

## P-limited bacteria but N and P co-limited phytoplankton in the Eastern Mediterranean—a microcosm experiment

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### Abstract

An on-board microcosm experiment was set up to test the hypothesis that the observed lack of phytoplankton biomass increase response to a mesoscale in situ P-enrichment experiment in the P-limited Eastern Mediterranean (Krom et al., 2005a) was a consequence of co-limitation by P and N availability in this ultraoligotrophic environment. Six microcosms were filled with subsurface seawater (ambient DIN: 90–100 nM) taken from inside a P-enriched patch (IN), which in the absence of biological activity would have had ca. 22 nM of  $\text{PO}_4^{3-}$ . Another six microcosms were filled with unfertilized ( $<2$  nM  $\text{PO}_4^{3-}$ ) subsurface seawater from outside the patch (OUT). The bottles were either supplemented with 1600 nM  $\text{NH}_4^+$  or not, incubated on-deck and subsampled daily, or at the first and last (fourth) day of the experiment, for a suite of biological parameters. The addition of N to OUT water did not induce cell abundance increases in either the phototrophic or heterotrophic sides of the food chain, in line with previous assessments that the Eastern Mediterranean is not purely N-limited. The IN and OUT treatments, to which no  $\text{NH}_4^+$  was added, mimicked the behavior of the in situ experiment, with an order of magnitude higher bacterial production of IN vs. OUT water, but no noticeable phytoplankton response. The addition of N to IN water, previously exposed to P, led to substantial responses of the entire microbial community, including 4 to 80-fold increases in chlorophyll, other pigments, bacterial activity, and the abundance of ciliates—relative to IN water to which N was not added. The ca. 10-fold increase in chlorophyll within 4 days was mostly due to a major increase in both abundance ( $\times 4$ ) and fluorescence per cell ( $\times 17$ ) of *Synechococcus*, whereas *Prochlorococcus* disappeared. These changes were accompanied by removal from the water of 570 nM of the added  $\text{NH}_4^+$ , equivalent to 570/22 or N:P ratio of 26:1, similar to the ratio measured for POM in the area. Possibly, non-Redfield ratios were maintained, still leaving by day 4 some 1100 nM of N that could not be used due to the lack of P. These results support our hypothesis that the lack of response of phytoplankton to the mesoscale P-enrichment was due to their concurrent N-starvation, i.e. N and

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P co-limitation. In contrast, bacteria could grow when only P was added, implying pure P-limitation. Thus, the heterotrophic and autotrophic components of the same aquatic community experienced different limitations.

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## 1. Introduction

An experimental way to demonstrate nutrient limitation in aquatic systems is to add the suspected limiting nutrient and demonstrate growth beyond that recorded for an unfertilized control. In most cases, either N or P will be limiting to phytoplankton growth (Tilman, 1977). However, in ultra-oligotrophic systems like the Eastern Mediterranean (Yacobi et al., 1995), where the ambient concentrations of both dissolved inorganic N and P are exceptionally low (Krom et al., 2003), co-limitation or near co-limitation is likely, i.e. that the addition of the primary limiting nutrient will immediately lead to limitation by the other, and substantial growth will occur only when both nutrients are added.

The Eastern Mediterranean is unusual in that its deep waters are characterized by high molar N:P ratios of ~28:1 (Krom et al., 1991; Kress et al., 2003), indicative of P-limitation. There is direct evidence that the Eastern Mediterranean is P-limited in winter (Zohary and Robarts, 1998), the season when phytoplankton biomass is highest (Krom et al., 2003). At the end of the bloom around March–April (Groom et al., 2005), while all of the phosphate has been consumed, there is measurable nitrate (~0.3 μM) remaining in the water column (Kress and Herut, 2001). However by early summer, when the CYCLOPS experiment took place, there is no longer any measurable nitrate present in the surface waters (Kress and Herut, 2001; Krom et al., 2005b), and the evidence for continued P-limitation is weaker. We have used an opportunity of a Lagrangian in situ P addition experiment conducted as part of the CYCLOPS program (Krom et al., 2005a) also to explore an alternative possibility of concurrent P and N limitations on phytoplankton and bacterial production in summer.

In the in situ P-addition experiment H<sub>3</sub>PO<sub>4</sub> combined with SF<sub>6</sub> tracer were injected into the surface water of the Cyprus Eddy in the Eastern Mediterranean over a 16-km<sup>2</sup> area on 17 May 2002, creating a patch of P-enriched water with maximum

concentrations of ca. 110 nM over 20 m depth (Law et al., 2005). The temporal dynamics of the chemical environment and the biota inside the P-enriched patch were then followed over the next nine days and compared to the dynamics in a control site outside the patch. Uptake and dispersal processes led to a rapid decline of the measured PO<sub>4</sub> concentration inside the patch (Law et al., 2005). Three days after the PO<sub>4</sub> addition, when the waters used in this microcosm experiment were sampled, the PO<sub>4</sub> concentration calculated from SF<sub>6</sub> concentrations in the patch was 22 nM while the measured concentration in this water was below detection limits (<2 nM), essentially the same as the background concentration outside the patch. The difference of ~20 nM was considered to be the amount of P taken up by the microbial community as a result of the P addition. This P uptake was confirmed by changes in P turnover times and increases in particulate P concentrations (Flaten et al., 2005). While some significant differences in the response of various food-chain components to the addition of P were reported (Pitta et al., 2005; Flaten et al., 2005; Pasternak et al., 2005), the most expected response of increase in phytoplankton biomass did not take place (Psarra et al., 2005).

Using a smaller scale, on-board microcosm experiment, we examined the hypothesis that the addition of NH<sub>4</sub><sup>+</sup>, a readily available form of nitrogen, to Eastern Mediterranean surface water that have previously been supplemented with P, will lead to an increase in phytoplankton biomass, suggesting N and P co-limitation, or close to co-limitation, of phytoplankton growth.

## 2. Methods

### 2.1. Experimental set up and sampling

Sea water from 8 m depth was collected at 14:00 on 20 May 2002, three days after the in situ P addition, from within the patch, pre-filtered through 200 μm mesh to exclude large predators, and used to fill six replicate 8-l low-density polyethylene Nalgene bottles. Another identical six bottles were filled

with pre-filtered 8 m depth water collected 2.5 h later outside the patch. The bottles, used as microcosms, were pre-washed with 2.5 l of 10% HCl, twice with MQ water and twice with fresh seawater from the rosette, using clean gloves throughout the procedure.  $\text{NH}_4^+$  was added (final conc. of addition: 1600 nM) as  $\text{NH}_4\text{Cl}$  to three of the six bottles containing in-patch water (IN) and to three of the six bottles containing out-of-patch water (OUT) to create a  $2 \times 2$  experimental design, with the following triplicated treatments: IN, IN+ $\text{NH}_4$ , OUT, OUT+ $\text{NH}_4$ . The bottles were shaken and incubated in a 2-m<sup>3</sup> on-deck flow-through water bath, covered by a screen that reduced the incident light by ca. 50%. Over the next four days samples were taken daily from each of the 12 bottles for determining a suite of chemical and biological variables. Bacterial activity and P-turnover times were determined daily, other parameters (see below) were determined at the start (day 0, or D0) and end (day 4 or D4) of the experiment, while some ( $\text{NH}_4^+$ , DOP, DOC, Chl *a*) were determined on day 2 (D2) as well. Inorganic nutrients other than ammonium were determined only on the water used for filling the bottles.

## 2.2. Analytical procedures

**Nutrients:** The methods used for the determination of nanomolar concentrations of nitrate, nitrite, ammonia, phosphate, and dissolved organic phosphorus (DOP), and for micromolar concentrations of silicate, dissolved organic carbon (DOC), and total dissolved nitrogen (TDN) are described in detail in Krom et al. (2005a, b). Briefly, nitrate, nitrite and phosphate were determined using a segmented flow analyzer with a long path-length liquid waveguide capillary cell detection system. Ammonia was determined using a Teflon membrane/fluorescence analysis procedure. DOP was determined as DIP after oxidation by UV light. Silicate was determined using an in-line/off-line 5 channel Technicon AAI segmented flow autoanalyzer. DOC/TDN were determined using a high-temperature catalytic oxidation procedure.

**P turnover times:** Were measured as detailed by Flaten et al. (2005), using <sup>33</sup>PO<sub>4</sub> as tracer.

**Bacterial activity:** The <sup>14</sup>C-Leucine incorporation method was used for assessing bacterial activity, using a modified micro-tube technique (Smith and Azam, 1993). L-[U-<sup>14</sup>C]Leucine (Amersham, CFB183, spec act 227 mCi/mmol) was added to

triplicate 1.8 ml samples in 2-ml micro-centrifuge tubes to give a final concentration of Leucine of ~100 nM. The choice of 100 nM Leucine was based on a concentration vs. incorporation pre-experiment, which showed saturation at about 70 nM. The samples were incubated at 23 ± 1 °C and subdued (laboratory) illumination for 4–8 h. Optimal incubation times (within the period of linearity but also exhibiting sample counts measurably higher than the killed controls) were determined in preliminary time series experiments. Additional replicates for subtraction of background and abiotic adsorption were fixed with 100 µl of 100% TCA per 1.8 ml sample before addition of the isotope. Incubation was stopped by the addition of 100% TCA, the micro-tubes were centrifuged on an ultra-centrifuge at 14,000 rpm for 10 min. The supernatant was then sucked out of the micro-tube and 1 ml of 5% TCA was added. After gentle mixing of the micro-tubes they were centrifuged again (same time and speed), the supernatant was sucked out, 1 ml scintillation Fluor (Ultimagold) was added, the micro-tubes were placed inside scintillation vials and counted on a Packard Scintillation counter.

**Bacterial counts:** 30-ml samples were fixed with 4.2 ml of filtered 5% Formaline and kept refrigerated until analysis within two months. DAPI-stained bacteria were counted with a fluorescent microscope (Porter and Feig, 1980).

**Chlorophyll:** 0.5–1 l of sample was filtered through 0.2-µm polycarbonate filters (Poretics, Ø 47 mm). Filters were placed in –20 °C immediately after filtration and kept frozen for six weeks until laboratory analyses took place. Filters were extracted in 90% acetone, and chlorophyll *a* concentration was determined fluorometrically according to Yentsch and Menzel (1963) with the use of a TURNER 112 fluorometer.

**Photosynthetic pigments by HPLC:** GF/F-filtered 2000-ml samples were immediately frozen at –70 °C for high performance liquid chromatographic (HPLC) analyses of chlorophylls and carotenoids. Pigment analyses involved reversed phase RP-HPLC developed at PML (Mantoura and Llewellyn, 1983) and adapted by Barlow et al. (1999).

**Picophytoplankton (Flow cytometry):** Duplicate samples (1.8 ml) were preserved with 140 µl of 25% glutaraldehyde (Sigma G-5882), deep-frozen in liquid N<sub>2</sub>, and kept at –80 °C until analysis about two months later using a FACScan (Becton Dickinson) flow cytometer. The samples were thawed for 1 min at 37 °C and analyzed at room temperature

( $23 \pm 2^\circ\text{C}$ ) by excitation with Argon laser at 488 nm. Forward light scatter (FSC), side scatter (SSC, a function of cell size), red fluorescence of chlorophyll above 630 nm (FL3), and orange fluorescence of phycoerythrin at 585 nm, were measured. Before running the sample, 0.93  $\mu\text{m}$  beads (make: Poly-Sciences) were added as an internal standard. The phytoplankton assemblages were composed mainly of two groups of cyanobacteria, *Synechococcus* sp. and *Prochlorococcus* spp., and a third group of diverse pico- and small nano-eukaryotes (referred to hereafter as picoeukaryotes), which were differentiated by their unique light scatter and auto fluorescence features. For each of these three groups, abundance and fluorescence per cell were recorded. A proxy of total chlorophyll fluorescence was obtained by summing the cell density of each group  $\times$  its mean FL3/cell. This proxy is referred to as Total FL3 fluorescence.

**Nanoflagellates microscopic counts:** Samples (50–100 ml), which were fixed with glutaraldehyde (final conc. 1%), were concentrated to ca. 10 ml on a 25-mm, 0.8- $\mu\text{m}$  pore sized polycarbonate black filter, stained with DAPI (final conc. 0.5  $\mu\text{g ml}^{-1}$ ) for 10 min, and filtered (Porter and Feig, 1980). Autotrophic and heterotrophic nanoflagellates (ANF and HNF, respectively) were counted using an ocular micrometer under epifluorescence microscopy.

**Ciliates and microphytoplankton:** 500-ml samples preserved with acid Lugol's solution for microphytoplankton counts and 100-ml samples preserved with alkaline Lugol's solution for ciliate counts (final conc. 2% for both) were stored at  $4^\circ\text{C}$  and examined with an inverted microscope after sedimentation in settling chambers. For further details see Pitta et al. (2005) (ciliates) and Psarra et al.

(2005) (microphytoplankton). Cell sizes were measured with an ocular micrometer and converted into cell volumes using appropriate geometric formulae.

**Statistical analysis:** For comparison between treatments on D4, 1-way ANOVA was used, with replicates and factor being the treatments. To minimize the possibility of getting significant results by chance alone due to the great number of ANOVAs conducted, the multiple comparisons between treatments were tested using the Bonferroni test. For parameters sampled every day, time was used as an additional factor, giving a day-by-day comparison, similar to a paired *t*-test.

### 3. Results

At the start of the experiment and prior to  $\text{NH}_4$  addition (D0; Table 1) phosphate concentrations were 3.9 nM (IN) and 1.3 nM (OUT). Calculations based on the  $\text{SF}_6$  concentration in the water used to fill the IN bottles (Law et al., 2005) indicate that, had there not been any biological activity, those water would have had ca. 22 nM of  $\text{PO}_4$ . Thus, about 18 nM P were taken up from the IN water by the biota during the course of three days between P addition and filling our bottles.

At the start of the experiment the microcosms also had: ca. 80 nM of  $\text{NH}_4$  in both IN and OUT treatments; 17.9 and 9.7 nM of nitrate in IN and OUT water, respectively, nitrite concentrations below detection limit of 1 nM; abundant Si, at ca. 1.4  $\mu\text{M}$ . The dissolved organic N and P concentrations were considerably high, several tens of nanomolars of DOP were present in both types of water, DON ranged from 2.2 to 2.3  $\mu\text{M}$ . DOC levels were moderate, 113  $\mu\text{M}$  (IN) and 77  $\mu\text{M}$  (OUT). These

Table 1

Starting (D0) nutrient and chlorophyll concentrations (determined by the fluorometric method and by HPLC), bacterial activity (Leucine incorporation rates,  $\text{pmol Leu l}^{-1} \text{h}^{-1}$ ) and abundances ( $\text{cells ml}^{-1}$ ) of the major taxonomic groups of the autotrophs and heterotrophs in the water used for filling the experimental bottles

Water source	$\text{PO}_4$ , nM	$\text{NH}_4$ , nM	$\text{NO}_3$ , nM	$\text{NO}_2$ , nM	$\text{SiO}_2$ , $\mu\text{M}$	DOP (UV) nM	DON, $\mu\text{M}$	DOC, $\mu\text{M}$	Chl <i>a</i> , $\text{ng l}^{-1}$	Chl <i>a</i> -HPLC, $\text{ng l}^{-1}$
IN	3.9	77.9	17.9	<1	1.43	58	2.3	113	18.2	12.0
OUT	1.3	82.0	9.7	<1	1.40	70	2.2	77	16.7	16.3
Water source	Bac Act	Pro $\text{ml}^{-1}$	Syn $\text{ml}^{-1}$	Euk $\text{ml}^{-1}$	Cocco $\text{ml}^{-1}$	Diat $\text{ml}^{-1}$	Dino $\text{ml}^{-1}$	Bac $\text{ml}^{-1}$	HNF $\text{ml}^{-1}$	Cil $\text{ml}^{-1}$
IN	22.0	46	2769	382	1.12	0.49	1.83	98,000	334	0.168
OUT	13.7	63	2949	311	2.59	0.44	1.09	121,000	326	0.182

Bac Act—bacterial activity, Pro—*Prochlorococcus*, Syn—*Synechococcus*, Euk—picoeukaryotes, Cocco—coccolithophores, Diat—diatoms, Dino—dinoflagellates, Bac—heterotrophic bacteria, HNF—heterotrophic nanoflagellates, Cil—ciliates.

results of dissolved inorganic and organic nutrients are similar to the more extensive dataset sampled in the area at this time (Krom et al., 2005a, b).

The two sources of water were quite similar in terms of their initial (fluorometrically determined) chlorophyll *a* content (IN: 18.2 ng Chl l<sup>-1</sup>; OUT: 16.7 ng Chl l<sup>-1</sup>) and in their phytoplankton species composition (Table 1). In both IN and OUT water, *Synechococcus* was numerically dominant, close to 3000 cells ml<sup>-1</sup>, followed by picoeukaryotes that numbered >300 cells ml<sup>-1</sup>. *Prochlorococcus* was less abundant, only tens per ml, which is typical for shallow depths in this region (Li et al., 1993; Zohary et al., 1998). Larger phytoplankton taxa (coccolithophores, diatoms, dinoflagellates) were relatively rare, with densities in the order of 1 cell from each taxonomic group per ml. The data suggest that possibly dinoflagellates were more abundant in IN water while coccolithophores were more abundant in OUT water (Table 1). The HPLC pigment analyses also showed that Chl *a* and zeaxanthin, indicative of *Synechococcus* type cyanobacteria, dominated the phytobiomass. The second most abundant pigment, 19'-hexanoyl-oxy-fucoxanthin, indicative of prymnesiophytes (coccolithophores) was more abundant in the OUT water (3.8 ng l<sup>-1</sup> as opposed to 2.8 ng l<sup>-1</sup> in IN water), confirming the microscopic observations. In terms of the heterotrophic community, noticeably higher bacterial activity was found in IN vs. OUT water while heterotroph abundances were quite similar in both waters.

The most striking experimental result was that, in support of our hypothesis, the IN + NH<sub>4</sub> treatment showed an exceptionally strong phytoplankton response relative to all other treatments, in the form of a 10-fold increase in chlorophyll from D0 to D4 (Fig. 1A, Table 2). Chlorophyll increased with time in the other treatments, but by only ca. 2-fold in the IN and OUT treatments, and by 3-fold in OUT + NH<sub>4</sub>. On D4, chlorophyll concentrations in IN + NH<sub>4</sub> were significantly higher than in the other three treatments, a response that was repeated by independent HPLC and flow-cytometry measurements (Table 2, Fig. 2A,B). Bearing this key response in mind, let us now go through the entire suite of results in a consistent manner.

### 3.1. Comparison of IN vs. OUT without NH<sub>4</sub> addition, mimicking the "in situ experiment"

The temporal dynamics of ammonium concentrations in both treatments were similar, and the

treatments were not significantly different from each other (Fig. 1B): ammonia, initially ca. 80 nM, was removed from the IN and the OUT water over the first two days declining to <30 nM, then increased (probably due to recycling) to ca. 40 nM by D4.

We have no measurements of the temporal changes of other N species and of inorganic P concentrations in the experimental bottles, but can learn about the availability of P from the dynamics of P turnover times. These were relatively high, ca. 100 h, at all sampling times in IN bottles, as opposed to less than 10 h at all sampling times in the OUT bottles (Fig. 1C), suggesting that P availability was higher in the IN bottles, and mimicking the patterns observed in the in situ experiment. The difference between the two treatments was significant ( $p < 0.01$ ). There were no systematic or significant trends in DOP concentrations during the experiment (data not shown).

Both IN and OUT treatments without NH<sub>4</sub><sup>+</sup> showed about 2–3-fold increases in chlorophyll concentrations from D0 to D4, as seen by both fluorometric and HPLC methods (Table 2). On D4, there was no significant difference between the chlorophyll concentrations of IN and OUT treatments for both the regular fluorometric and HPLC determinations (Figs. 1A, 2A).

The numerically dominant *Synechococcus* increased from D0 to D4 (Table 2) in both treatments by ca. 10–20%. Picoplanktonic eukaryote abundance increased from D0 to D4 only in the OUT bottles. *Prochlorococcus* declined from D0 to D4, the decline was more pronounced in the IN bottles. The abundance of the larger phytoplankton taxa declined from D0 to D4 in both treatments. On D4, there was no significant difference between the two treatments in the abundance of any of the taxonomic groups, nor in any of the pigment parameters (Fig. 2C–M). A 1.2–2.6-fold increase from D0 to D4 in fluorescence per cell was found for *Synechococcus* and *Prochlorococcus*, both in IN and OUT bottles whereas no respective change was found with time for eukaryotes.

On D0, bacterial cell numbers were ca.  $1.0 \times 10^5$  ml<sup>-1</sup> in IN water, increasing to  $1.4 \times 10^5$  ml<sup>-1</sup> by D4 (Fig. 2N). In OUT water bacterial densities at the start of the experiment were somewhat higher than those of the IN water, ca.  $1.2 \times 10^5$  ml<sup>-1</sup>, but remained at the same densities by D4. Their grazers, the heterotrophic flagellates and ciliates declined from D0 to D4 in the OUT treatment (Table 2). In the IN treatment, ciliate abundance did not change

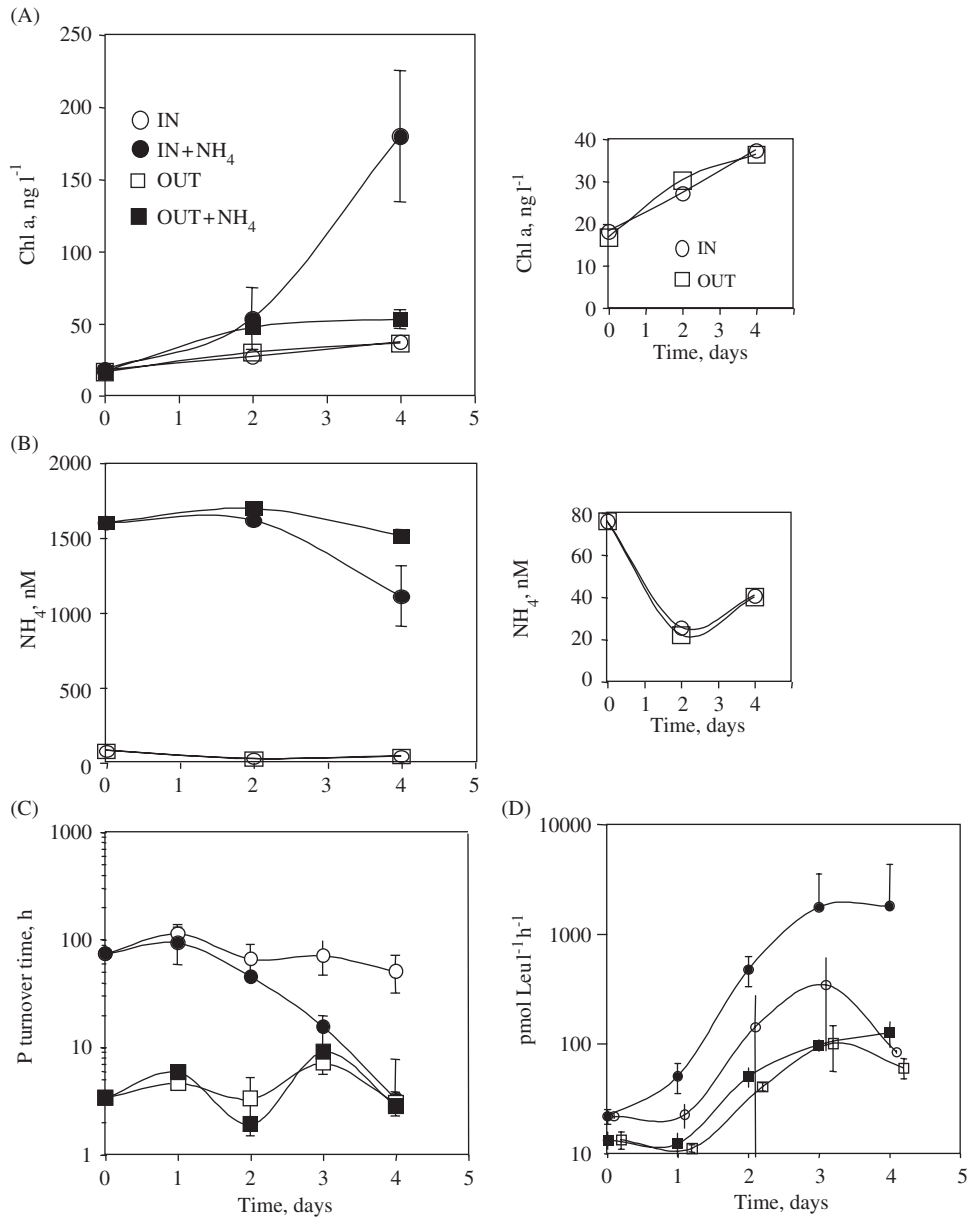


Fig. 1. Temporal dynamics in the experimental treatments (means of three true replicates  $\pm$ SD) of (A) Chlorophyll *a*, determined fluorometrically after acetone extraction, (B) NH<sub>4</sub><sup>+</sup> concentrations, (C) phosphorus turnover times, (D) bacterial activity, as rates of Leucine incorporation. Small figures to the right of (A) and (B) show the data for the treatments without NH<sub>4</sub><sup>+</sup> re-plotted on a smaller-range Y-scale. In (D) different treatments are spaced by 0.1 days to prevent overlapping of error bars.

from D0 to D4 although average cell size (and therefore biomass) increased (data not shown), while HNF cell density declined over time. On D4, there was no significant difference in cell densities of the 3 heterotrophic groups (bacteria, HNF and ciliates) between IN and OUT treatments (Fig. 2N–P).

On D0 bacterial activity in IN bottles was nearly double that in OUT bottles (Fig. 1D), mimicking the situation in the open-sea experiment. In both treatments, bacterial activity increased with time peaking on D3, then declined somewhat by D4. On average bacterial activity in IN bottles was always higher than in OUT bottles, but due to the large

Table 2

The ratio D4/D0 for the measured parameters in the four experimental treatments. Day 4 values are means of 3 values from the triplicated experimental bottles. Day 0 values are means of 1–4 determinations, depending on the parameter

Parameter	IN	IN+NH <sub>4</sub>	OUT	OUT+NH <sub>4</sub>
NH <sub>4</sub> <sup>+</sup>	0.5	0.7	0.5	0.9
Total DOP (UV)	1.0	1.0	1.2	0.9
DOC	1.1	1.2	1.4	1.5
P turnover time	0.7	0.0	0.9	0.8
Bacterial activity	3.9	82.4	4.5	9.3
Chlorophyll <i>a</i> (fluorometric)	2.1	9.9	2.2	3.2
Chlorophyll <i>a</i> (HPLC)	3.1	16.7	2.6	4.6
Zeaxanthin	1.6	9.5	1.3	2.7
19-hexanoyl-oxy-fucoanthin	3.6	26.4	2.9	6.8
pico- and nano-eukaryotes/ml	0.9	1.2	1.3	1.2
<i>Prochlorococcus</i> /ml	0.5	–	0.9	1.0
<i>Synechococcus</i> /ml	1.2	4.1	1.1	1.3
Eukaryotes, fluorescence/cell	0.9	1.1	0.9	1.0
<i>Prochlorococcus</i> , fluorescence/cell	1.2	–	1.5	2.4
<i>Synechococcus</i> , fluorescence/cell	1.6	17.2	2.6	6.4
Total Chl fluorescence (FC)	1.1	12.0	1.6	2.3
Coccolithophores/ml	0.5	0.7	0.3	0.4
Diatoms/ml	0.4	0.7	0.5	0.8
Dinoflagellates/ml	0.1	0.3	0.4	0.4
Bacteria/ml	1.4	1.6	1.0	0.9
Heterotrophic nanoflagellates/ml	0.8	2.1	0.9	0.7
Ciliates/ml	1.0	3.0	0.6	0.8

variability between replicate bottles the differences were not significant.

### 3.2. Comparison of treatment IN+NH<sub>4</sub> with all other treatments

The outstanding increase in chlorophyll concentration by D4 in the IN+NH<sub>4</sub> treatment reported earlier (Fig. 1A) also was manifested in the form of sharp increases, much greater than in all other treatments, in all HPLC-determined pigments and in total FL3 fluorescence (Fig. 2A–D). The D4 chlorophyll concentration of the IN+NH<sub>4</sub> treatment was significantly higher than the chlorophyll concentration in the other three treatments ( $p < 0.01$ ), and so were the total FC fluorescence, the zeaxanthin and 19 hexanoyl-oxy-fucoanthin concentrations. The phytoplankton group contributing most to this increase in the IN+NH<sub>4</sub> treatment was *Synechococcus*. These cyanobacteria increased both in cell densities and in their cellular chlorophyll content (Fig. 2E,F). The increase of cell densities was ca. 4-fold, from 2770 ml<sup>-1</sup> on D0 to 11,320 ± 860 ml<sup>-1</sup> on D4, the increase in cellular pigment content was even more substantial, a 17-fold increase (Table 2). None of the other treat-

ments displayed such substantial increases as *Synechococcus* did. An independent confirmation of the major increase in *Synechococcus* was recorded as a nearly 10-fold increase in zeaxanthin in the IN+NH<sub>4</sub> treatment, much greater than the respective increases of this pigment in any other treatment (Fig. 2D).

Another distinct change in the phytoplankton was the disappearance of *Prochlorococcus* in the IN+NH<sub>4</sub> treatment, which did not occur in any of the other treatments, although a decline in their abundance to about half the D0 value was recorded for the IN bottles (Table 2).

The increase in total chlorophyll and in *Synechococcus* abundance in the IN+NH<sub>4</sub> bottles was associated with a 570 nM decline of NH<sub>4</sub><sup>+</sup>, from 1680 to 1110 ± 203 nM (Fig. 1B). Thus, some but not all of the added ammonium was taken up and utilized by the biota. In the OUT+NH<sub>4</sub> bottles a much smaller decline was recorded, on D4 NH<sub>4</sub><sup>+</sup> concentration was 1515 ± 38 nM.

Phosphorus turnover time in IN+NH<sub>4</sub> bottles, initially at ca. 100 h indicating relative P sufficiency, declined with time to the same level as in the no-NH<sub>4</sub> treatments, reaching by D4 a level of 3.3 ± 4.4 h (Fig. 1C), which was significantly

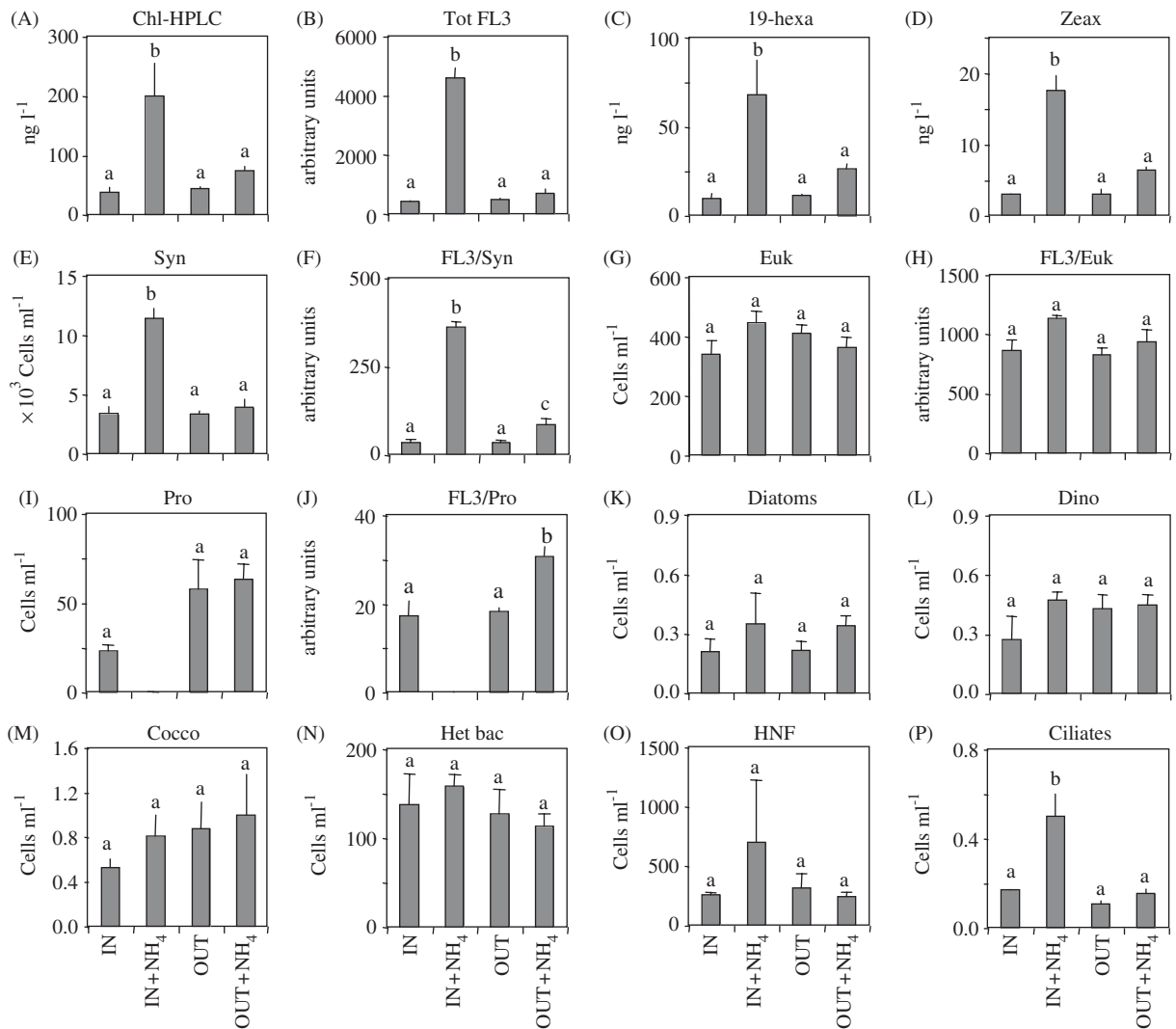


Fig. 2. Comparison between responses of the different treatments at the end (D4) of the experiment. Parameters plotted include pigment concentrations, abundance of the various phototrophic and heterotrophic groups (cells ml<sup>-1</sup>), and fluorescence per cell (flow-cytometer determined groups). Values are means of 3 replicate experimental bottles ± SD. Treatment means were compared using one-way ANOVA and a Bonferroni test. Columns labeled by different letters (A, B or C) are significantly different at  $p < 0.01$ , columns labeled by the same letter are not significantly different. Tot FL3—total fluorescence (Flow cytometric determination), 19-hexa—19-hexanoyl-oxy-fucoxanthin, Zeax—zeaxanthin, Syn—*Synechococcus*, FL3/cell—fluorescence per cell, Euk—picoplanktonic eukaryotes, Pro—*Prochlorococcus*, Dino—dinoflagellates, Cocco—coccolithophores, Het Bac—heterotrophic bacteria, HNF—heterotrophic nanoflagellates.

different from the IN value ( $51 \pm 19$  h). This sharp decline with time in P turnover time, concurrent with a decline in ambient NH<sub>4</sub><sup>+</sup>, suggests that as N was taken up and internally stored P was used, external P became again limiting. In both OUT and OUT + NH<sub>4</sub> treatments, P turnover time remained low (<10 h) at all sampling times, with some oscillations (Fig. 1C).

Bacterial numbers on D4 were rather similar in all four treatments (Fig. 2N). The abundance of HNF

tended to be higher in the IN + NH<sub>4</sub> treatment, though not significantly, but ciliates were significantly higher on D4 in IN + NH<sub>4</sub> than in the three other treatments (Fig. 2O,P), increasing their standing stock 3-fold, from 170 cells l<sup>-1</sup> on D0 to 500 cells l<sup>-1</sup> on D4. The high ciliate abundance in the IN + NH<sub>4</sub> matches with the increase in this treatment of the abundance of their major prey organisms, *Synechococcus*, HNF and eukaryotes. Within the ciliate community, on D4, tintinnids

increased their relative importance in terms of biomass in all treatments compared to D0 (from 22% of total ciliate biomass in IN on D0, to 73% and 68% in IN and IN + NH<sub>4</sub>, respectively, on D4) and even more in the OUT and OUT + NH<sub>4</sub> treatments (from 12% on D0 to 86% and 85%, respectively, on D4).

While bacterial abundance did not show significant changes, bacterial activity increased substantially in all treatments. The most prominent increase occurred in the IN + NH<sub>4</sub> bottles, where an average of 82-fold increase in activity was recorded from D0 to D4 (Table 2). The average increase in activity (D4/D0) for the other treatments was  $\times 4$ ,  $\times 4$  and  $\times 9$  for the IN, OUT and OUT + NH<sub>4</sub> treatments, respectively. It should be noted that large variability was recorded for bacterial activity rates between replicate bottles of the IN + NH<sub>4</sub> treatment (SD > mean), nevertheless, at any point in time the activity in each of the three replicate bottles of IN + NH<sub>4</sub> was higher than in any of the other nine bottles.

### 3.3. Comparison of treatments OUT with OUT + NH<sub>4</sub> to examine the effect of the addition of N alone

The addition of ammonia to OUT water led to minor responses beyond the response of controls, to which ammonium was not added (Figs. 1, 2, Table 2). In the OUT + NH<sub>4</sub> bottles, the dynamics of ammonium concentrations did not show substantial changes with time. Phosphorus turnover times remained similar to those at the start of the experiment. Both the above suggest that the added NH<sub>4</sub><sup>+</sup> could not be utilized and its addition did not have a direct or indirect effect on P availability. Day 4 chlorophyll levels were mildly higher in the OUT + NH<sub>4</sub> than in the OUT treatment (53 + 6.4 ng Chl1<sup>-1</sup> as opposed to 36.4 + 5.2 ng Chl1<sup>-1</sup>), but this difference was not statistically significant. In terms of species composition, the only noticeable difference between the two treatments was an apparent increase in the abundance of diatoms in the OUT + NH<sub>4</sub> as opposed to their decrease in the OUT bottles but the change was not significant. However, the average fluorescence per cell of *Synechococcus* was significantly higher (Fig. 2F) in the OUT + NH<sub>4</sub> (6-fold increase) as opposed to the OUT (2.6-fold increase) bottles.

In the heterotrophic component of the food web, no significant differences were found on D4 between

the OUT and OUT + NH<sub>4</sub> treatments in terms of bacterial, HNF and ciliate abundance, and bacterial activity.

## 4. Discussion

Results from microcosm experiments should be interpreted with caution because bottle effects and other experimental artifacts are considered to potentially confound the results. In our case, we had the rare opportunity to compare the results of a microcosm scale experiment with those of a concurrent in situ ocean-scale experiment conducted using the same water. The comparison was exceptionally encouraging, the responses observed in our IN vs. OUT bottles were very similar to those reported to occur in the in situ P addition experiment (Flaten et al., 2005; Pitta et al., 2005; Psarra et al., 2005). Just as in the in situ experiment, the addition of P alone (IN water) was *not* translated into an increase in phytoplankton biomass. In both experiments, bacterial activity increased several fold, with no apparent increase in bacterial abundance. In both experiments, this bacterial response was transferred higher up the food chain and recorded as an increase in ciliate abundance; ammonia concentrations declined initially and then increased to a level lower than the initial concentration; P turnover times were substantially longer in water that were exposed to added P. This resemblance between the results of the small scale and in situ experiments gives us confidence that the results regarding the IN + NH<sub>4</sub> and OUT + NH<sub>4</sub> treatments, which were not repeated in situ, may represent reasonably well the likely response of the open ocean to added NH<sub>4</sub><sup>+</sup> with or without earlier exposure to added P.

The addition of N alone, in the form of NH<sub>4</sub><sup>+</sup>, to Eastern Mediterranean water did not lead to significant abundance increases in either the phototrophic or the heterotrophic sides of the food chain, in line with previous observations and assessments (Krom et al., 1991; Bethoux et al., 1992; Thingstad and Rassoulzadegan, 1995; Zohary and Robarts, 1998) that unlike many of the world's oceans, this marine environment is not N-limited. While N addition did not invoke new growth, some uptake and storage of N did take place, since ammonia concentration in the OUT + NH<sub>4</sub> bottles decreased from 1680 nM immediately after the addition to 1515 ± 38 nM by Day 4, i.e. by 165 nM, as opposed to a 40 nM decline in the OUT water. In particular,

*Synechococcus* could take advantage of the added N, responding with a 6-fold increase in cellular chlorophyll content (4 N atoms per chlorophyll molecule), significantly higher than the increase in OUT water without  $\text{NH}_4^+$  added (Fig. 2F). This response was not as great as the 17-fold increase in *Synechococcus* cellular chlorophyll when subjected to both N and P additions (Table 2). Nevertheless, it represents an opportunistic capability of an organism well adapted to an ultraoligotrophic environment (Palenik et al., 2003). *Prochlorococcus* or the picoeukaryotes did not show this response.

Subtle differences in chemical composition of IN and OUT water used for filling the experimental bottles suggest that already at the start of the experiment some processes induced by the earlier addition of P were in progress. Notably, nearly all of the in situ added  $\text{PO}_4$  had disappeared prior to filling our microcosms with IN water, which in exchange were richer in DOC than the OUT water. Thingstad and Mantoura (2005) have shown that the Eastern Mediterranean surface water at that time had the capacity to consume between 10 and 25 nM of phosphate. Thus, upon addition of  $\text{PO}_4$  alone, phytoplankton and bacteria assimilated P, as indicated by an increase in particulate P (Flaten et al., 2005) but the phytoplankton did not grow until N was added too. While phytoplankton primary production was not measured during this experiment, it was measured in the in situ experiment, which demonstrated no significantly higher productivity in the P-enriched patch (Psarra et al., 2005). Apparently, the 2–3  $\mu\text{M}$  of dissolved organic N present in these surface water (Krom et al., 2005a) were mostly unavailable for phytoplankton growth, and nitrogen fixation could not supply the missing N at a time scale that could lead to a measurable phytoplankton biomass increase. New biomass could not be built and some of the photosynthetically fixed carbon leached or was excreted as DOC. Krom et al. (2005a) also report increases of DOC following the addition of P in the in situ experiment. Only after both P and N were added, did the Eastern Mediterranean water respond by a phytoplankton bloom, with a major increase in phytoplankton abundance, the various phytoplankton pigments, and all related indices. The experiment thus suggests that the phytoplankton were co-limited by N and P.

Bacteria, however, showed significantly higher Leucine-incorporation rates with the addition of P alone compared with the no-addition control, or the

addition of N alone, suggesting pure P-limitation. Still, the bacterial response to both N and P was much more pronounced than their response to P alone (Fig. 1D). Our interpretation is that, with a relatively small pool of available-N in excess of P, the experimental P addition supposedly caused the bacteria to rapidly flip from being P-limited to being N-limited, thus allowing only a moderate population increase without also adding N.

Differential nutrient-limitation was demonstrated also in a pristine coastal wetland, where the phytoplankton was N-limited but the bacteria were P-limited (Sundareshwar et al., 2003). This differential limitation experienced by phytoplankton and bacteria in the same Eastern Mediterranean system is possible if, e.g., bacteria are more efficient than phytoplankton in using DON as a nitrogen source. This conflicts somewhat with the growing body of literature demonstrating that phytoplankton and especially cyanobacteria do utilize DON (reviewed by Berman and Bronk, 2003), but this issue cannot be resolved until we know more about the composition and utilization of DON in the Eastern Mediterranean.

The increased bacterial activity when N and P were added was not matched by increases in bacterial cell numbers, suggesting that the standing crop was controlled by grazers, such as heterotrophic nanoflagellates and ciliates. The significantly higher D4 ciliate cell abundance in the IN +  $\text{NH}_4$  treatment relative to all other treatments supports this top-down interpretation. Another line of evidence in support of intense top-down control in this region is the much higher ammonium (ca. 80 nM) than nitrate (<10–18 nM) concentrations in the surface water, suggesting most inorganic N comes from recycling.

The main group of phototrophs to take advantage of the added P and N was *Synechococcus*, which not only increased in abundance but also used the sudden nutrient pulses to increase cellular pigment content. In contrast, *Prochlorococcus* lost this competition and disappeared from the water. A similar in situ increase of *Synechococcus* and disappearance of *Prochlorococcus* was recorded immediately after a Saharan dust storm during a cruise to the Cyprus Eddy in May 2001, and was repeated in a microcosm experiment in which dust was added to Eastern Mediterranean water (Herut et al., 2005). Saharan dust fertilizes the ocean surface water with both N and P (e.g. Migon et al., 2001; Herut et al., 2002; Ridame and Guieu,

2002) and therefore it is not a surprise that it triggers responses similar to those seen in our microcosm experiment.

Marine unicellular cyanobacteria of the *Synechococcus* group occupy an important position at the base of the marine food web: they are abundant, contributing an estimated 20–40% of chlorophyll biomass and carbon fixation in the world's oceans (Waterbury et al., 1986). Some strains of *Synechococcus* possess a unique type of swimming motility not seen in any other type of microorganism: they propel themselves through seawater at speeds of up to  $25 \mu\text{m s}^{-1}$  in the absence of any demonstrable external organelle. They use this motility to respond to sub-nanomolar gradients of nitrogenous compounds (Willey and Waterbury, 1989). Moutin et al. (2002) and Tanaka et al. (2003) have shown that *Synechococcus* has  $\text{PO}_4$  uptake kinetics (affinity, maximum uptake rate) superior to those of bacteria and eukaryotic algae, which allows it to outcompete other species when  $\text{PO}_4$  becomes available.

Apparently only  $1680 - 1110 = 570 \text{ nM}$ , about a third of the added ammonium, was taken up by the biota. Dividing  $570 \text{ nM}$  of N by  $22 \text{ nM}$  of P, the amount computed (from  $\text{SF}_6$ ) to have been initially added to the water with the in situ P addition, we get  $\text{N} : \text{P} = 26$ , higher than Redfield. Similar  $\text{N} : \text{P}$  ratios were observed for both  $\text{PON} : \text{POP}$  in the upper water column and for the calculated decomposed organic matter in the deep water, suggesting that this  $\text{N} : \text{P}$  ratio is characteristic of phytoplankton growth in the region (Krom et al., 2005b). Haldal et al. (2003) have shown that  $\text{N} : \text{P}$  ratio of marine *Synechococcus* cultures may exceed the Redfield ratio and have argued that this capacity will be advantageous in oligotrophic, P-limited oceans. Furthermore, cyanobacteria have a high demand for N. Bertilsson et al. (2003) reported  $\text{N} : \text{P}$  ranging between 21 and 33 for P-replete isolates of *Synechococcus*, and much higher ratios, 59–109, for P-deplete cultures. Their results suggest that *Prochlorococcus* and *Synechococcus* may have relatively low P requirements. Apparently, plasticity in  $\text{C} : \text{N} : \text{P}$  ratios of nutrient-limited cells is common in marine phytoplankton (Geider and La Roche, 2002) and bacteria (Martinussen and Thingstad, 1987), thus our results are not surprising.

To conclude, our experiment shows that in summer, when the surface water of the Eastern Mediterranean are depleted of both P and N, neither bacteria nor phytoplankton are limited by N alone. The bacteria are P-limited, respond to the

addition of P alone by enhanced growth, but flip to N-limitation after a small addition of P. The phytoplankton does not increase in biomass without the addition of both nutrients and can therefore be considered as being limited by both P and N. *Synechococcus* is the dominant player in the response of the phytoplankton.

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