

## Titrating excess nitrogen content of phosphorous-deficient eastern Mediterranean surface water using alkaline phosphatase activity as a bio-indicator

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### Abstract

From both an academic and a management point of view, not only the question of which element limits production, but also the excess amount of the next potentially limiting element, is of importance. Activity of the enzyme alkaline phosphatase (APA) produced during phosphorous (P) starvation has been used as a technically easy and sensitive indicator of P limitation. We explore an extension of this technique to estimate also the excess of nitrogen (N) by titrating samples with phosphate until APA disappears. In ultra-oligotrophic eastern Mediterranean surface water, this technique indicated P limitation with a nitrogen excess of  $230 \pm 60$  nM N. Adding ammonia as internal standard gave a molar ratio of  $15 \pm 2$  for balancing amounts of added N and P. Taking advantage of a Lagrangian experiment with in situ phosphate addition, we also used the mirror technique of titrating with ammonia until APA appeared. Excess P inside the experimental patch was estimated to be  $33 \pm 2$  nM 4 days after the addition of 110 nM  $\text{PO}_4$  P. In this case, however, a molar N:P ratio of only  $3.0 \pm 0.2$  was determined for balancing amounts of added ammonia and phosphate. The technique should, in principle, estimate excess nutrients accessible on a time scale of ca. 3 d (the incubation time used here). The method applies regardless of the form the excess nutrients are in as long as they are bioavailable. It does not presume that the organisms exhibiting the limitation belong to any particular taxon or functional group as long as they produce APA when P-limited.

An understanding of which nutrient(s) limit growth rates and biomass in the pelagic photic zone is essential whether the perspective is one from management of marine systems or one from basic research in any of the disciplines of ocean biogeochemistry, pelagic food-webs, or the cell physiology of phytoplankton and bacterioplankton. Also important, not least in the management and biogeochemical contexts, is the question of what surplus there is of the next potentially limiting nutrient, i.e., how much of the primary limiting nutrient could be added to the system before it transits into another state with another secondary type of limitation. If this distance between the primary and secondary limiting element is small, the question of which one is pri-

mary and secondary would seem to be of little practical management importance.

Phytoplankton growth in the marine pelagic has traditionally been considered primarily to be nitrogen limited (Smith 1984). The picture has, however, become increasingly complex with the demonstration of iron (Coale et al. 1996; Kolber et al. 1994) or phosphate (Ammerman et al. 2003) limitation of phytoplankton and/or heterotrophic bacteria in large and important marine regions. As indicated by the traditional terminology, iron limitation in the so-called high nutrient–low chlorophyll areas occurs in areas where there is a large excess of bioavailable phosphate and nitrogen over the limiting concentration of iron (Kolber et al. 1994). In other areas, orthophosphate as well as mineral forms of nitrogen may be very low, in which case it is not obvious what element is limiting. Neither is it obvious in these cases what excess there may exist in the system of other bioavailable forms of the secondary limiting element. In systems where winter concentrations of phosphate and nitrate are skewed relative to the 16:1 (molar) Redfield ratio, the residual excess mineral forms tend to disappear as the most limiting forms approach zero (Redfield et al. 1963). Absence of the free mineral forms routinely measured

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does therefore not preclude that nutrients may be stored in the system, e.g., as extracellular dissolved organic forms or as intracellular storage materials. There is a large amount of literature on different types of bioassays used to determine nutrient limitation. In Mediterranean surface waters, such techniques based on physiological responses (bioassays) have been used to infer phosphorous (P) limitation of both phytoplankton (Bonin et al. 1989; Diaz et al. 2001) and bacterioplankton (Thingstad et al. 1998; Van Wambeke et al. 2002; Zohary and Robarts 1998), a conclusion also supported by biogeochemical evidence (Krom et al. 1991). While biogeochemical data (e.g., deep-water concentrations of mineral nutrients) may provide information regarding how the balance between different potentially limiting elements is skewed, we are not aware of bioassays that focus on determining the amount of the secondary limiting nutrient. We here explore the potential use of the enzyme alkaline phosphatase (AP) for this purpose.

The genes coding for AP belong to the *pho* operon present in both prokaryotic and eukaryotic organisms (see Torriani-Gorini et al. 1994 and references therein). AP activity (APA) has often been used as a molecular indicator of P limitation, or at least P starvation, in planktonic systems (e.g., Boekel and Veldhuis 1990; Carlsson and Caron 2001; Davies and Smith 1988; Jamet et al. 2001; Mykkestad and Sakshaug 1983; Møller et al. 1975; Nyman and Graneli 1983; Perry 1972; Petterson 1980; Pick 1987; Sakshaug et al. 1984; Thingstad et al. 1998). Using an artificial substrate with an organic part that increases in fluorescence yield when the phosphate-ester bond is split, the presence of APA can be readily detected with a sensitivity sufficient to measure APA in natural environments (Perry 1972).

With the ability to detect APA in natural samples from P-deficient pelagic systems, a potential exists for exploiting this to determine the system's reservoir of excess secondary limiting nutrient. In the Mediterranean, with its supply of iron via Saharan dust (Herut et al. 1999), this secondary limiting nutrient would be expected to be nitrogen. By adding orthophosphate to samples from such systems until the organisms no longer experience P deficiency, one would expect production of AP to be repressed. Upon degradation of the AP present, APA in the sample should then disappear. We explore the potential of using this principle to titrate the pool of excess nitrogen in a natural surface sample from the eastern Mediterranean, an environment suggested to be P-limited both from biogeochemical (Krom et al. 1991) and from biological evidence (Bonin et al. 1989; Zohary and Robarts 1998). Taking advantage of an in situ addition experiment of excess orthophosphate, we also explored the possible reciprocal titration of excess P by adding ammonia-nitrogen (N) to the sample until APA increased.

### Materials and procedures

This work was done during a cruise with R/V *Aegeo* to the warm-core Cyprus gyre in the eastern Mediterranean arranged as part of the EU project CYCLOPS (a more detailed description of the eddy can be found in Krom et al. 1992). Samples were

collected at 12-m depth using 10-L Niskin bottles and a rosette sampler. Experiments 1 and 2, establishing the effects of incubation with  $\text{PO}_4$  or  $\text{NH}_4$  amendments on APA, were done with water collected 15 May 2002 from Station 2CYC22 (33°20.23'N 32°17.35'E). Detailed experiments to determine excess concentrations of secondary limiting nutrients were performed on two samples collected 21 May 2002. One (OUT) sample, representing the natural situation, was collected at Station 2CYC44 (33°02.45'N 32°17.43'E). The other (IN) sample was collected at Station 2CYC43 (33°09.08'N 32°22.22'E), inside the experimental patch of a Lagrangian experiment where a nominal concentration of 110 nM  $\text{PO}_4$  P had been added to the upper mixed layer in a 4 × 4 km area on 17 May 2002. For further details of the experimental setup, see Laws et al. (in press).

Alkaline phosphatase activity was measured fluorometrically according to Perry (1972), using  $\beta$ -fluorescein phosphate as the artificial substrate. Samples supplemented with substrate were incubated in disposable cuvettes and fluorescence measured at 1 to 2 h intervals using a Turner Quantech™ filter fluorometer to determine the rate of increase over time. Stability of the substrate was checked using autoclaved seawater. Incubations were done in on-deck incubators with running surface seawater and subdued natural illumination.

For titration curves aimed at assessing excess N in the OUT water, the four-parameter decreasing sigmoidal function

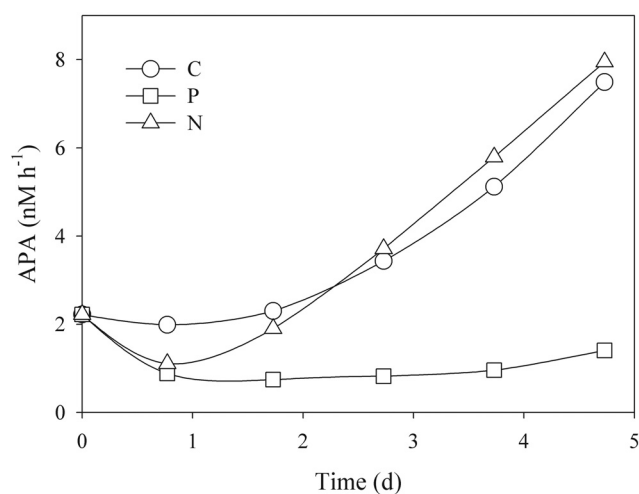
$$y = B + \frac{A - B}{1 + \left(\frac{x}{EC_{50}}\right)^\sigma} \quad (1)$$

was fitted to the part of the curve containing transition from high (A) to low (B) values of APA as a function of  $x$ , which indicates added  $\text{PO}_4$  P.  $EC_{50}$  corresponds to the effective concentration at which APA is reduced by 50%, and  $\sigma$  is the slope at  $EC_{50}$ . The initial part of the curves showed an increase in APA with increased  $\text{PO}_4$  addition, interpreted as stimulated AP-production when adding P to water from a P-limited environment (see Assessment). This part of the curve was not used for the fitting procedure.

For the mirror experiment adding  $\text{NH}_4$  N to assess excess P in IN water, the increasing sigmoidal function

$$y = A - \frac{A - B}{1 + \left(\frac{x}{EC_{50}}\right)^\sigma} \quad (2)$$

was fitted to the transition from low to high APA values for  $x$  as added  $\text{NH}_4$  N. y-axis intercepts of linear regression of  $EC_{50}$  values against the  $\text{NH}_4$  (OUT sample) or  $\text{PO}_4$  (IN sample) concentrations added as internal standards were used as the estimates of  $\text{PO}_4$  addition required to shift the OUT system to N limitation and the  $\text{NH}_4$  concentration required to shift the IN system to P limitation, respectively. The regression line slopes representing the N:P stoichiometric ratios in the two cases were used to convert the intercept values to excess N in the OUT system and excess P in the IN system, respectively. Fitting was done using built-in algorithms for pharmacology in the Sigmaplot



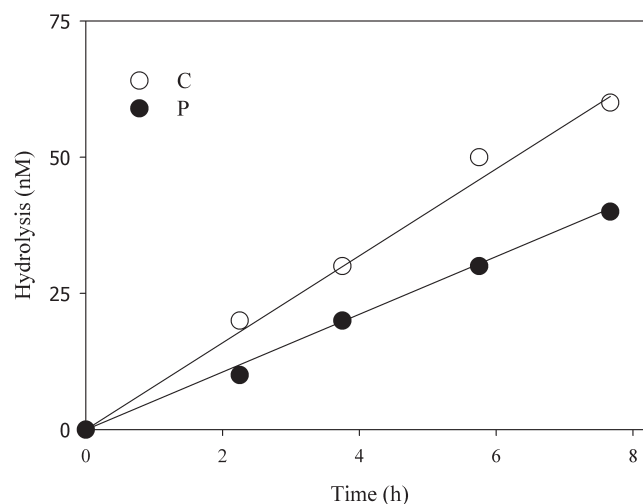
**Fig. 1.** Development of APA in a sample from 12-m depth in the Cyprus gyre incubated with 0.5  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$  (P), with 8  $\mu\text{M}$   $\text{NH}_4\text{Cl}$  (N), and without additions (C).

v.8.0 software (SPSS Inc.), providing parameter estimates with standard error. Further details are explained for each experiment.

### Assessment

*Experiment 1: APA activity in natural samples and the effects of incubating with excess phosphate or ammonia*—Not knowing the nutrient status in the water, this initial experiment was done to discern between two hypothesized possible situations: (1) The water contained detectable APA, and this would disappear upon addition of excess phosphate, or (2) there was no initial APA, but AP production could be initiated upon addition of ammonia. Situations 1 and 2 would be interpreted as indicating P or N limitation, respectively. Three 1-L polyethylene bottles were filled with water from station 2CYC22 on 15 May 2002. One was amended with 0.5  $\mu\text{M}$  final concentration  $\text{K}_2\text{HPO}_4$ , one with 8  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , and one was kept as control. An initial APA of 2.2  $\text{nM P h}^{-1}$  increased in the control bottle over 5 d of incubation to 7.5  $\text{nM P h}^{-1}$  (Fig. 1). In the phosphate-amended treatment, however, APA was reduced to 0.7  $\text{nM P h}^{-1}$  after 2 d, slowly recovering to 1.4  $\text{nM P h}^{-1}$  after 5 d incubation. A reduction in the ammonia-amended treatment after 1 d incubation recovered rapidly to regain the same levels as in the control for the rest of the experiment.

*Experiment 2: Inhibition or repression?*—Experiment 1 did not tell us whether the reduction in APA upon addition of phosphate was caused by an inhibition of the activity of enzymes present or a repression of enzyme production combined with inactivation of enzymes already present. This was explored by adding 0.5  $\mu\text{M}$   $\text{K}_2\text{PO}_4$  to water from the control bottle of experiment containing the high APA observed after 6 days of incubation. This resulted in a reduction in the slope from 8.0 to 5.3  $\text{nM P h}^{-1}$ , i.e., to a 34% inhibition of activity (Fig. 2). Most of the reduction in APA observed upon phos-

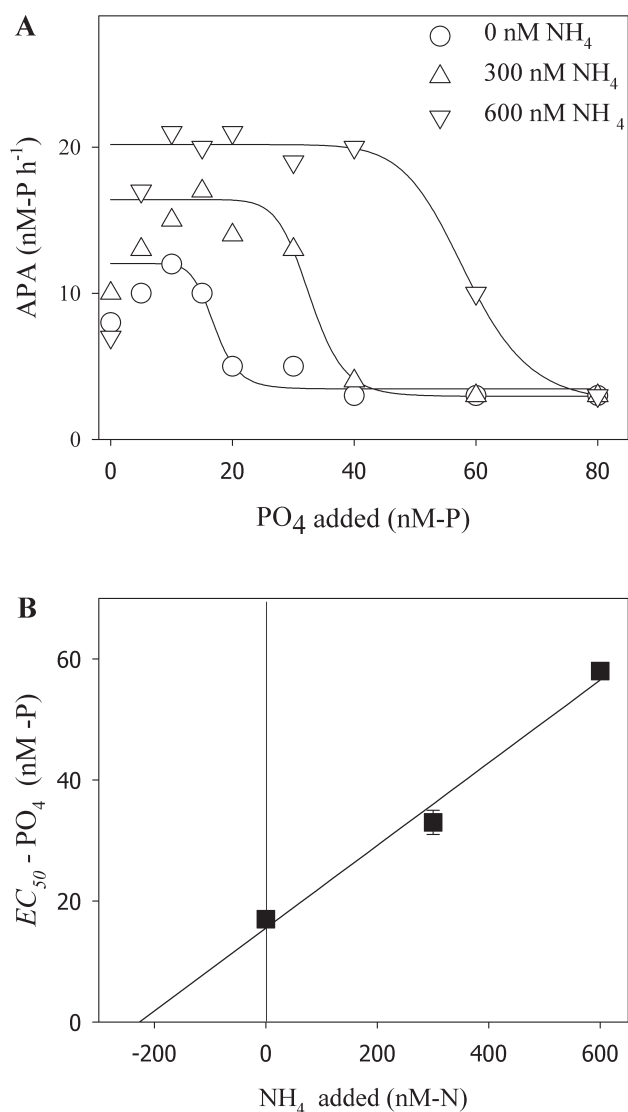


**Fig. 2.** Time course of hydrolysis after addition of the artificial substrate ( $\beta$ -fluorescein- $\text{PO}_4$ ) to an unamended subsample from the control bottle of Experiment 1 after 5 d of on-deck incubation (C), and one subsample with 0.5  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$  added at time 0 (P).

phate addition in Experiment 1 thus seems to be caused by repression of AP production, combined with inactivation of the existing enzymes.

*Experiment 3: Titration of excess nitrogen*—Experiment 1 demonstrated that addition of 500 nM phosphate was sufficient to stop AP production in the samples. To determine the critical concentration, water from the OUT sample was distributed into 15-mL Falcon tubes arranged in an array of 9 (columns)  $\times$  3 (rows). This arrangement allowed three 9-point gradients in added  $\text{K}_2\text{HPO}_4$  ranging from 0 to 80 nM P. All tubes in each of the three gradients were amended with  $\text{NH}_4\text{Cl}$  to give 0, 300, and 600 nM N, respectively, and incubated for 3 d. In the lower part of all three gradients with  $\text{PO}_4$  addition less than ca. 10 nM  $\text{PO}_4$ , APA increased with increasing phosphate additions. For higher P additions APA culminated and then dropped to background levels (Fig. 3A). As expected, the concentration of  $\text{PO}_4$  required to produce this reduction in APA increased with increased concentration of added  $\text{NH}_4$  (Fig. 3A). Disregarding the initial increasing parts of the observed curves, an  $EC_{50}$  value was obtained for each gradient by fitting decreasing sigmoidal functions (Eq.1). Linear regression of the three  $EC_{50}$  values against the  $\text{NH}_4$  concentrations added to the corresponding gradients (Fig. 3B, Table 1) gave a slope corresponding to a molar N:P ratio of  $15 \pm 2$ , well in agreement with the Redfield ratio of 16. The x-axis intercept of the regression line corresponds to an estimated  $230 \pm 60$  nM excess N in this system.

*Experiment 4: Addition of  $\text{NH}_4$  N to water amended with  $\text{PO}_4$  P in situ*—This investigation took place during a cruise primarily designed for an experiment with in situ addition of phosphate to surface waters in the Cyprus gyre. Taking advantage of this, we used a sample collected inside the experimental area (IN sample) 4 d after the in situ addition to explore the possible



**Fig. 3.** Natural water sample from 12 m. (A) Response in APA after 3 d incubation plotted as function of added PO<sub>4</sub> P. Experimental points for each of the three series amended with 0, 300, and 600 nM NH<sub>4</sub> N, respectively. Lines represent the fitted decreasing sigmoidal functions (Eq.1) as described in the text. (B) EC<sub>50</sub> values from the fitted sigmoidal functions in panel A plotted as function of the added NH<sub>4</sub> N concentrations. Error bars indicate the standard error of the EC<sub>50</sub> estimates.

induction of APA by adding NH<sub>4</sub> N to a potentially N-limited system. For this purpose, we used a design mirroring Experiment 3, but this time using an array with high resolution (7 columns) of the ammonia gradient ranging from 0 to 640 nM NH<sub>4</sub> N, and low resolution (3 rows) for phosphate additions of 0, 20, and 40 nM PO<sub>4</sub> P, respectively.

For all three gradients (rows), addition of NH<sub>4</sub> led to an increase in APA with progressively more NH<sub>4</sub> required as more PO<sub>4</sub> was added (Fig. 4A). Fitting the increasing sigmoidal function (Eq. 2) to each gradient (regression results summarized in Table 1) and performing a linear regression of the three EC<sub>50</sub> val-

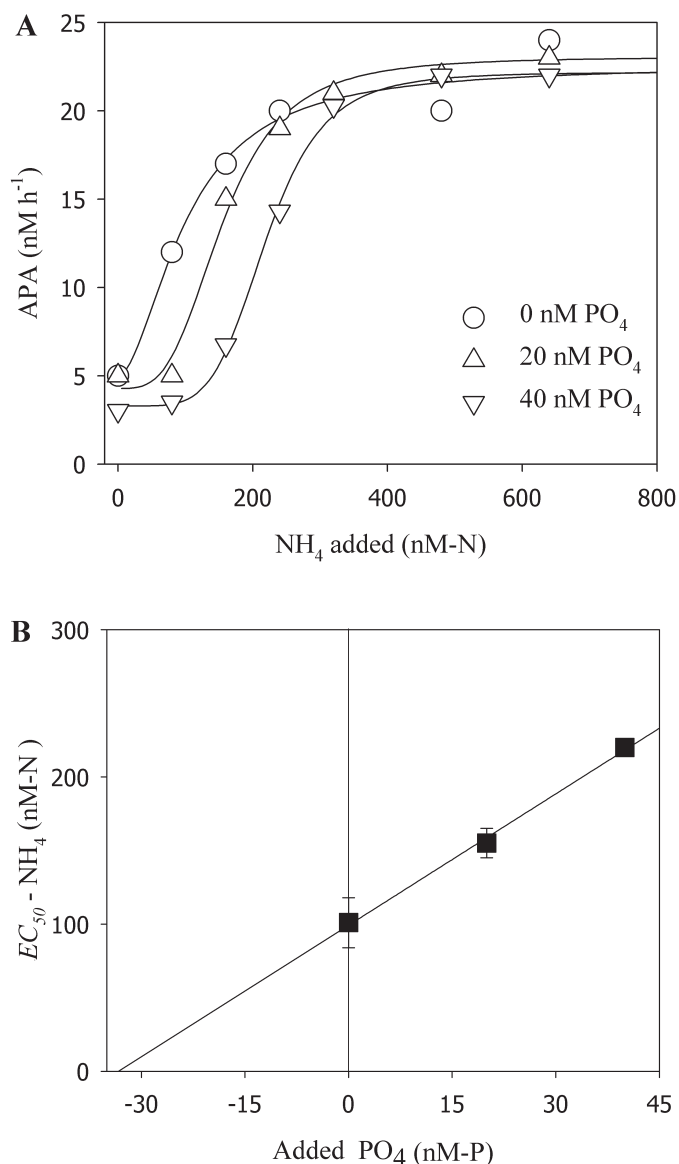
**Table 1.** Values for EC<sub>50</sub>\* and σ\* obtained from nonlinear regression of Eqs. 1 and 2 to the data shown in Figs. 3A and 4A, respectively. y-Intercept (y0) and slope (a) of the subsequent linear regressions of EC<sub>50</sub> versus added ammonia or phosphate in Figs. 3B and 4B, respectively

OUT sample: Sigmoidal regressions (Fig. 3A)					
nM NH <sub>4</sub>	EC <sub>50</sub> (nM PO <sub>4</sub> )	SE	σ	SE	R <sup>2</sup>
0	17	1	9	3	0.979
300	33	2	12	5	0.979
600	58	1	11	8	0.991
Linear regression of EC <sub>50</sub> against added NH <sub>4</sub> (Fig. 3B)					
y0 (nM P)	SE	a	SE	R <sup>2</sup>	
16	3	0.068	0.009	0.984	
IN sample: Sigmoidal regressions (Fig. 4A)					
nM PO <sub>4</sub>	EC <sub>50</sub> (nM NH <sub>4</sub> )	SE	σ	SE	R <sup>2</sup>
0	101	17	1.8	0.6	0.975
20	155	10	3.3	0.6	0.990
40	220	4	5.1	0.4	0.999
Linear regression of EC <sub>50</sub> against added PO <sub>4</sub> (Fig. 4B)					
y0 nM N	SE	a	SE	R <sup>2</sup>	
99	4	3.0	0.2	0.997	

\*EC<sub>50</sub> corresponds to the effective concentration at which APA is reduced by 50%, and σ is the slope at EC<sub>50</sub>.

ues against the PO<sub>4</sub> concentrations added to the corresponding gradients, resulted in an estimated excess P of 33 ± 3 nM, which can be compared to the 110 nM PO<sub>4</sub> P added 4 d previously. In this case an N:P-stoichiometry of 3.0 ± 0.2 was obtained, and for unknown reasons, was well below the Redfield value of 16.

*General comments on results*—A potential activity in the undisturbed water of 2.2 nM P h<sup>-1</sup> (Fig. 1) released from dissolved organic phosphorous (DOP) by alkaline phosphatase activity may appear low. Considering, however, that total particulate P in this ultra-oligotrophic region was only ca. 8 nM (Flaten et al. in press), this potential, if realized, could supply an amount of phosphate equivalent to the total (autotrophic + heterotrophic) microbial biomass in 3 to 4 h, a value comparable to that estimated for the much more eutrophic P-limited brackish layer of a Norwegian fjord (Thingstad et al. 1993). The presence of APA, disappearing when incubated with excess PO<sub>4</sub> (Fig. 1), thus adds an extra element to the existing series of evidence for P limitation in the eastern Mediterranean, which otherwise includes high nitrate:phosphate ratios in deep waters (Krom et al. 1991), the presence of a nitrate residue of 300 to 1000 nM NO<sub>3</sub> N but undetectable phosphate at the end of the spring bloom (Krom et al. 1991; Kress and Herut 2001), and bioassays demonstrating phytoplankton (Bonin et al. 1989) and bacterial (Zohary and Robarts 1998) responses to phosphate addition. The dominating P reservoir (> 50 nM) in this region is the DOP pool, but this is considered not bioavailable (Table 2). Knowing neither the enzyme kinetics nor the in situ concentration of AP substrates, we have not attempted to quantify the role of AP hydrolysis of DOP in the P cycle of the area. Neither do we know from this



**Fig. 4.** Water collected at 12 m inside the experimental patch 4 d after in situ addition of PO<sub>4</sub> P. (A) APA after 3 d incubation plotted as function of added NH<sub>4</sub> N. Series amended with 0, 20, and 40 nM PO<sub>4</sub> P, respectively. Lines represent the fitted decreasing sigmoidal functions (Eq. 2) as described in the text. (B) EC<sub>50</sub> values from the fitted sigmoidal functions in panel A plotted as function of the added NH<sub>4</sub> N concentrations. Error bars indicate the standard error of the EC<sub>50</sub> estimates.

investigation which organisms produced the AP detected and cannot specifically attribute the P stress to heterotrophic bacteria, phytoplankton, or species within such trophic groups.

The specific objective of this investigation was not to collect additional evidence for the P-limited status of the eastern Mediterranean, but to explore the possible use of APA as indicator for titrating excess N in this P-limited environment. At the time of this experiment, the post-spring bloom nitrate residual of 300 to 1000 nM nitrate had disappeared. The ~230 nM bioavailable excess N estimated to remain could be stored

in the system as ammonia, bioavailable DON, or inside the biota. With ~70 nM NH<sub>4</sub> N (Table 2), ~160 nM N thus appears to remain in the system in some of the other bioavailable forms. The deficit (~70 – 770 nM-N), may have been lost either to unavailable DON or exported via sedimentation. At PO<sub>4</sub> additions less than ca. 10 nM PO<sub>4</sub>, we found an increase in APA with increasing PO<sub>4</sub> dose (Fig. 3A). We interpret this as growth of the originally P-starved organisms during 3-d incubation of the samples. Using a Redfield 16:1 ratio, growth on 10 nM added PO<sub>4</sub> would require ca. 160 nM N. For higher PO<sub>4</sub> additions, we found the expected decrease in APA. Free phosphate will both inhibit the activity of existing AP and repress the production of new enzyme. We estimated the immediate inhibition effect of orthophosphate on APA to be 34% (Fig. 2). APA has been shown to degrade over a time scale of 1 d, particularly when subject to UV radiation (Garde and Gustavson 1999). Consistent with this, we found the main activity drop to have occurred 1 d after adding excess phosphate (Fig. 1). Using an incubation time of 3 d for the titration experiments thus seemed sufficient to remove existing AP when production of new enzyme has been repressed. Ammonia additions increased the amount of PO<sub>4</sub> required to remove APA in the expected 16:1 molar ratio between NH<sub>4</sub> and PO<sub>4</sub> additions. Both the observed pattern in Fig. 3 and the reasonable magnitude of the estimated excess N thus leads us to conclude that the proposed methodology could be used for the purpose of estimating excess N.

In the mirror experiment using IN-patch water from the Lagrangian experiment, we estimated excess P to be 30 ± 3 nM. This corresponds reasonably well with the ca. 20 nM PO<sub>4</sub> P that can be estimated to remain out of the initial in situ addition of ca. 110 nM PO<sub>4</sub> P to the patch 4 d earlier, taking into account the subsequent dilution of the patch as estimated from the SF<sub>6</sub>-tracer added along with the phosphate (Law et al. in press). The method thus also seems to work for estimating excess P in N-limited situations. For reasons not well understood, the stoichiometry between NH<sub>4</sub> and PO<sub>4</sub> additions in this case was 3, i.e., much lower than the expected Redfield ratio of 16. A shift in community composition toward organisms with low biomass N:P ratio as a response to the PO<sub>4</sub> addition may be suggested as one possible underlying mechanism. Stimulation of heterotrophic bacteria with their generally lower than Redfield biomass N:P ratio (Fagerbakke et al. 1996) would be one such possibility.

### Comments and recommendations

A method has been presented that uses the physiological response of alkaline phosphatase induction/repression to identify not only the most limiting substrate, but also the amount available of the next potentially limiting substrate. The method was tested in the ultra-oligotrophic eastern Mediterranean. As presented here the method is somewhat labor intensive. We here used one 15-mL Falcon tube for each point in the arrays of combined ammonia-phosphate additions and read the samples manually. Provided the biological

**Table 2.** Summary of nutrient information for the investigated area

Nutrient	Concentration	Reference
PO <sub>4</sub> *	< 5 nM P	Krom et al. in press
UV oxidizable DOP*	50 to 60 nM P	Krom et al. in press
NO <sub>3</sub> *	< 10 nM N	Krom et al. in press
NH <sub>4</sub> *	50 to 80 nM N	Krom et al. in press
DON*	5 × 10 <sup>3</sup> to 10 × 10 <sup>3</sup> μM N	Krom et al. in press
Particulate P*	ca. 8 nM P	Flaten et al. in press
Excess N*	230 ± 60 nM N	This method
Estimated remaining P of the 110 nM PO <sub>4</sub> added. Calculated from patch diffusion.†	23 nM P	Law et al. in press
Measured PO <sub>4</sub> by nanomolar technique†	1.5 nM P	Law et al. in press
Estimated excess P†	30 ± 3 nM P	This method

\*Outside the experimental patch of the Lagrangian experiment.

†Station (2CYC43) used for Experiment 3 inside the experimental patch.

reactions work well in micro-well plates, modification of the method to assays based on micro-well plates read in a fluorescence plate reader should be straightforward, making the method suitable for monitoring programs, for example.

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