environmental microbiology

Environmental Microbiology (2010) **12**(11), 2988–2997 doi:10.1111/j.1462-2920.2010.02276.x

Leucine-to-carbon empirical conversion factor experiments: does bacterial community structure have an influence?

Laura Alonso-Sáez,[1*†] Jarone Pinhassi,[2] Jakob Pernthaler[3] and Josep M. Gasol[1]
[1]*Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar, CSIC, 08003-Barcelona, Catalunya, Spain.*
[2]*Marine Microbiology, School of Natural Sciences, Linnaeus University, 39182 Kalmar, Sweden.*
[3]*Limnological station, Institute of Plant biology, University of Zurich, Seestrasse 187, 8802 Kilchberg, Switzerland.*

Summary

The suitability of applying empirical conversion factors (eCFs) to determine bacterial biomass production remains unclear because seawater cultures are usually overtaken by phylotypes that are not abundant *in situ*. While eCFs vary across environments, it has not been tested whether differences in eCFs are driven by changes in bacterial community composition or by *in situ* environmental conditions. We carried out seawater cultures throughout a year to analyse the correlation between eCFs and bacterial community structure, analysed by catalysed reporter deposition fluorescence *in situ* hybridization. *Gammaproteobacteria* usually dominated seawater cultures, but their abundance exhibited a wide range (25–73% of cell counts) and significantly increased with inorganic nutrient enrichment. *Flavobacteria* were less abundant but increased up to 40% of cells counts in winter seawater cultures, when *in situ* chlorophyll a was high. The correlations between eCFs and the abundance of the main broad phylogenetic groups (*Gamma-, Alphaproteobacteria* and *Flavobacteria*) were significant, albeit weak, while more specific groups (*Alteromonadaceae* and *Rhodobacteraceae*) were not significantly correlated. Our results show that the frequent development of the fast-growing group *Alteromonadaceae* in seawater cultures does not strongly drive the observed variations in eCFs. Rather, the results imply that environmental conditions and the growth of specific phylotypes interact to determine eCFs.

Received 12 February 2010; accepted 4 May 2010. *For correspondence. E-mail laura.alonso@gi.ieo.es; Tel. (+34) 985 309 780; Fax (+34) 985 326 277. †Present address: Centro Oceanográfico de Xixón, Instituto Español de Oceanografía, Camín del L'Arbeyal s/n, E-33212 Xixón, Spain.

Introduction

Radiotracers such as [^{3}H or ^{14}C]-Leucine and ^{3}H-thymidine offer a highly sensitive and relatively easy way to estimate rates of bacterial heterotrophic production. Therefore, their use was included into the common toolbox to study *in situ* bacterial processes. Since the incorporation of these techniques (Fuhrman and Azam, 1980; Kirchman *et al.*, 1985), a great amount of Leucine (Leu) and thymidine uptake rates has been reported for marine and freshwater environments, improving our understanding of the role of bacteria in carbon processing in aquatic ecosystems (Ducklow and Carlson, 1992). However, the conversion of Leu or thymidine uptake rates into rates of biomass production requires the use of conversion factors (CFs), which have received much less attention despite their crucial influence on the quantification of heterotrophic production and carbon fluxes (Ducklow and Carlson, 1992; Alonso-Sáez *et al.*, 2007a; Gasol *et al.*, 2008; Calvo-Díaz and Morán, 2009).

In the case of Leu uptake, a theoretical CF was proposed by Simon and Azam (1989) based on the protein content of an average bacterial cell and the ratio of carbon to protein content. According to their estimates, 1.55 kg of carbon should be produced per mol of Leu incorporated if no isotope dilution was assumed (i.e. no exogenous Leu was present in the environment). Thereafter, this value has been systematically applied in most studies despite it might be highly sensitive to different factors that have rarely been quantified *in situ*, such as the *per cell* protein content (but see Zubkov *et al.*, 1999).

Kirchman and Ducklow (1993) recommended determining CFs empirically in order to obtain reliable values for the particular environment under study. This determination consists in an experiment that promotes the growth of the *in situ* bacterial assemblage, commonly generated by a dilution of the sample and/or removal of grazers by filtration. Cell biomass production and total Leu incorporated during the incubation are measured before any

© 2010 Society for Applied Microbiology and Blackwell Publishing Ltd

flagellate development occurs, and thus, empirical CFs (eCFs, i.e. yield of bacterial biomass per unit of Leu incorporated) can be derived. In some cases, inorganic nutrients have also been added with the aim of releasing bacteria of inorganic nutrient limitation, even if it is not clear how nutrient enrichment may affect the CFs (Coveney and Wetzel, 1988; Kirchman, 1992).

The range of eCFs reported for marine and freshwater environments is surprisingly wide, suggesting that the use of a single theoretical CF can lead to large over- or underestimations of bacterial biomass production (e.g. Gasol *et al.*, 2008). Nevertheless, the use of eCFs to derive rates of biomass production is not free of criticisms. Ideally, bacterial growth in seawater dilution cultures (SWCs) should occur without changes in composition or metabolism, providing trustworthy measurements of biomass production and Leu incorporation as they occur *in situ*. However, in practice, this is seldom the case. SWCs usually promote the growth of phylotypes different from those dominant in the inoculum (but see Teira *et al.*, 2009), and this has been raised as the main argument against the validity of eCFs (Fuchs *et al.*, 2000; Massana *et al.*, 2001). To what extent the metabolic performance of these 'artificial' communities can be equated to that of the *in situ* assemblages, and thus, how representative are eCFs obtained from SWCs remains unclear.

Here, we explored the link between eCFs and bacterial community structure in a set of experiments throughout a year in a coastal Mediterranean site with the aim of testing: (i) whether the same bacterial taxa developed in the eCF experiments under different environmental conditions, (ii) whether the calculated eCFs are related to the phylogenetic composition of the communities growing in the incubations and (iii) whether inorganic nutrient addition (and the subsequent changes in bacterial community composition caused by the enrichments) affected the estimations of eCFs.

Results

Empirical conversion factors

The range of empirical Leu-to-carbon CFs obtained from unamended dilution cultures was 0.98–3.62 KgC mol Leu^{-1} (average ± SD: 1.79 ± 0.89 kgC mol Leu^{-1}) and the results of replicated bottles were very consistent throughout the year (Fig. 1). eCFs were fairly constant over summer and fall (average ± SD: 1.25 ± 0.14 kgC mol Leu^{-1}), while peaks were observed at times during spring (2.2 kgC mol Leu^{-1} in May) and winter (*c.* 3.5 kgC mol Leu^{-1}).

The SWCs amended with inorganic nutrients exhibited significantly higher rates of Leu uptake during exponential growth (*t*-test, $P < 0.05$) throughout the year. Maximal growth rates and biomass yields were also substantially higher in enriched SWCs during spring and summer periods but similar at other times of the year (Fig. 1). The resulting eCFs in the enriched SWCs (average ± SD: 1.24 ± 0.69 kgC mol Leu^{-1}) were lower than in unamended incubations (average ± SD: 1.79 ± 0.89 kgC mol Leu^{-1}, Wilcoxon signed-rank test, $n = 12$ $P = 0.04$), with an average ratio amended/unamended eCFs (± SD) of 0.7 ± 0.3.

Bacterial community composition in seawater cultures

Catalysed reporter deposition fluorescence *in situ* hybridization (CARDFISH) samples were analysed at stationary phase of the SWCs in April 2003 and from July 2003 to March 2004 (although in the two latest sampling points there are no data available to compute eCFs). On average, 95% of the total cell counts were identified with the set of general bacterial probes EubI-II-III (results not shown), and 86% of the Eub+ cells could be assigned to one of the three main bacterial groups, *Alpha-, Gammaproteobacteria* or *Flavobacteria*. Only in one experiment (March 2004), the addition of the abundance of cells targeted with these three broad probes exceeded the abundance of Eub+ cells, probably due to unspecificity of the probes (Loy *et al.*, 2007).

The abundance of these three main groups in the SWCs showed substantial changes throughout the year (Fig. 2). In the unamended SWCs, *Gammaproteobacteria* were generally the dominant group (24–68% of total cell counts), followed by *Alphaproteobacteria* (7–23% of total cell counts, Fig. 2). The abundance of both groups varied *c.* twofold throughout the year but did not show clear seasonal patterns. Conversely, *Flavobacteria* had the largest variations in abundance (21-fold, from 2% to 43% of total cell counts) and greatly increased during winter, co-dominating the assemblage composition with *Gammaproteobacteria*. The group *Alteromonadaceae* accounted for most *Gammaproteobacteria* (generally over 80% of gammaproteobacterial cells), and *Rhodobacteraceae* accounted for a highly variable fraction of the alphaproteobacterial cells, from 8% to 98% (1–18% of total cell counts, Fig. 2).

The addition of inorganic nutrients to the SWCs did not result in marked changes in the relative abundance of *Alphaproteobacteria* or *Flavobacteria*, while *Gammaproteobacteria* significantly increased their abundances (Wilcoxon signed-rank test, $n = 9$, $P = 0.04$), reaching on average 59% of total cell counts (Fig. 2). Since the percentage of *Bacteria* was similar in unamended and enriched SWCs (93% and 96% respectively), the increase in the abundance of *Gammaproteobacteria* produced a decrease in the fraction of

© 2010 Society for Applied Microbiology and Blackwell Publishing Ltd,

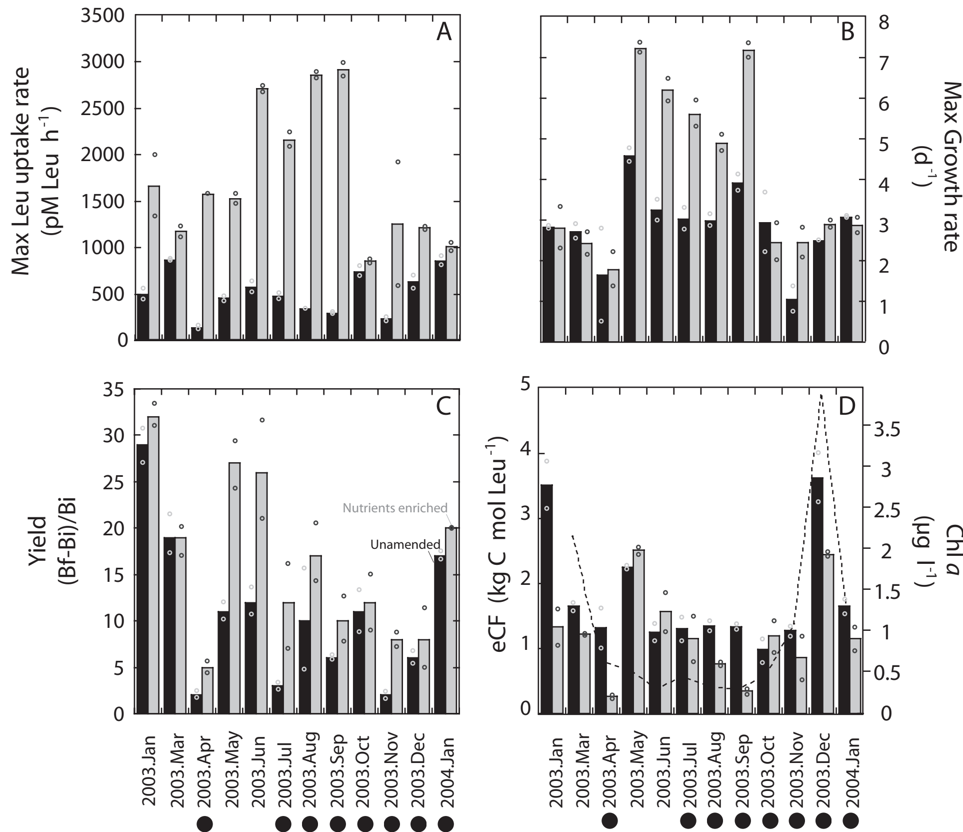

Fig. 1. Maximum Leucine uptake rates, (A) maximum growth rates, (B) biomass yield calculated as the increment of biomass (i.e. final biomass minus initial biomass: Bf-Bi) divided by the initial biomass, (C) and empirical conversion factors (eCF, D) obtained in the seasonal seawater cultures (SWCs). Maximum growth rates were calculated from bacterial biomass data during exponential phase. Panel D includes values of *in situ* Chl a concentration (dotted line). Black and gray bars represent values for unamended and nutrient enriched SWCs respectively. Open dots represent individual measurements of replicate seawater cultures. Black dots in *X*-axis indicate experiments in which bacterial community composition was analysed.

unidentified *Bacteria* in enriched cultures (from 19% in unamended to 7% of cell counts in enriched SWCs). The addition of nutrients did not significantly influence the abundance of the groups *Rhodobacteraceae* and *Alteromonadaceae* over the year. However, during the summer (July and August), *Rhodobacteraceae* increased their abundance after nutrient addition from less than 10% to 17–27% of total cell counts.

When the *in situ* abundance of the different bacteria groups in the inocula were compared with their abundance in SWCs at stationary phase, large variations were found (Fig. 3). *Gammaproteobacteria*, and specifically *Alteromonadaceae*, were in very low abundance *in situ* and largely overrepresented in the SWCs. *Alphaproteobacteria* and *Flavobacteria* showed more proportionate abundances (i.e. they were closer to the 1:1 line, Fig. 3), with a tendency to being underrepresented in the SWCs. However, during winter, *Flavobacteria* reached higher abundances in the SWCs than *in situ*.

Relationship between bacterial community composition, eCFs and environmental parameters

The influence of bacterial community composition on eCFs was assessed by a correlative approach. Since most parameters did not fit to a normal distribution (Shapiro-Wilk test, $P > 0.05$), we used non-parametric Spearman rank correlation analyses. eCFs were significantly but weakly correlated with the relative abundance of the three broad phylogenetic groups analysed. The correlation between eCFs and *Gammaproteobacteria* was negative (Spearman $rho = -0.47$, $P = 0.0084$, $n = 31$). On the contrary, *Alphaproteobacteria* (Spearman $rho = 0.42$, $P = 0.0179$, $n = 31$) and *Flavobacteria* (Spearman $rho = 0.48$, $P = 0.0079$, $n = 30$) showed positive correlations with eCFs (Fig. 4). No significant relationships were found between more specific groups, such as *Alteromonadaceae* and *Rhodobacteraceae*, and eCFs ($P > 0.05$, Fig. 4).

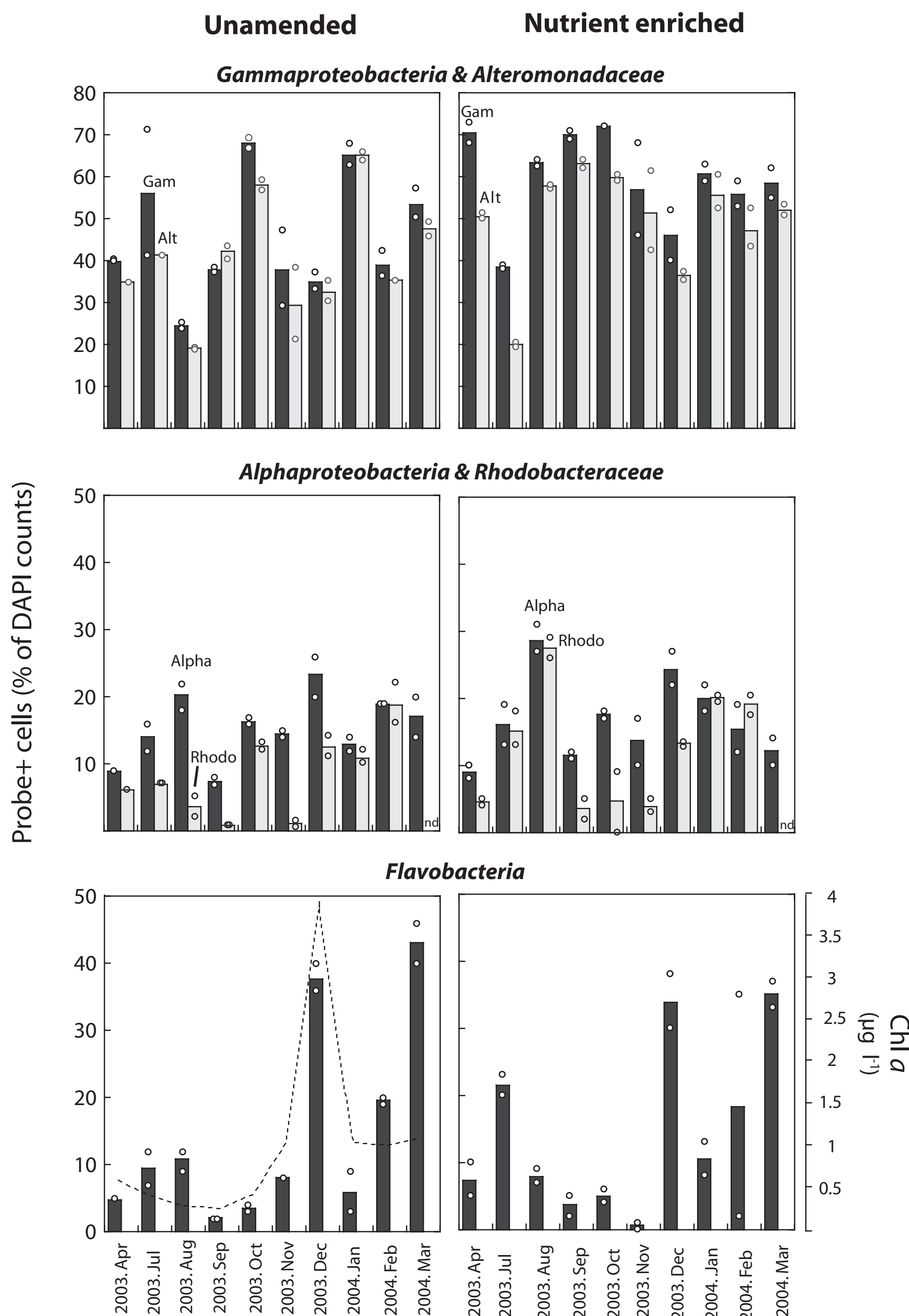

Fig. 2. Bacterial community composition as assessed by catalysed reporter deposition fluorescence *in situ* hybridization (CARDFISH) in the unamended (left panels) and nutrient enriched (right panels) seawater cultures. Dark gray bars represent the abundance of *Gammaproteobacteria* (probe Gam42a), *Alphaproteobacteria* (probe Alf968) and *Flavobacteria* (probe CFB319) in the upper, middle and lower panel respectively. Light gray bars represent the abundance of *Alteromonadaceae* (probe Alt1413) and *Rhodobacteraceae* (probe Ros537) in the upper and middle panels respectively. Dots represent individual measurements of replicate seawater cultures. Values of *in situ* Chl a concentration (dotted line) are shown in the right lower panel. Nd, non determined.

© 2010 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*, **12**, 2988–2997

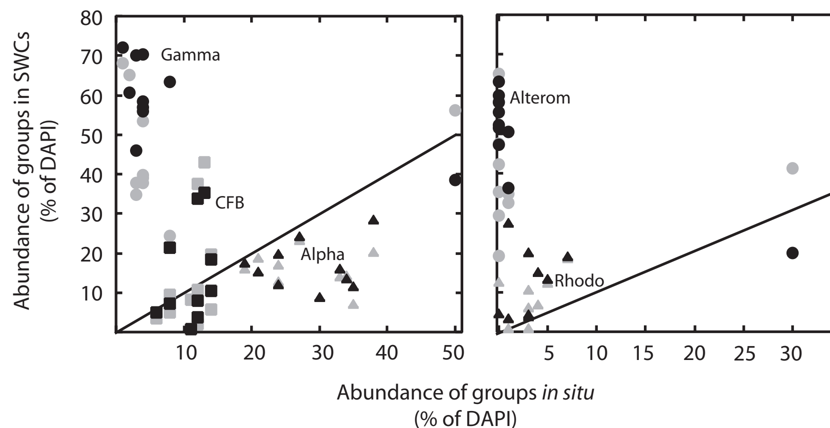

Fig. 3. Contribution of phylogenetic bacterial groups to total cell counts at stationary phase of the seawater cultures (SWCs) versus their abundance *in situ* (in the inoculum), as assessed by catalysed reporter deposition fluorescence *in situ* hybridization (CARDFISH). Percentages were calculated relative to total DAPI counts. For an easy visualization of the groups which are under or overrepresented in the SWCs as compared with *in situ* 1:1 lines have been drawn in the panels. Gamma: *Gammaproteobacteria* (probe Gam42a), Alpha: *Alphaproteobacteria* (probe Alf968), CFB: *Flavobacteria* (probe CFB319), Alterom: *Alteromonadaceae* (probe Alt1413) and Rhodo: *Rhodobacteraceae* (probe Ros537). Gray and black symbols represent values for unamended and nutrient enriched incubations, respectively.

The abundance of bacterial groups in SWCs was also significantly correlated with some *in situ* environmental parameters (Table S1). *Gammaproteobacteria* and *Rhodobacteraceae* were significantly correlated with the concentration of ammonia and nitrate respectively ($P < 0.05$, $n = 10$ and 9 respectively). Conversely, *Flavobacteria* showed a strong correlation with *in situ* primary production (Spearman $rho = 0.78$, $P = 0.007$, $n = 10$, Fig. 4) and a negative correlation with dissolved organic carbon (DOC, Spearman $rho = -0.70$, $P = 0.025$, $n = 10$, Table S1).

Discussion

The fact that bacterial community composition in dilution cultures is commonly overtaken by phylotypes which were not dominant *in situ* has been raised as the main argument against the use of empirical Leu-to-carbon CFs or, in fact, any biogeochemical determination based on long-term incubations (Fuchs *et al*., 2000; Massana *et al*., 2001). However, to our knowledge, there are no previous studies that have systematically investigated whether eCFs are influenced by the BCC emerging in the SWCs. Our initial hypothesis was that if eCFs were determined by the presence (or abundance) of specific phylogenetic bacterial groups, different bacterial communities should result in different eCFs. On the contrary, if eCFs were mainly determined by environmental factors their value would be independent of the changes in BCC occurring in the SWC. In a set of seasonal SWCs we observed significant correlations between the abundance of three broad phylogenetic groups (*Alpha-, Gammaproteobacteria* and *Flavobacteria*) and eCFs indicating that they are, at least, partly driven by changes in bacterial community structure. However, the correlations were rather weak (explaining less than 25% of eCFs variability), and there was a lot of scattering in the data (Fig. 4), suggesting that BCC is not a strong driver of eCFs as commonly assumed.

Interestingly, the correlations between eCF and the three broad phylogenetic groups showed different trends: when *Gammaproteobacteria* were dominant in the SWCs, eCFs were lower, while when *Alphaproteobacteria* and *Flavobacteria* were in higher proportions, eCFs were higher (Fig. 4). These results suggest that the incorporation of Leu into bacterial biomass are likely to differ among broad bacterial taxa, a point that, to our knowledge, has never been tested in experiments with isolates. Analyses through culture independent approaches such as microautoradiography combined with FISH have shown that *Flavobacteria* have low activity in the uptake of amino acids at trace concentration, while they dominate the consumption of high-molecular weight compounds (Cottrell and Kirchman, 2000). In principle, the low uptake of Leu by *Flavobacteria* could explain the higher eCFs observed when this group dominates the SWCs. If their biomass production originates mainly from other organic sources not represented by the Leu tracer, the ratio biomass produced per unit Leu incorporated would increase. However, it should be noted that when Leu is added at higher concentrations (as typically for bacterial heterotrophic production assays) *Flavobacteria* showed higher activity in the uptake of this amino acid (Cottrell and Kirchman, 2003). Indeed, in experiments carried out along a salinity gradient, it was shown that all major bacterial groups assimilated Leu and Thymidine (Cottrell and Kirchman, 2003). Thus, the distinct trends found for the three broad phylogenetic groups should be mainly due to metabolic differences of these taxa.

© 2010 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*, **12**, 2988–2997

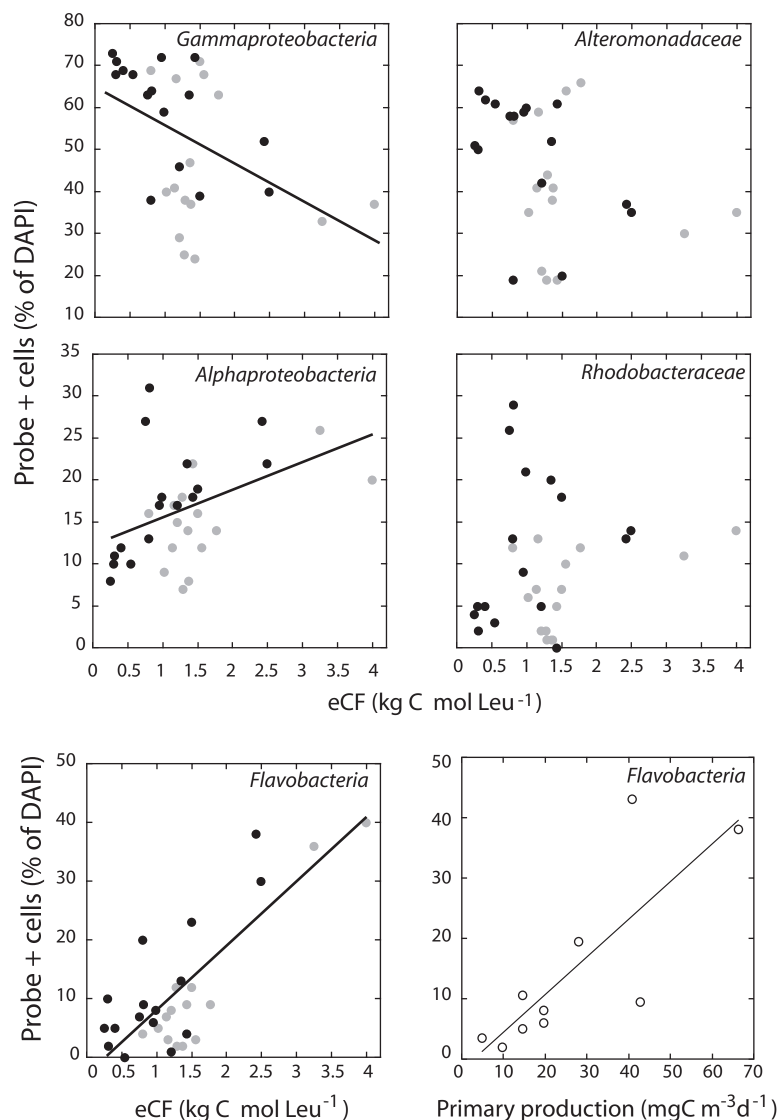

Fig. 4. Correlations between empirical Leucine-to-carbon conversion factors (eCF) and the relative abundance of the different phylogenetic groups in the experiments (expressed as percentage of DAPI counts). Gray and black circles represent values for unamended and nutrient enriched seawater cultures respectively. The lower right panel shows the correlation between the abundance of *Flavobacteria* (average values in unamended seawater cultures) and *in situ* primary production. Tendency lines are shown only for significant correlations (Spearman Rank correlation, $P < 0.05$).

At a finer phylogenetic resolution, eCFs were not significantly correlated with the abundance of more specific groups, i.e, *Rhodobacteraceae* or *Alteromonadaceae*, even if the later became dominant in most of the experiments. The weak relationship found between eCFs and bacterial community composition suggests that other factors (i.e. ecological factors, probably related with the characteristics of the waters) were influencing the eCFs. In a previous study on the same seasonal cycle, the correlations between eCFs and a wide range of environmental parameters (including temperature, DOC and inorganic nutrient concentrations, Table S1) were analysed and, remarkably, only a positive tendency with Chl a was found (Fig. 1D, Alonso-Sáez *et al.*, 2008). A similar lack of correlation between eCFs and typically measured environmental factors was found in a seasonal study in a subtropical estuary (Murrell, 2003). However, significant effects of *in situ* concentration of DOC in lakes (Pulido-Villena and Reche, 2003) or phosphate in marine waters (Calvo-Díaz and Morán, 2009) on eCFs have been detected, suggesting that we could have missed environmental parameters that were relevant in determining eCFs.

Interestingly, inorganic nutrient additions lead to significantly lower eCFs throughout the year, although eCF were not particularly sensitive to nutrient additions as compared with bacterial growth rates or biomass yields (Fig. 1). A couple of studies in Pacific and Antarctic waters did not detect a significant effect of the addition of organic and inorganic nutrients on eCF (Kirchman, 1992; Pedrós-Alió *et al.*, 2002), but other studies have also shown that nutrient enrichments tend to lower eCFs (Coveney and Wetzel, 1988). While nutrients have been added in some studies with the aim of releasing bacteria from potential

bottom-up limitation (Pulido-Villena and Reche, 2003), our results suggest that the enrichment should be avoided in this type of experiments. The release of inorganic nutrient limitation may lead to a less efficient growth by the bacterial communities or to a situation of C limitation, in which bacteria could metabolize Leu to obtain energy rather than use it for biomass production, explaining the observed decrease of the eCFs (Alonso-Sáez *et al.*, 2007a).

A common assumption also challenged by our dataset is that SWCs always promote the growth of the same bacterial groups, resulting in important skews in the eCF values. Particularly, the group *Gammaproteobacteria* contains fast-growing members, such as *Alteromonadaceae*, well known for their ability to outcompete other bacterial groups in SWCs (Fuchs *et al.*, 2000). In our set of seasonal experiments, *Alteromonadaceae* were in very low abundance in the inocula and became dominant in most of the SWCs. However, their abundance showed important variations throughout the year and this variability did not affect the eCFs in a comparable manner. For example, during the summer-autumn period from July to November, the abundance of *Alteromonadaceae* ranged between 19% and 58% in the SWCs (threefold variation, Fig. 2), while eCF remained stable (from 1.0 to 1.3 kgC mol Leu^{-1}, Fig. 1). Also, at different sampling points, other groups co-dominated with Gammaproteobacteria, such as *Alphaproteobacteria* in August or *Flavobacteria* in December. Dominance of *Alphaproteobacteria* and *Flavobacteria* in SWCs have also been reported before (Fuchs *et al.*, 2000; Teira *et al.*, 2009), suggesting that members from all broad phylogenetic groups can overtake in dilution cultures depending on different characteristics of the waters. It should be noted that the addition of inorganic nutrients significantly changed the bacterial community structure in the eCF experiments, significantly increasing the abundance of *Gammaproteobacteria* in most of the incubations (Fig. 2). In our view, this reinforces the idea that the addition of nutrients should be avoided in eCF experiments.

In general, the good reproducibility of the abundance of bacterial groups in duplicate cultures throughout the seasonal study indicates that the growth of the different taxa in the experiments was not a result of stochastic variability (Fig. 2). Instead, as pointed out above, it suggests that environmental conditions were influencing the growth of different phyla in the SWCs. Indeed, we detected some significant correlations between the abundance of bacterial groups and some environmental parameters (Table S1), in agreement with a recent study by Teira and colleagues (2009). Altogether, these observations suggest that some changes in bacterial community structure during SWC incubations are linked to environmental factors, and are not just an artifact of the so-called 'bottle effects'.

Flavobacteria showed the largest variation in abundance in the SWCs (from 2% to 43%) following the trend of *in situ* Chl a (Fig. 2) and being strongly correlated with primary production (Fig. 4, Table S1). Similarly, a positive correlation between Chl a and the growth rate of *Flavobacteria* was detected in Pacific coastal waters (Yokokawa and Nagata, 2005) and a *Flavobacteria* lineage (FLAV2) was shown to double their growth rates in SWCs during the period of maximum Chl a in a temperate lake (Zeder *et al.*, 2009). These results suggest that the growth of members of *Flavobacteria* in SWCs can be stimulated by phytoplankton exudates in the waters. *Flavobacteria* was also the group that showed a strongest positive correlation with eCFs. The covariation between Chl a, eCFs and the abundance of *Flavobacteria* in SWCs indicates that the correlation between the abundance of *Flavobacteria* and eCFs was not independent of the environment.

In summary, in the seasonal unamended SWCs we obtained a range of eCFs similar to the values commonly found in field studies (1.0–3.6 kgC mol Leu^{-1}). Notably, the variability in the eCFs (global coefficient of variation of 30% for unamended cultures) was much lower than the variability in the abundance of some bacterial groups in the SWCs (global coefficients of variation of 73% and 100% for *Rhodobacteraceae* and *Flavobacteria* respectively). The significant but weak correlations between the abundance of broad phylogenetic groups and CF values suggest that changes in bacterial community composition partly explain eCFs, but do not drive most of the variation. This does not imply that eCFs can be unequivocally applied to *in situ* communities, since the eCFs for phylotypes that dominate in the environment (e.g. SAR11) remain unknown. However, the fact that the abundance of the group which most strongly reacted in the SWCs (i.e. *Alteromonadaceae*) was not significantly correlated with the eCFs indicates that the development of this fast-growing phylotype did not strongly drive changes in eCFs. The observed covariation between eCF, *in situ* Chl a and the abundance of *Flavobacteria* suggests that both, environmental conditions and some changes in bacterial community composition in the SWCs interact to determine the measured eCFs. Our results indicate that seasonal changes in eCFs, partly reflecting changes in growth conditions and community composition, are relevant when interpreting field measurements of leucine incorporation in the frame of carbon cycling studies.

Experimental procedures

Seawater dilution cultures for determining eCFs

Duplicate SWCs were prepared monthly between January 2003 and March 2004 with samples from the Blanes Bay Microbial Observatory in the NW Mediterranean Sea

(41°40'N, 2°48'E). Background information from this site exists on bacterial heterotrophic activity (Alonso-Sáez *et al*., 2008), nutrient dependence (Pinhassi *et al*., 2006) and diversity (Alonso-Sáez *et al*., 2007b). Environmental parameters during the time of study can be found in Table S2. Beside unamended incubations, a set of nutrient enriched cultures received final concentrations of 2 μM N (NH_4Cl) and 0.6 μM P (Na_2HPO_4). Details about the preparation of the dilution cultures can be found in Pinhassi and colleagues (2006). In short, for each duplicate bottle, 1900 ml of sample was filtered through a 0.2 μm pore size Sterivex filter capsule (Millipore) using a peristaltic pump. The inocula (100 ml) were prepared by gravity filtration (0.8 μm pore-size polycarbonate filter, Nuclepore) and added to obtain a 20-fold dilution of bacterial abundance. SWCs were incubated at *in situ* temperatures for 2–4 days, when bacterial biomass was observed to reach stationary phase. SWCs were incubated in the dark in order to avoid interactions with phototrophic organisms, as recommended by Kirchman and Ducklow (1993). The *in situ* abundance of *Prochlorococcus*, which has been reported to actively consume leucine (Zubkov *et al*., 2004), was very low in Blanes Bay throughout the period of study (January 2003–March 2004, average ± SD: 0.51 ± 0.76% of prokaryotes). Thus, we consider that the potential presence of these cyanobacteria did not affect our results. Samples for bacterial abundance and Leu uptake determination were taken every 8–24 h from the duplicate unamended and nutrient enriched seawater cultures. Empirical CFs were computed with the cumulative method, which maximizes the use of data (Bjørnsen and Kuparinen, 1991).

Bacterial abundance and biomass

Samples (1.6 ml) were preserved with 1% paraformaldehyde + 0.05% glutaraldehyde (final concentrations). Bacterial abundance was analysed by flow cytometry (FACSCalibur cytometer, BectonDickinson) after staining with Syto13 (2.5 μM final, Molecular probes). Bacteria were detected by their signature in a plot of side scatter (SSC) versus FL1 (green fluorescence) as explained in Gasol and Giorgio (2000). Picocyanobacteria were excluded in a plot of FL1 versus FL3 (red fluorescence). Flow cytometric counts were calibrated with epifluorescence microscopic counts using 4'-6'-diamidino-2-phenylindole (DAPI). Bacterial cell size (biovolume) was estimated using the relationship between average bacterial size (obtained by image analysis of DAPI preparations following common procedures) and average fluorescence of the SYTO-13 stained sample relative to beads as shown by Gasol and Giorgio (2000). Bacterial biomass was calculated by using the volume-to-carbon relationship derived by Norland (1993): pgC $cell^{-1} = 0.12$ pg $(\mu m^3\ cell^{-1})^{0.7}$.

Leucine incorporation

We used [^{3}H]-Leu following standard protocols (Kirchman *et al*., 1985). For each sample, triplicate or quadruplicate aliquots (1.2 ml) and one or two TCA killed controls were incubated with 40 nmol l^{-1} Leu for about 2 h at *in situ* temperature in the dark. The incorporation was stopped with the addition of 120 μl of cold TCA 50% to the vials and samples were kept frozen at −20°C until processing, which was carried out by the centrifugation method of Smith and Azam (1992). Finally, the samples were counted on a Beckman scintillation counter, 24 h after addition of 1 ml of scintillation cocktail (Optiphase Hisafe2, Perkin Elmer). The average coefficient of variation between replicates was 8%.

CARDFISH

The *in situ* abundance of different bacterial populations determined by CARDFISH (Pernthaler *et al*., 2004) was analysed in duplicate bottles in 10 experiments (i.e. April 2003, and monthly from July 2003 to March 2004). eCF computed from the same SWCs are available for eight out of these 10 experiments. Samples for CARDFISH were analysed from the inoculum and from a time point placed near the early stationary phase of bacterial growth (after 24–55 h). In this way, we aimed at minimizing the risk of changes in bacterial community composition that could occur in later stages of stationary phase. The samples were fixed with paraformaldehyde (2% final concentrations, overnight at 4°C) and filtered onto 0.2 μm polycarbonate filters. The filters were permeabilized with lysozyme (37°C, 1 h) and hybridization was performed at 35°C for a minimum of 2 h. Horseradish peroxidase (HRP) labelled probes (50 ng μl^{-1}) were added to the hybridization buffer (HB, 1:300) containing the following percentages of formamide: 60% for Alt1413 (Eilers *et al*., 2000) and 55% for Eub338-II-III (Amann *et al*., 1990; Daims *et al*., 1999), Ros537 (Eilers *et al*., 2001), Gam42a and CF319a (Amann *et al*., 1990). We used higher concentration of probes in the HB (1:100, 45% formamide) and overnight hybridization to detect the cells with Alf968 probe (Neef, 1997). The Eub antisense probe Non338 (Wallner *et al*., 1993) was used as negative control. For the amplification, we used tyramide labelled with Alexa 488. Counter-staining of CARDFISH preparations was done with DAPI (final concentrations 1 $\mu g\ ml^{-1}$). DAPI and CARDFISH-stained cells were counted by automated image analysis (Pernthaler *et al*., 2003).

Acknowledgements

This work was supported by the Spanish projects GENμMAR (CTM2004-02586/MAR), MODIVUS (CTM2005-04975/MAR), SUMMER (CTM2008-03309/MAR), EU project BASICS (EVK3-CT-2002-00078) and NoE MARBEF. Financial support was provided by a PhD fellowship from the Spanish government to L.A.S. We thank V. Balague, C. Cardelús and I. Forn for their help with field sampling.

References

Alonso-Sáez, L., Gasol, J.M., Arístegui, J., Vilas, J.C., Vaqué, D., Duarte, C.M., and Agustí, S. (2007a) Large-scale variability in surface bacterial carbon demand and growth efficiency in the subtropical North East Atlantic Ocean. *Limnol Oceanogr* **52:** 533–546.

Alonso-Sáez, L., Balagué, V., Sà, E.L., Sánchez, O., González, J.M., Pinhassi, J., *et al*. (2007b) Seasonality in bacterial diversity in NW Mediterranean coastal waters: assessment through clone libraries, fingerprinting and

© 2010 Society for Applied Microbiology and Blackwell Publishing Ltd,

fluorescence in situ hybridization. *FEMS Microbiol Ecol* **60:** 98–112.

Alonso-Sáez, L., Vázquez-Domínguez, E., Cardelús, C., Pinhassi, J., Sala, M.M., Lekunberri, I., *et al*. (2008) Factors controlling the year round variability in carbon flux through bacteria in a coastal marine system. *Ecosystems* **11:** 397–409.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotice probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56:** 1919–1925.

Bjørnsen, P. K., and Kuparinen, J. (1991) Determination of bacterioplankton biomass, net production and growth efficiency in the Southern Ocean. *Mar Ecol Prog Ser* **71:** 185–194.

Calvo-Díaz, A., and Morán, X.A.G. (2009) Empirical Leucine-to carbon conversion factors for estimating bacterial heterotrophic production: seasonality and predictability in a temperate coastal ecosystem. *Appl Environ Microbiol* **75:** 3216–3221.

Cottrell, M.T., and Kirchman, D.L. (2000) Natural assemblages of marine Proteobacteria and members of the Cytophaga-Flavobacter cluster consuming low- and high-molecular-weight dissolved organic matter. *Appl Environ Microbiol* **66:** 1692–1697.

Cottrell, M.T., and Kirchman, D.L. (2003) Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware estuary. *Limnol Oceanogr* **48:** 168–178.

Coveney, M.F., and Wetzel, R.G. (1988) Experimental evaluation of conversion factors for the [^{3}H] thymidine incorporation assay of bacterial secondary productivity. *Appl Environ Microbiol* **54:** 2018–2026.

Daims, H., Bruhl, A., Amann, R., Schleifer, K.H., and Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22:** 434–444.

Ducklow, H. W., and Carlson, C. A. (1992) Oceanic bacterial production. *Adv Microb Ecol* **12:** 113–181.

Eilers, H., Pernthaler, J., Glöckner, F.O., and Amann, R. (2000) Culturability and in situ abundance of pelagic bacteria from the North Sea. *Appl Environ Microbiol* **66:** 3044–3051.

Eilers, H., Pernthaler, J., Peplies, P., Glöckner, F.O., Gerdts, G., and Amann, R. (2001) Isolation of novel pelagic bacteria from the German Bight and their seasonal contribution to surface picoplankton. *Appl Environ Microbiol* **67:** 5134–5142.

Fuchs, B.M., Zubkov, M.V., Sahm, K., Burkill, P.H., and Amann, R. (2000) Changes in community composition during dilution cultures of marine bacterioplankton as assessed by flow cytometric and molecular biological techniques. *Environ Microbiol* **2:** 191–202.

Fuhrman, J.A., and Azam, F. (1980) Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. *Appl Environ Microbiol* **39:** 1085–1095.

Gasol, J. M., and Giorgio, P. A. (2000) Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci Mar* **64:** 197–224.

Gasol, J.M., Pinhassi, J., Alonso-Sáez, L., Ducklow, H., Herndl, G.J., Koblížek, M., *et al*. (2008) Towards a better understanding of microbial carbon flux in the sea. *Aquat Microb Ecol* **53:** 21–38.

Kirchman, D.L. (1992) Incorporation of thymidine and leucine in the subarctic Pacific: application to estimating bacterial production. *Mar Ecol Prog Ser* **82:** 301–309.

Kirchman, D.L., and Ducklow, H.W. (1993) Estimating conversion factors for the thymidine and leucine method for measuring bacterial production. In *Current Methods in Aquatic Microbial Ecology*. Kemp, P.F., Sherr, B.F., Sherr, E.B., and Cole, J.J. (eds). New York, NY, USA: Lewis Publishers, pp. 513–519.

Kirchman, D. L., K'nees, E., and Hodson, R. (1985) Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic ecosystems. *Appl Environ Microbiol* **49:** 599–607.

Loy, A., Maixner, F., Wagner, M., and Horn, M. (2007) Probe-Base – an online resource for rRNA-targeted oligonucleotide probes: new feautures 2007. *Nucleic Acids Res* **35:** D800–D804.

Massana, R., Pedrós-Alió, C., Casamayor, E.O., and Gasol, J.M. (2001) Changes in marine bacterioplankton phylogenetic composition during incubations designed to measure biogeochemically significant parameters. *Limnol Oceanogr* **46:** 1181–1188.

Murrell, M.C. (2003) Bacterioplankton dynamics in a subtropical estuary: evidence for substrate limitation. *Aquat Microb Ecol* **32:** 239–250.

Neef, A. (1997) Anwendung der in situ-Einzelzell-Identifizierung von Bakterien zur Populationsanlayse in komplexen mikrobiellen biozönosen. PhD Thesis, Technische Universität München. Munich, Germany.

Norland, S. (1993) The relationship between biomass and volume of bacteria. In *Handbook of Methods in Aquatic Microbial Ecology*. Kemp, P., Sherr, B.F., Sherr, E., and Cole, J.J. (eds). Boca Raton, FL, USA: Lewis Publishing, pp. 303–307.

Pedrós-Alió, C., Vaqué, D., Guixa-Boixereu, N., and Gasol, J.M. (2002) Prokaryotic plankton biomass and heterotrophic production in western Antarctic waters during the 1995–1996 Austral summer. *Deep Sea Res II* **49:** 805–825.

Pernthaler, J., Pernthaler, A., and Amann, R. (2003) Automated enumeration of groups of marine picoplankton after fluorescence in situ hybridization. *Appl Environ Microbiol* **69:** 2631–2637.

Pernthaler, A., Pernthaler, J., and Amann, R. (2004) Sensitive multi-color fluorescence in situ hybridization for the identification of environmental microorganisms. In *Molecular Microbial Ecology Manual*, Vol. 3.11, 2nd edn. Kowalchuk, G.E.A., De Bruijn, F.J., Head, I.M., Akkermans, A.D.L., and van Elsas, J.D. (eds). Dordrecht, the Netherlands: Kluwer Academic Publishers, pp. 711–726.

Pinhassi, J., Gómez-Consarnau, L., Alonso-Sáez, L., Sala, M.M., Vidal, M., Pedrós-Alió, C., and Gasol, J.M. (2006) Seasonal changes in bacterioplankton nutrient limitation and their effects on bacterial community composition in the NW Mediterranean Sea. *Aquat Microb Ecol* **44:** 241–252.

Pulido-Villena, E., and Reche, I. (2003) Exploring bacterioplankton growth and protein synthesis to determine conversion factors accross a gradient of dissolved organic matter. *Microb Ecol* **46:** 3–42.
Simon, M., and Azam, F. (1989) Protein content and protein synthesis rates of planktonic marine bacteria. *Mar Ecol Prog Ser* **51:** 201–213.
Smith, D.C., and Azam, F. (1992) A simple, economical method for measuring bacterial protein synthesis rates in seawater using ^{3}H-leucine. *Mar Microb Food Webs* **6:** 107–114.
Teira, E., Martinez-Garcia, S., Lønborg, C., and Álvarez-Salgado, X.A. (2009) Growth rates of different phylogenetic bacterioplankton groups in a coastal upwelling system. *Environ Microbiol Rep* **1:** 545–554.
Wallner, G., Amann, R., and Beisker, W. (1993) Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14:** 136–143.
Yokokawa, T., and Nagata, T. (2005) Growth and grazing mortality rates of phylogenetic groups of bacterioplankton in coastal marine environments. *Appl Environ Microbiol* **71:** 6799–6807.
Zeder, M., Peter, S., Shabarova, T., and Pernthaler, J. (2009) A small population of planktonic Flavobacteria with disproportionally high growth during the spring phytoplankton bloom in a prealpine lake. *Environ Microbiol* **11:** 2676–2686.
Zubkov, M.V., Fuchs, B.M., Eilers, H., Burkill, P.H., and Amann, R. (1999) Determination of total protein content of bacterial cells by SYPRO staining and flow cytometry. *Appl Environ Microbiol* **65:** 3251–3257.
Zubkov, M.V., Tarran, G.A., and Fuchs, B.M. (2004) Depth related amino acid uptake by Prochlorococcus cyanobacteria in the Southern Atlantic tropical gyre. *FEMS Microbiol Ecol* **50:** 153–161.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Spearman Rank correlations between the relative abundance of bacterial phylogenetic groups (% of cell counts) at stationary phase in unamended seawater cultures and the environmental parameters in the inocula (shown in Table S1). Only correlations with $P < 0.1$ are shown. Correlations with $P < 0.1$, $P < 0.05$ and $P < 0.01$ are marked with *, ** and *** respectively. *N* varies between 9 and 10.
Table S2. Environmental parameters at the initial time of the empirical conversion factor experiments. Further details about the study site and the methods used for the measurement of the environmental variables can be found in Alonso-Sáez and colleagues (2008). nd, non determined.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Supplementary Table 1. Spearman Rank correlations between the relative abundance of bacterial phylogenetic groups (% of cell counts) at stationary phase in unamended seawater cultures and the environmental parameters in the inocula (shown in Table S1). Only correlations with $p < 0.1$ are shown. Correlations with $p < 0.1$, $p < 0.05$ and $p < 0.01$ are marked with *, **, and ***, respectively. N varies between 9 and 10.

Phylogenetic bacterial group	**Environmental parameter**	**Spearman *Rho***	**Prob > *Rho***
Bacteroidetes	Primary production	0.78	0.007 ***
	DOC	-0.70	0.025 **
	Chl *a*	0.60	0.067 *
Rhodobacteraceae	NO_3	0.83	0.006 ***
	PO_4	0.61	0.080 *
Gammaproteobacteria	NH_4	0.71	0.022 **

Supplementary Table 2. Environmental parameters at the inicial time of the empirical conversion factor experiments. Further details about the study site and the methods used for the measurement of the environmental variables can be found in Alonso-Sáez *et al*. 2008. nd: non determined.

Date	***Temperature*** *(ºC)*	***Salinity*** *(psu)*	***Chl a*** *(µg L^{-1})*	***NH_4*** *(µM)*	***NO_2*** *(µM)*	***NO_3*** *(µM)*	***Si*** *(µM)*	***PO_4*** *(µM)*	***DOC*** *(µM)*	***Primary production*** *(mg C m^{-3} d^{-1})*
28-01-03	14	37.5	nd	4.78	0.62	0.74	1.29	nd	nd	nd
04-03-03	11	36.1	2.15	0.82	0.55	7.11	4.95	nd	176.9	48.5
22-04-03	14.5	37.7	0.62	0.79	0.28	0.63	1.975	0.029	112.5	14.7
13-05-03	17	37	0.53	0.25	0.13	0.82	1.42	0.036	94.2	13.3
25-06-03	25	37.9	0.29	1.20	0.11	0.09	1.58	0.035	102.5	4.6
14-07-03	25.2	37.2	0.43	1.08	0.06	0.08	0.97	0.013	85	42.8
04-08-03	25.2	37.8	0.31	0.12	0.06	0.04	0.85	0.015	88.1	14.6
16-09-03	23	nd	0.28	0.38	0.05	0.03	1.07	0.057	117.1	9.8
21-10-03	18	37.5	0.44	1.02	0.16	5.54	7.59	0.201	112.9	5.0
25-11-03	16	37.6	1.08	0.2	0.2	0.77	1.09	0.027	91.7	19.6
16-12-03	14.5	36.1	3.93	0.13	0.65	3.88	5.93	0.106	102.1	66.3
26-01-04	14	38.6	1.06	0.29	0.28	1.53	1.8	0.068	103.5	19.6
23-02-04	12.9	37.9	1.04	0.67	0.23	1.56	0.84	0.064	82.8	28.0
22-03-04	12.9	37.9	1.13	0.28	0.28	2.82	2.47	0.102	98.8	40.9

Reference:

Alonso-Sáez, L., Vázquez-Domínguez, E., Cardelús, C., Pinhassi, J., Sala, M.M., Lekunberri, I., Balagué, V., Vila-Costa, M., Unrein, F., Massana, R., Simó, R., Gasol, J.M. Factors controlling the year-round variability in carbon flux through bacteria in a coastal marine system. 2008. *Ecosystems*: 11: 397-409