

SHORT COMMUNICATION

Heterogeneity in the nutrient limitation of different bacterioplankton groups in the Eastern Mediterranean Sea

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The heterotrophic bacterial community of the Eastern Mediterranean Sea is believed to be limited by phosphorus (P) availability. This observation assumes that all bacterial groups are equally limited, something that has not been hitherto examined. To test this hypothesis, we performed nutrient addition experiments and investigated the response of probe-identified groups using microautoradiography combined with catalyzed reporter deposition fluorescence *in situ* hybridization. Our results show contrasting responses between the bacterial groups, with *Gammaproteobacteria* being the group more affected by P availability. The *Roseobacter* clade was likely colimited by P and nitrogen (N), whereas *Bacteroidetes* by P, N and organic carbon (C). In contrast, SAR11 cells were active regardless of the nutrient concentration. These results indicate that there is high heterogeneity in the nutrient limitation of the different components of the bacterioplankton community.

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Although inorganic nutrient limitation of bacterioplankton was considered rare about a decade ago (Caron *et al.*, 2000), we now know it frequently occurs in oligotrophic waters. Phosphorus (P), for example, is often the primary limiting nutrient in the Atlantic and Mediterranean (Cotner *et al.*, 1997; Pinhassi *et al.*, 2006). Nevertheless, most studies have considered heterotrophic bacteria as a homogeneous black box, while the bacterial community is composed by cells expressing high metabolic diversity (for example, Musat *et al.*, 2008; Alonso-Saéz *et al.*, 2012), which likely experience different degrees of limitation and stress. The fact that nutrient availability plays an important role in niche partitioning supports this hypothesis (Pinhassi *et al.*, 2006), but the variability in the stress responses among different bacterial groups has been hitherto ignored.

The Eastern Mediterranean is one of the most oligotrophic and P-starved marine systems on Earth (for example, Tanaka *et al.*, 2007), where bacterioplankton is often P-limited (for example, Thingstad *et al.*, 2005). Here we assessed whether nitrogen (N)

and/or organic carbon (C) could be colimiting the bacterial community, and whether different bacterial groups responded similarly to the various nutrient additions (see Supplementary material for details).

Heterotrophic bacterial activity was significantly stimulated ($P < 0.05$, Dunnett's test) in all the treatments that contained P (Figure 1). Bacterial activity doubled in the P and PC treatments, and additional increases occurred in the NP ($5 \times$ higher than the control) and NPC treatments ($10 \times$ higher). These findings indicate that the heterotrophic bacterial community as a whole was primarily limited by P, but that these waters are a nearly balanced system, where addition of P leads to shifts from one type of limitation to another. This hypothesis is supported by the results obtained with the phosphate turnover time (Supplementary Figure S1).

To investigate whether all the bacterial groups responded equally to the nutrient additions, we used microautoradiography combined with catalyzed reporter deposition fluorescence *in situ* hybridization (MARFISH). Community composition 2 days after inoculation was not strongly affected by the nutrient amendments, and was similar to that at the beginning of the experiment, that is, SAR11 dominated in all the treatments, followed by *Gammaproteobacteria* (Supplementary Figure S2). Non-EUB cells decreased dramatically in the NP and NPC treatments (from 20% to 0%), suggesting that

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dormant cells may have become active upon nutrient additions, as observed in other studies (for example, Eilers *et al.*, 2000).

The different bacterial groups showed very contrasting responses. *Gammaproteobacteria* activity (in terms of % cells taking up leucine) was stimulated in all treatments containing P (Dunnnett's test, $P < 0.05$, Figure 2). This group was the one responding more clearly to P additions, suggesting that *Gammaproteobacteria* experienced more severe P limitation than the other groups. Indeed, recent metatranscriptomic data show that *Gammaproteobacteria* transcript pools are enriched in genes for P acquisition when compared with other phylogenetic groups (Gifford *et al.*, 2012). Nevertheless, the further increase observed in the total number of active cells in the PC, NP and NPC treatments (Supplementary Figure S3) indicated that

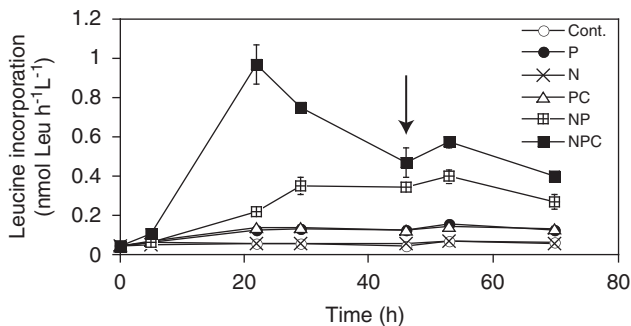


Figure 1 Bacterial heterotrophic activity in the addition experiments. Samples were amended with phosphate (P), ammonia (N) and organic carbon (C), or with combinations of these nutrients (PC, NP and NPC). Cont.: control treatment (no amendments). Each data point represents the average of the two replicates. Error bars represent the s.d. Arrow highlights the time-point when samples were taken for the MARFISH analyses.

Gammaproteobacteria were also able to rapidly take advantage of additional inputs of N and C, and increase their abundance and production. The strong relationship found between the bulk incorporation of leucine and the abundance of leucine-incorporating gammaproteobacterial cells (Supplementary Figure S4) suggests that *Gammaproteobacteria* likely accounted for the activity changes of the bulk bacterial community. MARFISH micrographs showed that gammaproteobacterial cells had larger per-cell silver grain clusters than the dominant SAR11 (Figure S5), which indicates higher assimilation per cell (Nielsen *et al.*, 2003), and were notably larger in the nutrient-enriched treatments. *Gammaproteobacteria* were dominated by the NOR5/OM60 clade in the control, P and N treatments, whereas *Alteromonadaceae* dominated in the NPC treatments (Figure S6).

The percentage of cells taking up leucine within the *Bacteroidetes* group was generally low, due to their known preference for high-molecular-weight compounds (Cottrell and Kirchman, 2000). The activity of this group was only significantly stimulated in the NPC treatment, although on average the activity increased in all the treatments that contained P. Therefore, *Bacteroidetes* cells were likely limited by P, but secondarily colimited by N and C.

Roseobacter activity was stimulated ca. twofold in the P and PC treatments and eightfold and fivefold in the NP and NPC treatments, respectively (Figure 2). However, this stimulation was only significant in the NP and NPC treatments, suggesting that this group was colimited by P and N. Nevertheless, the *Roseobacter* clade dominates among algal-associated bacteria (Buchan *et al.*, 2005), and phytoplankton at the moment of the

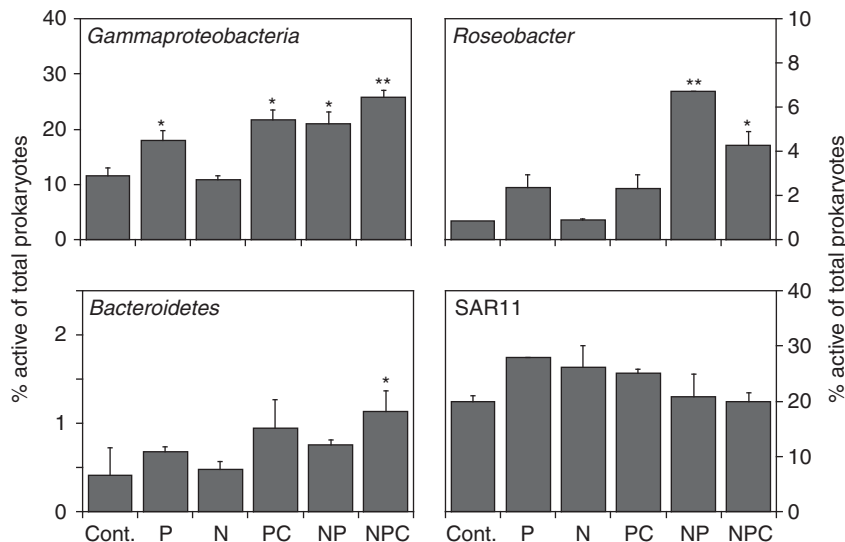


Figure 2 Percentage of active cells within each probe-identified group in relation to total prokaryotes (DAPI counts). Labels as defined in Figure 1. Asterisks denote significant differences in relation to the control ($*P < 0.05$, $**P < 0.001$). Note the different y axis scale for *Roseobacter* and *Bacteroidetes*.

study was colimited by P and N (Pitta *et al.*, unpublished). Hence, it is possible that the strong positive response of this group to N and P additions is a consequence of dissolved organic carbon release by the stimulated phytoplankton.

SAR11 activity, expressed as percentage of active cells, was relatively high in all the treatments (Figure 2). The fact that this group was active regardless of the nutrient concentration contradicts the common belief that SAR11 cells are strict oligotrophs. Instead, we believe that the conservative metabolism of SAR11 cells allows them to thrive well in a wide range of trophic conditions. This is consistent with a recent metatranscriptomic study showing that SAR11 cells have limited capacity to sense and respond to environmental changes, which suggests that this phylogenetic group has evolved to maintain consistent growth independent of environmental conditions (Gifford *et al.*, 2012), something that had already been hinted in studies with a cultured representative of this clade (for example, Rappé *et al.*, 2002). The cell-associated silver grain clusters were not larger in the nutrient amended treatments (Figure S5), suggesting that the per-cell activity within the SAR11 group did not notably increase, again agreeing with the view that SAR11 cells would not take advantage of nutrient pulses (Gifford *et al.*, 2012) and in general have low nutrient requirements (Sebastián *et al.*, 2012). Yet, it should be noted that at least certain SAR11 phylotypes may display high growth rates (Malmstrom, *et al.*, 2005, Campbell *et al.*, 2011).

For many decades, the Liebig's law of the minimum, which states that only a single resource is limiting, was the dominant theory shaping how oceanographers viewed phyto- and bacterioplankton ecology and their impact on biochemical cycles (Arrigo, 2004). This view has been recently challenged by the concept of colimitation or multiple nutrient limitations (Saito *et al.*, 2008). Here we show that the reality is even more complex, with different bacterial groups experiencing different types of limitations under the same environmental conditions.

Conflict of Interest

The authors declare no conflict of interest.

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