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.
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' 30"N, 03° 15' 50"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 cm², 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 filtered 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 (NO₃⁻), nitrite (NO₂⁻) and phosphate (PO₄³⁻) 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 (H₃PO₄, 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-gluc-
pyranoside and 4-methylumbelliferyl-β-D-glucopy-
ranoside (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 NH₄OH) (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 European 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 Chimera-Slayer (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 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. 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$^5$ 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

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<th>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</th>
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<td><strong>Input medium</strong></td>
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<td><strong>Culture</strong></td>
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<td>DOC (µM)</td>
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<td>Bacterial abundance (10$^6$ cell ml$^{-1}$)</td>
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<td>α-glucosidase activity (amol cell$^{-1}$ h$^{-1}$)</td>
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<td>β-glucosidase activity (amol cell$^{-1}$ h$^{-1}$)</td>
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the continuous cultures reached a plateau, curves of the bacterial communities grown in Chao1 index for bacterial richness and the by the DOM addition, we calculated the tion in structuring bacterial communities in data indicate the importance of the DOM addi-
2 experimental conditions were due to a re-
in bacterial community structure between the differences were 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. 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.](image)

![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](image)
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: Alphamonadales, 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
