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Phosphate addition has minimal short-term effects on bacterioplankton community structure of the P-starved Eastern Mediterranean

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ABSTRACT: The effect of the addition of a limiting nutrient to the composition and structure of bacterial communities in oligotrophic environments remains an interesting question. We conducted a mesocosm experiment to study short-term effects of phosphorus addition on bacterioplankton communities in the Eastern Mediterranean, which is a phosphorus-starved ecosystem. Inorganic phosphorus was added to 10 m deep pelagic water which was transported from offshore Crete, Greece, to mesocosms to yield a final concentration of 100 nM. Using 454 16S amplicon sequencing, we found that phosphorus addition during the first 72 h of the experiment had a minimal effect on bacterioplankton community composition and structure, affecting mostly the abundance of the 'non-unique' members of the community.

KEY WORDS: Mediterranean · Phosphorus · Mesocosm · Bacterioplankton communities

INTRODUCTION

The Eastern Mediterranean Sea is one of the world's most oligotrophic marine areas (Krom et al. 2004). Bacterial production and growth in the Eastern Mediterranean are considered to be phosphorus limited (Van Wambeke et al. 2002, Thingstad et al. 2005), with phosphorus concentrations typically varying between 20 and 90 nM (Zohary & Robarts 1998). Phosphorus addition during a Lagrangian *in situ* experiment in the Eastern Mediterranean showed that there was substantial increase in bacterial production, but not bacterial biomass, within 3 d after the treatment (Pitta et al. 2005), indicating phosphorus limitation. A microcosm experiment showed nitrogen and phosphorus co-limitation of the phytoplankton community (Zohary et al. 2005), but particulate primary production was found to be unaffected by phosphorus addition alone (Lagaria et al. 2011).

In 2009, an *ex-situ* P-addition experiment was conducted in mesocosm scale in the Eastern Mediterranean (Crete, Greece). This experiment verified that different bacterial phylogroups had differential phosphorus uptake rates: members of the SAR11 clade had fulfilled their needs for phosphate by Day 1, but *Gammaproteobacteria* and *Roseobacter* clades were actively taking up phosphate until Day 4. Moreover, an increase of 65 and 44 % was observed in bacterial biomass and production respectively, on Day 3 in the P-treated bags compared to the controls (Sebastián et al. 2012). In the same experiment, we investigated changes in community composition and structure that may had occurred in bacterioplankton after phosphorus addition. We examined these changes during the first 3 d of the same experiment

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in a fine scale, using 16S rRNA pyrosequencing amplicon analysis of the V3 hypervariable region.

MATERIALS AND METHODS

Experimental design and sampling

The mesocosm experiment took place in September 2009 at the CRETACOSMOS mesocosm facilities of the Hellenic Centre for Marine Research, Crete, Greece. Briefly, water for the experiment (ca. 18 m^3) was collected on 14 September 2009, aboard the R/V 'Philia' (Hellenic Centre for Marine Research), from a site 170 m deep and about 5 nautical miles north of Heraklion, Crete, Greece (35° 24.957′ N, 25° 14.441′ E). Water from ca. 10 m depth was pumped into high-density polyethylene (PE) tanks of 1 m^3 using a submersible water pump. All equipment had been submerged for at least 1 wk in tap water; the tanks were washed with hydrochloric acid (HCl) (10%) and rinsed with deionized water. The water was gravity poured into 6 mesocosms (made of transparent PE bags with a diameter of 1.32 m and a depth of 2.3 m) of 3 m^3 each, all incubated in a 350 m^3 concrete tank with running seawater at *in situ* temperature (25.6°C) during the entire experiment in order to avoid an increase in temperature. The water was left overnight to settle, and on the next day (Day 0), phosphate was added (once) to 3 mesocosms to a final concentration of 100 nM, in the form of monobasic potassium phosphate (KH_2PO_4). The other 3 mesocosms were used as controls (no P addition). Phosphate was below the detection limit of the micromolar method at the beginning of the experiment. After the addition of phosphate (Day 0) to the 3 mesocosms, the concentration of phosphate decreased during Day 1 of the experiment to a concentration of 40 nM (data not shown). The concentration continued to drop over the next 2 d and remained steadily below the detection limit of the method. In the control mesocosms, phosphate concentrations remained consistently low (<10 nM) throughout the experiment. Concentrations of nitrate and ammonium varied little and were always close to the detection limit of the method used (data not shown).

The samples for this study were collected at the beginning (Day 0) and on the third day (Day 3) of the experiment. Seawater (2 l) was filtered with Sterivex GP filters (0.22 µm pore size) with the aid of a peristaltic pump under aseptic conditions to avoid contamination. Filters were immediately stored at −80°C until DNA extraction.

DNA extraction, PCR and sequencing

DNA extraction was performed according to Massana et al. (1997), as modified by Tzahor et al. (2009) without adding lysis buffer to the sterivex filters prior storing them at −80°C. DNA yield was quantified by a NanoDrop 2000 spectrophotometer (Thermo Scientific), and DNA quality was visualized in agarose gel electrophoresis of 5 µl diluted DNA. DNA extracts were stored at 4°C until use.

Polymerase chain reaction (PCR) was carried out in a Biometra Tpersonal Thermocycler (Biometra biomedizinische Analytik) under aseptic conditions. Sterile water was used as a negative control in all PCR reactions. A ~150 bp fragment of the V3 region of the bacterial 16S rRNA locus was amplified using the universal bacterial primers 329f (5′-ACG GNC CAG ACT CCT AC-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′) (Sánchez et al. 2007). Each PCR reaction (50 µl) contained 0.3 mM of each primer, 50 ng of template DNA, 1× KAPAHiFi Fidelity Buffer (KAPA), which contained $MgCl_2$ at a concentration of 2 mM, 1 unit of KAPAHiFi HotStart DNA polymerase (KAPA) and 0.3 mM of each dNTP. PCR conditions were as follows: 95°C for 4 min followed by 25 cycles of 98°C for 20 s, 55°C for 15 s and 72°C for 30 s, with a final extension step of 5 min at 72°C.

The methodology for analyzing the 16S amplicons has been described in detail elsewhere (Sogin et al. 2006, Huber et al. 2007, Huse et al. 2007, 2008, Galand et al. 2009, Quince et al. 2009, 2011). Pyrosequencing was performed using the GS-FLX Titanium platform of the Institute of Marine Biology and Genetics (IMBG) at the Hellenic Centre for Marine Research. Raw sequences have been uploaded as .sff files to the NCBI SRA database with accession number SRA059200.

The AmpliconNoise v1.4 algorithm package (Quince et al. 2011) was used for cleaning the pyrosequencing reads from sequencing errors, PCR single base substitutions and PCR chimeras. The remaining clean sequences were assigned into samples according to their barcode. Reads sequenced with the reverse primer were 'reverse complemented' using a custom script written in RealBasic (release 7, Real Software). An abundance matrix (operational taxonomic unit [OTU] table) was formed by clustering the clean sequences into OTUs of 97% similarity or more using complete linkage hierarchical clustering (FCluster script of the AmpliconNoise package). Phylogenetic affiliation of the clean and clustered sequences was performed in QIIME (Caporaso et al. 2010) using the 'uclust' algorithm (Edgar 2010)

against the Greengenes reference dataset (McDonald et al. 2012). The reference dataset was first trimmed using V-Xtractor v2.0 (Hartmann et al. 2010) as this procedure has been shown to improve taxonomic classification (Werner et al. 2012). For some OTUs where the resulting phylogenetic resolution was low (e.g. stopping at the level of '*Bacteria*'), manual BLAST of the OTU's consensus sequence against the NCBI non-redundant (nr) nucleotide collection database was performed and the phylogeny of the OTU was reassigned if possible at a lower level, based on 99% or more sequence identity. Pyrosequencing resulted a total of 125 809 sequences. After removing the noisy (14.5%) and the chimeric (14.1%) sequences, we ended up with 92 978 clean sequences, which varied between 4250 and 13 574 per sample (see Table S1 in the Supplement at www.int-res.com/articles/suppl/a072p098_supp.pdf). Plastid sequences (68 to 136 sample^{-1}) were identified at this stage and removed before further analysis. Clean sequences clustered into 670 OTUs from which 247 OTUs were characterized as 'non-unique', i.e. they were present in at least 2 samples and consisted of 10 or more sequences (0.02 to 0.18% in terms of relative abundance). The 'non-unique' OTUs accounted for 95.96 to 99.35% of each sample's sequences. The remaining 423 OTUs were characterized as 'rare'.

Statistical analyses

For alpha diversity estimates, Shannon's index ($\log_2$-based, $H'_{\log2}$), Pielou's evenness (J') and the expected number of species corrected for the smallest sample size ($E(s)_{4250}$) were calculated using the vegan package (Oksanen et al. 2013) in R (R Core Team 2012). Cluster (group average hierarchical clustering) analysis and similarity percentage (SIMPER) analysis were performed using the PRIMER6 software for Windows (PRIMER-E) based on the Bray-Curtis (BC) similarity index, calculated after a standardization (dividing the initial OTU abundances by sample total) of the initial abundances. One-way ANOVA was performed using the appropriate QIIME scripts for separate OTU relative abundance comparisons. The Mann-Whitney *U*-test and Anderson-Darling normality test (for OTU abundances) were performed in PAST v2.17c (Hammer et al. 2001). The false discovery rate correction method (Benjamini & Hochberg 1995) was applied when multiple comparisons were performed. Graphical representation of the rarefaction curves (see Fig. S1 in the Supplement) and the phylogenetic composition of each sample were performed using Microsoft Excel.

RESULTS AND DISCUSSION

Phylogenetic composition of P-treated and control samples

For this analysis, OTUs were grouped at the level of 'Phylum' or 'Class' for *Proteobacteria*. Four major bacterial groups accounted for ~90% of the total sequences in each sample: *Alphaproteobacteria* (33.4 to 55.7% per sample), consisting mainly of SAR11 and *Rhodobacterales*; *Gammaproteobacteria* (20.4 to 30.7% per sample), consisting mainly of *Alteromonadales* and *Pseudomonadales*; *Bacteroidetes* (8.1 to 14.8% per sample), consisting mainly of *Flavobacteriia* and *Sphingobacteriia*; and *Cyanobacteria* (5.1 to 16.4% per sample), consisting mainly of *Synechococcales*. The rest of the community was composed of several other low-abundant (<5%) groups, such as *Actinobacteria*, *Firmicutes*, *Tenericutes*, *Verrucomicrobia*, *Betaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria*. Detailed phylogenetic composition of each sample (after pooling replicates) is presented in Table S2 in the Supplement, whereas the cumulative relative abundances of each of the major phylogenetic groups are shown in Fig. 1. Composition was similar to what has been reported for Day 4 of the same experiment in a previous study using group-specific probes (Sebastián et al. 2012) as well as in previous studies examining the bacterioplankton composition of the upper water column of the Mediterranean Sea (Alonso-Sáez et al. 2007, Feingersch et al. 2010, Ferrera et al. 2011).

Community changes induced by P addition

We examined the impact of P addition on α-diversity (i.e. $H'_{\log2}$, $E(s)_{4250}$ and J'), and β-diversity (i.e. the BC similarity among samples). $H'_{\log2}$, $E(s)_{4250}$ and J' did not differ significantly among P-treated and control samples (Mann-Whitney *U*-test p = 0.936, 0.298 and 0.809 respectively, n = 6) (Table 1). Cluster analysis was performed on the BC similarity matrix for estimating changes in β-diversity. We observed 2 major clusters with 72.34% similarity, each consisting of samples from the same day (Fig. 2). Moreover, 2 control and 2 P-treated samples clustered together within the Day 3 cluster: P1D3-P3D3 and C1D3-C3D3 (Fig. 2). The analysis was also performed using the UniFrac metric (Lozupone et al. 2011) which incorporates phylogeny, and the clustering was identical (data not shown).

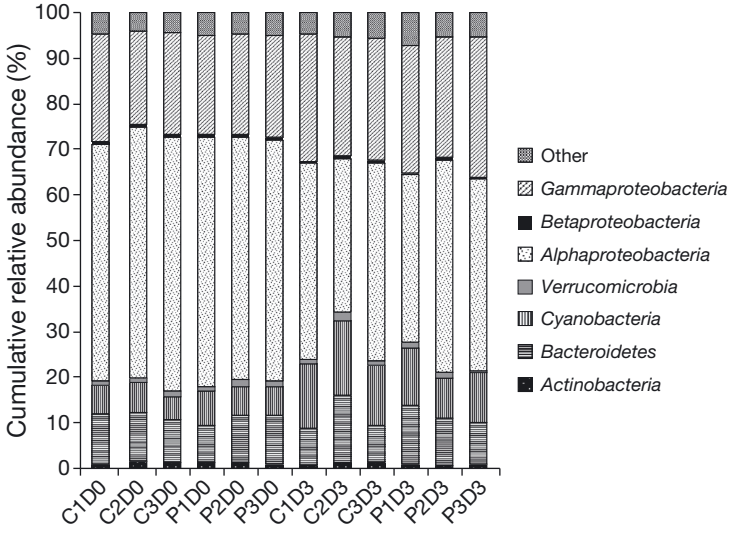

Fig. 1. Relative abundances of the major bacterial phylogroups (Phylum and Class level for *Proteobacteria*). P: P-treated samples; C: control samples. P and C are followed by the replicate number. D0: samples from experimental Day 0; D3: samples from experimental Day 3. Other: unassigned sequences or very small phylogroups

The temporal variation that we observed may be the result of natural short-term changes (Hewson et al. 2006, Ottesen et al. 2011, Yao et al. 2011), confinement effects occurring in mesocosms (Beardsley et al. 2003, Eiler et al. 2003, Allers et al. 2007, Lekunberri et al. 2010), or a mixed effect of the 2. The sub-clustering within the Day 3 cluster suggests that P addition induced some changes in community structure on Day 3, but these changes were minimal as the average dissimilarity between the 2 sub-clusters was low (22.8%) and no changes in α-diversity were observed. We used SIMPER analysis to determine each OTU's contribution to community variation between these 2 sub-clusters (Table 2). We observed that most (89.14%) variation between the sub-clusters could be attributed to 'non-unique' OTUs. In a recent study at the Baltic Sea, members of the 'rare' community became dominant after environmental disturbance (Sjöstedt et al. 2012). In our study, no such recruitment of 'rare' OTUs was observed. Moreover, the changes in abundances were not statistically significant (ANOVA, $p > 0.1$ for all OTUs, $n = 3$), and many OTUs contributed similarly to community variation between the 2 sub-clusters on Day 3 (Table 2). Thus, the observed changes in community structure on Day 3 might have resulted from subtle changes in many OTUs' abundances at P-treated samples.

Resistant communities are defined as communities with unaltered composition after a disturbance event (Allison & Martiny 2008). The minimal changes in composition that we observed between P-treated and control samples on Day 3 when the added phosphate was depleted, suggest that the communities we studied were resistant to P addition. This is probably related to the ultra-oligotrophic nature of the Eastern Mediterranean (Krom et al. 2004) and highlights the capacity of this region to rapidly absorb the externally added phosphate without significant impact to the community structure of bacterioplankton. Nevertheless, we have to note that the added phosphate in the beginning of the experiment (100 nM) is comparable to the background levels that have been recorded in this region (Zohary & Robarts 1998). Therefore, the added phosphate may not have been enough to induce changes in the bacterioplankton community, despite the overall increase in bacterioplankton biomass and production (Sebastián et al. 2012). There are, however, examples of

Table 1. Mean (±SE) values for Pielou's evenness (J'), expected number of species corrected for sample size ($E(s)_{4250}$) and Shannon's index ($\log_2$-based, $H'_{\log 2}$). Replicates have been pooled. P: P-treated samples; C: control samples; D0: samples from experimental Day 0; D3: samples from experimental Day 3

Sample	J'	$E(s)_{4250}$	$H'_{\log 2}$
CD0	0.66 ± 0.002	197.73 ± 7.35	5.42 ± 0.05
PD0	0.67 ± 0.006	205.93 ± 3.34	5.40 ± 0.04
CD3	0.72 ± 0.01	213.80 ± 7.97	5.64 ± 0.11
PD3	0.72 ± 0.009	223.70 ± 2.59	5.59 ± 0.06

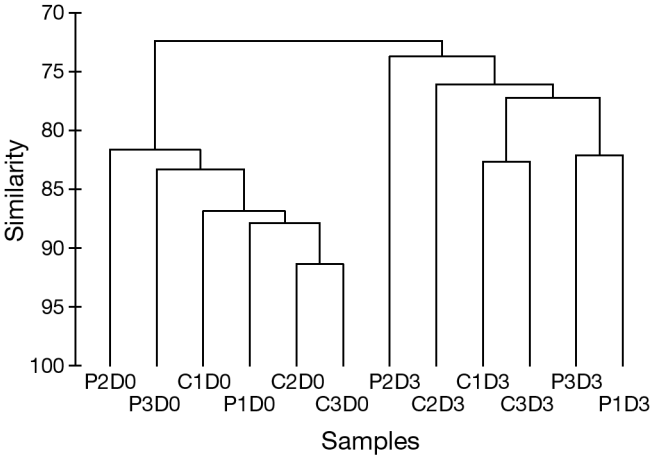

Fig. 2. Cluster analysis plot. P: P-treated samples; C: control samples. P and C followed by the replicate number. D0: samples from experimental Day 0; D3: samples from experimental Day 3

Table 2. Results from SIMPER analysis regarding the differences between the P1D3-P3D3 and C1D3-C3D3 sub-clusters. First column: operational taxonomic unit (OTU) identification number. Second column: change in the relative abundance of the OTU in the P1D3-P3D3 sub-cluster. Third column: contribution (%) of the OTU to the total variation between the 2 sub-clusters. Fourth column: phylogenetic affiliation of the OTU (at the lowest possible level). The OTUs contributing less than 0.5 % to total variation between the 2 sub-clusters are not shown

OTU ID	Relative abundance change (%)	Contribution (%)	Phylogenetic affiliation
48	3.83	8.4	*Gammaproteobacteria*
0	−3.53	7.72	SAR11
27	−2.43	5.35	*Synechococcus* sp.
7	2.17	4.77	*Gammaproteobacteria*
26	−1.95	4.27	SAR11
12	1.52	3.35	*Alphaproteobacteria*
23	−1.11	2.43	*Marinobacter* sp.
49	1.02	2.25	*Flavobacteriaceae*
3	1.02	2.24	*Oceanospirillum* sp.
5	−0.91	2	*Oceanospirillales*
31	0.76	1.66	*Lutimonas* sp.
141	−0.65	1.51	*Alteromonas* sp.
20	−0.55	1.4	*Gammaproteobacteria*
74	−0.42	1.34	*Alphaproteobacteria*
11	0.58	1.29	SAR116
17	0.09	1.24	SAR116
21	0.54	1.18	*Alphaproteobacteria*
8	0.13	1.11	*Gammaproteobacteria*
61	−0.13	0.96	*Candidatus Actinomarina minuta*
40	−0.17	0.84	*Verrucomicrobia*
63	−0.31	0.83	*Alphaproteobacteria*
90	0.36	0.79	*Flavobacteriaceae*
77	0.35	0.77	*Flavobacteriaceae*
443	0.34	0.74	*Gammaproteobacteria*
267	0.15	0.71	*Gammaproteobacteria*
33	−0.21	0.7	*Pseudomonas* sp.
56	−0.31	0.68	*Oceanospirillales*
25	0.02	0.65	*Gammaproteobacteria*
67	−0.29	0.65	*Gammaproteobacteria*
6	0.2	0.64	SAR11
15	0.13	0.59	*Proteobacteria*
13	−0.15	0.57	*Gammaproteobacteria*
39	0.15	0.57	*Alphaproteobacteria*
30	−0.01	0.57	*Alphaproteobacteria*
413	0.26	0.57	*Oleibacter marinus*
10	−0.25	0.55	*Roseovarius* sp.
122	0.25	0.54	*Bacteroidetes*
4	−0.25	0.54	*Bacteroidetes*
142	−0.24	0.54	*Vibrio* sp.
1	0.13	0.52	*Bacteroidetes*
70	−0.23	0.5	*Flavobacteriaceae*
68	0.11	0.5	*Vibrio* sp.

similar nutrient addition experiments in mesocosms, where phosphate was added at the µmolar range but still no changes where observed in bacterioplankton community composition (Lekunberri et al. 2010, Teira et al. 2011). Alternatively, a shift in the nutrient limitation among different phylogroups may have followed the initial increase in bacterial biomass and productivity after P addition, as has been recently reported in a nutrient addition experiment in the Eastern Mediterranean (Sebastián & Gasol 2013).

CONCLUSIONS

In this study, we examined the effects of P addition to bacterioplankton community structure in a mesocosm setup using a next high resolution phylogenetic screening. The results of this study showed that phosphorus addition had minimal effects on the bacterioplankton community structure of the P-starved Eastern Mediterranean, indicating that bacterioplankton communities in this region are highly

resistant to that kind of disturbance. However, we cannot exclude the possibility that P addition in higher concentrations and/or for a longer period may induce significant changes in bacterioplankton community composition and structure.

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