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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
Barcelona, Spain

* Corresponding author: clara.ruiz.glez@gmail.com

Condensed running head: Diel activity of distinct bacterial taxa

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34

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-day periods separated by 2 days. Twice a day, the single-cell activity of major bacterial groups was further analysed 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 ^{3}H-leucine recovered the diel pattern after two days, mostly driven by the activity of *Gammaproteobacteria*. Among the possibilities considered, the observed night-time 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.

Introduction

Day-night variations of biological parameters in the ocean are originally a consequence of the relation between sunlight and marine organisms. Since the different components of the microbial food web are continuously interacting, any synchronization of life cycles and behaviours, 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 the photosynthetic release or the excretion through grazing activities are thought to be major sources of dissolved organic matter (DOM) for marine bacterial use in oceanic environments (Nagata 2000). Since 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 mainly been addressed at the community level, while 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 NW Mediterranean waters during two consecutive, 72 h cycles, along with bulk bacterial heterotrophic activity measured both as ^{3}H-leucine and ^{3}H-thymidine incorporation. Microautoradiography combined with catalyzed reported deposition-fluorescence in situ hybridization (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 NW Mediterranean Sea, located 800 m offshore of Blanes, Catalonia, Spain (41º39.90'N, 2º48.03E). Experiments were performed in two blocks, on 20 to 23 February (first cycle) and on 26 February to 01 March (second cycle), 2007. Surface water samples (0.5 m depth) were collected with polycarbonate carboys every 4 hours during these two 72 h periods, and transported to the lab under dim light. Incubations were started less than 30 minutes after water collection. The sample of the third day at noon could not be collected due to rough sea conditions. The temperature and salinity of the sampled waters were obtained with a SAIV A-S 204 conductivity, temperature, depth (CTD) 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

124 at 4 m above sea level. The station recorded arithmetically averaged hourly air
125 temperature and relative humidity at 1.5 m above ground, vector-averaged hourly
126 wind speed and direction and global irradiance at 2 m, and accumulated rainfall at 1
127 m. Wave height data were collected from a scalar buoy (DATAWELL, Waverider)
128 placed at 41º 38.82´N, 02º 48.93'E over a depth of 74 m (XIOM Network,
129 www.boiescat.org).

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

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

145 *Bacterial heterotrophic activity*. Bacterial activity was estimated every 4 hours
146 from both radioactive ^{3}H-leucine and ^{3}H-thymidine incorporation. For leucine we
147 used the ^{3}H-leucine assimilation method described by Kirchman et al. (1985) with the

148 modifications of Smith and Azam (1992). Briefly, 4 aliquots (1.2 mL) and 2 TCA-
149 killed controls were incubated with radiolabelled leucine (40 nmol L^{-1}, final
150 concentration, 160 Ci $mmol^{-1}$) for about 2 hours in the dark at in situ temperature. The
151 incorporation was then stopped by adding 120 μL of cold TCA 50% to the samples,
152 which were stored at –20ºC until processing by the centrifugation method of Smith
153 and Azam (1992). Bacterial activity was also measured as ^{3}H-thymidine incorporation
154 following Fuhrman and Azam (1980) with the modifications of Smith and Azam
155 (1992). Samples were incubated with 10 nmol L^{-1} ^{3}H-thymidine (final concentration)
156 and processed like the ^{3}H-leucine samples.

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

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

172 *Microautoradiography combined with catalyzed reporter deposition-*
173 *fluorescence in situ hybridization (MAR-CARD-FISH).* Twice a day (GMT 13:00 h
174 and 01:00 h) 30 mL water samples were incubated with added radioactive ^{3}H-leucine
175 (160 Ci mmol^{-1}, 0.5 nmol L^{-1} final concentration) for 4 hours in the dark at in situ
176 temperature. Controls killed with paraformaldehyde (PFA) were also run
177 simultaneously with all live incubations. After 4 h the live samples were fixed
178 overnight with PFA (1% final concentration) at 4ºC in the dark.

179 For the analysis of the single-cell bacterial activity, we followed the protocol
180 described in Alonso-Sáez and Gasol (2007). Aliquots of 10 mL were gently filtered
181 through 0.2 μm polycarbonate filters (GTTP, Millipore), rinsed with milliQ water, air
182 dried and stored at –20ºC until processing. Hybridization of the filters was done
183 following the CARD-FISH protocol. Several horseradish peroxidase (HRP)-probes
184 were used to characterize the composition of the bacterial community in the water
185 samples: Eub338-II-III for most *Eubacteria* (Daims et al. 1999), Gam42a for most
186 *Gammaproteobacteria* (Manz et al. 1992), CF319 for many clades belonging to the
187 *Bacteroidetes* group (Manz et al. 1996), Ros537 for the *Roseobacter* clade (Eilers et
188 al. 2001), SAR11-441R for the SAR11 cluster (Morris et al. 2002) and Syn405 for the
189 cyanobacterial genus *Synechococcus* (West et al. 2001). The Eub antisense probe
190 Non338 (Wallner et al. 1993) was used as a negative control. All probes were
191 purchased from biomers.net (Ulm).

192 Cell walls were first permeabilized with lysozyme (37ºC, 1 h) and
193 achromopeptidase (37ºC, 0.5 h) before the hybridization. Hybridizations were carried
194 out on sections of the filters at 35ºC overnight, and specific hybridization conditions
195 were established by addition of formamide to the hybridization buffers (45%
196 formamide for SAR11 probe, 20% for Non338, 60% for Syn405, and 55% for the rest

of 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 46ºC 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 4ºC until development. The optimal exposure time was determined for all the samples and resulted in an average of 8 days. 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 the Levene's test for homogeneity of variance were applied prior to analysis, and either one-way ANOVA or the non-parametric 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 the Pearson's correlation coefficient. These statistical analyses were performed using the 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

221 formula for the propagation of error as described by Bevington and Robinson (2003).

222 Results

223 *Background information.* Sampling was done at the coldest period of the year
224 (average temperature ~ 13.4ºC), and temperature did not vary significantly ($p > 0.05$)
225 between both weeks. The water column was totally mixed (data not shown) as it is
226 typical for this area in winter pre-bloom conditions (Estrada et al. 1985). Chlorophyll
227 *a* concentration was only estimated for the first sampling time of each cycle, and it
228 almost doubled (from 0.47 to 0.89 μg L^{-1}) between the first and the second cycle
229 (Table 1). Whereas the average number of heterotrophic bacteria did not significantly
230 change between both cycles, averaged *Synechococcus*, *Prochlorococcus*, and PPeuk
231 abundances significantly ($p < 0.05$) increased from the first to the second week (Table
232 1), in concordance with the higher chlorophyll *a* concentrations found in the second
233 week. During the unsampled days between the two cycles, wind direction changed
234 (shifting from South and Southwest to East) causing increased sea turbulence and
235 wave action (Fig. 1B), with simultaneous rainfall and low-light conditions (Fig. 1A).

236 *Diel variations in bulk bacterial activity.* Both ^{3}H-leucine and ^{3}H -thymidine
237 incorporation rates were measured every 4 hours during two periods of 72 h in order
238 to assess the effect of daily light changes on bacterial heterotrophic activity. During
239 the first cycle (20 to 23 February) bacterial activity showed a marked diel pattern with
240 higher ^{3}H-leucine and ^{3}H-thymidine incorporation values at night and lower values
241 during the day (Fig. 2A). Throughout the 72 h there were extremely large variations in
242 bacterial activity among sampling times. ^{3}H-leucine incorporation increased from
243 53.8 to 314.2 pmol L^{-1} h^{-1} between 13:00 h on 20 February and 05:00 h on 21
244 February, meaning a ~ 6-fold increase in less than 24 h. On 21 and 22 February the

245 peaks reached 269.8 and 243.9 pmol L^{-1} h^{-1}, respectively, and they appeared to be
246 displaced in time with respect to the first day, because they both were reached at
247 21:00 h instead of at 05:00 h. Minimum values were around 50 and 75 pmol ^{3}H-
248 leucine L^{-1} h^{-1}. ^{3}H–thymidine incorporation was also higher at night than during the
249 day and it very closely paralleled the ^{3}H-leucine incorporation pattern. Maximum ^{3}H–
250 thymidine incorporation values ranged from 80.4 pmol L^{-1} h^{-1} on 21 February to 59.4
251 pmol L^{-1} h^{-1} on 22 February. Minimum values were obtained during daytime at around
252 5 and 20 pmol L^{-1} h^{-1} (Fig. 2A).

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

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

264 The cycle in bacterial abundances was less pronounced (T. Lefort unpubl.) and,
265 as a result, bacterial specific activities (Fig. 3) followed the same cycles as those of
266 bulk bacterial activity. During the second week, however, bacterial specific activity
267 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 as for bulk incorporation (Fig. 3B).

On 20, 21, 26, and 28 February (days 1 to 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 (2 to 4-fold) on days 2 and 3 and no significant differences between treatments on days 1 and 4.

Diel variability of the grazing activity of heterotrophic nanoflagellates (HNF) on photosynthetic picoeukaryotes (PPeuk). 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 PPeuk 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 be explained by the availability of preys, 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-hour period ($r = 0.99$, $p < 0.0001$, $n = 6$) compared to the other two days ($r = 0.88$ 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 HNF, which had increased their numbers, had caused a significant decrease in PPeuk abundances. Unfortunately, we

293 did not determine the grazing activity during the second cycle, and HNF cells were
294 only quantified in six different sampling points (Fig. 5C). However, it seemed that
295 they were relatively less abundant during this cycle, and PPeuk, instead, appeared to
296 restore a diel pattern in their abundances from the second day onwards, showing
297 greater numbers at night than during the day (Fig. 5C).

298 *Diurnal variations in community composition and single-cell activities.* The
299 composition of the bacterial community during both cycles was analyzed by CARD-
300 FISH for day (13:00 h) and night (01:00 h) samples (Table 2). The fraction of DAPI-
301 stained cells hybridized with as *Eubacteria* ranged from 81% to 88% during the first
302 week and from 78% to 88% during the second week. Hybridization with specific
303 probes showed that the bacterial community was mainly dominated by the SAR11
304 clade of the *Alphaproteobacteria,* which accounted for 31% to 42% of the total DAPI
305 counts. Also *Bacteroidetes* comprised an important fraction of the bacterial
306 community, with percentages ranging from 14% to 24%. The rest of the groups
307 (*Gammaproteobacteria, Roseobacter,* and *Synechococcus)* were always below 11%.
308 No significant differences in the average relative abundances of groups were found
309 between the two cycles except for *Roseobacter,* which showed significantly higher
310 numbers in cycle 1 than in cycle 2 (8% vs. 5% of DAPI counts, respectively, $p <$
311 0.05).

312 The relative abundances of the studied groups remained constant throughout the
313 first week; only *Gammaproteobacteria* showed significantly higher numbers at 01:00
314 h on 21 February compared to the rest of the sampling times (Table 2A). On the
315 contrary, the percentages of bacterial groups during the second week were more
316 variable (Table 2B). Whereas *Roseobacter* or SAR11 did not show changes, total
317 bacteria occurred at slightly lower percentages at 13:00 h on 27 February with respect

318 to some of the sampling times. Conversely, *Gammaproteobacteria* and *Bacteroidetes*
319 had significantly higher contributions to total abundance at 13:00 h on 28 February,
320 but remained more or less constant during the rest of the cycle.

321 The diurnal variations in single-cell activity of the different phylogenetic groups
322 of bacteria were investigated in the two cycles using the MAR-CARD-FISH
323 technique. On average, 28% (19-34%) of total *Eubacteria* were labelled (active) in
324 ^{3}H-leucine uptake during the first week, and 20% (14-27%) during the second week.
325 In general, most of the studied groups showed no significant differences in averaged
326 percentages of active cells between the two cycles. Only members of
327 *Gammaproteobacteria* presented on average many more active cells during the first
328 cycle (39-87%, mean 65%) than during the second cycle (14-53%, mean 27%), even
329 if the number of labelled cells tended to increase throughout the sampling period.

330 Considering the two weeks together, the average proportions of active cells
331 were 6% (3-12%) for *Bacteroidetes*, 23% (13-41%) for SAR11, 82% (68-93%) for
332 *Roseobacter*, and 7% (5-11%) for *Synechococcus*.

333 In the first week, the majority of the bacterial groups showed an obvious and
334 similar diurnal pattern of activity, with greater percentages of active cells at night than
335 during the day (Fig. 6). These changes between day and night were significant ($p <$
336 0.05) in all cases. Instead, no clear diurnal pattern was registered during the second
337 cycle: just *Gammaproteobacteria* seemed to start to recover the same diel trend from
338 the second night onwards (Fig. 7C).

339 With regard to variations in single-cell activity throughout the day in the first
340 cycle (Fig. 6), we found that the percentage of labelled *Eubacteria* increased at nigh
341 and decreased during the day by an average factor of ~ 1.5 (Fig. 6A). When we

342 focused on specific bacterial groups, a similar response was generally found. Most
343 groups showed a stronger increase in the percentage of active bacteria from 13:00 h
344 on 20 February to 01:00 h on 21 February (the first night) than during the second
345 night. This was in agreement with the lower rates of ^{3}H-leucine incorporation
346 registered at 01:00 h on 22 February compared to the first peak at 05:00 h on 21
347 February (Fig. 2A). *Gammaproteobacteria, Roseobacter,* and SAR11 showed
348 night:day increases of 80%, 32%, and 93% during the first night, and 40%, 20%, and
349 55% during the second night, respectively. This night stimulation of the number of
350 active cells was more similar between both nights for *Bacteroidetes* (108% and 91%
351 increase, respectively), and *Synechococcus* did also show this nocturnal activation of
352 their heterotrophic activities, although conversely to the rest of the groups, its
353 stimulation was greater during the second night (57%) than during the first one (24%).

354 During the second cycle (26 February to 01 March) we could not find any
355 consistent diel pattern, but there was a general increase in the number of active cells
356 towards the end of the sampling period (Fig. 7). Only *Bacteroidetes,* which remained
357 constant during the whole cycle, did not show this significant increase in activity (Fig.
358 7E). Members of the *Gammaproteobacteria* cluster, instead, exhibited some diel
359 pattern with higher percentages of active cells at night than during the last sampled
360 day (from 18% at 13:00 h on 28 February to 53% at 01:00 h on 01 March, Fig. 7C).
361 Interestingly, whereas bulk ^{3}H-leucine incorporation did not significantly correlate
362 with prokaryote abundances (as measured by flow cytometry) when considering the
363 two cycles together, it did correlate nicely with the number of MAR- active
364 *Eubacteria* ($r = 0.83$, $p < 0.005$, $n = 10$).

365 Figure 8 shows the percentage contribution of each group to the total bacteria
366 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 increases in the fraction of active cells of most groups during the first cycle. Since 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 two days, 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 NW 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-day 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 (Chròst and Riemann 1994) and were undersampled with the small volumes used for bacterial activity assays and flow cytometry analyses. Unfortunately, since 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 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 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) had 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 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

440 coast, supporting the idea that under more oligotrophic conditions, the coupling of
441 phytoplankton and bacterial activities should be more discernible. Indeed, most
442 studies of diel variations in bacteria have suggested this link between primary
443 production and bacterial use of the released DOM, usually leading to increased
444 bacterial abundances or activity during daytime with maxima values in late afternoon
445 and minima at night (Fuhrman et al. 1985; Herndl and Malacic 1987).

446 Other authors have reported only weak or inconsistent diel patterns (Riemann
447 and Søndergaard 1984; Torreton and Dufour 1996) but very few have observed this
448 nocturnal stimulation of bacterial activity (Jeffrey et al. 1996; Shiah 1999; Kuipers et
449 al. 2000). Remarkably, in these latter studies, bacterial activity was measured only in
450 terms of deoxyribonucleic acid (DNA) synthesis (not as protein synthesis) and the
451 night increase was suggested as a mechanism of avoidance of UVR-damage to DNA
452 during the day. During our first cycle, however, the incorporation of ^{3}H-leucine
453 followed this trend of nocturnal increases too. This opposite pattern of protein
454 synthesis and primary production suggests that causes other than photosynthetic DOM
455 might be driving the changes in bacterial activity.

456 All in all, multiple potential explanations for the nocturnal increase (or diurnal
457 decrease) in heterotrophic bacterial activity could be invoked: 1) experimental
458 artifacts such a radioisotope dilution; 2) UVR-inflicted photoinhibition or DNA
459 damage; 3) lagged response to photosynthetic DOM release; 4) light-enhanced
460 competition of phytoplankton for nutrient and DOM uptake; 5) rapid response to
461 grazing-derived DOM; 6) diel changes in free-living and attached bacteria; 7) diel
462 changes in bacterial growth and mortality. Hereafter we will examine these potential
463 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 ^{3}H-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 (HNA)-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, VanWambeke et al. (2009) observed that increases in the daily UVB to UVA 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 since 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 ^{3}H-leucine incorporation rates (C. Ruiz-González unpubl.).

Lagged response of bacteria to photosynthetic DOM release. Shiah (1999) also found higher ^{3}H-thymidine incorporation rates at night but in UVR-devoid experiments. He associated this nocturnal increase in bacterial activity indirectly to 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. 2008*b*), a late response of bacteria to photosyntate would still be possible. Since 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 two and a half years (C. Ruiz-González unpubl.), the consistent stimulation observed of bulk ^{3}H-leucine incorporation rates under an artificial light source (without UVR) was not due to neither eukaryotic algae nor cyanobacteria, but to *Gammaproteobacteria* (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 since 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-day 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 due to 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,

537 which dominate oligotrophic environments such as the Blanes Bay and graze on small
538 phytoplankton or bacteria, play a major role in the release of DOM rich in different
539 nutrients (Nagata and Kirchman 1991; Strom et al. 1997). Since carbon is the
540 principal limiting element for bacteria in winter Blanes Bay waters (Pinhassi et al.
541 2006), it is plausible that grazing-derived labile DOM explained the strong
542 synchronization between micrograzing on the abundant PPeuk and bacterial activity
543 during the first week. After the stormy unsampled days, though, *Synechococcus*
544 became dominant among picophytoplankton and the abundance of HNF decreased, so
545 these community composition changes could have altered the amount and quality of
546 grazing-derived DOM, at least at the beginning of the cycle.

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

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

559 *Diel changes in bacterial growth and mortality rates.* The observed diel cycles in
560 ^{3}H-leucine incorporation rates might not translate into real bacterial production cycles

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

572 *Diurnal variations in single-cell activities of dominant bacterial groups.* Several
573 studies have tested the variations in bulk bacterial activity at daily scales, but very few
574 have analyzed the changes within specific phylogenetic groups. Pernthaler and
575 Pernthaler (2005) studied cell proliferation of three bacterial taxa (*Roseobacter,*
576 SAR86, and NOR5) looking for diurnal patterns of DNA synthesis within them, but
577 they did not find any clear diel trends. Using flow cytometric cell sorting, Mary et al.
578 (2008*a*) found obvious diurnal rhythms in ^{3}H-leucine and ^{35}S-methionine uptake by
579 *Prochlorococcus* cells in the tropical Atlantic, with maximum values at dusk and
580 minimum at midday, and Chen et al. (1991) described a circadian clock regulating
581 amino acid uptake in freshwater *Synechococcus,* which presented the highest uptake
582 rates during the light period. To our knowledge, however, this is the first report
583 showing clearly defined diel patterns in the activities of dominant hetero- and
584 mixotrophic bacterial groups.

585 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). Whereas 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 less 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 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 non-growth, which would allow them to rapidly initiate growth at changing conditions (Eilers et al. 2000).

Remarkably, besides the fact that the studied groups may harbour 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

611 DOM supply, the released compounds might have been suitable for a fraction of cells
612 within all groups. Instead, if bacteria had been more tightly dependant on
613 phytoplankton DOM, we might have found some favoured taxa, such as some
614 *Roseobacter* closely related to blooms of particular phytoplankton species and thus to
615 short-term fluctuations of primary production (Pinhassi et al. 2004). Similarly, if solar
616 radiation had played a significant direct role, we could have found some differential
617 activation or inhibition of bacteria throughout the daylight hours depending on each
618 group's sensitivity to sunlight (Alonso-Sáez et al. 2006) or their potential capabilities
619 to derive energy from light (Béjà et al. 2000; Kolber et al. 2000).

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

634 Dark standard measurements of bulk and single-cell bacterial activities may
635 underestimate bacterial production during the day or neglect any potential UVR-

636 driven effect, so future experiments under real light conditions will be needed in order
637 to assess the actual magnitude of these diel variations. Moreover, since the relative
638 activities of bacterial groups from Blanes Bay vary between winter and summer
639 (Alonso-Sáez and Gasol 2007), as does the availability of DOM (Alonso-Sáez et al.
640 2008*b*), we cannot discard that completely different bacterial cycles occur at different
641 times of the year, as shown by Ghiglione et al. (2007) between spring and summer
642 samples. Further research on the relative contribution of the key players in DOM
643 supply and the diel activities of different bacterial taxa will be required for better
644 comprehension of their contribution to daily variations in carbon fluxes.

645

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Tables

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

	1st cycle	2nd cycle
Temperature (ºC)	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 leu 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*

Table 2. Bacterial assemblage structure described as percentage of hybridized cells with specific probes by CARD-FISH in five samples of (A) the first cycle and (B) the second cycle. Eub338-II-III (*Eubacteria*), CF319a (*Bacteroidetes)*, Gam42a (*Gammaproteobacteria*), Ros537 (*Roseobacter*), SAR11-441R (SAR11 clade), and Syn405 (*Synechococcus*). SAR11and *Roseobacter* are subgroups of *Alphaproteobacteria*. Values are expressed as percentage of the total DAPI counts (± standard error of 10-40 fields). Letters refers 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.

A		Fraction (%) of total DAPI counts detected with CARD-FISH probe					
Date	Time	Eub338-II-III	CF319a	Gam42a	Ros537	Sar11-441R	Syn405
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}
	Avg ± SE	84 ± 1	20 ± 1	6 ± 1	8 ± 1	39 ± 2	< 1

B		Fraction (%) of total DAPI counts detected with CARD-FISH probe					
Date	Time	Eub338-II-III	CF319a	Gam42a	Ros537	Sar11-441R	Syn405
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}
	Avg ± SE	83 ± 2	17 ± 1	5 ± 1	5 ± 0.4	35 ± 1	1.3 ± 0.2

Figure legends

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 centre 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 4 replicate measurements. Arrows indicate when incubations for MAR-CARD-FISH analyses were taken. Solid bars on top axis represent dark periods.

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.

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 heterotrophic nanoflagellates (HNF) on photosynthetic picoeukaryotes (PPeuk) 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 (C) cycles. Solid bars on top axis represent dark periods. HNF during the second cycle were only counted in six different occasions.

Fig. 6. Percentage of positively hybridized cells taking up ^{3}H-leucine as measured by MAR-CARD-FISH during the first cycle (20 to 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 due to bad weather conditions.

Fig. 7. Percentage of positively hybridized cells taking up ^{3}H-leucine as measured by MAR-CARD-FISH during the second week (26 February to 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 ^{3}H–leucine incorporation rates for comparison as displayed in Fig. 2A. Solid bars on top axis represent dark periods.

Fig. 8. Contributions of various phylogenetic groups (SAR11, *Roseobacter* [Ros], *Gammaproteobacteria* [Gam], *Bacteroidetes* [Bcdt], and *Synechococcus* [Syn]) to the number of cells active in ^{3}H-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).