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Changes in bacterial diversity in response to dissolved organic matter supply in a continuous culture experiment

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ABSTRACT: Heterotrophic bacterial metabolism is tightly linked to the availability of dissolved organic matter (DOM), but the relationship between substrate supply and bacterial diversity remains unresolved. The objective of the present study was to determine how an increase in the supply of bioavailable DOM affects bacterial metabolism and diversity. We addressed this issue by growing a natural bacterial community over 5 generations in continuous cultures either on seawater (dissolved organic carbon concentration equal to 74 µM) or on seawater amended with 13 µM of diatom-derived DOM. Bacteria markedly responded to the additional DOM supply, as revealed by 1.6-fold higher bacterial abundances and 10-fold higher cell-specific α- and β-glucosidase activities in the +DOM treatment than in the control throughout the experimental period. Analysis of diversity as determined by tag pyrosequencing of 16S rRNA genes after 3 and 5 bacterial generations showed that the bacterial communities in the +DOM treatment were more similar to each other than to communities in the control. Diversity indices such as richness and phylogenetic distance were higher in the DOM-amended cultures than in the control. *Gammaproteobacteria* dominated the communities selected in our cultures, representing between 87 and 99% of the sequences. The addition of DOM led to a higher relative abundance of several bacterial groups, in particular *Alphaproteobacteria*, *Bacteroidetes*, and *Verrucomicrobia*, which accounted for up to 14% of the sequences, compared to the control in which they represented <5% of the sequences. Our results demonstrate that in this experimental context, an increased supply of bioavailable DOM sustained a higher bacterial diversity.

KEY WORDS: Bacterial diversity · Dissolved organic matter · Continuous cultures · 454 pyrosequencing

INTRODUCTION

Aquatic environments harbor a vast bacterial diversity (Giovannoni & Rappé 2000, Sogin et al. 2006). The factors that shape this bacterial diversity and in turn how the bacterial community contributes to ecosystem functioning have become a subject of intense research. The variability in bacterial community structure on different temporal and spatial scales has been shown to correlate with changes in biotic and abiotic environmental factors, indicating their selective control of bacterial communities (Ramette 2007, Logue et al. 2012). These studies have further provided insight into the co-occurrence of different phylotypes, suggesting environmentally driven interactions among bacterial groups (Fuhrman et al. 2006, Gilbert et al. 2012). However, the question of how patterns in phylogenetic diversity are linked to patterns in functional capabilities remains unanswered.

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A key role of heterotrophic bacteria in the aquatic environment is the degradation of dissolved organic matter (DOM). Previous studies have provided some evidence that bacterial community composition varies with DOM source, indicating the structuring effect of DOM (Van Hannen et al. 1999, Covert & Moran 2001, Kirchman et al. 2004). Different metabolic capabilities to degrade and utilize DOM among bacterial groups is one of the hypotheses that has been developed to explain these observations, an idea that was first supported by results from fluorescence *in situ* hybridization coupled to microautoradiography (Cottrell & Kirchman 2000, Teira et al. 2004, Vila-Costa et al. 2007). Single-cell analyses have demonstrated that polymeric substances are preferentially degraded by *Bacteroidetes* (Cottrell & Kirchman 2000), a finding that was later confirmed by genomic analyses of several members of this group (Bauer et al. 2006, Gómez-Pereira et al. 2012). Metatranscriptomic analyses of bacterial communities grown on different DOM sources further reveal taxon-specific expression of genes related to carbon metabolism (McCarren et al. 2010, Poretsky et al. 2010, Rinta-Kanto et al. 2012). These studies provide some evidence for a link between resource supply and bacterial community composition, suggesting some specialization of bacterial groups over a range of phylogenetic levels.

If, as suggested by these previous studies, DOM structures bacterial community composition by favoring the development of certain bacterial groups, the way bacterial communities come into contact with DOM might modulate the bacterial response. Aquatic systems receive DOM through different modes and at variable concentrations. River discharges and atmospheric deposition provide episodic pulses of organic matter supply on time scales of hours to days (Naudin et al. 1997, Jurado et al. 2008), in contrast to the overall continuous supply of DOM by phytoplankton. While an episodic pulse of organic matter is likely to have short-term consequences predominantly on the active part of the bacterial community, DOM that continuously supports bacterial growth probably affects bacterial diversity over a longer time scale.

To address the question of how bacterial diversity is linked to DOM supply, we investigated the response of a natural bacterial community to the sustained input of a small amount of bioavailable DOM under constant environmental conditions. We expected an increase in bulk bacterial abundance without any changes in bacterial diversity if the added DOM stimulates all bacterial groups. In contrast, distinct diversity patterns between experimental conditions would be observed if the added DOM stimulates different bacterial groups. Our results show an increase in bacterial diversity, including richness and phylogenetic diversity, in response to the addition of bioavailable DOM.

MATERIALS AND METHODS

Experimental design

To explore DOM–bacteria interactions, we designed an experiment using continuous cultures. Seawater for the continuous cultures was collected in the NW Mediterranean Sea, 5 nautical miles off Banyuls-sur-Mer (42° 28′ 300″ N, 03° 15′ 500″ E; 95 m overall depth). For the medium, 300 l of seawater were collected in September 2009 at 3 m using an All-PFA Double-Bellows Pump (Asti) connected to 50 m PVC tubing. Seawater was 20 µm filtered and stored in 1 polycarbonate (PC) carboy in the dark at room temperature for 6 wk to allow degradation of the readily bioavailable DOM. One day before the start of the experiment, the 300 l were sequentially filtered through 0.4 and 0.2 µm filters using a Sartobran cartridge (150 cm^2, Sartorious), and the filtrate was stored at 4°C in the dark in acid-washed (10% HCl), Milli-Q water rinsed 20 l polycarbonate carboys. For the initial cultures, seawater (10 l) was collected at the start of the experiment on 6 November 2009 at the site described above at 5 m. The seawater was 0.8 µm filtered (PC, Nuclepore), 2 l of the filtrate were used as initial bacterial inoculum, and 8 l were filtered on a 0.2 µm cartridge (Sterivex, Millipore) and stored at −80°C for further analyses.

The exudate of the diatom *Phaeodactylum tricornutum* was used as a source of bioavailable DOM in one of the 2 experimental conditions. The *P. tricornutum* cells were grown in low (15 µM) concentrations of dissolved organic carbon (DOC) artificial seawater amended with nutrients in Guillard's F/2 medium. We did not autoclave the artificial seawater to avoid potential DOC contamination, and thus, the phytoplankton culture was not axenic. As a consequence, the most labile fraction of the DOM was probably degraded in the phytoplankton culture, and the exudate recovered at the end of the exponential growth phase was most likely dominated by semi-labile DOM. The culture was grown at 18°C under continuous light. A total culture volume of 12 l was prepared. Cell growth was followed by measuring the ab-

sorbance at 660 nm. The culture was sequentially filtered through a combusted GF/F filter (90 mm diameter, Whatman) and an acid-washed, Milli-Q rinsed 0.2 µm PC filter (90 mm diameter, Nuclepore). The filtrate was recovered in acid-washed, Milli-Q rinsed 4 l PC carboys and stored at 2°C during the entire experimental period. The DOC concentration in the exudate was 151 µM.

The continuous cultures were run under 2 conditions, each conducted in duplicate. In the control, the initial bacterial community was grown on seawater only, while in the +DOM treatment, the bacterial community was grown on seawater amended with DOM from the *Phaeodactylum tricornutum* culture. At the start of the experiment, the initial bacterial community was diluted 1:10 either with seawater (74 µM DOC concentration) or with a mixture of seawater and phytoplankton-derived DOM (87 µM final DOC concentration) prepared as described above. We added 13 µM of phytoplankton DOC to seawater because in the ocean this bioavailable resource makes up a relatively small fraction of bulk DOM (Benner 2002). The final volume of each of the bacterial cultures was 6.5 l. To ensure that organic carbon was the limiting factor for bacterial growth, nitrogen and phosphorus were added in excess to both the control and the +DOM treatment. The final concentrations in the initial cultures were 94 µM for nitrite and nitrate and 11 µM for phosphate. The input media for each of the 2 conditions consisted of either seawater or a mixture of seawater with phytoplankton-derived DOM. The concentration of inorganic nutrients was identical in both types of media (94 µM for nitrite and nitrate and 11 µM for phosphate). We prepared 4 l of each type of medium every 2 d and kept them in acid-washed PC carboys at 2°C before use. The 2 replicate cultures were supplied by a single PC carboy containing the input medium.

The cultures were run in a closed system consisting of 4 all-glass vessels (15 cm diameter, 50 cm height) connected to the medium by Teflon tubing. The continuous supply of input medium and the output of the cultures were conducted using a peristaltic pump (Gilson). The cultures were bubbled with filter-sterilized air (Sartorios). The bacterial cultures were run as a batch culture until the stationary phase was reached before starting the inflow of input medium and the outflow of culture. The dilution rate was 0.27 d^{-1}, resulting in a generation time of 2.6 d. We selected this dilution rate based on growth rates of 0.3 d^{-1} determined *in situ* for coastal Mediterranean waters (Obernosterer et al. 2010). The continuous cultures were run in a temperature-controlled room (18°C) in the dark.

The cultures were sampled daily during the 15 d experimental period. Samples were taken with glass syringes (Hamilton) connected with a Luer lock to Teflon tubes that reached the bottom of the cultures. This system allowed us to sample without opening the culture containers. The glass syringes were extensively rinsed with Milli-Q water prior to sampling. A total of ~100 ml was sampled every day in each culture, representing <2% of the total volume of the cultures.

Inorganic nutrients and dissolved organic carbon

For inorganic nutrients, subsamples (5 ml) were collected in 20 ml acid-washed polyethylene vials. Concentrations of nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) were determined immediately after sampling using the automated colorimetric technique on a Skalar autoanalyzer following the protocol of Tréguer & Le Corre (1975). For DOC, triplicate 15 ml subsamples were filtered through 2 precombusted GF/F filters (Whatman) and stored after acidification with orthophosphoric acid (H_3PO_4, final pH 2) in precombusted glass vials with Teflon-lined caps in the dark. DOC analyses were performed by high temperature catalytic oxidation on a Shimadzu TOC-V-CSH analyzer (Benner & Strom 1993). The analytical precision was 1 to 2% (coefficient of variation).

Enumeration of heterotrophic bacteria

For heterotrophic bacterial abundance, duplicate subsamples (1.8 ml) were fixed with formaldehyde (2% final concentration). The samples were incubated at 4°C for 30 min and stored at −80°C until flow cytometric analysis. Samples were thawed and stained with SYBR Green I (Invitrogen–Molecular Probes) at 0.025% (v/v) final concentration for 15 min at room temperature in the dark. Counts were performed with a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled argon laser (488 nm, 15 mW) (Lebaron et al. 2001).

Enzymatic activities

We measured the hydrolysis of the fluorogenic substrate analogues 4-methylumbelliferyl-α-D-gluco-

pyranoside and 4-methylumbelliferyl-β-D-glucopyranoside (MUF-α-Glc and MUF-β-Glc, respectively, Sigma Aldrich) to estimate potential activity rates of α- and β-glucosidases (Hoppe 1983). We followed a protocol from Van Wambeke et al. (2009) with some modifications. Triplicate subsamples (380 µl) of each culture were incubated with MUF-α-Glc and MUF-β-Glc prepared at 10 mM in methyl cellosolve after determining saturating concentrations. The final concentration of the substrate was 500 µM. The samples were incubated for 8 h in the dark at the same temperature as the cultures. For the 2 enzymes, a blank was prepared each time using sterile seawater and incubated under the same conditions as the samples from the cultures. Incubations were stopped by addition of alkaline (pH 10.5) glycine-ammonium hydroxide buffer (0.05 M glycine + 0.2 M NH_4OH) (10% final concentration). The samples were stored at −20°C until analysis. The samples were thawed at +55°C, and 550 µl of borate buffer (pH 10, Sigma-Aldrich) were added. Fluorescence was determined at 460 nm under 364 nm excitation on a fluorescence spectrophotometer (Hitachi F-2500).

Bacterial diversity

After ~3 bacterial generations (Day 8), samples for bacterial diversity (200 ml) were collected from the outflow in autoclaved PC flasks. Bacterial cells were collected on a 0.2 µm PC filter (47 mm diameter, Nuclepore), and the filters were stored at −80°C until further analysis. At the end of the experiment, corresponding to ~5 bacterial generations (Day 15), samples (1500 to 2300 ml) were collected in autoclaved PC flasks, concentrated on a 0.2 µm cartridge (Sterivex, Millipore) and stored at −80°C until further analysis.

DNA was extracted as described by Laghdass et al. (2011). Briefly, the bacterial cells collected on the filters were lysed using lysis buffer (40 mM EDTA, 50 mM Tris and 0.75 M sucrose) and performing 3 freeze-thaw cycles with liquid nitrogen and a water bath at 65°C. After denaturation and degradation of proteins using sodium dodecyl sulfate (1%) and Proteinase K (0.2 mg ml^{-1}), the DNA was purified using a Qiagen AllPrep DNA/RNA extraction kit according to the manufacturer's instructions. The molecular size and purity of the DNA were analyzed using agarose gel electrophoresis (1%). In total, 7 samples were extracted, and for each sample, a total amount of 600 ng was sent to the Research and Testing Laboratory (www.researchandtesting.com) for 454 tag pyrosequencing.

The bacterial 16S rRNA genes were sequenced using the FLX-Titanium platform as described by Wolcott et al. (2009). The regions V2 and V3 of the 16S rRNA gene were amplified using primers targeting 410 bp between positions 121 and 531 on the *Escherichia coli* reference sequence (Baker et al. 2003). For the 7 samples that were analyzed, a total number of 88 121 sequences was obtained. The raw data are deposited in the Euopean Nucleotide Archive under accession number ERP002553 and can be accessed at www.ebi.ac.uk/ena/data/view/ERP002553. All further analyses were conducted using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (http://qiime.org) (Caporaso et al. 2010). The sequences were quality filtered, and all sequences shorter than 200 bp or containing ambiguous bases were eliminated from the data set. Sequences were then attributed to each sample according to their barcode sequence. These operations led to the removal of ~25% of the sequences with either bad quality scores or ambiguous barcode sequences. The sequencing errors were corrected among the remaining sequences using the QIIME denoiser algorithm, and barcodes and primers were removed (Caporaso et al. 2010). Operational taxonomic units (OTUs) were identified using the uclust picking method and defined at a 97% sequence similarity level, and only the most abundant sequence was conserved as a representative. Taxonomy was assigned using the RDP classifier program (Wang et al. 2007) with the most recent Greengenes reference data set released in October 2012. The sequences were aligned using the PyNast algorithm (DeSantis et al. 2006, Caporaso et al. 2010). The chimeric sequences were identified and removed with ChimeraSlayer (Caporaso et al. 2010).

A phylogenetic tree was constructed using the FastTree method (Price et al. 2009), and an OTU table was created. To enable comparisons between samples, the OTU table was subsampled to ensure an equal number of sequences in each sample. The resulting OTU table comprised 3416 sequences per sample. All further analyses, such as alpha and beta diversity estimations, were conducted with this OTU table. Phylogenetic differences between samples were tested using Andrew Martin's phylogenetic test (P-test) (Martin 2002, Eckburg et al. 2005). The clustering of samples was checked for robustness with jackknife analyses and for significance with an ANOSIM. All statistics were performed using the QIIME pipeline.

RESULTS

Nutrient conditions and bulk bacterial response in the continuous cultures

The chemical parameters were stable for the 15 d experimental period. The sum of the concentrations of NO_3^- and NO_2^- was 93 ± 2 and 90 ± 3 µM (mean ± SD, n = 15) in the control and the +DOM treatment, respectively. The mean concentrations of PO_4^{3-} (11 ± 1 µM) were not different under the 2 conditions (data not shown). DOC concentrations in the cultures remained stable over time, with mean values of 74 ± 2 and 75 ± 2 µM in the control and the +DOM treatment, respectively (Table 1).

Organic carbon appeared to be the primary limiting factor of bacterial growth in the cultures. Ratios of DOC:(NO_3^- + NO_2^-) and DOC:PO_4^{3-} in the cultures were 0.8 and 7, respectively, throughout the experiment and thus well below the C:N:P ratios of heterotrophic bacteria (45:9:1) (Goldman et al. 1987). Concentrations of DOC in the input media were 74 ± 3 and 87 ± 7 µM for the control and the +DOM treatment, respectively. The difference in the DOC concentrations between the input medium and the DOM-amended cultures demonstrates that bacteria rapidly utilized the added DOC. In the control, DOC utilization was below our limit of detection.

Bacterial abundances increased exponentially in all cultures during the first 24 h when the system was maintained as a batch. After the initial growth phase, bacterial abundances stabilized in 3 out of 4 cultures.

Table 1. Basic biogeochemical parameters in the continuous culture system. Concentrations of dissolved organic carbon (DOC) are given for the input media and the cultures. Mean values ± SD of measurements performed during the 15 d experimental period are given. For the +DOM treatment, the mean value of the replicate incubations was used at each time point

	Control	+DOM
Input medium		
DOC (µM)	74 ± 3 (n = 4)	87 ± 7 (n = 4)
Culture		
DOC (µM)	74 ± 2 (n = 10)	75 ± 2 (n = 10)
Bacterial abundance (10^6 cell ml^{-1})	2.6 ± 0.8 (n = 15)	4.3 ± 0.5 (n = 15)
α-glucosidase activity (amol $cell^{-1}$ h^{-1})	0.13 ± 0.1 (n = 8)	1.17 ± 0.2 (n = 8)
β-glucosidase activity (amol $cell^{-1}$ h^{-1})	0.18 ± 0.1 (n = 8)	1.23 ± 0.2 (n = 8)

In one of the control cultures, bacterial abundance decreased over time, and this replicate had to be discarded for further analysis. Bacterial abundances were 3.9 ± 0.9 × 10^6 and 4.6 ± 0.7 × 10^6 cells ml^{-1} in Replicates 1 and 2 of the +DOM treatment, respectively (mean ± SD, n = 15). The bacterial abundance in each of the replicate +DOM cultures was significantly higher (1.5- and 1.7-fold, respectively; Wilcoxon test, $p < 0.008$) than the bacterial abundance in the control culture (2.6 ± 0.8 × 10^6 cells ml^{-1}) (Table 1). This response of heterotrophic bacteria to the addition of DOM was also reflected in bacterial enzymatic activities. α- and β-glucosidase activities were significantly different between conditions (Wilcoxon test, $p < 0.004$), with roughly 10-fold higher cell-specific activities in the +DOM treatment (Table 1).

Effect of DOM addition on the bacterial diversity

We explored bacterial diversity and composition after 3 and 5 bacterial generations by tag pyrosequencing the 16S rRNA gene and clustering the sequences into OTUs at >97% identity. In total, 507 OTUs were obtained from the clustering of the sequences, and the total number of OTUs for the initial community was 254. In the control, the number of OTUs was 62 and 67 after 3 and 5 generations, respectively. A higher number of OTUs was observed in the +DOM treatment both after 3 generations (118 in Replicate 1 and 119 in Replicate 2) and after 5 generations (80 in Replicate 1 and 109 in Replicate 2). A dendrogram based on a weighted UNIFRAC similarity matrix, using the relative abundance of each OTU and phylogenetic affiliation, illustrates that time was the major factor in determining the differences among the bacterial communities. At each time point, however, the bacterial communities grown on the DOM-amended seawater clustered separately from the bacterial communities grown on seawater alone (Fig. 1). This clustering was robust, as confirmed by jackknife analyses and statistical examination (ANOSIM, $p = 0.01$).

We further compared the dissimilarity between replicate communities and also between the communities in the DOM-amended cultures versus the control, using the P-test (Martin 2002). At both time points, the within-treatment variability between the 2 DOM-amended replicate cultures (7 and 3% after 3 and 5 generations, respectively) was not significant (P-test; $p > 0.08$). In contrast, the dissimilarity between

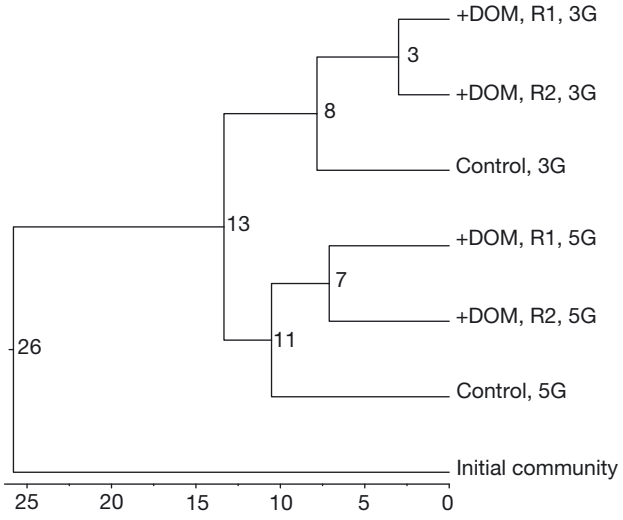

Fig. 1. UPGMA analysis of bacterial communities sampled in the continuous cultures after 3 (3G) and 5 generations (5G). The dendrogram was constructed using the average linkage method on a weighted UNIFRAC similarity matrix. R1 and R2: Replicates 1 and 2 for the +DOM treatment. Values on the scale bar and the nodes indicate the percentage of dissimilarity

the DOM-amended cultures and the control was significant for Replicates 1 and 2 after 3 generations and for Replicate 1 after 5 generations ($p < 0.02$). Taken together, our results indicate that at each time point, the differences in bacterial community structure between the 2 experimental conditions were due to a response to the DOM addition. Interestingly, a dendrogram constructed from an unweighted UNIFRAC similarity matrix, which does not consider the relative abundance of each OTU but considers only its presence or absence and phylogenetic affiliation, revealed an inverse pattern in which communities clustered first according to the experimental condition and then according to time (data not shown). This pattern suggests that the presence of OTUs was mainly determined by the experimental condition, whereas their abundance was mainly determined by time. With either analysis, the data indicate the importance of the DOM addition in structuring bacterial communities in these continuous cultures.

To better understand the changes induced by the DOM addition, we calculated the Chao1 index for bacterial richness and the phylogenetic distance. Overall, the rarefaction curves of the bacterial communities grown in the continuous cultures reached a plateau, indicating that the sampling was deep enough to cover most of the diversity (Fig. 2). The indices for bacterial richness and phylogenetic distance were higher in the DOM-amended cultures than in the control culture at both time points. Richness decreased between the third and fifth generation, but the DOM-amended cultures were still more diverse (Fig. 2a). Phylogenetic distance was almost twice as high in the DOM-amended cultures than in the control culture, with remarkable steadiness over time (Fig. 2b). Overall, these indices were lower in the cultures compared to the inoculum (Chao1: 753; phylogenetic distance: 28).

We investigated the OTUs that were potentially selected by the DOM addition. The identification of an OTU deemed specific to one experimental condition was based on its presence in one condition and its absence from the other. OTUs present in all 3 cultures were considered common. The phylogenetic diversity of the OTUs specific to the +DOM treatment was higher than in the control (Fig. 3). *Alphaproteobacteria*, *Bacteroidetes*, *Verrucomicrobia*, and other groups made up the largest part (50% after 3 and

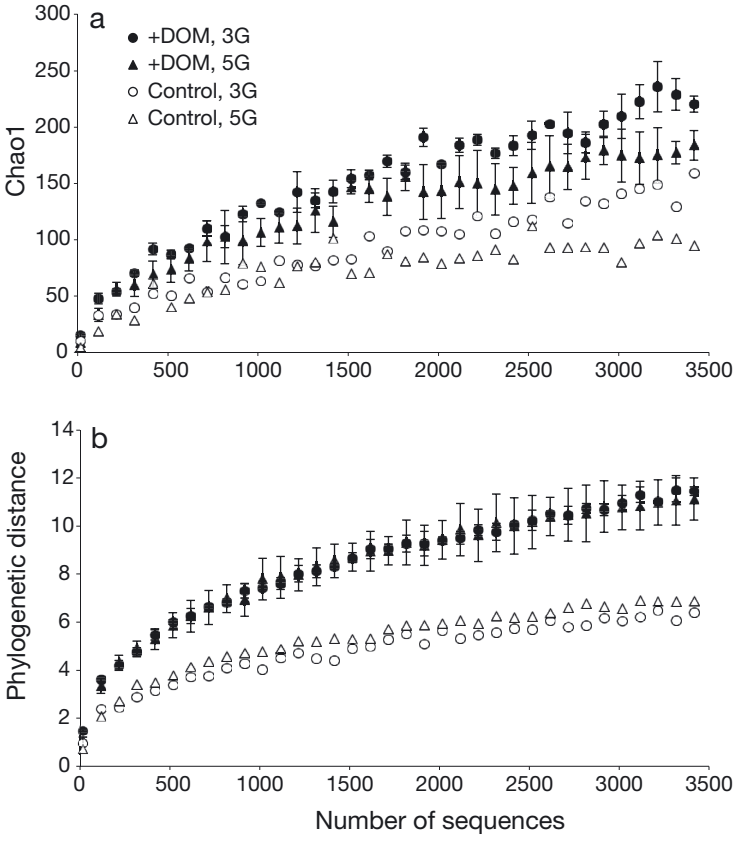

Fig. 2. Rarefaction curves of the diversity indices (a) Chao1 and (b) phylogenetic distance after 3 (3G) and 5 generations (5G). For the +DOM treatment, mean values ± SD are given

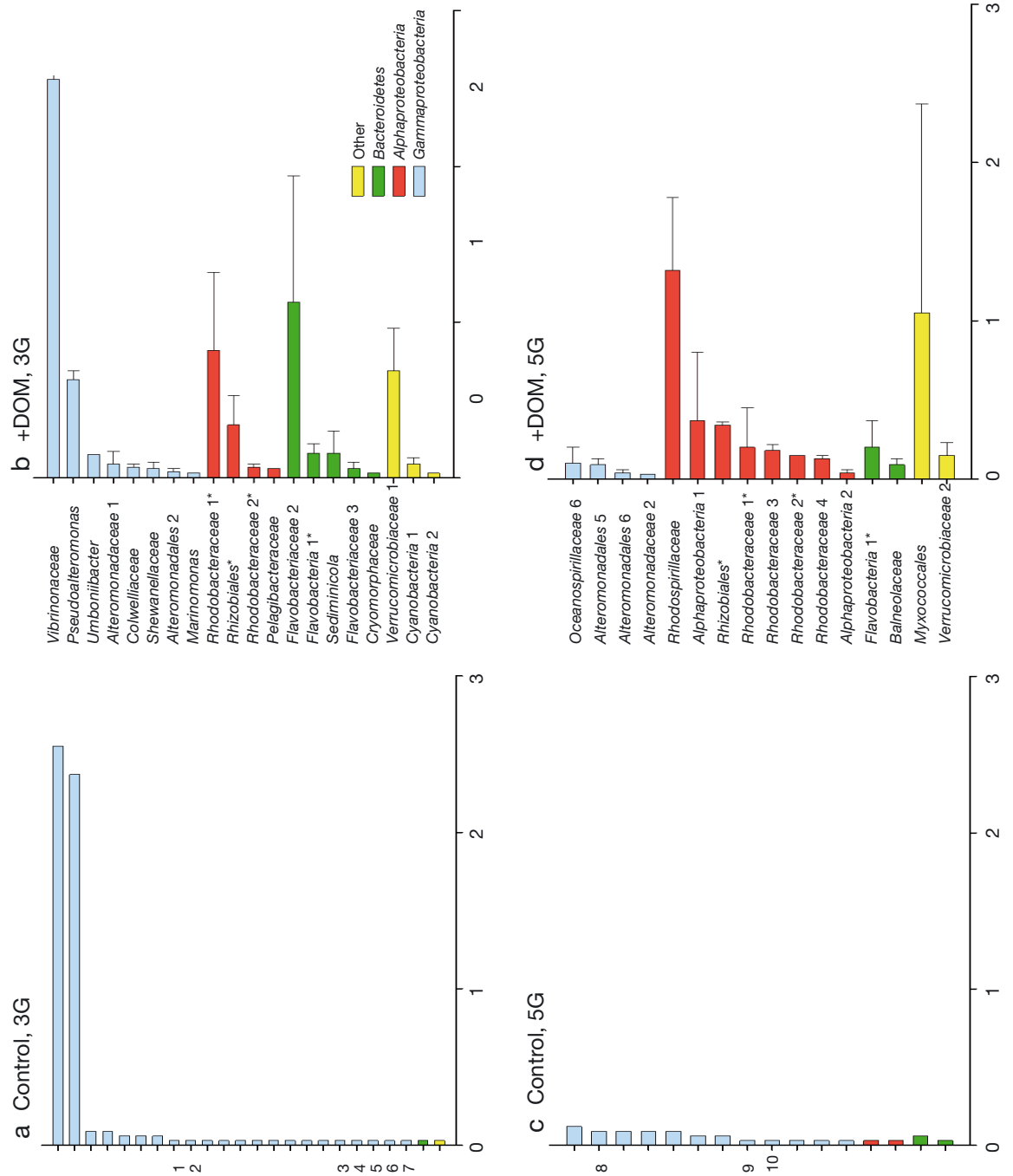

Fig. 3. Percentage of the total number of sequences (3416) of operational taxonomic units (OTUs) specific to (a,c) the control or to (b,d) the +DOM cultures after (a,b) 3 and (c,d) 5 generations (3G and 5G, respectively). For the +DOM treatment, mean values ± SD between replicates are given. The name of each OTU corresponds to its taxonomic assignment. The OTUs with identical taxonomy are differentiated by numbers. OTUs that are marked with an asterisk (*) are those that were found specific at both time points

94% after 5 generations) of the OTUs specific to the DOM-amended treatment. In contrast, *Gammaproteobacteria* accounted for 99 and 83% of the control-specific OTUs after 3 and 5 generations, respectively.

Bacterial community composition

As indicated by the clustering of samples (Fig. 1), the communities selected by our experimental system largely differed from the initial community (Fig. 4, see Table S1 in the Supplement at www.int-res.com/articles/suppl/a069p157_supp.pdf). The bacterial communities present in both the control and the +DOM treatment were dominated by *Gammaproteobacteria*, belonging mainly to 3 orders: *Alteromonadales*, *Oceanospirillales*, and *Thiotrichales* (Fig. 4a, Table S2 in the Supplement). However, their relative abundance was lower in the DOM-amended cultures than in the control due to a more pronounced response of other bacterial groups to the culture conditions. The relative contribution of *Alphaproteobacteria* after 3 and 5 generations was 3- and 2-fold higher in the DOM-amended treatment than in the control (Fig. 4b, Table S3 in the Supplement). This pattern was observed for the *Rhodobacterales*, *Rhodospirillales*, and other groups within the *Alphaproteobacteria*. The proportion of sequences belonging to several other bacterial groups, in particular *Bacteroidetes* and *Verrucomicrobia*, was also noticeably enhanced in the +DOM treatment (Fig. 4c,d, Tables S4 & S5 in the Supplement). A maximum of 0.1% of the sequences detected in a single sample could not be properly characterized even to the phylum level. Overall, the taxonomic affiliation of the sequences retrieved from our cultures confirms the higher phylogenetic diversity in the DOM-amended cultures illustrated in Fig. 2b.

DISCUSSION

Bacterial diversity, including richness and phylogenetic diversity, increased in response to an enhanced supply in bioavailable DOM in our experiment. We chose continuous cultures to provide insight into how a sustained input of DOM under C-limited conditions affects the structure of a natural bacterial community. We were successful in maintaining both stable abiotic conditions and organic carbon as the growth-limiting factor throughout the experimental period. As a selective tool, continuous cultures set a lower limit for the growth rate through the dilution rate (Kovárová-Kovar & Egli 1998), restricting bacterial growth to the groups that are most responsive or adapted to the culture conditions, as reflected by the lower diversity in the cultures compared to the inoculum. Our result thus reflects the adaptation of a bacterial community after 3 and 5 generation times to a small increase in a mixture of organic carbon substrates of varying bioavailability and chemical composition.

Continuous cultures are technically challenging, and the large volumes used for each culture in the present study together with the duration of the experiment made it difficult to set up more than 2 replicate conditions. The patterns in diversity were repeatable for the replicate +DOM cultures and for the samples taken after 3 and 5 generations of each condition, indicating that our conclusions are robust. The observation that the response to the DOM addition in terms of bacterial biomass, metabolism, and diversity was the same over time supports the hypothesis that differences between the +DOM and the control were real rather than due to chance. For all these reasons, we have confidence in the validity of our conclusions, despite the loss of 1 control replicate.

The surprising finding of the present study is the increase in diversity, including richness and phylogenetic diversity, of the bacterial communities in the phytoplankton DOM-amended cultures. We consider 2 possible explanations for this observation. The increase in bacterial richness could be linked to the higher DOC concentrations due to the DOM addition. A previous study using a DNA fingerprinting method found changes in bacterial diversity with increasing DOC (40 to 540 µM) (Eiler et al. 2003). In the present study, the total concentration only partly explains our results because the addition of DOM changed DOC concentrations only slightly (13 µM added to the original 74 µM). By contrast, the phytoplankton DOM most likely had a different chemical composition and bioavailability than the seawater DOM that had been aged for 6 wk prior to the start of the experiment. Carbohydrates contribute substantially to DOM from phytoplankton (Biddanda & Benner 1997, Aluwihare & Repeta 1999). Molecular-level analyses have identified the same individual sugars in phytoplankton DOM and surface DOM; however, their relative contributions and in particular the DOC yields vary greatly between DOM sources (Biersmith & Benner 1998, Aluwihare & Repeta 1999). The higher glucosidase activities are thus likely a response to higher concentrations of oligo- and polysaccharides in our DOM-amended cultures, supporting the idea that DOM composition was different between the condi-

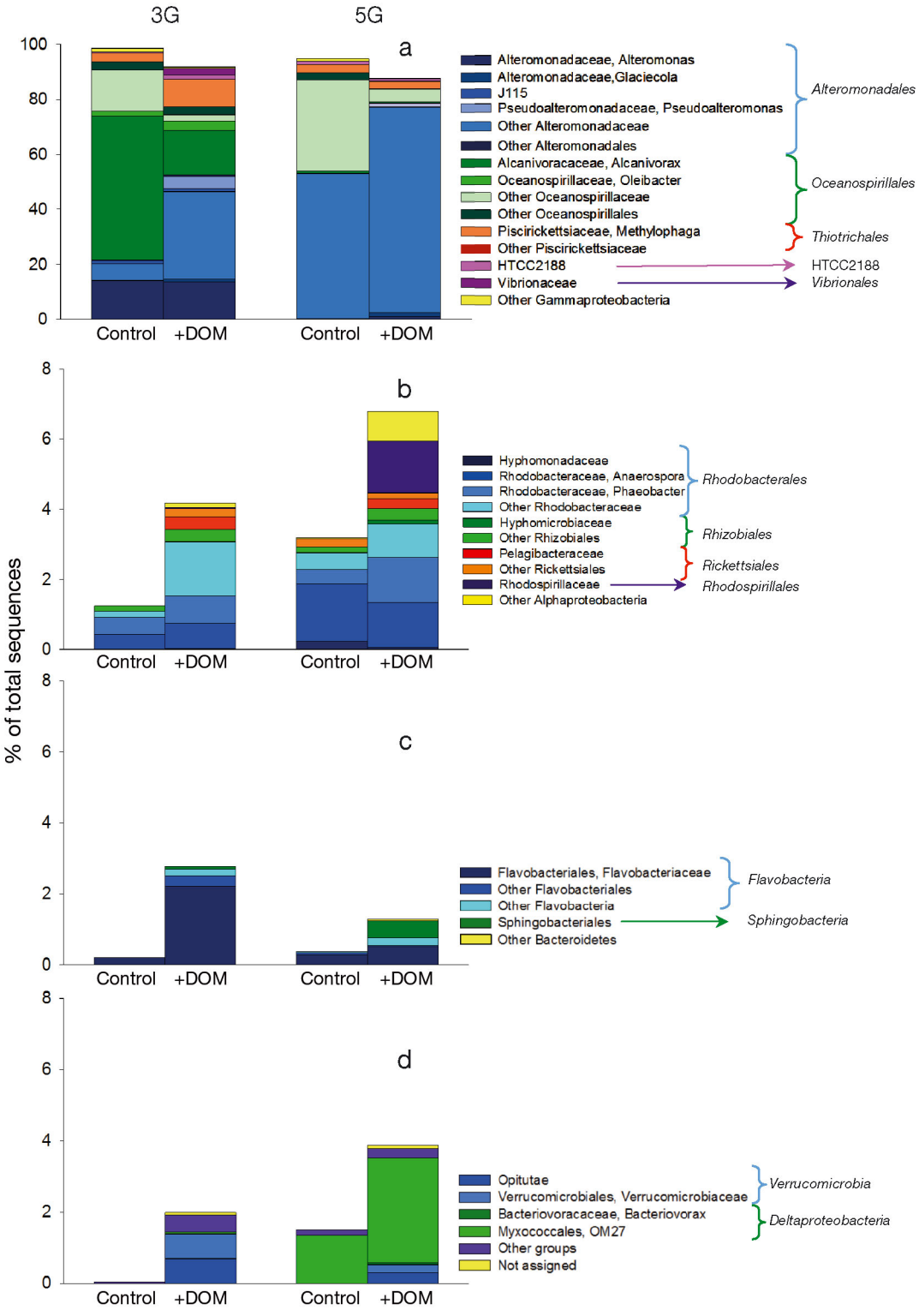

Fig. 4. Percentage of the total made up by the indicated bacterial groups in the communities present in the continuous cultures after 3 and 5 generations (3G and 5G, respectively). Bacterial groups belonging to (a) *Gammaproteobacteria*, (b) *Alphaproteobacteria*, (c) *Bacteroidetes*, and (d) other groups are shown. For (a) *Gammaproteobacteria* and (b) *Alphaproteobacteria*, the different colors correspond to different orders within these classes. For (c) *Bacteroidetes* and (d) other groups, different colors correspond to different phyla and classes. For the +DOM treatment, the mean percentage of the 2 replicates was calculated. Note the different scales on the *y*-axis. Detailed phylogenetic information is given in Tables S2 to S5 in the Supplement

tions. The compounds present in the phytoplankton DOM could act as new niches, based on different chemical composition and higher concentrations, sustaining growth of bacterial groups that were less successful in the culture provided with seawater only. The higher number of OTUs in response to an increase in bioavailable DOM could be due to a direct link between the identity and diversity of the substrates present in the added DOM and functional properties of the selected bacterial groups.

Independent of treatment, *Gammaproteobacteria* represented the majority of bacteria selected by our experimental system. This result is not surprising because *Gammaproteobacteria*, such as *Vibrio* and *Alteromonas*, are frequently observed in high abundances in incubation experiments (Eilers et al. 2000, Pinhassi & Berman 2003, Nelson & Carlson 2012). These previous studies and the present one clearly point to their opportunistic life style, characterized by rapid growth and their ability to quickly take up a range of substrates (Lauro et al. 2009). *Gammaproteobacteria* highly expressed transporter genes linked to broad carbon uptake patterns during an *in situ* phytoplankton bloom (Teeling et al. 2012), and members of this phylum had some of the most diverse transcriptomes for a variety of metabolic processes in a coastal bacterial community (Gifford et al. 2012). The high abundance of *Gammaproteobacteria* in our continuous cultures and their low contribution to the +DOM-specific community indicate that this group responded to the culture conditions rather than to the DOM input. This likely reflects their capability to rapidly adapt to a variety of compounds in the original seawater DOM and also in the phytoplankton DOM.

Rhodobacterales, *Rhodospirillales*, and *Rickettsiales* belonging to *Alphaproteobacteria*, *Flavobacteria* belonging to *Bacteroidetes*, and *Verrucomicrobia* responded most to the DOM addition. These bacterial groups have been shown to respond to *in situ* phytoplankton blooms and blooms simulated in mesocosm studies (Pinhassi et al. 2004, Rink et al. 2007, West et al. 2008). Communities specific to a given diatom culture were mainly composed of *Alphaproteobacteria* and *Bacteroidetes* and to a lesser extent of *Gamma-* and *Betaproteobacteria* (Schäfer et al. 2002, Grossart et al. 2005). The *Roseobacter* clade contributed substantially to the overall carbohydrate uptake during a mesocosm experiment with phytoplankton DOM, as evidenced by highly expressed DOC-related transporter genes (Poretsky et al. 2010). *Bacteroidetes* and in particular *Flavobacteria* were found to produce specific enzymes, such as fucosidases and sulfatases, involved in the degradation of polysaccharides produced during an *in situ* diatom bloom (Teeling et al. 2012). Using stable isotope probing, Nelson & Carlson (2012) observed that OTUs belonging to *Flavobacteria* and *Verrucomicrobia* substantially contributed to the incorporation of a *Synechococcus* exudate. These latter studies suggest a function-related response of bacterial groups to phytoplankton DOM.

The question of how specific the utilization of DOM is to a given bacterial group has been addressed in several previous studies (Malmstrom et al. 2005, Alonso-Sáez & Gasol 2007, Mou et al. 2008, Gómez-Consarnau et al. 2012, Sarmento & Gasol 2012). These studies demonstrated that the potential to utilize a variety of simple, monomeric substrates is widespread among bacterial groups but that affinities for a given substrate might vary (Alonso-Sáez & Gasol 2007, Mou et al. 2008, Gómez-Consarnau et al. 2012). Mixed substrate sources of high biological availability, such as DOM from phytoplankton exudates (Nelson & Carlson 2012, Sarmento & Gasol 2012) or extracellular polymeric substances (Elifantz et al. 2005), are utilized by a diverse bacterial community, perhaps reflecting substrate complexity. By investigating how a natural DOM source selects from a bacterial community in a constantly C-limited system over several generation times, our experiment complements these previous studies. Our results provide a first mechanistic insight into this issue, by investigating the potential adaption of a bacterial community at both the community and the OTU level to a natural DOM source.

Bacterial diversity in aquatic systems is maintained by several bottom-up and top-down factors. Resource competition is common in the oligotrophic ocean, and access to bioavailable organic carbon often limits bacterial growth (Kirchman & Rich 1997, Church et al. 2000, Van Wambeke et al. 2002). We wanted to test the idea of a DOM-stimulated modification of bacterial diversity using an experimental approach that focuses on DOM–bacteria interaction, while maintaining most other potential driving factors constant. The increase in diversity we observed in our experiment might be counteracted in the environment by factors such as top-down control, inorganic nutrient limitation, or a combination of these or other factors. Whether the results from our experiment performed with a bacterial community from the NW Mediterranean Sea are valid for other marine environments and the extent to which different sources of DOM contribute to the maintenance of bacterial diversity should be the focus of future research.

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LITERATURE CITED

Alonso-Sáez L, Gasol JM (2007) Seasonal variations in the contributions of different bacterial groups to the uptake of low-molecular-weight compounds in Northwestern Mediterranean Coastal Waters. Appl Environ Microbiol 73:3528–3535

Aluwihare LI, Repeta DJ (1999) A comparison of the chemical characteristics of oceanic DOM and extracellular DOM produced by marine algae. Mar Ecol Prog Ser 186: 105–117

Baker GC, Smith JJ, Cowan DA (2003) Review and re-analysis of domain-specific 16S primers. J Microbiol Methods 55:541–555

Bauer M, Kube M, Teeling H, Richter M and others (2006) Whole genome analysis of the marine *Bacteroidetes* '*Gramella forsetii*' reveals adaptations to degradation of polymeric organic matter. Environ Microbiol 8:2201–2213

Benner R (2002) Chemical composition and reactivity. In: Hansell DA, Carlson CA (eds) Biogeochemistry of marine dissolved organic matter. Academic Press, San Diego, CA, p 59–90

Benner R, Strom M (1993) A critical evaluation of the analytical blank associated with DOC measurements by high-temperature catalytic oxidation. Mar Chem 41:153–160

Biddanda B, Benner R (1997) Carbon, nitrogen, and carbohydrate fluxes during the production of particulate and dissolved organic matter by marine phytoplankton. Limnol Oceanogr 42:506–518

Biersmith A, Benner R (1998) Carbohydrates in phytoplankton and freshly produced dissolved organic matter. Mar Chem 63:131–144

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K and others (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336

Church MJ, Hutchins DA, Ducklow HW (2000) The limitation of bacterial growth by dissolved organic matter and iron in the Southern Ocean. Appl Environ Microbiol 66: 455–466

Cottrell MT, Kirchman DL (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

Covert JS, Moran MA (2001) Molecular characterization of estuarine bacterial communities that use high-and low-molecular weight fractions of dissolved organic carbon. Aquat Microb Ecol 25:127–139

DeSantis TZ Jr, Hugenholtz P, Keller K, Brodie EL and others (2006) NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Res 34:W394–W399

Eckburg PB, Bik EM, Bernstein CN, Purdom E and others (2005) Diversity of the human intestinal microbial flora. Science 308:1635–1638

Eiler A, Langenheder S, Bertilsson S, Tranvik LJ (2003) Heterotrophic bacterial growth efficiency and community structure at different natural organic carbon concentrations. Appl Environ Microbiol 69:3701–3709

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

Elifantz H, Malmstrom RR, Cottrell MT, Kirchman DL (2005) Assimilation of polysaccharides and glucose by major bacterial groups in the Delaware Estuary. Appl Environ Microbiol 71:7799–7805

Fuhrman JA, Hewson I, Schwalbach MS, Steele JA, Brown MV, Naeem S (2006) Annually reoccurring bacterial communities are predictable from ocean conditions. Proc Natl Acad Sci USA 103:13104–13109

Gifford SM, Sharma S, Booth M, Moran MA (2013) Expression patterns reveal niche diversification in a marine microbial assemblage. ISME J 7:281–298

Gilbert JA, Steele JA, Caporaso JG, Steinbrück L and others (2012) Defining seasonal marine microbial community dynamics. ISME J 6:298–308

Giovannoni S, Rappé MS (2000) Evolution, diversity, and molecular ecology of marine prokaryotes. In: Kirchman DL (ed) Microbial ecology of the oceans. Wiley-Blackwell, Hoboken, NJ, p 47–84

Goldman JC, Caron DA, Dennett MR (1987) Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C:N ratio. Limnol Oceanogr 32: 1239–1252

Gómez-Consarnau L, Lindh MV, Gasol JM, Pinhassi J (2012) Structuring of bacterioplankton communities by specific dissolved organic carbon compounds. Environ Microbiol 14:2361–2378

Gómez-Pereira PR, Schüler M, Fuchs BM, Bennke C and others (2012) Genomic content of uncultured *Bacteroidetes* from contrasting oceanic provinces in the North Atlantic Ocean. Environ Microbiol 14:52–66

Grossart HP, Levold F, Allgaier M, Simon M, Brinkhoff T (2005) Marine diatom species harbour distinct bacterial communities. Environ Microbiol 7:860–873

Hoppe HG (1983) Significance of exoenzymatic activities in the ecology of brackish water: measurements by means of methylumbelliferyl-substrates. Mar Ecol Prog Ser 11: 299–308

Jurado E, Dachs J, Duarte CM, Simó R (2008) Atmospheric deposition of organic and black carbon to the global oceans. Atmos Environ 42:7931–7939

Kirchman DL, Rich J (1997) Regulation of bacterial growth rates by dissolved organic carbon and temperature in the equatorial Pacific Ocean. Microb Ecol 33:11–20

Kirchman DL, Dittel AI, Findlay SEG, Fischer D (2004) Changes in bacterial activity and community structure in response to dissolved organic matter in the Hudson River, New York. Aquat Microb Ecol 35:243–257

Kovárová-Kovar K, Egli T (1998) Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. Microbiol Mol Biol Rev 62:646–666

Laghdass M, Blain S, Besseling M, Catala P, Guieu C, Obernosterer I (2011) Effects of Saharan dust on the microbial community during a large *in situ* mesocosm experiment in the NW Mediterranean Sea. Aquat Microb Ecol 62: 201–213

Lauro FM, McDougald D, Thomas T, Williams TJ and others (2009) The genomic basis of trophic strategy in marine bacteria. Proc Natl Acad Sci USA 106:15527–15533

Lebaron P, Servais P, Agogue H, Courties C, Joux F (2001) Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? Appl Environ Microbiol 67:1775–1782

Logue JB, Langenheder S, Andersson AF, Bertilsson S and others (2012) Freshwater bacterioplankton richness in oligotrophic lakes depends on nutrient availability rather than on species–area relationships. ISME J 6:1127–1136

Malmstrom RR, Cottrell MT, Elifantz H, Kirchman DL (2005) Biomass production and assimilation of dissolved organic matter by SAR11 bacteria in the Northwest Atlantic Ocean. Appl Environ Microbiol 71:2979–2986

Martin AP (2002) Phylogenetic approaches for describing and comparing the diversity of microbial communities. Appl Environ Microbiol 68:3673–3682

McCarren J, Becker JW, Repeta DJ, Shi Y and others (2010) Microbial community transcriptomes reveal microbes and metabolic pathways associated with dissolved organic matter turnover in the sea. Proc Natl Acad Sci USA 107:16420–16427

Mou X, Sun S, Edwards RA, Hodson RE, Moran MA (2008) Bacterial carbon processing by generalist species in the coastal ocean. Nature 451:708–711

Naudin JJ, Cauwet G, Chrétiennot-Dinet MJ, Deniaux B, Devenon JL, Pauc H (1997) River discharge and wind influence upon particulate transfer at the land–ocean interaction: case study of the Rhone River plume. Estuar Coast Shelf Sci 45:303–316

Nelson CE, Carlson CA (2012) Tracking differential incorporation of dissolved organic carbon types among diverse lineages of Sargasso Sea bacterioplankton. Environ Microbiol 14:1500–1516

Obernosterer I, Lami R, Larcher M, Batailler N, Catala P, Lebaron P (2010) Linkage between bacterial carbon processing and the structure of the active bacterial community at a coastal site in the NW Mediterranean Sea. Microb Ecol 59:428–435

Pinhassi J, Berman T (2003) Differential growth response of colony-forming α- and γ-proteobacteria in dilution culture and nutrient addition experiments from Lake Kinneret (Israel), the eastern Mediterranean Sea, and the Gulf of Eilat. Appl Environ Microbiol 69:199–211

Pinhassi J, Sala MM, Havskum H, Peters F and others (2004) Changes in bacterioplankton composition under different phytoplankton regimens. Appl Environ Microbiol 70: 6753–6766

Poretsky RS, Sun S, Mou X, Moran MA (2010) Transporter genes expressed by coastal bacterioplankton in response to dissolved organic carbon. Environ Microbiol 12: 616–627

Price MN, Dehal PS, Arkin AP (2009) FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol 26:1641–1650

Ramette A (2007) Multivariate analyses in microbial ecology. FEMS Microbiol Ecol 62:142–160

Rink B, Seeberger S, Martens T, Duerselen CD, Simon M, Brinkhoff T (2007) Effects of phytoplankton bloom in a coastal ecosystem on the composition of bacterial communities. Aquat Microb Ecol 48:47–60

Rinta-Kanto JM, Sun S, Sharma S, Kiene RP, Moran MA (2012) Bacterial community transcription patterns during a marine phytoplankton bloom. Environ Microbiol 14: 228–239

Sarmento H, Gasol JM (2012) Use of phytoplankton-derived dissolved organic carbon by different types of bacterioplankton. Environ Microbiol 14:2348–2360

Schäfer H, Abbas B, Witte H, Muyzer G (2002) Genetic diversity of 'satellite' bacteria present in cultures of marine diatoms. FEMS Microbiol Ecol 42:25–35

Sogin ML, Morrison HG, Huber JA, Welch DM and others (2006) Microbial diversity in the deep sea and the underexplored 'rare biosphere'. Proc Natl Acad Sci USA 103: 12115–12120

Teeling H, Fuchs B, Becher D, Klockow C and others (2012) Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. Science 336:608–611

Teira E, Reinthaler T, Pernthaler A, Pernthaler J, Herndl G (2004) Combining catalyzed reporter deposition-fluorescence *in situ* hybridization and microautoradiography to detect substrate utilization by *Bacteria* and *Archaea* in the deep ocean. Appl Environ Microbiol 70: 4411–4414

Tréguer P, Le Corre P (1975) Manuel d'analyse des sels nutritifs dans l'eau de mer (utilisation de l'autoanalyzer II Technicon R). UBO, Brest

Van Hannen EJ, Mooij W, Van Agterveld MP, Gons HJ, Laanbroek HJ (1999) Detritus-dependent development of the microbial community in an experimental system: qualitative analysis by denaturing gradient gel electrophoresis. Appl Environ Microbiol 65:2478–2484

Van Wambeke F, Christaki U, Giannakourou A, Moutin T, Souvemerzoglou K (2002) Longitudinal and vertical trends of bacterial limitation by phosphorus and carbon in the Mediterranean Sea. Microb Ecol 43:119–133

Van Wambeke F, Ghiglione JF, Nedoma J, Mével G, Raimbault P (2009) Bottom up effects on bacterioplankton growth and composition during summer-autumn transition in the open NW Mediterranean Sea. Biogeosciences 6:705–720

Vila-Costa M, Pinhassi J, Alonso C, Pernthaler J, Simó R (2007) An annual cycle of dimethylsulfoniopropionate-sulfur and leucine assimilating bacterioplankton in the coastal NW Mediterranean. Environ Microbiol 9: 2451–2463

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267

West NJ, Obernosterer I, Zemb O, Lebaron P (2008) Major differences of bacterial diversity and activity inside and outside of a natural iron-fertilized phytoplankton bloom in the Southern Ocean. Environ Microbiol 10:738–756

Wolcott RD, Gontcharova V, Sun Y, Dowd SE (2009) Evaluation of the bacterial diversity among and within individual venous leg ulcers using bacterial tag-encoded FLX and Titanium amplicon pyrosequencing and metagenomics approaches. BMC Microbiol 9:226

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