

Studies of the microbial P-cycle during a Lagrangian phosphate-addition experiment in the Eastern Mediterranean

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Abstract

Microbial uptake of orthophosphate was studied before and during a Lagrangian experiment where orthophosphate was added to the surface mixed layer in the Cyprus Gyre, Eastern Mediterranean, a region previously hypothesized to be characterized by P-limited growth of both phytoplankton and heterotrophic bacteria. The addition of ca. 110 nM orthophosphate to a ca. 16 km² patch in situ led, within 1 day, to an increase in particulate-P from 8 to ca. 15 nM, a result in good agreement with a previous microcosm bioassay indicating this system to have a maximum capacity for orthophosphate consumption of between 10 and 25 nM phosphate. In samples of unperturbed water taken before the addition, outside, or below the experimental patch, orthophosphate turnover time (T_t) was <4 h, argued to be consistent with the assumption of diffusion-limited phytoplankton growth. Upon addition, T_t increased to 94 h. Estimates of maximum potential uptake rate (V^{\max}) for orthophosphate in unperturbed water exceeded by more than one order of magnitude the biological P-requirement (v) as obtained from stoichiometric conversion of C-based primary and bacterial production values to estimated P-requirement. Upon addition of orthophosphate, V^{\max} decreased to a level comparable to v . The observations are consistent with the assumption of P-starved cells before and P-replete cells with excess external orthophosphate after the addition. Orthophosphate uptake in unperturbed water was dominated by <1 μm organisms (mean \pm SD between samples $0.56 \pm 0.03 \mu\text{m}$). In samples with higher turnover time, orthophosphate uptake was shifted towards larger organisms, culminating after 5 days with a near doubling in mean size (1.08 μm). The size distribution of particulate-P standing stock had a mean size of 10 μm , indicating the presence of a substantial biomass of micro-organisms larger than those involved in P-uptake. Comparison of the measured particulate-P with microscope-based biomass estimates indicated a microbial food web dominated by heterotrophic organisms (70% of particulate-P), distributed with ca. 25% of total particulate-P in heterotrophic bacteria, ca. 40% in heterotrophic flagellates, and ca. 5% in ciliates.

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Concentration of bioavailable phosphate (S_n) estimated from the relationship $S_n = vT_i$ indicated S_n values < 1 nM PO_4 before the addition, increasing afterwards. Estimates of the sum $K_t + S_n$ for the 0.6–0.2 μm size fraction were in the range 1–7 nM PO_4 before and outside patch, suggesting this sum to be dominated by the half-saturation constant K_t . $K_t + S_n$ increased to 69 nM after addition, then dropped over the following week back to background levels. As reported elsewhere in this volume, there was a decline in the observed chlorophyll concentrations, but a positive response in copepods. Less clear than the effects at the level of osmotroph physiology were the subsequent responses expected in the food web. Two possible mechanisms are discussed: (1) a positive response in bacterial production and the subsequent food chain of bacterial predators, and (2) a positive response in phytoplankton predators due to a shift in food quality rather than in food quantity.

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1. Introduction

In the pelagic ecosystem, phosphorus circulates through the food web via a number of steps (Fig. 1). Following the numbering in Fig. 1 and starting from the free mineral form of orthophosphate (Step 1), uptake (Step 2) allows intracellular storage (Step 3) in osmotrophic micro-organisms (phytoplankton and heterotrophic bacteria) which, provided other essential factors like, e.g., nitrogen, organic substrates (for heterotrophic bacteria), and light (for phytoplankton) are available, is followed by growth (Step 4) and cell division with increase in osmotroph cell number (Step 5) and abundance, subsequent transport (Step 6) via trophic interactions to predators and parasites (e.g. viruses) (Step 7), accompanied by release either directly as orthophosphate (Step 8) or via dissolved organic forms (Step 9) that can be hydrolyzed enzymatically

to orthophosphate (Step 10). Perturbing this dynamic system at Step 1 by adding orthophosphate would, with time, be expected to be transmitted around this circle in a pattern dependent upon the turnover time for P in the different pools, the manner in which the food web is connected by trophic interactions, and the characteristic response time of these interactions. The effect of the perturbation would also be strongly influenced by the size of the perturbation relative to the available amounts of the other limiting factors potentially blocking the transmission at Step 4.

In the high-nutrient–low-chlorophyll (HNLC) regions the available amounts of such other potentially limiting factors appear in large excess relative to the limiting iron, and in situ addition of iron has been shown able to induce large phytoplankton blooms (Behrenfeld et al., 1996; Cavender-Bares et al., 1999; Gall et al., 2001). The time scale

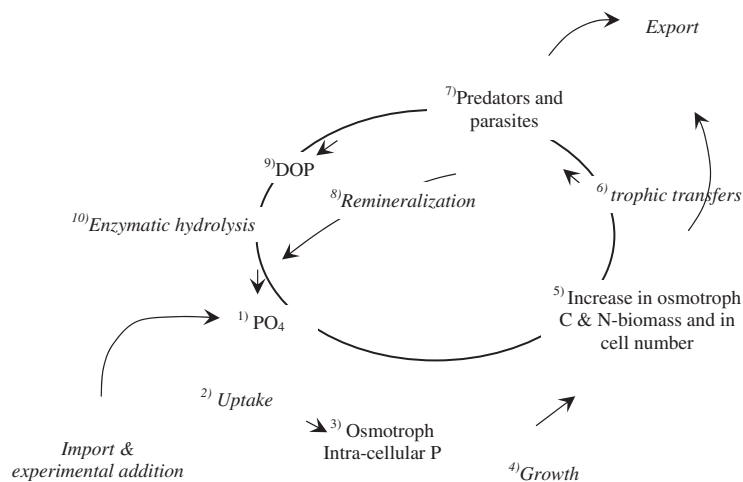


Fig. 1. Processes (*italic font*) assumed to transfer phosphorus between pools (regular font) in the pelagic food web. Numbers to assist reference in the text.

for further transmission to Steps 6 and 7 then depends on the response time of the subsequent predator which presumably is long if production of new copepod individuals is required, shorter for heterotrophic dinoflagellates and fairly rapid if the blooming species can be grazed by ciliates or even heterotrophic flagellates.

The traditional paradigm that nitrogen is the proximate (*sensu* Tyrrell, 1999) limiting factor for biological biomass or growth rate in the ocean's surface layer has been challenged over the last decade. Not only has iron been shown to be the limiting factor in HNLC areas, but there are also increasing evidence of P-limitation in large, truly marine areas such as e.g. the Atlantic (Ammerman et al., 2003; Vidal et al., 2003). In the Eastern Mediterranean, bioassays both for phytoplankton (Bonin et al., 1989) and for heterotrophic bacteria (Zohary and Robarts, 1998) have suggested P-limitation for both groups of osmotrophs. P-limitation in this region has also been inferred from high nitrate-to-phosphate ratios in the deep water of the Levantine Basin (Krom et al., 1991). This proposed P-limitation is a feature that comes in addition to the west-to-east general oligotrophication, traditionally explained by the negative thermo-haline (anti-estuarine) Mediterranean circulation (Redfield et al., 1963), leading to a typical chlorophyll levels in the Levantine Basin around $20 \mu\text{g chl m}^{-3}$ in summer (see, e.g. <http://seawifs.gsfc.nasa.gov/SEAWIFS.html>).

The effects to be expected from adding P to a presumably P-limited system are less obvious than those expected from adding Fe to a HNLC situation. Starting from a pre-bloom situation with a skewed ratio in nitrate to phosphate, Redfield et al. (1963) noted consumption to follow the 16:1 ratio until one element was depleted. Then the mineral form of the excess nutrient was consumed, leading to a situation with both nitrate and phosphate eventually being depleted. Situations with the mineral forms of more than one potentially limiting element being depleted is thus probably more a rule than an exception in the ocean's surface layer. In the Cyprus Gyre, ca. 300 nM $\text{NO}_3\text{-N}$ remains after the spring bloom (Krom et al., 1992). At the time of the experiment reported here (mid-May), the nitrate was below detection limits of conventional automated technology (Krom et al., 2005). The amount of bioavailable, excess N was, at this point, estimated from bioassays (Thingstad and Mantoura, 2005) to be ca. 230 ± 60 nM N. With

ammonia levels around 80 nM and undetectable nitrate, ca. 150 nM bioavailable N may be estimated as stored either as DON or as enlarged cell quotas of N in the biota. Using a 16:1 ratio, an addition between 15 and 20 nM phosphate would thus be expected to drive such a system to pure N-limitation, thus constraining the magnitude of a phosphate perturbation that can pass through Step 4. Assuming further a N:Chl ratio of 0.3 mol N consumed per g Chl produced, this would correspond to a maximum production of around $800 \mu\text{g chl m}^{-3}$. In this oligotrophic region this potential production would, if ungrazed and undiluted, correspond to a ca. 40-fold increase in phytoplankton biomass. The chlorophyll response that a priori might be expected from saturating this biological system with phosphate is thus, in relative terms, large.

In the Lagrangian iron-addition experiments, larger-sized osmotrophs responded with the blooming phytoplankton community dominated by diatoms (Cavender-Bares et al., 1999; Gall et al., 2001). When phosphate concentrations are experimentally increased in bottle incubations (Suttle et al., 1990), and when phosphate transport over the nutricline increase seasonally (Dolan et al., 1995), the orthophosphate uptake distribution also shifts towards larger-size fractions. Such a pattern fits the predictions of theoretical and experimental (Thingstad and Sakshaug, 1990; Bohannan and Lenski, 2000) models where competition-specialized, in this case presumably small, organisms dominate under nutrient poor conditions, while predator defence-specialized, in this case presumably large, organisms dominate under high-nutrient regimes.

Transfer of P from orthophosphate into organisms has been studied in the Mediterranean with the methodology combining radioactive tracers (^{32}P or ^{33}P) with collection of organisms on polycarbonate filters with defined pore sizes (Dolan et al., 1995; Thingstad et al., 1996a; Thingstad et al., 1998; Zohary and Robarts, 1998; Moutin et al., 2002; Tanaka et al., 2003). Such studies basically provide information on the size-distribution of the uptake and on the turnover time (T_t) of the bioavailable orthophosphate pool. Turnover time, concentration (S_n) and rate of uptake (V) are connected through the relationship $T_t = S_n/V$. From any two of these values, the third can thus be calculated. With S_n in P-limited environments usually below detection limit of conventional chemical techniques, V cannot be directly calculated. Inverting this relationship to

calculate S_n from an independent estimate of V has been attempted by Thingstad et al. (1996a) using the loss rate of label from the bacterial size fraction, and by Moutin et al. (2002) by conversion of the carbon-flow estimates from primary and bacterial production measurements into P-flow values using fixed stoichiometric ratios. Both methods suggested S_n values slightly below 1 nM, values in the higher end of the range suggested for freshwater by Hudson et al. (2000). Turnover time T_t also has the potential to reveal how a pulse of orthophosphate is transmitted around the circle in Fig. 1. Initially one would expect T_t to increase as the perturbation remains at Step 1, then a decrease to low values as the perturbation is transmitted to Step 5 since then there will be many competitors for low external concentrations. This phase should be followed by an increase in T_t as biomass is transferred along to Steps 6 and 7 with less competition and more recycling as the result. If however phosphate is added in excess of what the system can absorb, one would expect T_t to remain high. Since orthophosphate turnover time can be determined relatively easily and precisely, we expected T_t to be a sensitive indicator for the general transmission of the perturbation through the food web.

Additional information can be obtained using the Rigler bioassay (Rigler, 1956) based on isotope dilution and the assumption that uptake follows Michaelis–Menten kinetics. This allows estimation of $K_t + S_n$, and V^{\max} , where K_t is the half-saturation constant for orthophosphate uptake, and the populations' maximum potential uptake rate V^{\max} for high orthophosphate. In P-starved cells, the potential for uptake can be much higher than the potential for growth, leading to a temporal separation of Steps 3 and 4 with cell quotas of P remaining high until Step 4 is initiated (Nissen et al., 1987). With the capacity for internal storage fully exploited, uptake must be reduced until growth and cell division allow new uptake (Parslow et al., 1984).

Studies of dynamic properties of the microbial food web of Mediterranean waters has been studied in micro or mesocosms (e.g. Thingstad et al., 1999; Agawin et al., 2000; Olsen et al., 2001; Kress et al., 2005). We here also report the use of a microcosm experiment to assess how much orthophosphate the biological system could absorb before being restricted by other factors. Such studies will, however, unavoidably be hampered by the questions of potential effects from water manipulation and

confinement. We here also report studies on the microbial P-cycle from an experiment with in situ addition of orthophosphate.

2. Material and methods

The 2001 microcosm bioassay reported here was performed during a pilot experiment in the Cyprus gyre, the same area as the main experiment, at the same time of year in 2001. Water collected at 15 m was distributed in 81 acid-cleaned and sea water-rinsed polyethylene carboys. Orthophosphate was added as KH_2PO_4 to final concentrations of 0, 10, 25, 50 and 200 nM PO_4 . The carboys were incubated under reduced natural sunlight at in situ temperature using an on deck tank with running seawater and covered with a neutral light screen. Turnover time for orthophosphate was measured daily as described below.

The Lagrangian experiment was performed in the Cyprus gyre, a permanent anti-cyclonic gyre south of Cyprus in the ultra-oligotrophic Eastern Mediterranean in the period 17–27 May 2002, here referred to as day 0–day 10. Using the vessel R/V Aegeo, ca. 110 nM orthophosphate was added as diluted phosphoric acid to the surface mixed layer in a ca. 16 km² patch, together with the tracer SF_6 . For further details of the Lagrangian experiment and the hydrographic and chemical characteristics of the experimental area, see Law et al. (2005).

Samples were collected using a rosette sampler equipped with 101 Niskin bottles. The data reported here either refer to samples taken from 12 m depth, or averaged over four samples taken at 4, 8, 12 and 16 m. Samples are characterized as BEFORE if taken before the experimental in situ addition of orthophosphate and the tracer SF_6 on 17 May 2002, day 0, or as OUT or BELOW, if taken later but outside or below the patch as confirmed by lack of SF_6 . Samples are characterized as IN samples if taken inside the patch.

Orthophosphate turnover time was measured by adding ca. $5 \times 10^3 \text{ Bq ml}^{-1}$ specific activity ($\sim 1.1 \times 10^{11} \text{ Bq nmol}^{-1}$) $^{33}\text{PO}_4^{3-}$ (Amersham code BF1003) to 10 ml water samples in sterile disposable 15 ml Falcon test tubes. 0.1 ml was collected for counting of added radioactivity. Following incubation in the laboratory for 0.25–1 h depending on expected turnover time, samples were filtered on 0.2 μm pore-sized 25 mm polycarbonate filters. A Millipore 12-hole filtration manifold was used. 25 mm Whatman GF/C filters, saturated with

100 mM “cold” KH_2PO_4 were used as support filters. The samples were filtered under low (<0.2 bar) suction until all liquid had passed through the filters. Suction was then increased to maximum (ca. 0.6 bar) to suck remaining ^{33}P -containing liquid out of the polycarbonate filters. To avoid loss of label taken up, no washing steps were used. Background from isotope solution or a-biological adsorption to particles was checked using samples fixed before isotope addition with 100 μl glutaraldehyde, but found negligible. Radioactivity was counted on-board by liquid scintillation counting in mini vials containing 2 ml Ultima-Gold scintillation liquid (Packard Biosciences) using a Lumi-One portable luminometer (Bioscan Inc.). Orthophosphate was calculated as $T_t = -t/\ln(1-f)$ where f is the fraction of added isotope collected on the $0.2\ \mu\text{m}$ filter after the incubation time t .

Size spectrum of orthophosphate uptake was determined using the same technique as for determination of T_t , but a 15 ml sample was split after incubation between eight polycarbonate filters of 0.2, 0.4, 0.6, 0.8, 1, 2, 5 and $10\ \mu\text{m}$ pore sizes. The cumulative gamma-function $\Gamma(X-0.2, \alpha, \beta)$ was fitted to the experimental data-pairs $X_i, 1-r_i$ where r_i is the ratio between radioactivity on filter with pore size i to radioactivity on the $0.2\ \mu\text{m}$ filter by minimizing the squared sum of residuals using the built-in Γ -function and the Solver option in ExcelTM. The mean size of phosphate consuming cells was calculated as $\alpha\beta$ and the median using Excel's inverse Γ -function with 0.5 probability. There is no biological argument for using this function other than that it fits the data well.

P-kinetics and PO_4 concentration. Maximum potential uptake rate V^{max} and the sum $K_t + S_n$ of half-saturation constant K_t and concentration S_n of bioavailable phosphate were estimated from isotope dilution (Rigler bioassay). Isotope (as above) and cold orthophosphate were added to six sterile 15 ml Falcon test tubes prior to adding water samples. KH_2PO_4 in sterile Milli-Q-rinsed water was added to give final concentrations of 0, 10, 20, 40, 60 and 80 nM $^{31}\text{PO}_4\text{-P}$, respectively, in the six tubes after filling with 10 ml sample. Uptake was measured by filtering 5 ml sub-samples on 0.2 and $0.6\ \mu\text{m}$ pore-sized polycarbonate filters and determining fraction of added label on each filter as described above. Uptake in the $0.6\text{--}0.2$ size fraction was obtained by subtraction and the fraction f of uptake in the $0.6\text{--}0.2\ \mu\text{m}$ size fraction calculated. Parameters

were determined for the $0.6\text{--}0.2\ \mu\text{m}$ and $>0.6\ \mu\text{m}$ size fractions by linear regression of T_t/f and $T_t/(1-f)$, respectively, versus S_a as described by Thingstad et al. (1993). Osmotroph P-requirement was estimated according to Moutin et al. (2002) by converting C-based values for primary (Psarra et al., 2005), and bacterial (Pitta et al., 2005) production assuming a C:P ratio of 106 and 50 in phytoplankton and bacterial biomass, respectively. Bioavailable orthophosphate concentration S_n was then calculated from the relationship $S_n = vT_t$.

Particulate phosphate was measured by collecting 250 ml sample on each of eight 47 mm polycarbonate filters with the same pore sizes as used for determining size spectrum of uptake. The filters were suspended in 5 ml Milli-QTM water in 10 ml polypropylene test tubes and wet-oxidized in acid persulphate (Koroleff, 1983). Particulate-P was measured spectrophotometrically as liberated orthophosphate with the standard molybdenum blue technique using a 5 cm cuvette. Unused filters in 5 ml Milli-QTM water were treated identically and OD values subtracted as blanks.

3. Results

3.1. 2001 microcosm bioassay

In the microcosm with 10 nM orthophosphate added, we observed an oscillating pattern in T_t as

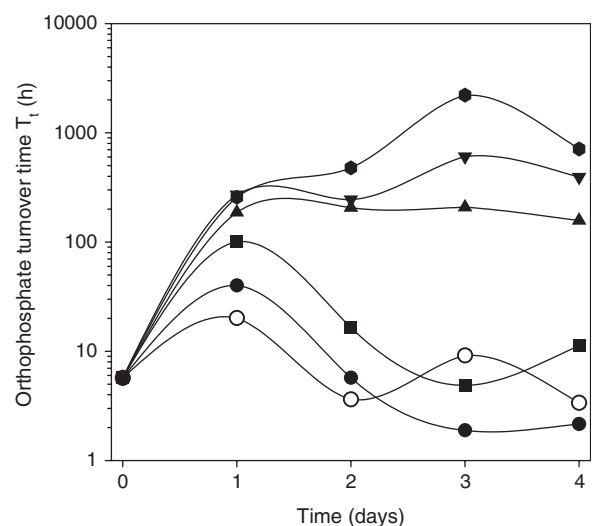


Fig. 2. Orthophosphate turnover time in 81 deck-incubated microcosms amended with 0 (○), 10 (●), 25 (▲), 50 (▼), and 200 (●) nM PO_4 .

expected from adding orthophosphate to a P-limited system (Fig. 2). Adding 25, 50 or 200 nM, however, T_t increased initially, then remained high for the rest of the experimental period of 4 days. The pattern is consistent with the assumption that the system could consume our 10 nM dose, but with 25 nM or more orthophosphate added, other factors became limiting. Also in the control microcosms we found an oscillating pattern, less pronounced than in the carboy with 10 nM addition. Whether these reflect natural oscillations in the food web, or whether we caused a minor perturbation of the food web by a <10 nM contamination or by disturbing some of the organisms during water manipulation and confinement is not known.

3.2. Orthophosphate turnover time T_t

In BEFORE, OUT, and BELOW samples from the main sampling depth (12 m), T_t values were between 2 and 5 h. Upon addition, T_t increased to 94 h. Values remained elevated for 1 week, then returned to background values 8 days after the addition (Fig. 3). Such a reduction could theoretically be caused either by a biological response delayed 1 week after addition, or by dilution of the patch with outside water returning the system towards its initial state. T_t correlated well (correlation coefficient $\rho = 0.92$) with measured concentrations of the conservative tracer SF_6 (Fig. 3) added together with the phosphate as described by Law et al. (2005), suggesting the latter mechanism to be dominant.

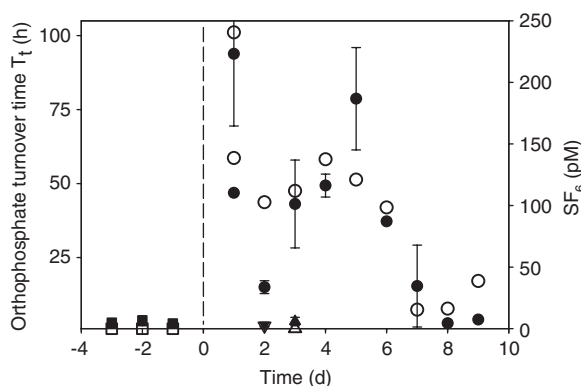


Fig. 3. Orthophosphate turnover time T_t (filled symbols) and concentration of SF_6 (open symbols) at the main depth on stations sampled before (squares), inside (circles), outside (triangle up), or below (triangle down) the experimental patch.

3.3. Concentration estimates for bioavailable orthophosphate

Except for high values in the two first days after addition, our $K_t + S_n$ values corresponded well with chemically measured phosphate as reported by Law et al. (2005) (Fig. 4). Considering the high initial dilution the $K_t + S_n$ value of 69 nM PO_4 , obtained on the day after addition compares well with the nominal added concentration of 110 nM PO_4 -P.

S_n estimated from combining turnover time with the biological P-requirement obtained by stoichiometric conversion of primary (Psarra et al., 2005) and bacterial production (Pitta et al., 2005) gave somewhat lower estimates than the chemically measured SRP and the $K_t + S_n$ values the first days after addition, but consistently higher estimates than the other two for a period 5–6 days after addition. All methods returned towards low values (ca. 1 nM PO_4 -P) 1 week after the addition. If phosphate uptake and carbon biomass production were out of phase with phosphate uptake relatively high the first 2–3 days and relatively low the following 3–4 days after addition, our method would underestimate S_n in the first period and overestimate it in the second.

3.4. Particulate-P

Upon P-addition, particulate-P values increased from a level around 8 to about 15 nM P (Fig. 5). Elevated values lasted for 1 week, then returned to

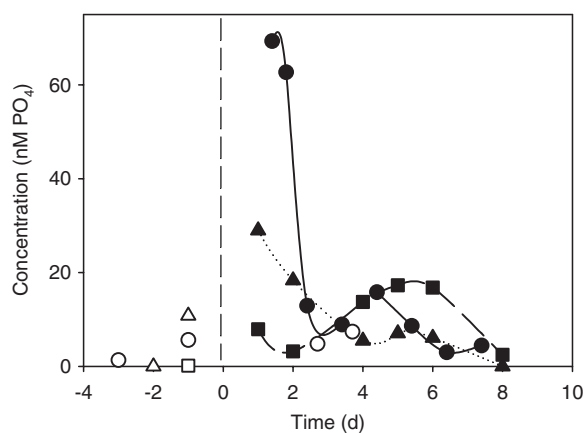


Fig. 4. Estimates of bioavailable orthophosphate S_n (squares) obtained from turnover time and production estimates averaged over the upper 16 m, sum $K_t + S_n$ (circles) at 12 m, compared to chemically measured phosphate concentrations (triangles) at 12 m as reported by Law et al. (2005). Values from IN (filled symbols) and BEFORE, OUT or BELOW (open symbols) samples.

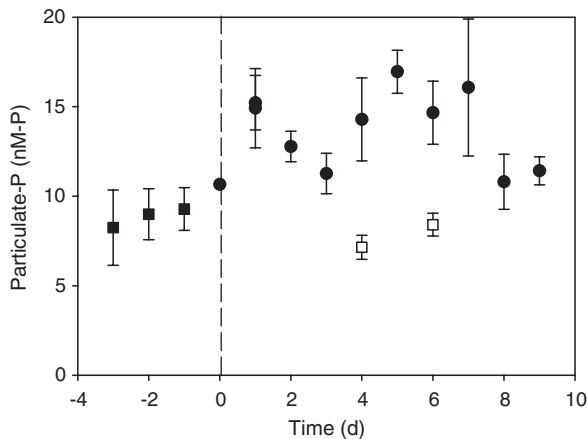


Fig. 5. Particulate P averaged over depths ≤ 16 m for samples taken before (filled squares), inside (filled circles) or outside (open squares) the experimental patch. Error bars indicate the standard error of determinations at four depths.

background level. The return of both particulate-P and T_t values to background levels after 1 week supports the suggestion that dilution of the patch then had reached the level where the system returned to its pre-perturbation state, rather than a biological uptake of the added orthophosphate at this late stage.

The size spectrum for particulate-P in unperturbed water before addition had a mean of $9.7 \mu\text{m}$ and a median of $3.7 \mu\text{m}$, as calculated from the fitted γ -distribution (Fig. 6A). Considering the precision in the experimental data, we regard the somewhat arbitrary choice of the γ -distribution as a satisfactory choice for smoothing the data (examples shown in Fig. 6A and B). The data suggest a minimum mean and median of the fitted γ -distributions ca. 3 days after the addition (Fig. 6C). With uptake dominated by much smaller organisms (see section on orthophosphate uptake), a major P-uptake would be expected initially to shift the spectrum of particulate-P towards smaller organisms, followed by an increase as both predation moved P up the food chain and dilution with OUT-water returned the system towards the initial conditions.

3.5. Phosphate uptake

Orthophosphate uptake in samples from unperturbed water (example in Fig. 7A) was concentrated in a narrow range between 0.6 and $1.0 \mu\text{m}$, with median and mean size of 0.50 ± 0.02 and $0.56 \pm 0.03 \mu\text{m}$, respectively (mean and SD of five samples). The size spectrum of uptake thus differed

considerably from the size-distribution of standing-stock particulate-P, the latter indicate presence of a significant amount of larger micro-organisms (Fig. 6). Following the addition, the uptake distributions shifted progressively towards larger organisms, reaching approximately a doubling in mean size 1 week after addition, then followed by a restoration of the original shape of the distribution (Fig. 7B and C).

Maximum potential uptake rate for orthophosphate V^{max} can be compared to estimated P-requirement v (Fig. 8). In BEFORE, BELOW and OUT samples, V^{max} (range 1.3 – 1.8 nM P h^{-1}) was always higher than the v -value of 0.7 nM P h^{-1} estimated before addition, as would be expected for P-starved cells. In IN samples up to day 6 after addition, the two estimates were similar with overlapping V^{max} and v ranges (0.2 – 0.7 and 0.1 – 0.3 nM P h^{-1} , respectively), as expected in a system with P-replete osmotrophs. About 6 and 7 days after the addition, V^{max} values returned to high values, but then, for unknown reasons, two relatively low values were again found at the end of the experimental period (Fig. 8).

Average size-fractionated measurements of primary production (Psarra et al., 2005) in IN samples from the main sampling depth indicated gave a distribution of 55% and 45% ($n = 7$) in the 2 – $0.2 \mu\text{m}$, and $>2 \mu\text{m}$ size fractions, respectively, corresponding well with the estimated distribution of chlorophyll (16%, 44%, 40%) in the 0.6 – 0.2 , 2 – 0.6 and $>2 \mu\text{m}$ size fractions, respectively.

4. Discussion

4.1. Structure of the pre-perturbation P-flow

Combining our measurements of P-stocks and flows with other observations reported in this volume, we have tried to construct a picture of the P-flow through the microbial food web for the unperturbed situation (Fig. 9). The chlorophyll concentration of ca. $18 \mu\text{g m}^{-3}$ could be converted to an estimate of autotroph biomass P using a standard C:chl ratio and Redfield C:P stoichiometry. The validity of such a conversion would, however, be uncertain since both conversion factors may have been affected considerably by the supposed P-limitation and extreme light conditions of this environment. Also C-content in *Synechococcus*, one of the groups dominating phytoplankton biomass in this system (Pitta et al., 2005) appears to

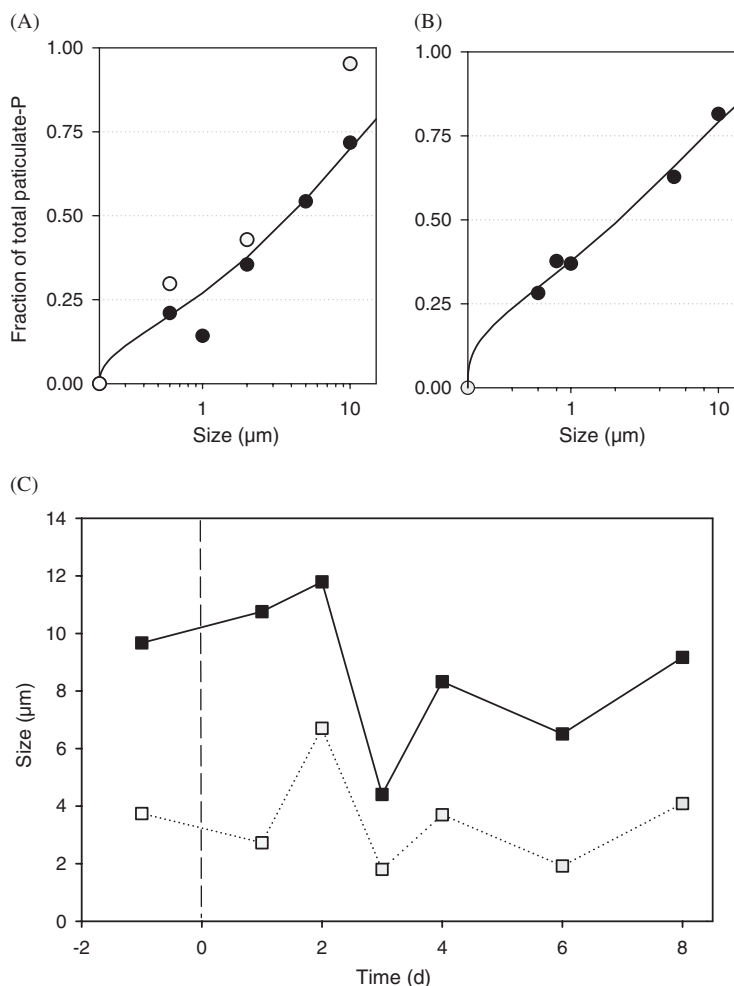


Fig. 6. Size distribution of particulate-P. Examples of observed cumulative (filled circles) and fitted cumulative γ -distributions (solid lines). Samples from (A) before perturbation and (B) inside the experimental patch. Panel C shows development over time in mean (solid squares) and median (open squares) in IN samples from the 12 m main sampling depth. The cumulative size distribution corresponding to the P-inventory estimated is shown in Panel A (open circles).

be generally high (Bertilsson et al., 2003; Heldal et al., 2003). The net outcome of combining the two conversion factors to one chl:P ratio would thus be difficult to evaluate. Using as basis microscopic counts converted to C (Pitta et al., 2005), and arguing that biomass element composition of phagotrophic protozoa may have been less stressed than phytoplankton by the presumed P-shortage in this system, a Redfield C:P stoichiometry of 106 was used to give 3.4 and 0.4 nM P in 2–10 μm heterotrophic flagellates and in ciliates, respectively (Fig. 9). Assuming 20 fg C cell⁻¹ for heterotrophic bacteria (Zohary et al., 2005), combined with a C:P ratio of 50 for bacteria (Fagerbakke et al., 1996) corresponds to 0.033 fmol P cell⁻¹. Arguing that bacterial cells in this oligotrophic environment

may have been small and that the presumed P-limitation would be expected to favour a low P per cell content (Martinussen and Thingstad, 1987; Vadstein and Olsen, 1989), we have, somewhat arbitrarily, reduced this value to 0.02 fmol P cell⁻¹. This gives a P-content of 2.1 nM P in heterotrophic bacteria. Subtracting these heterotrophic groups from the total particulate-P of 8.4 nM P leaves 2.5 nM P, or ca. 30% of particulate-P, for the autotrophs. Assuming further a fixed chl:P ratio in all autotrophs, the measured distribution of chlorophyll with 16, 44 and 40% in the 0.6–0.2 μm , 2–0.6 μm , and >2 μm size fractions, was used to distribute autotroph-P correspondingly, giving 1.0 and 1.5 nM P, respectively for the two groups of “autotrophic flagellates” (>2 μm) and “autotrophic

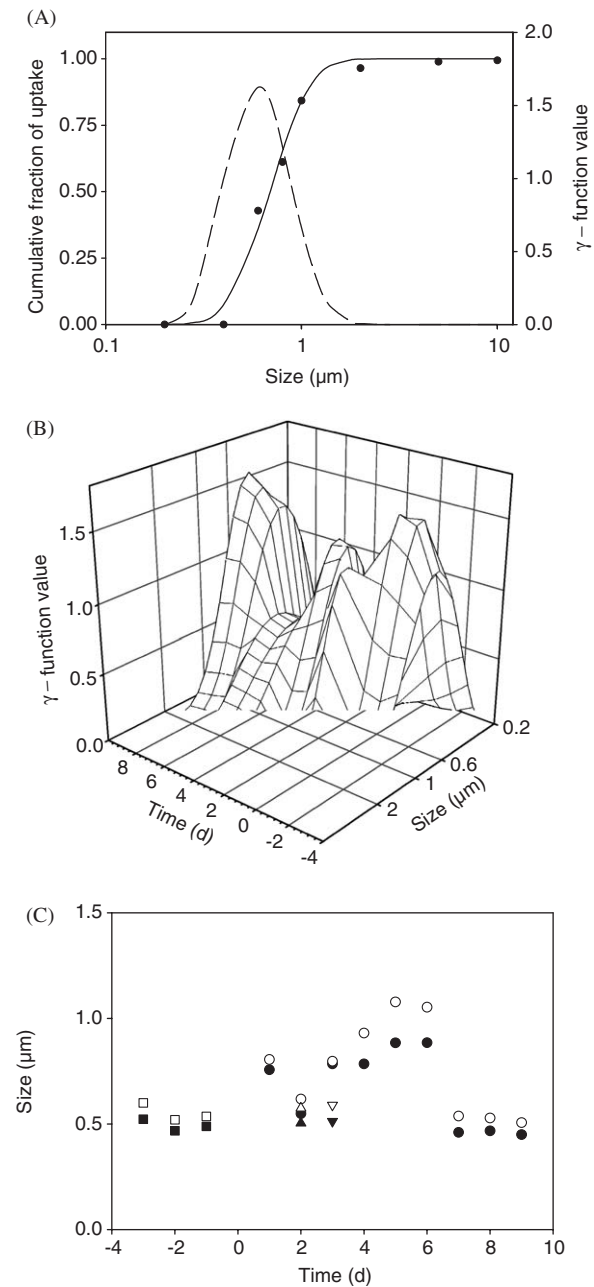


Fig. 7. Size distribution of orthophosphate uptake. (A) Example from main sampling depth before addition on Day-1 2002 with measured cumulative distribution (filled circles), fitted cumulative γ -distribution (solid line) and the corresponding γ -distribution (dotted line). (B) Development over time in the fitted γ -distributions, and (C) The median (filled symbols) and mean (open symbols) of BEFORE (squares), IN (circles), OUT (up triangles) and BELOW (down triangles) samples from the main sampling depth.

picoplankton" ($<2\mu\text{m}$). On the basis of the chlorophyll distribution, our "autotrophic picoplankton" distributed with 73% in the $2\text{--}0.6\mu\text{m}$

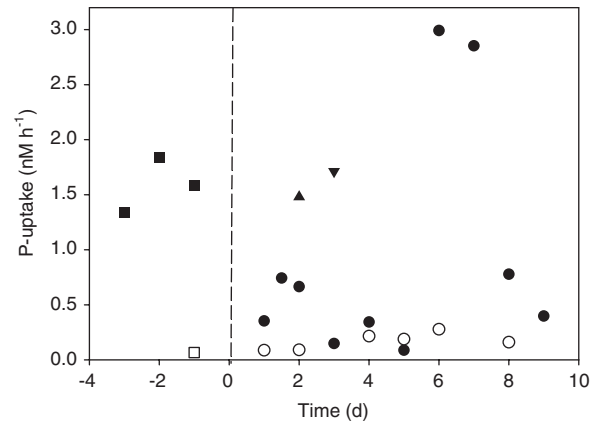


Fig. 8. Estimates of maximum orthophosphate uptake potential V_{max} (filled symbols) and P-requirement estimated from primary and bacterial production (open symbols) in samples taken BEFORE (squares), IN (circles), OUT of (up triangle), or BELOW (down triangle) the experimental patch.

and 27% in the $0.6\text{--}0.2\mu\text{m}$ size classes. It should be noted that a combination of these calculations with the microscopy-based estimate of ca. 3 mg C m^{-3} in autotrophic $2\text{--}10\mu\text{m}$ nanoflagellates (Pitta et al., 2005) leads to C:P (molar) and C:chl (w:w) values of ca. 250 and 417, respectively, for the $2\text{--}10\mu\text{m}$ autotrophs. While both of these are high compared to standard conversion factors, high values would be expected in an environment characterized by a combination of P-limitation and extreme light conditions. Marine *Synechococcus* have been shown to have C:P ratios up to 779 (molar) when grown under P-limited conditions (Bertilsson et al., 2003) and C:chl values up to 668 (w:w) have been found in *Synechococcus* grown under high light conditions (Six et al., 2004). Assuming all heterotrophic bacteria to be in the $0.6\text{--}0.2\mu\text{m}$ size range, and ciliates to be the only $>10\mu\text{m}$ micro-organisms, the cumulative size distribution of particulate phosphorus corresponding to Fig. 9 can be calculated. The result obtained by these indirect estimates is shown in Fig. 6, Panel A, together with the measured size distribution of particulate-P for a pre-perturbation station. The main discrepancy is for the higher size classes, suggesting that the budget in Fig. 9 under-estimates particulate P in size classes $>10\mu\text{m}$ to the extent that an extra ca. 20% of the particulate P should be in such larger organisms. An interesting aspect of Fig. 9 is the relative dominance of heterotrophs in the P-budget (70%), with 25% of total part P in heterotrophic bacteria. Obviously, there are large uncertainties linked to all conversions used, but the general trend of an increasing

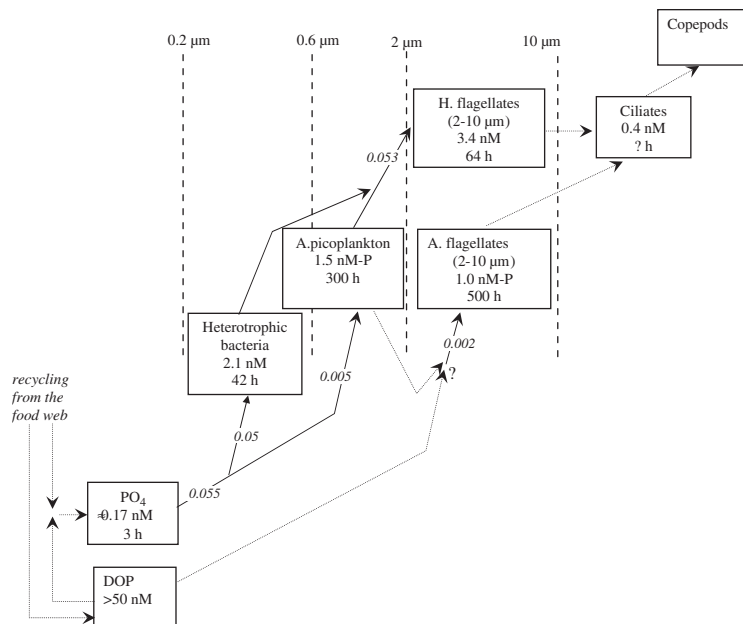


Fig. 9. Estimated structure of P-flow through the microbial food in unperturbed water. Concentrations and turnover time given for the different pools, flows (italics) in nM P h^{-1} . Dotted lines indicate pathways for which estimation was not attempted. See discussion for further details.

relative dominance of heterotrophic bacteria as one moves from eutrophic to oligotrophic systems is in agreement with what has been previously reported (Fuhrman et al., 1989; Simon et al., 1992).

A somewhat paradoxical aspect of these calculations seem to emerge if one considers that the size fraction $>2\mu\text{m}$ contributed substantially to primary production, but not to orthophosphate uptake. Using the mean for all measurements ($n = 7$) about 45% of primary production, corresponding to about $5\mu\text{g C m}^{-3}\text{ h}^{-1}$ was estimated for the $>2\mu\text{m}$ size fraction. With an estimated C:P ratio of 250 for autotrophs in the $>2\mu\text{m}$ size fraction (see above), their corresponding P-requirement would be 0.002 nM P h^{-1} . This corresponds to ca. 4% of total P-uptake estimated (Fig. 9). Uptake by these larger osmotrophs at periods in the diel cycle outside our time of measurement may have left uptake in this group undetected with our techniques. Alternatively, this eukaryotic part of the phytoplankton community could have obtained their P from other sources. Organic-P in dissolved or particulate (via mixotrophy) form are both potential candidates. This system contained ca. 50 nM UV-oxidizable DOP-P (Krom et al., 2005). Since UV-oxidizable DOP probably was only a part of total DOP, there was at least $6 \times$ more P bound

up as DOP than in all the other pools of Fig. 9 together. A hydrolysis rate of only 0.3% of this DOP pool per hour would then produce enough P to support all biological needs as estimated in Fig. 9. This of course introduces a major uncertainty in our attempts to understand the P-cycle of this system. As an illustration, even a very small rate of photo-oxidation of this pool could thus be speculated to have a major controlling effect on the P-flow and thus presumably be central to the control of biological activity in this system. No such photoliberation of orthophosphate was, however, detected (V. Kitidis, personal communication). If the phosphate part liberated from DOP passes through a common pool of orthophosphate before being taken up by organisms, the uptake should have been detected by our method. The tracer added as $^{33}\text{PO}_4$ is assumed to mix instantaneously with such a common pool of orthophosphate, and uptake in all organisms using orthophosphate from this common pool should have been detected. In P-limited environments, however, hydrolysis has been found to be so tightly bound to the cell surface that some of the released phosphate is taken up directly (Tamminen, 1989), possibly escaping detection. Another intriguing potential P-source for this photosynthetic group would be through

mixotrophy. Such phagotrophic ingestion of other organisms as a source of “pelleted” P has been suggested to be stimulated by P-limitation (Nygaard and Tobiesen, 1991). Mixotrophic nanoflagellates have been reported in the Eastern Mediterranean (Christaki et al., 1999), though not found to give any substantial contribution to bacterial predation. Pitta et al. (2005) reports, however, a substantial (44%) contribution of mixotrophs to ciliate biomass.

In the unperturbed system we found a turnover time of 2.3–3.7 h, slightly higher than found by Moutin et al. (2002) (min. 1.1 h) in the Eastern Mediterranean. Since turnover times in P-limited limnic systems may be <10 min (e.g. Millard et al., 1996), one may wonder whether the level observed here really is indicative of a P-limited system. To evaluate this, one need to consider the osmotroph biomass involved. Assuming that uptake at low external concentrations is approximately proportional to concentration S_n , uptake rate V by the whole osmotroph community containing a P-biomass B_P becomes $V = \alpha S_n B_P$. The proportionality constant α is then a mean biomass-specific affinity for the whole osmotroph community. Insertion of this in the expression $T_t = S_n/V$ gives $T_t = 1/\alpha B_P$, or $\alpha = 1/T_t B_P$. With an osmotroph biomass <2 μm of 3.6 nM P (Fig. 9) and a turnover time of 3 h, this gives an estimated mean specific affinity of 0.093 nM P⁻¹ h⁻¹. Starting from the assumption of diffusion limitation, Thingstad and Rassoulzadegan (1999) estimated a theoretical maximum affinity for a 2 μm -diameter spherical cell to be about 0.05 nM P⁻¹ h⁻¹. Turnover times in the range observed for the unperturbed system thus seem well reconcilable with the hypothesis of P-limitation of the osmotroph community.

The estimates of P-requirement (v) based on standard C:P conversion of 106 and 50 for primary and bacterial production, respectively, gave a v -value of 0.07 nM P h⁻¹ in unperturbed water. Combined with a turnover time of 3 h this corresponds to a bioavailable orthophosphate concentration $S_n \approx 0.21$ nM PO₄. This may, however, be suspected to be an over-estimation since v was obtained including primary production >2 μm , and also assuming standard C:P ratios in phytoplankton and heterotrophic bacteria, respectively. Primary production <2 μm accounted for ca. 55% of a total of ca. 12 $\mu\text{gC m}^{-3} \text{h}^{-1}$. Retaining the assumption of a C:P ratio of 106 for the autotrophic picoplankton, gives an estimated requirement of 0.005 nM P h⁻¹.

Likewise, a bacterial production of 63 $\mu\text{gC m}^{-3} \text{h}^{-1}$ corresponds, with a C:P of 50, to a P-requirement of 0.05 nM P h⁻¹, summing up to 0.055 nM P h⁻¹. Assuming all this to come from the common pool of free orthophosphate, combination with an orthophosphate turnover time of 3 h gives an estimated concentration S_n of bioavailable orthophosphate of 0.17 nM PO₄-P. This reconciles well with conclusions from the observation that chemical determinations with a nanomolar technique (Krom et al., 2005) were below detection limit of ca. 1 nM on several stations in unperturbed water. It also agrees with the conclusion from limnic studies that concentrations of bioavailable orthophosphate in P-limited systems are below 1 nM (Hudson et al., 2000). A turnover time of the entire system can be calculated by using the P-requirement of 0.055 nM P h⁻¹ and the ca. 8 nM of particulate phosphate. Together these numbers give a turnover time of ca. 6 days. Hudson et al. (1999) and (2000) found turnover rates of ca. 5 days for particulate P in freshwater lakes.

It is worth noting that the structure suggested for microbial P-flow in Fig. 9 is very different from that of the associated C-flow. In heterotrophic bacteria, P-incorporation is relatively dominating when compared to C-incorporation since we have assumed a C:P of 50 for this process, P-requirement in phytoplankton >2 μm is small (4% of total) due to the very high C:P ratio implicit in the calculations for this fraction (see above). With 44% of ciliate biomass in mixotrophic species, there may also be an extra C-input via photosynthesis to the ciliate compartment. Another interesting aspect of the C flow emerges if one compares the bacterial production of ca. 30 $\mu\text{gC m}^{-3} \text{h}^{-1}$ with the total primary production of ca. 110 $\mu\text{gC m}^{-3} \text{h}^{-1}$. With a growth efficiency of 22% as estimated for the Eastern Mediterranean by Turley et al. (2000), bacterial respiration would be 106 $\mu\text{gC m}^{-3} \text{h}^{-1}$, almost twice the measured primary production. Our study thus adds to the cases reported with bacterial respiration exceeding phytoplankton production (del Giorgio et al., 1997).

4.2. Effects of the perturbation

The 2001 microcosm bioassay experiment suggests that the system outlined in Fig. 9 only has a capacity to consume not more than between 10 and 25 nM orthophosphate before some mechanism stops further consumption. Using this value for

our 2002 in situ experiment, the ca. 110 nM $\text{PO}_4\text{-P}$ addition should thus be well in excess of the system's capacity. With these numbers, a roughly $10 \times$ dilution with OUT water would be expected to be required before all excess P could be consumed and the system return towards pre-perturbation conditions. In 9 days, the patch spread from ca. 16 to $>400 \text{ km}^2$ (Law et al., 2005), i.e. with a volume increase of ca. $25 \times$ when negligible vertical mixing is assumed. Return to pre-perturbation limiting conditions should thus be expected before these 9 days. In general accordance with this estimate, most of our measurements suggest a shift back to pre-perturbation conditions around day 7–8.

As reported elsewhere (Zohary et al., 2005) the observation that T_i remained high when 25 nM PO_4 or more was added to microcosms was confirmed when water collected at an IN station was incubated in on-deck microcosms. Adding ammonia to such IN water, however, led to a rapid increase in chlorophyll and a concomitant reduction in T_i . This is the response expected if the in situ perturbation was halted at Step 4 (Fig. 1) due to lack of nitrogen required for biomass production and cell division.

Our measurements of particulate P suggested an approximate doubling from 8.4 to between 15 and 16 nM P, a level reached already on the first day after addition. This is an increase slightly below the consumption capacity of 10–25 nM phosphate that was estimated from microcosm experiments the previous year. Of the ca. 110 nM $\text{PO}_4\text{-P}$ added, ca. 8 nM thus apparently passed Step 2 in Fig. 1. With a Redfield ratio of N:P = 16, conversion of 8 nM P to biomass would correspond to an N-requirement of 128 nM N. This is a bit more than half the $230 \pm 60 \text{ nM N}$ estimated from bioassays as being the concentration of N in excess of P in unperturbed water (Thingstad and Mantoura, 2005). Based on this estimate there should thus be sufficient N for about 14–15 nM of the added $\text{PO}_4\text{-P}$ to pass on through Step 4 in Fig. 1. A decline in chlorophyll was, however, observed (Psarra et al., 2005). The relatively constant elevation over background level in particulate-P level observed for 1 week after the addition does also not suggest any substantial growth, supporting the same interpretation as the microcosm experiment referred to above; i.e. that another factor (presumably N) was blocking any major transmittal of the perturbation through Step 4. Note, however, that viruses are believed to release most of the host material as DOM and detritus (Bratbak et al., 1992). A rapid removal of produced

biomass by viral lysis, not included in Fig. 9 is thus in principle possible without any continued accumulation of particulate P.

Luxury consumption, i.e. nutrient uptake in excess of the immediate requirement for growth, is well described for both phytoplankton, including *Synechococcus* (Falkner et al., 1998) and for heterotrophic bacteria (Nissen et al., 1987). For both P-starved (Jansson, 1993) and N-starved (Cochlan and Harrison, 1991) cells, a pulse of the limiting nutrient results in a surge uptake, followed by a reduction in uptake rate as the internal cell quotas are filled up (Ducobu et al., 1998). If any subsequent increase in growth rate is prevented by the N supply, further uptake of P will only be expected to occur to maintain constant cell quotas. If this occurred here, one would for ca. 1 week have a system where, by turbulent diffusion, P-replete cells in IN water are exchanged with P-starved cells from OUT water. For particulate P to remain constant, there must have been a reservoir of free phosphate in the IN water from which the imported P-starved cells could fill their cell quota. Our finding of a reduction in IN water of the maximum uptake potential V^{max} to the level of the estimated P-requirement v , is consistent with such a scenario. Before addition V^{max} estimates were in the range $1\text{--}2 \text{ nM P h}^{-1}$ while the estimated P-requirement for growth was about an order of magnitude lower. With our method based on diluting the added tracer with “cold” orthophosphate, V^{max} values may have been underestimated since the time span for surge uptake may potentially have been exceeded at the higher concentrations. The ratio between v and V^{max} in unperturbed water may thus have been >10 . With V^{max} values found, 2–3 h would suffice for the uptake of the observed ca. 8 nM increase in particulate-P. Interestingly, there are some elevated values of V^{max} in the period where a transition back to P-starved conditions is suggested to occur (day 6–day 8, Fig. 8). Whether these are indicative of a short window in time when mixing of IN water containing excess-P with OUT water containing excess-N, had reached the balanced point, leaving the system with both nutrient in excess, is not known.

Estimates of V^{max} from uptake of $^{33}\text{PO}_4$ is a procedure independent of the estimation of v from stoichiometric conversion of primary and bacterial C-production estimates. With the differences in methodology and the many conversion factors involved, and also considering the fact that the

two cellular processes of P-uptake and C-biomass production in principle may be separated in time, comparison of V^{\max} and v may seem risky. More detailed studies of the stoichiometry of C and P uptake (Thingstad et al., 1996b) and comparison of S_n -estimates obtained as $S_n = vT_t$ (Moutin et al., 2002) have, however, indicated the feasibility of this approach. If biased, one would expect C:P ratios to be high in this P-limited systems, and our v -estimates to be too high. The real difference between V^{\max} and v in the unperturbed state would then be even higher, thus strengthening the conclusion of strongly P-starved osmotrophs in the unperturbed state of this system.

In the N-limited period, the P-uptake shifted clearly towards larger organisms. Whether this was caused by osmotroph organisms, for unknown reasons, becoming larger, or by a shift in uptake towards other osmotroph species with larger cells, is not known. If it is larger-celled phytoplankton already present that take over more of the orthophosphate uptake, the discrepancy between primary production and P-uptake in the $>2\mu\text{m}$ size fraction in the supposedly P-limited situation prior to PO_4 addition, becomes less pronounced in the supposedly N-limited post-perturbation period.

With the physiological state of the osmotrophs changed after PO_4 addition to one with filled cell quotas for P, and growth limited by nitrogen, further development depends crucially on which micro-organisms that, in this new situation, can get access to the N previously in excess. The traditional view has been that central organic N compounds like amino acids are primarily used by heterotrophic bacteria. In this environment, however, where the dominant phytoplankters are prokaryotic, this should perhaps not taken for granted (Zubkov et al., 2003). Transfer of some of the phosphate pulse via heterotrophic bacteria would be expected since bacteria in this region have been suggested to be P-limited (Zohary and Robarts, 1998), and the observation of a positive response in bacterial production suggests a response in production of bacterial C-biomass (Zohary et al., 2005). Such a response in heterotrophic bacteria would also require an excess pool of degradable organic substrates. The unperturbed system contained a large concentration of DOC (Krom et al., 2005). How much of this that is easily accessible to bacterial consumption is however unknown.

Increased production of heterotrophic bacteria would be expected to be followed by a rapid response in the predator food chain, presumably through the heterotrophic flagellates–ciliate link. In this system where the dominant phytoplankter is the small-celled ($<1\mu\text{m}$) *Synechococcus*, the consequence is somewhat unpredictable since this would rapidly stimulate predators that also feed on dominant fractions of the phytoplankton community (Fig. 9). A reduction of the phytoplankton community is thus a theoretical possibility.

Another interesting aspect of the response is that we apparently have a situation where the evidence for a change in osmotroph biomass *composition* are quite strong, but there is no evidence for any positive net changes in their *abundance or biomass*. The chlorophyll concentration actually went down, an a priori somewhat unexpected response to adding phosphate to a P-limited, or at least P-stressed, system. The consequence of shifting the system state from low to high cell quotas of P in osmotrophs is that food quality for predators (and parasites) changed, probably more than food concentration. A positive response was observed in fecal pellet and egg production, and also in gut content of herbivorous and omnivorous copepods (Pasternak et al., 2005). Elevated phosphate concentrations, decoupled from the SF_6 , were also found below the pycnocline at 16m, this could reflect the release of phosphate during remineralization of particulate material (Law et al., 2005). It is thus tempting to speculate that higher predators in this system may have been limited by food quality, e.g. lack of P, more than by food concentration in terms of e.g., carbon biomass, energy or prey abundance. Such limitation has been a central research issue in limnic research on *Daphnia* (e.g. DeMott et al., 2001) and also been suggested for marine copepods when phytoplankton are N-limited (Kiorboe, 1989). Cladocerans can prey on small sized micro-organisms (Burns and Schallenberg, 1996) while copepods seem to be inefficient at prey-sizes below $5\mu\text{m}$ (Levinsen et al., 2000; Turner et al., 2001). Unless copepods have adapted to particularly small prey in this ultra-oligotrophic environment, the effect may seem to have been transmitted through the ciliate link as indicated by the trophic structure suggested in Fig. 9. Interestingly, a small, but positive response was found in ciliate abundance (Pitta et al., 2005).

In conclusion, our observations are consistent with the hypothesis that, before addition, the

osmotrophs were P-limited, or at least, severely P-starved. In the time-span between addition and our first sampling, the osmotrophs changed physiological state and biomass composition from that characteristic of P-stressed cells, to a state consistent with N-limitation. Two not mutually exclusive hypotheses for further transmittal of the P-pulse through the food web are suggested: (1) a positive response in bacterial production and the subsequent food chain of bacterial predators, and (2) a predator response based on changes in food quality, rather than in food quantity. Using a mathematical model, the two mechanisms are analyzed in more detail elsewhere (Thingstad, 2005).

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