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An annual cycle of dimethylsulfoniopropionate-sulfur and leucine assimilating bacterioplankton in the coastal NW Mediterranean

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Summary

The contribution of major phylogenetic groups to heterotrophic bacteria assimilating sulfur from dissolved dimethylsulfoniopropionate (DMSP) and assimilating leucine was analysed in surface seawaters from Blanes Bay (NW Mediterranean) over an annual study between March 2003 and April 2004. The percentage of bacteria assimilating DMSP-S showed a strong seasonal pattern, with a steady increase from winter (8 ± 5%) to summer (23 ± 3%). The same seasonal pattern was observed for the rate of DMSP-S assimilation. The annual average percentage of DMSP-S-assimilating bacteria (16 ± 8%) was lower than the corresponding percentage of leucine-assimilating cells (35 ± 16%), suggesting that not all bacteria synthesizing protein incorporated DMSP-S. Smaller differences between both percentages were recorded in summer. Members of the *Alphaproteobacteria* (*Roseobacter* and SAR11) and *Gammaproteobacteria* groups accounted for most of bacterial DMSP-S-assimilating cells over the year. All major bacterial groups showed an increase of the percentage of cells assimilating DMSP-S during summer, and contributed to the increase of the DMSP-S assimilation rate in this period. In these primarily P-limited waters, enrichment with P + DMSP resulted in a stimulation of bacterial heterotrophic production comparable to, or higher than, that with P + glucose in summer, while during the rest of the year P + glucose induced a stronger response. This suggested that DMSP was more important a S and C source for bacteria in the warm stratified season. Overall, our results suggest that DMSP-S assimilation is controlled by the contribution of DMSP to S (and C) sources rather than by the phylogenetic composition of the bacterioplankton.

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Introduction

Microorganisms control biogeochemical cycles in the sea. One of the current challenges in marine microbial ecology consists of identifying what phylogenetic groups mediate particular key biogeochemical processes in the ocean, to increase our understanding of how these processes are regulated over various temporal and spatial scales. For instance, if relatively few studies have addressed the seasonal variability of bacterial diversity in marine waters (Pinhassi and Hagström, 2000; Eilers *et al.*, 2001; Schauer *et al.*, 2003; Brown *et al.*, 2005; Ghiglione *et al.*, 2005; Morris *et al.*, 2005; Mary *et al.*, 2006; Alonso-Sáez *et al.*, 2007), to our knowledge only one has analysed the seasonality in the processing of different components of the dissolved organic matter (DOM) pool (Alonso-Sáez and Gasol, 2007).

Novel methods to link bacterial diversity and biogeochemical activity include the search and affiliation of specific genes in metagenome analyses (e.g. Béjà *et al.*, 2002) and the single cell biogeochemistry analysis techniques. Among the latter, the techniques that have been applied with greatest success involve the use of radioisotope substrates: MARFISH, a combination of microautoradiography (MAR) and fluorescence *in situ* hybridization of RNA (FISH) (Ouverney and Furhman, 1999; Lee *et al.*, 1999; Cottrell and Kirchman, 2000), and flow cytometry cell sorting (e.g. Servais *et al.*, 2003). A critical issue of these techniques is the choice of model molecules from the complex mixture of the DOM pool, which are representative of major matter fluxes in the sea.

Dimethylsulfoniopropionate (DMSP) is an ubiquitous compound in the upper ocean, where it represents a

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major pool of reduced sulfur (Bates *et al.*, 1994). This compatible solute is produced by a number of phytoplankton taxa, with suggested roles as osmoregulator, antioxidant and overflow of reducing power (Malin and Kirst, 1997; Stefels, 2000; Sunda *et al.*, 2002). Dimethylsulfoniopropionate is a significant component of the organic matter that flows through the microbial food-web. During blooms of DMSP-producing phytoplankton, it can represent ≥50% of sulfur and ~ 10% of carbon fluxes through various trophic levels, namely phytoplankton, bacterioplankton and microzooplankton (Burkill *et al.*, 2002; Simó *et al.*, 2002). In addition, DMSP is the biogenic precursor of dimethylsulfide (DMS). Dimethylsulfide is a climatically active gas released by the oceans to the atmosphere where its oxidation products (sulfate and sulfonate aerosols) increase cloud albedo and thus play an opposite effect to that of greenhouse gases (Charlson *et al.*, 1987). The existence of convenient methods to measure trace aqueous concentrations of DMSP confers to this substance an advantage over other DOM components. The study of DMSP transformations can serve as a model to better understand the microbial cycling of labile DOM and possible linkages between marine microbiota and atmospheric composition.

Dimethylsulfoniopropionate is released into the dissolved pool through algal senescence, viral lysis, and grazing by micro- and mesozooplankton (Simó, 2001). Dissolved DMSP (DMSPd) is mainly metabolized by heterotrophic bacteria and partly by non-DMSP-producing phytoplankton (Vila-Costa *et al.*, 2006). Bacteria degrade DMSP through two pathways: (i) cleavage to give rise to DMS production, or (ii) demethylation. This latter route can be followed by a second demethylation, resulting in non-volatile sulfur compounds (Visscher *et al.*, 1992), or by a demethiolation that leads to methanethiol (MeSH) formation, which goes primarily into bacterial macromolecules (proteins) (Kiene *et al.*, 1999; Kiene and Linn, 2000a). Thus, bacterioplankton are a key component that largely mediates the switch between DMS–producing and non-DMS-producing DMSP transformations.

Non-DMS-producing DMSP transformations are the dominant fate of bacterial DMSPd degradation in most studied systems (Kiene *et al.*, 2000). Because a portion of total DMSPd demethylation ends up as assimilated sulfur, the identification of the factors that drive DMSP assimilation is of key importance to understand the biotic control of DMS production. Recent studies have suggested that, although DMSP-S assimilation is a widespread feature among groups of bacterioplankton (Malmstrom *et al.*, 2004a; Vila *et al.*, 2004). not all bacteria uniformly assimilate DMSPd (Malmstrom *et al.*, 2004a,b). Probably, taxonomic composition is one of the factors influencing the extent of DMSPd assimilation by bacterioplankton assemblages and the preference of bacteria for either the cleavage or the demethylation route, thus influencing the DMS production yield. This hypothesis needs to be verified with studies over year-round time scales. Contrasting with the seasonality of DMS and DMSP concentrations, which has been described in a few different sites (e.g. Leck *et al.*, 1990; Turner *et al.*, 1996; Dacey *et al.*, 1998; Simó and Pedrós-Alió, 1999; Uher *et al.*, 2000; M. Vila-Costa *et al.*, submitted), the seasonality of the microorganisms involved has not been attempted so far.

Members of two clades within the *Alphaproteobacteria* group, *Roseobacter* and SAR11, when abundant, seem to have a prominent yet not unique role in DMSP-S assimilation in the sea (Malmstrom *et al.*, 2004a,b; Vila *et al.*, 2004). During blooms of DMSP-producing phytoplankton, indeed, the associated bacterial assemblages were dominated by *Roseobacter* taxa, and their abundance was positively correlated with DMSP concentration or DMSP turnover (González *et al.*, 2000; Zubkov *et al.*, 2002). This high capability for DMSP metabolism was also observed in isolates of *Roseobacter* (González *et al.*, 1999), and, very recently, the gene encoding for DMSP demethylation has been found in the genome of two representatives of *Roseobacter* and SAR11 taxa (Howard *et al.*, 2006).

In Blanes Bay (NW Mediterranean) the seasonal succession of bacterial groups has been described in two independent studies (Schauer *et al.*, 2003; Alonso-Sáez *et al.*, 2007). In 2003, abundances of the SAR11 and *Roseobacter* clades showed opposite patterns: SAR11 were abundant in summer when nutrient limitation is pronounced while *Roseobacter* abundances followed chlorophyll a (Chla) concentrations more closely (Alonso-Sáez *et al.*, 2007). That same year, the dynamics of dimethylated sulfur compounds were analysed. Dimethylsulfide concentration showed a strong seasonal pattern, with maximum concentrations in summer, whereas DMSP concentration peaked in March Bacterial DMSP consumption rates were higher in summer, suggesting that the summer bacterial assemblage had a higher capability for DMSP use (M. Vila-Costa *et al.*, submitted).

In the time series study from early 2003 to early 2004, we used an improved protocol of the MARFISH method (see Alonso and Pernthaler, 2005) to address the link between the succession of bacterial groups and DMSP dynamics. The objectives of this work were: (i) to determine the seasonality of bacterial DMSP-S assimilation, (ii) to identify the main DMSP-S-assimilating bacteria using phylogenetic probes for the main broad groups, namely *Alphaproteobacteria*, *Gammaproteobacteria*, Bacteroidetes (or CFB: Cytophaga-Flavobacterium-Bacteroides), and specifically for *Roseobacter* and SAR11 phyla and (iii) to analyse the seasonal variation of the contribution of each of these groups to DMSP-S assimilation. We compared DMSP-S-assimilation results

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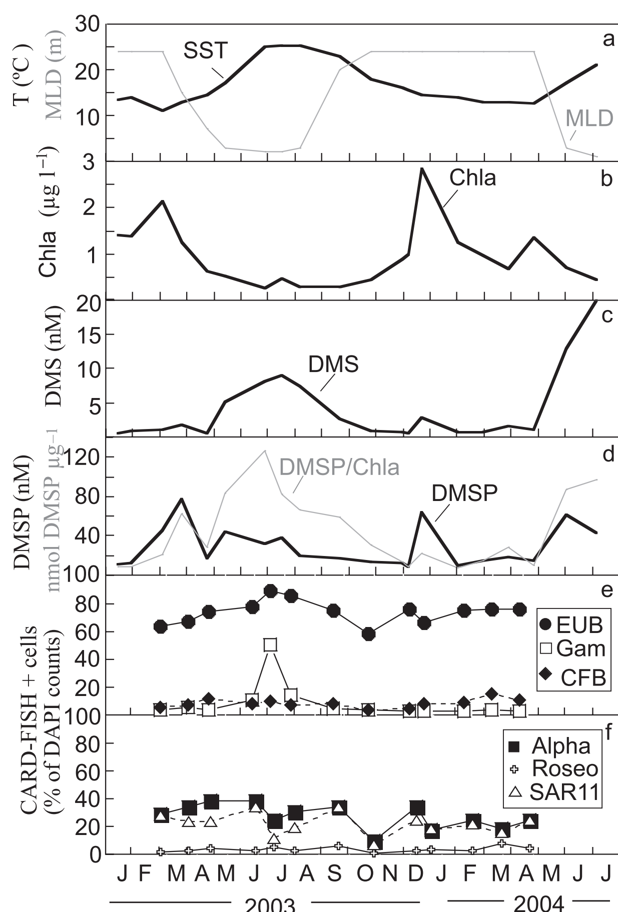

Fig. 1. Monthly series of (a) sea-surface temperature (SST) and mixing layer depth (MLD, defined as the depth at which temperature was 0.2°C lower than that at surface), (b) chlorophyll *a* (Chla) concentrations, (c) DMS concentrations, and (d) DMSPt and Chla-normalized DMSPt concentrations (data from Vila-Costa *et al.*, submitted). Percentages (with respect to total DAPI counts) of Eubacteria (EUB) and the main broad groups of marine bacteria, namely *Alphaproteobacteria* (Alpha), *Gammaproteobacteria* (Gam), and Bacteroidetes (CFB), as detected by CARDFISH are shown in (e). Within the Alpha group, percentages of the *Roseobacter* (Roseo) and SAR11 clades are also shown (f).

with assimilation of leucine, which is used as a universal substrate by heterotrophic bacteria.

Results

Chlorophyll a*, DMSP and bacterioplankton production and composition*

The strong seasonality of the air-to-sea heat flux in Blanes Bay resulted in a marked seasonality of the sea-surface temperature and a shallow stratification (2–3 m of mixing depth) in the summer period (from May to August) (Fig. 1a). Concentrations of Chla in winter (December to February) averaged 1.7 ± 0.7 μg l^{-1}, and decreased to 0.4 ± 0.1 μg l^{-1} in summer (Fig. 1b).

Dimethysulfoniopropionate concentrations did not follow Chla variation over the year (Fig. 1b and d). However, a strong seasonality was observed for the ratio DMSP/Chla, reaching maximum values in the summer months (Fig. 1d). This higher DMSP content per unit of Chla in summer has been regarded as an indication of the dominance of DMSP-producing phytoplankton acclimated to high irradiances (M. Vila-Costa *et al.*, submitted). This same seasonal pattern was observed for DMS concentrations (Fig. 1c).

The seasonality of the bacterial assemblage composition was determined by FISH with horseradish peroxidase-labelled oligonucleotide probes and catalysed reporter deposition (CARDFISH), and described by Alonso-Sáez and colleagues (2007). The CARDFISH numbers obtained from our MARFISH filters after 18 h of incubation were not statistically different (*t*-test, $P > 0.05$) than the *in situ* CARDFISH numbers determined by Alonso-Sáez and colleagues (2007). The averages between both determinations are shown in Fig. 1e and f. *Alphaproteobacteria* dominated total prokaryotic abundance over the seasonal cycle. This group consisted mainly of SAR11, whereas *Roseobacter* occurred at higher abundances coinciding with Chla concentration peaks but never reached values higher than 8% of total prokaryotic cells. The second most abundant group was Bacteroidetes, with constant numbers throughout the year (~11%). Finally, the third most abundant group was *Gammaproteobacteria*, the proportion of which correlated significantly with sea-surface temperature (Spearman $r = 0.68$, $P < 0.05$), with abundances ranging from 1 to 8% (Fig. 1e).

The sampling in July 2003 occurred during an unusual event that seems to deviate from the seasonal patterns observed in precedent years. Bacterial production was exceptionally high and the bacterial community was clearly dominated (30%) by only one phylotype: *Glacieocola* sp., Alteromonadaceae, *Gammaproteobacteria* (Alonso-Sáez *et al.*, 2007).

Seasonality of DMSP-S assimilation rate and proportion of total bacteria assimilating DMSP-S

The rate of DMSP-S assimilation showed a strong seasonal pattern with significantly higher values ($P < 0.05$) in summer compared with the rest of the year (Fig. 2a). From January to April, assimilation rates averaged 0.6 ± 0.1 nM DMSP/d, representing 8% of bacterial DMSPd consumption. Coinciding with the onset of water column stratification in May, assimilation rates increased and remained high (6.2 ± 2 nM day^{-1}) over the summer, accounting for 33% of bacterial DMSPd consumption rates. In September, when the stratification broke up, values decreased again and settled to ~0.5 nM day^{-1} until March 2004 (Fig. 2a).

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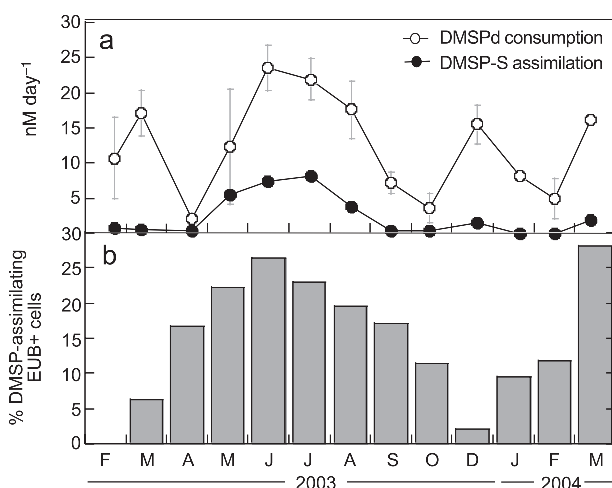

Fig. 2. Monthly series of bacterial DMSP consumption and DMSP-S assimilation rates (a) and percentage of DMSP-S-assimilating Eubacteria (b).

In general, the percentage of EUB+ cells (cells that hybridized with the universal EUB probe EUBI–III) that assimilated DMSP-S (i.e. 100 × (EUB + DMSP+)/(EUB+), or %EUB + DMSP+, Fig. 2b showed a similar seasonal pattern (Spearman correlation $r = 0.55$, $P < 0.05$) to that of the DMSP-S assimilation rate (Fig. 2a). From March to June, the percentage of EUB + DMSP+ increased from 6% to 26% and then decreased until December 2003 when they increased again. This latter increase was comparatively higher than that observed for the DMSP-S assimilation rate. On an annual average, the percentage of EUB + DMSP+ cells was 16 ± 8%.

The percentage of EUB+ cells that assimilated leucine (i.e. %EUB + lEU+) was equal to, or higher than EUB+ cells assimilating DMSP (%EUB + lEU+, Fig. 3a). In the summer period, %EUB + lEU+ cells showed the same trend as %EUB + DMSP+ cells, peaking in June and July 2003. However, the steady increase of %EUB + LEU+ cells from September to January was not observed for %EUB + DMSP+ cells, as the latter decreased (Fig. 3a). On an annual average, the percentage of EUB + lEU+ cells was 35 ± 16%.

Seasonality of DMSP-S and leucine-assimilating bacteria within the phylogenetic groups

The proportion of *Alphaproteobacteria* actively assimilating DMSP-S (%ALPHA + DMSP+) did not follow a clear seasonal pattern but similar values over the year except in March 2004 (annual average excluding this month's value: 12 ± 8%, Fig. 3b). Much higher percentages were recorded for %ALPHA + LEU+ cells (annual average: 53 ± 18%, Fig. 3b). These values decreased dramatically in September 2003, a month characterized by the highest phosphorus limitation of bacterial production (Pinhassi *et al.*, 2006). Among subgroups of *Alphaproteobacteria*, the percentages of DMSP-S-assimilating *Roseobacter* cells (%ROSEO + DMSP+) increased in May 2003 and stayed at similar or even higher values than those assimilating leucine (%ROSEO + LEU+) over the summer (Fig. 3c). During the rest of the year, percentages of ROSEO + DMSP+ were low (8 ± 7%). In contrast, the fraction of ROSEO + LEU + was consistently high over the year (56 ± 20%). Due to methodological problems, the percentage of SAR11 cells assimilating DMSP-S could only be measured in 7 months (Fig. 3d). In 5 out of these 7 months, values of %SAR11 + DMSP+ (7–14%) were lower than those of %SAR11 + lEU+ (15–60%). Only in May and June 2003 %SAR11 + DMSP+ cells increased

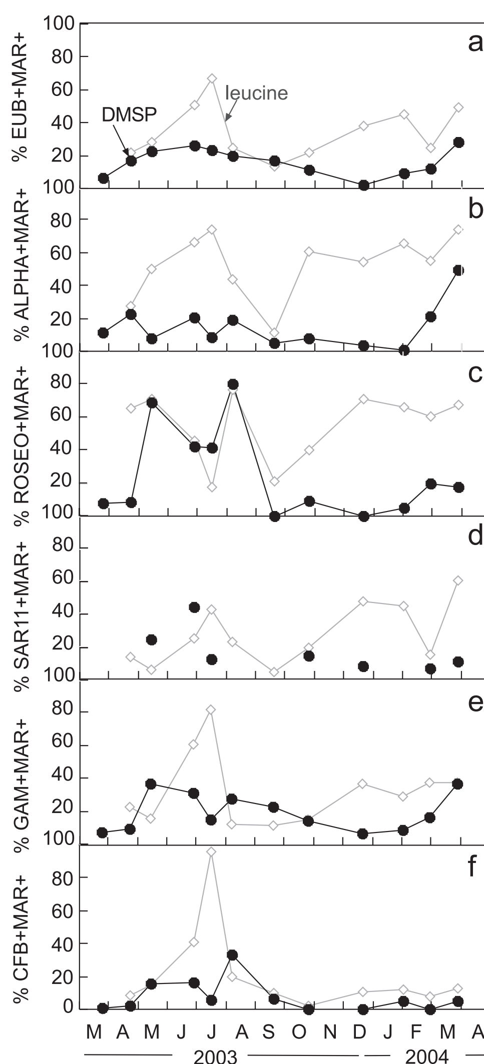

Fig. 3. Monthly series of the percentage of DMSP-S-assimilating (filled circles) and leucine-assimilating cells (open circles) within each bacterial group: Eubacteria (EUB, a), *Alphaproteobacteria* (ALPHA, b), *Roseobacter* (ROSEO, c), SAR11 (d), *Gammaproteobacteria* (GAM, e), and Bacteroidetes (CFB, f).

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to 24% and 44%, respectively, and were higher than %SAR11 + lEU+ cells (6% and 25%).

The fraction of *Gammaproteobacteria* actively assimilating DMSP-S (%GAMMA + DMSP+) presented a marked seasonality with consistently higher numbers (28 ± 9%) during summer and September, a decrease towards December, and a further increase into March 2004 (Fig. 3e). The proportion of GAMMA + LEU+ peaked in July 2003, coincident with the dominance of a single phylotype of *Gammaproteobacteria* (Alonso-Sáez *et al.*, 2007). A similar trend was observed for Bacteroidetes (Fig. 3f). The percentages of CFB + DMSP+ cells (18 ± 11%) and CFB + lEU+ cells (43 ± 36%) in summer were clearly higher than those in winter (2 ± 2% and 7 ± 4% respectively).

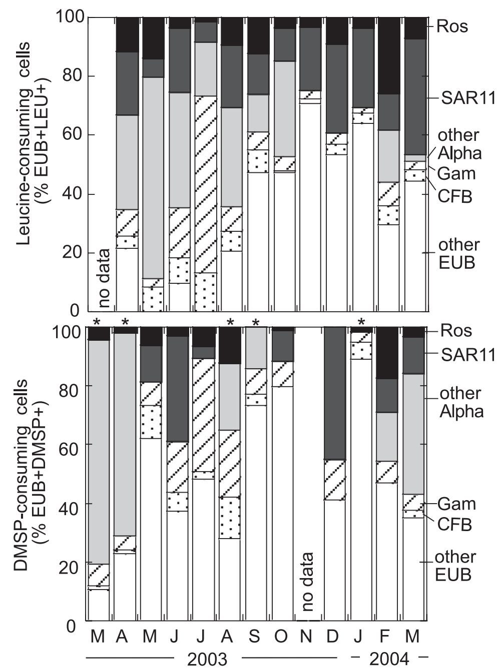

Fig. 4. Monthly series of the contribution of each phylogenetic group [*Alphaproteobacteria* (Alpha), *Gammaproteobacteria* (Gam), Bacteroidetes (CFB)] to total substrate assimilation (leucine, top; DMSP, bottom). The Alpha group is divided among *Roseobacter* (Ros), SAR11, and unidentified Alpha (other Alpha). *Months when microautoradiographs of SAR11 could not be performed.

Seasonal variation in DMSP-S and leucine assimilation among bacterial groups

The dominance of *Alphaproteobacteria* among DMSP-S and leucine assimilating cells was a common feature all over the year, except in July 2003 when the numbers of cells assimilating both substrates were dominated by *Gammaproteobacteria* (Fig. 4). In the summer samples, when hybridizations of SAR11 were performed, *Roseobacter* and SAR11 explained almost all of the Alphaproteobacterial cells assimilating DMSP-S (Fig. 5). On an annual basis, members of the SAR11 clade were responsible for most of DMSP-S-assimilating Alphaproteobacterial cells (62 ± 33%) while *Roseobacter* accounted for

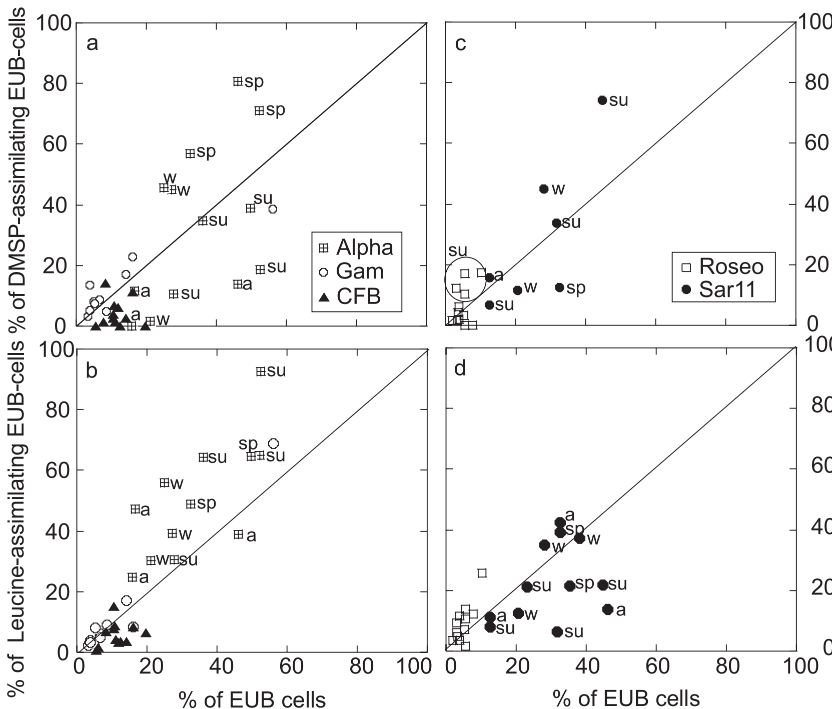

Fig. 5. Contribution of each bacterial phylogenetic group (*Alphaproteobacteria* (Alpha), *Gammaproteobacteria* (Gam), Bacteroidetes (CFB), *Roseobacter* (Roseo), and SAR11) to the number of cells assimilating DMSP-S (a, c) or leucine (b, d) versus the relative abundance of the group (calculated with respect to Eubacteria (EUB) cells). Data points on the 1:1 line indicate contribution to cells assimilating substrate corresponding to their contribution to abundance. Labels by the data points indicate the sampling season: spring (sp., March–April), summer (su, May–August), autumn (a, September–November), winter (w, December–February).

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25 ± 25%. However, a clear seasonal pattern could not be determined. Similarly, SAR11 accounted for the largest fraction of *Alphaproteobacteria* assimilating leucine over the year, with higher contributions in the winter period (63 ± 35%) than in the summer period (25 ± 12%). The contributions of the *Roseobacter* group were lower in summer (10 ± 5%) than in winter (27 ± 18%).

A seasonal pattern was observed in the contribution of *Gammaproteobacteria* to DMSP-S-assimilating cells, with higher contributions in summer (17 ± 15%) and lower values over the rest of the year (7 ± 3%). The highest Gammaproteobacterial contributions to leucine-assimilating cells were observed in June (17%) and July (68%), with values averaging ~5 ± 2% during the rest of the year.

Although Bacteroidetes was the second most abundant group, it accounted for the smallest proportion of the cells assimilating both substrates on an annual basis (4 ± 1% and 6 ± 1% of DMSP-S and leucine-assimilating cells respectively). However, Bacteroidetes showed a slightly higher contribution to leucine assimilating cells in summer (10 ± 4%), which was only observed in August in DMSP-S assimilation.

On annual average, ~40% (± 24) of the cells that had assimilated DMSP-S or leucine could not be attributed a phylogenetic identity with the here used probes for large phylogenetic groups. In general, this fraction was higher in winter and lower in summer for both compounds.

Contribution of each group to cells assimilating substrate relative to their abundances

The contribution of each group to the total number of cells assimilating a substrate was calculated from the fraction of active cells in the group and its abundance (with respect to total EUB+ cells) relative to the percentage of total bacteria (EUB+ cells) active at substrate assimilation. For example, the contribution of *Alphaproteobacteria* to the total number of cells assimilating DMSP-S was calculated as:

$$\{[(\text{ALPHA}+\text{DMSP+})/(\text{ALPHA+})]\times[(\text{ALPHA+})/(\text{EUB+})]\}/[(\text{EUB}+\text{DMSP+})/(\text{EUB+})]$$

where (ALPHA + DMSP+)/(ALPHA+) is the percentage of DMSP-S-assimilating *Alphaproteobacteria* cells in the *Alphaproteobacteria* group, i.e. %ALPHA + DMSP+ (ALPHA+)/(EUB+) is the abundance of *Alphaproteobacteria* with respect to EUB-hybridized cells; and (EUB + DMSP+)/(EUB+) is the percentage of DMSP-S-assimilating EUB-hybridized cells among total EUB-hybridized cells, i.e. %EUB + DMSP + 0. Simplifying, the equation becomes:

$$(\text{ALPHA}+\text{DMSP+})/(\text{EUB}+\text{DMSP+})$$

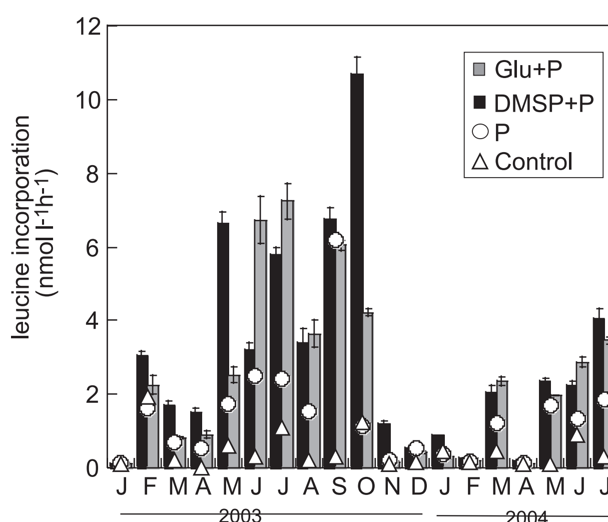

Fig. 6. Monthly series of bacterial production responses to phosphate and organic carbon enrichments. Bacterial production is measured as leucine incorporation rates in samples incubated for 24 h following enrichment with P (0.6 μM Na_2HPO_4), P plus glucose, or P plus DMSP (24 μM C + 0.6 μM Na_2HPO_4). No-addition controls are shown as a reference.

that is, the number of DMSP-S-assimilating *Alphaproteobacteria* divided by the number of DMSP-S-assimilating, EUB-hybridized bacteria.

The comparison of these group contributions to total DMSP-S- and leucine-assimilating cells with their contribution to bacterial abundances [in our example: (ALPHA+)/(EUB+)] is shown in Fig. 5. For *Gammaproteobacteria*, most data points were on or near the 1:1 line, i.e. their contribution to the cells assimilating both substrates corresponding to their relative abundance (Fig. 5a and b). The *Alphaproteobacteria* were underrepresented among DMSP-S-assimilating cells in summer and overrepresented in spring (Fig. 5a), while they were generally overrepresented among leucine-assimilating cells (Fig. 5b). Within this group, *Roseobacter* was overrepresented for DMSP-S in summer and for leucine over most of the year (Fig. 5c and d), whereas SAR11 exhibited a variable representation among DMSP-S-assimilating cells and were generally underrepresented among leucine-assimilating cells (Fig. 5c and d). Fewer CFB cells than their relative abundance assimilated either substrate over most of the year (Fig. 5a and b).

Short-term nutrient limitation bioassays

Enrichment with P substantially increased bacterial heterotrophic production (leucine incorporation), as compared with controls without enrichment, on 12 sampling occasions (Fig. 6). Addition of glucose in combination with P caused increases in bacterial heterotrophic production (leucine incorporation) that, in 14 out of 19 monthly

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experiments, were significantly higher than those after addition of P only. This suggests that over most of the year a secondary C limitation occurred associated with a strong and consistent P limitation of bacterial growth. In 10 experiments, addition of DMSP + P stimulated bacterial production over that with P only, particularly from April to October 2003 and from March to July 2004 (Fig. 6). That is, DMSP was an efficient C source during spring-summer periods. Interestingly, in three experiments (June and July 2003, and June 2004) combined additions of DMSP + P caused significantly higher bacterial heterotrophic production than combined additions of glucose + P.

Discussion

We aimed at describing the seasonality of the number and identity of major groups of DMSP-assimilating bacteria in order to assess the importance of bacterial assemblage composition in controlling the fate of DMSP in the pelagic marine environment. During the summer period bacteria not only significantly increased their rate of DMSP-S assimilation by a factor of 15 with respect to winter values (Fig. 2a) but also increased the percentage of DMSP-S-assimilating cells (Fig. 2b), and the two variables were well correlated. The seasonal pattern observed for the ratio DMSP : Chla (Fig. 1d) suggests that the succession of phytoplankton assemblages towards higher-DMSP producers in summer (M. Vila-Costa *et al.*, submitted) could have increased the contribution of DMSP to the fluxes of reduced S compounds, thus increasing the role of DMSP as a S source for bacteria. In order to determine whether the summer bacterial assemblage was more suited to use DMSP as a S source, we used the MAR-CARD-FISH technique.

DMSP-S was assimilated by members of the three main groups: *Alphaproteobacteria*, *Gammaproteobacteria* and Bacteroidetes. This provides further evidence that the capacity to take up and incorporate DMSP-S into biomass is widespread among marine heterotrophic bacterioplankton, in agreement with the results of previous studies (Malmstrom *et al.*, 2004a; Vila *et al.*, 2004). Differences in the contribution of each group also agree with the previous studies: *Alphaproteobacteria* dominated DMSP-S assimilation over the year, as was observed in the Gulf of Maine and the Sargasso Sea (Malmstrom *et al.*, 2004a,b) and at the same sampling site (Blanes Bay) in January and February 2003 (Vila *et al.*, 2004). This group also dominated leucine incorporation over the year, in support of the observation that *Alphaproteobacteria* preferentially take up low-molecular-weight compounds (LMW) (Cottrell and Kirchman, 2000). *Gammaproteobacteria* was the second main contributor to DMSP assimilation, while Bacteroidetes had a minor contribution.

A remarkable finding of this work is that all tested groups increased their percentages of cells assimilating DMSP-S in the summer period, contrasting with the seasonality of leucine-assimilating cells, which showed high percentages also in winter. Thus, the increase of DMSP-S assimilation in summer cannot be attributed exclusively to one group. The level of resolution of the CARDFISH probes used provided no information about potential succession patterns within the groups that might be related to specific affinity to DMSP-S assimilation. An exhaustive characterization of the bacterial composition in the same Blanes Bay series using a combination of molecular techniques (Alonso-Sáez *et al.*, 2007) revealed a high diversity within some groups (such as SAR11) but did not show any clear seasonal succession pattern in any group. Thus, it seems that factors other than bacterial assemblage composition were causing the increase of DMSP-S assimilation in summer.

The use of the SAR11 and *Roseobacter* probes is justified because these two abundant subgroups of marine *Alphaproteobacteria* have shown a high capability for DMSP transformations, including assimilation (Moran *et al.*, 2003; Malmstrom *et al.*, 2004b). Interestingly, the gene responsible for DMSP demethylation has recently been identified in cultured representatives of both clades (Howard *et al.*, 2006). In samples from the Gulf of Mexico and Sargasso Sea collected in April, Malmstrom and colleagues (2004a) observed that SAR11 accounted for 31–47% of total DMSP-S assimilation. We obtained similar high values only in the summer samples, whereas over the rest of the year SAR11 contributed ~12% of total DMSP-S assimilation. This finding seems not to be related to the degree of oligotrophy of the sampled seawater. Other studies in different trophic regions indicate that the contribution of SAR11 to the fluxes of monomers is substantial because of their high abundances rather than their average specific activities, that are relatively low (< 30% of active cells; Elifantz *et al.*, 2005; Alonso and Pernthaler, 2006a; Alonso-Sáez and Gasol, 2007). Remarkably, in Blanes Bay, SAR11 presented a relatively low and seasonally constant percentage of cells active at the uptake of glucose, amino acids and ATP (Alonso-Sáez and Gasol, 2007).

Members of the *Roseobacter* group are frequently found associated with algal blooms (Buchan *et al.*, 2005). In blooms of DMSP producers, such as the coccolithophore *Emiliania huxleyi*, *Roseobacter* abundances were positively correlated with DMSP concentrations (González *et al.*, 2000) or DMSP consumption rates (Zubkov *et al.*, 2002), thus reinforcing their prominent role as DMSP consumers that had been observed with isolates (González *et al.*, 1999). In Blanes Bay, the phytoplankton succession to high DMSP producers in summer was not followed by an increase in *Roseobacter*

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abundance, which rather matched total Chla concentrations (Alonso-Sáez *et al.*, 2007). Despite the lack of marked and consistent patterns in the seasonality of *Roseobacter* phylotypes as seen by DGGE and clone libraries in this same study (Alonso-Sáez *et al.*, 2007), it is interesting to note that the increase in the percentage of DMSP-S-assimilating *Roseobacter* cells in summer coincided with the presence of a DGGE band that had the closest match to another DGGE band found in a previous microcosm nutrient-enrichment study where 87% of *Roseobacter* cells assimilated DMSP-S (Vila *et al.*, 2004; Pinhassi *et al.*, 2005). However, due to the very low abundances of *Roseobacter* in summer, their contribution to DMSP-S assimilation during this period was not significantly increased.

Our results agree with the conclusions of previous MARFISH studies in that *Roseobacter* alone could not explain all DMSP-S assimilation (Malmstrom *et al.*, 2004a; Vila *et al.*, 2004). Further recent studies have observed the competitively advantageous capacity of this group to take up leucine at substantially lower concentrations than other groups (Alonso and Pernthaler, 2006b). Additionally, in Blanes Bay, *Roseobacter* showed a high percentage of cells active at the uptake of glucose, amino acids, and ATP over the year (Alonso-Sáez and Gasol, 2007). The consistent high percentages (56%) of *Roseobacter* cells assimilating leucine over the year compared with the average 23% of DMSP-assimilating *Roseobacter* cells reflect the high capacity of the group (as a whole) to take up LMW-DOM components in general rather than showing any preference and particular affinity for DMSP-S assimilation.

The increased proportion of *Roseobacter* and SAR11 cells assimilating DMSP-S during summer was not reflected in an increase of DMSP-S-assimilating *Alphaproteobacteria* cells. This could be explained because the probe used for *Alphaproteobacteria* (ALF968) has a mismatch against the SAR11 clones from Blanes Bay samples, as discussed by Alonso-Sáez and colleagues (2007). The ALF968 probe can thus underestimate the abundances of *Alphaproteobacteria* because some SAR11 cells do not get hybridized. This effect could be especially important during the summer period when the abundances of SAR11 cells were the highest.

Remarkably, *Gammaproteobacteria* contributed to total DMSP-S-assimilating cells proportionally to their abundance over the year. In general, this group contributed equally to DMSP-S assimilation and to leucine assimilation. This finding confirms the prominent role of *Gammaproteobacteria* in DMSP-S assimilation suggested by Vila and colleagues (2004). As a matter of fact, in blooms of DMSP-producing phytoplankton where the bacterial communities were dominated by *Alphaproteobacteria*, members of *Gammaproteobacteria* were also present at significant numbers (González *et al.*, 2000; Zubkov *et al.*, 2002). Moreover, Mou and colleagues (2005) found that 16S rRNA sequences belonging to the *Gammaproteobacteria* group contributed 14–21% of the abundance of DMSP-enriched communities in two of four samples collected from salt marsh waters. Similarly, enrichment of estuarine and salt marsh waters with DMSP resulted in the predominance (80%) of *Gammaproteobacteria* isolates (Ansede *et al.*, 2001). Thus, our results provide evidence for more important a role of *Gammaproteobacteria* in DMSP consumption in sea-surface waters than previously considered.

Bacteroidetes did not contribute significantly to DMSP-S assimilation in eight of the 12 monthly samples, and they were consistently underrepresented in the assimilation of DMSP-S relative to their abundance *in situ*. This result is in agreement with previous observations (Malmstrom *et al.*, 2004a; Vila *et al.*, 2004) and is consistent with the suggestion by Cottrell and Kirchman (2000) of a preference of this group for degrading high-molecular-weight compounds.

Similar values of total DMSP-S- and leucine-assimilating cells were observed only in four out of 11 sampling months (Fig. 3a). Over the entire period, the average of leucine-active cells was ~35 $\pm$ 16%, in the range of typical values obtained in other marine sites (Smith and del Giorgio, 2003). The average percentage of DMSP-S-assimilating cells was lower (15 $\pm$ 9%), and significantly lower than values observed in the Gulf of Maine and Sargasso Sea (48 $\pm$ 10%, Malmstrom *et al.*, 2004a). The assimilation of leucine can be taken as an indicator of cell activity and growth, because leucine is widely used for bacterial protein synthesis (Kirchman, 2001). Reduced percentages of DMSP-S- versus leucine-assimilating cells in all the major bacterial groups during much of the year seems to suggest that not all bacteria synthesizing protein assimilated DMSP-S. This finding contrasts with the good correlation found between DMSPd consumption rates and leucine incorporation rates found in temperate open-ocean and subtropical coastal waters (Kiene and Linn, 2000b; Malmstrom *et al.*, 2004a). It must be noted, however, that DMSPd consumption rates and both DMSP-S and leucine MARFISH are performed with trace concentrations of substrates ($<$ 0.5 nM) whereas leucine incorporation for bacterial production measurements is done with 20–40 nM of ^{3}H-leucine. Although sulfate is very abundant in seawater and bacteria have the capability to reduce it, so that they never get limited by this element, the use of available S in a reduced form would be energetically advantageous. Kiene and colleagues (1999) demonstrated the incorporation of the CH_3SH moiety from DMSP into S-amino acids, so that it is very plausible that bacterioplankton are well suited to use DMSP, when it is available, to save energy for protein

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synthesis. The excellent seasonal correlation we found between bacterial heterotrophic production (^{3}H-leucine incorporation) and ^{35}S-DMSP assimilation rates in this same study (M. Vila-Costa *et al.*, submitted) suggests that our determinations of the percentage of DMSP-S-assimilating cells with MAR might be underestimates if cells were not accumulating enough ^{35}S to be visually detected once their S demands were satisfied.

The results of the enrichment experiments highlight the role of DMSP as a source of S and C. The elevated bacterial production caused by combined additions of DMSP + P, relative to additions of P-only was observed during spring and summer, coinciding with an increase in the proportion of bacterioplankton that assimilated DMSP-S. This coincided with the period of increasing DMSP : Chla ratios, which can thus be proposed as a proxy for the quantitative role of DMSP as a supply of reduced S and C to bacterial S and C demands (M. Vila-Costa *et al.*, submitted). On several occasions in summer the stimulation of DMSP + P additions was as large as, or even larger than the stimulation caused by additions of glucose + P, indicating that DMSP is a highly labile organic compound for marine bacteria to use. This corroborates recent findings from the C-limited coastal Gulf of Mexico, where the addition of DMSP alone triggered as large an increase in bacterial leucine incorporation as glucose addition (Pinhassi *et al.*, 2005).

Conclusions

In Blanes Bay, both the DMSP-S assimilation rates and the percentage of DMSP-S-assimilating cells showed a strong seasonal pattern with maximum values in summer, concomitant with higher percentages of leucine-assimilating cells. Furthermore, summer was the period when additions of DMSP plus phosphate produced stronger stimulation of bacterial production. All this indicated that either DMSP-S was being assimilated by a very broad and diverse fraction of the bacterial assemblage or DMSP specialists, if occurring, were very abundant in summer. Our results confirm the widespread capacity of the main groups of heterotrophic bacteria to assimilate DMSP-S, although *Gammaproteobacteria* and, on occasions, *Alphaproteobacteria* contributed the most to the DMSP-S-assimilating cells relative to their abundance. With respect to leucine assimilation, *Alphaproteobacteria* generally were the main contributors to the number of assimilating cells, clearly above their contribution to total cell abundance. The seasonality observed in DMSP-S assimilation rates and number of DMSP-S-assimilating cells were not related to seasonal shifts in the abundances of major bacterial groups. Rather, DMSP-S was more assimilated when it contributed more to S sources for bacteria, independently of the coarse composition of the bacterial assemblage. This study highlights the importance of DMSP as a S source for marine bacteria under oligo- to mesotrophic conditions, particularly in summer highly irradiated waters.

Experimental procedures

Sampling

Surface seawater samples were collected 1 km offshore in Blanes Bay (the Blanes Bay Microbial Observatory, 41°40′N, 2°48′E) by submerging a rinsed amber glass bottle (2.5 l) an arm length into the water. Samples were kept in the dark at *in situ* temperature until being processed in the laboratory (usually ~2 h after sampling). Samples were collected monthly from 25 March 2003–22 March 2004.

Chemical analyses

Chla was measured by fluorometry in 90% acetone extracts (extraction overnight at 4°C) from 150 ml samples filtered through GF/F. Dimethylsulfide in 40–50 ml of GF/F filtered water was measured using a purge and cryotrapping system and sulfur-specific gas chromatography as described by Simó and colleagues (1996). Total DMSPt concentrations were determined in 20–40 ml of whole seawater by alkaline hydrolysis overnight and analysis of the evolved DMS.

Bacterial DMSPd consumption and DMSP-S assimilation rates

Bacterial DMSP consumption was measured following the protocol described in M. Vila-Costa *et al.* (submitted). In brief, 30-ml seawater samples were incubated in the dark at *in situ* temperature with tracer levels of ^{35}S-DMSP (~0.002 nM, 1000 dpm ml^{-1}, 5.79 Ci pmol-1). Subsamples of 4 ml were taken over a short-term time-course (< 2 h), acidified, and stored at 4°C for 24 h in the dark. Untransformed ^{35}S-DMSP was converted into volatile ^{35}S-DMS by injection of NaOH into sealed vials, and trapped in 3% H_2O_2-soaked wicks suspended in cups. Dimethylsulfoniopropionate loss rate constant was calculated from the exponential loss of ^{35}S-DMSP with time. Bacterial DMSPd consumption rate was calculated as the product of the DMSPd concentration and the loss rate constant.

For DMSP-S assimilation rates (Kiene and Linn, 2000a), a 15-ml subsample of whole seawater was incubated in the dark at *in situ* temperature without headspace for 18 h with a trace addition of ^{35}S-DMSP (~0.002 nM; 1000 dpm ml^{-1}; 5.79 Ci pmol^{-1}). Triplicate aliquots (5 ml) were filtered through nylon filters (GN, Millipore, 0.2-μm pore size) using a gentle vacuum (< 5 cm Hg) and rinsed with 0.2 μm-filtered seawater (FSW). Macromolecules were precipitated by treating filters with cold aliquots (5 ml) of trichloroacetic acid (TCA 5%) for 5 min. Filters were then rinsed twice with MilliQ water. Radioactivity in the TCA-rinsed filters was determined in 5 ml of scintillation cocktail (Optimal HiSafe) using a Beckman scintillation counter. The precision (%SE) of triplicate measurements averaged ~ 1%. Incorporation of ^{35}S-DMSP in formalin-killed controls was ≤1.5% that in live samples.

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The percentage of incorporated ^{35}S into macromolecules from initially added ^{35}S-DMSP was multiplied by the DMSPd consumption rate (nM DMSPd/d) to calculate the DMSP assimilation rate (see M. Vila-Costa *et al.*, submitted). It is assumed that all initial ^{35}S-DMSP added to the sample (1000 dpm ml^{-1} = 0.0004 nM) was consumed during the 18 h of incubation.

Quantification of populations by CARDFISH

The abundance of bacterial populations was determined by FISH with horseradish peroxidase-label nucleotide probes (HRP probes) and catalysed reporter deposition (CARD-FISH) following the protocol described by Pernthaler and colleagues (2004) with the only modification that samples were fixed with formaldehyde (4% final conc., overnight at 4°C) instead of paraformaldehyde. To permeabilize the cells, filters were treated with lysozyme (37°C, 1 h) and then with Achromopeptidase (37°C, 30 min). HRP probes used were the following (in parenthesis the percentage of formamide of the hybridization): EUBI–III, to target most *Bacteria* (55%; Daims *et al.*, 1999), ALF968, to target most *Alphaproteobacteria* (45%; Neef, 1997), GAM42am to target most *Gammaproteobacteria* (55%; Manz *et al.*, 1992) and CF319 to target most Bacteroidetes (CFB 55%, Manz *et al.*, 1996). Two subgroups of *Alphaproteobacteria* were also selected: ROS537 (*Roseobacter* clade, 55%; Eilers *et al.*, 2001) and SAR11–441R (SAR11 cluster, 45%; Morris *et al.*, 2002). Hybridizations were carried out for 2 h at 35°C, except for SAR11–441R, ALF968 and EUBI–III that were incubated overnight to improve the signal. Non-specific binding was examined by hybridizing with NON338 probe (Amann *et al.*, 1995). Counterstaining of CARDFISH preparations was done with 4,6-diamidino-2-phenylindole (DAPI, 1 μg ml^{-1}). An image analysis system (described by Cottrell and Kirchman, 2003) was used to count a minimum of 1000 DAPI-positive cells. These counts were verified with manual counts for a subset of samples for all probes used.

MARFISH

We followed the recent developed MARFISH method that combines MAR and CARDFISH to follow radioactive substrate incorporation by specific phylogenetic groups (Alonso and Pernthaler, 2005). Briefly, 30 ml samples were incubated in the dark at *in situ* temperature for 18 h with trace additions of ^{35}S-DMSP (≤ 0.4 nM, specific activity: 5.79 Ci $pmol^{-1}$) and ^{3}H-leucine (0.5 nM, specific activity: 161 Ci $mmol^{-1}$). Incubations were stopped with formaldehyde (final con*c*. 4%) and fixed overnight at 4°C. A formaldehyde-killed control was prepared for each incubation. Aliquots (5 ml) were filtered using a gentle vacuum (< 5 cm Hg) through 0.2-μm pore size polycarbonate filters (Millipore, GTTP, 25 mm diameter) and rinsed twice with 0.2 μm-FSW. No differences were observed if filters were rinsed with 0.2 μm-filtered Milli-Q water instead. Filters were air dried and stored at −20°C until processed. Filter sections were hybridized following the CARDFISH procedure and glued onto microscope slides using a two-component epoxy adhesive (UHU plus sofortfest; UHU GmbH, Bühl, Germany). CARDFISH counts were performed cutting a small piece of these filters. For the MAR procedure, in the darkroom, slides were dipped in melted (46°C) NTB-2 nuclear track emulsion (diluted 1:1 with agarose 1%, Kodak). Slides were kept for ~10 min on a metal ice cold bar until solidification of the emulsion and then exposed in the dark at 4°C. The exposure times were tested individually in the most contrasted samples regarding ^{35}S-DMSP incorporation and bacterial production (6 out of 12). The optimal exposure time was 20 days for ^{35}S-DMSP filters and 14 days for ^{3}H-leucine. The July month presented exceptionally high activity and exposures were stopped after 2 days for both radioisotopes. The microautoradiographs were developed for 3 min in Kodak D19 developer, followed by 30 s of rinsing with distilled water and 3 min in Kodak Tmax fixer. Slides were washed in tap water for 10 min and dried overnight in a desiccator. Cells were stained with DAPI (1 μg ml^{-1}) for 3 min at 4°C, washed with Milli-Q water and ethanol (80%), and then stored at −20°C until counting. Total radiolabelled cell counts were determined manually with a Olympus BX61 epifluorescence microscope. Killed-controls were evaluated with probe EUBI–III.

Short-term nutrient limitation bioassays

To study the effect of nutrient and substrate availability on the growth of heterotrophic bacteria, enrichment experiments were performed monthly from January 2003 to July 2004. Seawater was transferred to acid-rinsed 250-ml polycarbonate bottles (Nalgene) that had been rinsed thoroughly with MilliQ-water and sample water. Phosphate was added as Na_2HPO_4 to final concentrations of 0.6 μM P, singly, or in combination with 24 μM C as DMSP or glucose, in duplicates. After incubation for 24 h at *in situ* temperature in the dark, bacterial production was measured as ^{3}H-leucine (20 nM) incorporation following the protocol of Smith and Azam (1992). For details on bacterioplankton C, N and P limitation in the Bay of Blanes see Pinhassi and colleagues (2006).

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