Biogeosciences Discuss., 7, 8143–8176, 2010
www.biogeosciences-discuss.net/7/8143/2010/
doi:10.5194/bgd-7-8143-2010
© Author(s) 2010. CC Attribution 3.0 License.

Biogeosciences Discussions

This discussion paper is/has been under review for the journal Biogeosciences (BG).
Please refer to the corresponding final paper in BG if available.

N-limited or N and P co-limited indications in the surface waters of three Mediterranean basins

T. Tanaka[1,*], T. F. Thingstad[2], U. Christaki[3], J. Colombet[4], V. Cornet-Barthaux[1], C. Courties[5,6], J.-D. Grattepanche[3], A. Lagaria[3,7], J. Nedoma[8], L. Oriol[9,10], S. Psarra[7], M. Pujo-Pay[9,10], and F. Van Wambeke[11]

[1]Université de la Méditerranée, CNRS, Laboratoire d'Océanographie Physique et Biogéochimique – UMR 6535, OSU/Centre d'Océanologie de Marseille, Campus de Luminy-Case 901, 13288 Marseille cedex 09, France
[2]Department of Biology, University of Bergen, Thormøhlens gate 55, 5006 Bergen, Norway
[3]Université Lille Nord de France, ULCO, LOG, CNRS, UMR 8187, 32 Avenue Foch, 62930 Wimereux, France
[4]Université Blaise Pascal (Clermont-Ferrand II), Laboratoire Microorganismes, Génome et Environnement, UMR CNRS 6023, 63177 Aubière cedex, France
[5]Université Pierre et Marie Curie-Paris 6, UMS 2348, Observatoire Océanologique, 66651 Banyuls sur mer, France
[6]CNRS, UMS 2348, Observatoire Océanologique, 66651 Banyuls sur mer, France

CC BY

[7]Hellenic Centre for Marine Research, Institute of Oceanography, 71003 Heraklion, Crete, Greece
[8]Biology Centre ASCR, v.v.i., Institute of Hydrobiology, 37005 Ceske Budejovice, Czech Republic
[9]Université Pierre et Marie Curie-Paris 6, UMR 7621, Laboratoire d'Océanographie Microbienne, Observatoire Océanologique, 66651 Banyuls sur mer, France
[10]CNRS, UMR 7621, Laboratoire d'Océanographie Microbienne, Observatoire Océanologique, 66651 Banyuls sur mer, France
[11]Université de la Méditerranée, CNRS, Laboratoire de Microbiologie, Géochimie et Ecologie Marines-UMR 6117, OSU/Centre d'Océanologie de Marseille, Campus de Luminy-Case 901, 13288 Marseille, France
[*]now at: CNRS, UMR 7093, Laboratoire Océanologique de Villefranche-sur-mer, 06230 Villefranche-sur-mer, France, and Université Pierre et Marie Curie-Paris 6, Observatoire Océanologique de Villefranche-sur-mer, 06230 Villefranche-sur-mer, France

Received: 26 October 2010 – Accepted: 27 October 2010 – Published: 5 November 2010

Correspondence to: T. Tanaka (tsuneo.tanaka@obs-vlfr.fr)

Published by Copernicus Publications on behalf of the European Geosciences Union.

BGD
7, 8143–8176, 2010

Abstract

The limiting nutrient for the pelagic microbial food web in the Mediterranean Sea was investigated in the nutrient manipulated microcosms during summer 2008. Surface waters were collected into 12 carboys at a center of anticyclonic eddy at the Western Basin, the Ionian Basin, and the Levantine Basin, respectively. As compared to the Redfield ratio, the ratio of N to P in the collected waters was always smaller in the dissolved inorganic fraction but higher in both dissolved and particulate organic fractions. Four different treatments in triplicates (addition of ammonium, phosphate, a combination of both, and the unamended control) were set up for the carboys. Responses of chemical and biological parameters in these different treatments were measured during the incubation (3–4 days). Temporal changes of turnover time of phosphate and ATP, and alkaline phosphatase activity during the incubation suggested that the phytoplankton and heterotrophic prokaryotes (Hprok) communities were not purely P-limited at any studied stations. Statistical comparison between the treatments for a given parameter measured at the end of the incubation did not find pure P-limitation in any chemical and biological parameters at three study sites. Primary production was consistently limited by N, and Hprok growth was not limited by N nor P in the Western Basin, but N-limited in the Ionian Basin, and N and P co-limited in the Levantine Basin. Our results demonstrated the gap between biogeochemical features and biological responses in terms of the limiting nutrient. We question the general notion that Mediterranean surface waters are limited by P alone during the stratified period.

1 Introduction

A large portion of the oceanic pelagic waters is characterized as low nutrient and low chlorophyll (LNLC), i.e. oligotrophic or ultra-oligotrophic. In such waters, the pelagic productivity is generally limited by the availability of inorganic nutrients (e.g. N, P), and the microbial food web plays a significant role in carbon flux through the pelagic

BGD

7, 8143–8176, 2010

N-limited or N and P co-limited indications in the surface waters

T. Tanaka et al.

Discussion Paper | Discussion Paper | Discussion Paper | Discussion Paper |

plankton food web. In order to better understand biogeochemical cycling of C in the ocean, it is important to identify which nutrient is the most limiting factor of the pelagic productivity and how the structure and function of the plankton food web is affected by the availability of the limiting nutrient.

The Mediterranean Sea is a unique oceanic regime in terms of its hydrography and biogeochemistry (reviewed by Krom et al., 2003). Nutrient concentrations, the integrated chlorophyll and primary production in the epipelagic layer, and the POC export from the epipelagic layer, all decrease in the Mediterranean Sea from west to east on the basin scale (Moutin and Raimbault, 2002). The low nutrient status is caused by anti-estuarine circulation in which nutrient-poor surface water flows eastward through the Straits of Gibraltar and Sicily getting progressively more saline to the east, while a counter-current of (relatively) nutrient-rich water flows out of the basin. The deep waters have a nitrate to phosphate ratio of 20–25:1 in the Western Mediterranean Sea (Béthoux et al., 1992; Marty et al., 2002; Pujo-Pay et al., 2010) and 25–30:1 in the Eastern Mediterranean Sea (Krom et al., 1991; Kress and Herut, 2001; Pujo-Pay et al., 2010). In other words, the biogeochemical evidence suggests that the Mediterranean Sea is P-starved compared to other oceanic regions whose ratios of nitrate to phosphate conform to what is known as the Redfield ratio of 16.

Nutrient enrichment studies suggest that, in the lower part of the plankton food web, growth of phytoplankton and heterotrophic prokaryotes (hereafter, Hprok) is generally limited by the availability of phosphate in Mediterranean surface waters during the stratified period (Jacques et al., 1973; Fiala et al., 1976; Zweifel et al., 1993; Vaulot et al., 1996; Thingstad et al., 1998; Zohary and Robarts, 1998; Sala et al., 2002; Van Wambeke et al., 2002; Pinhassi et al., 2006; Tanaka et al., 2009). The specific affinity for phosphate uptake by phytoplankton and Hprok, which is the specific phosphate uptake rate normalized to biomass, is a useful diagnostic tool to measure the extent of phosphate availability (Thingstad and Rassoulzadegan, 1999; Tanaka et al., 2006). In Mediterranean surface waters during the stratified period, specific phosphate affinities by Hprok and pico- and nanophytoplankton, respectively, are close to their respective

BGD
7, 8143–8176, 2010

theoretical maxima which are predicted from the assumption that molecular diffusion to the cell surface is the rate-limiting step (Moutin et al., 2002; Tanaka et al., 2003, 2004; Flaten et al., 2005). These results suggest that the biologically available phosphate pool in Mediterranean surface waters is reduced to a level so low that molecular transport by diffusion towards cells becomes the most limiting step for phosphate uptake.

There are however indications of limiting nutrient other than P. Data on concentration ratios of NO_3+NH_4 to PO_4 and assimilation ratios of organic C to inorganic nitrogen suggest the potential N limitation of phytoplankton production in the NW Mediterranean Sea, although this study was done during the winter overturn (January 1989) in the region of the plume of the Rhone River (Owens et al., 1989). Van Wambeke et al. (2002) report that prokaryotic heterotrophic production (PHP) in surface waters was stimulated by addition of phosphate at 13 sites, but also stimulated by addition of nitrate at 2 sites and by addition of organic carbon at 5 sites of the 18 sites during the stratified period in the Mediterranean Sea. These studies suggest that phytoplankton and Hprok may experience growth limitation by substrates other than P in the Mediterranean Sea.

Phosphate addition to surface waters of the P-starved ultra-oligotrophic Eastern Mediterranean Sea in a Lagrangian experiment caused unexpected ecosystem responses (Thingstad et al., 2005). The added phosphate rapidly disappeared, resulting in an increase of particulate P (i.e., biological P uptake). Moreover, a decline in chlorophyll-*a*, no significant change in biomass of Hprok, but an increase in PHP, ciliate biomass, and copepod egg abundance were observed after a short lag (ca. 2 days). To explain these responses, Thingstad et al. (2005) proposed two not mutually exclusive mechanisms: because of P-limited Hprok and N and P co-limited phytoplankton, (i) the added P was transferred "around" the phytoplankton compartment to copepods (i.e., microbial heterotrophic/mixotrophic pathway), and (ii) the added P was rapidly taken up into P-starved phytoplankton and Hprok, by which the stoichiometry of prey organisms was rapidly changed from P-poor to P-rich (luxury uptake). Results from a microcosm experiment, which was done in parallel with the Lagrangian experiment, support P-limited Hprok and N and P co-limited phytoplankton (Zohary et al., 2005).

It is generally considered that element transfer from lower to higher trophic levels is accompanied by biomass oscillations between prey and predator, and ecological efficiency becomes lower in more oligotrophic system due to increased steps of the trophic level. However, the above counter-intuitive observation suggests that, once the limiting nutrient (P) is added to a P-starved ultra-oligotrophic system, the microbial food webs can immediately contribute to copepod production, thus retrieve part of the primary production which would otherwise be lost through dissipation inside a microbial loop. The study of Thingstad et al. (2005) suggests that it is necessary to investigate the food web level, rather than the osmotroph level (phytoplankton and Hprok) alone, in response to nutrient manipulation for better understanding the structure and function of the plankton food web in P-starved oligotrophic system.

The objective of this study was to examine which nutrient is the most limiting factor for the pelagic microbial food web in the Mediterranean Sea, how the structure of the pelagic microbial food web responds to enrichment of the most limiting nutrient, and if the bypass and tunneling mechanisms for P exist at different basins in the Mediterranean Sea. We performed on-board microcosm experiments that manipulated availability of inorganic N and P in surface offshore waters collected at three Mediterranean basins. A suite of chemical and biological variables was measured during the experiments to determine the effect of the nutrient manipulation.

2 Materials and methods

2.1 Experimental set up and sampling

Microcosm experiments were performed at the three long-duration stations in the Western Basin (Stn A), the Ionian Basin (Stn B), and the Levantine Basin (Stn C) of the Mediterranean Sea (Table 1) during the BOUM (Biogeochemistry from the Oligotrophic to the Ultra-oligotrophic Mediterranean) cruise from 16 June 2008 to 20 July 2008 on the RV *L'Atalante* (Moutin et al., 2010). These stations are located at the center of

BGD
7, 8143–8176, 2010

anticyclonic eddies. Water samples for the experiments were collected at 8 m depths using a multi-sampler/carousel rosette system equipped with Niskin bottles and a CTD, and were poured into polycarbonate carboys (20 l volume, Nalgene). Four different treatments were set up to examine the limiting nutrient for the plankton community. The treatments in triplicate carboys consisted of the unamended treatment, N enrichment, P enrichment, and N and P enrichment (hereafter, Control, +N, +P, and +NP, respectively). +N received 1600 nM-N at Stn A and Stn B and 3200 nM-N at Stn C, +P received 100 nM-P at all stations, and +NP received 1600 nM-N and 100 nM-P at Stn A and Stn B and 3200 nM-N and 100 nM-P at Stn C at the start of the experiment. N and P were added as NH_4Cl and KH_2PO_4, respectively. The carboys were incubated for 3 or 4 days in on-deck flow-through incubators, which were covered by a screen to reduce the incident light by ca. 50%. During the incubation, samples were taken from each of 12 carboys to measure a suite of chemical and biological variables. The carboys used as microcosms were washed with 10% of HCl, and rinsed thoroughly with Milli-Q water and three times with the water samples from the Niskin bottles. To minimize contamination, clean gloves were always used during the experimental set up and sampling.

2.2 Dissolved and particulate nutrients

Concentrations of nitrate+nitrite (NO_{3+2}) and soluble reactive phosphorus (SRP) were measured with an autoanalyzer (Bran+Luebbe) (Tréguer and Le Corre, 1975), and those of ammonium (NH_4) were measured with a fluorimetry (Fluorimeter Jasco FP-2020) (Holmes et al., 1999). SRP does not necessarily represent dissolved inorganic phosphorus or phosphate (e.g., Murphy and Riley, 1962), however we use the term phosphate (PO_4) in this paper. The detection limit and the precision were, respectively, 3 and 2 nM for NH_4, 0.02 and 0.02 µM for NO_{3+2}, and 0.01 and 0.01 µM for PO_4. Concentrations of DOC were measured with a Shimadzu TOC-V analyzer by high temperature catalytic oxidation (Cauwet, 1994, 1999). Samples for dissolved organic nitrogen and phosphorus (DON and DOP, respectively) were oxidized and measured

BGD
7, 8143–8176, 2010
N-limited or N and P co-limited indications in the surface waters
T. Tanaka et al.

spectrophotometrically (Pujo-Pay and Raimbault, 1994; Pujo-Pay et al., 1997). Particulate organic carbon (POC) were collected on pre-combusted glass fiber filters (Whatman GF/F), dried, and measured with a CHN analyzer (Perkin Elmer 2400). Particulate organic nitrogen (PON) and phosphorus (POP) were collected on 25 mm GF/F filters. After oxidization of PON and POP, liberated N and P were measured spectrophotometrically (Pujo-Pay and Raimbault, 1994). See also Pujo-Pay et al. (2010) for details.

2.3 Chlorophyll-*a* concentration

Chlorophyll-*a* was measured fluorometrically, according to Yentsch and Menzel (1963). For each sample, samples (500 ml) were filtered through 0.2 µm polycarbonate filters. Filters were kept frozen in the dark until extraction in 90% acetone solution overnight. Measurements were performed on board with a Shimadzu RF5301 spectrofluorometer.

2.4 Abundance of microbial components

Samples for enumeration of viruses (2 ml) and heterotrophic prokaryotes (Hprok) plus small phytoplankton (3 ml) were respectively fixed with glutaraldehyde (final concentration, 0.5% for the former and 1% for the latter), frozen in liquid nitrogen, and stored at −80 °C until analysis (e.g., Troussellier et al., 1995; Marie et al., 2000; Lebaron et al., 2001; Brussaard, 2004). After quick thawing at room temperature, samples for viruses and Hprok, respectively, were stained with SYBR Green I (Molecular Probes) and analyzed based on right-angle light scatter properties (approximate cell size) and green and red fluorescences using FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with an air-cooled argon laser (488 nm, 15 mW) and a standard filter setup. Thus, the term Hprok used in this paper does not include cyanobacterial cells such as *Synechococcus* and *Prochlorococcus*. Using the same model of the flow cytometer mentioned above, three different groups of small phytoplankton (*Synechococcus,* picoeukaryotes, and autotrophic nanoplankton (ANP)) were discriminated and counted based on right-angle light scatter properties and orange and red fluorescences.

BGD
7, 8143–8176, 2010

To enumerate heterotrophic nanoflagellates (HNF), samples (20–30 ml) were fixed with formaldehyde (final concentration of 1%). Samples were filtered onto black Nuclepore filters (pore size, 0.8 µm) and stained with 4′6-diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980) within 5 h of sampling and stored at −20 °C until counting. HNF were enumerated using an epifluorescence microscope (Leica FW4000) at 1000x. To distinguish between ANP and HNF, autofluorescence (chlorophyll) was determined under blue light excitation. For ciliate enumeration, samples (500 ml) were fixed with acid Lugol's solution (final concentration, 2%) and stored at 4 °C in the dark until analysis. Samples were by gravity pre-concentrated and settled in Utermöhl chamber. Ciliate enumeration was done by an inverted microscope (Nikon Eclipse TE2000-S) at 400x.

2.5 Uptake of $^{33}PO_4$ and ATP

Uptake rate of orthophosphate and ATP was measured using ^{33}P-orthophosphate and adenosine 5′-[γ−^{33}P]triphosphate, respectively (Thingstad et al., 1993). Carrier-free ^{33}P-orthophosphate (PerkinElmer, 370 MBq ml^{-1}) was added to samples at a final concentration of 20–79 pM. Samples for the subtraction of the background and abiotic adsorption were fixed with 100% TCA before isotope addition. Samples were incubated under subdued (laboratory) illumination. The incubation time varied between 15 and 20 min: short enough to assure a linear relationship between the fraction of isotope adsorbed vs. the incubation time but it was long enough to reliably detect isotope uptake above background levels. Incubation was stopped by a cold chase of 100 mM KH_2PO_4 (final conc. 1 mM). Subsamples on Day 0 were filtered in parallel onto 25 mm polycarbonate filters with 2, 0.6, and 0.2 µm pore sizes, and subsamples on Days 1–4 were filtered onto 25 mm polycarbonate filters with 0.2 µm pore size. All filters were placed on a Millipore 12 place manifold with Whatman (GF/C) glass fiber filters saturated with 100 mM KH_2PO_4 as support. After filtration, filters were placed in polyethylene scintillation vials with Ultima Gold (Packard), and radio-assayed. After the radioactivities of the filter were corrected for those of the blank filter obtained from fixed samples, phosphate turnover time ($T_{[PO_4]}$: h) was calculated as $T_{[PO_4]} = -t/\ln(1\text{-}f)$ where f is the fraction (no

dimension) of added isotope collected on the 0.2 µm filter after the incubation time (*t*: h).

Adenosine 5′-[$\gamma-^{33}$P]triphosphate (AT^{33}P: PerkinElmer, specific activity 111 TBq mmol^{-1}) was added to samples at a near-tracer concentration of 5–25 pM and incubated under laboratory temperature and illumination for 1–2 h. Incubations were terminated by filtration through 25 mm polycarbonate filters with 0.2 µm pore size and washed with 2 ml of 0.2 µm filtrate of seawater. Samples for the subtraction of the background and abiotic adsorption were immediately (within 30 s) filtered after ATP-γ^{33}P addition (i.e. blank filter). Filters were placed in scintillation vials with Ultima Gold (Packard) scintillation cocktail, and radio-assayed. After the radioactivities of the filter were corrected for those of the blank filter, ATP turnover time ($T_{[ATP]}$: h) was calculated as mentioned above for $T_{[PO_4]}$. Since the initial value of ATP turnover time at Stn B was accidentally lost, a datum obtained from the surface water (5 m), which was chronologically closest to the start of the microcosm experiment, was used in this study.

The specific affinity for phosphate uptake was calculated by normalizing phosphate uptake rates (inverse of phosphate turnover times) to the summed P-biomass of phytoplankton and Hprok (Tanaka et al., 2006). P-biomass of phytoplankton was estimated from Chl-*a* concentration with an assumption of C:Chl-*a* ratio of 50 and C:P ratio of 106, that of Hprok was estimated from abundance of Hprok with an assumption of cell carbon content of 15 fg cell^{-1} (Caron et al., 1995) and C:P ratio of 50 (Fagerbakke et al., 1996).

2.6 Alkaline phosphatase activity (APA)

APA was measured fluorometrically using 3-0-methylfluorescein-phosphate as substrate (Perry, 1972). Fluorescence in the samples mixed with the substrate (final concentration, 0.1 µM) was measured immediately after the addition of the substrate solution and at three or four subsequent times according to the fluorescence increase. After correcting fluorescence values of samples to those of blank, APA (nM-P h^{-1}) was

BGD
7, 8143–8176, 2010

calculated by using a linear regression of fluorescence values versus incubation time.

2.7 Primary production and prokaryotic heterotrophic activity

Primary production (PP) and prokaryotic heterotrophic activity (PHA) were measured as a measure of growth rate of phytoplankton and Hprok, respectively. PP was measured by the ^{14}C incorporation method of Steemann Nielsen (1952). Three light and one dark 170 ml polycarbonate bottles were filled up with sample water from each microcosm. Each bottle was inoculated with 20 µCi of $NaH^{14}CO_3$, and all bottles were incubated in the on-deck incubator for 4 hours around midday. After the incubation, samples were filtered through 0.2 µm polycarbonate filters under low vacuum pressure (<200 mmHg). Filters were put in scintillation vials, fumed with 1 ml of 1% HCl in order to remove excess ^{14}C-bicarbonate over night, and radioassayed with scintillation cocktail. PP (nM-C h^{-1}) was calculated by subtracting the radioactivity in dark bottles from that in light bottles under the assumption that DIC is 2×10^6 nM-C and a correction factor for the lower uptake of ^{14}C as compared to ^{12}C is 1.05 (See Lagaria et al, 2010 for details).

PHA (pM-leucine h^{-1}) was measured as ^3H-leucine incorporation rate into TCA-insoluble fraction by the centrifuge method (Smith and Azam, 1992). For each sample, duplicate aliquots (1.5 ml) and one TCA-killed control were incubated with 22 nM of leucine (a mixture of 8 nM of ^3H-leucine and 14 nM of cold leucine) for 2 h at in situ temperature in the dark. The incorporation was stopped with the addition of TCA (final concentration, 5%). Samples were centrifuged, aspired, and washed 3 times (see Van Wambeke et al., 2010 for details).

2.8 Statistical analysis

Student t-test was used to compare values of parameters between the start and the end of the incubation. One-way analysis of variance (ANOVA) was used to test the effect of different nutrient treatments on a given parameter on the last day of the incubation at

each station. For comparison of each parameter between different nutrient treatments, statistical analysis was done by post hoc Tukey Honestly significant difference (HSD) test after ANOVA test. Before statistical comparison, data were $\log_{10}$ transformed to meet homogeneity of variance. When dataset included zero, log transformation was done using the equation: $\log_{10}$ (x+1) (x: data). All statistical analyses were done using the *R* software (http://www.r-project.org/).

3 Results

3.1 Initial status of the sample waters

Concentrations of NO_{3+2} and PO_4 were initially close to or below the detection limit of the conventional method at all stations (Table 1). NH_4 concentrations were always above the detection limit of the nanomolar analytical technique and in a range of 15–34 nM. Ratios of DIN to PO_4 ranged from 1.8 to 12.2, although these ratios include certain elements of uncertainty (see Discussion). Concentrations of DOC, DON, and DOP were 66–73, 4.5–8.4, and 0.04–0.06 µM, respectively. Ratios of DOC : DON : DOP were 1012–2102 : 111–217 : 1. Concentrations of POC, PON, and POP were 2.6–3.1, 0.29–0.32, and 0.01 µM, respectively. Ratios of POC : PON : POP were 202–307 : 21–30 : 1. That is, mean ratios of DON : DOP were 114–190, and those of PON : POP ratios were 23-28 at the three study sites (Table 1).

PO_4 turnover time was in a range of 4.1–6 h. Uptake of $^{33}PO_4$ was always dominated by the 0.6–2 µm fraction, and its dominance increased from west to east (65% at Stn A to 87% at Stn C, data not shown). ATP turnover time was one to two orders of magnitude longer than PO_4 turnover time. Specific PO_4 affinity ranged from 0.017 to 0.029 l nmol-P^{-1} h^{-1}. APA was always at measurable level in the collected waters. Chl-*a* concentrations and Hprok abundances were initially 0.03–0.06 µg l^{-1} and 1.8×10^5–3.4×10^5 cells ml^{-1}, respectively.

3.2 Temporal variations of dissolved inorganic N and P, turnover times of PO_4 and ATP, and APA

NH_4 concentrations in +N and +NP at all stations significantly decreased between the start and the end of the incubation (t-test, $P < 0.05$) (Fig. 1). PO_4 concentrations significantly decreased during the incubation in +N, +P, and +NP at Stn A (t-test, $P < 0.05$). In contrast, significant increase of PO_4 concentration was detected in the Control, +N, and +P at Stn B, and in +N and +P at Stn C (t-test, $P < 0.05$). No significant change of NO_{3+2} concentration was detected between the start and the end of the incubation in 8 out of 12 cases (t-test, $P > 0.05$, data not shown).

PO_4 turnover time decreased to 1–2 h in the Control and +N at Stn A and Stn C and in +N at Stn B during the incubation (Fig. 2). In the Control at Stn B, turnover time oscillated between 1.7 and 6.5 h. The shortest turnover time at the end of the incubation was found in +N at Stn A, in +N and +NP at Stn B, and in the Control and +N at Stn C (Tukey HSD test, $P < 0.05$). PO_4 addition resulted in increase of turnover time in both +P and +NP during the first 1–2 days. The extent of the increase of turnover time after the PO_4 addition was smallest at Stn A. From Day 1 or 2 to the end of the incubation, PO_4 turnover time in +P and +NP decreased. At the end of the incubation, turnover time was longest in +P and +NP at Stn A and in +P at Stn B and Stn C (Tukey HSD test, $P < 0.05$). ATP turnover times in all nutrient treatments tended to change little or decrease during the incubation. The effect of the PO_4 addition alone resulted in at most 1.6 time increase of turnover time of ATP, whereas we observed up to a 10 times increase of PO_4 turnover time. ATP turnover time in +N was shortest on Day 2 at Stn A and on Days 2 and 4 at Stn B and Stn C (Tukey HSD test, $P < 0.05$).

APAs in +N continuously increased but those in the other treatments varied little at all stations. APAs were significantly higher in +N than all the other treatments on Days 1–3 at Stn A, on Days 1–2 at Stn B, and on Days 3–4 at Stn C (Tukey HSD test, $P < 0.05$). No statistical difference between the treatments was detected on Days 3–4 at Stn B because of increased variations between the triplicates in +N.

BGD
7, 8143–8176, 2010

3.3 Responses of organic C, N, and P, and microbial components

Effects of nutrient addition on a given parameter were tested by comparing parameter values between the treatments at the end of the incubation. Concentrations of POC were highest in +NP at all three stations, and significantly higher in +N than the Control at Stn B (Fig. 3, Tukey HSD test, $P < 0.05$). Only a significant effect of nutrient addition on DOC concentration was detected as smaller concentration in +NP than the Control at Stn C. PON concentrations were significantly higher in +N than the Control and +P and highest in +NP at all stations (Tukey HSD test, $P < 0.05$). No significant difference of DON concentration was detected between the Control and the nutrient addition treatments at all stations. POP concentrations were highest in +NP at all stations (Tukey HSD test, $P < 0.05$). At Stn B and Stn C, POP concentrations in +P were significantly higher compared to those in the Control but not to those in +N (Tukey HSD test, $P < 0.05$). Nutrient addition did not affect DOP concentration at all stations.

Chl-*a* concentrations were higher in +N than the Control and +P, and highest in +NP at Stn A and Stn B, and higher in +NP at Stn C (Fig. 4, Tukey HSD test, $P < 0.05$). PP was significantly higher in +N than the Control and +P, and highest in +NP at all stations (Tukey HSD test, $P < 0.05$). No significant difference between the treatments was detected for Hprok abundance and PHA at Stn A (Fig. 4). At Stn B, Hprok abundances were significantly smaller in +N and +NP than the Control and +P, while PHA was significantly higher in +N than the Control and +P, and highest in +NP (Tukey HSD test, $P < 0.05$). At Stn C, Hprok abundances were significantly smaller in +N than the other treatments, and PHA was significantly higher in +NP than the other treatments (Tukey HSD test, $P < 0.05$).

Abundances of *Synechococccus* were significantly higher in +N than the Control and +P, and highest in +NP at Stn A and Stn B (Fig. 5, Tukey HSD test, $P < 0.05$). At Stn C, *Synechococcus* abundance was significantly higher in +NP than the others. No significant difference of picoeukaryotes abundance between the treatments was detected at all stations. Significant effect of nutrient addition on ANP abundance was

BGD
7, 8143–8176, 2010

detected at Stn B and Stn C. ANP abundance was higher in +N than the Control and +P, and highest in +NP at Stn B (Tukey HSD test, $P < 0.05$). At Stn C, ANP abundance was significantly higher in +NP than the others. No significant difference of viral particles between the treatments was detected at all stations (Fig. 6). Significant difference of HNF abundance between the treatments was detected at only two cases: higher in +N than the Control at Stn A, and higher in +NP than the Control at Stn B (Tukey HSD test, $P < 0.05$). Ciliate abundances were significantly higher in +NP than the others at Stn A and Stn C (Tukey HSD test, $P < 0.05$), while no significant effect of nutrient addition was detected at Stn B.

4 Discussion

Our study is the first on-board nutrient-manipulated microcosm study that examined major biogenic elements (C, N, P), the extent of availability of PO_4 and labile DOP, abundances of major functional groups (viruses to ciliates) in the microbial food web, and growth of phytoplankton and Hprok in response to different nutrient additions at three Mediterranean Basins. Our results are the first that demonstrate that the lower part of the pelagic plankton food web did not experience any pure P-limitation during the stratified period at three Mediterranean Basins.

The waters used in this study initially showed smaller ratios of DIN to PO_4 (1.8–12.2) but higher ratios of DON to DOP (111–217) and of PON to POP (21–30) compared to the Redfield ratio at all three stations (Table 1). Chl-*a* concentrations, PP, Hprok abundance, and PHA in the waters used in this study were within the previously reported range in offshore surface waters of the Mediterranean Sea during the stratified period (see the compiled data by Siokou-Frangou et al., 2010). This suggests that the studied waters were initially oligotrophic or ultra-oligotrophic with a mixed signal of N or P starved feature. However, these DIN to PO_4 ratios include certain elements of uncertainty. Although concentrations of NH_4 were low but well above the detection limit of the nanomolar analytical technique, concentrations of NO_{3+2} and PO_4 were close to or

BGD

7, 8143–8176, 2010

N-limited or N and P co-limited indications in the surface waters

T. Tanaka et al.

below the detection limit of the conventional technique used in this study. The molybdenum blue reaction method measures not only PO_4 but also has a potential background such as from acid labile DOP and arsenate (reviewed by Karl and Björkman, 2002). On the other hand, Marty et al. (2002) report that the NO_3 to PO_4 ratio in surface waters was highly variable in a range of 1-60 at the DYFAMED time-series station (an offshore site in the NW Mediterranean Sea), when only the data on concentration of NO_3 and PO_4 that exceeds 0.1 µM (i.e., twice their analytical detection limit) were analyzed for the period of 1991–1999. The NO_3 to PO_4 ratios in Mediterranean surface waters which are lower than the Redfield ratio is a contrast with the NO_3 to PO_4 ratios in Mediterranean intermediate and deep waters (22–28: Krom et al., 1991; Béthoux et al., 1992; Kress and Herut, 2001; Pujo-Pay et al., 2010). This may suggest that surface oligotrophic waters with low DIN to PO_4 ratios occur on limited spatial and temporal scales during the stratified period in the Mediterranean Sea.

The P-starved status was supported by the indication from specific PO_4 affinity which is a measure of P availability for osmotrophs (cf. Thingstad and Rassoulzadegan, 1999). Tanaka et al. (2006) have proposed that a specific PO_4 affinity > 0.02 l nmol-P^{-1} h^{-1} indicates P limitation, i.e., the growth rate of the existing osmotrophs (here, phytoplankton and Hprok community) is reduced because of the reduced P availability. According to this, the phytoplankton and Hprok community was initially P-limited at all studied stations, and the extent of P limitation tended to increase from west to east (Table 1). The P biomass estimate, which was used to determine specific PO_4 affinity, includes elements of uncertainty (see Materials and methods). If the specific PO_4 affinity was recalculated by assuming C:Chl-*a* ratio of 100 (e.g., Malone et al., 1993), and C:P ratio of 250 for phytoplankton, which is similar to POC:POP ratios in the collected waters (Table 1), and C:P ratio of 150 for Hprok, which was found in P-limited conditions (e.g., Vrede et al., 2002), we found ca. 2 times increase of specific affinity (i.e., an indication of enhanced P limitation) at all stations. This suggests that the phytoplankton and Hprok community was initially P-limited at all three stations, despite of the uncertainty in our estimate of specific PO_4 affinity. However, note that

BGD
7, 8143–8176, 2010

specific PO_4 affinity for the phytoplankton and Hprok "community" can diagnose only the aspect of phosphate availability. In other words, the extent of P availability based on specific PO_4 affinity values does not necessarily exclude the possibility that growth of osmotroph community was co-limited by P and other substrate nor the situation that some osmotroph groups were limited by substrate(s) other than P in our study.

Our study demonstrated that no chemical and biological parameters measured during the microcosm experiments indicated pure P-limitation at any station. If the osmotroph community were purely limited by P, turnover times of PO_4 and ATP would have been similar between the Control and +N, and APA would not have been enhanced by NH_4 addition (Fig. 2). Responses of PO_4 turnover time and APA were similar between the Control and the NH_4 addition treatments, when surface waters of the eastern Mediterranean Sea were investigated in May 2002 (Flaten et al., 2005; Thingstad and Mantoura, 2005). At that time, Hprok growth was P-limited and PP was N and P co-limited (Thingstad et al., 2005; Zohary et al., 2005). Our results suggest that the waters initially contained a certain amount of PO_4 pool that became available for osmotrophs only after NH_4 addition, i.e. potential N-limitation at the three stations. Hence, an NH_4 addition to the waters collected in this study enhanced the P requirement by the osmotroph community, by which turnover times of PO_4 and ATP decreased and APA increased in +N. Whenever significant difference between the different nutrient treatments was statistically detected in a given parameter, the indication was almost always N-limitation or N and P co-limitation (Figs. 3–6).

We found that PP was consistently limited by N, while the limiting nutrient for Hprok growth was variable between the study sites: no nutrient limitation in the Western Basin, N-limitation in the Ionian Basin, and N and P co-limitation in the Levantine Basin (Fig. 4). However, Ridame et al. (personal communication, 2010) found that P addition in their experiment significantly enhanced PP at 48 h at Stn B and Hprok growth at 24 h at Stn A during 48 h incubation on the same cruise. While both our study and Ridame et al. study collected waters at the same depth at the same sites, the experimental design and treatment were different. Hence, these differences in response of phytoplankton

and Hprok between two studies remained to be explained. Ternon et al. (2010) found significant increases of PP in both the aerosol and the Saharan dust analog addition treatments compared to the Control at Stn A, B, and C in the same cruise. Since both analogs include N and P as well as other elements (e.g. Fe, Al), the limiting element was not evident in their study.

Responses to the nutrient addition were also different between the stock parameters and among the study sites in our study (Figs. 3–6). An NH_4 addition resulted in an increase of PON, Chl-*a*, *Synechococcus*, and HNF at Stn A, while at Stn B, an NH_4 addition resulted in an increase of POC, PON, Chl-*a*, *Synechococcus*, and ANP but a decrease of Hprok abundance. At Stn C, an NH_4 addition alone resulted in an increase of PON and POP, but a decrease of Hprok abundance. A combined addition of NH_4 and PO_4 resulted in an increase of POC, PON, POP, Chl-*a*, *Synechococcus*, ANP, and ciliates, but no change of Hprok abundance. Interestingly, despite N-limitation or N and P co-limitation of Hprok growth, an NH_4 addition resulted in significantly smaller abundance of Hprok compared to the Control (Fig. 4). Hprok were dominated by a low DNA containing subpopulation at the start of the experiment, and the significantly smaller abundances of Hprok in +N and +NP compared to the Control were mostly because of little change or decrease of this subpopulation during the incubation (data not shown). Abundances of viruses, HNF, and ciliates were similar between the Control and +N at Stn B and Stn C (Fig. 6), while ANP abundance was significantly higher in +N than the Control at Stn B (Fig. 5). This may suggest a tight trophic coupling in the heterotrophic compartments and more benefit for ANP (more growth and/or less grazing) over a short time.

The limiting nutrient in our sample waters was not the same as in the water studied during the CYCLOPS Lagrangian P-addition experiment in the eastern Mediterranean Sea (see Introduction, Thingstad et al., 2005). Hence it was impossible to test in this study if the bypass and tunneling mechanisms for P exist in different sites of the Mediterranean Sea. On the other hand, the responses at the trophic level of osmotrophs in +N at Stn C (an increase of PP, no change of Chl-*a* and PHA, but a

BGD
7, 8143–8176, 2010

decrease of Hprok abundance) seem a mirror image of the results in the CYCLOPS experiment (an increase of PHA, no change of Hprok abundance and PP, but a decrease of Chl-*a*; Psarra et al., 2005). However, a rapid transfer of a limiting nutrient (N) to higher trophic level such as ciliates was not evident at Stn C. An existence of the bypass and tunneling mechanisms for N under N limited condition needs to be tested in future study.

In the eastern Mediterranean Sea, high amount of NO_3 and PO_4 with N:P ratios of 22–28 is supplied to the epipelagic layer during the winter overturn, and annual phytoplankton bloom (November-March) results in an exhaustion of PO_4 and a NO_3 residual in the epipelagic layer (Krom et al., 1991, 2003, 2010). In the same area, summer conditions are firmly established with a strong thermocline and a well-developed deep chlorophyll maximum by May (Krom et al., 2005). That is, LNLC conditions have developed in the euphotic zone above the chlorophyll maximum with concentrations of NH_4 (30–80 nM), NO_{3+2} (<1–10 nM), and PO_4 (<2-4 nM) which were only detectable by nanomolar methods (Krom et al., 2005). Note again that Hprok were not limited by N likely because of the presence of measurable NH_4 and of bioavailable DON, while phytoplankton were N and P co-limited in surface waters in May 2002 in the same area (Thingstad et al., 2005; Zohary et al., 2005). The nutrient-manipulated bioassay experiments done in July and September 1999 along the longitudinal transect in the Mediterranean Sea indicated that Hprok growth is generally limited by P in surface waters (Van Wambeke et al., 2002). Our study done in June/July indicated that PP was consistently limited by N, while Hprok growth was no nutrient limited in the Western Basin, N-limited in the Ionian Basin, and N and P co-limited in the Levantine Basin (Fig. 4). Specific PO_4 affinities indicated P-limitation of the phytoplankton and Hprok community in the collected waters (Table 1). An explanation for these results would be that, as inorganic N and P were getting very low in concentration along the stratified period, the surface ecosystem became or close to N and P co-limitation, in which growth of ostmotroph groups was limited by different nutrients in a same system, and the most limiting nutrient for osmotrophs' growth could shift seasonally or sporadically

BGD

7, 8143–8176, 2010

N-limited or N and P co-limited indications in the surface waters

T. Tanaka et al.

among N, P and N+P.

We have presented that, whereas the C:N:P ratio of particulate organic matter consistently indicated P-starved status compared to the Redfield ratio, whenever nutrient limitation was detected, phytoplankton and Hprok experienced N-limitation or N and P co-limitation but never indicated pure P-limitation in surface waters in the center of anticyclonic eddy at the three Mediterranean Basins. These results demonstrated the gap between biogeochemical features and biological responses in terms of the limiting nutrient, and we question the general notion that Mediterranean surface waters are limited by P alone. Our results require mechanism or condition to create the following two situations: (1) the skewed PON:POP ratio but the microbial food web being N-limitation or N and P co-limitation, and (2) N and P co-limited Hpok but N-limited phytoplankton in the same water. Such situations may be a result of either turnover time of organic N longer than that of organic P (cf. Thingstad and Rassoulzadegan, 1995), or N:P ratio in phytoplankton community much higher than that in Hprok community (i.e. higher P requirement per cell volume by Hprok) (reviewed by Sterner and Elser, 2002), or both, in surface waters. On the other hand, surface waters in N-limitation or N and P co-limitation seem to create a niche for N_2 fixers that have recently been found in the Mediterranean Sea (Sandroni et al., 2007; Bar Zeev et al., 2008; Bonnet et al, 2010). Mediterranean surface waters during the stratified period become LNLC that is a dominant mode in the world oceans. Our results are likely relevant to better understand and predict the biogeochemical cycling of carbon in the Mediterranean pelagic system. Future research needs to investigate how the most limiting element for the pelagic plankton food web varies with time (spring phytoplankton bloom, development of the stratification, and decline of the stratification) and space (different depth layers in the euphotic zone, anticyclonic and cyclonic eddies, coast-offshore gradients, different basins in the Mediterranean Sea).

BGD
7, 8143–8176, 2010

Acknowledgements. This is a contribution to the BOUM (Biogeochemistry from the Oligotrophic to the Ultraoligotrophic Mediterranean) project financed by the French national LEFE-CYBER program and the European commission SESAME IP. The BOUM project was coordinated by the Institut des Sciences de l'Univers (INSU) and managed by the Centre National de la Recherche Scientifique (CNRS). The authors thank the crew of the R/V *L'Atalante* for outstanding shipboard operation, chief scientists T. Moutin, F. Van Wambeke, and L. Prieur for the cruise organization, C. Marec and L. Prieur for CTD and rosette operation, and D. Lefèvre, M. Le Moal, A. Nowaczyk, and K. Popendorf for microcosm setup and sampling.

The publication of this article is financed by CNRS-INSU.

References

Bar Zeev, E., Yogev, T., Man-Aharonovich, D., Kress, N., Herut, B., Beja, O., and Berman-Frank, I.: Seasonal dynamics of the endosymbiotic, nitrogen-fixing cyanobacterium Richelia intracellularis in the eastern Mediterranean Sea, The ISME Journal, 2, 911–923, 2008.

Béthoux, J. P., Morin, P., Madec, C., and Gentili, B.: Phosphorus and nitrogen behaviour in the Mediterranean Sea, Deep-Sea Res., 39, 1641–1654, 1992.

Bonnet, S., Grosso, O., and Moutin, T.: Nitrogen fixation in the Mediterranean Sea: a major biogeochemical process during the stratified period? Biogeosciences Discussion, in prep., 2010.

Brussaard, C. P. D.: Optimisation of procedures for counting viruses by flow cytometry, Appl. Environ. Microbiol., 70, 1506–1513, 2004.

Caron, D. A., Dam, H. G., Kremer, P., Lessard, E. J., Madin, L. P., Malone, T. C., Napp, J. M., Peele, E. R., Roman, M. R., and Youngbluth, M. J.: The contribution of microorganisms

to particulate carbon and nitrogen in surface waters of the Sargasso Sea near Bermuda, Deep-Sea Res. Pt I, 42, 943–972, 1995.

Cauwet, G: HTCO method for dissolved organic carbon analysis in sea water: influence of catalyst on blank estimation, Mar. Chem., 47, 55–64, 1994.

Cauwet, G.: Determination of dissolved organic carbon (DOC) and nitrogen (DON) by high temperature combustion, edited by: Grashoff, K., Kremling, K., and Ehrhard, M., Weinheim, New York, USA, 407–420, 1999.

Copin-Montégut, C. and Copin-Montégut, G.: Stoichiometry of carbon, nitrogen, and phosphorus in marine particulate matter, Deep-Sea Res., 30, 31–46, 1983.

Fagerbakke, K. M., Heldal, M., and Norland, S.: Content of carbon, nitrogen, oxygen, sulfur and phosphorus in native aquatic and cultured bacteria, Aquat. Microb. Ecol., 10, 15–27, 1996.

Fiala, M., Cahet, G., Jacques, G., Neveux, J., and Panouse, M.: Fertilisation de communautés phytoplanctonique. I. Cas d'un milieu oligotrophe: Mediterranée nord-occidentale, J. Exp. Mar. Biol. Ecol., 24, 151–163, 1976.

Flaten, G. A. F., Skjoldal, E. F., Krom, M. D., Law, C. S., Mantoura, R. F. C., Pitta, P., Psarra, S., Tanaka, T., Tselepides, A, Woodward, E. M. S., Zohary, T., and Thingstad, T. F.: Studies of the microbial P-cycle during a Lagrangian phosphate-addition experiment in the Eastern Mediterranean, Deep-Sea Res. Pt II, 52, 2928–2943, 2005.

Holmes, M. R., Aminot, A., Kérouel, R., Hooker, B. A., and Peterson, B. J.: A simple and precise method for measuring ammonium in marine and freshwater ecosystems, Can. J. Fish. Aquat. Sci., 56, 1801–1808, 1999.

Jacques, G., Minas, H. J., Minas, M., and Nival, P.: Influence des conditions hivernales sur les productions phyto- et zooplanctoniques en Mediterranee Nord-occidentale. II. Biomasse et production phytoplanctonique, Mar. Biol., 23, 251–265, 1973.

Karl, D. M. and Björkman, K. M.: Dynamics of DOP, edited by: Hansell, D. A. and Carlson, C. A., Elsevier Science, 249–366, 2002.

Kress, N. and Herut, B.: Spatial and seasonal evolution of dissolved oxygen and nutrients in the Southern Levantine Basin (Eastern Mediterranean Sea): chemical characterization of the water masses and inferences on the N : P ratios, Deep-Sea Res. Pt. I, 48, 2347–2372, 2001.

Krom, M. D., Emeis K.-C., and van-Cappellan, P.: Why is the Eastern Mediterranean P limited? Prog. Oceanogr., 85, 236–244, 2010.

Krom, M. D., Groom, S., and Zohary, T.: The eastern Mediterranean, in: Biogeochemistry of

BGD
7, 8143–8176, 2010

Marine Systems, edited by: Black, K. D. and Shimmield, G. B., Blackwell Publishing, Oxford, 91–126, 2003.
Krom, M. D., Kress, N., Brenner, S., and Gordon, L. I.: Phosphorus limitation of primary productivity in the eastern Mediterranean-Sea, Limnol. Oceanogr., 36, 424–432, 1991.
Krom, M. D., Woodward, E. M. S., Herut, B., Kress, N., Carbo, P., Mantoura, R. F. C., Spyres, G., Thingstad, T. F., Wassmann, P., Wexels-Riser, C., Kitidis, V., Law, C. S., and Zodiatis, G.: Nutrient cycling in the south east Levantine basin of the eastern Mediterranean: Results from a phosphorus starved system, Deep-Sea Res. Pt. II, 52, 2879–2896, 2005.
Lagaria, A., Psarra, S., Lefèvre, D., Van Wambeke, F., Courties, C., Pujo-Pay, M., Oriol, L., Tanaka, T., and Christaki, C.: Particulate and dissolved primary production and metabolic shifts upon nutrient additions in microcosm experiments related to trophic conditions in three Mediterranean basins, Biogeosciences Discussion, in prep., 2010.
Lebaron, P., Servais, P., Agogué, H., Courties, C., and Joux, F.: Does the high nucleic-acid content of individual bacterial cells allow to discriminate active cells in aquatic systems?, Appl. Environ. Microbiol., 67, 1775–1782, 2001.
Marie, D., Partensky, F., Simon, N., Guillou, L., and Vaulot, D.: Flow cytometry analysis of marine picoplankton, edited by: Diamond, R. A., and DeMaggio, S., Springer Verlag, 421–454, 2000.
Marty, J. C.: The DYFAMED time-series program (French-JGOFS), Deep-Sea Res. Pt II, 49, 1963–1964, 2002.
Moutin, T., Van Wambeke, F., and Prieur, L.: Introduction to the biogeochemistry from the oligotrophic to the ultraoligotrophic Mediterranean experiment: the BOUM program, Biogeosciences Discussion, in prep., 2010.
Moutin, T. and Raimbault, P.: Primary production, carbon export and nutrients availability in western and eastern Mediterranean Sea in early summer 1996 (MINOS cruise), J. Mar. Sys., (33–34), 273–288, 2002.
Moutin, T., Thingstad. T. F., Van Wambeke, F., Marie, D., Slawyk, G., Raimbault, P., and Claustre, H.: Does competition for nanomolar phosphate supply explain the predominance of the cyanobacteirum Synechococcus? Limnol. Oceanogr., 47, 1562–1567, 2002.
Murphy, J. and Riley, J. P.: A modified single solution method for the determination of phosphate in natural waters, Anal. Chim. Acta, 27, 31–36, 1962.
Owens, N. J. P., Rees, A. P., Woodward, E. M. S., and Mantoura, R. F. C.: Size-fractionated

primary production and nitrogen assimilation in the North-western Mediterranean Sea during January 1989, edited by: Martin, J. M. and Barth, H., Paris, 126–134, 1989.
Perry, M. J.: Alkaline phosphatase activity in subtropical central north Pacific waters using a sensitive fluorometric method, Mar. Biol., 15, 113–119, 1972.
5 Pinhassi, J., Gómez-Consarnau, L., Alonso-Sáez, L., Sala, M. M., Vidal, M., Pedros-Alió, C., and Gasol, J. M.: Seasonal changes in bacterioplankton nutrient limitation and their effects on bacterial community composition in the NW Mediterranean Sea, Aquat. Microb. Ecol., 44, 241–252, 2006.
Porter, K. G. and Feig, Y. S.: The use of DAPI for identifying and counting aquatic microflora,
10 Limnol. Oceanogr., 25, 943–948, 1980.
Psarra, S., Tselepides, A., and Ignatiades, L.: Primary productivity in the oligotrophic Cretan Sea (NE Mediterranean): seasonal and interannual variability, Prog. Oceanogr., 46, 187–204, 2000.
Psarra, S., Zohary, T., Krom, M. D., Mantoura, R. F. C., Polychronaki, T., Stambler, N., Tanaka,
15 T., Tselepides, A., and Thingstad, T. F.: Phytoplankton response to a Lagrangian phosphate addition in the Levantine Sea (Eastern Mediterranean), Deep-Sea Res. Pt. II, 52, 2944–2960, 2005.
Pujo-Pay, M., Conan, P., and Raimbault, P.: Particulate and dissolved organic nitrogen and phosphorus in the north western Mediterranean Sea (EROS 2000 – Discovery cruise 1993),
20 edited by: Martin, J. M. and Barth, H., 79–86, 1995.
Pujo-Pay, M., Conan, P., and Raimbault, P.: Excretion of dissolved organic nitrogen by phytoplankton assessed by wet oxidation and N-15 tracer procedures, Mar. Ecol. Prog. Ser., 153, 99–111, 1997.
Pujo-Pay, M. and Raimbault, P.: Improvment of the wet-oxydation procedure for simultaneous
25 determination of particulate organic nitrogen and phosphorus collected on filters, Mar. Ecol. Prog. Ser., 105, 203–207, 1994.
Pujo-Pay, M., Conan, P., Oriol, L., Cornet-Barthaux, V., Falco, C., Ghiglione, J.-F., Goyet, C., Moutin, T., and Prieur, L.: Integrated survey of elemental stoichiometry (C, N, P) from the Western to Eastern Mediterranean Sea, Biogeosciences Discuss., 7, 7315–7358,
30 doi:10.5194/bgd-7-7315-2010, 2010.
Sala, M. M., Peters, F., Gasol, J. M., Pedrós-Alió, C., Marrasé, C., and Vaqué, D.: Seasonal and spatial variations in the nutrient limitation of bacterioplankton growth in the northewestern Mediterranean, Aquat. Microb. Ecol., 27, 47–56, 2002.

Sandroni, V., Raimbault, P., Migon, C., Garcia, N., and Gouze, E.: Dry atmospheric deposition and diazotrophy as sources of new nitrogen to northwestern Mediterranean oligotrophic surface waters, Deep-Sea Res. Pt. I, 54, 1859–1870, 2007.

Siokou-Frangou, I., Christaki, U., Mazzocchi, M. G., Montresor, M., Ribera d'Alcalá, M., Vaqué, D., and Zingone, A.: Plankton in the open Mediterranean Sea: a review, Biogeosciences, 7, 1543–1586, doi:10.5194/bg-7-1543-2010, 2010.

Smith, D. C. and Azam, F.: A simple, economical method for measuring bacterial protein synthesis rates in seawater using ^{3}H-leucine, Marine Microbial Food Webs, 6, 107–114, 1992.

Steemann Nielsen, E.: The use of radio-active carbon (^{14}C) for measuring organic production in the sea, Journal du Conseil – Conseil International pour l'Exploration de la Mer, 18, 117–140, 1952.

Tanaka, T., Henriksen, P., Lignell. R., Olli, K., Seppälä, J., Tamminen, T., and Thingstad, T. F.: Specific affinity for phosphate uptake and specific alkaline phosphatase activity as diagnostic tools for detecting P-limited phytoplankton and bacteria, Estuar. Coast., 29, 1226–1241, 2006.

Tanaka, T., Rassoulzadegan, F., and Thingstad, T. F.: Measurements of phosphate affinity constants and phosphorus release rates from the microbial food web in Villefranche Bay, northwestern Mediterranean, Limnol. Oceanogr., 48, 1150–1160, 2003.

Tanaka, T., Rassoulzadegan, F., and Thingstad, T. F.: Orthophosphate uptake by heterotrophic bacteria, cyanobacteria, and autotrophic nanoflagellates in Villefranche Bay, northwestern Mediterranean: Vertical, seasonal, and short-term variations of the competitive relationship for phosphorus, Limnol. Oceanogr., 49, 1063–1072, 2004.

Tanaka, T., Thingstad, T. F., Gasol, J. M., Cardelús, C., Jezbera, J., Sala, M. M., Simek, K., and Unrein, F.: Determining the availability of phosphate and glucose for bacteria in P-limited mesocosms of northwestern Mediterranean surface waters, Aquat. Microb. Ecol., 56, 81–91, 2009.

Ternon, E., Guieu, C., Ridame, C., L'Helguen, S., and Catala, P.: Longitudinal variability of the biogeochemical role of Mediterranean aerosols in the Mediterranean Sea, Biogeosciences Discussion, in prep., 2010.

Thingstad, T. F., Krom, M. D., Mantoura, R. F. C., Flaten, G. A. F., Groom, S., Herut, B., Kress, N., Law, C. S., Pasternak, A., Pitta, P., Psarra, S., Rassoulzadegan, F., Tanaka, T., Tselepides, A., Wassmann, P., Woodward, E. M. S., Wexels Riser, C., Zodiatis, G., and Zohary, T.: Nature of Phosphorus Limitation in the Ultraoligotrophic Eastern Mediterranean, 309,

1068–1071, 2005.
Thingstad, T. F. and Mantoura, R. F. C.: Titrating excess nitrogen content of phosphorous-deficient eastern Mediterranean surface water using alkaline phosphatase activity as a bio-indicator, Limnol. Oceanogr., 3, 94–100, 2005.
Thingstad, T. F. and Rassoulzadegan, F.: Conceptual models for the biogeochemical role of the photic zone microbial food web, with particular reference to the Mediterranean Sea, Prog. Oceanogr., 44, 271–286, 1999.
Thingstad, T. F., Skjoldal, E. F., and Bohne, R. A.: Phosphorus cycling and algal-bacterial competition in Sandsfjord, western Norway, Mar. Ecol. Prog. Ser., 99, 239–259, 1993.
Thingstad, T. F., Zweifel. U. L., and Rassoulzadegan, F.: P limitation of heterotrophic bacteria and phytoplankton in the northwest Mediterranean, Limnol. Oceanogr., 43, 88–94. 1998.
Tréguer, P. and Le Corre, P.: Manuel d'analyses des sels nutritifs dans l'eau de mer, edited by: Chimique, L. d. O., Université de Bretagne Occidentale, Brest, France, 1975.
Troussellier, M., Courties, C., and Zettelmaier, S.: Flow cytometric analysis of coastal lagoon bacterioplankton and picophytoplankton: Fixation and storage effects, Estuarine, Coastal and Shelf Science, 40, 621–633, 1995.
Van Wambeke, F., Christaki, U., Giannakourou, A., Moutin, T., and Souvemerzoglou, K.: Longitudinal and vertical trends of bacterial limitation by phosphorus and carbon in the Mediterranean Sea, Microb. Ecol., 43, 119–133, 2002.
Van Wambeke, F., Catala, P., and Lebaron, P.: Relationships between cytometric characteristics of high and low nucleic-acid bacterioplankton cells, bacterial produciton and environmental parameters along a longitudinal gradient across the Mediterranean Sea in early summer, Biogeosciences Discusion, in prep., 2010.
Vaulot, D., LeBot, N., Marie, D., and Fukai, E.: Effects of phosphorus on the *Synechococcus* cell cycle in surface Mediterranean waters during summer, Appl. Environ. Microbiol., 62, 2527–2533, 1996.
Yacobi, Y. Z., Zohary, T., Kress, N., Hecht, A., Robarts, R. D., Waiser, M., Wood, A. M., and Li, L. W. L.: Chlorophyll distribution throughout the southeastern Mediterranean in relation to the physical structure of the water mass, J. Marine Syst., 6, 179–190, 1995.
Yentsch, C. S. and Menzel, D. W.: A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence, Deep-Sea Res., 10, 221–231, 1963.
Zohary, T., Herut, B., Krom, M. D., Mantoura, R. F. C., Pitta, P., Psarra, S., Rassoulzadegan, F., Stambler, N., Tanaka, T., Thingstad, T. F., and Woodward, E. M. S.: P-limited bacteria but

BGD
7, 8143–8176, 2010

N-limited or N and P co-limited indications in the surface waters

T. Tanaka et al.

N and P co-limited phytoplankton in the Eastern Mediterranean—a microcosm experiment, Deep-Sea Res. Pt. II, 52, 3011–3023, 2005.

Zohary, T. and Robarts, R. D.: Experimental study of microbial P limitation in the eastern Mediterranean, Limnol Oceanogr., 43, 38–395, 1998.

5 Zweifel, U. L., Norrman, B., and Hagström, A.: Consumption of dissolved organic carbon by marine bacteria and demand for inorganic nutrients, Mar. Ecol. Prog. Ser., 101, 23–32, 1993.

Table 1. Experimental period and location, water temperature, and initial status of chemical and biological parameters before different nutrient treatments.

Study site	Stn A	Stn B	Stn C
Experimental period in 2008	14–17 July	4–8 July	27 June–1 July
Location of water sample	39°5.96 N, 5°21.00 E	34°8.20 N, 18°26.70 E	33°37.50 N, 32°39.20 E
Parameter [a]			
Water temperature (°C)	24.5	25.1	25.2
NH_4 (nM)	34 ± 11	49 ± 22	15 ± 5
NO_{3+2} (nM)	< 20	37 ± 21	40 ± 20
PO_4 (nM)	< 10	< 10	30 ± 2
DOC (µM)	66 ± 0.6	67 ± 1	73 ± 0.2
DON (µM)	4.5 ± 0.3	6.3 ± 0.1	8.4 ± 1.6
DOP (µM)	0.04 ± 0.00	0.06 ± 0.00	0.05 ± 0.02
POC (µM)	3.1 ± 0.1	2.6 ± 0.3	2.9 ± 0.2
PON (µM)	0.29 ± 0.03	0.29 ± 0.01	0.32 ± 0.05
POP (µM)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
DIN:PO_4[b]	6.5:1	12.2:1	1.8:1
DOC:DON:DOP	1876:130:1	1215:114:1	1712:190:1
POC:PON:POP	245:23:1	251:28:1	227:24:1
PO_4 turnover time (h)	5.8 ± 0.8	4.1 ± 0.3	6.0 ± 0.8
ATP turnover time (h)	32.5 ± 12.0	110 [c]	22.9 ± 12.2
APA (nM-P h^{-1})	1.0 ± 0.2	1.9 ± 0.1	1.2 ± 0.0
Specific PO_4 affinity (l nmol-P^{-1} h^{-1})	0.017 ± 0.004	0.024 ± 0.003	0.029 ± 0.004
chlorophyll-*a* (µg l^{-1})	0.06 ± 0.00	0.05 ± 0.00	0.03 ± 0.00
Primary production (nM-C h^{-1})	24.3 ± 1.8	18.9 ± 1.2	7.3 ± 0.0
Bacteria ($\times 10^5$ cells ml^{-1})	3.3 ± 0.3	3.4 ± 0.4	1.8 ± 0.1
Bacterial activity (pM-leucine h^{-1})	12.5 ± 0.1	16.7 ± 1.7	7.7 ± 2.0
Synechococcus (cells ml^{-1})	3465 ± 1402	8552 ± 198	1381 ± 5
Picoeukaryotes (cells ml^{-1})	67 ± 55	373 ± 24	180 ± 20
Autotrophic nanoplankton (cells ml^{-1})	63 ± 53	238 ± 48	280 ± 52
Viruses ($\times 10^5$ ml^{-1})	21 ± 0.6	2.4 ± 0.1	9.5 ± 0.5
Heterotrophic nanoflagellates (cells ml^{-1})	2736	1755	918
Ciliates (cells ml^{-1})	151 ± 38	216 ± 49	122 ± 29

[a] Values are shown as mean ± SD ($n = 3$) except for water temperature, nutrient stoichiometry, and heterotrophic nanoflagellates.
[b] DIN denotes dissolved inorganic nitrogen and defined as the sum of NH_4, NO_2, and NO_3.
[c] The value was a datum obtained from the surface water (5 m) which was chronologically closest to the start of the microcosm experiment.

BGD
7, 8143–8176, 2010
N-limited or N and P co-limited indications in the surface waters
T. Tanaka et al.

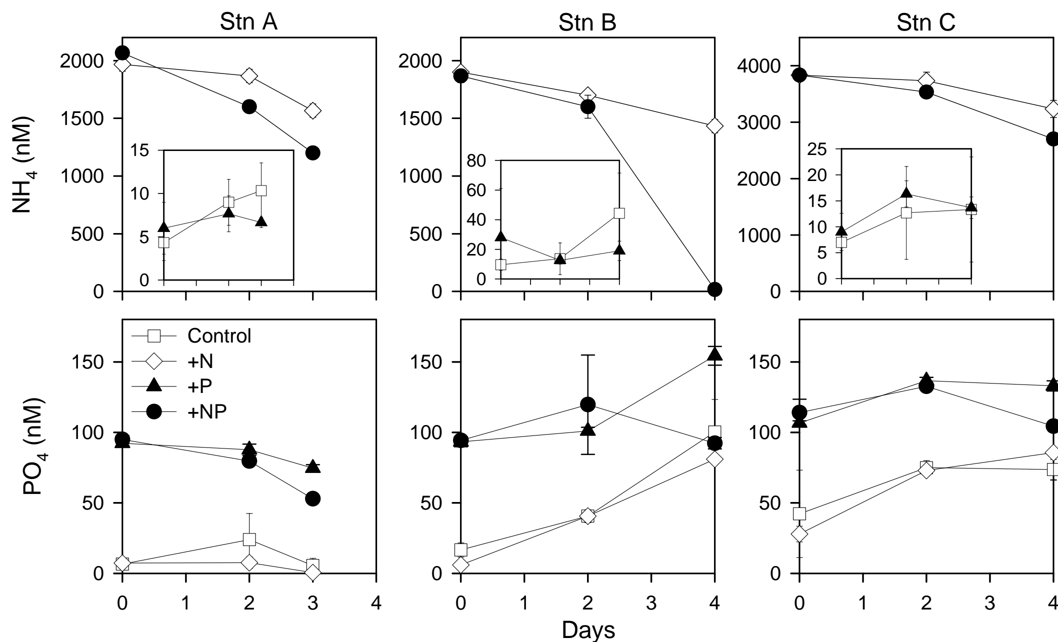

Fig. 1. Temporal changes in concentration of NH_4 and PO_4 (nM) in different treatments (mean ± SD). Open square, open diamond, closed triangle, and closed circle denote Control, +N, +P, and +NP, respectively. Inlets show NH_4 concentrations (nM) in Control and +P.

BGD

7, 8143–8176, 2010

N-limited or N and P co-limited indications in the surface waters

T. Tanaka et al.

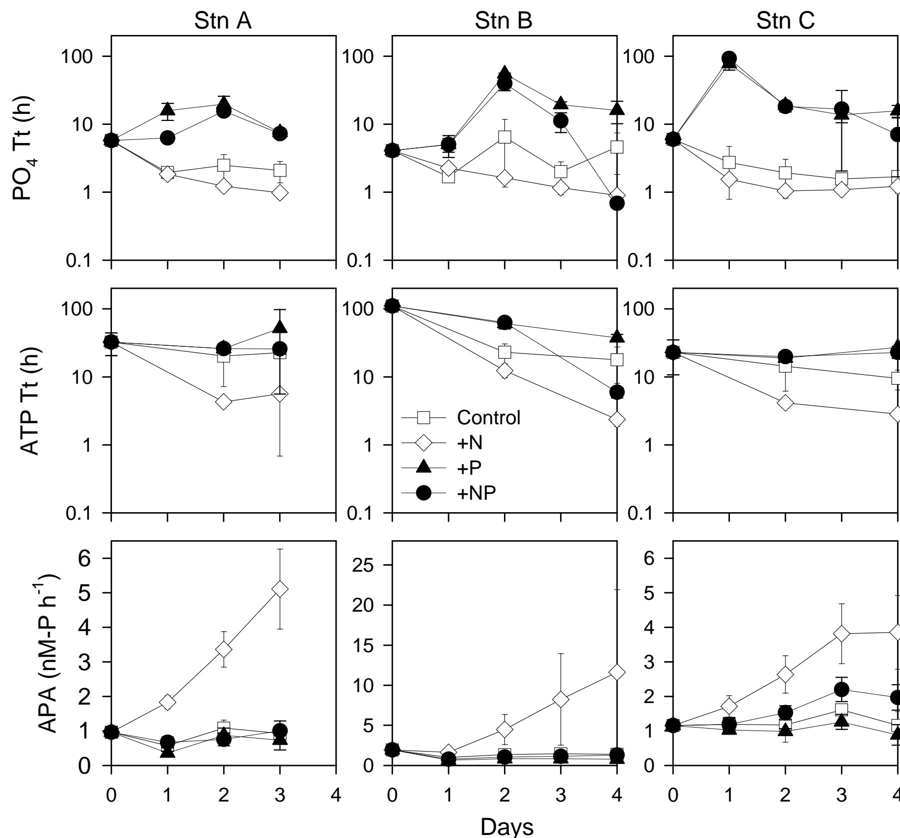

Fig. 2. Temporal changes in turnover time (h) of PO_4 and ATP, and alkaline phosphatase activity (APA: nM-P h^{-1}) in different treatments (mean ± SD). Open square ,open diamond, closed triangle, and closed circle denote Control, +N, +P, and +NP, respectively. The value of ATP turnover on Day 0 at Stn B was a datum obtained from the surface water (5 m) which was chronologically closest to the start of the microcosm experiment.

BGD
7, 8143–8176, 2010

CC BY

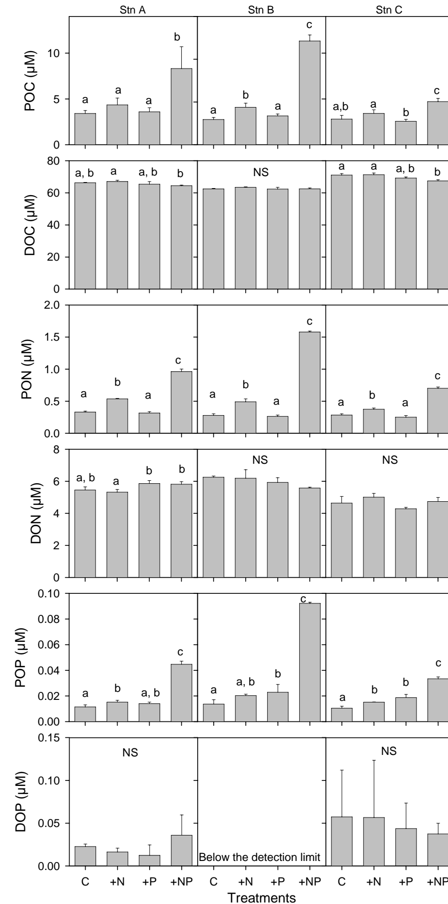

Fig. 3. Comparison of responses of concentration (µM) of POC, DOC, PON, DON, POP, and DOP between the different treatments at the last day of the experiment. Values are shown as mean ± SD. C, +N, +P, and +NP denote Control, NH_4 addition, PO_4 addition, NH_4+PO_4 addition, respectively. Columns labeled by different letters (a, b or c) are significantly different at $P < 0.05$. NS denotes no significant effect of different nutrient treatments on the parameter measured. See Materials and methods for details.

BGD
7, 8143–8176, 2010

N-limited or N and P co-limited indications in the surface waters

T. Tanaka et al.

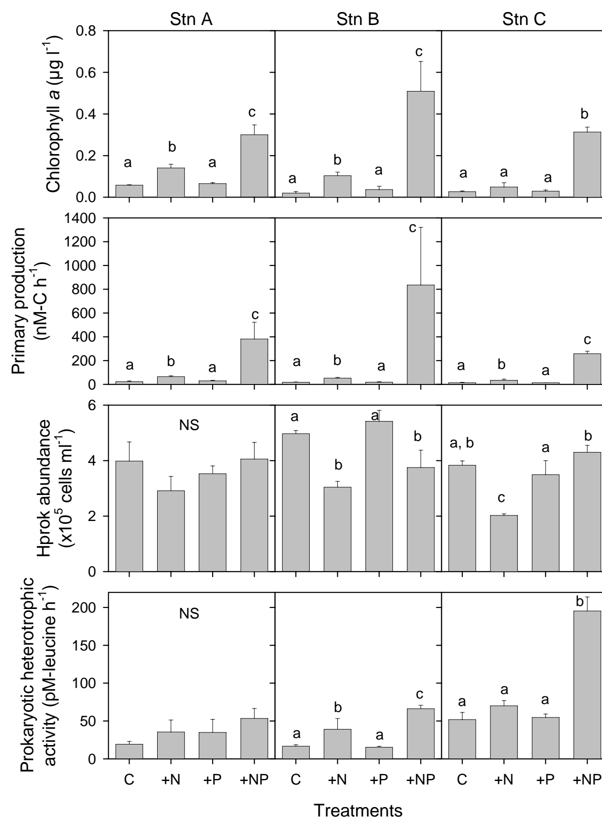

Fig. 4. Comparison of responses of chlorophyll *a* (µg l^{-1}), primary production (nM-C h^{-1}), abundance of heterotrophic prokaryotes (Hprok) (cells ml^{-1}), and prokaryotic heterotrophic activity (pM-leucine h^{-1}) between the different treatments at the last day of the experiment. Values are shown as mean ± SD. C, +N, +P, and +NP denote Control, NH_4 addition, PO_4 addition, NH_4+PO_4 addition, respectively. Columns labeled by different letters (a, b or c) are significantly different at $P < 0.05$. NS denotes no significant effect of different nutrient treatments on the parameter measured. See Materials and methods for details.

BGD

7, 8143–8176, 2010

N-limited or N and P co-limited indications in the surface waters

T. Tanaka et al.

CC BY

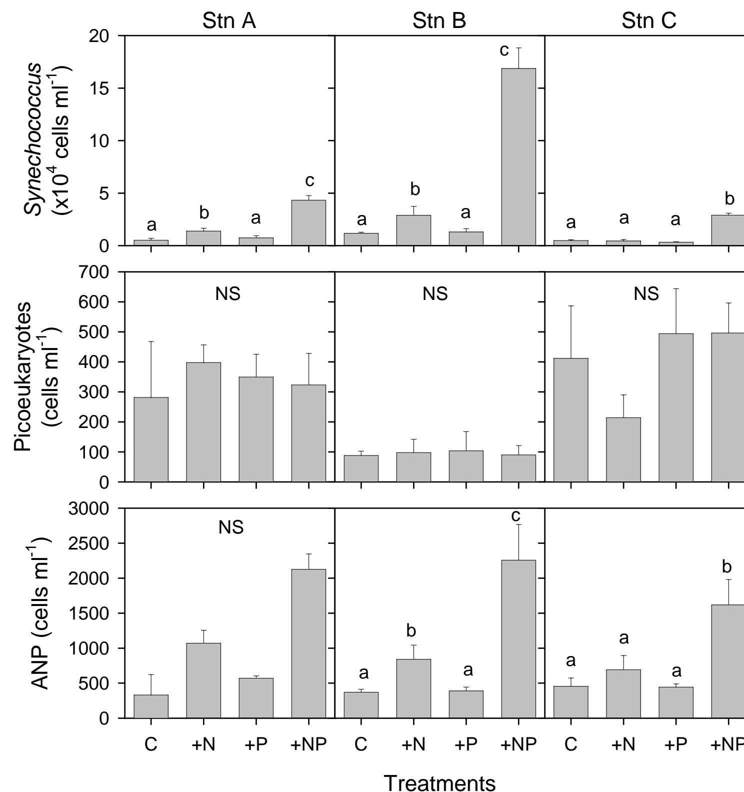

Fig. 5. Comparison of responses of abundance (cells ml^{-1}) of *Synechococcus*, picoeukaryotes, and autotrophic nanoplankton (ANP) between the different treatments at the last day of the experiment. Values are shown as mean ± SD. C, +N, +P, and +NP denote Control, NH_4 addition, PO_4 addition, NH_4+PO_4 addition, respectively. Columns labeled by different letters (a, b or c) are significantly different at $P < 0.05$. NS denotes no significant effect of different nutrient treatments on the parameter measured. See Materials and methods for details.

BGD
7, 8143–8176, 2010

CC BY

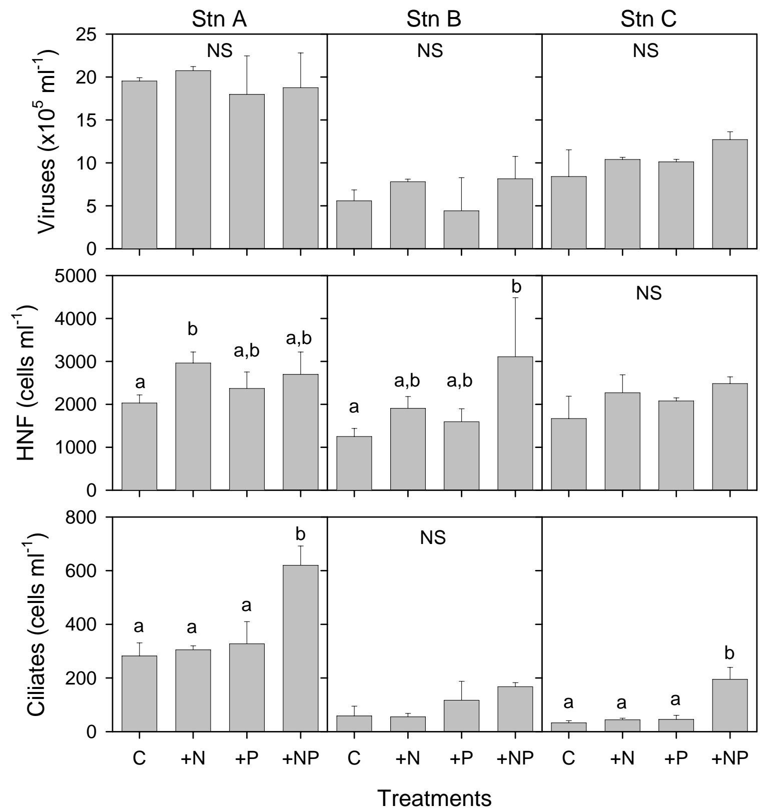

Fig. 6. Comparison of responses of abundance (particles or cells ml^{-1}) of viruses, heterotrophic nanoflagellates (HNF), and ciliates between the different treatments at the last day of the experiment except for viruses (data from Day 2). Values are shown as mean ± SD. C, +N, +P, and +NP denote Control, NH_4 addition, PO_4 addition, NH_4+PO_4 addition, respectively. Columns labeled by different letters (a, b or c) are significantly different at $P < 0.05$. NS denotes no significant effect of different nutrient treatments on the parameter measured. See Materials and methods for details.

BGD
7, 8143–8176, 2010

N-limited or N and P co-limited indications in the surface waters

T. Tanaka et al.

CC BY