

Lack-of P-limitation of phytoplankton and heterotrophic prokaryotes in surface waters of three anticyclonic eddies in the stratified Mediterranean Sea

Tsuneo Tanaka, T. F. Thingstad, Urania Christaki, J. Colombet, Veronique Cornet, Claude Courties, J.D. Grattepanche, Anna Lagaria, Jiri Nedoma, Louise Oriol, et al.

▶ **To cite this version:**

Tsuneo Tanaka, T. F. Thingstad, Urania Christaki, J. Colombet, Veronique Cornet, et al.. Lack-of P-limitation of phytoplankton and heterotrophic prokaryotes in surface waters of three anticyclonic eddies in the stratified Mediterranean Sea. Biogeosciences, 2011, 8, pp.525-538. 10.5194/bg-8-525-2011 . hal-00573163

HAL Id: hal-00573163
https://hal.science/hal-00573163

Submitted on 18 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NoDerivatives 4.0 International License

Biogeosciences, 8, 525–538, 2011
www.biogeosciences.net/8/525/2011/
doi:10.5194/bg-8-525-2011
© Author(s) 2011. CC Attribution 3.0 License.

Biogeosciences

Lack of P-limitation of phytoplankton and heterotrophic prokaryotes in surface waters of three anticyclonic eddies in the stratified Mediterranean Sea

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
[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

Received: 26 October 2010 – Published in Biogeosciences Discuss.: 5 November 2010
Revised: 14 February 2011 – Accepted: 17 February 2011 – Published: 28 February 2011

Abstract. We investigated the identity of the limiting nutrient of the pelagic microbial food web in the Mediterranean Sea using nutrient manipulated microcosms during summer 2008. Experiments were carried out with surface waters at the center of anticyclonic eddies in the Western Basin, the Ionian Basin, and the Levantine Basin. In situ, the ratio of N to P was always higher in both dissolved and particulate organic fractions compared to the Redfield ratio, suggesting a relative P-starvation. In each experiment, four different treatments in triplicates (addition of ammonium, phosphate, a combination of both, and the unamended control) were employed and chemical and biological parameters monitored throughout a 3–4 day incubation. 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 P-limited at the sites. Furthermore, statistical comparison among treatments at the end of the incubation did not support a hypothesis of P-limitation at the three study sites. In contrast, 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 (an apparent P-starved status) and biological responses (no apparent P-limitation). We question the general notion that Mediterranean surface waters are limited by P alone during the stratified period.

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

1 Introduction

A large portion of oceanic pelagic waters are characterized as "low nutrient and low chlorophyll" (LNLC), i.e. oligotrophic. In such waters, pelagic productivity is generally limited by the availability of inorganic nutrients (e.g. nitrogen (N), phosphorus (P)), and the microbial food web plays

a significant role in carbon flux through the pelagic plankton food web (reviewed by Karl, 2007). In order to better understand the biogeochemical cycling of carbon (C) in the ocean, it is important to identify which nutrient is the most limiting factor of 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 marine system in terms of its hydrography and biogeochemistry (reviewed by Krom et al., 2003). Nutrient concentrations, integrated chlorophyll and primary production in the epipelagic layer, and the export of particulate organic carbon (POC) 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 nutrient-rich water flows out of the basin. The waters below the epipelagic layer have a nitrate (NO_3) to phosphate (PO_4) 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). The biogeochemical parameter of the N to P ratio suggests that the Mediterranean Sea is P-starved compared to other oceanic regions whose ratios of NO_3 to PO_4 conform to what is known as the Redfield ratio of 16.

Nutrient enrichment studies have shown that growth of phytoplankton and heterotrophic prokaryotes (hereafter, Hprok) is often limited by the availability of PO_4 in Mediterranean surface waters during the stratified period (Jacques et al., 1973; Fiala et al., 1976; Zweifel et al., 1993; Dolan et al., 1995; 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 PO_4 uptake by phytoplankton and Hprok, which is the specific PO_4 uptake rate normalized to biomass, is a useful diagnostic tool to measure the extent of PO_4 availability (Thingstad and Rassoulzadegan, 1999; Tanaka et al., 2006). In Mediterranean surface waters during the stratified period, specific PO_4 affinities are close to their respective 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 would then indicate that the biologically available PO_4 concentrations are so low that molecular transport by diffusion towards cells is the rate-limiting process for PO_4 uptake in Mediterranean surface waters.

However, there are indications of the limiting nutrient being other than PO_4. Data on concentration ratios of $NO_3 + NH_4$ to PO_4 and assimilation ratios of organic C to inorganic N suggested a 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) reported the results of Mediterranean transect study in which prokaryotic heterotrophic production (PHP) was stimulated by addition of PO_4 at 13 sites but also stimulated by addition of NO_3 at 2 sites and by addition of organic C at 5 sites of the 18 sites. These studies suggest that phytoplankton and Hprok may experience growth limitation by substrates other than P in the Mediterranean Sea.

PO_4 addition to surface waters of the P-starved ultra-oligotrophic Eastern Mediterranean Sea in a Lagrangian experiment resulted in unexpected ecosystem responses (Thingstad et al., 2005): the added PO_4 rapidly disappeared, resulting in an increase of particulate P (i.e. biological P uptake). 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 PO_4 was transferred "around" the phytoplankton compartment to copepods (bypass mechanism), and (ii) the added PO_4 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 (i.e. luxury uptake) (tunneling mechanisms). 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 numbers of the trophic levels. 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) showed that it is necessary to investigate the multiple levels of the food web in response to nutrient manipulation, rather than the osmotroph level (phytoplankton and Hprok) alone, to understand the structure and function of the plankton food web in P-starved oligotrophic systems.

The objectives of this study were to identify which nutrient is the most limiting for the pelagic microbial food web in the Mediterranean Sea, and determine how the structure of the pelagic microbial food web responds to enrichment of the most limiting nutrient. 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 R/V *L'Atalante* (Moutin et al., 2011). These stations are located at the center of 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. The sampling depth located at the lower part of the surface mixed layer (13.5 m at Stn A, 8.5 m at Stn B, 11.5 m at Stn C; Moutin et al., 2011). The collected waters were poured into polycarbonate carboys (20 l, Nalgene). The four treatments in triplicate microcosms 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 level of nutrient amendments were chosen with the aim of satisfying the requirement of either N or P or both by the natural Hprok and phytoplankton community during the experimental period (i.e. 3–4 days). The N:P ratios (16 and 32) were used as a rough approximation of those below the epipelagic layer of the Western and the Eastern Basins, respectively (see Introduction). Since previous studies have shown that Hprok and small phytoplankton (pico- and nanophytoplankton) use ammonium (NH_4) rather than NO_3 (e.g., Lipschultz, 1995; Wheeler and Kirchman, 1986), we used NH_4 as an inorganic N source in this study.

The microcosms were then 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 microcosms to measure a suite of chemical and biological variables (see below for the sampling frequency of each parameter). 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

Samples for inorganic nutrients were collected on Day 0, Day 2, and at the end of the experiment. 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 NH_4 were measured with a fluorimeter (Fluorimeter Jasco FP-2020) (Holmes et al., 1999). Concentrations of dissolved inorganic nitrogen (DIN) were calculated by summing concentrations of NO_{3+2} and NH_4. 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.

Samples for organic nutrients were collected at the start and the end of the experiment. 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 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* (Chl-*a*) concentration

Samples for Chl-*a* were collected on Day 0, Day 2, and at the end of the experiment. Chl-*a* was measured fluorometrically, according to Yentsch and Menzel (1963). For each sample, 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 microbial populations except ciliates were collected on Day 0, Day 2, and at the end of the experiment. Ciliate samples were collected at the start and the end of the experiment. Samples for enumeration of viruses (2 ml) and heterotrophic prokaryotes (Hprok) plus small phytoplankton (3 ml) were fixed, respectively, with glutaraldehyde and formaldehyde (final conc. 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 were stained with SYBR Green I (Molecular Probes) and analyzed based on right-angle light scatter properties (approximate cell size) and green and red fluorescence using FACScan flow cytometer (BD Biosciences, San Jose, CA, USA)

Table 1. Initial condition at the three sampling sites. Parameter values are shown as mean ± SD ($n = 3$) except for water temperature and heterotrophic nanoflagellates ($n = 1$).

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			
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[a,b]	–	–	1.8:1
DOC/DON/DOP[b]	1876:130:1	1215:114:1	1712:190:1
POC/PON/POP[b]	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
Heterotrophic prokaryotes ($\times 10^5$ cells ml^{-1})	3.3 ± 0.3	3.4 ± 0.4	1.8 ± 0.1
Prokaryotic heterotrophic 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] DIN denotes dissolved inorganic nitrogen and presented as the sum of NH_4, NO_2, and NO_3.
[b] Only the mean value is shown for nutrient stoichiomotry for the simplicity.
[c] The value was a datum obtained from the surface mixed water (5 m) which was chronologically closest to the start of the microcosm experiment.

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 fluorescence.

To enumerate heterotrophic nanoflagellates (HNF), samples (20–30 ml) were fixed with formaldehyde (final conc. 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 1000×. 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 conc. 2%) and stored at 4 °C in the dark until analysis. Ciliates were pre-concentrated by gravity in the sample bottles, and then settled in Utermöhl chamber. Ciliate enumeration was done by an inverted microscope (Nikon Eclipse TE2000-S) at 400×.

2.5 Turnover times of PO_4 and adenosine 5′-triphosphate (ATP)

Samples for turnover times of PO_4 and ATP were collected every day and measured using ^{33}P-orthophosphate and adenosine 5′-[γ-^{33}P]triphosphate (AT^{33}P), 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% trichloroacetic acid (TCA) (final conc. 0.5%) before isotope addition. Samples were incubated under subdued (laboratory) illumination. The incubation time varied between 15 and 20 minutes: 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. The radioactivity from blanks accounted for on average 10.7% ($n = 141$) of that from live samples. 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 $T_{[PO_4]} = -t/\ln(1-f)$ where f is the fraction (no dimension) of added isotope collected on the 0.2 µM filter after the incubation time (t:h).

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 AT^{33}P addition (i.e. blank filter). The radioactivity from blanks accounted for on average 8.5% ($n = 78$) of that from live samples. 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 sample for the initial value of ATP turnover time at Stn B was 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 specific 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). The POP data present a sum of living particles and detritus so that we estimated P-biomass based on Chl-*a* and cell concentration. The estimated P-biomass was 45–98% ($n = 7$) of POP except for two occasions (105 and 115% of POP).

2.6 Alkaline phosphatase activity (APA)

Samples for APA were collected every day. APA was measured fluorometrically using 3-*O*-methylfluorescein-phosphate as substrate (Perry, 1972). Fluorescence in the samples mixed with the substrate (final conc. 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 calculated by using a linear regression of fluorescence values versus incubation time.

2.7 Primary production (PP) and prokaryotic heterotrophic activity (PHA)

Samples for PP and PHA were collected on Day 0, Day 2, and at the end of the experiment. PP and 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 Na$H^{14}CO_3$, and all bottles were incubated in the on-deck incubator for 4 h around midday. After the incubation, samples were filtered through 0.2 µm polycarbonate filters under low vacuum pressure (< 200µmHg). 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 ^{3}H-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 ^{3}H-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 conc. 5%). Bovine serum album was added to each sample (final conc. 100 mg l^{-1}) prior to

the first centrifugation. After aspirating the supernatant, 5% TCA was mixed with pellet, and then the sample was centrifuged again. Supernatant was discarded, and a last centrifuge treatment was done after addition of 80% ethanol. After removing the supernatant, and addition of scintillation cocktail, the sample was radioassayed (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 the requirement of homogeneity of variance. When the dataset included zero values, data were log transformed using the equation: $\log_{10}(x+1)$ (x: data). Virus samples collected at the end of the experiment at Stn A and Stn B were lost; consequently, the statistical test for viral particles employed data from Day 2 for all three stations. All statistical analyses were done using the R software (http://www.r-project.org/).

The following four "responses" to nutrient additions were defined and used to interpret results of the statistical comparison of parameter values on the last day of the incubation:

1. No limitation: No statistical difference in concentration or rate of a given parameter detected among the four treatments.

2. N-limitation: concentration or rate of a given parameter is statistically higher in +N than the Control.

3. P-limitation: concentration or rate of a given parameter is statistically higher in +P than the Control.

4. N and P co-limitation: Concentration or rate of a given parameter is statistically higher in +NP than the others (i.e. the Control, +N, and +P). Moreover, no statistical difference in concentration or rate of a given parameter is detected between the Control, +N, and +P.

3 Results

3.1 Initial characteristics of the study site waters

Concentrations of NO_{3+2} and PO_4 were initially close to, or below, the detection limit of conventional analytical methods 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. Ranges of 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. Ranges of 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. The mean ratios of DON/DOP were in a range of 114–190, and those of PON/POP ratios were in a range of 23–28 at the three study sites (Table 1).

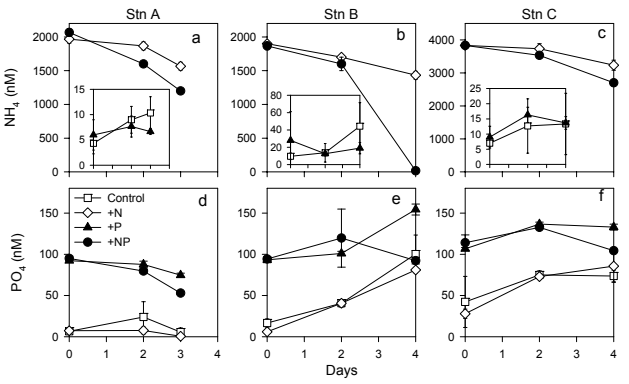


Fig. 1. Temporal changes in concentration (nM) of NH_4 (**a, b, c**) and PO_4 (**d, e, f**) in different treatments (mean ± SD). Open square, open diamond, closed triangle, and closed circle denote Control, +N, +P, and +NP, respectively. Inlets show NH_4 concentrations (nM) in Control and +P.

PO_4 turnover time ranged from 4.1 h to 6 h. Uptake of $^{33}PO_4$ was always dominated by the 0.6–2 µM fraction, and the dominance of this size-fraction 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 levels in situ. 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 decreased significantly from the start to the end of the experiments at all three stations (Fig. 1a, b, c; t-test, $P < 0.05$). These decreases corresponded to 16–99% of the initial NH_4 concentration. Significant decreases of PO_4 concentrations in +P and +NP (19% and 44%, respectively, of the initial concentration) were observed at Stn A during the incubation (Fig. 1d; t-test, $P < 0.05$). In contrast, a significant increase of PO_4 concentration (1.2–6 times) was detected in the Control, +N, and +P at Stn B, and in +N and +P at Stn C (Fig. 1e, f; t-test, $P < 0.05$). Significant changes in NO_{3+2} concentration were detected between the start and the end of the incubation in 4 out of 12 occasions (t-test, $P < 0.05$, data not shown): increase in the Control, +N, and +NP at Stn A (60–70 nM

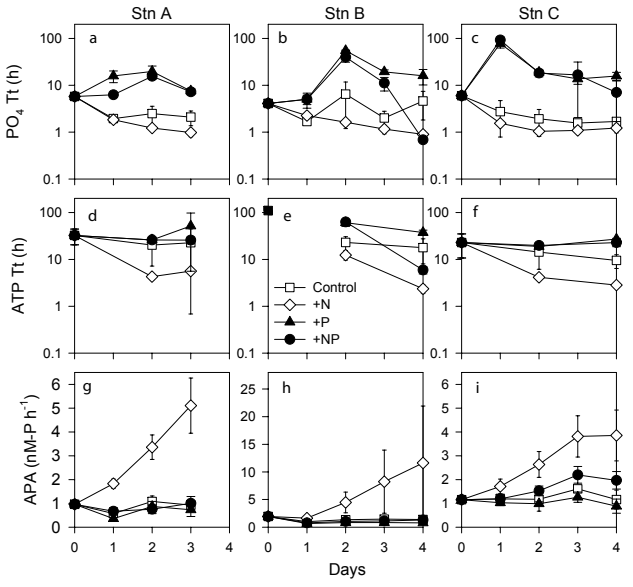


Fig. 2. Temporal changes in turnover time (h) of PO_4 (**a, b, c**) and ATP (**d, e, f**), and alkaline phosphatase activity (APA: nM-P h^{-1}) (**g, h, i**) in different treatments (mean ± SD). Open square, open diamond, closed triangle, and closed circle denote Control, +N, +P, and +NP, respectively. Closed square symbol denotes a datum obtained from the surface mixed water (5 m) which was chronologically closest to the start of the microcosm experiment.

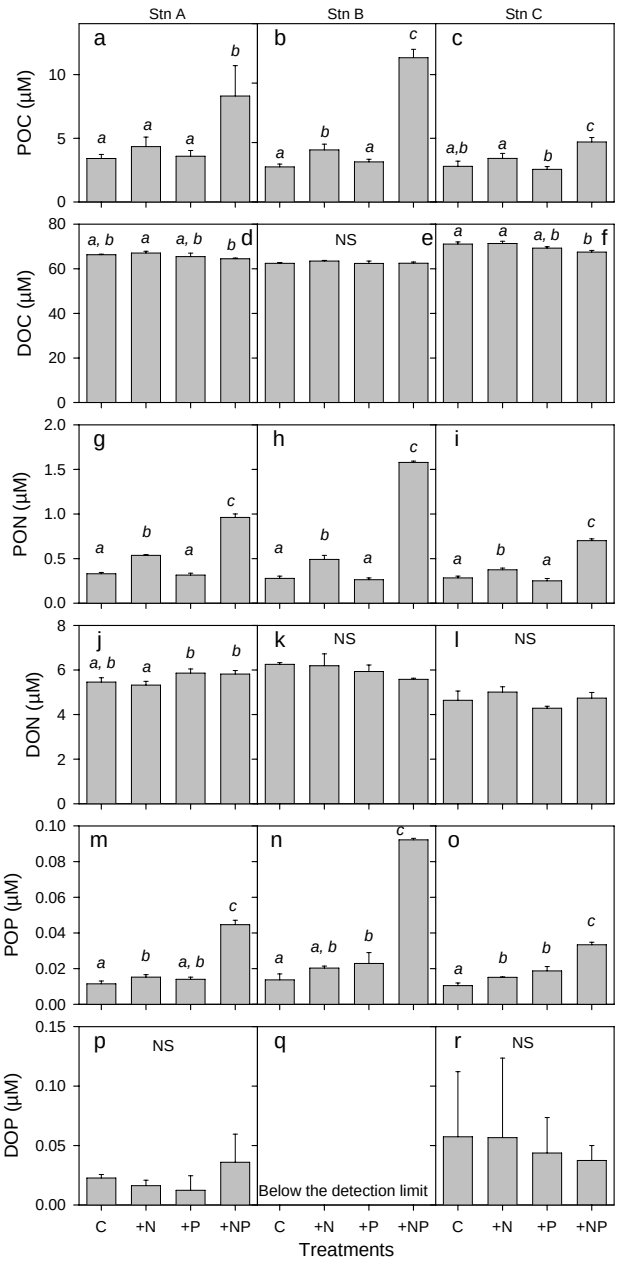


Fig. 3. Comparison of responses of concentration (μM) of POC (**a, b, c**) DOC (**d, e, f**), PON (**g, h, i**), DON (**j, k, l**), POP (**m, n, o**), and DOP (**p, q, r**) between the different treatments at the last day of the experiment. Values are shown as mean ± SD. C, +N, +P, and +NP denote Control, NH_4 addition, PO_4 addition, $NH_4 + PO_4$ addition, respectively. Columns labeled by different italic letters (*a* or *b*) 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.

on Day 3), and decrease in +N at Stn B (below the detection limit on Day 4).

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. 2a, b, c). 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 in turnover time in both +P and +NP during the first 1–2 days. 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 (Fig. 2d, e, f). The effect of the PO_4 addition alone resulted in, at most, a 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 continuously increased in +N treatments but in the other treatments varied little at all stations (Fig. 2g, h, i). 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.

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

Concentrations of POC were highest in the +NP treatments at all three stations (Fig. 3a, b, c), and significantly higher in +N than the Control at Stn B (Tukey HSD test, $P < 0.05$). An effect of nutrient addition on DOC concentration was detected only as a significantly lower concentration in +NP than the Control at Stn C (Fig. 3d, e, f). PON concentrations were significantly higher in +N than the Control and +P and highest in +NP at all stations (Fig. 3g, h, i; 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 (Fig. 3j, k, l). POP concentrations were highest in +NP at all stations (Fig. 3m, n, o; 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 (Tukey HSD test, $P < 0.05$) but not compared to those in +N. Nutrient addition did not affect DOP concentration at all stations (Fig. 3p, q, r).

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 than the others at Stn C (Fig. 4a, b, c; Tukey HSD test, $P < 0.05$). PP was significantly higher in +N than the Control and +P, and highest in +NP at all stations (Fig. 4d, e, f; Tukey HSD test, $P < 0.05$). No significant difference between the treatments was detected for Hprok abundance and PHA at Stn A (Fig. 4g, j). At Stn B, Hprok abundances were significantly lower 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 (Fig. 4h, k; Tukey HSD test, $P < 0.05$). At Stn C, Hprok abundances were significantly lower in +N than the other treatments, and PHA was significantly higher in +NP than the other treatments (Fig. 4i, l; 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. 5a, b; Tukey HSD test, $P < 0.05$). At Stn C, *Synechococcus* abundance was significantly higher in +NP than the others (Fig. 5c). No significant differences in picoeukaryotes abundance between the treatments were detected at any station (Fig. 5d, e, f). A significant effect of nutrient addition on ANP abundance was detected at both Stn B and Stn C (Fig. 5h, i). 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 in concentration of viral particles between the treatments was detected at any station (Fig. 6a, b, c). Significant differences in HNF abundance between the treatments were detected only on twice: higher in +N than the Control at Stn A, and higher in +NP than the Control at Stn B (Fig. 6d, e; Tukey HSD test, $P < 0.05$). Ciliate abundances were significantly higher in +NP than the others at Stn A and Stn C (Fig. 6g, i; Tukey HSD test, $P < 0.05$), while no significant effect of nutrient addition was detected at Stn B (Fig. 6h).

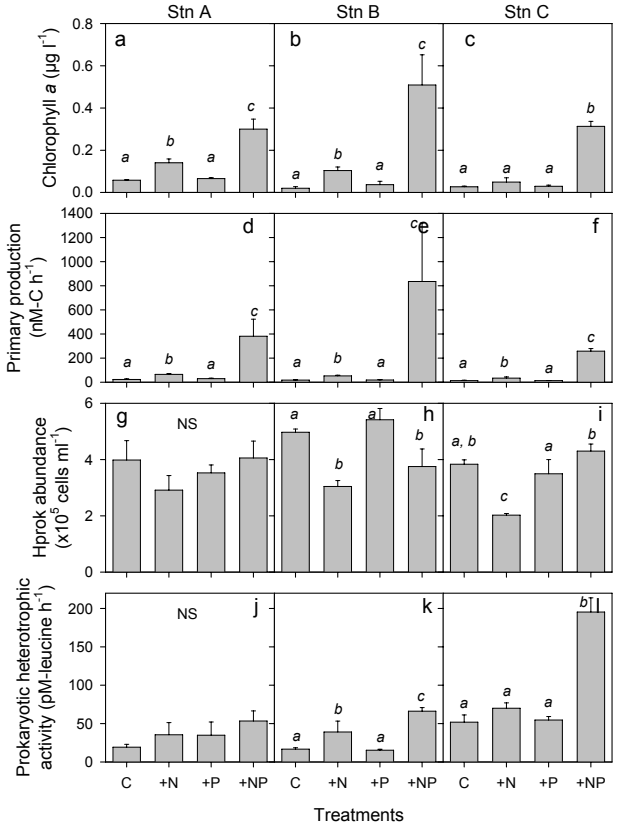


Fig. 4. Comparison of responses of chlorophyll-*a* ($\mu g\,l^{-1}$) (**a, b, c**), primary production (nM-C h^{-1}) (**d, e, f**), abundance of heterotrophic prokaryotes (Hprok) (cells ml^{-1}) (**g, h, i**), and prokaryotic heterotrophic activity (pM-leucine h^{-1}) (**j, k, l**) between the different 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 italic letters (*a* or *b*) 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.

4 Discussion

Our investigation, at three offshore stations in the Mediterranean Sea, is the first on-board study of nutrient-manipulated microcosms that examined simultaneously 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. Our results are the first that demonstrate that a P-starved status, as indicated by relative *in situ* concentrations, may not correspond with P-limitation. We found no significant differences indicative of P-limitation

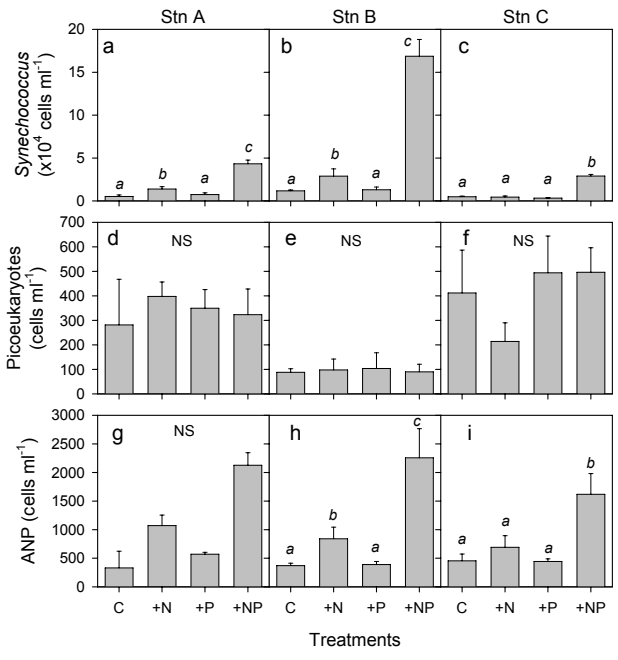


Fig. 5. Comparison of responses of abundance (cells ml^{-1}) of *Synechococcus* (**a, b, c**), picoeukaryotes (**d, e, f**), and autotrophic nanoplankton (ANP) (**g, h, i**) between the different treatments at the last day of the experiment. Values are shown as mean ± SD. C, +N, +P, and +NP denote Control, NH_4 addition, PO_4 addition, NH_4+PO_4 addition, respectively. Columns labeled by different italic 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.

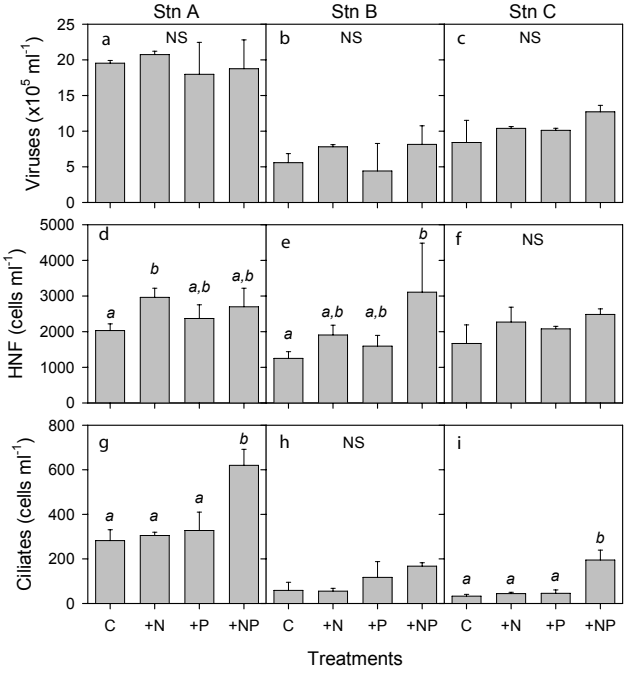


Fig. 6. Comparison of responses of abundance (particles or cells ml^{-1}) of viruses (**a, b, c**), heterotrophic nanoflagellates (HNF) (**d, e, f**), and ciliates (**g, h, i**) between the different treatments at the last day of the experiment except for viruses (data from Day 2). Values are shown as 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 italic 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.

in any measured biological parameters of the microbial food web after P-addition in three anticyclonic eddies of the Western, the Ionian, and the Levantine Basins.

In all three experiments, the initial ratios of N to P were higher than the Redfield Ratio at all three stations with regard to both DON to DOP (111–217) and PON to POP (21–30) (Table 1). In contrast, the ratio of DIN to PO_4 (1.8) at Stn C was much lower than the Redfield ratio. 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 in Siokou-Frangou et al., 2010). This suggests that the sites studied were initially oligotrophic or ultra-oligotrophic with a mixed signal of either N or P limitation. However, the estimated DIN to PO_4 ratio at Stn C includes 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 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 of the DYFAMED time-series station (an offshore site in the NW Mediterranean Sea) was highly variable in a range of 1-60 (observation period: 1991-1999), even when only the data on concentration of NO_3 and PO_4 that exceeds 0.1 µM (i.e. more than twice their analytical detection limits) were taken into account. The NO_3 to PO_4 ratios in Mediterranean surface waters which are lower than the Redfield ratio are a contrast with the NO_3 to PO_4 ratios in waters below the epipelagic layer (22–28, Krom et al., 1991; Béthoux et al., 1992; Kress and Herut, 2001; Pujo-Pay et al., 2010). This may suggest that surface offshore waters with low DIN to PO_4 ratios occur on limited spatial and temporal scales during the stratified period in the Mediterranean Sea.

A P-starved status at all three sites was suggested by data on specific PO_4 affinity which is a measure of P availability for osmotrophs (cf. Thingstad and Rassoulzadegan, 1999). Tanaka et al. (2006) proposed that a specific PO_4 affinity $> 0.02\,l\,nmol\text{-}P^{-1}\,h^{-1}$ indicates P limitation, i.e. the growth rate of the existing osmotrophs (here, phytoplankton and Hprok community) is reduced because of low P availability.

According to this criterion, the phytoplankton and Hprok communites were initially P-limited at all three 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, also includes elements of uncertainty (see Materials and methods). If specific PO_4 affinity is 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 situ (Table 1), and C/P ratio of 150 for Hprok, which was found in P-limited conditions (e.g., Vrede et al., 2002), the estimate of specific affinity would increase by a factor of 2 at all three stations (i.e. an indication of enhanced P limitation). This suggests that the phytoplankton and Hprok community was initially P-limited at all three stations, despite the uncertainty in our estimate of specific PO_4 affinity. However, it should be noted that specific PO_4 affinity for the phytoplankton and Hprok "community" is a measure only of PO_4 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 the whole osmotroph community was co-limited by P and other substrate nor the situation that specific osmotroph groups were limited by substrate(s) other than P in our study.

Increases of PO_4 concentration in the Control and +N at Stn B during the incubation were unexpectedly high (up to ca. 6 times, Fig. 1e), and were not consistent with variations of organic P pools. PO_4 turnover times were relatively stable in the Control and decreased in +N at Stn B (Fig. 2b). Hence, we speculate that PO_4 contamination in these samples occurred outside rather than inside the microcosms. With the analytical detection limit and the precision for the NO_{3+2} measurement in this study, significant changes of NO_{3+2} concentration remained to be explained.

Our study demonstrated that no chemical and biological parameters measured during the microcosm experiments indicated P-limitation at any station. If the osmotroph community were limited by P alone, 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). In a previous study, responses of PO_4 turnover time and APA were similar in the Control and the NH_4 addition treatments, when surface offshore waters of the Eastern Mediterranean Sea were manipulated by an in situ PO_4 addition in May 2002 (Flaten et al., 2005; Thingstad and Mantoura, 2005). In that study, 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 initially the availability of the PO_4 pool for osmotrophs was controlled by the availability of NH_4 (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. In our experiments, whenever a significant difference between the different nutrient treatments was 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 among 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, 2011) found that P addition in their experiment significantly enhanced PP at 48 h at Stn B and Hprok growth at 24 h but not at 48 h at Stn A during 2-day incubation at the same cruise. While both our study and Ridame et al. study employed water collected at the same depth at the same sites, the experimental design and treatments were different (e.g., nutrient concentration and microcosm size). 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 nutrient additions were also different among the stock parameters and between the study sites in our investigation (Figs. 3–6). The NH_4 addition resulted in increases of PON, Chl-*a*, *Synechococcus*, and HNF at Stn A, while at Stn B the NH_4 addition resulted in an increase of POC, PON, Chl-*a*, *Synechococcus*, and ANP but a decrease of Hprok abundance. At Stn C, the 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 at Stn C resulted in an increase of POC, PON, POP, Chl-*a*, *Synechococcus*, ANP, and ciliates, but no change in Hprok abundance. Interestingly, despite N-limitation or N and P co-limitation of Hprok growth, the abundance of Hprok was significantly smaller in the NH_4 addition compared to the Control (Fig. 4h, i). 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). From the same experiment done at Stn B, Talarmin et al. (2011) report that cell-specific PHAs of two high DNA containing subpopulations were 2 times higher in +N and +NP than the Control, but the proportion of these subpopulations to total Hprok abundance was similar between the Control, +N, and +NP at the end of the incubation. This suggests rapid top-down control on Hprok abundance. However, 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. 5h). This may indicate a tight trophic coupling in the heterotrophic compartments and a relative 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 offshore 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 osmotrophs community level in +N treatment at Stn C (an increase of PP, no change of Chl-*a* and PHA, but a 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 studies.

In the Eastern Mediterranean Sea, high amounts 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 when the water column stratification is established (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). LNLC conditions develop 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 are only detectable using 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 offshore waters in May 2002 in the same area (Thingstad et al., 2005; Zohary et al., 2005). The previous study which employed nutrient-manipulated bioassay experiments in July and September 1999 along the longitudinal transect in the Mediterranean Sea, found that Hprok growth is generally limited by P in surface offshore waters (Van Wambeke et al., 2002). Our study done in June/July indicated that PP was consistently limited by N, while Hprok growth was not 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 initial community (Table 1). A possible scenario for these seemingly disparate results would be that (1) as the stratified period progresses, the available pool of both inorganic N and P becomes very small in the surface mixed layer, (2) the plankton food web shifts to N and P co-limitation. Thus, growth of osmotroph groups is limited by different nutrients in a same system, and the most limiting nutrient for osmotroph growth shifts seasonally or sporadically among N, P and N + P (cf. Hecky and Kilham, 1988).

Our results show that while 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 never experienced P-limitation in surface waters in the center of anticyclonic eddies at the three Mediterranean Basins. These results suggest a large gap between biogeochemical features (P-starved status) and biological responses (no P-limitation). We question the general notion that Mediterranean surface waters are limited by P alone during the stratified period. However, surface waters in N-limitation or N and P co-limitation may 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., 2011). If indeed organic matter in the surface layer is P-poor (skewed ratios of DON/DOP and PON/POP), but the microbial food web is either N-limited or N and P co-limited, are conditions which suggests a turnover time of organic N longer than that of organic P (cf. Thingstad and Rassoulzadegan, 1995). A required condition that permits N and P co-limited Hpok but N-limited phytoplankton in the same water is that N:P ratio in phytoplankton community is much higher than that in Hprok community (i.e. greater P requirement per cell volume by Hprok) (reviewed by Vadstein, 2000; Sterner and Elser, 2002) in surface waters. Further research is needed 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, and different basins) in the Mediterranean Sea.

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 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. Thanks are also due to two reviewers for useful comments, and J. Dolan for the linguistic correction.

Edited by: T. Moutin



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.: Planktonic dinitrogen fixation in the Mediterranean Sea: a major biogeochemical process during the stratified period?, Biogeosciences Discuss., 8, 1197–1225, doi:10.5194/bgd-8-1197-2011, 2011.

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. Part 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.

Dolan, J. R., Thingstad, T. F., and Rassoulzadegan, F.: Phosphate transfer between microbial size-fractions in Villefranche Bay (N.W. Mediterranean Sea) France in autumn 1992, Ophelia, 41, 71–85, 1995.

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. Part II, 52, 2928–2943, 2005.

Hecky, R. E. and Kilham, P.: Nutrient limitation of phytoplankton in freshwater and marine environments: A review of recent evidence on the effects of enrichment, Limnol. Oceanogr., 33, 796–822, 1988.

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.: Microbial oceanography: paradigms, processes and promise, Nature Rev. Microbiol., 5, 759–769, 2007.

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. Part I, 48, 2347–2372, 2001.

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., Groom, S., and Zohary, T.: The eastern Mediterranean, in: Biogeochemistry of Marine Systems, edited by: Black, K. D. and Shimmield, G. B., Blackwell Publishing, Oxford, 91–126, 2003.

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. Part II, 52, 2879–2896, 2005.

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

Lagaria, A., Psarra, S., Lefèvre, D., Van Wambeke, F., Courties, C., Pujo-Pay, M., Oriol, L., Tanaka, T., and Christaki, U.: The effects of nutrient additions on particulate and dissolved primary production in surface waters of three Mediterranean eddies, Biogeosciences Discuss., 7, 8919–8952, doi:10.5194/bgd-7-8919-2010, 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.

Lipschultz, F.: Nitrogen-specific uptake rates of marine phytoplankton isolated from natural populations of particles by flow cytometry, Mar. Ecol. Prog. Ser., 123, 245–258, 1995.

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., Chiaverini, J., Pizay, M. D., and Avril, B.: Seasonal and interannual dynamics of nutrients and phytoplankton pigments in the western Mediterranean Sea at the DYFAMED time-series station (1991–1999), Deep-Sea Res., 49, 1965-1985, 2002.

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.

Moutin, T., Van Wambeke, F., and Prieur, L.: Introduction to the Biogeochemistry from the Oligotrophic to the Ultraoligotrophic Mediterranean (BOUM) experiment, Biogeosciences Discuss., in preparation, 2011.

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.

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, Limnol. Oceanogr., 25, 943–948, 1980.

Psarra, S., Zohary, T., Krom, M. D., Mantoura, R. F. C., Polychronaki, T., Stambler, N., Tanaka, T., Tselepides, A., and Thingstad, T. F.: Phytoplankton response to a Lagrangian phosphate addition in the Levantine Sea (Eastern Mediterranean), Deep-Sea Res. Part II, 52, 2944–2960, 2005.

Pujo-Pay, M. and Raimbault, P.: Improvment of the wet-oxydation procedure for simultaneous determination of particulate organic nitrogen and phosphorus collected on filters, Mar. Ecol. Prog. Ser., 105, 203–207, 1994.

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., 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, 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. Part 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, Mar. Microb. 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.

Sterner, R. W. and Elser, J. J.: Ecological stoichiometry, Princeton University Press, Princeton, 439 pp., 2002.

Talarmin, A., Van Wambeke, F., Catala, P., Courties, C., and Lebaron, P.: Flow cytometric assessment of specific leucine incorporation in the open Mediterranean, Biogeosciences, 8, 253–265, doi:10.5194/bg-8-253-2011, 2011.

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., 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., 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 Discuss., 7, 8087–8121, doi:10.5194/bgd-7-8087-2010, 2010.

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.: Methods, 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.

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, Science, 309, 1068–1071, 2005.

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.

Vadstein, O.: Heterotrophic, planktonic bacteria and cycling of phosphorus: Phosphorus requirements, competitive ability, and food web interactions, Adv. Microb. Ecol., 16, 115–167, 2000.

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 production and environmental parameters along a longitudinal gradient across the Mediterranean Sea, Biogeosciences Discuss., 7, 8245–8279, doi:10.5194/bgd-7-8245-2010, 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.

Vrede, K., Heldal, M., Norland, S., and Bratbak, G.: Elemental composition (C, N, P) and cell volume of exponentially growing and nutrient-limited bacterioplankton, Appl. Environ. Microbiol., 68, 2965-2971, 2002.

Wheeler, P. A. and Kirchman, D. L.: Utilization of inorganic and organic nitrogen by bacteria in marine systems, Limnol. Oceanog., 31, 998–1009, 1986.

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 N and P co-limited phytoplankton in the Eastern Mediterranean – a microcosm experiment, Deep-Sea Res. Part 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.

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.