

Phytoplankton response to a Lagrangian phosphate addition in the Levantine Sea (Eastern Mediterranean)

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Abstract

In order to test the hypothesis of P-limitation on primary production and microbial biomass, a mesoscale Lagrangian phosphate-enrichment experiment was performed in the warm core of the Cyprus Eddy (Eastern Mediterranean Sea) in May 2002. This study reports the effects of the phosphate addition on the phytoplankton community, by measuring induced changes in biomass, primary production, and community structure. Prior to the addition, primary production and chlorophyll *a* were very low ($0.107 \mu\text{gC l}^{-1} \text{h}^{-1}$ and 18 ng l^{-1} , respectively), typical of the ultra-oligotrophic conditions prevailing in the area. The autotrophic community was dominated in terms of both abundance and biomass (60% of total chl-*a*) by picoplankton (mostly *Synechococcus*). More than 90% of the autotrophic biomass was confined to particles $< 10 \mu\text{m}$, while larger phytoplankton (diatoms, dinoflagellates, coccolithophores) were very scarce. Unexpectedly, the addition of P resulted in a decrease in phytoplankton biomass. Total HPLC-chlorophyll slightly declined to 11 ng l^{-1} in the P-enriched patch, five days after the P-addition. As the patch was diluted away (7–9 days), chlorophyll concentrations returned to background levels. Similar trends were observed in chlorophyll determined fluorometrically and in primary production. Picophytoplankton and smaller nanophytoplankton abundance declined (by 49 and 65%, respectively) within the first four days of the experiment. At the same time, there was a small increase of larger nanophytoplankton ($10\text{--}20 \mu\text{m}$) and microphytoplankton ($> 20 \mu\text{m}$) species. Small nanophytoplankton ($2\text{--}10 \mu\text{m}$) were the group most affected by the addition. These results together with an onboard microcosm experiment (reported elsewhere in this issue), in which ammonia was added to the P-enriched water, indicate that the system was N and P co-limited for phytoplankton. The decrease in pico and smaller nanophytoplankton was probably caused by increased predation by micrograzers that became more active because of the increased heterotrophic bacterial activity and/or the increased P content of their prey. The

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immediate decrease in *Synechococcus* numbers, as the system became temporarily N-limited implies that N-fixation caused by this particular cyanobacteria was unlikely to occur.

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1. Introduction

The Eastern Mediterranean Sea is an extreme oligotrophic environment (Krom et al., 2003), at the far end of a prominent west–east increasing oligotrophy gradient (Turley et al., 2000). Its ultra-oligotrophic status is reflected in the exceptional water clarity (Berman et al., 1984; Ignatiades, 1998), low concentrations of nutrients, extremely low values for all phytoplankton related variables, including chlorophyll *a*, primary production and cell abundance (Sournia, 1973; Berman et al., 1984a,b; Dowidar, 1984; Azov, 1986; Bonin et al., 1989; Psarra et al., 2000; Christaki et al., 2001), dominance of small-size phytoplankton (Li et al., 1993; Yacobi et al., 1995; Ignatiades, 1998; Ignatiades et al., 2002), and outstandingly low bacterial abundance and production (Robarts et al., 1996). This extreme “poverty” has also been verified by satellite imagery of sea-surface chlorophylls (Antoine et al., 1995; Bosc et al., 2004). Such ultra-oligotrophic regimes lead to conditions of diffusion limitation of osmotrophs (Thingstad and Rassoulzadegan, 1999). The typical dominance of small-size phytoplankton in the Levantine Basin has been reported even during the annual phytoplankton bloom (Vidussi et al., 2001), which takes place in winter, when deep mixing introduces nutrients into the surface waters (Krom et al., 1993, 2003). During the rest of the year, the water column is stratified and the Levantine Basin is characterized by a prominent deep chlorophyll maximum at 100–130 m depth (Li et al., 1993), with very low phytoplankton biomass ($\ll 50 \text{ ng chl-}a \text{ l}^{-1}$) in the surface layers (Yacobi et al., 1995; Vidussi et al., 2001).

The Eastern Mediterranean and particularly the Levantine Basin is unusual in having a highly skewed deep-water nutrient ratio (Krom et al., 2003). McGill (1965, 1969) found the nitrate:phosphate ratio to be $\geq 16:1$, which was later confirmed by Krom et al. (1991) and Kress and Herut (2001). In winter, after the annual phytoplankton bloom, phosphate concentrations rapidly decrease to below detection limits while measurable ($\sim 500 \text{ nM}$) nitrate remains in the surface waters (Kress and Herut, 2001). This residual nitrate is

isotopically heavy and the PON isotopically light, both characteristics of a phytoplankton bloom that ceased as a result of P-limitation (Struck et al., 2001). This evidence together with various bottle-incubation experiments (e.g. Bonin et al., 1989; Zohary and Robarts, 1998; Christaki et al., 1999) suggested that the Eastern Mediterranean heterotrophic bacteria and phytoplankton is conventionally P-limited in winter.

In situ additions have been applied as the ultimate tool for demonstrating nutrient limitation in natural ecosystems. Langrangian Fe-addition experiments in high-nutrients low-chlorophyll (HNLC) regions of the Pacific and Southern Oceans demonstrated unequivocally the existence of iron-limited phytoplankton growth (Martin et al., 1994; Coale et al., 1996; Boyd et al., 2000).

The CYCLOPS (CYCLing Of PhoSphorus in the Mediterranean) experiment (Krom et al., 2005), of which this study is part, was the first in situ addition experiment involving a possible limiting nutrient other than iron. The principle aim of the CYCLOPS experiment was to test the hypothesis that P was the nutrient limiting phytoplankton growth in the Eastern Mediterranean, by following a range of biological and chemical responses to an in situ P-addition. It was anticipated that following P-addition phytoplankton biomass would increase substantially, to levels significantly higher than those encountered in the un-enriched surrounding waters.

In this paper we report on the responses of the autotrophic community to this in situ P-addition. This is accomplished by comparing a suite of biological parameters (primary productivity, phytoplankton community composition, chlorophyll and other phytopigments) both from water to which P was added as well as from un-enriched surrounding waters.

2. Materials and methods

2.1. Experimental design and sampling

An inert tracer (SF_6) was added with the phosphate, enabling the patch to be tracked even

after the phosphate concentration declined to detection limits, and provided quantitative estimates of mixing, dilution and patch volume (Law et al., 2005). The experiment was conducted aboard the R.V. AEGAEON during the CYCLOPS cruise (14 to 26 May 2002) to the Cyprus Eddy (33.3°N, 32.3°E), in the Levantine Basin of the Eastern Mediterranean. On the afternoon of May 17, NaHCO₃-buffered phosphoric acid combined with the SF₆ tracer was added to 4 × 4 km of surface water in the core of the eddy. This resulted in an initial concentration of ca. 110 nM of phosphate throughout the surface mixed layer (0–16 m depth). A series of biological variables were determined in samples collected from the core of the eddy before the P-addition (PRE stations) and over a period of nine days after the P-addition, from both within (IN) and outside (OUT) the P-enriched patch, as defined from the SF₆ and phosphate concentrations. Sampling for chemical and biological parameters was performed at seven standard depths down to 45 m (4, 8, 12, 16, 20, 30, 45 m) using a multi-sampler/Rosette system (General Oceanics) with twelve 10-l Niskin bottles. The 12-m depth layer was chosen as a reference depth at which size fractionation of chlorophyll *a* and primary production was performed. In this study we concentrate on data from the upper 20 m because the surface mixed layer, within which the phosphate patch was confined, varied between 16 and 20 m during the course of the experiment (Law et al., 2005). The location of sampling stations together with a mean surface mixed layer (SML) temperature contour of the sampling area are presented in Fig. 1. Further station details are given in Table 1. A more comprehensive description of the experimental design, the sampling strategy, and the evolution and tracking of the phosphate patch by the use of the gaseous SF₆ tracer are presented by Law et al. (2005). CTD data were collected from 16 casts in the center of the eddy, using the Sea Bird Electronics (SBE911plus) CTD profiler, equipped with oxygen and fluorometer sensors.

2.2. Analytical methods

Chlorophyll *a*—fluorometric method—A variable amount of water (500–1000 ml depending on phytoplankton biomass) was filtered through 0.2-μm polycarbonate filters (Poretics, Ø47 mm) for the estimation of total chlorophyll *a* (Tchl-*a*). Filters were stored at –20 °C immediately after

filtration and kept frozen for six weeks until laboratory analyses took place. Chlorophyll-*a* was extracted in 90% acetone and concentrations were determined fluorometrically according to Yentsch and Menzel (1963), using a TURNER TD-700 fluorometer.

In addition, up to 1 l of water sample, collected from 12 m depth, was filtered onto 2.0-μm polycarbonate filters for the determination of the size distribution of chl-*a*. Apart from total (>0.2 μm) chl-*a*, the fractions presented in the results section are those of picoplankton (0.2–2.0 μm) and nano + microplankton (>2.0 μm).

Photosynthetic pigments by HPLC—From the same Niskin bottles used for fluorometry water samples (21) were filtered through GF/F filters for high-performance liquid chromatography (HPLC) analyses of chlorophylls, carotenoids and other diagnostic pigments (Table 2). Pigment GFF filters were immediately stored on board at –20 °C, then transported under dry ice (–78 °C) and kept in an ultra low freezer (–80 °C) until HPLC analyses took place. Mantoura et al. (1997) have shown that little or no degradation of HPLC detectable pigments occurs under these conditions. Pigment analyses involved reversed phase RP-HPLC developed at PML (Mantoura and Llewellyn, 1983) and adapted by Barlow et al. (1999). The chemotaxonomic pigments were used as size class markers (Table 2) and the results were grouped in order to correspond with size classification as follows:

Picoplankton: Zea + dvchl-*a* + dvchl-*b*, (1)

Nanoplankton: 19'-HF + 19'-BF
+ chl *b* chl *c*_{1/2} + chl *c*₃, (2)

Microplankton: Fuco + Peri, (3)

where Zea = zeaxanthin, dvchl-*a* = divinyl chlorophylla, dvchl-*b* = divinyl chlorophyll *b*, 19'-HF = 19'-hexanoyloxyfucoxanthin, 19'-BF = 19'-Butanoyloxyfucoxanthin, chl-*b* = chlorophyll *b*, chl *c*_{1/2} = chlorophyll *c*_{1/2}, chl *c*₃ = chlorophyll *c*₃, Fuco = Fucoxanthin, and Peri = Peridinin.

In order to follow the dynamics of the various diagnostic pigments, their ratio to Tchl-*a* was calculated assuming a constant proportion of each diagnostic pigment relative to chl-*a* (Vidussi et al., 2001). This was done although it is known that variations may occur between species of a certain group and/or according to physiological state (Kana and Glibert, 1987). The Vidussi et al.

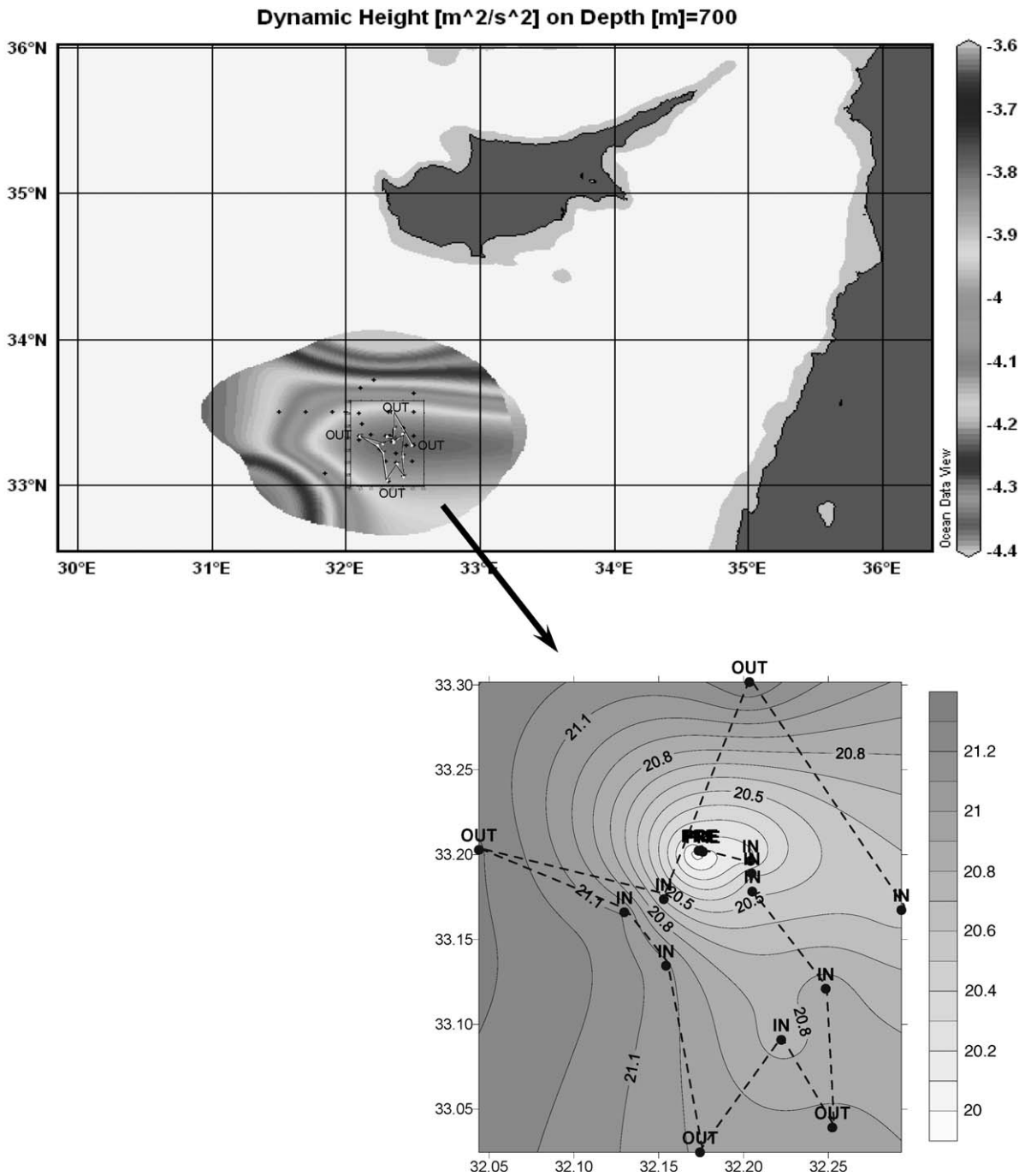


Fig. 1. (Top): Location of stations in the Cyprus Eddy plotted over a dynamic height contour (Drakopoulos, personal communication). (Bottom): The sampling stations over a mean temperature (°C) contour in the surface mixed layer.

(2001) approach was selected since it was already used for the Eastern Mediterranean thus providing continuity and robustness to the treatment of these data.

Primary production (PP)—Photosynthetic carbon fixation rates were estimated by means of the ¹⁴C technique of Steemann-Nielsen (1952), as modified for the ultra-oligotrophic waters of the Eastern

Table 1
Dates and time of sampling, station locations and type of stations

Date, May 2002	Days after P-addition	GMT (h)	Longitude	Latitude	Station type
14	(−3)	1510	3217.47	3320.25	PRE
15	(−2)	1250	3217.35	3320.23	PRE
16	(−1)	0915	3217.60	3320.17	PRE
18	1	0900	3220.41	3319.63	IN
19	2	0850	3220.51	3317.82	IN
20	3	0824	3224.84	3312.09	IN
20	3	1505	3225.23	3303.91	OUT
21	4	0820	3222.22	3309.08	IN
21	4	0945	3217.43	3302.45	OUT
22	5	0855	3215.43	3313.45	IN
23	6	0900	3212.97	3316.60	IN
23	6	1420	3204.40	3320.28	OUT
24	7	0900	3215.30	3317.37	IN
25	8	0845	3220.48	3318.89	IN
25	8	1410	3220.34	3330.17	OUT
26	9	0910	3229.30	3316.73	IN

PRE =before P-addition, IN = inside P-patch, OUT = outside the patch. P-addition was conducted on the 17 May 2002.

Table 2
Taxonomic pigments detected by HPLC and their correspondence to phytoplanktonic groups and size class (adapted from Vidussi et al., 2001 and references therein)

Pigments	Abbreviations	Taxonomic group	Size class (μm)
Zeaxanthin	Zea	cyanobacteria and prochlorophytes	pico (<2)
Divinyl-chlorophyll <i>a</i>	Dv-chl <i>a</i>		
Divinyl-chlorophyll <i>b</i>	Dv-chl <i>b</i>		
Chlorophyll <i>b</i>	chl <i>b</i>	green flagellates prymnesiophytes	nano (2–20)
19'	19'-HF		
hexanoyloxyfucoxanthin	19'-BF		
butanoyloxyfucoxanthin		prymnesiophytes, chrysophytes	
Chlorophyll <i>c</i> _{1/2}	Chl <i>c</i> _{1/2}	diatoms, dinoflagellates, prymnesiophytes, chrysophytes, raphidophytes	nano + micro (>20)
Chlorophyll <i>c</i> ₃	Chl <i>c</i> ₃		
Fucoxanthin	Fuco		
Peridinin	Peri		
Pheophorbide- <i>a</i> like	Pheo		
		feacel pellets (chl- <i>a</i> degradation products)	

Mediterranean by Psarra et al. (2000) and Ignatiades et al. (2002). Samples were placed in 250-ml polycarbonate bottles (three light and one dark for each depth), inoculated with 5 μCi of $\text{NaH}^{14}\text{CO}_3$ tracer and incubated in situ, for ca. 2 h. This was

done at noon (11:00–13:00 local time), when the incident irradiance is maximum yielding maximum primary production rates. At the end of the incubation, samples were immediately stored in the dark until completion of filtration (ca. 2.5 h).

Samples were filtered onto 0.2- μm pore-size polycarbonate filters (Poretics) at <100 mm Hg vacuum pressure. Similar to chlorophyll, size fractionation onto 0.2- and 2.0- μm polycarbonate filters (parallel filtration) was performed only on samples from 12 m depth. Due to technical constraints the use of replicates for samples dedicated to size fractionation was not possible, and thus the results are single sample measurements. Deionized water and pre-filtered (through 0.2 μm) seawater also were processed and used as blanks. Killing of samples and rinsing of the filters at the end of the filtration were avoided in order to minimize exudation losses of organic matter. All materials were acid-cleaned prior to use. To remove excess ^{14}C -bicarbonate, filters were soaked in 1 ml 0.1-N HCl and allowed to stand in uncapped polyethylene 5-ml vials (Packard) overnight. After the addition of 4 ml of BSF scintillation cocktail (Packard), the activity was measured using a Packard Tri-Card 4000 Liquid Scintillation Counter. Daily primary production rates integrated in the SML were estimated using the model proposed by Moutin et al. (1999). Growth rates were calculated by dividing primary production rates by phytoplankton biomass as derived from a C:Chl-ratio of 40 (w:w), using fluorometric chl-*a* determinations.

Phytoplankton cell abundance and biomass—Counts of phytoplankton cells were performed with three different methods, each one employed for a different size class: thus, autotrophic picoplankton (0.2–2.0 μm) counts were performed by flow cytometry, while small nanoplankton (2–10 μm) were measured by epifluorescence microscopy and larger nano and microplankton ($\geq\sim 7\mu\text{m}$) were counted by inverted microscopy. It is, however, anticipated that an inevitable small overlap between size classes and methods may have occurred, mostly related to the small nanoplankton cells measured by epifluorescence microscopy. Thus within this group, the smaller ones may overlap with pico-eukaryotes measured by flow cytometry involving organisms with a mean diameter of 3–4 μm , whereas the larger ones (7–10 μm) may overlap with nanoplankton counted under the inverted microscope.

Picoplankton (Flow cytometry)—Duplicate samples (1.8 ml) were preserved with 140 μl of 25% glutaraldehyde, deep-frozen in liquid nitrogen and kept at -80°C until further analysis, using a FACScan (Becton Dickinson) flow cytometer. For further details see Zohary et al. (2005). Cell counts for *Synechococcus* (SYN) and *Prochlorococcus*

(PRO) were converted to C units using the factor 250 fg C cell $^{-1}$ (Kana and Glibert, 1987) and 50 fg C cell $^{-1}$ (Campbell et al., 1994), respectively.

Small nanoflagellates (Epifluorescence microscopy)—Samples for nanoflagellates were fixed with glutaraldehyde (final conc. 1%). Subsamples (50–100 ml) were concentrated to ca. 10 ml on a 25-mm diameter, 0.8- μm pore sized polycarbonate black filter, stained with DAPI for 10 min (final conc. 0.5 μgml^{-1}), and filtered. Filtration was completed within each sampling day. Small autotrophic nanoflagellates (SNANO) were counted in different size classes (2–5 and 5–10 μm) using an ocular micrometer under blue excitation filters. Cell counts were then converted to C units using the mean cell volume of 35 μm^3 cell $^{-1}$ for 2–5 μm size and 294 μm^3 cell $^{-1}$ for the 5–10 μm size. In both cases a conversion factor of 183 fg C μm^{-3} was used (Caron et al., 1995).

Large nanoplankton and microplankton (Inverted microscopy)—Determinations of the abundance, biomass and taxonomy of larger nano- and micro-phytoplankton (LNANO + MICRO, $\geq\sim 7\mu\text{m}$ cell size) were performed on samples taken from PRE and IN stations, preserved in alkaline Lugol's solution, and refrigerated. Subsamples of 100 ml were analyzed after sedimentation for 24 h according to Utermöhl's (1958) inverted microscope method. A total of ca. 100–600 cells were identified and counted per sample. The mean cell volume for each species was calculated simulating similar geometrical shapes (Hillebrand et al., 1999). The approximate carbon content of each species was calculated using appropriate conversion factors (Strathmann, 1967; Rocha and Duncan, 1985; Verity et al., 1992; Montagnes et al., 1994) and then summed for the major taxonomic groups.

2.3. Statistical analyses

In order to check whether the P-addition had a significant effect on the autotrophic community, one-way Analysis of Variance (ANOVA, Sokal and Rohlf, 1981) was applied to chl-*a* and primary production values, using “stations” as a main source of variability. Measurements from different stations were grouped into PRE, IN and OUT of the patch, selecting those obtained before any biological changes would be diluted away (Law et al., 2005). This means that the IN patch data were considered only for days 1–5 after the addition.

2.4. Predicted biological responses as a result of the dynamics of the patch

The P-modified patch of water rapidly expanded as the patch was diluted with surrounding water. As a result, many biological observations in this study are a combination of the biological dynamics (growth rate, grazing rate etc.) and the patch dilution rate. This meant that most of the biological parameters affected by the P-addition reached a maximum after 3–4 days and then decreased towards background levels after seven days.

3. Results

3.1. Chlorophyll *a*, primary production

The most prominent, as well as unexpected, result of the P-addition experiment was a lack of increase of chlorophyll in the P-enriched water in comparison to both the Pre-addition state (PRE) and to the unenriched water, located several kilometers away from the P-patch (OUT). In fact, during the first six days after the addition, a decrease was recorded in both the HPLC and fluorometrically determined chl-*a* measurements (Fig. 2). HPLC-chl-*a* decreased gradually from $18 \pm 0.6 \text{ ng l}^{-1}$ prior to the P-addition to a minimum of 11 ng l^{-1} , by day 5 of the experiment (Fig. 2A). The lowest HPLC-chl-*a* values in the patch were recorded from day 3 to 6, only to return to background levels within ca. one week. Fluorometrically determined chl-*a* was $18 \pm 1.1 \text{ ng l}^{-1}$ prior to the P-addition. Within six days after the P-addition it declined by ca. 20%, returning to background levels within a week (Fig. 2B). Comparisons of chl-*a* concentration between stations sampled prior to (PRE) and after the P-addition (IN) showed significant differences for the HPLC measurements (ANOVA, $p < 0.01$). The fluorometric measurements showed similar trends, but these were not statistically significant. The same comparisons between inside (IN) and outside (OUT) the P-patch were not significant for either chl-*a* measurement (ANOVA, $p > 0.05$). It was, however, found that the average HPLC chl-*a* in the IN stations was less than the average OUT data on both of the occasions when HPLC data for the OUT stations were available (Fig. 2A).

Primary production rates averaged in the upper 20 m (usually $N = 5$) were very low, with an overall mean of $0.091 \text{ (SD } \pm 0.014) \mu\text{g C l}^{-1} \text{ h}^{-1}$, fairly close to the analytical limits of $0.05 \mu\text{g C l}^{-1} \text{ h}^{-1}$ (Strick-

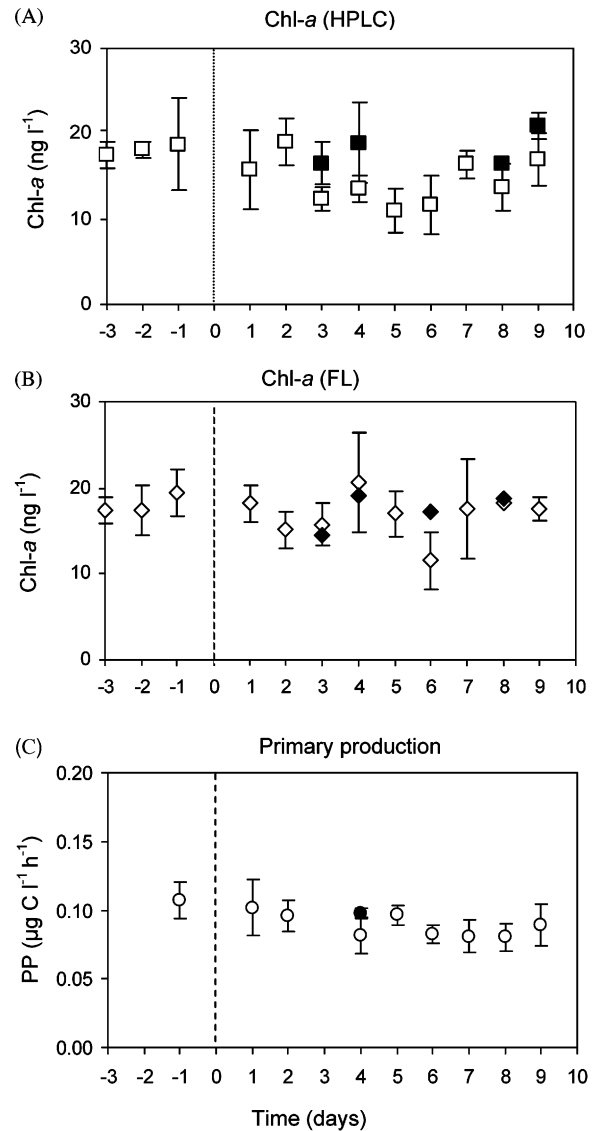


Fig. 2. Temporal dynamics of: (A) chlorophyll-*a* measurements (ng l^{-1}) by HPLC, (B) chl-*a* by fluorometry (FL, ng l^{-1}) and (C) primary production rates ($\text{mg C m}^{-3} \text{ h}^{-1}$) (IN) and outside (OUT) the P-patch, before and after the P-addition. OUT stations are marked with solid markers. Data points represent means of all measurements between 0–20 m (usually $N = 5$) in each cast \pm SD. The dashed line indicates the day of the P-addition (17/5/02).

land and Parsons, 1972). The highest daily primary production rates ($0.35 \mu\text{g C l}^{-1} \text{ day}^{-1}$) were recorded one day prior to the addition, while a net decrease of ca. 25% was recorded following the addition (until day 6), after which primary production rates remained more or less unchanged (Fig. 2C). Despite this trend, primary production variability between “PRE-IN”, “IN-OUT” and “PRE-OUT” stations

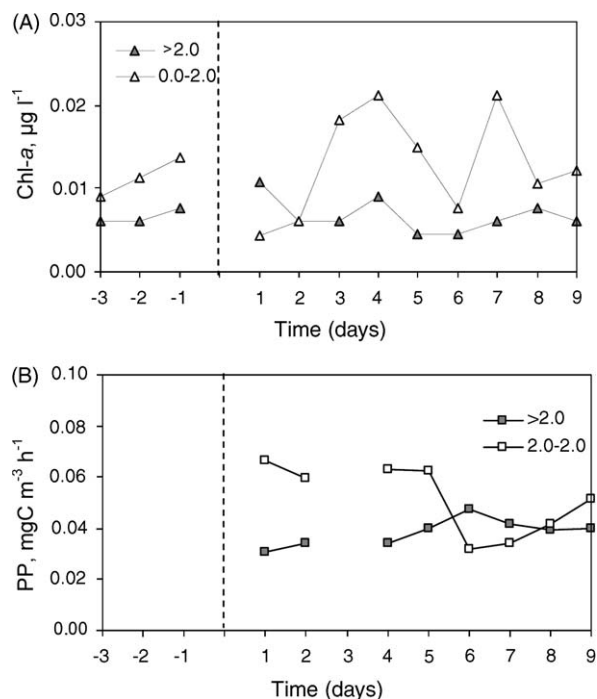


Fig. 3. Temporal evolution of size fractionated chl-*a* (A) and primary production (B), corresponding to pico- (0.2–2.0 µm) and nano- and microphytoplankton (>2 µm). The dashed line indicates the day of the P-addition (17/5/02).

was not significant (ANOVA, $p > 0.05$). Growth rates were ca. 1 d^{-1} prior to the addition, while four days later they were reduced to 0.7 d^{-1} , thereafter remaining at this level.

3.2. Size fractionation of chl-*a*, primary production

Prior to the P-addition, the 0.2–2.0 µm size fraction (autotrophic picoplankton) accounted for 60–65% of Tchl-*a* (Fig. 3A, Table 3). Following the P-addition, chlorophyll content and primary production changes were observed mainly in this dominant picoplankton fraction, while the >2.0-µm fraction displayed little change (Fig. 3). Interestingly, on day 1 the decrease in Tchl-*a* was accompanied by a relative 35% decrease of the picoplankton size fraction. In the following days, picoplankton regained their dominant role (to >60% of Tchl-*a*).

For primary production, the picture was different, revealing a slower decrease (as compared to day 1) of the dominant role of picoplankton (>60% of PP), with a parallel moderate increase in nano- and microplankton both culminating at day 6, by which

time their relative contributions were reversed, with nano + microplankton contributing 60% of PP (Fig. 3B, Table 3).

3.3. Chemotaxonomic pigment analysis—HPLC measurements

A first observation of chemotaxonomic pigment analysis was that the sum of the ten diagnostic pigments (DP) averaged over the 0–20 m water column (without Pheophorbide *a*, which is a degradation product of chl-*a*) was linearly correlated to Tchl-*a* ($r^2 = 0.7, n = 17$), showing that DP is a relatively good proxy for Tchl-*a* (Fig. 4A).

The most abundant pigment was 19'-hexanoyloxyfucoxanthin (19'-HF), which is indicative of the nanophytoplanktonic group of prymnesiophytes, representing overall $37 \pm 5\%$ of DP and $24 \pm 5\%$ of Tchl-*a*. 19'-HF:chl-*a* ratio drastically decreased the first day after the addition but quickly regained its initial levels by day 2–3. During the following days (day 4–8), it fluctuated around the initial values (Fig. 4B).

Among the pigments corresponding to the picophytoplanktonic groups, zeaxanthin is a signature pigment of *Synechococcus* and surface *Prochlorococcus*. Since flow cytometry analysis found abundances of *Prochlorococcus* and *Synechococcus* to be low and high, respectively, zeaxanthin results here are considered to represent mainly the second group. Zeaxanthin (Zea) was the second most abundant pigment, representing 24% of DP and 16% of Tchl-*a*. With the exception of a small increase on day 4 (Fig. 4C), the temporal dynamics of the Zea: chl-*a* ratio revealed high values immediately prior to the addition and a gradual decrease immediately after the addition (until day 5), recovering to close to initial levels ca. one week after the addition.

All the remaining pigments analyzed represented on average $\leq 8\%$ of DP. 19'-Butanoyloxyfucoxanthin (19'-BF), also belonging to prymnesiophytes, together with chlorophyll $c_{1/2}$, non-specific for nano- and microphytoplankton (prymnesiophytes, chrysophytes, diatoms, dinoflagellates, Table 2), were the next most important photosynthetic pigments, representing each 5% of DP. The dynamic trend of 19'-BF:chl-*a* was similar to that of 19'-HF:chl-*a*. By contrast, concentrations of Divinyl chl-*a* (dvchl-*a*) and Divinyl chl-*b* (dvchl-*b*), both signature pigments of *Prochlorococcus*, were very low ($< 1 \text{ ng l}^{-1}$), denoting a weak presence of

Table 3

Synthesis of phytoplankton community size structure results as derived from three different methods measuring three biomass indicators: Fluorometric determination of size fractionated (SF) chl-*a*, chemotaxonomic pigments' size correspondence by HPLC and total community carbon partitioning by conversion of cell counts (involving flow cytometry, epifluorescence and inverted microscopy) to biomass (for details see methods)

sta	PP (SF)			Chlorophyll- <i>a</i> (SF fluorometry)		Chemotaxonomic pigments (HPLC)				Cell count based Carbon content (Flow cytometry, microscopy)			
	Days	p	n + m	p	n + m	p	n	m	n + m	p	sn	ln + m	n + m
PRE	-3			60	40	18	71	11	82	24	69	7	76
PRE	-2			65	35	32	62	6	68	25	75		75
PRE	-1			64	36	30	63	7	70	16	78	6	84
	Avg.			63	37	27	65	8	73	22	74	7	78
IN	1	64	36	29	71	45	46	9	55	19	72	9	81
IN	2	63	37	50	50	33	61	5	67	17	70	12	83
IN	3			75	25	31	62	7	69	17	65	18	83
IN	4	65	35	70	30	30	62	8	70	28	45	27	72
IN	5	61	39	77	23	23	69	8	77	23	51	25	77
IN	6	40	60	63	38	31	63	7	69	20	65	15	80
IN	7	45	55	78	22	30	65	6	70				
IN	8	51	49	58	42	40	55	5	60				
IN	9	56	44	67	33	25	70	5	75	19	81		81
	Avg.	56	44	63	37	32	61	7	68	20	64	18	80

Primary production (PP) size fractionation results are also shown. Results are grouped so as to represent the size classes of pico- (p), nano- (n) and microplankton (m) organisms and the relative contributions (%) of each size group estimated by the three approaches are compared between them. Nanoplankton derived from microscopic counts are further divided into small (sn, 2–10 µm) and large (ln, 10–20 µm). avg.: average

prochlorophytes. Both *dvchl-a: chl-a* and *dvchl-b: chl-a* ratios did not show any response throughout the experiment, remaining at very low background levels (Fig. 4C).

Marker pigments of larger cells in the micro-phytoplankton are Fucoxanthin (Fuco) and Peridinin (Peri), representing diatoms and dinoflagellates, respectively. These represented only 4 and 2% of total diagnostic pigments, respectively. The Fuco: chl:*a* ratio demonstrated a similar temporal pattern with that of 19'-HF:chl-*a* and 19'-BF: chl-*a*, with a net initial decrease during the first day after the addition, followed by a recovery of the initial values during the next days (i.e. until day 5), to finally balance out at lower levels towards the end of the experiment (Fig. 4D). Peridinin:chl-*a* (Peri:chl-*a*) ratio did not show any significant response to the addition, fluctuating around values <2% (Fig. 4D). However, relative to values prior to the addition, a slight increase was observed starting from day 1 and culminating on day 4, to return to initial levels within a week after the addition. Photosynthetic pigments in the OUT stations did not present a consistent

pattern as compared to the IN and PRE-release stations (Fig. 4B–D).

In order to represent the three major size classes of autotrophs (pico-, nano- and micro- phytoplankton), the above results were regrouped (Table 3) by size and chemotaxonomic correspondence according to Table 2. According to the HPLC grouped data and contrary to the size fractionated results of fluorometric chl-*a* measurements, pigments corresponding to nanoplankton comprised the dominant fraction by ca. 60% whereas pigments assigned to picoplankton only conferred <30% of the DP.

3.4. Phytoplankton community structure

The pico-phytoplankton assemblages (PICO, Fig. 5A) were composed of three groups, *Synechococcus*-like cyanobacteria composed by *Synechococcus* (SYN) and *Prochlorococcus* (PRO), and diverse picoeukaryotes (EUK), characterized by differences in light scatter (size and granularity) and auto fluorescence. *Synechococcus* and *Prochlorococcus* were smaller than the eukaryotes, based on forward light scattering, with less chlorophyll per cell. In all

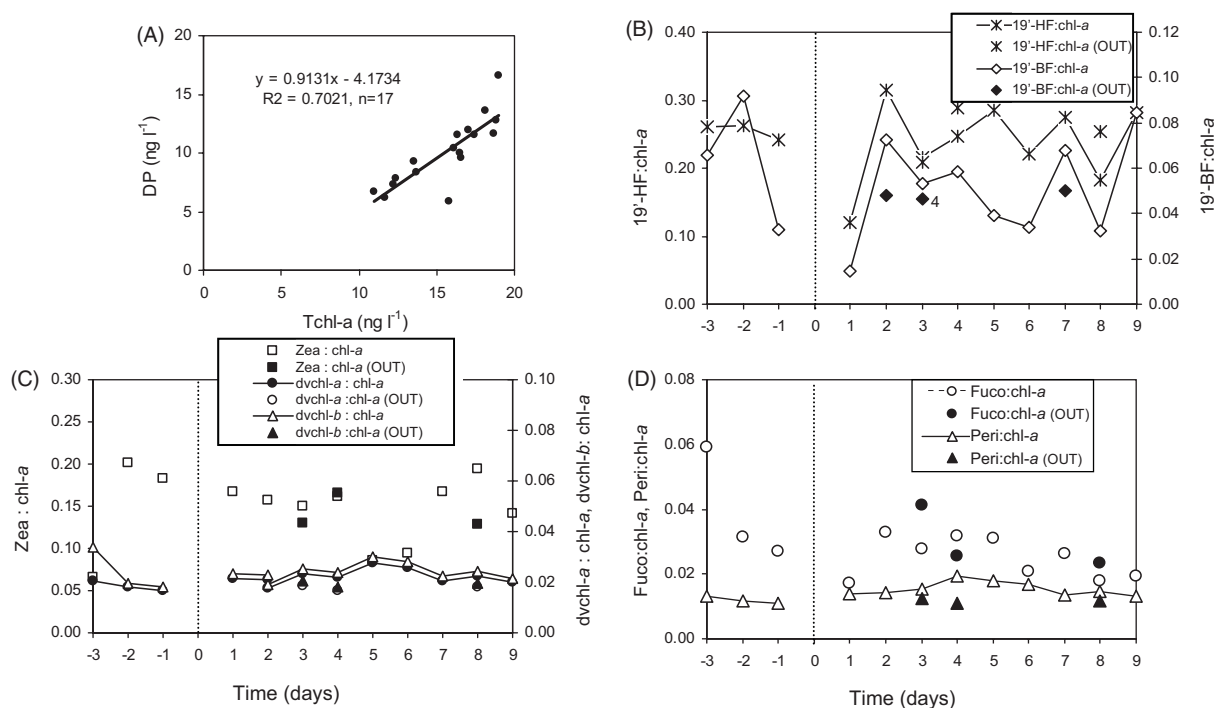


Fig. 4. Chemotaxonomic composition averaged for the SML (0–20 m): (A) Correlation between concentrations of Tchl-*a* and DP (sum of diagnostic pigments measured); (B) ratios of picoplankton marker pigments to Tchl-*a*; (C) ratios of nanophytoplankton and (D) ratios of microphytoplankton marker pigments to Tchl-*a*. In (B), (C), (D) the most representative marker pigments of the respective size classes are shown. Zea: Zeaxanthin, dvchl-*a*: divinyl chl-*a*, dvchl-*b*: divinyl chl-*b*, 19'-HF: 19' hexanoyloxufucoanthin, 19'-BF: 19' butanoylxu-fucoanthin, Fuco: Fucoxanthin, Peri: Peridinin.

PRE, IN and OUT stations *Synechococcus* were numerically dominant, close to 4000, 3000 and 2400 cells ml⁻¹, respectively, followed by picoeukaryotes that numbered ca. 300 cells ml⁻¹ (Table 4). *Prochlorococcus* were less abundant, $\ll 100$ cells ml⁻¹ before and after the addition, while in the OUT stations they were much less, $\ll 50$ cells ml⁻¹ (Fig. 5A). After the P-addition there was a net decrease of both abundance and biomass of *Synechococcus* compared to values prior to the addition. They both reached minimum values on day 3, then increased towards initial values by day 6. The abundance of picoeukaryotes and *Prochlorococcus* remained relatively unchanged throughout the addition experiment. While the details of the dynamics of *Synechococcus* abundance and zea: chl-*a* ratio did not follow a similar trend, both measures did show a decrease when compared to the values of the two days prior to the addition.

Out of all phytoplankton groups analyzed, small nanoplankton (SNANO, 2–10 μ m), displayed the most clear and dramatic decrease in abundance and biomass immediately after the P-addition, reaching

minima on days 4 and 5 and showing recovery ca. a week after the addition (Fig. 5B). Abundance was an order of magnitude lower than that of picophytoplankton, < 400 cells ml⁻¹ (Table 4). Small autotrophic nanoflagellates were numerically dominated (87%) in the smaller fraction of 2–5 μ m cell size, the fraction of 5–10 μ m cell size being less abundant (13%) (Fig. 5B). The contribution of the two size sub-fractions in terms of biomass was 43 and 57%, respectively. This nanoplankton group included all four prymnesiophyte classes with the exception of coccolithophores that include coccolithophores which were mostly > 10 μ m in cell size.

Phytoplankton taxa > 7 μ m (LNANO + MICRO) comprising coccolithophores, diatoms, dinoflagellates and various flagellates were relatively rare, ca. 1 cell from each taxonomic group per ml. However, the most striking result is that, contrary to trends displayed by all smaller-size classes, LNANO + MICRO increased in abundance and biomass after the addition. This was expressed with a 2–3-fold increase by day 4, which was mainly attributed to the increase of small (< 20 μ m) dinoflagellates (Fig. 5C).

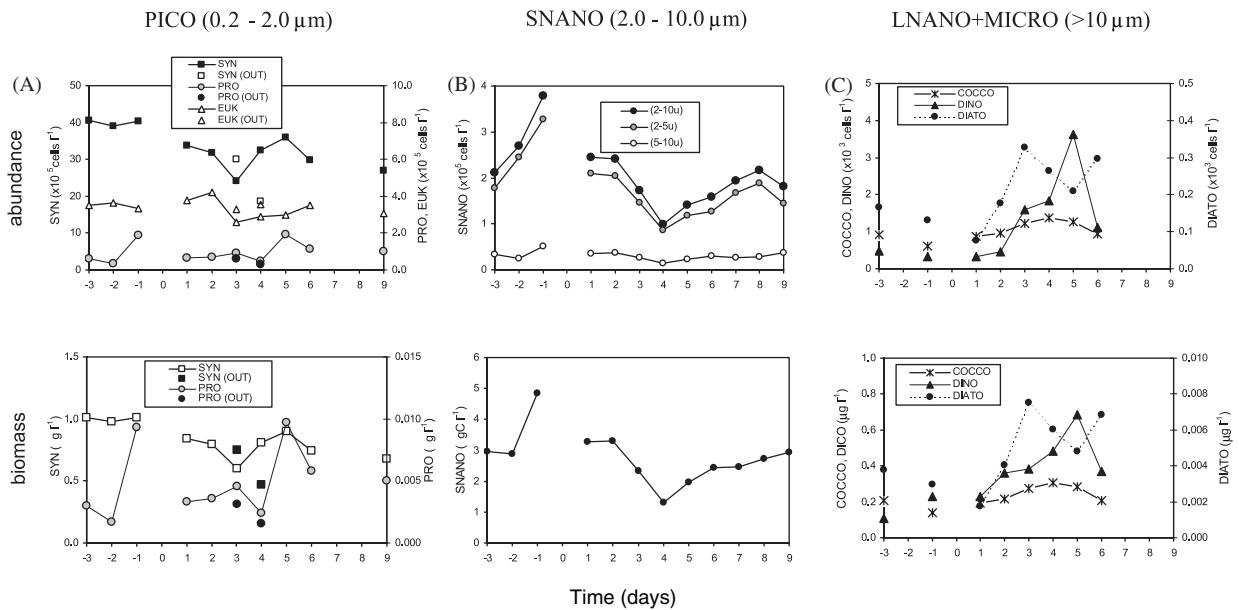


Fig. 5. Dynamics of all phytoplankton groups analyzed: (A) picoplankton—PICO (0.2–2.0 μm), by flow cytometry (left), (B) small nanoplankton—SNANO (2.0–10 μm), by epifluorescence microscopy (center) and (C) large nano + microplankton—LNANO + MICRO, by inverted microscopy (right). The upper panel of figures shows the temporal dynamics of abundances of the various groups whereas on the lower one, abundances are converted to C biomass (μg C l⁻¹) using appropriate factors and transformations described in the methods. COCCO: coccolithophores, DINO: dinoflagellates, DIATO: diatoms.

Table 4

Abundance of different groups of phytoplankton in the surface mixed layer of the Cyprus warm-core Eddy in May 2002 prior (PRE), after (IN) and outside (OUT) the P-addition area

Groups of Phytoplankton	PRE (cells ml ⁻¹)	IN* (cells ml ⁻¹)	OUT (cells ml ⁻¹)
<i>Synechococcus</i>	4000	3065	2430
<i>Prochlorococcus</i>	90	100	420
pico-eukaryotes	350	330	340
Autotrophic nanoflagellates	300	180	200
Heterotrophic nanoflagellates	350	340	280
<i>Coccolithophores</i>	0.8	1.1	—
Dinoflagellates	0.4	1.5	—
Diatoms	0.1	0.2	—

* mean of days 1–5.

Prior to the addition and during the following two days, the coccolithophores dominated larger phytoplankton cells representing on average 64% of total abundance, followed by dinoflagellates (28%). After the third day, their relative contribution was inverted, with coccolithophores and dinoflagellates representing 30 and 62%, respectively. Diatoms, during the experiment occupied on average 9% of the total abundance and were represented (98%) by a single pennate species (*Thalassionema frauenfeldii*). Though of low abundance (<0.3 cells ml⁻¹, Table 4), they also displayed a net increase, culminating on day 3.

Thus diatoms showed a faster response than coccolithophores and dinoflagellates, which attained maxima on days 4 and 5, respectively (Fig. 5C). Interestingly, the pronounced 11-fold increase in dinoflagellate abundance by day 5 represented only a 3-fold increase in biomass since it was attributed to smaller species (<20 μm) that contributed less in biomass. Their relative contribution increased from <10% on day 2 to ca. 70% of total dinoflagellate abundance by day 5.

Biomass calculations derived from cell counts were regrouped into the three major size classes

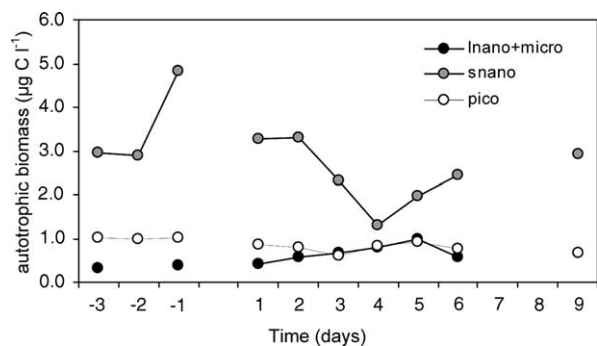


Fig. 6. Temporal variation in autotrophic biomass ($\mu\text{g C l}^{-1}$) considering all phytoplankton groups studied. Biomass data in the upper 20 m inside the P-patch, before and after the P-release. PICO: *Synechococcus* + *Prochlorococcus*, SNANO: small autotrophic nanoflagellates (2–10 μm), LNANO + MICRO: large nanoflagellates + microphytoplankton (>10 μm).

defined and presented together in order to assess total phytoplankton community evolution (Fig. 6, Table 3). Presented this way, the P-addition effect was mostly reflected in the biomass decrease of the dominant small nanoplankton group (>70%) and to a lesser extent in the decrease of picoplankton, which represented only 30% of the total biomass. At the same time, large nanoplankton and microplankton, which were originally <10% of the total biomass, increased and even outcompeted picoplankton in biomass from the second day until day 5.

4. Discussion

The Levantine basin of the Eastern Mediterranean is phosphate-limited in the conventional sense in winter. During this period the phytoplankton bloom ceases when phosphate is entirely consumed, leaving a measurable residual of nitrate (Krom et al., 2003). Despite the fact that in May the surface waters of the Eastern Mediterranean were below detection limit for both nitrate and phosphate (Kress and Herut, 2001), it was expected that, when phosphate was added to a patch of surface water, a phytoplankton bloom would be instigated, as had happened in the east central Pacific and the Southern Ocean (HNLC areas) when iron (the limiting nutrient in those regions) was added (Martin et al., 1994; Boyd et al., 2004). This was not what happened when P was added to the Eastern Mediterranean. The results presented here show that the first response of the phytoplankton to the addition of phosphate was far from being a significant increase in chlorophyll. Instead, there

was a small but measurable decrease in both phytoplankton biomass and C-uptake rates.

4.1. Phytoplankton response to P-addition

Chlorophyll-*a*, as determined by both conventional fluorometry and HPLC, and primary production decreased in the P-enriched patch during the first days of the experiment (Fig. 2). There was also a pronounced decrease in cell counts, with a 25% and 65% decrease in the abundance and biomass of *Synechococcus* and autotrophic nanoflagellates, over the first two and four days, respectively (Fig. 5). This change was accompanied by a parallel decrease in zeaxanthin, the pigment associated with cyanobacteria (HPLC pigment analysis, Fig. 4). Flow cytometry analysis showed that there was no detectable decrease in chlorophyll per cell (Zohary et al., 2005). The above observation indicated that these changes were due to a decrease in *Synechococcus* abundance and not simply a decrease in their chlorophyll content. At the same time there was a small but gradual increase in large nanoplankton and microplankton after the P-addition (Fig. 5). While this was relatively small in terms of total community abundance, it was relatively large in carbon biomass so as to balance out the observed decrease in the biomass of picoplankton within five days after the addition (Fig. 6). The changes observed in the patch were probably due to a short time increase in phosphate, caused by the artificial addition, and an increase in ammonia (bioavailable N), caused by the increase in grazing (Krom et al., 2005), to levels sufficient to allow microplankton to start growing. The pulse of P was sufficient to trigger the microbial loop and favour microbial grazers, which are the important grazers in such an extreme oligotrophic system (Pitta et al., 2005). The larger cells were thus able to “escape” the enhanced micrograzing pressure that was responsible for the decrease in the smaller pico and nanoplankton cells. The above hypothesis was partly confirmed by the increase in the corresponding marker pigment dynamics (Fig. 4) and to a lesser extent by a moderate increase in the >2.0- μm size fraction in both chl-*a* and primary production (Fig. 3).

4.2. Phytoplankton dynamics, community structure and methodological comparisons

The important variability in group-specific parameters such as fluorescence/chl-*a* per cell or per unit

biomass and the existence of allometric relationships between size and abundance make direct comparisons between the various methods employed in phytoplankton analysis not always feasible. In order to follow the response of the different phytoplankton groups and thus facilitate the interpretation of the complex microbial food web dynamics evoked by this experiment a comparison was undertaken synthesizing all autotrophic biomass related parameters. According to size-fractionated fluorometric determinations of chl-*a*, picoplankton cells contained ca. 60% of total chl-*a* (Table 3). However, although picoplankton were also numerically dominant, as shown by flow cytometry and microscopic counts, they were less important in terms of biomass. The HPLC chemotaxonomic biomass size fractionation revealed a different community structure, with nanoplankton dominating (ca. 60%) autotrophic biomass, followed by pico- (>30%) and microplankton (<10%) (Table 3).

The discrepancy between the HPLC/cell count derived biomass proportions and the fluorometric size-fractionated data might be basically related to higher cellular chl-*a* concentrations (chl-*a* per unit-C) of picoplankton cells. This is also reflected in the observed higher productivity rates (Table 3) as compared to those of larger phytoplankton cells. In this respect, it seems that size fractionation of chl-*a* alone is not a good proxy for biomass partitioning of the phytoplankton community, especially in such extreme oligotrophic environments and that a combination of techniques is more appropriate. Other technical issues to be considered is the fact that the HPLC analysis data were averaged over 0–20 m depth whereas size fractionated chl-*a* were derived from 12 m. The above mentioned discrepancy also could be accounted for by the low volume of water filtered for the larger chl-*a* size fraction (>2.0 μm) determinations.

When cell counts of all autotrophic groups analyzed were converted to C-biomass, the relative contributions of the three major size classes confirmed the HPLC-derived community structure with nanophytoplankton carbon as dominant (70–80%), followed by picoplankton (ca. 20%) and microplankton (ca. 10%) (Fig. 6, Table 3).

The above techniques, though widely applied, are not often combined to address both species diversity and size class/taxonomy related biomass measurements. Recent studies have combined HPLC chemotaxonomic analysis and flow cytometric

picoplankton determinations with genetic fingerprint patterns (Veldhuis and Kraay, 2004) and with conventional microscopic techniques (Estrada et al., 2004). These techniques have been applied in order to test whether autotrophic population analysis, based on different properties of the same group, offers consistent results, and to get an indication of the limitations and possibilities of each method applied separately as well as to reveal the potential of their combined use. To our best knowledge, this is one of the first studies applying HPLC and flow cytometry in addition to conventional microscopy techniques in an ultra-oligotrophic environment such as the Levantine Sea.

4.3. Mechanisms of nutrient limitation and grazing controlling phytoplankton

It is possible to estimate how much extra chlorophyll could have been generated in the patch by the P-addition using some simple assumptions. Thingstad and Mantoura (2005) measured the bioavailable excess-N in the system using an alkali-phosphatase titration and found it to be $230 \pm 60 \text{ nM-N}$, which is close to the excess values of 300–500 nM of nitrate remaining in surface waters at the end of the annual phytoplankton bloom (Kress and Herut, 2001). Using the Redfield N:P-ratio of 16 (molar), the above estimated bioavailable excess-N corresponds to ca. 14.4 nM P. Using a N:chl conversion factor of 0.31 nmol-N consumed per ng chl-*a* formed (Bertilsson et al., 2003), the estimated excess-N together with the added P could theoretically have allowed a production of $741 \text{ ng chl-}a \text{ l}^{-1}$, almost a 40-fold increase above the background level of 20 ng l^{-1} . The simplest explanation for the lack of increase in chlorophyll was that, at this time of the year, there was very little N available for phytoplankton growth. At the time of the experiment there were essentially undetectable concentrations of both nitrate and phosphate in the surface waters (Krom et al., 2005). Thus, the ca. 230 nM of excess N determined by Thingstad and Mantoura (2005) were in a form which was not immediately available to the phytoplankton community.

The anticipated N-limited status of the system after P-addition was confirmed by an on-deck microcosm experiment. In this experiment when ammonia was added to water collected inside the P-enriched patch, a massive phytoplankton bloom was initiated with 5-fold and 3-fold increases in

chlorophyll and *Synechococcus* cell density, respectively. There was no corresponding increase in chlorophyll when water collected outside the P-patch was supplemented with NH_4 (Zohary et al., 2005). These data taken together suggest that the system was N and P co-limited for phytoplankton growth during the CYCLOPS experiment.

Extensive N fixation in the eastern basin has been used to explain the unusual N:P ratio (28:1) in the deep water (Bonin et al., 1989; Sachs and Repeta, 1999; Béthoux et al., 2002). *Trichodesmium* species has not been observed so far in the Eastern Mediterranean despite the conditions being within the temperature, light and iron ranges considered conducive for this important N-fixing organism. The most abundant phytoplankton organism known to be present in the system, which can fix nitrogen is *Synechococcus*. Zehr et al. (2001) reported potential N-fixation ability of unicellular cyanobacteria in the subtropical North Pacific Ocean. It would thus be expected that as the system switched to N-limitation, if *Synechococcus* was an important N-fixing organism, it would be able to outcompete other phytoplankton in the system (Tyrell, 1999; Mills et al., 2004). This did not happen. In the patch, the first response to the N-limitation induced by P-addition was a decrease in the numbers of *Synechococcus* by ~25%. In the microcosm experiments *Synechococcus* increased only when both P and N were added (Zohary et al., 2005). These results might either reinforce the suggestion that any N fixation by *Synechococcus* or other similar cyanobacteria is P-limited (Mills et al., 2004) or simply that these *Synechococcus* strains thriving in the basin are no nitrogen fixers. If, however, the presence of *Synechococcus* as the dominant phytoplankton, was because of its unusually high N:P elemental ratio (Bertilsson et al., 2003; Haldal et al., 2003), then one would expect *Synechococcus* to be outcompeted by other groups when the N:P ratio dropped, as was actually observed in this experiment.

Symbiotic cyanobacteria have been reported as potential source of recently fixed N associated with certain open-ocean diatoms (Villareal, 1994) or other phytoplankters (Carpenter and Foster, 2002). However, this study shows that at this location diatom numbers ($\ll 1$ cells/ml) and Fucoxanthin pigment amounts (4% of DP) are so small that it is unlikely that N-fixation by diatoms with N-fixing symbionts could be significant. It is noted, however, that the core of a warm-core eddy in

summer is probably the most oligotrophic, nutrient-depleted area of the basin and thus microplankton are likely to be particularly scarce. Thus, if nitrogen fixation is an important process at this location it is likely to be due to some as yet unidentified cyanobacterial species.

After the P-addition in the Levantine basin, phytoplankton biomass decreased, most likely as a result of increased grazing. A rapid response of the grazing community to the addition of P was reported by both Pitta et al. (2005) and Pasternak et al. (2005). Heterotrophic nanoflagellates, increased over the first two days after the addition, then decreased for two days and then increased again. Ciliates showed a significant increase in abundance soon after the P-addition. A positive response in copepod gut fullness and egg abundance also was recorded (Pasternak et al., 2005). Taken together, this pattern was interpreted as being caused by a differing response to P-addition of various parts of this low-nutrient low-chlorophyll (LNLC) microbial system (Thingstad et al., 2005; Krom et al., 2005). After phosphate was added to the system, both phytoplankton and bacteria responded by taking up P into their cells, as shown by a rapid increase in particulate P (Flaten et al., 2005; Thingstad et al., 2005). Bacteria were able to access part of the large pool of DON present in surface waters and thus increase their production (Flaten et al., 2005) while phytoplankton were unable to access this pool of N and thus did not grow. Thus, the phytoplankton became P-rich food for the P-starved grazers. Once provided with P-rich food, the latter increased their grazing rate upon both bacteria and phytoplankton. This increased P allowed them to increase in numbers and, in the case of zooplankton, increase egg production (Thingstad et al., 2005).

Overall therefore, the response in this LNLC ultraoligotrophic area was very different to that observed in the HNLC areas (Martin et al., 1994; Boyd et al., 2004). In the HNLC areas, the addition of iron, the limiting nutrient, enabled the large pool of N and P to become available to the phytoplankton, particularly diatoms and other large eukaryotes. This resulted in a large and relatively long-term increase in phytoplankton biomass, which was observed both in the field and by remote sensing. By contrast, in the Eastern Mediterranean there was no significant pool of bioavailable N available to the phytoplankton even when excess phosphate was added. The immediate response was an increase in

the P-starved heterotrophic community of bacteria and micrograzers. This resulted in an increased grazing rate that caused the numbers and biomass of the dominant picoplankton to decrease. Although there was a small increase in larger nanoplankton and microplankton as ammonia was released by grazers, their initial numbers were so small that these changes were relatively insignificant. After seven days all these biological changes disappeared as the patch relaxed and was diluted away.

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