

# Effect of P and N addition to oligotrophic Eastern Mediterranean waters influenced by near-shore waters: A microcosm experiment

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## Abstract

Phosphate (P), nitrate (N) or P+N added in a microcosm experiment to oligotrophic waters of the Eastern Mediterranean influenced by near-shore waters triggered a range of responses in the autotrophic and heterotrophic compartments of the system. Chlorophyll *a* increased in all treatments, including the no-addition control, implying that nutrients became available also from internal sources (recycling). Larger and faster biomass increase as well as a larger P utilization took place in the P+N treatments. Diatoms bloomed in the P+N treatments whereas coccolithophores bloomed following the addition of P ultimately reaching N-limitation. Bacterial activity responded with a transient peak to both low P-alone and N-alone additions (0.01 and 1  $\mu\text{M}$ , respectively). For reasons not well understood, no such response was observed at higher P-alone additions (0.05 and 0.5  $\mu\text{M}$ ), whereas at the two highest P+N additions the positive response was delayed. We therefore were unable to conclude conclusively on bacterial limitation. In most cases, the increase in bacterial activity was not matched by an increase in abundance, suggesting a tight top-down control of the biomass. Instead, heterotrophic nanoflagellate and ciliate abundances increased in all treatments. A slightly elevated orthophosphate turnover-time ( $T_t$ ) (32 h) in the initial waters did not give a clear indication of P-limitation, although the system could absorb the lowest P-addition (0.01  $\mu\text{M}$ ) without increase in  $T_t$ . N alone lead to a reduction in  $T_t$  as would be expected in an N-limited system consuming existing surplus P after N-addition.

The response of the near-shore influenced system used in this study was in accord with the ‘classical’ response to nutrient introduction—increase in chlorophyll *a* and in large size phytoplankton. In contrast, in the ultraoligotrophic Cyprus Eddy [Krom, Thingstad, Carbo, Drakopoulos, Fileman, Flaten, Groom, Herut, Kitides, Kress, Law, Liddicoact, Mantoura,

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E-mail address: [nurit@ocean.org.il](mailto:nurit@ocean.org.il) (N. Kress).

Pasternak, Pitta, Polychronaki, Psarra, Rassoulzadegan, Skjoldal, Spyres, Tanaka, Tselepidis, Wassmann, Wexels-Riser, Woodward, Zodiatis, Zohary, 2005. Overview of the CYCLOPS P addition lagrangian experiment in the Eastern Mediterranean. Deep-Sea Research II, this volume.], the short  $T_L$  (<4 h) indicated P-limitation, the combined addition of P and N (as ammonium) induced a bloom of picocyanobacteria [Zohary, Herut, Krom, Mantoura, Pitta, Psarra, Rassoulzadegan, Stambler, Tanaka, Thingstad, Woodward, 2005. P-limited bacteria but N&P co-limited phytoplankton in the Eastern Mediterranean—a microcosm experiment. Deep-Sea Research II, this volume.] and the in situ P alone addition led to a decrease in chlorophyll.

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## 1. Introduction

Offshore and near-shore waters of the same marine environment can differ substantially due to proximity of the latter to natural and anthropogenic sources. Generally, the near-shore waters are more eutrophic, with higher nutrient and chlorophyll concentrations, different species composition and community size structure distribution. Thus, the responses of both systems to added nutrients are likely to differ. In the ultra-oligotrophic Levantine basin of the Eastern Mediterranean, the offshore waters have exceptionally low nutrient concentrations and primary production (Krom et al., 1991; Kress and Herut, 2001; Psarra et al., 2005), and the phytoplankton community is dominated by the pico and nano fractions which are heavily grazed (Yacobi et al., 1995; Zohary et al., 1998; Christaki et al., 2001; Pitta et al., 2001; Psarra et al., 2005). The coastal waters, on the other hand, are characterized by somewhat higher nutrient and chlorophyll concentrations, higher primary production and a greater abundance of larger size phytoplankton (Berman et al., 1984; Azov, 1986; Kimor et al., 1987; Bonin et al., 1989; Herut et al., 2000).

Bioassays in offshore and coastal waters in the eastern Mediterranean (Bonin et al., 1989; Zohary and Robarts, 1998) as well as nutrient stoichiometry (Krom et al., 1991; Kress and Herut, 2001) have suggested that biological production in the region is limited by phosphorus (P) availability. The CYCLOPS program was set up to investigate the cycling of P in the Eastern Mediterranean by ways of a series of P-addition experiments, both in microcosms and in situ, with the main focus on the latter (Krom et al., 2005). In this study we describe the chemical and biological characteristics of the oligotrophic near-shore waters of the Levantine basin, and their response to nutrient addition in the form of a gradient of increasing concentration of P and P+N. We compare the

responses of the autotrophic and heterotrophic community components in near-shore waters to those observed when similar additions were performed in situ in Levantine Basin's open sea water (Flaten et al., 2005; Pitta et al., 2005; Psarra et al., 2005) and in on-deck microcosm experiments (Zohary et al., 2005), in the framework of the CYCLOPS project.

## 2. Methods

### 2.1. Experimental design

Nine different treatments were carried out in the microcosm experiment (Table 1). Each treatment was performed in triplicate, thus giving a total of 27 experimental carboys. Treatment #1 was the control (no additions), treatments #2–4 had increasing phosphate (P) additions, and treatments #5–8, increasing phosphate + nitrate (P+N) additions.

Table 1  
Experimental set-up of the microcosm nutrient addition experiment, Haifa, 14–20 May 2000. Each treatment was performed in triplicate, giving a total of 27 experimental carboys

Addition	Treatments			
Phosphate	#1	#2	#3	#4
	0 $\mu\text{M}$ P	0.01 $\mu\text{M}$ P	0.05 $\mu\text{M}$ P	0.5 $\mu\text{M}$ P
Phosphate + nitrate <sup>a</sup>	#5	#6	#7	#8
	0 $\mu\text{M}$ P	0.01 $\mu\text{M}$ P	0.05 $\mu\text{M}$ P	0.5 $\mu\text{M}$ P
	1 $\mu\text{M}$ N	1.2 $\mu\text{M}$ N	2 $\mu\text{M}$ N	11 $\mu\text{M}$ N
Glucose	#9			
	0 $\mu\text{M}$ P			
	0 $\mu\text{M}$ N			
	60 $\mu\text{M}$ C			

<sup>a</sup>The logic behind the N additions was to have surplus N (N:P higher than 16) in these treatments so that one could compare the pairs #1–5, #2–6, #3–7, and #4–8.

The latter should in principle represent surplus N, thus preventing N-limitation in order to enable comparison of the pairs: #1–5, #2–6, #3–7, and #4–8. Glucose was added to treatment #9 in order to check possible C-limitation of bacteria. The P and N concentrations are in the range found in the open waters of the Levantine basin of the Eastern Mediterranean, except for the high P+N addition to #8 in which the concentrations are much higher than natural (Kress and Herut, 2001).

## 2.2. Collection of sea water and set-up of the experiment

Eastern Mediterranean water for the experiment (ca. 1000 L) was collected on 14 May 2000, from ca. 10 m depth at a site located about 30 km west of Haifa (32°54.79N, 34°48.11E, 980 m bottom depth)

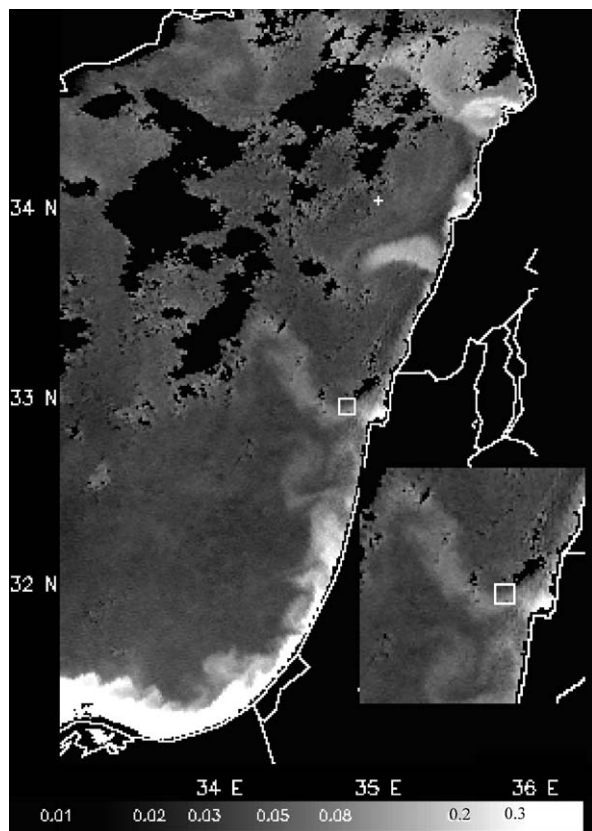


Fig. 1. SeaWiFS chlorophyll-*a* image for the 14 May 2000, computed using the Bricaud et al. (2002) Mediterranean-specific algorithm (Groom et al., 2005). It shows a filament of higher chl-*a* near-shore water extending offshore. The centre of the white square marks the position where the water was sampled for the microcosm experiment. At the sampling location the computed chl-*a* was  $0.084 \text{ mg m}^{-3} \pm 0.015$ .

(Fig. 1). Seawater was pumped using three FLOJET pumps into acid-precleaned 25-L carboys. Prior to filling, the carboys were washed three times with the pumped water passed through a 125- $\mu\text{M}$  mesh net (to remove the large predators) and then filled with filtered (125- $\mu\text{M}$ ) water. The carboys were split into three groups, so that each treatment would have one carboy from each group. A single pump was used to fill the carboys in each group to reduce possible effects due to the pump and its relative position in the ship during filling. In each group, one quarter of each carboy was filled in sequential order and the procedure repeated until filling all carboys to achieve a homogenous sample. Two replicate water samples for nutrient determinations were sampled from each pump in 15-mL acid-washed plastic scintillation vials and kept refrigerated until determination in the laboratory later on the same day. The carboys (30 experimental + nine for spare and rinse water) were brought ashore, where they were incubated, half floating, in a 6-m diameter circular out-door pond with running seawater at constant temperature of 21 °C. The incident light was reduced by 50% with screens placed on top of the pond with a further reduction of 30% due to the carboy itself.

## 2.3. Nutrient additions and daily sampling

Stock solutions of 1 mM of phosphate, 10 mM nitrate and 10 mM glucose were prepared from reagent grade  $\text{KH}_2\text{PO}_4$ ,  $\text{KNO}_3$  and glucose. One day after sea water collection, on the morning of 15 May, “time zero” or Day 0 (D0 hereafter), nutrients were added to the carboys in a single addition to reach the final concentrations listed in Table 1. Subsequent days are referred to as Day 1 (D1) up to Day 5 (D5) on 20 May. In addition, on D0, samples were collected from three extra carboys to which no nutrients were added.

From D1 until D5, the carboys were sampled every morning at 9 a.m. Each carboy was taken out of the pond, rinsed externally and then mixed well, before a 4.5-L sample was poured into a 5-L pre-cleaned container that was taken to the laboratory and sub-sampled for the different analyses. Special care was taken to prevent contamination of the water in the carboys or containers. In order to shorten the sampling time, three sampling teams worked in parallel, each responsible for the sampling of nine carboys. Sampling was completed in 1 h. On D5, the approximately 7 L remaining in the carboys were made available for analyses.

#### 2.4. Variables measured

The following variables were measured at each sampling day in all carboys: inorganic nutrients (phosphate, nitrate + nitrite and silicic acid), total chlorophyll *a* (chl *a*) and size fractionated chl *a*, photosynthetic rate, leucine incorporation (bacterial activity), bacterial abundance, orthophosphate turnover time. Size distribution of phosphate uptake and of particulate P, and nanoflagellates abundance and biomass were measured up to D4. Microscopic determination of phytoplankton and size-fractionated photosynthetic rate were determined at the start and end of the experiment (D0 and D5, respectively). Microscopic determination of ciliates was performed on D0, D3 and D5.

#### 2.5. Analytical methods

**Inorganic nutrients:** Duplicate water samples were collected in 15-mL acid-washed plastic scintillation vials. One replicate was analyzed on the day of sampling and one was frozen for later analysis. They did not differ significantly and the reported concentrations are averages. Nutrients were determined using a segmented flow Technicon AutoAnalyser II (AA-II) system as described by Krom et al. (1991) and Kress and Herut (2001). The precision of nitrate + nitrite, phosphate and silicic acid measurements was 0.02, 0.003 and 0.06  $\mu\text{M}$ , respectively. The limit of detection (2 times the standard deviation of the blank) was 0.075  $\mu\text{M}$  for nitrate + nitrite, 0.008  $\mu\text{M}$  for phosphate and 0.03  $\mu\text{M}$  for silicic acid. For simplicity, in the text we refer to nitrate + nitrite as nitrate.

**Phosphate size-distribution of orthophosphate uptake:** The fraction of uptake in size-fractions  $>5$ , 5–1, and 1–0.2  $\mu\text{m}$  was calculated by subtraction and using background-corrected radioactivity on the 0.2- $\mu\text{m}$  filter as the total uptake. Isotope dilution was estimated from experiments where isotope plus 0, 10, 25, 50, and 75 nmol  $\text{PO}_4 \text{ PL}^{-1}$ , respectively, of unlabelled  $\text{KH}_2\text{PO}_4$  was added to the Falcon tubes prior to addition of the sample.

**Size-distribution of particulate P:** 200–500 mL of sample was filtered sequentially through 47-mm polycarbonate filters (Poretics<sup>®</sup>) of 5.0, 1.0, and 0.2  $\mu\text{m}$  pore-size. The filters were transferred to polypropylene test tubes, and 5 mL of distilled water added. Following acid persulphate oxidation at 120 °C, orthophosphate liberated was measured

spectrophotometrically using the molybdenum blue reaction (Flaten et al., 2005).

**Chlorophyll *a*:** 0.5–1 L of sample was filtered through GF/F for total chlorophyll *a* (chl *a*). Filters were placed in  $-20^\circ\text{C}$  immediately after filtration and kept frozen for six weeks until laboratory analyses took place. Chl *a* concentrations were determined fluorometrically after acetone extraction according to Yentsch and Menzel (1963) using a TURNER 112 fluorometer.

**Microscopic determinations:** Quantitative and qualitative determinations of micro- and nanophytoplankton populations were performed by the methods described in Pitta et al. (2005) and Psarra et al. (2005). Briefly, samples for heterotrophic bacteria, microphytoplankton and ciliates counting were fixed with formaldehyde and for nanoflagellates with glutaraldehyde. Bacteria and nanoflagellates (2–10  $\mu\text{m}$ ) were counted on DAPI-stained samples using an epifluorescence microscope while microphytoplankton and ciliates were counted after sedimentation using an inverted microscope. Carbon biomass of nanoflagellates was calculated assuming constant volumes of 35 and 294  $\mu\text{m}^3 \text{ cell}^{-1}$  for the 2–5 and 5–10  $\mu\text{m}$  size class, respectively, and a conversion factor of 183  $\text{fg C } \mu\text{m}^{-3}$  (Caron et al., 1995). Carbon biomass of ciliates was calculated by the method detailed in Pitta et al. (2005).

**Photosynthetic carbon fixation rates** were estimated by means of the  $^{14}\text{C}$  technique of Steemann Nielsen (1952) as modified by Psarra et al. (2000). Samples were incubated while floating in the outdoor pond, next to the carboys, for 2.5–3.5 h around noon (11:30 to 14:30 local time). During incubation the bottles were placed under a screen that reduced incident light by 50%. Turbidity of the circulating water reduced further the light by 20%. Incident irradiance was measured by a Licor LI185B Quantum meter. At the end of incubation samples were treated as described in detail in Psarra et al. (2005).

**Leucine incorporation** as a measure of bacterial activity was determined following the protocol of Simon and Azam (1989).  $^{14}\text{C}$  Leucine (Amersham) was added to triplicate 10-mL samples in sterile Steriline<sup>®</sup> tubes to give a final concentration of leucine of 30 nM. The samples were incubated at  $24 \pm 1^\circ\text{C}$  and subdued (laboratory) illumination for 2–2.5 h. Additional replicates for subtraction of background and abiotic adsorption were fixed with 200  $\mu\text{L}$  of concentrated formaldehyde (12%) per

10 mL sample before addition of the isotope. Incubation was stopped by the addition of 200  $\mu\text{L}$  of formaldehyde, and the contents of the test-tube were filtered onto 25-cm diameter, 0.2- $\mu\text{m}$  pore-size Milipore filters. The suction was then stopped and 5 mL of 5% TCA were placed on top of the filters. After 5 min, the TCA was filtered through, the filters washed twice with 2.5 mL of 80% ethanol and placed in a scintillation vial to which 0.5 mL ethyl acetate was added (to dissolve the filter). Five mL scintillation Fluor (Ultimagold) were added and the filters were counted on a Packard Scintillation counter. Bacterial activity ( $\text{pmol leucine L}^{-1} \text{h}^{-1}$ ) was calculated as: Activity = ( $\text{dpm's on filter/dpm added}$ )  $\times$  ( $\text{pmol/mL}$ )  $\times$  ( $1000/\text{incubation time}$ ).

**Orthophosphate turnover-time ( $T_t$ ):** Carrierfree  $\text{H}_3^{33}\text{PO}_4$  was added to 12-mL samples in 15-mL Falcon<sup>®</sup> tubes to give a final radioactive concentration of ca.  $10^6 \text{ DPM mL}^{-1}$ . The samples were incubated at  $24 \pm 1^\circ\text{C}$  in subdued illumination from 15 min to 4.5 h according to the expected turnover-time. Samples for subtraction of background and abiotic adsorption were fixed with glutaraldehyde before addition of the isotope. Incubation was stopped by a cold chase of  $100 \mu\text{mol KH}_2\text{PO}_4 \text{ L}^{-1}$  and 3-mL aliquots filtered in parallel on 25-cm polycarbonate (Poretics<sup>®</sup>) filters of 5.0, 1.0, and 0.2  $\mu\text{m}$  pore-size, respectively, within 1 h. The filters were placed on GF/C filters soaked in  $10 \text{ mmol L}^{-1} \text{KH}_2\text{PO}_2$  using a Millipore 12-place filter manifold. The needle valve of the suction pump was kept open until all water had passed the 5.0- and 1.0- $\mu\text{m}$  filters, the suction was then increased to  $<0.2$  bar until all water had passed the 0.2- $\mu\text{m}$  filters, and the needle valve then closed to increase the suction to  $>0.6$  bar to remove any water remaining in the filters. No washing was performed. Radioactivity was counted by scintillation counting and turnover-time calculated from  $T_t = -t/\ln(l-f)$ , where  $f$  is the fraction of added isotope recovered on the 0.2- $\mu\text{m}$  filter (corrected for background), and  $t$  is the incubation time.

### 2.6. Statistical analysis

T test and ANOVA, at the 95% confidence level were used to assess the significance of differences among days and treatments. Most of the triplicates for each treatment agreed well, and the results presented here are the averages of the three replicates. In treatment #8 one replicate clearly departed from the others in most variables measured and was therefore excluded.

## 3. Results

### 3.1. Initial state

The water used in the experiment was collected from 30 km off the Israeli coast. While quite distant from shore, remote sensing has confirmed that the location at which the carboys were filled was within a meander of near-shore waters clearly seen in the SeaWiFS-derived chlorophyll concentrations map (Fig. 1). Such an occurrence, previously unreported in this area, was since then identified again (Karabashev et al., 2002). The state of the system in this water (D0, Table 2) was oligotrophic ( $0.009 \pm 0.007$ ,  $0.08 \pm 0.09$ , and  $1.06 \pm 0.11 \mu\text{M}$  for phosphate, nitrate, and silicic acid, respectively, chl  $a$  concentration of  $0.06 \pm 0.01 \text{ mg m}^{-3}$  and photosynthetic rate of  $0.85 \pm 0.53 \mu\text{g C L}^{-1} \text{h}^{-1}$ ). Most of the chl- $a$  was in the two smallest size fractions, 0.2–1 and 1–5  $\mu\text{m}$  with 34% and 42% of the total chl  $a$ , respectively. Only 23% was contributed by cells  $>5 \mu\text{m}$ . Orthophosphate turnover-time was  $32 \pm 3 \text{ h}$ , and the concentration of particulate P was  $0.018 \pm 0.001 \mu\text{M}$ , of which most belonged to the smallest size fraction (1–0.2  $\mu\text{m}$ ). Diatoms dominated the  $>5\text{-}\mu\text{m}$  fraction (48% of cell abundance,  $11.1 \text{ cells mL}^{-1}$ ), followed by dinoflagellates (29%,  $4.3 \text{ cells mL}^{-1}$ ) and coccolithophorides (22%,  $3.5 \text{ cells mL}^{-1}$ ). HPLC pigment analysis (RFC Mantoura, unpublished results) showed that 40% of the pigments were attributable to picocyanobacteria. Heterotrophic bacterial abundance was  $4.4 \pm 0.4 \times 10^8 \text{ cells L}^{-1}$ , with a leucine incorporation rate of  $170 \pm 35 \text{ pmol L}^{-1} \text{h}^{-1}$ . ANF and HNF abundance were  $0.71 \times 10^5$  and  $1.19 \times 10^5 \text{ cells L}^{-1}$ , respectively, while the total ciliate abundance was only  $73 \text{ cells L}^{-1}$ .

### 3.2. Temporal evolution

The addition of glucose (#9) caused no discernible effects in the system, behaving in general as the control (#1). There was no indication of C-limitation of bacteria. Therefore, this treatment will be treated hereafter as an additional control and not referred to specifically. The values for all variables measured during the experiment on D0 and D5 are available at [www.earth.leeds.ac.uk/cyclops](http://www.earth.leeds.ac.uk/cyclops).

#### 3.2.1. Inorganic nutrients

The concentrations of phosphate and nitrate during collection at sea were  $0.009 \pm 0.007$  and

Table 2  
Initial status of the system in the different experiments of the CYCLOPS program

Parameter	Microcosma <sup>a</sup> 2000	Microcosm <sup>b</sup> 2001	In situ <sup>c</sup> 2002
PO <sub>4</sub> (μM) <sup>d</sup>	<0.008	<0.008	<0.005
NO <sub>3</sub> (μM) <sup>d</sup>	<0.075	<0.075	<0.010
Si(OH) <sub>4</sub> (μM)	1.06±0.11	0.87±0.02	1.00
Chl <i>a</i> (mg m <sup>-3</sup> )	0.06±0.01	0.029	0.018±0.001
Bacterial abundance (cells L <sup>-1</sup> )	4.4±0.4 × 10 <sup>8</sup>		0.6–1.4 × 10 <sup>8</sup>
Leucine incorporation (pmol leucine L <sup>-1</sup> h <sup>-1</sup> )	170±35	8.4±2.1	15
Orthophosphate turnover-time (h)	32±3	2–5	2–4
Photosynthetic rate (μg C L <sup>-1</sup> h <sup>-1</sup> )	0.85±0.53		0.091±0.014
Particulate P (nM)	18±1	8	8–10
ANF (cells L <sup>-1</sup> )	0.71 × 10 <sup>5</sup>	2.14 × 10 <sup>5</sup>	1.6–4.8 × 10 <sup>5</sup>
HNF (cells L <sup>-1</sup> )	1.19 × 10 <sup>5</sup>	3.69 × 10 <sup>5</sup>	1.9–5.6 × 10 <sup>5</sup>
Large phytoplankton (cells mL <sup>-1</sup> )	CC—3.5 DF—4.3 DT—11.1		CC—0.8 DF—0.4 DT—0.1
Ciliate abundance (cells L <sup>-1</sup> )	73	40	53–94

CC—Coccolithophores, DF—Dinoflagellates, DT—Diatoms.

<sup>a</sup>This study.

<sup>b</sup>May 2001 (CYCLOPS, unpublished results).

<sup>c</sup>May 2002.

<sup>d</sup>Concentrations at the day of collection at sea in this study were 0.009±0.007 and 0.08±0.09 μM for PO<sub>4</sub> and NO<sub>3</sub>, respectively.

0.08±0.09 μM, respectively. On D0, 24 h after collection but prior to nutrient addition, the two nutrients were below the detection limit, suggesting their rapid uptake. Due to an instrumental malfunction silicic acid was not determined until D1. Background concentration was therefore determined from the results of treatments #1 and #9 that remained constant during the experiment yielding 1.06±0.11 μM ( $n = 28$ , Fig. 2). On D1 concentrations of phosphate and nitrate were detectable in all treatments (Fig. 2), with average concentrations of 0.018±0.011 μM and 0.21±0.10 for phosphate and nitrate, respectively, in the controls. In the carboys to which nutrients were added, there was generally a good agreement between the calculated and measured values on D1.

Phosphate concentrations decreased with time in all treatments to which P was added (Fig. 2). In the treatments with P alone, all the phosphate was utilized (concentration added—concentration left in solution) by D5 at the lowest addition (#2), whereas at the higher additions, #3 and #4, only 71% and 20% were utilized (0.04 and 0.10 μM respectively), implying that the P-additions were sufficient to force the system to N limitation. However, 2.7 times more phosphate was utilized in #4 than in #3, yet the latter still had excess dissolved phosphate by D5. In the P+N treatments #6 and #7, all the added P was utilized (0.01 and 0.05 μM, respectively)

whereas in treatment #8, 79% of P was utilized (0.40 μM) compared to only 0.10 μM in treatment #4.

Nitrate concentrations generally declined with time in all treatments, from D3 in treatments with P alone (#2–4), being depleted by D5 except for the lowest P alone (Fig. 2). In the N alone or P+N treatments, the decrease in nitrate concentration started on D2 and reached minimum concentration on D5. In the N alone treatment (#5), nitrate was reduced by 68% (0.68 μM) and in the high P+N treatment (#8) by 79% (6.6 μM). The entire N added in treatments #6 and #7 was utilized (1.2 and 2 μM, respectively).

Silicic acid remained essentially constant along the experiment in all treatments except at the P+N additions (#6–8) where it decreased significantly by ca. 50% by D4 (Fig. 2).

### 3.2.2. Standing stocks

The particulate-P concentration remained essentially constant in the controls while the P-alone additions increased the concentrations slightly (Fig. 3). P+N additions caused however, a clear increase in particulate P, particularly at higher additions (#7 and #8). Fitting an exponential curve to the data of treatment #8 ( $y = 0.017e^{0.51t}$ ,  $R^2 = 0.991$ , where  $y$  is particulate P in nM P and  $t$  is time in days) the calculated concentration on D5

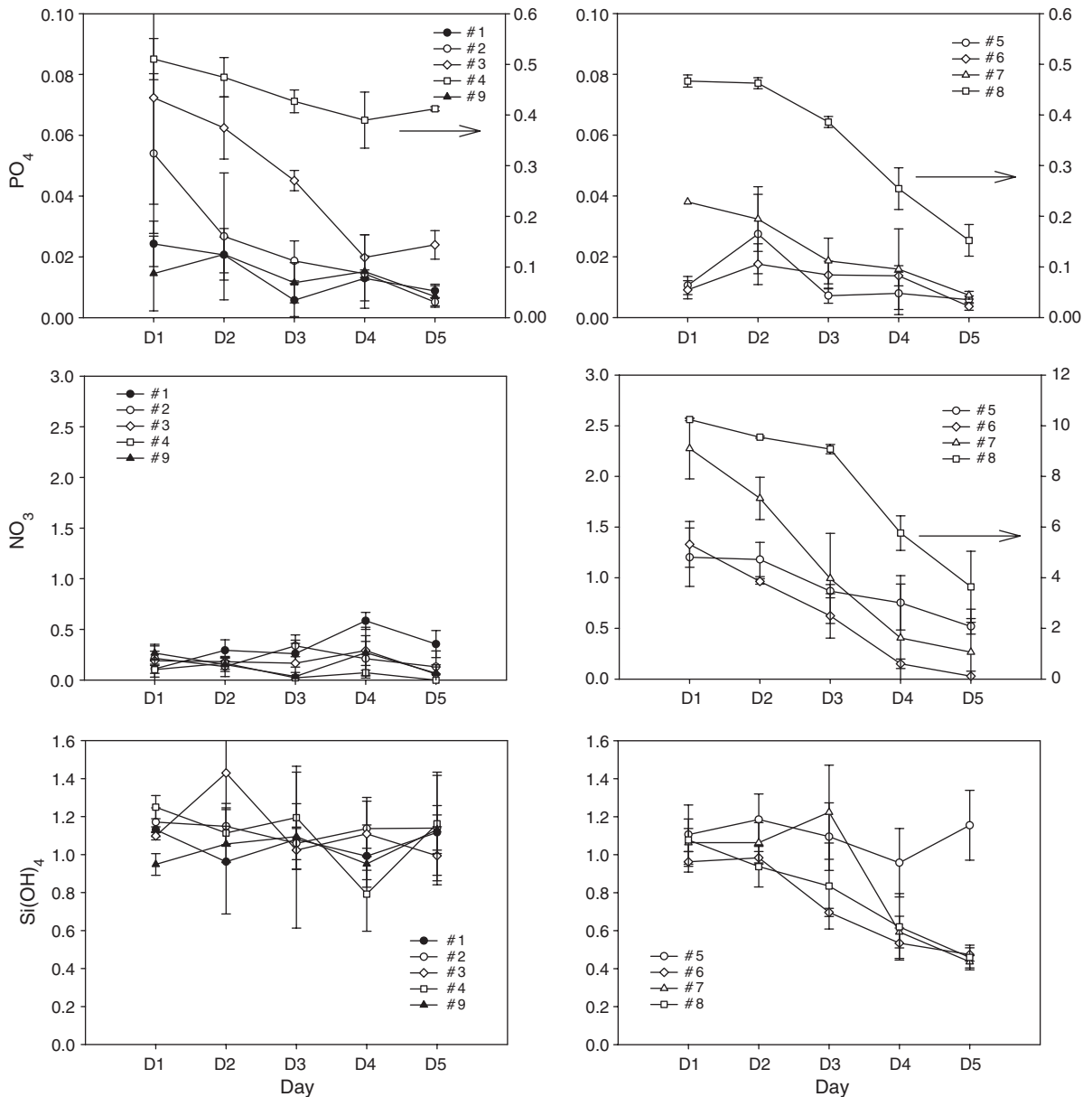


Fig. 2. Nutrients concentrations ( $\mu\text{M}$ ) in the different treatments as a function of time. Concentration of phosphate and nitrate on D0 were  $<0.008$  and  $<0.075 \mu\text{M}$ , respectively. The arrow indicates that data for treatment #4 (phosphate) and #8 (phosphate and nitrate) refer to the secondary Y axis on the right.

is  $0.22 \mu\text{M P}$ , corresponding to an increase of  $0.20 \mu\text{M P}$  from D0. The amount of phosphate utilized during the experiment in #8 was  $0.40 \mu\text{M P}$  (Fig. 2), suggesting that ca.  $0.2 \mu\text{M P}$  was converted to dissolved organic forms.

Particulate P on D0 was dominated by the smallest ( $1\text{--}0.2 \mu\text{m}$ ) size-fraction and remained the same on D4 in the treatments with P-alone addition (#2–4). There was a slight increase in the smallest

size fraction in the N-alone addition (#5). The relative importance of the larger size fraction ( $>5 \mu\text{m}$ ) increased with increasing addition when P+N were added together (#6 < 7 < 8) (Fig. 4).

Uptake of  $^{33}\text{PO}_4$  at different concentrations of added phosphate gave different kinetic parameters for the three size fraction.  $K_t + S_n$  increased with increasing size fraction (Table 3). The smallest value,  $27 \text{ nmol P L}^{-1}$ , was thus the estimated upper

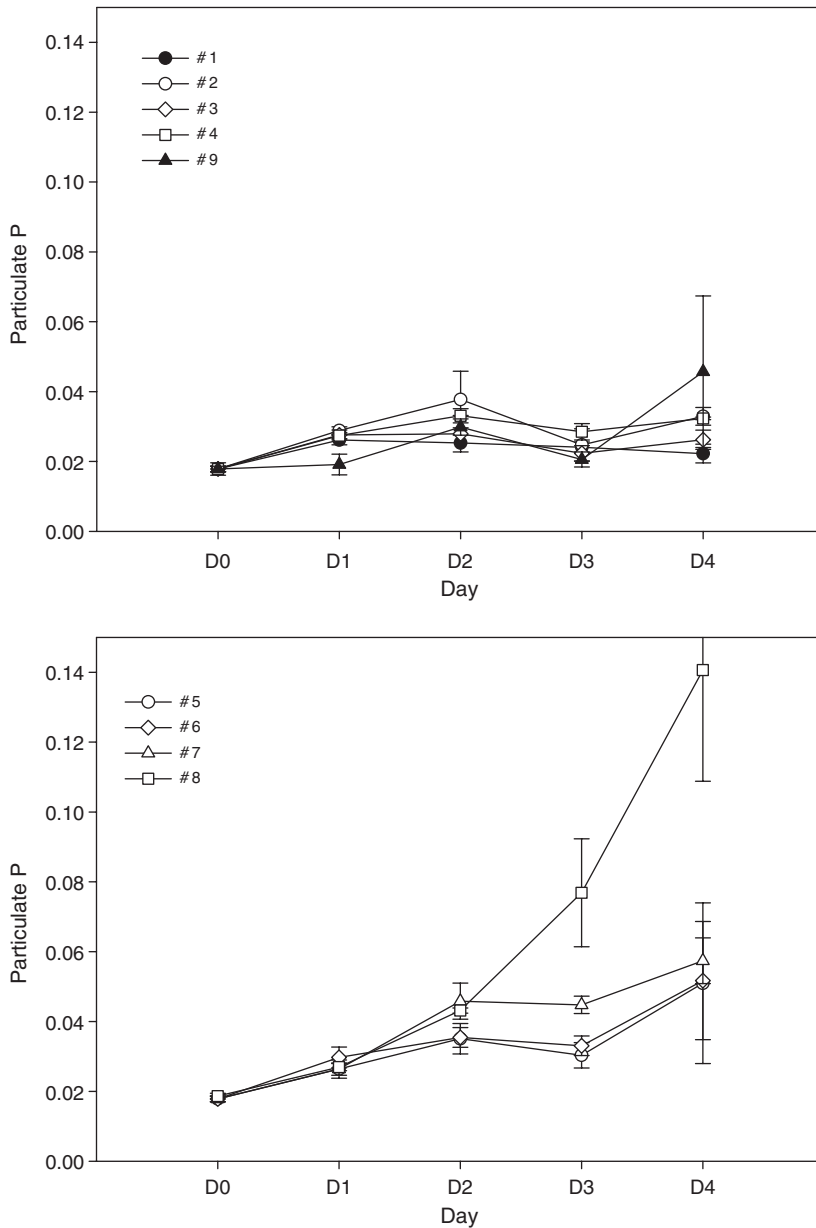


Fig. 3. Particulate P ( $\mu\text{M}$ ) in the different treatments as a function of time.

bound on the concentration (i.e. assuming  $K_t$  to be negligible compared to  $S_n$ ) of bioavailable P on D0. As for particulate P, the distribution on D0 was dominated by uptake in the small phytoplankton and heterotrophic bacteria with 69% in the smallest particulate size-fraction (1–0.2  $\mu\text{m}$ ) (Fig. 4). A shift towards uptake by the larger size-fractions was observed in the controls, the two highest P-alone treatments (#3–4) and P+N treatments (#6–#8). The latter shifted the relative uptake from the

1–0.2  $\mu\text{m}$  to the >5  $\mu\text{m}$  size-fraction, the shift increasing with increasing additions (#6 < 7 < 8). The lowest P-alone (#2) and N-alone (#5) additions did not change the original size-distribution.

Chl *a* increased from D0 to D5 in all treatments, including the controls (Fig. 5). In all treatments, except for those to which P+N were added (#6–8), the increase in chl *a* started on D3 and continued until the end of the experiment. In the P+N treatments, the increase in chl *a* started earlier, on

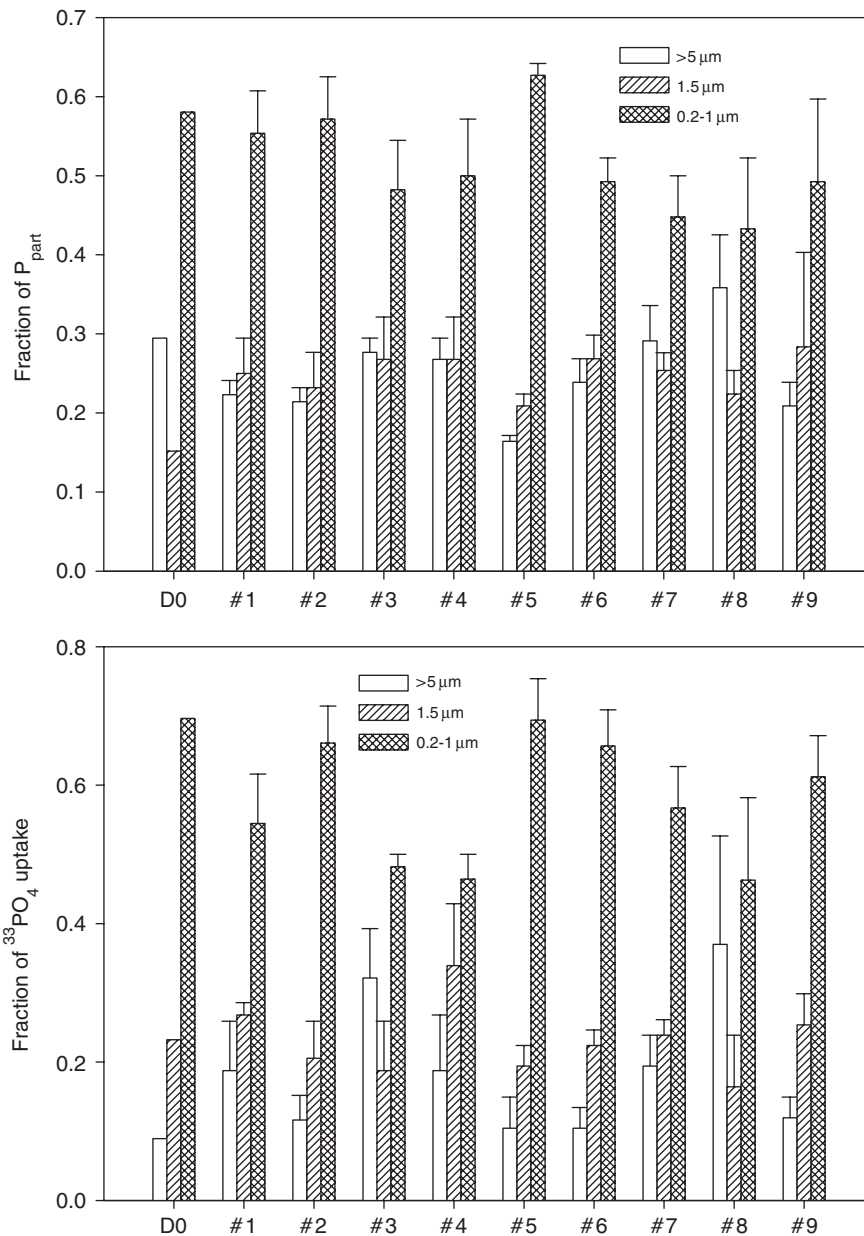


Fig. 4. Relative size-distribution of particulate P and <sup>33</sup>PO<sub>4</sub>-uptake on D0 and in the different treatments on D5.

Table 3

Day 0: Particulate P and kinetic parameters for phosphate uptake in size fractions

Size fraction	Particulate P nmol PL <sup>-1</sup>	SE ( <i>n</i> = 3)	<i>K<sub>t</sub></i> + <i>S<sub>n</sub></i> nmol-P L <sup>-1</sup>	SE	<i>V<sub>max</sub></i> nmol PL <sup>-1</sup> h <sup>-1</sup>	SE
> 5 μm	5.1	0.6	170	80	0.39	0.18
5–1 μm	2.6	0.5	45	11	0.27	0.05
1–0.2 μm	10.1	0.7	27	3	0.53	0.03

SE—standard error, *K<sub>t</sub>*—half saturation constant for phosphate uptake, *S<sub>n</sub>*—bioavailable phosphate concentration, *V<sub>max</sub>*—maximum uptake potential (Flaten et al., 2005).

D1 and reached a maximum on D3 (#6 and #7) or on D5 (#8). Comparison between the treatments with same P-additions (with and without N) indicated that in the two lowest additions, maximum chl *a* concentrations were twice as high when also N was added. In the highest addition, chl *a* was 5 times higher when N was added (#8 compared to #4). In all P-alone additions the maximum chl *a* concentration occurred later in the experiment compared to the equivalent P + N treatments.

Generally, the abundance of the large phytoplankton cells (>5 μm) increased from D0 to D5 and the community composition changed. In the controls there was an increase in the relative contribution of coccolithophorids (to ca. 60%) accompanied by a decrease in diatom and dinoflagellate abundance (to ca. 20% and 11%, respectively). In the P-alone additions (#2–4), coccolithophorids comprised more than 80% of the community on D5. Their abundance increased by up to 25-fold

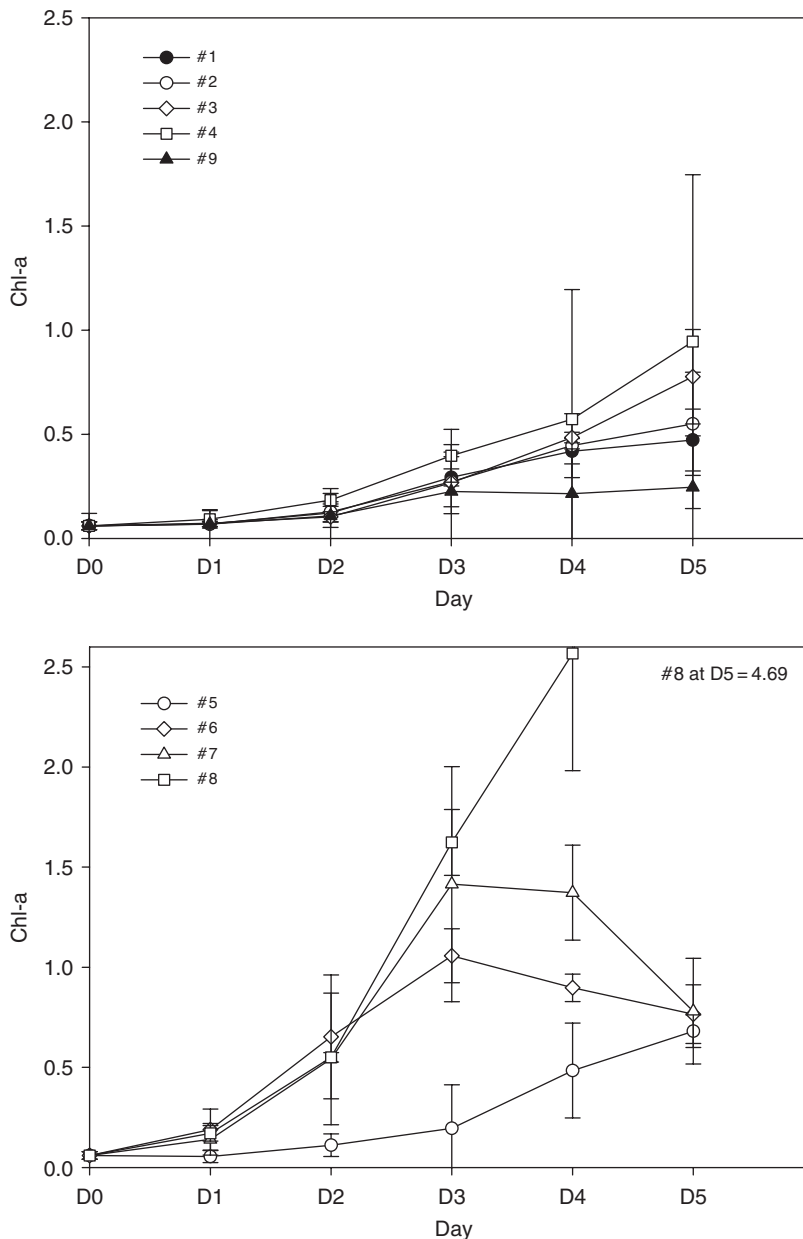


Fig. 5. Chl-*a* concentrations (mgm<sup>-3</sup>) in the different treatments as a function of time.

(26, 57, and 90 cells mL<sup>-1</sup> in #2, 3 and 4, respectively). The N-alone addition (#5) showed a moderate increase in coccolithophorids (44%) and a decrease in diatoms and dinoflagellates (33% and 20%, respectively). In all treatments with P+N additions (#6–8) diatoms were clearly predominant

on D5 (74–86%, 391, 542, and 2097 cells mL<sup>-1</sup> in #6, 7, and 8, respectively). This is in agreement with the chl *a* increase in the larger size fraction (not shown) and the decrease in silicic acid concentration (Fig. 2).

No specific trend was found in heterotrophic bacterial abundance. In all treatments, bacterial

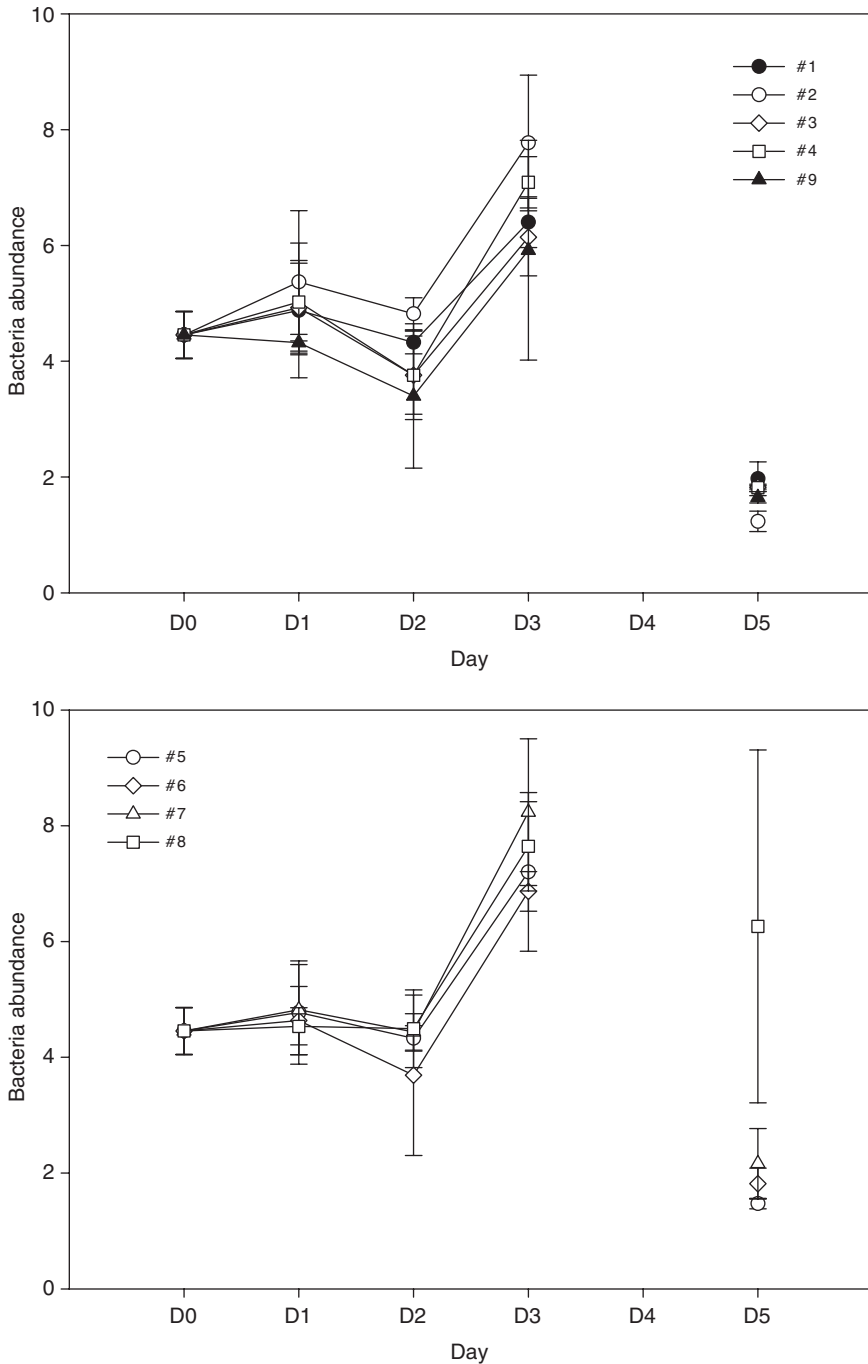


Fig. 6. Bacterial abundance (10<sup>8</sup> cells L<sup>-1</sup>) in the different treatments as a function of time.

abundance remained relatively stable around  $4.4 \pm 0.4 \times 10^8 \text{ L}^{-1}$  for D0–D1, increasing to about  $6\text{--}8 \times 10^8 \text{ L}^{-1}$  on D3 (Fig. 6). The maximum abundance was found in treatments with the lowest P- or N-alone additions (#2 and #5, respectively), and in the highest P+N additions (#7 and #8). On D5 (samples for D4 were unfortunately lost) there was a reduction in bacterial abundance in all treatments to ca.  $1.75 \pm 0.3 \times 10^8 \text{ L}^{-1}$  except for #8 in which the abundance was to  $6.4 \pm 3.1 \times 10^8 \text{ L}^{-1}$ .

There was an increasing, but not statistical significant, trend for autotrophic nanoflagellates abundance in the N-alone or P+N treatments (#5–8) as the experiment evolved. Biomass increased slightly in all treatments (Fig. 7), with the largest effect at the highest P-addition, with or without N (#8 and #4) on D4. The biomass of heterotrophic nanoflagellates increased slightly on D3 in all treatments, and continued to increase up to D4 in

the lowest P-alone, N-alone and P+N additions (#2, 5–8) (Fig. 7).

Total ciliate abundance increased in all treatments from the very low initial value ( $73 \text{ cells L}^{-1}$ , Table 2). On D5, in the control (#1) ciliates increased by one order of magnitude ( $857 \text{ cells L}^{-1}$ ). Their abundance increased more in the treatments with P-addition ( $1917 \text{ cells L}^{-1}$  in the highest P-addition, #4) and by two orders of magnitude in the treatments with P+N addition ( $4510, 7403,$  and  $14,197 \text{ cells L}^{-1}$  in treatments #6, 7, and 8, respectively). A similar trend was found for biomass, i.e. an increase with increased P-addition, more pronounced when P+N were added together; however, with variations between parallel carboys (Fig. 8). Aloricates made up 34% of the total ciliate biomass on D0. On D5, aloricates dominated the ciliate community in all treatments: control (75%), P alone (69%) and P+N (97%).

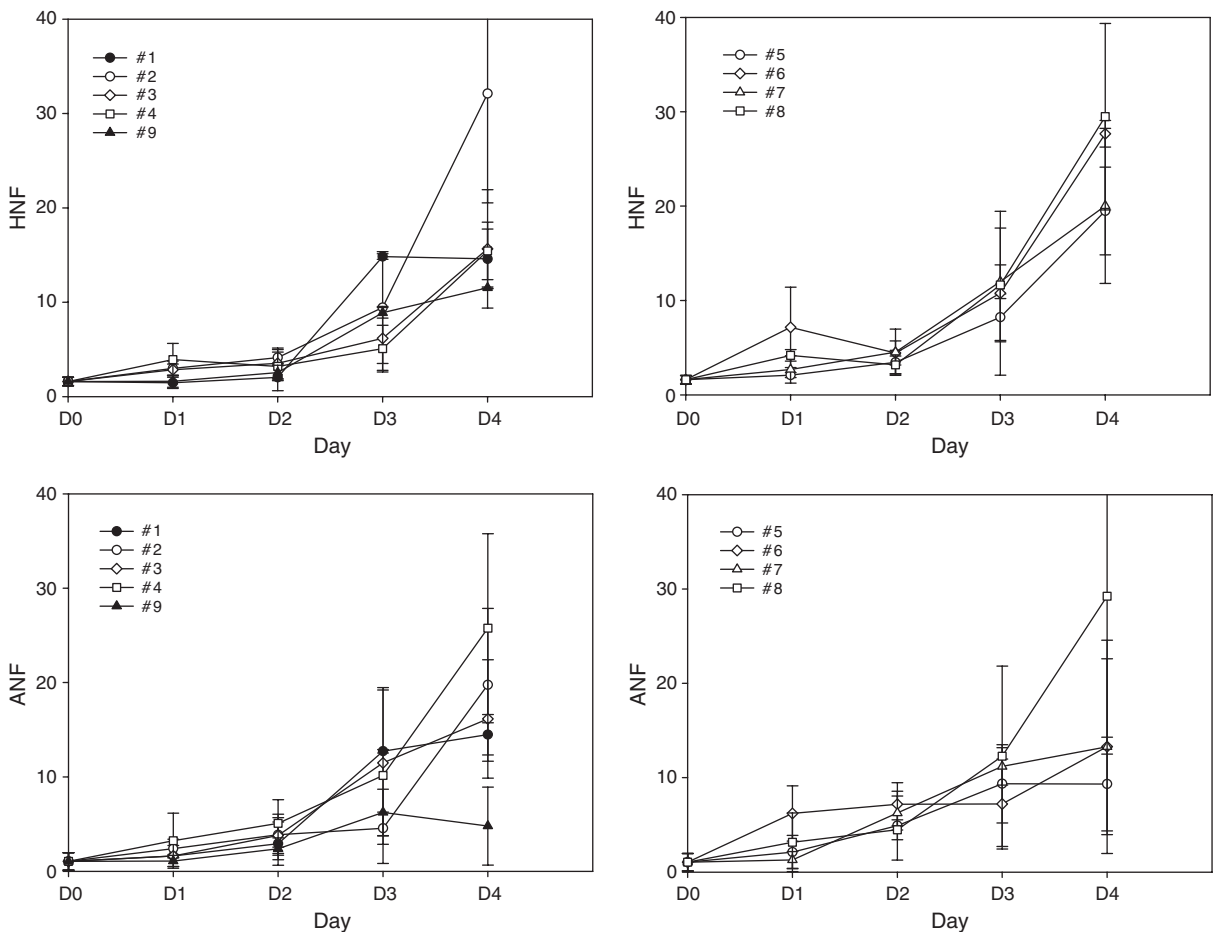


Fig. 7. Heterotrophic (HNF) and autotrophic (ANF) nanoflagellate biomass ( $\mu\text{g CL}^{-1}$ ) in the different treatments as a function of time.

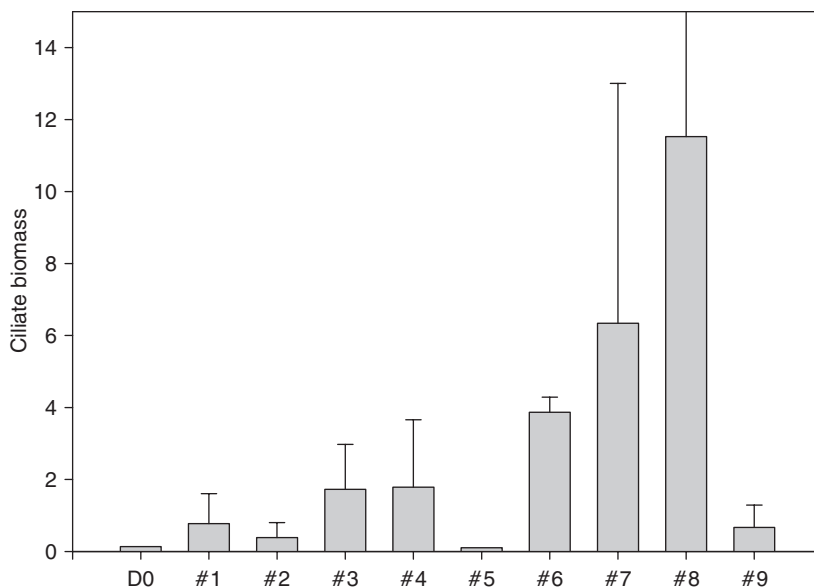


Fig. 8. Ciliates biomass ( $\mu\text{g C L}^{-1}$ ) on D0 and in the different treatments on D5.

### 3.2.3. Rates

The photosynthetic rate in the controls nearly doubled over the course of the experiment, increasing gradually until D5. Addition of phosphate alone (#2–4) stimulated photosynthetic rate, more in the medium and high P (#3 and #4) than the low P addition (#2) (Fig. 9). Addition of N-alone (#5) also stimulated photosynthetic rate in a similar way as in the lowest P addition (#2). The highest response of photosynthetic rate occurred by adding P+N (#6–8). In these treatments, photosynthetic rate started to increase already on D1 reaching maximal values on D3 (#6) and D4 (#7 and #8). Maximum values were 10 times higher than with the P-alone additions.

Size-fractionation of photosynthetic rate on D5 indicated a major shift towards larger size-classes in all treatments with added phosphate (#3, 4, 6, 7, and 8) except for the lowest P alone (#2). N alone (#5) had no major effect on the size fraction structure of photosynthetic rate (Fig. 10).

Essentially no changes occurred in bacterial activity during the experiment in the controls, high P-alone additions (#3, #4), and low P+N addition (#6) (Fig. 11). The average ( $n = 18$ ) leucine incorporation on D5 in these treatments was  $136 \pm 27$  pmol leucine  $\text{L}^{-1} \text{h}^{-1}$ . A fairly complex pattern was found for leucine incorporation in the other treatments. In the lowest P addition (#2) leucine incorporation reached a maximum (600 pmol leucine  $\text{L}^{-1} \text{h}^{-1}$ ) on D3,

decreasing to 210 pmol leucine  $\text{L}^{-1} \text{h}^{-1}$  on D5. A similar behavior was observed for the low N-addition (#5), with a maximum of 565–590 pmol leucine  $\text{L}^{-1} \text{h}^{-1}$  on D2–D3 and then decreasing to 160 pmol leucine  $\text{L}^{-1} \text{h}^{-1}$  on D5. In the highest P+N additions (#7 and #8) the increase in leucine incorporation started on D2, reaching a maximum on D4 (365 pmol leucine  $\text{L}^{-1} \text{h}^{-1}$ , #7) and on D5 (2420 pmol leucine  $\text{L}^{-1} \text{h}^{-1}$ , #8). The highest P+N addition yielded the highest incorporation rate.

Orthophosphate turnover time ( $T_t$ ) ranged across more than three orders of magnitude in the course of the experiment (Fig. 12). In the controls  $T_t$  remained relatively constant at 10–30 h. In the lowest P-addition (#2)  $T_t$  was slightly lower than the controls as would be expected if the added P was taken up by the biota without driving the system to N-limitation. In the higher P-additions (#3 and #4)  $T_t$  increased reaching, on D2, relatively constant levels of ca. 200 and 1000 h, respectively, as expected when a system is unable to assimilate the added P. When only N was added (#5) and with the lowest P+N addition (#6),  $T_t$  decreased reaching a minimum of ca. 1 h on D3, suggesting that the addition of N shifted a N limited system to a P-limited one. In the higher P+N additions (#7, 8), there was an initial increase in  $T_t$  on D1 followed by a decrease to minimum values of 0.96 and 48 h for treatments #7 and #8, respectively, on D5.

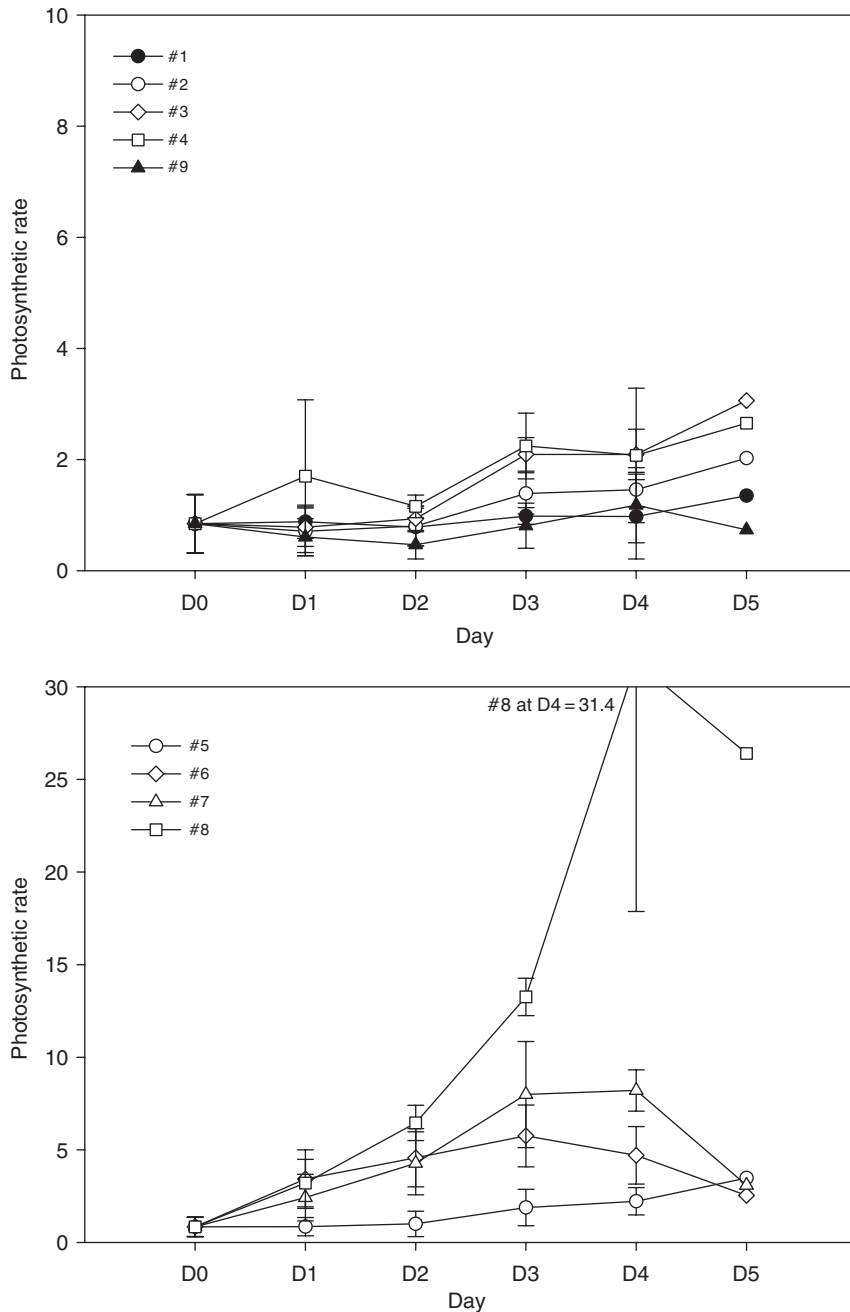


Fig. 9. Photosynthetic rate ( $\mu\text{gCL}^{-1}\text{h}^{-1}$ ) in the different treatments as a function of time. Note different y scales in upper and lower panels.

#### 4. Discussion

The CYCLOPS program was set up to investigate the cycling of P in the Eastern Mediterranean by ways of in situ and microcosms P-addition experiments (Krom et al., 2005). This microcosm experiment was designed to study a dose-response

relationship for additions of the limiting nutrient, hypothesized to be P, to an oligotrophic near-shore community, providing a valuable basis of comparison to subsequent experiments performed in the ultra-oligotrophic Cyprus Gyre (Krom et al., 2005). The low-nutrient (0.009 phosphate and 0.08  $\mu\text{M}$  nitrate) and chl *a* concentrations ( $0.06\mu\text{g L}^{-1}$ )

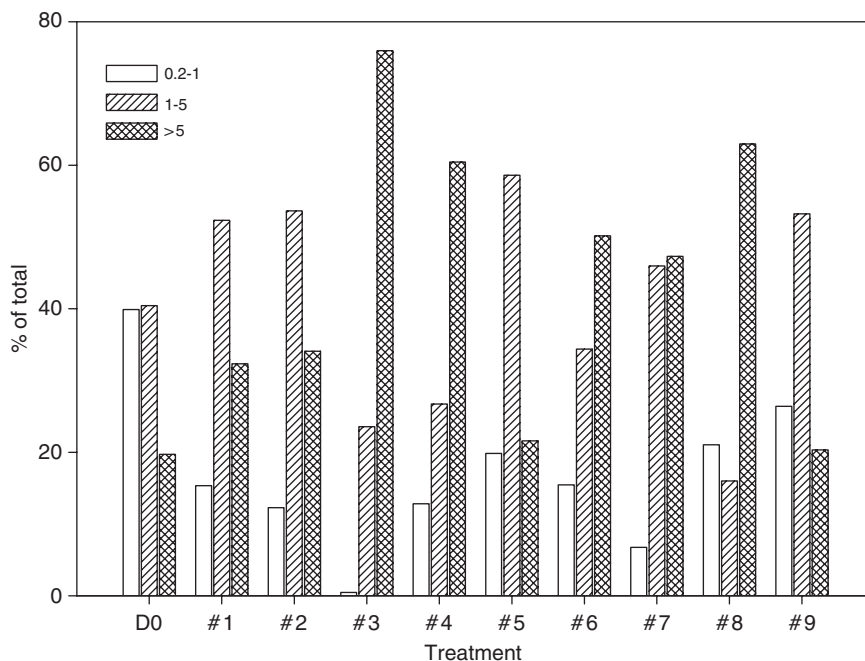


Fig. 10. Relative (%) size-fractionated photosynthetic rate on D0 and in the different treatments on D5.

confirmed the oligotrophic nature of the water used in the experiment that was sampled some 30 km offshore but from a meander of near-shore waters (Fig. 1). Contrary to our expectations, at the start of the experiment there was no clear indication of P-limitation. Orthophosphate turnover-time,  $T_t$ , was ca. 30 h, longer than the <7 h proposed by Zohary and Roberts (1998) as being indicative of P-limitation in the area, and longer than the <4 h measured in the in situ experiments in the Cyprus Eddy (Table 2, Flaten et al., 2005; Zohary et al., 2005).

#### 4.1. Response of near-shore oligotrophic waters nutrient additions

The similar responses of the system to the lowest P-alone and N-alone additions (#2 and #5, respectively) emphasized the question of which is the limiting nutrient in these near-shore waters. As would be expected in a P-limited system (Flaten et al., 2005), the lowest P-addition did not produce any sustained increase in  $T_t$ . On the other hand, the decrease in  $T_t$  after the addition of N-alone is expected in a N-limited system (Fig. 12). Moreover, chl *a* concentrations increased with time in both cases suggesting P and N co-limitation of the phytoplankton (Fig. 5). These responses imply that nutrients were made available to the system both

from internal sources (recycled nutrients), and from external sources (addition). The initial increase in bacterial activity was replaced by an increase in chl *a* concentrations on D3 (Figs. 11, 5), representing the time it took the bacteria to mobilize and make the internal nutrients available to the phytoplankton. Bacteria are more efficient than phytoplankton in utilizing nutrients at low concentrations and so could out-compete the phytoplankton. They are also heavily grazed, thereby increasing the recycling of nutrients (Harris, 1986; Strom, 2000). And indeed, there was an increase in the biomass of heterotrophic nanoflagellates, known to graze on bacteria, indicating a top-down control (Fig. 7). A shift from osmotrophs towards phagotrophs (minimum in  $T_t$ , Fig. 12) started on D3, more emphasized in the N-alone treatment.

The higher additions of P-alone ( $\geq 0.05 \mu\text{M}$ , treatments #3–4) probably pushed the system to N-limitation.  $T_t$  increased immediately and remained high until the end of the experiment (Fig. 12) and the N:P ratio of the nutrients taken up was lower than the Redfield ratio (ca. 7). No significant changes occurred in heterotrophic bacterial activity (Fig. 11), probably a result of competition for P by the coccolithophores. In fact, coccolithophores are good competitors when nutrients are available (Egge and Heimdal, 1994), can

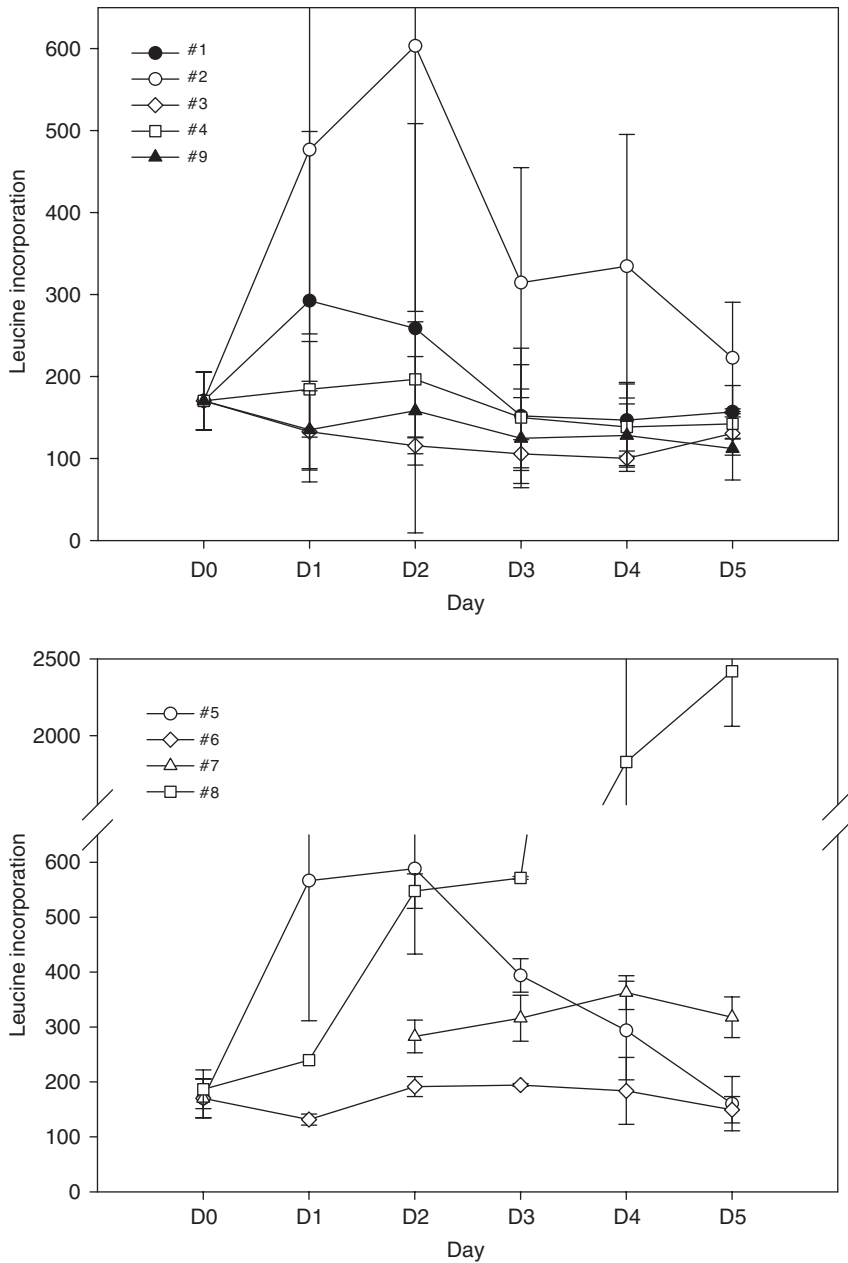


Fig. 11. Leucine incorporation ( $\text{pmol leucine L}^{-1} \text{h}^{-1}$ ) in the different treatments as a function of time.

store extra phosphate in their tests and access N from internal sources (Fanning, 1992).

Only the addition of P+N (#6–8) induced an immediate uptake of the nutrients added together with the ambient silicic acid (Fig. 2), an increase in chl *a* and photosynthetic rate, proportional to the concentrations added (Figs. 5, 9) and an increase in particulate P (Fig. 3). These treatments were characterized by a considerable bloom of diatoms

that took advantage of the adequate availability of all nutrients and of the lack of large grazers. The system reached P-limitation at the two lowest P+N additions (#6–7):  $T_t$  decreased to less than 1 h (Fig. 12), lower than the initial value in this experiment and similar to those found in oligotrophic open sea waters of the eastern Mediterranean (Table 2, Zohary and Robarts, 1998; Flaten et al., 2005) and the N:P ratio of the utilized nutrients at the end

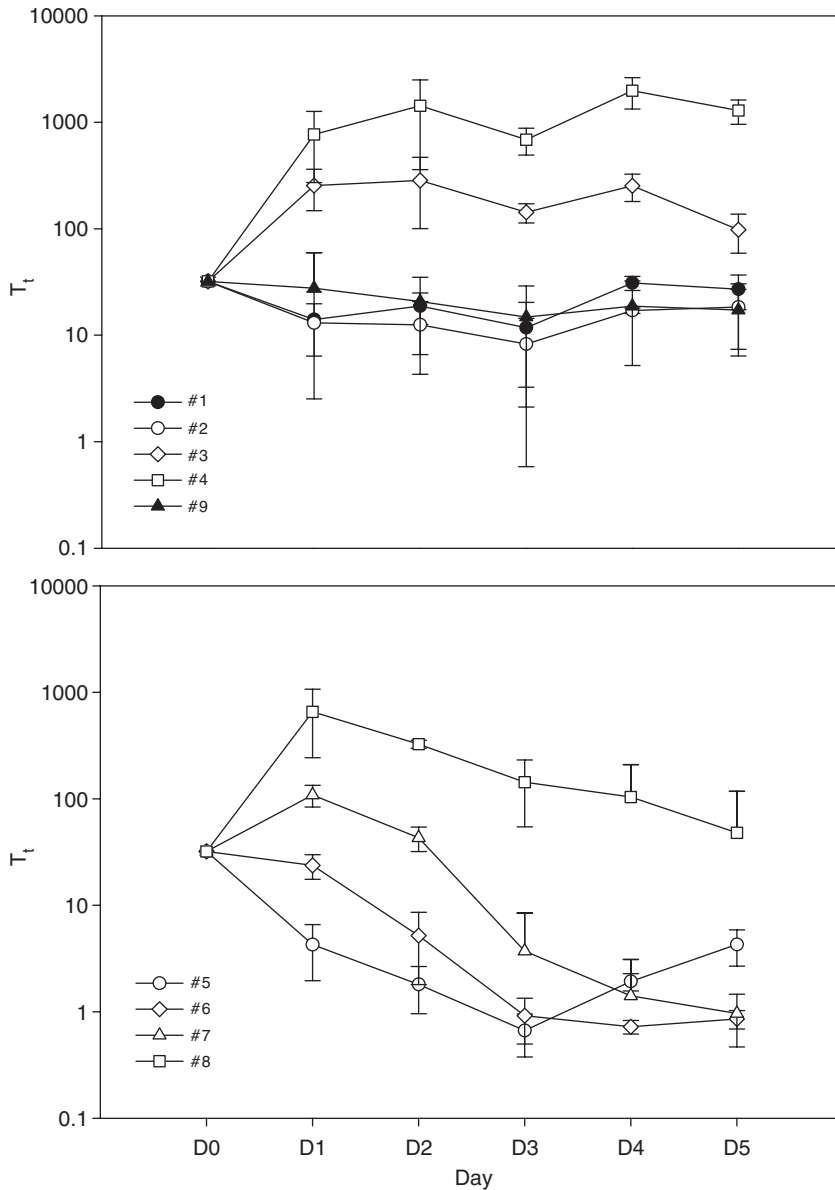


Fig. 12. Orthophosphate turnover-time ( $T_t$ , h) in the different treatments as a function of time. Note logarithmic Y scale.

of the experiment (ca. 100% nutrient utilization) were higher than the Redfield ratio (221 and 47 in #6 and #7, respectively). Only at the highest P+N addition (#8), when both nutrients were supplied in excess and inorganic nutrients remained in solution, it seems that nutrients did not limit production in the system:  $T_t$  remained high (Fig. 12) and the molar ratio N:P of the consumed nutrients ( $18 \pm 3$ ) was very close to the Redfield ratio. The molar Si:N ratio of utilized nutrients in these treatments was much lower than the expected ratio of 1 in nutrient-

replete diatoms (Brzezinski, 1985), implying that the diatoms were not heavily silicified. Similar low Si:N ratios have been found when Fe was added to waters off South America or in the subantarctic Southern Ocean (Hutchins et al., 2002).

Bacterial activity increased only when sufficient P+N were added (#7–8), following the phytoplankton (Figs. 11, 5). Bacterial abundance stayed almost constant with time while HNF and ciliate abundance increased, suggesting top-down control of bacterial abundance. Only in treatment #8 in which

the P+N addition transformed the starved oligotrophic system into a eutrophic one, there was an increase in ANF abundance. However, by the end of the experiment, the biomass of nanoflagellates increased 2–3-fold whereas ciliate biomass increased by two orders of magnitude indicating grazing of nanoflagellates by ciliates (Figs. 6, 7). Thus, the ciliates were able to grow and develop their populations in the absence of larger predators, i.e. copepods. They took advantage of the growth of their prey's populations and kept them in relatively low abundance.

Similar but not identical responses were observed when P, N or P+N were added to oligotrophic waters of the Mediterranean Sea in other studies. Bonin et al. (1989) found stimulation of algal growth (mainly cyanobacteria) as a result of P-alone addition (1  $\mu\text{M}$ ) to coastal waters off Israel, but not as a result of adding N alone. The addition of P+N induced increased abundance of eukaryotes and heterotrophic bacteria. Community biomass increased in large mesocosm experiments performed off the Mediterranean coast of Spain, together with a shift from initial picophytoplankton dominance to that of the microphytoplankton. Variations of N:P ratios in the additions showed increased diatoms' biomass at high N:P (excess N). *Synechococcus* sp. increased as well but the response to different N:P additions was complex with a strong top-down control by grazing (Duarte et al., 2000; Agawin et al., 2004).

#### 4.2. Comparison to open sea waters

The responses to nutrient introduction observed in this study represent those of a planktonic microbial community affected by near-shore waters and can be contrasted with those observed in the subsequent CYCLOPS studies conducted in open sea waters (Flaten et al., 2005; Krom et al., 2005; Pitta et al., 2005; Psarra et al., 2005; Zohary et al., 2005). While the initial nutrient concentrations were similar and very low,  $T_t$ , chl *a*, photosynthetic rate, large phytoplankton abundance, bacterial abundance and activity, were higher in this study (Table 2). Autotrophic and heterotrophic nanoflagellates initial abundance in near-shore influenced water was lower than in open sea water, yet ciliate abundance was similar.

There were some similarities in the response of this system to P-addition to those in the in situ experiment. The 0.12  $\mu\text{M}$  P added in situ to open-

sea oligotrophic waters were utilized rapidly by the biota, accompanied by a decline in chl *a* concentration (Krom et al., 2005; Pitta et al., 2005; Psarra et al., 2005). Similarly, at the low P-addition in this study (#2, 0.01  $\mu\text{M}$ ) there was rapid initial utilization of the P through the heterotrophic pathway. However, at the end of the experiment chl *a* increased slightly, contrary to the in situ experiment. Moreover, at the higher P-alone additions (#3–4, 0.05 and 0.5  $\mu\text{M}$ , respectively) bacterial activity did not increase while chl *a* concentrations increased.

A more extensive comparison can be made between this study and the on-deck microcosm experiment run in parallel to the in situ addition experiment in 2002 (Zohary et al., 2005). Ammonium was added to seawater sampled from the P-enriched patch water and from water outside the patch, enabling a comparison to the low P-alone, N-alone, low P+N, and control treatments in this study. The responses of the open-sea system in the on-deck microcosm exhibited some similar responses to those in near-shore waters: (a) chl *a* increased in all treatments from the initial values, with the highest increase when both P and N were added; (b)  $T_t$  decreased in all treatments, (c) bacterial activity increased in all treatments in the on-deck experiment, while in this study it increased at the beginning of the experiment in the control, low P and low N, but not in the low P+N addition.

The main difference between the on-deck experiment and this study was in the identity of the blooming phytoplankton: the picocyanobacterium *Synechococcus* in open sea water as opposed to larger phytoplankton in near-shore influenced open sea water (coccolithophorids when P alone was added and diatoms when P+N were added). The response of the system in this study was more in accord with the 'classical' expected result of nutrient introduction—increase in large size phytoplankton and chl *a*, as seen also in the response to iron introduction to high-nutrient low-chlorophyll areas (Coale et al., 1996, 2004). The differences between this study and the on-deck microcosm probably reflect the differences in seed population between near-shore influenced and pure open sea waters in the Eastern Mediterranean. In the on-deck microcosm experiment, large phytoplankton were relatively rare (ca. 1 cell  $\text{mL}^{-1}$  for each taxonomic group), while in this experiment the initial cell density was 3.5, 11.1, 4.3 cells  $\text{mL}^{-1}$  for coccolithophores, diatoms and dinoflagellates, respectively.

These taxa are known to take advantage of plentiful nutrient and out-compete the smaller phytoplankton.

In conclusion, this study and other nutrient-addition experiments performed with coastal Mediterranean waters (Bonin et al., 1989; Duarte et al., 2000; Sala et al., 2002; Agawin et al., 2004) show that, the joint addition of P and N elicits the major responses indicating that in these oligotrophic areas limitation by one nutrient can switch rapidly to limitation by a different nutrient. It also can shift the relative predominance of the planktonic population. The CYCLOPS in situ experiment showed that shifts in nutrient-limitation are not confined to coastal areas and take place in open sea waters as well (Flaten et al., 2005; Krom et al., 2005; Zohary et al., 2005). A natural P+N-enrichment experiment, similar to this microcosm, was documented at the open waters in the Rhodes Gyre, when deep winter mixing brought up nutrients from the deep layers, increasing chl *a* concentrations and bringing  $T_t$  to more than 3000 h (Zohary and Robarts, 1998).

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