

Measurement of ectoenzyme activities as an indication of inorganic nutrient imbalance in microbial communities

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ABSTRACT: Assessment of nutrient limitation in microbial populations in marine environments is an important topic. Existing methodologies generally demand long incubations in an experimental assay manipulating nutrients. We suggest a novel approach using the ratio between 2 ectoenzymatic activities, alkaline phosphatase (APA) and aminopeptidase (AMA), to evaluate nutrient limitation of microbial communities *in situ*. APA is used as an indication of P limitation and AMA of N limitation. The ratio bypasses the use of biomass estimations to calculate specific activities, which are error-prone and time-consuming. Our results from enrichment experiments have shown that the ratio APA:AMA increases in P-deficient treatments, and decreases after inorganic P addition. After P-deficient nutrient addition (n treatments) the ratio APA:AMA increased to 90–230%, whereas following N+P balanced additions the ratio decreased to 30–70% of the initial value. These results demonstrate that this approach is easy, quick and gives a general insight into the nitrogen versus phosphorus nutrient balance of the communities without the need for biomass estimations or measurement of actual nutrient concentrations.

KEY WORDS: Alkaline phosphate · Aminopeptidase · N:P ratio · Microbial community

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INTRODUCTION

Nutrient deficiency of microorganisms has been assessed in various environments, especially in aquatic ecosystems, where variations of phosphorus (P) or nitrogen (N) deficiency may be important in the growth and collapse of blooms (Healey & Hendzel 1976). There is recent controversy about which is the nutrient commonly limiting primary production in the oceans (Downing 1997, Tyrrell & Law 1997, Tyrrell 1999, Guildford & Hecky 2000). However, in some localized areas, P has been identified as the limiting nutrient (Cotner et al. 1997, Wu et al. 2000), e.g. in the Mediterranean (Berland et al. 1980, Bonin et al. 1989, Krom et al. 1991, Thingstad et al. 1998, Zohary &

Robarts 1998). In spite of many attempts to find suitable indicators, there is no optimal approach for rapidly discerning between N and P nutrient limitation in natural planktonic communities.

A possible indirect way of rapidly evaluating nutrient deficiency of cells is by the determination of ectoenzyme activities. Nutrient limitation may induce cells to synthesize ectoenzymes to enable the acquisition of the limiting nutrient (Hoppe 1983). Ectoenzyme activities may thus serve as a proxy to nutrient limitation, not by indicating the amount of the proper substrate present, but by pointing towards the physiological state of the cell or of the total microbial population. Specifically, alkaline phosphatase activity (APA) has been used in many environments as an indicator of phosphorus deficiency (Pettersson 1980, Smith & Kalff 1981, Gage & Gorham 1985, Vrba et al. 1995, Rose & Axler 1998). Aminopeptidases (AMA) hydro-

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lyze peptides and proteins, which comprise a major part (and probably the most utilizable fraction) of the marine organic N pool (Henrichs et al. 1984, Coffin 1989, Keil & Kirchman 1991). Recently, Patel et al. (2000) have shown negative correlations between inorganic nitrogen concentrations and AMA activities. Leucine-methyl coumarinamide, an artificial fluorogenic substrate suitable for estimating aminopeptidase activities (Hoppe et al. 1988), may likewise serve as an indicator of N limitation. These enzymes, however, can be partially constitutive and should be corrected by the biomass of the producers. They can be synthesized by bacteria and by phytoplankton (Hooper & Hughes 1992, Martinez & Azam 1993, Berges & Falkowski 1996). Therefore, accurate biomass estimations of all organisms producing 1 enzyme class to yield biomass-specific enzyme activities are further complicated. In order to bypass the use of biomass estimations, we suggest using the ratio between alkaline phosphatase and aminopeptidase activities (APA:AMA) as an indicator of N versus P limitation of the whole microbial community. We expect APA to be relatively more important than AMA in P-limiting conditions and the other way around when N is the limiting nutrient. Therefore, the APA:AMA ratio should be relatively high when P limitation prevails and relatively low when N limitation is more severe than P limitation. These changes in community enzyme activity could be due either to physiological adaptation or to changes in species composition. This reasoning leads us to hypothesize that nutrient additions with different N:P ratios should result in alterations of the APA:AMA relationship.

To test the validity of this approach, we carried out microcosm experiments to examine the response of natural plankton communities to different nutrient inputs.

MATERIAL AND METHODS

Description of the experiments. Long-term microcosm experiments: We performed 2 long-term microcosm experiments with water from the Catalan coast (Masnou, 20 km north of Barcelona) and from ca 1 km offshore. The first experiment (continuous-enrichment) was carried out in October 1997, and the second (single-pulse enrichment) in March 1998.

Surface water (0.5 m depth) was collected in plastic (75 l) containers previously washed with a sodium hypochlorite solution. The water was taken to the laboratory in less than 1 h and prescreened with a Nylon mesh (150 μm). Microcosms were constituted by six 15 l cylindrical containers incubated at *in situ* temperature a 12 h light:12 h dark cycle. Light intensity was 250 $\mu\text{E m}^{-2} \text{s}^{-1}$. Turbulence was generated in the microcosms by vertically oscillating plastic grids to avoid set-

ling of organisms. This allowed us to generate turbulence at levels up to 0.055 $\text{cm}^{-2} \text{s}^{-3}$, similar to strong wind events in coastal waters (Peters & Redondo 1997). Duplicate treatments consisted of N and N+P enrichment (n and np), and a control (k) without additions. Sampling took place daily using a glass tube connected to a silicone tube. The first milliliters of each subsample were discarded.

Microcosms were incubated for 4 d in the continuous-enrichment and for 7 d in the single-pulse experiment. In the latter, after 7 d, P was added to 1 of the replicates of Treatments n and np, and the response of the community to the new nutrient conditions was monitored every 6 h.

Short-term single-pulse experiments: Five short-term experiments (with 4 treatments) were run on 5 consecutive days in September 1994. Treatments in duplicates consisted of additions of N (n), P (p), N+P (np), and a control (k) without additions. Water was taken at 10 m depth at the 'Point B' sampling station, a few hundred meters off the coast at Villefranche-sur-Mer, France, Northwestern Mediterranean Sea. Samples were incubated for 1 d in 1 l acid-washed bottles at seawater surface temperature (flow-through deck incubators) with natural light.

Nutrient additions: In the long-term continuous-enrichment experiment, nutrients were added daily and consisted of 1 μM NaNO_3 in n treatments (P-deficient), and 1 μM NaNO_3 and 0.06 μM Na_2HPO_4 in np treatments (nutrient-balanced). To both n and np treatments, silicate (2 μM $\text{Na}_2\text{SiO}_3 \cdot \text{H}_2\text{O}$) and a trace-metal mix (Guillard 1975) were also added. Controls (k) were left unamended.

In the long-term single-pulse experiment, nutrients were added in a single input before Time 0 (t_0). Final concentrations of the pulse were 16 μM NaNO_3 , 0.1 μM phosphorus Na_2HPO_4 (n treatments), and 16 μM NaNO_3 , 1.0 μM Na_2HPO_4 in np treatments. The N:P ratios of the additions were 160 for n and 16 for np. Treatment np was therefore, nutrient balanced while Treatment n was P deficient. Silicate (30 μM $\text{Na}_2\text{SiO}_3 \cdot \text{H}_2\text{O}$ final concentration) and a trace-metal mix were added to both n and np treatments. After 7 d, an additional P (1 μM) pulse was added to one of the replicates (a) of Treatments n and np, while the b replicates were unaltered. The experiment was then continued for 30 h with 6 h subsampling intervals for microbial parameters to evaluate the response of this second pulse.

Additions in the short-term enrichment experiment had final concentrations of 1 μM PO_4 (p treatments), 5 μM $\text{NO}_3 + \text{NH}_4$ equally distributed (n treatments), and both nutrient additions in the np treatments. Nutrients were added in a single-pulse at t_0 .

Parameters measured. Chlorophyll *a* (chl *a*) was estimated fluorimetrically in the long-term experiments

(Yentsch & Menzel 1963). Between 15 and 20 ml samples were filtered through Whatmann GF/F filters. The filters were ground in 90% acetone and left for at least 2 h in the dark at room temperature for chl *a* extraction. The fluorescence of the extract was measured with a Turner Designs fluorometer.

Inorganic nutrients (PO_4 , NO_3 , NO_2 , NH_4) were measured following the standard procedure described by Grasshoff et al. (1983). Total inorganic nitrogen (TIN) was calculated by adding the molarity of NO_3 , NO_2 , NH_4 . The N:P ratio is molar and is calculated by dividing TIN values by PO_4 values.

Bacterial concentration in the long-term experiments was estimated by flow cytometry following a methodology described in Gasol & del Giorgio (2000). Samples (1.5 ml) were fixed with 0.15 ml of a 10% paraformaldehyde and 0.5% glutaraldehyde mixture and stored at -80°C . At the time of determination, samples were thawed and run through a FACScalibur (Becton and Dickinson, San Jose, CA, USA) flow cytometer with laser emission of 488 nm. Bacteria had previously been stained with SYTO13 (Molecular Probes Inc., Eugene, OR, USA) at 1.6 μM . Samples were run at low flow speed (about 12 $\mu\text{l min}^{-1}$) and data acquisition was in log mode until about 10 000 events were acquired. As an internal standard we added 10 μl of a solution containing yellow-green 0.92 μm latex beads (Polysciences Inc., Warrington, PA, USA) at a concentration of 10^6 beads ml^{-1} . Bacteria were detected by their signature in a plot of side-light scatter versus green fluorescence. In the short-term experiments, bacterial numbers were obtained by epifluorescence microscopy (Zeiss Axioplan, 100 \times objective) after 2% formaldehyde-fixing and DAPI staining of seawater filtrate on 0.2 μm polycarbonate filters (Porter & Feig 1980).

In the long-term experiments, as an estimation of bacterial activity we measured the rate of incorporation of tritiated leucine into protein following the method of Kirchman et al. (1985) with the Smith & Azam (1992) modifications as described by Kirchman (1993). Tritiated leucine from Amersham (151 mCi mmol^{-1}) was brought to a concentration of 1 μM by mixing with non-radioactive leucine at 1:9 hot:cold, and dispensed (final conc. 40 nM) to 1.2 ml of sample. This concentration was enough to saturate incorporation according to the curves obtained during the experiment. We used 3 replicates and 2 blanks killed with 50% TCA, and incubated them for between 1 and 2 h in the dark at 18°C . The incubation was stopped with 50% TCA. Samples were rinsed twice by centrifugation in 5% TCA and then counted in a Packard scintillation counter after addition of Optiphase Hisafe II scintillation cocktail.

For determination of the APA and AMA activities in the long-term experiments, we used fluorogenic sub-

strates (Hoppe 1983, 1993) and followed a modification of the methodology described by Sala & Güde (1996, 1998). The substrates 4-MUF-P-phosphate and leucine 7-amido-4 methylcoumarin were dissolved in a 1:10 methylcellosolve:sterile water solution, or in sterile water, respectively, to 2.4 mM. Substrates were added at saturating concentrations (200 μM final conc.) to 1 ml subsamples in replicates. Fluorescence in the samples was measured immediately after addition of the substrate (t_0), and after an incubation of 1 h (t_1). Incubation was in the dark at room temperature. Fluorescence was read on a Shimadzu spectrofluorometer RF-540 at 365 nm emission and 446 nm excitation wavelengths. Increase of fluorescence units during the incubation time was converted into activity by preparing a standard curve with the end product of the reaction, 4-methylumbelliferone for APA, and 7-amino-4-methylcoumarin for aminopeptidase. In the short-term experiment, APA and AMA were measured following a similar procedure to that described above, but using final concentrations of 1.5, 3, 6, 12, 24 and 48 μM of the same substrates in 5 ml samples, and calculating V_{max} using a non-linear model assuming Michaelis-Menten kinetics.

Specific activities were calculated by dividing ectoenzyme activities by the sums of bacterial and algal biomass in terms of carbon. Bacterial concentration was converted to biomass by the conversion factor of 12 fg C cell $^{-1}$ (Simon & Azam 1989). Chlorophyll concentration was converted to algal biomass by applying the conversion factor 1 g chl *a* = 50 g C, which is the mean value of the measurements of Antia et al. (1963).

The ratio APA:AMA was calculated by dividing alkaline phosphatase by aminopeptidase activity.

RESULTS

Initial conditions of the water used for each experiment are shown in Table 1.

Initial nutrient concentrations in long-term experiments were lower in the continuous-enrichment than in the single-pulse experiments. The single-pulse experiment started with the highest density of microorganisms.

Specific activities, both APA and AMA, were higher in the continuous-enrichment than in the long-term single-pulse experiments. The APA:AMA ratio was about 3 to 6 times lower in the short-term than in the long-term experiments.

Long-term continuous-enrichment experiment

Nutrients, chl *a* and bacterial concentrations, as well as ectoenzyme activities in the long-term continuous-

Table 1. Initial conditions of the water used for the experiments. bd: below detection; nd: not determined

Initial values	Long-term continuous	Long-term single-pulse	Short-term single-pulse
TIN (μM)	bd	0.97	nd
Phosphate (μM)	0.02	0.05	nd
N:P ratio	?	19.4	nd
Chlorophyll <i>a</i> ($\mu\text{g l}^{-1}$)	0.32	0.90	nd
Bacterial conc. (cells ml^{-1})	1.13×10^6	1.78×10^6	$4.79\text{--}7.85 \times 10^5$
Bacterial production (pmol leucine $\text{l}^{-1} \text{h}^{-1}$)	nd	164	nd
Specific APA ($\mu\text{mol } \mu\text{g C}^{-1} \text{h}^{-1}$)	3.16	1.05	nd
Specific AMA ($\mu\text{mol } \mu\text{g C}^{-1} \text{h}^{-1}$)	1.66	0.70	nd
APA:AMA	1.90	1.79	0.22–0.59

enrichment experiment are shown in Fig. 1. A bloom of phytoplankton was formed only in the np treatment, with maximum chl *a* concentrations ($3.18 \mu\text{g l}^{-1}$) reached on Day 3. Bacteria in np achieved their maximum concentration on Day 2 ($3.22 \times 10^6 \text{ cell ml}^{-1}$) and stayed at this level for the rest of the experiment. Specific APA, as APA per total carbon content of bacteria and phytoplankton, showed clearly higher values in the treatments with no phosphorus addition (up to $11 \mu\text{mol } \mu\text{g C}^{-1} \text{h}^{-1}$) than in the treatment where P was added. In spite of inorganic phosphorus additions, P concentration in the np treatment was always below $0.1 \mu\text{M}$. Total nitrogen concentration in k controls was low (below $0.53 \mu\text{M}$) during the experiment. Nitrogen in the n treatments increased steadily due to the daily additions, reaching a maximum concentration of $4.4 \mu\text{M}$ on the last day. In the np treatments, however, nitrogen concentrations increased only slightly and then leveled off, with values between 0.68 and $1.16 \mu\text{M}$. Specific AMA did not show a clear pattern throughout the experiment, ranging from 1 to $3 \mu\text{mol } \mu\text{g C}^{-1} \text{h}^{-1}$.

The N:P ratio was clearly higher in the P-deficient (n) than in the other treatments during the experiment (between 24 and 89). The APA:AMA ratio was clearly lower in the np treatment than in the k and n treatments. The n treatment exhibited ratios twice as high as those in the np treatment throughout the experiment, with a maximum of 4.55.

Long-term single-pulse experiment

The results of the single-pulse enrichment experiment are shown in Fig. 2, and replicates are plotted separately in order to evaluate the effect of an extra P addition after Day 7 in one of the n and np replicates. As in the continuous-pulse experiment, the addition that clearly stimulated algal growth was np, and a peak was detected on Day 4 in both replicates (18 and $20 \mu\text{g chl } a \text{ l}^{-1}$), followed by a decrease to $5.4 \mu\text{g chl } a \text{ l}^{-1}$ on Day 7. N additions caused a slow increase in

chl *a* to 8 and $10 \mu\text{g chl } a \text{ l}^{-1}$ up to Day 7. Chl *a* concentrations stayed low in the controls (0.16 and $1.42 \mu\text{g l}^{-1}$).

Bacterial concentration initially increased in all treatments during the first 2 d of incubation and then decreased to a minimum on Day 5. In the np treatments, the collapse of the phytoplankton bloom up to Day 6 was followed by a steady increase in bacterial abundance, which reached 2.2 and $2.8 \times 10^6 \text{ cells ml}^{-1}$ on Day 7. In both the k and the n treatments, bacterial numbers showed a continuous decrease towards the end of the experiment. Bacterial production showed an initial peak in the n and np treatments of about $500 \text{ pmol leucine (leu) l}^{-1} \text{h}^{-1}$ on Days 1 and 2. Production then decreased and remained low in the n treatment, and increased to 1262 and $1527 \text{ pmol leu l}^{-1} \text{h}^{-1}$ in the np treatment.

Nutrient concentrations were always low in the controls, below $0.16 \mu\text{M N}$ and $0.12 \mu\text{M P}$. Initial N concentrations in the n and np treatments were ca $20 \mu\text{M}$, and decreased continuously. The decrease in N was more marked in the np treatments, declining to less than $0.2 \mu\text{M}$. The n treatments showed P levels similar to those in the k treatment during the experiment (below 0.11). In the np treatments, P decreased slowly from an initial $1.1 \mu\text{M}$ to almost undetectable concentrations on Day 5.

The APA:AMA ratio was clearly highest in the n treatments from Day 4 onwards. In the k treatments, values were between 1 and 2, and in the np treatments always below 1, suggesting that the consumption of both nutrients occurred at a similar rate and deficiency gradually increased simultaneously for both nutrients.

The highest specific APA was also found in k ($8.5 \mu\text{mol } \mu\text{g C}^{-1} \text{h}^{-1}$ at Day 7), and was the lowest in the np treatments, which had a P addition.

Response to additional phosphorus pulse

In the long-term single-pulse experiment, a high concentration of P was added to 1 replicate of the n and np

[n (a) and np (a)] treatments on Day 7 (Fig. 2). Addition of P to the np (a) container did not seem to have any clear effect, since no differences in chl *a* and bacterial numbers were found between np (a) and np (b). TIN concentration was close to the detection limit, and P in np (a) increased to almost 1 μM due to the addition, followed by a slight decrease to 0.8 after 30 h. Bacterial production showed a similar pattern in both treatments np (a) and np (b). The ratio APA:AMA remained low (≤ 0.5) after the P pulse. No changes in either specific AMA or APA were observed in any np treatment.

In the n treatments, however, the replicate with additional P pulse (a) did deviate from the replicate with no such addition. Chlorophyll *a* increased slightly (to 10.8 $\mu\text{g l}^{-1}$) in the n (a) container, while in n (b) no changes were

detected. The increase in bacterial numbers in n (a) was 5-fold compared to n (b) 30 h after the addition. Bacterial production reflected this increase in bacterial concentrations in the n (a) container by a 10-fold increase compared to n (b). N concentration in the n (a) decreased more rapidly than in n (b), probably due to a higher utilization of nitrogen in response to the addition of the deficient nutrient (phosphate). The added phosphate (1 μM) decreased steadily to a final concentration of 0.4 μM .

Short-term and long-term experiments

The results of 5 short-term experiments (4 treatments) are summarized in Fig. 3. They are represented as the

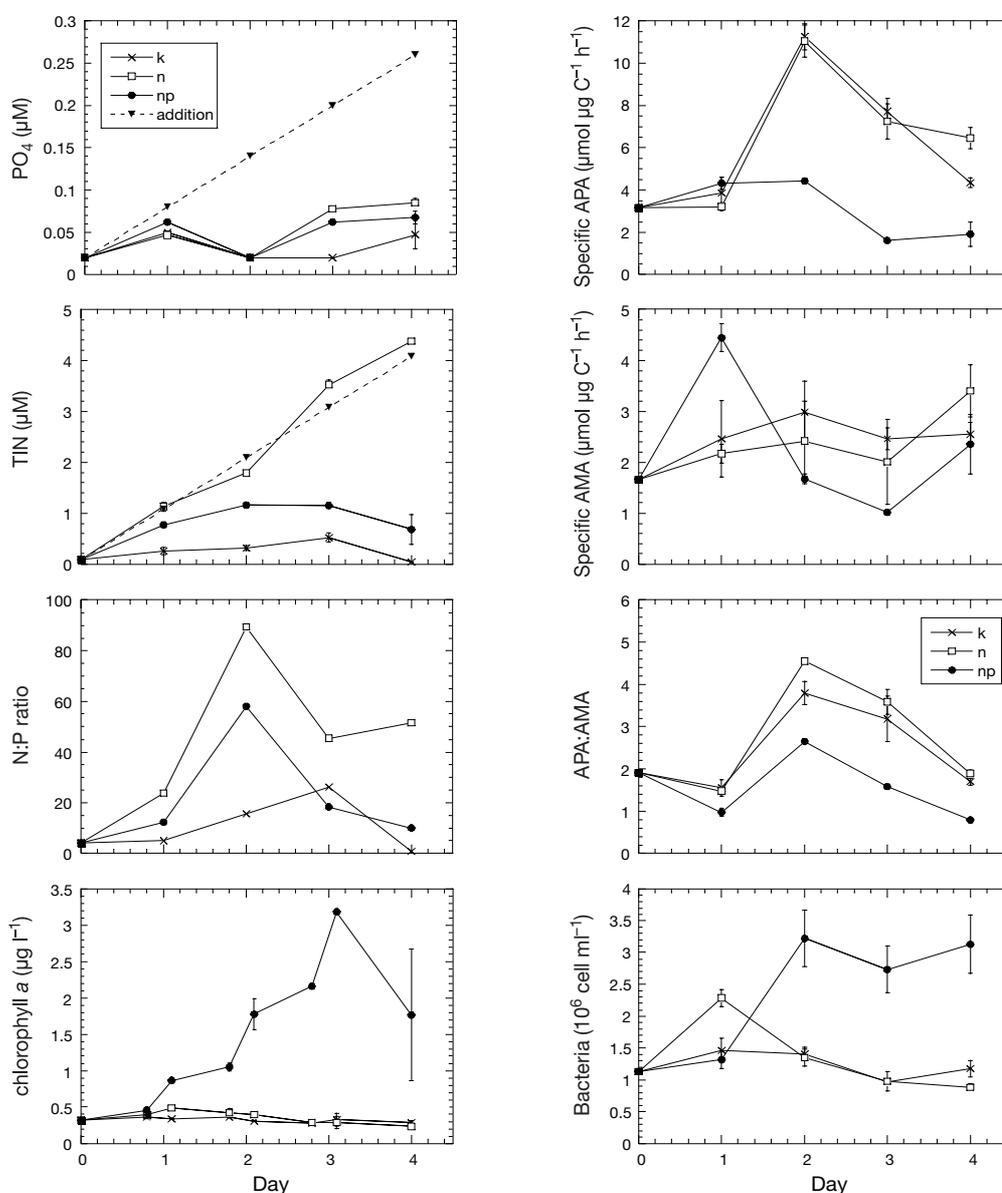


Fig. 1. Parameters measured in the long-term continuous-enrichment experiment. Mean of 2 replicate microcosms per treatment. k: control without addition; n: N addition; np: N and P addition. Additions were daily; dashed line (addition) shows the theoretical concentration after addition of the nutrient to the microcosms. TIN: total inorganic nitrogen; error bars = SE

percentage of the initial APA:AMA ratio for the treatments with N, P, or N+P addition (n, p and np), and the control (k). The results indicate that the addition of N modified the ratio only slightly compared to that without N addition. P addition, however, caused a clear de-

crease in the APA:AMA ratio, which was detectable whether the addition was P or P+N.

A similar summary of the APA:AMA ratio was made for the long-term experiments (Fig. 4a,b) using the pooled data of both experiments. In the long-term

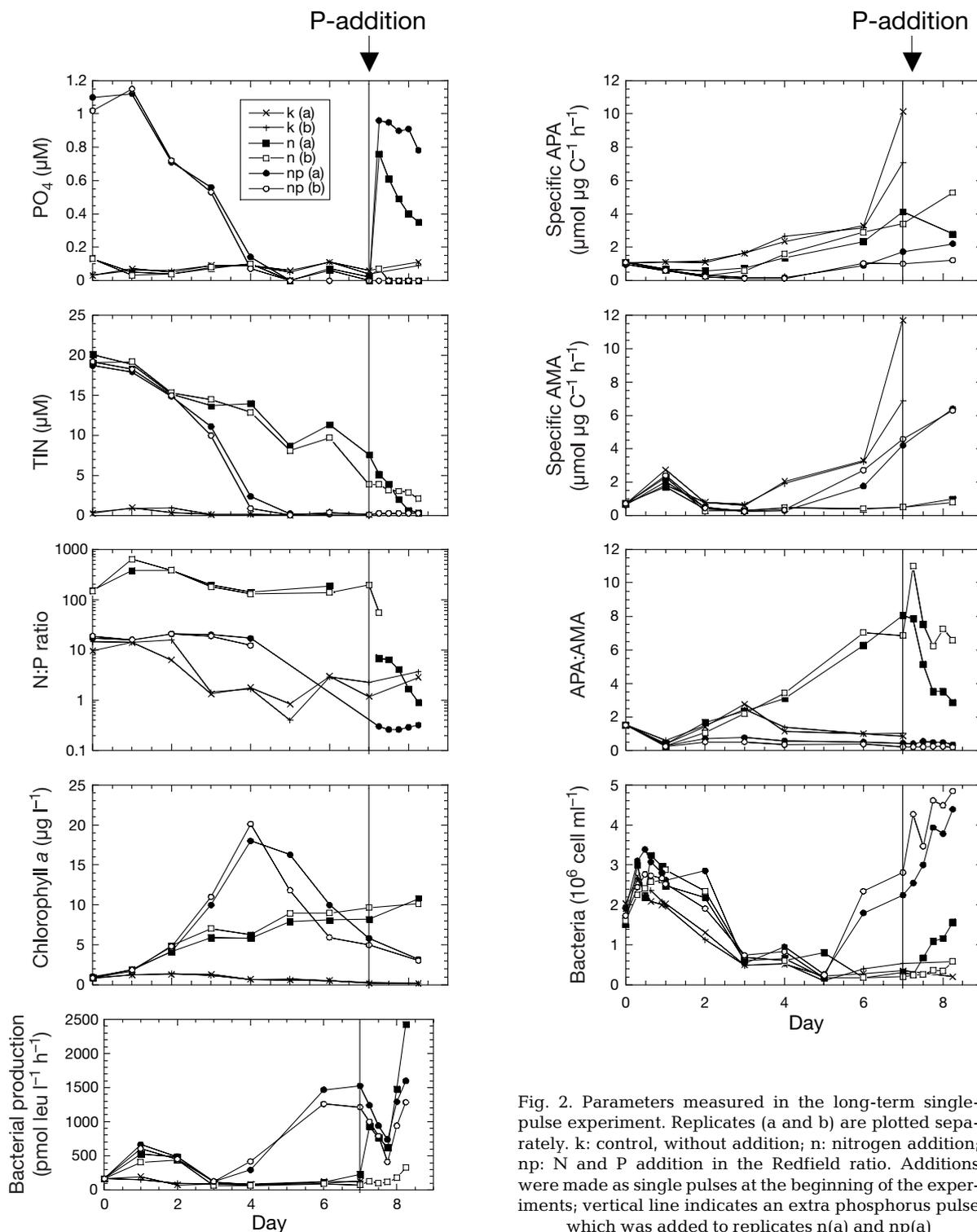


Fig. 2. Parameters measured in the long-term single-pulse experiment. Replicates (a and b) are plotted separately. k: control, without addition; n: nitrogen addition; np: N and P addition in the Redfield ratio. Additions were made as single pulses at the beginning of the experiments; vertical line indicates an extra phosphorus pulse which was added to replicates n(a) and np(a)

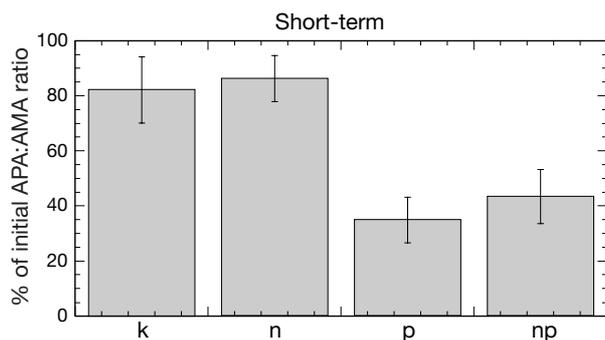


Fig. 3. Mean percent changes in APA:AMA ratios with respect to the initial ratio before nutrient additions during 5 short-term experiments (4 treatments: k, control without addition; n: N addition; p: P addition; np: N+P addition). Changes were calculated after 1 d incubation for each experiment. Error bars = SE of the 5 experiments

continuous-enrichment experiment we used data from Days 1 to 4, and in the single-pulse experiment data from Days 1 to 7. We calculated the percentage of increase in the ratio measured each day from the initial ratio, i.e. the ratio at t_0 . The results showed a clear decrease in the APA:AMA ratio when P was added due to repression of APA activity. Addition of N did not have a consistent effect on the APA:AMA ratio. In the continuous-enrichment experiment, no clear difference from the control could be seen, while in the single-pulse experiment a 3-fold increase was detected. From these responses, we interpret P to have been always limiting, while the degree of initial N limitation was more variable among the different experiments.

DISCUSSION

Extracellular enzyme activity is the initial response of a microbial community to environmental changes (Karner & Rassoulzadegan 1995) and our results indicate that ectoenzymes can indeed be appropriate physiological indicators of the nutritional status of a biological system. We believe that the APA:AMA ratio is a good example of this general principle and can be used successfully to assess N:P nutrient imbalance in aquatic communities.

In oligotrophic environments, N and P concentrations are often not measurable with standard methods. Further, in many cases more than 1 nutrient is below detection limits. Besides conceptual problems, this clearly presents a technical challenge in the study of nutrient limitation. A direct methodology indicating the degree of nutrient limitation of *in situ* microplanktonic populations through assessment of the cells' physiological condition would be a concrete asset. A

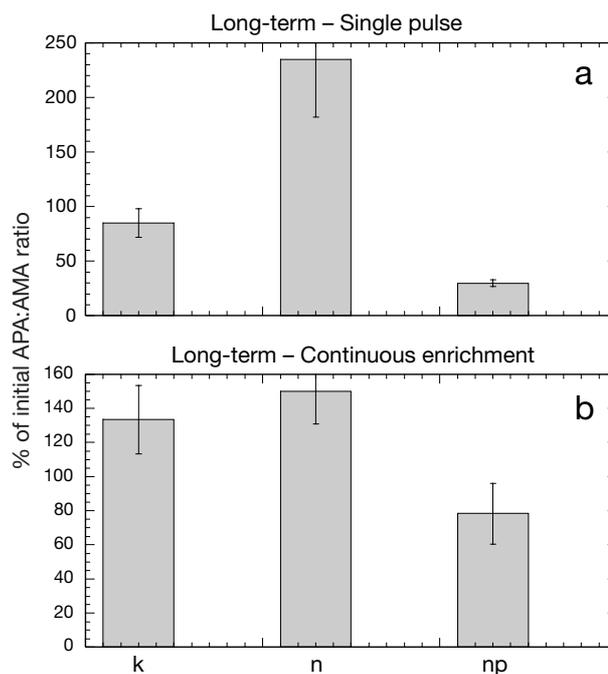


Fig. 4. Mean percent changes in APA:AMA ratios with respect to the initial ratio before nutrient additions in the long-term experiments. Changes were calculated throughout whole experiments: 7 d for the (a) single-pulse experiment, 4 d for the (b) continuous-enrichment experiment. k: control without addition; n: N addition; p: P addition; np: addition of both N and P. Error bars = SE of the daily measurements

large number of studies using different methodologies for the evaluation of nutrient limitations have been published. Biochemical studies generally include determinations of the elemental, macromolecular and pigment composition such as the protein:DNA index (Berdalet et al. 1996). However, current biochemical composition reflects the physiological history of a cell and therefore may not reflect current deficiency, i.e. the cells nutritional status (Istvánovics et al. 1992). Estimation of the physiological processes within the microbial populations seems to be the most suitable approach for evaluating the nutritional status of cells, since these reflect a cell's immediate environment. The most studied physiological processes are photosynthesis, carbon and nitrogen metabolism, and nutrient uptake and ectoenzyme activities. The most direct methodologies target nutrient turnover times, generally those of orthophosphate (Lean & Pick 1981, Thingstad et al. 1998) or, in the case of algae, the P-deficiency index (Istvánovics et al. 1992). These methodologies, however, are time-consuming and use radiolabelled substrates.

Other approaches are bioassays, which consist of measuring the response to an addition of the deficient nutrient as an increase in bacterial numbers or production (Schweitzer & Simon 1995, Thingstad et al. 1998),

or algal growth (Bonin et al. 1989). This usually requires long incubations in which the community might change and be altered by the enclosure.

The use of the APA:AMA ratio to assess nutrient limitation is a novel approach. Individually, however, both APA and AMA activities have been previously used as physiological indicators, especially APA in algae. Gage & Gorham (1985), studying Minnesota lakes, found that APA was inversely related to cellular phosphorus in the particulate organic matter. APA was significantly enhanced in phosphorus-deficient phytoplankton (Vrba et al. 1995), and has been shown to be a good indicator of phosphorus deficiency in lake-water cultures (Smith & Kalff 1981). APA is induced by phosphorus limitation in algae (Kuenzler & Perras 1965, Healey 1975). However, other authors (Jansson et al. 1988) have recommended that APA should not be used as an indicator of phosphorus deficiency in natural plankton assemblages unless other P-deficiency indicators are included. AMA has already been used as indicator of bacterial activity (Andersen-Elvehoy & Thingstad 1991) and of the flux of combined amino acid nitrogen (Jacobsen & Rai 1988).

Ectoenzyme activities alone may not give an idea of the real nutritional status of the community, since they are partially constitutive and the amount of biomass of the producers can thus play an important role in the total level of activity. Therefore, activities need to be related to other parameters which can indicate the biomass of potential enzyme producers. To calculate specific enzyme activities, one must first identify the organisms which, when undergoing certain nutrient deficiency, are able to synthesize the necessary ectoenzymes. Ectoenzymes can be produced by a large number of microorganisms, such as heterotrophic nanoflagellates (Karner et al. 1994) or zooplankton (Bochdansky et al. 1995, Jamet & Boge 1998), although their main producers are generally algae and bacteria. Several authors have investigated the relative importance of bacterial, algal and free enzymes by selective filtration and evaluation of the activity in each size fraction. Such selective filtration may include sources of error: (1) the size of the filter may not accurately separate the different functional groups; (2) filtration pressure may detach ectoenzymes from cells; and (3) some filters may retain proteins, and therefore enzymes, altering the relative contribution of each size fraction to the total activity (Karner & Rassoulzadegan 1995). The results in the literature on the relative contribution of functional microbial groups to the total activity of an enzyme show large variability, not only between environments, but also between seasons. AMA has been generally associated with heterotrophic bacteria (Vives-Rego et al. 1985, Chróst 1991, Münster et al. 1992). However, Chróst and co-authors

found the bacterial fraction to account for only up to 12% during a bloom breakdown in Lake Plußsee (northern Germany) (Chróst et al. 1989), or about 10% in nutrient-manipulated enclosures (Chróst & Rai 1993). Alkaline phosphatase has been reported to be produced both by algae and bacteria, although dissolved APA may contribute to a highly variable degree (Francko 1983). A large percentage of the total APA activity has been generally attributed to algae; nevertheless, this percentage may vary widely over the year (Chróst & Overbeck 1987). Chróst & Overbeck found that the algal fraction dominated APA production during bloom conditions, while bacteria dominated in winter. Münster et al. (1992) found about 50 to 70% of APA freely dissolved in the water column. All this uncertainty has led several authors to avoid cell counts as an estimate of biomass for the calculation of specific activity. Some have opted for normalizing APA by estimating total biomass with ATP (Healey & Hendzel 1980), or with total particulate organic matter, POM (Healey & Hendzel 1979, Pettersson 1980). POM, however, is not very appropriate for systems where detritus is abundant. Other studies have assumed that all activity is produced by bacteria in the case of AMA, or by algae in the case of APA, and have calculated specific activities using bacterial numbers or chl *a* concentration, respectively. However, such a procedure can only be valid when the production of an enzyme is almost entirely attributable to a particular group of organisms.

Our estimates of specific activities (Figs. 1 & 2) were calculated after bacterial counts and chl *a* concentrations had been converted to carbon and then summed. We thus assumed that enzyme activities were produced by both algae and bacteria. The results thus obtained provide information about the limitation status for each nutrient.

The complexity and assumptions required to calculate specific activities lead us to emphasize the utility of the APA:AMA. Our data clearly showed the usefulness of the ratio as an indicator of the dominance of N versus P deficiency. In Fig. 2, for example, we found the highest specific APA in k (control) conditions, where both N and P were limiting, while the highest ratio APA:AMA was detected in n enclosures (P-deficient treatment).

The ultimate aim of our study was to present an equation that would allow us to infer a N:P ratio from a APA:AMA ratio. However, when comparing N:P ratios with APA:AMA ratios in our microcosms, the r^2 value (0.459, $n = 66$) indicates that APA:AMA does not completely reflect N:P. It is not surprising that the residual nutrient concentration does not reflect the rate-limiting step in the chain of nutrient regeneration, since production and consumption are tightly balanced. Ecto-

zymes are produced to solve an anticipated shortage of N or P and rectify the nutrient imbalance. Additionally, the complex origin of enzyme activities includes constitutive enzymes and various possible enzyme-expression regulation mechanisms in various microbial subgroups. Similarly, the N:P ratio of the microbial environment certainly displays wide qualitative variability that remained undetected in our experiments—as in most other similar studies, even those focusing on nutrients exclusively. We can, however, show that the trends in APA:AMA ratios clearly did reflect the nutrient imbalances we experimentally induced.

Substrate induction for expression of certain microbial enzymes has been reported by Chróst (1989). It has also been demonstrated, however, that production of enzymes such as APA is likely to reflect deficiency of the hydrolysis end product (Chróst et al. 1989). Enzyme activity response to nutrient variability is rapid compared to changes in biomass and species composition, as shown in Fig. 2 for the second part of the long-term single-pulse enrichment experiment. After Day 7, the response to the extra P addition in the np containers differed from that in the n conditions. The extra P addition in the np (a) containers did not increase the APA:AMA ratios over those in the np (b) containers, to which P was not added. The lack of response in this ratio indicates that both N and P had already been consumed at that point of the experiment (concentration values were almost to undetectable values: Fig. 2), and therefore we can assume that both N and P were limiting. In contrast, the treatment designed to induce relative P deficiency, i.e., the n treatment, responded quickly to Day 7 P addition. After only 6 h, the APA:AMA ratio showed clear divergences, decreasing in n (a) containers to which P had been added, and displaying higher values in n (b) containers (without P addition) (Fig. 2). It seems that the first responders to the new nutrient condition were bacteria. Increases in bacterial numbers were evident as early as 6 h, increasing about 20-fold over the 24 h following the P pulse in this experiment. Conversely, chl *a* concentrations only increased 1.5-fold (Fig. 2). The bacterial response was also reflected in the 10-fold increase in bacterial production after P addition. Incidentally, excess N was consumed as rapidly as newly added P, indicating the dominance of P limitation over N limitation (Fig. 2). We believe that in the long-term experiments there must have been a change in bacterial and algal species. This change, however, must have been concomitant with changes in the nutrient concentration, and therefore, enzyme activities, as shown by Pinhassi et al. (1999).

Our results have shown that the ratio between the ectoenzyme activities alkaline phosphatase and aminopeptidase (APA:AMA) clearly reflects experimentally

induced nutrient imbalances. We believe that this approach is easy, quick, and provides insight into nutrient deficiency in the whole microbial ecosystem. The proposed ratio would be useful for monitoring short-term changes or fluctuations in nutrient availability. However, further research is needed to extend the application of this ratio to different systems.

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