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# Biological response to P addition in the Eastern Mediterranean Sea. The microbial race against time

Paraskevi Pitta[a,*], Noga Stambler[b], Tsuneo Tanaka[c], Tamar Zohary[d], Anastasios Tselepides[a], Fereidoun Rassoulzadegan[c]

[a]*Hellenic Centre for Marine Research, P.O. Box 2214, 71003 Heraklion, Crete, Greece*
[b]*Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel*
[c]*Station Zoologique, Villefranche-sur-mer, France*
[d]*IOLR, Kinneret Limnological Laboratory (KLL), Tiberias, Israel*

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**Abstract**

The response of the microbial food web to P-addition was studied during a 10-day Lagrangian experiment in the Eastern Mediterranean during which orthophosphate was added to the surface water of the Cyprus anticyclonic eddy. Very low levels of all microbial populations (heterotrophic bacteria, *Synechococcus*, *Prochlorococcus*, autotrophic and heterotrophic nanoflagellates, ciliates) and bacterial production were detected, verifying the extreme oligotrophic character of this area. The microbial biomass was dominated by heterotrophs and the heterotroph/autotroph ratio increased from 1.05 before to 2.8 after the P-addition, on day 4. Bacteria took advantage of the supply of the putative limiting factor (P) increasing their production but not their abundance. The heterotrophic nanoflagellates remained stable in numbers. By contrast, *Synechococcus* and autotrophic nanoflagellates decreased after the P addition. This is an indication of consumption by ciliates which were the only organisms that showed a significant increase in abundance during the first 4 days after the P addition, relative to the period before the addition as well as to the second phase of the experiment (days 5–9). In particular, the mixotrophic ciliate biomass increased by 50% after the P release. In environmental conditions of general resource scarcity as is the case of the Eastern Mediterranean, the addition of the putative limiting factor (P) was reflected in the increased abundance of microzooplankton shortly after the enrichment as a result of a quick transfer of energy (“heterotrophic by-pass”) through bacteria and heterotrophic nanoflagellates. A “mixotrophic by-pass” of phytoplankton primary producers also occurred, transferring the P-addition driven primary production to higher trophic levels through mixotrophic ciliates.

*Keywords:* Eastern Mediterranean; P addition; Bacteria; Picophytoplankton; Auto and heterotrophic nanoflagellates; Mixotrophic ciliates

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## 1. Introduction

The Mediterranean Sea is a water body with special characteristics such as anti-estuarine circulation, low nutrient concentration (Béthoux et al., 1992), increased transparency, low chlorophyll

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[*]Corresponding author. Tel.: +30 2810 337801; fax: +30 2810 33 7822.
*E-mail address:* vpitta@her.hcmr.gr (P. Pitta).

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concentration (Williams, 1998), a West to East gradient of increasing oligotrophy (Turley et al., 2000), dominance of the autotrophic component by ultraplankton (Yacobi et al., 1995) and a microbial food web dominated by the heterotrophic component (Christaki et al., 2001).

The Mediterranean is the largest water body in the world believed to be phosphorus and not nitrogen limited. Initially, the hypothesis of phosphorus limitation of the Eastern Mediterranean was based on observations of the cessation of phytoplankton bloom after the depletion of phosphate but not nitrate from the surface layer and on high ratios of inorganic N:P in the deep water (Krom et al., 1991).

The hypothesis of P limitation in the Eastern Mediterranean has been verified in bottle experiments for phytoplankton and heterotrophic bacteria. During an experiment with water from the coastal Israeli area (Bonin et al., 1989), P and P + N additions produced an increase in phytoplankton biomass which was mainly due to pico-cyanobacterial cells. In nutrient-enrichment bioassays using subsurface water from the Ionian and Levantine basins, P addition caused significant increases in bacterial production, bacterial numbers and chlorophyll *a* concentration relative to unenriched controls (Zohary and Robarts, 1998). In a similar experiment in the Cretan Sea, treatments including phosphorus resulted in increased bacterial production (Christaki et al., 1999). In a microcosm experiment using Eastern Mediterranean surface water, mixed indications were found as to whether the osmotroph organisms were P or N limited (Thingstad et al., 2001; Kress et al., 2005).

The first two mesoscale iron-enrichment experiments, IronEx I (October 1993) and IronEx II (May–June 1995) in the equatorial Pacific, demonstrated the feasibility of fertilising a patch in the ocean with a micronutrient, of marking the patch with an inert gaseous tracer (SF6) and of tracking and resampling the patch (Martin et al., 1994; Coale et al., 1996).

However, the equatorial Pacific is an environment completely different from the Mediterranean in that the former is a high-nutrient-low-chlorophyll (HNLC) area with excess nutrients except for the limiting factor (Fe), whereas the latter is a low-nutrient-low-chlorophyll (LNLC) environment characterised by scarcity in all nutrients (Béthoux et al., 1992).

The Mediterranean Sea and particularly the eastern basin is an extreme oligotrophic environment characterised by a microbial dominated food web consisting of small unicellular phytoplankton, bacteria, protozoa and viruses, connected by complex trophic interactions (Thingstad and Rassoulzadegan, 1999). In open ocean oligotrophic ecosystems, the role of the microbial organisms becomes crucial since carbon flows mainly through the microbial food web (Kiorboe et al., 1990; Longhurst, 1991). Recent studies in this area have verified the dominance of small autotrophs and heterotrophs and demonstrated the role of small grazers (Christaki et al., 2001; Pitta et al., 2001).

The hypothesis of little energy transfer to the higher trophic levels has been proposed for the Eastern Mediterranean (Turley et al., 2000). From this point of view, it is both necessary and intriguing to study the structure of the Eastern Mediterranean microbial food web as it is intimately linked to its function in terms of material cycling (Thingstad and Rassoulzadegan, 1999). This is a prerequisite in order to assess the effect of the addition of the putative limiting factor (P) to the fate of energy through the microbial food web.

In May 2002, the hypothesis of P limitation in the Eastern Mediterranean was tested by adding orthophosphate to the surface water of the Cyprus anticyclonic eddy over a $4\times4\,\mathrm{km}^2$ area (Law et al., 2005). The dynamics of physical, chemical and biological variables was followed for 9 days after P addition and compared to a control site outside the P patch as well as to the situation before the addition.

The goal of the present paper is to describe and assess the response of the microbial populations to the P addition, to compare their abundance and/or biomass and activity to the pre-release situation and to detect changes in the trophic relations inside this food web in order to follow the fate of energy through the trophic pathways after the addition of the putative limiting factor.

## 2. Materials and methods

### 2.1. Experimental design and sampling

The design of the P enrichment experiment in the Eastern Mediterranean has been described in detail elsewhere (Law et al., 2005). Briefly, the Lagrangian experiment was carried out in the centre of the Cyprus anticyclonic eddy, SE Levantine Basin (Fig. 1), by the R/V Aegaeo between 14 and 25 of May 2002.

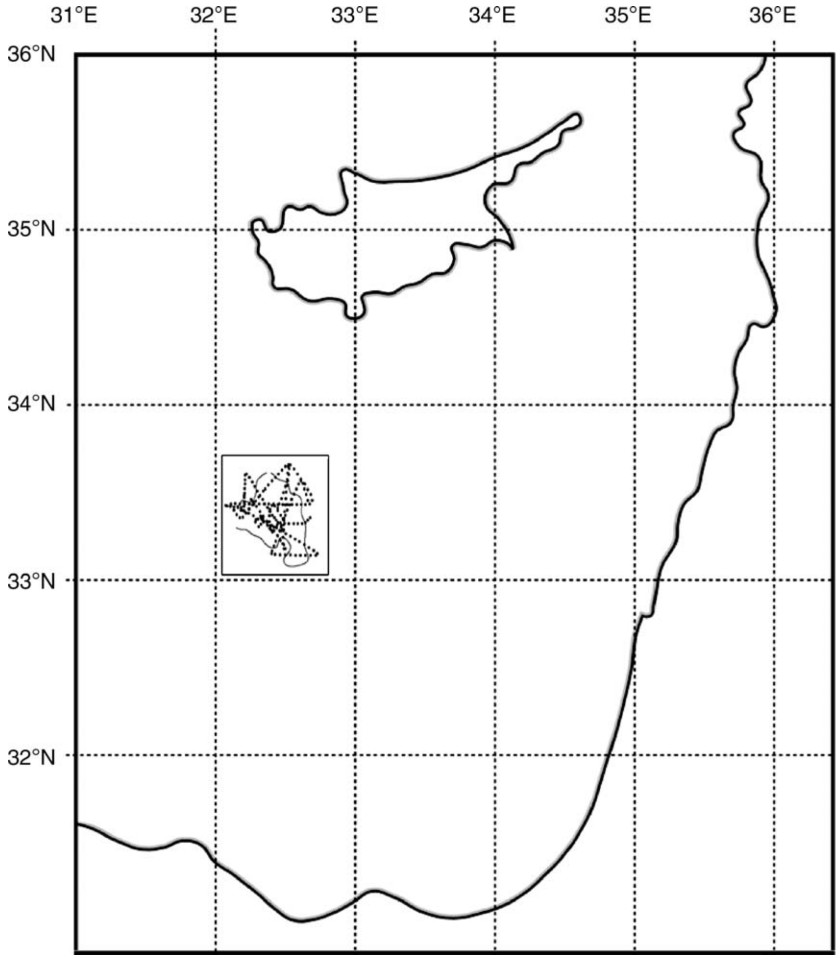

Fig. 1. Map showing location of the P addition patch close to the centre of the Cyprus Eddy. The dotted line shows $SF_6$ sampling locations and the continuous line shows the extent of the fertilised patch.

Eighteen tonnes of diluted and partially neutralised phosphoric acid, together with $SF_6$ as the inert tracer, were added to a 16 km$^2$ patch of water. This resulted in a mean initial phosphate concentration of 120 nmol l$^{-1}$. The patch, which was contained in the centre of the warm core eddy, was followed successfully for 9 days. Detailed biological, chemical and physical measurements were carried out on the patch water, and also on a series of stations outside the patch and before the P-release (referred to in the text as IN stations, OUT stations and PRE stations, respectively, Table 1).

Physical data was gathered using the Sea Bird Electronics (SBE911plus) CTD profiler, furnished with oxygen and fluorometer sensors. Water samples for nutrient and biological analysis were taken using a multi-sampler/Rosette system (General Oceanics). Samples were collected during daytime (11:00 to 12:00, local time) at 6 depths in the upper 45 m. In the present study, only data from the upper 20 m layer are reported since $SF_6$ data showed that the mixing of the added P was restricted to this layer (Law et al., 2005).

### 2.2. Bacterial production

Bacterial protein synthesis was determined by the $^{14}$C-leucine incorporation using a modified micro-tube technique (Smith and Azam, 1993). L-[U- $^{14}$C]Leucine (Amersham, CFB183, spec act 227 mCi/mmol) was added to triplicate 1.8 ml samples in 2 ml micro-centrifuge tubes to give a final concentration of leucine of ~100 nM. The choice of 100 nM leucine was based on a concentration vs.

Table 1
Station characteristics during the P-addition experiment: PRE, IN and OUT refer to the stations sampled before the experiment and to the ones inside and outside of the patch of the P-release

| | Date (2002) | Longitude | Latitude | Time elapsed after P-release (days) |
|---|---|---|---|---|
| PRE | 14 May | 32.1747 | 33.2025 | −3 |
| PRE | 15 May | 32.1735 | 33.2023 | −2 |
| PRE | 16 May | 32.1760 | 33.2017 | −1 |
| IN | 18 May | 32.2041 | 33.1963 | 1 |
| IN | 18 May | 32.2073 | 33.2350 | 1 |
| IN | 19 May | 32.2051 | 33.1782 | 2 |
| IN | 19 May | 32.2477 | 33.2121 | 2 |
| IN | 20 May | 32.2484 | 33.1209 | 3 |
| IN | 21 May | 32.2222 | 33.0908 | 4 |
| IN | 22 May | 32.1543 | 33.1345 | 5 |
| IN | 23 May | 32.1297 | 33.1660 | 6 |
| IN | 24 May | 32.1530 | 33.1737 | 7 |
| IN | 25 May | 32.2048 | 33.1889 | 8 |
| IN | 26 May | 32.2930 | 33.1673 | 9 |
| OUT | 20 May | 32.2523 | 33.0391 | “3” |
| OUT | 21 May | 32.1743 | 33.0245 | “4” |
| OUT | 23 May | 32.0440 | 33.2028 | “6” |
| OUT | 25 May | 32.2034 | 33.3017 | “8” |

incorporation pre-experiment which showed saturation of leucine incorporation at about 70 nM. The samples were incubated at 23 ± 1 °C under subdued (laboratory) illumination for 4–8 h. These incubation times were also based on pre-experiments showing that uptake was linear over at least the first 10 h of incubation. Additional replicates for subtraction of background and abiotic adsorption were fixed with 100 μl of 100% TCA per 1.8 ml sample before addition of the isotope. Incubation was stopped by the addition of 100% TCA, the micro-tubes were centrifuged on an ultra-centrifuge at 14,000 rpm for 10 min. The supernatant was then sucked out of the test-tube and 1 ml of 5% TCA was added. After gentle mixing of the micro-tubes, they were centrifuged again (same time and speed). Again, the supernatant was sucked out, 1 ml scintillation Fluor (Ultimagold) was added and the filters were counted on a Packard Scintillation counter. Leucine uptake was converted to carbon uptake using the formula of Simon and Azam (1989) with an isotope dilution factor of 1 (assuming that in the ultra-oligotrophic Eastern Mediterranean, ambient leucine concentrations are orders of magnitude lower than the 100 nM added, i.e. practically no dilution of the isotope takes place).

### 2.3. Population abundances and biomass

For bacterial counts, 30 ml samples were fixed with 4.2 ml of filtered 5% formalin. Counts were performed on DAPI-stained samples (Porter and Feig, 1980) filtered on 0.2 μm pore-size black polycarbonate (Poretics) filters using an epifluorescence microscope. Abundance data were converted into C biomass using 20 fg C cell$^{-1}$ (Lee and Fuhrman, 1987).

Duplicate samples (1.8 ml) for counts of prokaryotic *Prochlorococcus* and *Synechococcus* were preserved with 140 μl of 25% glutaraldehyde (Sigma G-5882), deep-frozen in liquid nitrogen and kept at −80 °C until analysis using a FACScan (Becton Dickinson) flow cytometer. The samples were thawed for 1 min at 37 °C and analysed at room temperature (23 ± 2 °C) by excitation with Argon laser at 488 nm. Forward light scatter (FSC), side scatter (SSC, a function of cell size), red fluorescence of chlorophyll above 630 nm (FL3), and orange fluorescence of phycoerythrn at 585 ± 15 nm (FL2) were measured. Before running the sample, 0.93 μm beads (produced by PolySciences) were added as an internal standard. The phytoplankton assemblages were composed mainly of two groups of cyanobacteria, *Synechococcus* sp. and *Prochlorococcus* spp., and a third group of diverse pico-eukaryotes which were differentiated by their unique light scatter (size and granularity) and auto fluorescence features. *Synechococcus* and *Prochlorococcus* abundance data were converted into C biomass using 250 fg C cell$^{-1}$ (Kana and Glibert, 1987) and 50 fg C cell$^{-1}$ (Campbell et al., 1994), respectively.

Samples for nanoflagellates were fixed with glutaraldehyde (final con. 1%). Subsamples (50–100 ml) were concentrated to ca. 10 ml on 25 mm, 0.8 μm pore sized polycarbonate black filters, stained with DAPI for 10 min (final conc. 0.5 μg ml$^{-1}$) and filtered. Filtration was completed within each sampling day. Autotrophic and heterotrophic nanoflagellates (ANF and HNF, respectively) were counted in different size classes (2–5 and 5–10 μm) using an ocular micrometer under UV and blue excitation filters (Porter and Feig, 1980). Carbon biomass of nanoflagellates was calculated under the assumption of a constant volume: 35 μm$^3$ cell$^{-1}$ for 2–5 μm and 294 μm$^3$ cell$^{-1}$ for 5–10 μm and the conversion factor of 183 fg C μm$^{-3}$ (Caron et al., 1995).

For ciliate enumeration and identification, 500 ml samples were preserved with borax-buffered formalin (final concentration 2% formaldehyde) and stored at 4 °C in the dark. Before examination, samples were left to settle in their bottles in the dark at 4 °C and after 48 h, the top 400 ml of the sample was slowly siphoned off. The bottom 100 ml of the sample was transferred into sedimentation chambers, allowed to settle for 24 h and was finally examined by means of an inverted microscope equipped for transmitted light, phase-contrast and epifluorescence microscopy (blue light excitation). Ciliates were counted and distinguished into size-classes, trophic modes (auto-, hetero-, mixotrophs) and major taxonomic groups (orders Oligotrichida, Choreotrichida and Tintinnida [Laval-Peuto, 1994; Laval-Peuto et al., 1994], the first two comprising aloricate and the third loricate species). Oligotrich and choreotrich ciliates were identified down to genus or species level where possible, following Maeda and Carey (1985), Maeda (1986), Laval-Peuto and Rassoulzadegan (1988), Lynn et al. (1988, 1991), Montagnes et al. (1988, 1990), Montagnes and Taylor (1994). Tintinnids were identified to species level, based on the lorica shape and dimensions after Jorgensen (1924) and Balech (1959). Mixotrophs were recognised by means of the algae chloroplasts "enslaved" within their cells. Ciliate cell sizes were measured with an ocular micrometer and converted into cell volumes using appropriate geometric formulae (Peuto-Moreau, 1991). For biomass estimation, the conversion factor 140 fg C $\mu$m$^{-3}$ was used, as has been suggested for ciliates fixed with 2% formaldehyde (Putt and Stoecker, 1989). Mixotrophic ciliates use both photosynthesis and particle ingestion to cover their energy needs. However, we do not know which proportion of the carbon budget of plastidic ciliates is contributed by photosynthesis, this proportion depending not only on the ciliate species, but also on food and light availability (Stoecker, 1991). For this reason, an arbitrary assumption was made and 50% of their biomass was assigned to the autotrophic and 50% to the heterotrophic component of the microbial biomass.

### 2.4. Statistical analysis

One- and two-way ANOVA were used to test whether significant differences existed between samples grouped according to certain criteria (i.e. before or after the P-addition). This type of analysis was performed on abundance of different groups of organisms and the bacterial production. The post hoc Tukey test was employed for multiple comparisons between days after the P addition. Correlation analysis was performed between the abundance of different groups of organisms using the Pearson correlation index. In order to test whether P addition had an impact on the ciliate community structure, affecting the species composition and/or the relative abundance of the species present in the community, multivariate analysis was performed on the species abundance data, using non-metric multidimensional scaling (MDS, Field et al., 1982) in the PRIMER software package. Similarities between samples were calculated by means of the Bray–Curtis index (Bray and Curtis, 1957) and a square root transformation was applied on the abundance data prior to the analysis in order to normalise the data and to avoid skewness.

## 3. Results

### 3.1. Abundance distribution of pico-, nano- and micro-plankton and bacterial production

In the upper 20 m layer, bacterial abundance was (before and after the P-release) $10^4$–$10^5$ cells ml$^{-1}$ (Fig. 2) whereas heterotrophic nanoflagellates (HNF) numbered $10^2$ cells ml$^{-1}$. *Synechococcus* dominated numerically the autotrophic component ($10^3$ cells ml$^{-1}$); *Prochlorococcus* concentration presented remarkably low values in this upper layer and varied from $10^1$ to $10^2$ cells ml$^{-1}$ and autotrophic nanoflagellates (ANF) were of the order of $10^2$ cells ml$^{-1}$. Ciliates also showed low values ($10^2$ cells l$^{-1}$) and varied little in the surface layer.

Bacterial production was 43.1 ng C l$^{-1}$ h$^{-1}$ on average in the PRE and the OUT stations (Fig. 2). Inside the fertilised patch and during the 9 days of the experiment after the P addition, bacterial production increased to 95 ng C l$^{-1}$ h$^{-1}$ on average.

One-way ANOVA was used to test for significant differences between before and after the P addition using two groups of stations: all the IN-patch stations, after the P-addition grouped together compared to the PRE stations, also grouped together. Only bacterial production was found to be significantly higher in the IN-patch stations compared to the PRE stations ($p<0.001$). By contrast, *Synechococcus* and autotrophic nanoflagellates (ANF) were significantly less abundant ($p<0.01$ and $p<0.0001$, respectively) at the IN-patch stations compared to the PRE ones.

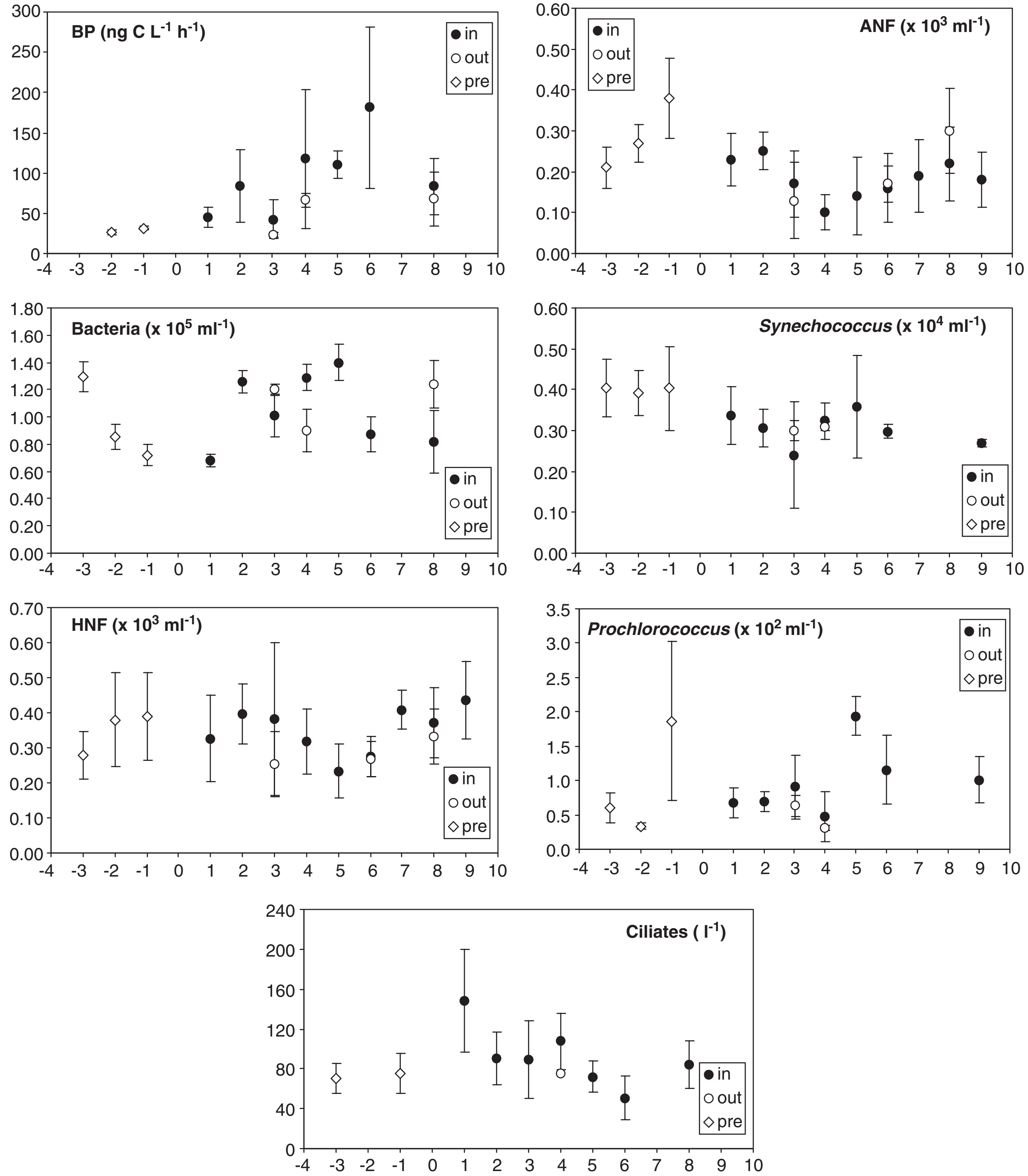

Fig. 2. Mean values (of 5 depths within the surface 20 m layer ±SE) of bacterial production and abundances of autotrophic and heterotrophic groups of organisms before (PRE stations) and after the P-addition, inside (IN stations) and outside (OUT) of the patch of the P-release. BP: bacterial production, ANF: autotrophic nanoflagellates, HNF: heterotrophic nanoflagellates.

Table 2
Results of ANOVA used to test for significant differences in abundance of different microbial populations among the 9 days which followed the P-release in relation to the PRE-release stations. Only significant differences between days are shown

| | D-3 | D-2 | D-1 | D1 | D2 | D3 | D4 | D5 | D6 | D7 | D8 | D9 | D3out | D4out | D8out |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D-3 | | | | | | | | | | | | | | | |
| D-2 | BACT** | | | | | | | | | | | | | | |
| D-1 | BACT** | | | | | | | | | | | | | | |
| D1 | BACT**<br>CIL** | | CIL* | | | | | | | | | | | | |
| D2 | | BACT** | BACT** | BACT** | | | | | | | | | | | |
| D3 | | | BACT*<br>ANF** | BACT** | | | | | | | | | | | |
| D4 | | BACT** | BACT**<br>ANF** | BACT** | | | | | | | | | | | |
| D5 | | BACT** | BACT**<br>ANF** | BACT**<br>CIL** | | BACT** | | | | | | | | | |
| D6 | BACT** | | ANF** | CIL** | BACT** | | BACT** | BACT** | | | | | | | |
| D7 | | | ANF* | | | | BACT** | BACT** | | | | | | | |
| D8 | BACT** | | | CIL* | BACT** | | | | | | | | | | |
| D9 | | | ANF* | | | | | | | | | | | | |
| D3out | | | BACT** | BACT** | | | | | BACT** | | BACT** | | | | |
| D4out | BACT**<br>PRO**<br>SYN** | PRO**<br>SYN* | PRO**<br>SYN** | PRO**<br>CIL* | BACT**<br>PRO** | PRO** | BACT**<br>PRO** | BACT**<br>PRO** | PRO** | | | PRO** | BACT**<br>PRO** | | |
| D8out | | BACT** | BACT** | BACT** | | | | | BACT** | | BACT** | | | BACT** | |

$^{**}$:$p<0.01$, $^{*}$:$p<0.05$. BACT: bacteria, ANF: autotrophic nanoflagellates, PROC: *Prochlorococcus*, SYN: *Synechococcus*, CIL: ciliates.

In addition, analysis of variance was used in order to study the temporal dynamics of all microbial variables as a result of the P addition, in other words to test for significant differences between the 9 days which followed the P release in relation to the PRE-release stations (Table 2). Only ciliate abundance showed a significant increase; in detail, on the first day after the addition, ciliate abundance was significantly higher than the abundance on days −3 and −1 before the fertilisation ($p<0.01$ and $p<0.05$, respectively), as well as days 5, 6 and 8 after the P release ($p<0.01$ in all cases) whereas ciliate abundance on day 1 did not differ from days 2, 3 and 4. On the other hand, the abundances of heterotrophic nanoflagellates as well as those of *Synechococcus* and *Prochlorococcus* did not show any significant differences in time during the experiment ($p>0.05$). As for autotrophic nanoflagellates, after the P release and particularly on days 3, 4, 5, 6, 7 and 9, their abundance decreased significantly ($p<0.01$) compared to day −1 before the release, during which an exceptionally high abundance of ANF was found. Regarding bacteria, their abundance presented high variability which resulted in differences between days without any pattern in terms of time (before vs. after the release or during the experiment) or space (in vs. out of the patch). This gave the previously mentioned result of lack of differences in bacterial abundance before and after the P addition when studied as a whole using one-way ANOVA.

Autotrophic nanoflagellate abundance was significantly correlated (negatively) with bacterial production; heterotrophic nanoflagellate abundance was negatively correlated with bacterial production and positively with autotrophic nanoflagellates (Table 3). *Prochlorococcus* numbers were correlated to *Synechococcus* abundance.

### 3.2. Community structure

Depth integration (down to 20 m) revealed a slight increase in microbial carbon biomass on day 2 after the P release followed by a return to the PRE-release levels by day 3 and 4 (Fig. 3). After the P release, the microbial food web was dominated by the heterotrophic component in terms of carbon biomass (Fig. 3). The ratio of the heterotrophic/autotrophic biomass increased from 1.05 on day −1 (Fig. 3) to 1.3 on day 1 after the P addition and reached the value of 2.8 by day 4; this was due to both increased biomass of the heterotrophic component, mainly of ciliates, and decreased biomass of the autotrophic component, mainly of ANF.

The autotrophic component of the food web represented on average 44% of the microbial biomass before and decreased to 35% after the release (Fig. 3). ANF dominated the autotrophic biomass (77% before and 69% after the P-addition, Fig. 4). *Synechococcus* biomass also decreased considerably during the first days after the P release compared to the PRE-release period. However, its

Table 3
Results of Pearson correlation analysis between abundances of measured microbial variables

| | BP | BACT | ANF | HNF | PROC | SYN |
|---|---|---|---|---|---|---|
| BACT | ns | | | | | |
| ANF | $-0.554^{***}$ | $-0.433^{*}$ | | | | |
| HNF | $-0.437^{*}$ | ns | $0.524^{*}$ | | | |
| PROC | ns | ns | ns | ns | | |
| SYN | ns | ns | ns | ns | $0.480^{*}$ | |
| CIL | ns | ns | ns | ns | ns | ns |

$^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$, ns: not significant. BP: bacterial production, BACT: bacteria, ANF: autotrophic nanoflagellates, HNF: heterotrophic nanoflagellates, PROC: *Prochlorococcus*, SYN: *Synechococcus*, CIL: ciliates.

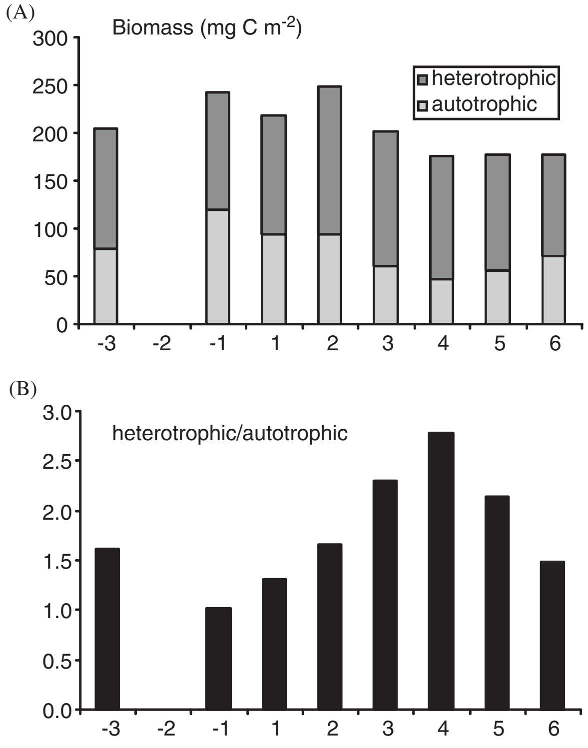

Fig. 3. (A) Integrated (down to 20 m) autotrophic and heterotrophic biomass before and after the P-release, (B) ratio of heterotrophic/autotrophic biomass.

relative importance to total autotrophic food web remained stable in terms of biomass (around 22%, Fig. 4). *Prochlorococcus* represented an extremely low component of the autotrophic food web in terms of biomass (0.1%).

Bacteria represented 32% of the heterotrophic carbon biomass before (Fig. 4) and 34% after the release. On the other hand, HNF increased slightly in biomass after the release. HNF dominated the heterotrophic biomass before (62%, Fig. 4) and after the release, but dropped to 58% of the heterotrophic biomass after the release. Although HNF increased in abundance after the release (which may explain the lack of increase in bacteria numbers despite the increase in bacterial production) their contribution to the heterotrophic component decreased after the release due to the considerable increase in ciliates. There was not a remarkable change in distribution of HNF into size classes before and after the P addition (data not shown).

Ciliates represented a substantial part of the heterotrophic biomass. The relative importance of ciliates increased from 6% of the heterotrophic biomass before to 8% IN the patch-area after the release (Fig. 4); these organisms reached 16% of the heterotrophic biomass on the first day after the addition. This increase was mainly due to aloricates, the contribution of which to ciliate carbon biomass increased by 20% after the P addition related to the PRE stations, representing 77% of ciliate biomass; as for size classes, large aloricates (>30 μm) made up 53% of aloricate abundance before and 60% at the IN-patch stations and more than 90% of aloricate biomass in both cases. In the case of ciliates, mixotrophic species were found to make a substantial part of both abundance and biomass. Before the P release, 25% of ciliate abundance and 44% of ciliate biomass were due to mixotrophic species; these numbers increased to 32% and 66%, respectively, at the IN-patch stations, after the release.

### 3.3. Species composition of ciliate community

A total of 84 ciliate taxa was identified and counted during this study. The ciliate assemblage included members of the orders Choreotrichida, Tintinnida and Oligotrichida. Aloricate forms dominated ciliate species (Choreotrichida and Oligotrichida) by 67% while tintinnids represented 33% of the total species number. Mixotrophs were represented by 21 species.

Ciliates ranged in length from 10 μm (a tiny *Strombidium* species) to 350 μm (two tintinnid species, *Cyttarocylis magna* and *Eutintinnus fraknoi*). Ten species were found in the nanoplankton fraction (<18 μm), 7 tintinnid species exceeded 200 μm in length, while the rest of the species

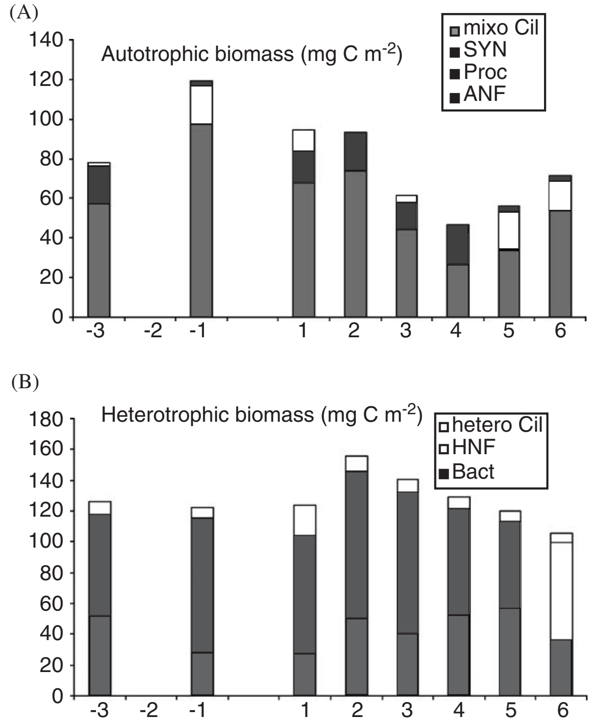

Fig. 4. Integrated (A) autotrophic and (B) heterotrophic biomass (down to 20 m) before and after the P-addition and their further analysis in major components: SYN: *Synechococcus*, Proc: *Prochlorococcus*, ANF: autotrophic nanoflagellates, cil: ciliates, HNF: heterotrophic nanoflagellates and Bact: bacteria.

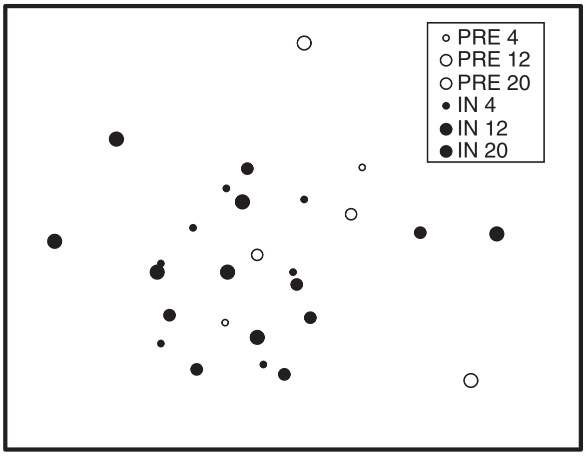

Fig. 5. Multidimensional Scaling Ordination (MDS) plot of ciliate species-abundance data at all PRE and IN stations and in all depths within the 0–20 m surface layer. PRE (open circles): stations sampled before the P-addition, IN (solid circles): stations sampled after the addition, in the P-patch. 4, 12 and 20 refer to the sampling depths: 4, 12 and 20 m, respectively.

belonged to the microplankton fraction (20 to 200 μm). In terms of equivalent spherical diameter (ESD), ciliate species ranged from 8 μm (a tiny *Strombidium* species) to 140 μm (the tintinnid *Cyttarocylis magna*).

Multivariate analysis of ciliate species abundance data for all the samples taken within the surface 20 m deep layer, before and after the P addition, revealed no major clustering corresponding to either P addition (before–after) or to time elapsed from the P addition (Fig. 5); in other words, the ciliate community structure did not change due to the P addition. The relatively high stress value (although below 0.3) is typical for samples which do not represent series along a gradient or grouping into clusters.

## 4. Discussion

### 4.1. Microbial stocks and bacterial production

Population abundances of the six microbial assemblages enumerated in this study (*Prochlorococcus*, *Synechococcus*, autotrophic nanoflagellates, heterotrophic bacteria, heterotrophic nanoflagellates and ciliates) ranged in similar or lower levels than reported in other studies conducted in the Eastern basin of the Mediterranean (Zohary and Robarts, 1992; Li et al., 1993; Robarts et al., 1996; Zohary et al., 1998; Christaki et al., 2001; Pitta et al., 2001). From this point of view, these values are among the lowest reported, even when compared with other oligotrophic environments such as the Sargasso Sea (Caron et al., 1995, 1999) or the Arabian Sea (Reckermann and Veldhuis, 1997). The ultra-oligotrophic character of this area is also confirmed by the low concentration of all nutrients (Krom et al., 2005) and the low phytoplankton production and biomass (Psarra et al., 2005).

In our study, *Prochlorococcus* showed remarkably low concentrations in the surface 20 m layer, though lower or comparable to earlier measurements in other areas of the Eastern Mediterranean (Li et al., 1993). Studies from the Mediterranean (Martin, 1997; Partensky et al., 1999) presented the distribution of *Prochlorococcus* as having a maximum at depth. However, it is not unlikely that the weak fluorescence of *Prochlorococcus* in increased oligotrophic conditions may lead to underestimates at the surface (Partensky et al., 1999). In the area of the present study, Li et al. (1993) were unable to measure any *Prochlorococcus* concentration in the uppermost 75 m.

### 4.2. Trophic state of the food web in the study area

The trophic food web was dominated by the heterotrophic component which is not surprising for an oligotrophic environment and has been found in other cases as well (Gasol et al., 1997). After the P addition, the ecosystem shifted to even more pronounced heterotrophy since the populations of autotrophs decreased while those of heterotrophs increased.

Bacterial production was found to increase significantly after the fertilisation of the patch and the bacterial growth rate doubled, from 1.1 to 2.3 $\text{day}^{-1}$, after the phosphorus addition compared to the PRE and the OUT stations. This is an indication that the heterotrophic bacteria were P limited and took advantage of the supply of the limiting nutrient which is not surprising when taking into account the excess semi-labile and labile dissolved organic carbon in the area (Krom et al., 2005). In contrast to bacterial production and growth rate, bacterial abundance remained at the same levels after the P release. This fact could be seen as an indication of heavy predation by microbial grazers, most probably the heterotrophic nanoflagellates. It is unlikely that ciliates contributed to keeping the bacteria stable in numbers because the bacteria populations were very scarce and consequently not sufficient for the feeding of ciliates (Fenchel, 1980). The role of viruses in controlling bacteria abundance was not measured during this study. However, Thingstad et al. (1996) have found viral activity to be low in the N.W. Mediterranean. Although grazing on bacteria-favoured by the P addition, the heterotrophic nanoflagellates did not increase in abundance possibly due to grazing by ciliates.

In contrast to bacterial production, phytoplankton did not show any increase after the P-addition in terms of chlorophyll *a* or primary production (Psarra et al., 2005). This could possibly be explained if we consider that phytoplankton was not P limited but N+P co-limited, as was demonstrated by an on-board microcosm experiment (Zohary et al., 2005). On the other hand, picophytoplankton, i.e. *Synechococcus* and *Prochlorococcus* as well as autotrophic nanoflagellates, decreased after the P release. This is also an indication of consumption by micrograzers, and mainly ciliates, which increased in abundance and biomass after the fertilisation. In a recent study that also took place in the Levantine Sea (Pitta et al., 2001), it was found that autotrophic nanoflagellates and *Synechococcus* were present in equal quantities in the food vacuoles of both tintinnids and aloricates which are the two main groups of planktonic ciliates. This has been interpreted as an indication of selection by ciliates for ANF taking into account their lower density by one to two orders of magnitude compared to *Synechococcus*. This also being true in the present study, one may explain the decrease in ANF and *Synechococcus* populations as a result of grazing by ciliates. Pitta et al. (2001) also showed that 54–88% of the "non-empty" ciliates, irrespective of size or group (aloricates and tintinnids) contained small prey ($<3\,\mu m$). This result reflects the environmental conditions of the open waters where *Synechococcus* dominate the phytoplankton population (90% of autotrophic abundance in the present study, Psarra et al., 2005).

Interestingly, ciliates were the only group of organisms which showed an increase in abundance after the addition of P. In fact, this took place very quickly, and on the first 3 days after the addition their populations were more abundant. This may reflect the state of starvation in which the system was and the quick passage of nutrients from bacteria towards ciliates via the heterotrophic nanoflagellates ("heterotrophic by-pass" of the phytoplankton). According to Flaten et al. (2005), the osmotrophs were P-starved before and P-replete after the addition. According to Thingstad (2005) the P pulse was transferred to higher trophic levels by a "trophic tunnelling" where the added P was rapidly taken by "luxury consumption" in P starved osmotrophs; then the P starved predators responded rapidly to this change in food quality and not quantity. If the previous process is the "phagotrophic" way of transferring nutrients to higher trophic levels, we have to take also into account the "photosynthetic" way. In fact, the considerable increase in mixotrophic ciliate populations after the P release (on average, 44% of ciliate biomass before and 66% after) brings to light another potential role of this functional group. Assuming that chloroplast-retaining ciliates are a functional component of the primary producers in the studied area, it would be reasonable to consider them as competitors of "regular phytoplankton". In this case, plastidic ciliates would reap greater benefit of P addition than phytoplankton ("mixotrophic by-pass" of the phytoplankton) because P addition gives only one possible direct nutrient source to the "regular phytoplankton", that is to fuel photosynthesis.

However, it may provide three possible nutrient sources to the "chloroplast-retaining ciliates": (a) fuelling photosynthesis performed by their "enslaved" chloroplasts, (b) direct ingestion of the "P addition favoured" bacterial growth by small sized ($<30\,\mu m$) ciliates (Rivier et al., 1985; Rassoulzadegan et al., 1988) and (c) ingestion of heterotrophic flagellates through food chain cascade (bacteria-->HF-->ciliates). Such competition would lead to the observed decrease of the "regular phytoplankton" as the function response to P addition.

In fact, the 4-fold increase in the biomass of chloroplast-retaining ciliates from day −1 to day 1, followed by an increase in bacterial production and in bacteria and HNF biomass on day 2, is reminiscent of a situation in a coastal environment, where added mineral nutrients are taken up mainly by diatoms and where the diatom bloom is followed by an increase in bacterial production and an increase in HNF biomass. In the present study, the role of the diatoms has been replaced by chloroplast-retaining ciliates. Like the diatoms, the ciliates in the present investigation were mainly $>30\,\mu m$ and only accessible to copepods which in fact showed a positive response at the end of the investigation period (Pasternak et al., 2005).

In any case, heterotrophic nanoflagellates and ciliates, both being protozoa and whose growth rates are as high as that of their prey, are capable of a quick response in case of excess food supply and they may double their populations within hours. By contrast, copepods, the main grazers of ciliates, are metazoa and therefore characterised by longer life cycles. In fact, this must be one of the reasons for which there was an accumulation of biomass in the ciliate compartment, at least during the first days after the enrichment (another reason could be the direct P uptake in the case of plastidic ciliates). Although copepods seem to have benefited from the increased ciliate populations (Pasternak et al., 2005) they were not capable of reacting quickly and controlling the ciliate abundance due to their scarce initial populations. In this case, we could refer to a "ciliate bloom" as opposed to the well-known "diatom bloom". The latter is typical of an ecosystem to which is supplied one limiting factor (light, nutrients) where there is adequate availability of other resources; this drives the system towards larger phytoplankton and an accumulation of phytoplankton biomass is usually observed due to inefficient control by copepods.

Mesozooplankton organisms have been recognised as potential grazers of microzooplankton in the oligotrophic open waters (Pierce and Turner, 1992; Calbet and Landry, 1999; Pitta and Giannakourou, 2000). During the present study, it is quite probable that a bottom-up control of the ciliate biomass took place during the first days after the enrichment during which the ciliates quickly increased their populations, taking advantage of the favourable environmental conditions (excess prey [bacteria and heterotrophic nanoflagellates] for the heterotrophic ciliates and P availability for the mixotrophic ciliates) whereas a top-down control of their populations by the mesozooplankton grazers followed and returned the biomass of ciliates to the levels before the enrichment. Besides grazing, the return of the ciliate biomass to pre-addition levels could be related to the dilution of the phosphorus added and the associated ecological signal, since in 9 days the patch spread from 16 to $>400\,km^2$ (Law et al., 2005).

During the IronEx I experiment, heterotrophic biomass, dominated by heterotrophic dinoflagellates and ciliates, increased by ~50% (Martin et al., 1994). During the IronEx II experiment, the biomass of microzooplankton, primarily small ciliates and flagellates, increased in step with the smaller autotrophs (Coale et al., 1996). Experimental dilution incubations (Landry et al., 1995) proved that the initial phytoplankton bloom after the Fe addition was due to the high specific growth rate of the phytoplankton community which was more than double the growth rate in ambient control waters, leaving a large imbalance between growth and grazing processes. The microzooplankton grazing rate increased by more than three times in the patch as chlorophyll reached its peak concentration and controlled the phytoplankton biomass during a later phase. During the IronEx II experiment, in environmental conditions of excess nutrients, a microzooplankton increase followed the phytoplankton bloom and controlled its biomass with a time lag as in a classic predator–prey relationship experiment with biomass oscillations. However, we have no information on the role of mixotrophic ciliates in any of the two IronEx experiments.

In our case of the P addition in the Eastern Mediterranean, i.e. in environmental conditions of general resource scarcity, heterotrophic bacteria took advantage of the supply of the putative limiting factor whereas pico- and nanophytoplankton proved unable to do so in the context of the scarcity of other necessary resources. Microzooplankton increased shortly after the enrichment as a result of a quick transfer of nutrients to higher trophic levels via two potential and complementary pathways: (a) "heterotrophic by-pass" of phytoplankton and transferring nutrients to higher trophic levels through grazing by heterotrophic ciliates on bacteria and HNF and (b) "mixotrophic by-pass" of phytoplankton primary producers and transferring P addition driven primary production to higher trophic levels through mixotrophic ciliates.

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