stramska et al. 1995) have also found that the diel variability of marine parameters can be often more important than the variability at the weekly scale, or even at the annual scale. This scale of variability is often neglected in sampling strategies, and thus the interpretation of seasonal data, particularly when sampling time varies, should carefully be considered.

The bacterial diel pattern found in this study is opposite to that reported by Gasol et al. (1998) in an offshore station near this area in June 1993 and 1995, although they sampled in summer instead of winter, which probably led
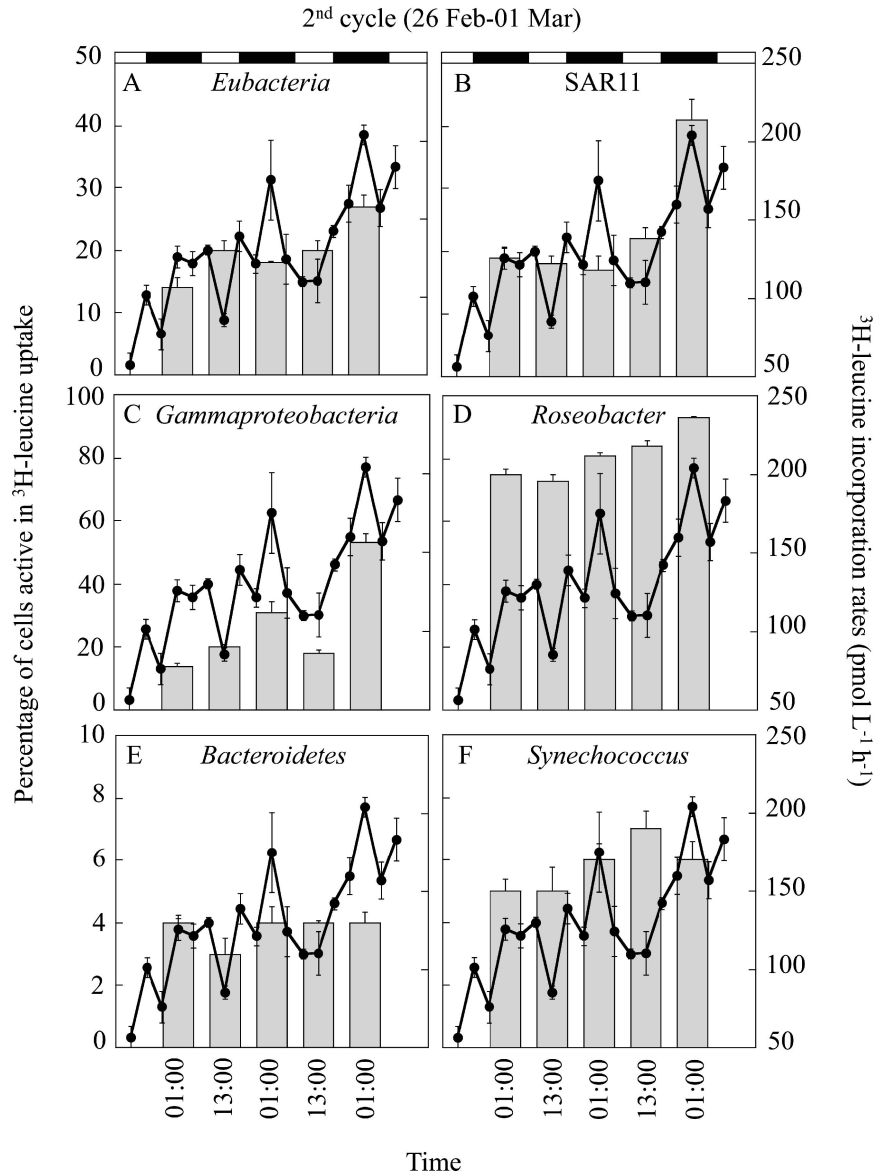
Fig. 7. Percentage of positively hybridized cells taking up ³H-leucine as measured by MAR–CARD–FISH during the second week (26 February–01 March) in both 13:00 h and 01:00 h samples. Columns represent means of duplicate samples and error bars indicate the range of variation between them. The dotted line behind shows bulk ³H-leucine incorporation rates for comparison as displayed in Fig. 2A. Solid bars on top axis represent dark periods.

to differences in DOM quality and/or bacterial community composition, in addition to the spatial divergence between coastal and offshore communities. These authors found that noon estimates of bacterial activity were more than twice the daily average in one oceanic station (~100 km offshore the Blanes Bay) and no clear trends were apparent near the coast, supporting the idea that under more oligotrophic conditions, the coupling of phytoplankton and bacterial activities should be more discernible. Indeed, most studies of diel variations in bacteria have suggested this link between primary production and bacterial use of the released DOM, usually leading to increased bacterial abundances or activity during daytime with maxima values

in late afternoon and minima at night (Fuhrman et al. 1985; Herndl and Malacic 1987).

Other authors have reported only weak or inconsistent diel patterns (Riemann and Søndergaard 1984; Torreton and Dufour 1996) but very few have observed this nocturnal stimulation of bacterial activity (Jeffrey et al. 1996; Shiah 1999; Kuipers et al. 2000). Remarkably, in these latter studies, bacterial activity was measured only in terms of deoxyribonucleic acid (DNA) synthesis (not as protein synthesis) and the night increase was suggested as a mechanism of avoidance of UVR damage to DNA during the day. During our first cycle, however, the incorporation of ³H-leucine followed this trend of nocturnal increases too.
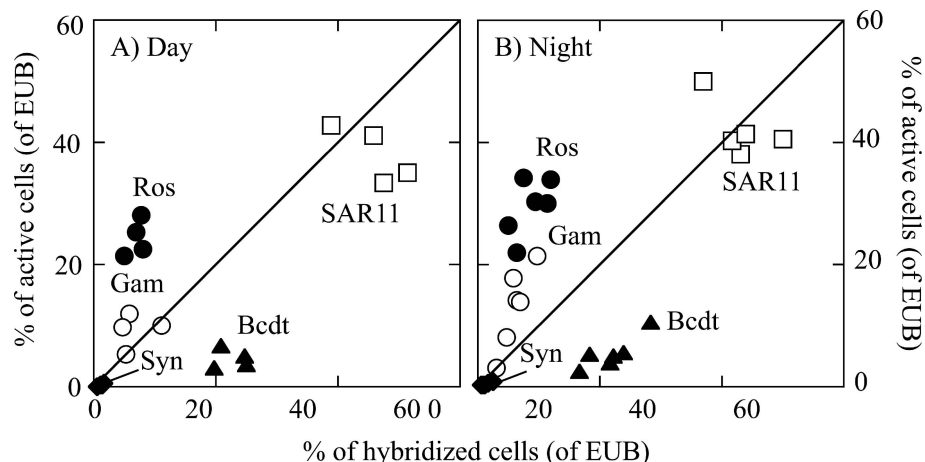
Fig. 8.   Contributions of various phylogenetic groups (SAR11, *Roseobacter* [Ros], Gamma-proteobacteria [Gam], Bacteroidetes [Bcdt], and *Synechococcus* [Syn]) to the number of cells active in ³H-leucine uptake, presented against their contribution to the assemblage composition (relative abundance) in both (A) day and (B) night samples. Samples from the two cycles are plotted together. Percentages were calculated relative to eubacterial cells (probes EUB338, -II and -III).

This opposite pattern of protein synthesis and primary production suggests that causes other than photosynthetic DOM might be driving the changes in bacterial activity.

All in all, multiple potential explanations for the nocturnal increase (or diurnal decrease) in heterotrophic bacterial activity could be invoked: (1) experimental artifacts such a radioisotope dilution, (2) UVR-inflicted photoinhibition or DNA damage, (3) lagged response to photosynthetic DOM release, (4) light-enhanced competition of phytoplankton for nutrient and DOM uptake, (5) rapid response to grazing-derived DOM, (6) diel changes in free-living and attached bacteria, and (7) diel changes in bacterial growth and mortality. Hereafter we will examine these potential explanations one by one.

Radioisotope dilution as an experimental artifact—Should diel variations in the release and concentration of dissolved free amino acids occur (Mopper and Lindroth 1982), this could result in an apparent diel pattern in ³H-leucine incorporation simply through dilution of the radiotracer. Although this artifact cannot be totally discarded, additional unpublished data from these same experiments, such as increased proportions of high-nucleic-acid–content cells or actively respiring cells at night (T. Lefort unpubl.), support the true occurrence of greater nocturnal activity.

UVR-inflicted bacterial photoinhibition or DNA damage—Photoinhibition of bacterial heterotrophic production due to UVR has been widely shown (Herndl et al. 1993; Alonso-Sáez et al. 2006), and some authors have concluded that it can be a significant factor in the diel cycling of organic matter in the euphotic zone. Sunlight-induced delay in the growth of surface bacteria has been reported (Sieracki and Sieburth 1986) as being the reason why the DOM released by algae is not immediately taken up by bacteria (Burney 1986). Diurnal accumulation of DNA damage and nocturnal recovery have been also reported

(Jeffrey et al. 1996; Booth et al. 2001). More recently, Van Wambeke et al. (2009) observed that increases in the daily ultraviolet B : ultraviolet A ratios were negatively correlated with bacterial activity in South Pacific clear waters.

In our case, a major involvement of UVR in setting the diel pattern of bacterial activity seems unlikely because the mixed-layer UVR doses registered in the Blanes Bay during winter are low (range 0.01–0.05 W m^{-2}, M. Galí unpubl.). In addition, a seasonal study at the same site has shown that winter UVR levels do not cause significant inhibition of ³H-leucine incorporation rates (C. Ruiz-González unpubl.).

Lagged response of bacteria to photosynthetic DOM release—Shiah (1999) also found higher ³H-thymidine incorporation rates at night but in UVR-devoid experiments. He associated this nocturnal increase in bacterial activity indirectly with light, by means of a diurnal DOM release by phytoplankton and an apparently lagged response of heterotrophic bacteria. In our study, however, we found that noon bacterial heterotrophic activity was higher in light than in dark incubations, and that the response was immediate (Fig. 4). Among the possibilities explaining such an effect, a rapid use of photoproducts by bacteria upon illumination would discard a potentially delayed response of bacteria like the one reported by Shiah. Instead, if the stimulation was due to light-enhanced activity of mixotrophic organisms (Rivkin and Putt 1987; Mary et al. 2008b), a late response of bacteria to photosynthate would still be possible. Because all our MAR–CARD–FISH incubations were performed in the dark, this hypothesis could not be tested. However, in a seasonal study carried out in Blanes Bay during 2.5 yr (C. Ruiz-González unpubl.), the consistent stimulation observed of bulk ³H-leucine incorporation rates under an artificial light source (without UVR) was not due either to eukaryotic algae or to cyanobacteria, but to Gammapro-

teobacteria (C. Ruiz-González unpubl.). It is therefore possible that the observed diel pattern had been different under sunlight exposure, yet even if including this light stimulated noon values in the cycle, the activity levels at night would still be much greater than during the day.

Diurnal competition of phytoplankton for nutrient uptake—Bacterial competition with phytoplankton for inorganic nutrients during the day might also offer an alternative explanation to the nocturnal stimulation of bacterial activity (Kuipers et al. 2000). Although most inorganic nutrients were at their annual maximum (details not shown), and bacteria from Blanes Bay winter waters are known to be mainly carbon limited (Pinhassi et al. 2006), we cannot discard this possibility because no information exists on diel changes in bacterial nutrient limitation in this area.

Rapid bacterial response to grazing-derived DOM—Greater grazing activity at night, as suggested by the marked increase in the number of ingested PPeuk cells by HNF, might have resulted in the nocturnal enhancement of bacterial activity through DOM release. Herbivore activity is a major source of labile DOM in oligotrophic waters (Nagata 2000). As reported for other regions (Vaulot and Marie 1999), in Blanes Bay PPeuk divided at the beginning of the night, thus leading to higher availability of prey for the HNF. Indeed, it is known that protozoa preferentially graze on larger, actively growing and dividing cells rather than on smaller ones (Sherr et al. 1992). However, although the increase in PPeuk abundances itself seemed to trigger the activity of grazers, by the end of the sampling period this enhanced predation appeared to cause a significant reduction in prey abundance. This suggests that the observed 3-d pattern would probably not be maintained over time and that, subsequently, the release of DOM and the associated bacterial activity would also be disrupted. It is remarkable, though, that during cycle 2, the abundances of PPeuk recovered the diel fluctuations, showing greater abundances at night than during the day, which might have been because of the restoration of the grazing activity by HNF. Should this activity release any DOM, it might explain that some opportunistic bacterial groups such as Gammaproteobacteria did immediately respond with an increase in their numbers of active cells by the end of the second cycle that appeared to drive the bulk activity patterns (*see* below).

The release of DOM by heterotrophic protists appears to be fully accounted for by egestion (Nagata 2000). This author proposed a model in which grazers were the dominant source of DOM over phytoplankton production. Among grazers, protozoa, which dominate oligotrophic environments such as the Blanes Bay and graze on small phytoplankton or bacteria, play a major role in the release of DOM rich in different nutrients (Nagata and Kirchman 1991; Strom et al. 1997). Because carbon is the principal limiting element for bacteria in winter Blanes Bay waters (Pinhassi et al. 2006), it is plausible that grazing-derived labile DOM explained the strong synchronization between micrograzing on the abundant PPeuk and bacterial activity

during the first week. After the stormy unsampled days, though, *Synechococcus* became dominant among picophytoplankton and the abundance of HNF decreased, so these community composition changes could have altered the amount and quality of grazing-derived DOM, at least at the beginning of the cycle.

Alternatively, it is possible that some DOM was released as a result of the processes of cell growth and division, because the abundances of most studied groups (PPeuk, *Synechococcus*, bacteria) increased at night and decreased during the day (T. Lefort unpubl.), yet little information is still available on this topic (but *see* Kawasaki and Benner 2006).

Diel changes in free-living and attached bacteria—Short-term changes in the relative contributions of free-living vs. attached bacteria to total bacterial production have been observed (Ghiglione et al. 2007). If the proportions of free-living and attached bacteria had changed over diel cycles, this might potentially cause apparent diel patterns in bacterial activity. We cannot test this hypothesis because the volumes we used to determine bacterial activity and abundances (1.2 mL) seem too small to obtain accurate quantification of attached bacteria.

Diel changes in bacterial growth and mortality rates—The observed diel cycles in ^{3}H-leucine incorporation rates might not translate into real bacterial production cycles if diel changes in the growth efficiency of bacteria on leucine (i.e., the leucine to carbon conversion factors) occurred. To our knowledge, no study has addressed this issue hitherto, and the determination of near-instantaneous growth rates represents a methodological challenge. Bacterial biovolumes did not significantly change in our study (T. Lefort unpubl.); therefore, the fact that bacterial activity varied much more profoundly than abundance might be indicative of diel changes in bacterial mortality rates. Both grazing and viral lysis are known to be relevant controlling factors in Blanes Bay (Boras et al. 2009). Unfortunately, we do not have information on their diel variations. The most we can say is that HNF and bacterial abundances were significantly correlated during the first cycle ($r = 0.81$, $p < 0.0001$, $n = 18$), indicating a likely coupling between predators and preys.

Diurnal variations in single-cell activities of dominant bacterial groups—Several studies have tested the variations in bulk bacterial activity at daily scales, but very few have analyzed the changes within specific phylogenetic groups. Pernthaler and Pernthaler (2005) studied cell proliferation of three bacterial taxa (*Roseobacter*, SAR86, and NOR5) looking for diurnal patterns of DNA synthesis within them, but they did not find any clear diel trends. Using flow cytometric cell sorting, Mary et al. (2008a) found obvious diurnal rhythms in ^{3}H-leucine and ^{35}S-methionine uptake by *Prochlorococcus* cells in the tropical Atlantic, with maximum values at dusk and minimum at midday, and Chen et al. (1991) described a circadian clock regulating amino acid uptake in freshwater *Synechococcus*, which presented the highest uptake rates during the light period.

To our knowledge, however, this is the first report showing clearly defined diel patterns in the activities of dominant hetero- and mixotrophic bacterial groups.

The composition of the bacterial community, as assessed with CARD–FISH probes, was within the compositional variability previously reported for this area (Alonso-Sáez et al. 2007). Although the relative abundances of the studied groups did not change on a daily scale, greater numbers of active cells were observed at night for all groups during the first cycle, in accordance with the pattern of bulk activity. During the second cycle, instead, only Gammaproteobacteria seemed to start recovering the trend again from the second night onwards, and interestingly, this appeared to drive bulk ^{3}H-leucine incorporation rates, despite being one of the less abundant groups. In fact, although Gammaproteobacteria presented on average much fewer active cells during the second week than during the first one, a progressive increase in the number of labeled Gammaproteobacteria cells was recorded towards the end of the week. Cells belonging to the Gammaproteobacteria clade have shown preference for amino acids rather than other compounds as a carbon source, with varying proportions of active cells depending on nutrient availability (Alonso-Sáez and Gasol 2007). It is possible that changes in the quality of DOM derived from the east wind episode (either by resuspension or by entrance of a different water mass) had negatively affected the activity of Gammaproteobacteria at the beginning of the cycle. However, the observed restoration in PPeuk abundance changes might suggest a recovery of the grazing activity patterns, thus explaining the responses of Gammaproteobacteria to this potential new DOM-supply cycle. Members of the Gammaproteobacteria group have been shown to maintain high levels of ribosomes during long periods of nongrowth, which would allow them to rapidly initiate growth at changing conditions (Eilers et al. 2000).

Remarkably, besides the fact that the studied groups may harbor a number of subgroups with rather different metabolic properties, all of them were responding in the same way, showing a general diel synchronization. Should this be due to a periodic DOM supply, the released compounds might have been suitable for a fraction of cells within all groups. Instead, if bacteria had been more tightly dependant on phytoplankton DOM, we might have found some favored taxa, such as some *Roseobacter* closely related to blooms of particular phytoplankton species and thus to short-term fluctuations of primary production (Pinhassi et al. 2004). Similarly, if solar radiation had played a significant direct role, we could have found some differential activation or inhibition of bacteria throughout the daylight hours depending on each group's sensitivity to sunlight (Alonso-Sáez et al. 2006) or their potential capabilities to derive energy from light (Béjà et al. 2000; Kolber et al. 2000).

When we compared the number of active cells within each group with their relative abundances, Gammaproteobacteria and specially *Roseobacter* were found to be overrepresented in terms of ^{3}H-leucine uptake, meaning that they contributed more to the total ^{3}H-leucine uptake than to the composition of the community, whereas Bacteroidetes was underrepresented, possibly explained by their lack of affinity for leucine and their preference for high molecular weight compounds. Cells belonging to the SAR11 clade participated in ^{3}H-leucine incorporation in proportion to their contribution to bulk abundance. This pattern, commonly found for these groups from this and other regions (Vila-Costa et al. 2007; Alonso-Sáez et al. 2008*a*), did not change between day and night, except for a general displacement towards higher numbers of active cells in night samples. The figure also illustrates that the day–night cycle affected the activity but not the composition of the bacterial community. *Synechococcus* remained basically the same in both day and night samples, showing negligible contributions to both activity and community composition.

Dark standard measurements of bulk and single-cell bacterial activities may underestimate bacterial production during the day or neglect any potential UVR-driven effect, so future experiments under real light conditions will be needed in order to assess the actual magnitude of these diel variations. Moreover, because the relative activities of bacterial groups from Blanes Bay vary between winter and summer (Alonso-Sáez and Gasol 2007), as does the availability of DOM (Alonso-Sáez et al. 2008*b*), we cannot discard that completely different bacterial cycles occur at different times of the year, as shown by Ghiglione et al. (2007) between spring and summer samples. Further research on the relative contribution of the key players in DOM supply and the diel activities of different bacterial taxa will be required for better comprehension of their contribution to daily variations in carbon fluxes.

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