Kinetics of alkaline phosphatase activity, and effect of phosphate enrichment: a case study in the NW African upwelling region

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ABSTRACT: Alkaline phosphatase (AP) kinetic experiments were performed in a broad range of trophic conditions in the transition zone between the North African upwelling and the open ocean, in order to investigate the effect of nutrient richness on kinetic parameters. Turnover times decreased from 224 h in the open ocean to 13 h in the upwelling region. $K + S_n$ showed the lowest values at the most eutrophic station (27 nM), while at this station V_m reached its maximum value of 2.1 nM h^{-1}. Nevertheless, as multiphasic kinetics occurred, kinetic parameter values depended largely on the fitting technique used. Response of AP activity (APA) to phosphate enrichment was also studied along a trophic gradient at the surface and the chlorophyll maximum level. Inhibition of APA reached 50% with only 0.1 µM of phosphate added, and rose to 80 to 96% inhibition by additions of 1 µM at the oligotrophic stations, whereas in the most eutrophic station, phosphate amendments had no effect. Inhibition was higher at the surface and towards oligotrophy. The lack of inhibition in the upwelling station and the low $K + S_n$ encountered suggest that the APA of eutrophic waters was mainly from bacterial origin. This supports the hypothesis of APA being mainly involved in supplying easily assimilable organic carbon to bacteria in eutrophic waters.

KEY WORDS: Alkaline phosphatase · Kinetic parameters · Phosphorus · Trophic gradient · Turnover

INTRODUCTION

Phosphorus in marine systems has received limited attention, since growth and production of planktonic organisms in the ocean have traditionally been considered as limited by the availability of nitrogen. Growing evidence that P is the limiting nutrient in some coastal systems (Thingstad et al. 1993) and oligotrophic oceans (Cotner et al. 1997, Karl & Yanagi 1997) have resulted in several recent studies being conducted (Rivkin & Anderson 1997, Thingstad et al. 1998, Zohary et al. 1998, Cañellas et al. 2000, Van Wambeke et al. 2002).

Phosphate is frequently exhausted in the euphotic zone of the oceans. It often represents less than 25% of the total dissolved phosphorus pool in these systems, and the remaining 75% occurs in the form of dissolved organic phosphorus and inorganic polyphosphate

compounds (Karl & Yanagi 1997). Consequently, the cycling of TDP could potentially control P availability and affect biomass and production of the phytoplankton and bacterial communities.

The importance of microbial enzymatic activities to the mobilisation, transformation and turnover of organic and inorganic compounds in aquatic environments has been proved during the last 2 decades (e.g. Hoppe 1983, Chrôst 1990, Cotner & Wetzel 1991). The use of combined P compounds requires the action of specific hydrolytic enzymes such as phosphatases (Perry 1972) and nucleotidases (Ammerman & Azam 1985). Alkaline phosphatase (AP) is the most characterised enzyme in studies of degradation of organic phosphorus compounds (e.g. Kobori & Taga 1979, Hino 1988, Ammerman & Azam 1991, Hantke et al. 1996).

Most of the studies dealing with AP have been conducted in freshwater systems (e.g. Chrôst & Overbeck 1987, Pick et al. 1987, Boavida & Heath 1988, Hernández et al. 1996) and in coastal or freshwater-influenced marine systems (Taft et al. 1977, Hoppe 1983, Thingstad et al. 1993, Nausch 1998, Cotner et al. 2000). However, data in marine oligotrophic environments are scarce (e.g. Perry 1972, Cotner et al. 1997, Thingstad et al. 1998, Cañellas et al. 2000, Van Wambeke et al. 2002). Studies carried out in several coastal marine habitats concluded that AP hydrolyses ~10 to 50% of the total soluble non-reactive phosphorus pool (Strickland & Solorzano 1966, Taft et al. 1977, Kobori & Taga 1979). Originally, AP was thought to be mainly synthesised by phytoplankton (Perry 1972, Chrôst & Overbeck 1987), but it is widespread in bacteria (Kobori & Taga 1979, Martinez et al. 1996) and is also produced by zooplankton (Boavida & Heath 1984, Jansson 1988). The phosphatase group of enzymes has broad substrate specificity and hydrolyses ester bonds between phosphates and dissolved organic molecules, making phosphate available for cellular assimilation. As they may be regulated (induced or repressed) by the occurring ambient inorganic phosphate concentration or by intracellular P concentrations (Chrôst & Overbeck 1987), alkaline phosphatase activity (APA) has been widely used as an index of P-status of phytoplankton cells. On the other hand, the mechanism of regulation of bacterial phosphatases seems to be linked to the C cycle, as bacteria can produce APA to supply their metabolism with the organic C substrates which are also final products of phosphoester hydrolysis (Chrôst 1990, Siuda & Güde 1994). Thus, APA seems to have a multiple function in aquatic environments by supplying the pools of phosphorus and available organic carbon simultaneously.

In order to provide more information about APA characteristics in the marine system, we studied this activity in different trophic scenarios. The Canary Islands region is characterised by an intricate hydrological pattern of mesoscale structures, which leads to the occurrence of a broad range of trophic conditions in short spatial scales, and thus provides an appropriate study area for testing APA behaviour in marine systems.

Here, we carried out kinetics experiments at stations of different eutrophic level, ranging from the oligotrophic open ocean to the upwelling area. We also examined the effects of phosphate amendments on hydrolysis rates to see the regulation of APA by phosphate along a trophic gradient.

MATERIALS AND METHODS

Field data were obtained on board BIO 'Hespérides', on cruise FAX-9908, from 5 to 27 August 1999. The cruise was carried out within the CANIGO EU project framework.

Hydrological data. At each station, a General Oceanics Mk III CTD was used to obtain vertical profiles of temperature, salinity, density and fluorescence. Water samples for nutrient and biological determinations were collected at discrete depths (from 0 to 500 m) with 24×12 l Niskin bottles from a rosette sampler attached to the CTD. Inorganic nutrients were measured colorimetrically by continuous flow analysis, using a Technicon-Bran Luebbe AA II AutoAnalyzer.

Chl a. Chl a (hereafter referred to as chl) was estimated fluorometrically by means of a Turner Designs bench fluorometer, previously calibrated with pure chl (Sigma), following the recommendations of Holm-Hansen et al. (1965). Seawater samples (500 ml) were filtered through Whatman GF/F fibre filters. Pigments were extracted on-board in cold acetone (90% v/v) for 24 h. Extracted chl samples were used to calibrate the voltage readings of the submersible fluorometer (Sea-Tech 57S) linked to the CTD unit. Water-column integration of chl concentration was performed with the Kaleida Graph Software.

Dissolved inorganic phosphorus (Pi), total phosphorus (TP) and particulate phosphorus (PP). For Pi and TP determination, water samples were immediately frozen at –20°C and preserved frozen until their analysis. Dissolved inorganic phosphorus was estimated as soluble reactive phosphorus by the malachite green method (Fernández et al. 1985). For PP determinations 2 l samples of water were filtered through Whatman GF/F fibre filters and then preserved frozen (–60°C) until their analysis. TP (water samples) and PP (filters) were digested by means of the perchloric-nitric acid procedure (Sommers & Nelson 1972) and afterwards estimated as soluble reactive phosphorus by the malachite green method (Fernández et al. 1985).

Diffusive Pi fluxes across the nutricline. Diffusive fluxes were estimated assuming that the only properties controlling the diffusion coefficient, Kz, are the dissipation rate of kinetic energy (ε) and the buoyancy frequency (N^2), thus:

$$Kz = A\varepsilon N^{-2} \quad (m^2 \ s^{-1})$$

where A is a empirical constant close to 0.25 (Linden 1971, Oakey 1982). ε, under pure wind-stress forcing, should follow:

$$\varepsilon(z) = u^{\star 3}/kz \quad (m^2 \ s^{-3})$$

where k ~ 0.4 is von Karman's constant, z is the vertical coordinate and $u^\star$ is the friction velocity, expressed as:

$$u^\star = fc U_{10} \quad (m \ s^{-1})$$

where fc is an empirical coefficient (0.00123, Oakey & Elliott 1982) and U_{10} is the mean wind speed 10 m

above the sea surface ($U_{10} \sim 10$ m s^{-1} for the cruise period).

N^2 can be derived directly from the CTD profiles according to the equation:

$$N^2 = (g/\rho_w)(\partial\rho/\partial z) \quad (s^{-1})$$

where g is the acceleration due to gravity (9.8 ms^{-2}), ρ_w is seawater density (1027 kg m^{-3}) and $\partial\rho/\partial z$ is the vertical density gradient.

Vertical phosphate fluxes across the nutricline were calculated from the product of the gradient of phosphate concentration across the nutricline, determined by linear regression analysis, and the estimated mean diffusion coefficient for the nutricline layer.

Proteins. Proteins were determined according to the Peterson's (1977) modification of the Lowry et al. (1951) method, using a protein assay kit provided by Sigma. Water samples (5 l) were concentrated on GF/F filters. Proteins were extracted by grinding the filters with Lowry reagent directly and diluting with water afterwards. Sodium dodecylsulfate included in the Lowry reagent facilitated the dissolution of relatively insoluble lipoproteins. Replicate assays were run for each sample. BSA standards with protein concentrations between 4 and 400 µg l^{-1} were run at the same time to obtain a calibration curve.

Bacterial abundance. Heterotrophic bacteria (HB) were counted by flow cytometry, using a FACScalibur (Becton & Dickinson). Samples (4 ml) were fixed with 2% final concentration of formaldehyde, incubated for 15 to 30 min at 4°C, and then stored frozen in liquid nitrogen until analysed. To count HB, 200 µl of sample was stained with a DMS-diluted SYTO-13 (Molecular Probes) stock (10:1) at 2.5 µM final concentration. Bacteria were identified by their signatures in a plot of side scatter (SSC) versus green fluorescence (FL1). Samples were run at low speed until 10 000 events were acquired. A solution of yellow-green 1 µm latex beads ($\sim10^6$ beads m l^{-1}) was added as an internal standard (Polysciences). Cell abundances were calculated from bead concentrations. The bead solution was checked daily through epifluorescence microscopy counting. The conversion factor for calculation of bacterial biomass was 20 fg C cell^{-1} (Lee & Fuhrman 1987).

Alkaline phosphatase activity (APA). APA was measured using the fluorogenic substrate methyl-umbelliferyl phosphate (MUF-P; Sigma). MUF-P was dissolved in cold sterile artificial seawater and then diluted to obtain the working solutions that were frozen until use on board. The methyllumbelliferon (MUF) produced after the assay was detected as increase in fluorescence by using a Shimadzu RF-3501 PC spectro-fluorometer. A standard curve with MUF (Sigma) was used to quantify the amount of MUF produced from APA.

Kinetic approach: Kinetic measurements were performed at the chl maximum level at 3 stations of different hydrographical characteristics to study regional variation: 1 open ocean station (Stn 2) located upstream of the islands near La Palma, one downstream Fuerteventura (Stn 102) and one to the south affected by the presence of a cyclonic eddy (Stn 45) (Fig. 1).

Assays were run in duplicate. Varying amounts of MUF-P were added to samples to obtain concentrations from 0 to 25 µM. Autoclaved or zero-time samples were used as blanks. Spontaneous breakdown of the MUF-substrate when added to the samples was observed; since this breakdown was observed to be proportional to its concentration, we did separate duplicate blanks for each amount of MUF-substrate added. Incubations lasted for 2 to 3 h.

Estimation of kinetic parameters, V_m and $K + S_n$: The relationship of enzymatic activity to substrate concentration can be generally described by the Michaelis-Menten equation:

$$V = \frac{V_m \times S}{K + S} \quad (1)$$

where V is the rate of uptake or metabolism (µM h^{-1}) of a substance present at a concentration of S (µM), V_m is the asymptote towards which V tends (maximum velocity), and K is the value of S for which V is half of V_m (half saturation constant).

Kinetic studies are performed by adding different substrate to concentrations. In many cases, the ambi-
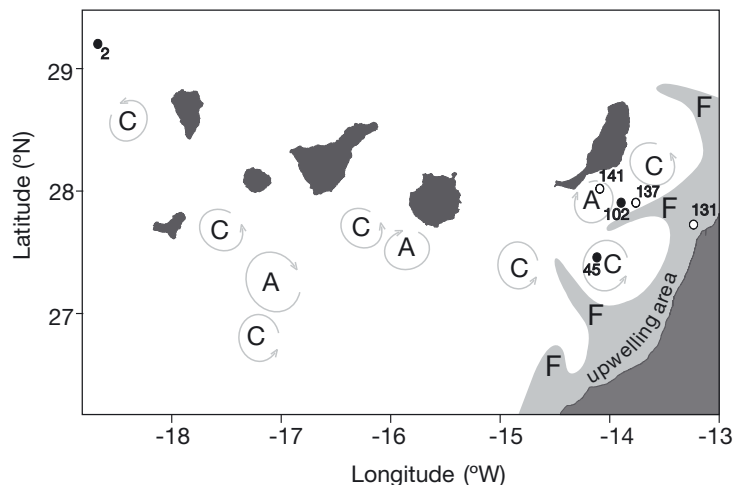

Fig. 1. Diagram of mesoscale structures (A: anticyclone, C: cyclone, F: upwelling filament) in the Canary Current upwelling system. Black dots: stations of kinetic experiments. White dots: stations of dissolved inorganic phosphorus (Pi) addition experiments

ent concentration of the substrate (S_n) is unknown. Thus, the effective concentration S is the sum of S_n and the added substrate concentration (S_a) and Eq. (1) becomes:

$$V = \frac{V_m \times (S_n + S_a)}{K + (S_n + S_a)} \qquad (2)$$

Following the procedure developed by Li (1983), this equation can be transformed to:

$$r = \frac{V_m}{K' + S_a} \qquad (3)$$

(hereafter referred to as the Li parameterisation or Li model) where r is turnover rate, and $K' = K + S_n$. According to standard practice, Eq. (2) can also be transformed to:

$$T = \frac{K'}{V_m} + \frac{S_a}{V_m} \qquad (4)$$

known as the Wright-Hobbie linearization (Wright & Hobbie 1966), where T is the turnover time.

However, Li (1983) compared the goodness-of-fits and precision of parameter estimates of both equations, and concluded that estimating parameters from a linear regression of unweighted T on S_a (Eq. 4) is statistically less satisfactory than from a direct nonlinear fit of r to S_a (Eq. 3). Therefore we chose the Li model to estimate kinetic parameters.

The turnover rate in unamended water samples (r_n) would be estimated as $V_m/(K+S_n)$, thus substituting $S_a = 0$ in the Li equation. The natural turnover time (T) is estimated as $1/r_n$.

The Li-model was fitted using S-plus software.

Effect of phosphate in APA. To test the effect of the addition of phosphate on APA, experiments were done at 3 selected stations along a transect extending from upwelled waters to warmer nutrient-poor stratified waters near the Fuerteventura Island. One station was located in the upwelling area (Stn 131), one in the channel between the African Coast and the island (Stn 137), and one near Fuerteventura (Stn 141) (Fig. 1). Samples were taken from surface and chl maximum (CM). As in the upwelled areas, the level of CM reached the surface, for those areas we obtained one sample from the surface and another from subsurface waters.

For APA determination, a working solution of MUF-P was added to water samples to give 100 nM final concentration. We decided to do near-tracer assays in order to estimate actual *in situ* substrate turnover times, and also because non-saturating substrate additions allow greater sensitivity than higher substrate additions (which have high background fluorescence).

In each experiment, APA assays were carried out in triplicate at *in situ* temperatures in the absence and presence of increasing Pi concentrations (i.e. 0.1, 0.25, 0.5, and 1 µM K_2HPO_4). Incubations lasted 4.25 h. The time course of MUF production (i.e. phosphate release) was observed to be linear for at least 9 h (Sebastian et al. unpubl.).

RESULTS

The upwelling/open ocean transition in the Canary Islands region is subjected to great mesoscale variability, as can be observed in Fig. 1. Upwelling filaments in the time of the study extended far offshore from the shelf, and a series of cyclonic and anticyclonic eddies appeared downstream of the islands of the Canary archipelago. The lateral entrainment of non-local near-surface waters by upwelling filaments, and the secondary vertical circulation produced by eddy mean flow interactions provide likely mechanisms of enrichment of offshore nutrient-depleted waters (Barton et al. unpubl., Sebastián et al. unpubl.).

As a result, high variability in biological variables in the area was also observed. Despite the fact that generally low mean values of chl were obtained (~0.6 µg chl l^{-1} in the CM), the occurrence of upwelling filaments and cyclonic eddies promoted the uplifting of the nutricline and the CM, and chl at this level increased (>1.2 µg chl l^{-1} in the CM).

Alkaline phosphatase kinetics

Temperature-salinity (T–S) diagrams of the stations sampled for kinetic experiments (Stns 2, 102 and 45, Fig. 1) are shown in Fig. 2. The 3 stations were located along a coastal/open ocean gradient, and showed very different T–S properties. Stn 2, the offshore station, showed higher salinity and temperature values than
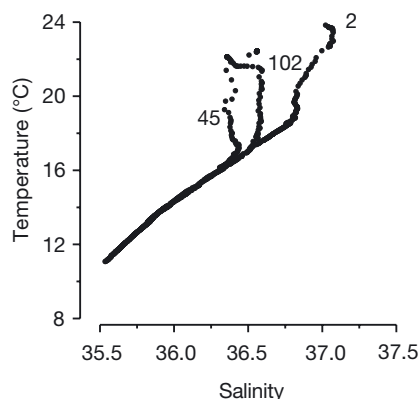

Fig. 2. Temperature–salinity plots of stations where kinetic experiments were done

Stn 102, which also showed higher values of salinity at surface than Stn 45. As a result, the 3 stations exhibited different trophic states, as reflected by the uplifting of the nutricline and the CM and the increase in water-column-integrated chl towards Stn 45 (Table 1). Stn 45 presented a clear eutrophic character compared to the other 2 stations, with the nutricline occurring more than 70 m shallower and with a much higher phosphate concentration at the CM level. Although Stn 45 is located slightly further offshore than Stn 102, its eutrophic character is due to the occurrence of a cyclonic eddy (Barton et al. unpubl.).

Kinetic analyses demonstrated varying results at the different stations tested. Results showed that APA followed a Michaelis-Menten (M-M) kinetic trend in all cases (Fig. 3a–c). Nevertheless, results from the eutrophic station (Stn 45) followed the M-M model better than the other ones, on which more variability was observed. A possible explanation for that is that at Stn 45, hydrolysis was carried out by a dominance of microorganisms with identical or very similar kinetic properties, while at the more oligotrophic stations, hydrolysis was carried out by mixed microbial populations, which gave rise to different values of V_m and $K + S_n$ depending on the range of substrate concentrations considered.

In addition to the great variability, V_m estimated by Li parameterisation (Table 2) is considerably lower than that expected, looking at the asymptote towards which APA apparently leans (Fig. 3a–c). When analysed in detail (Fig. 3d–f), the 2 more oligotrophic stations showed an apparent initial saturation at low concentrations (Fig. 3d,e). The asymptote of this initial saturation is similar to the V_m estimated by Li parameterisation using all data (Fig. 3d,e, dashed line). In accordance with this observation, Bentzen et al. (1992) reported that the Li equation seems to give better fits at low substrate concentrations and, therefore, occa-
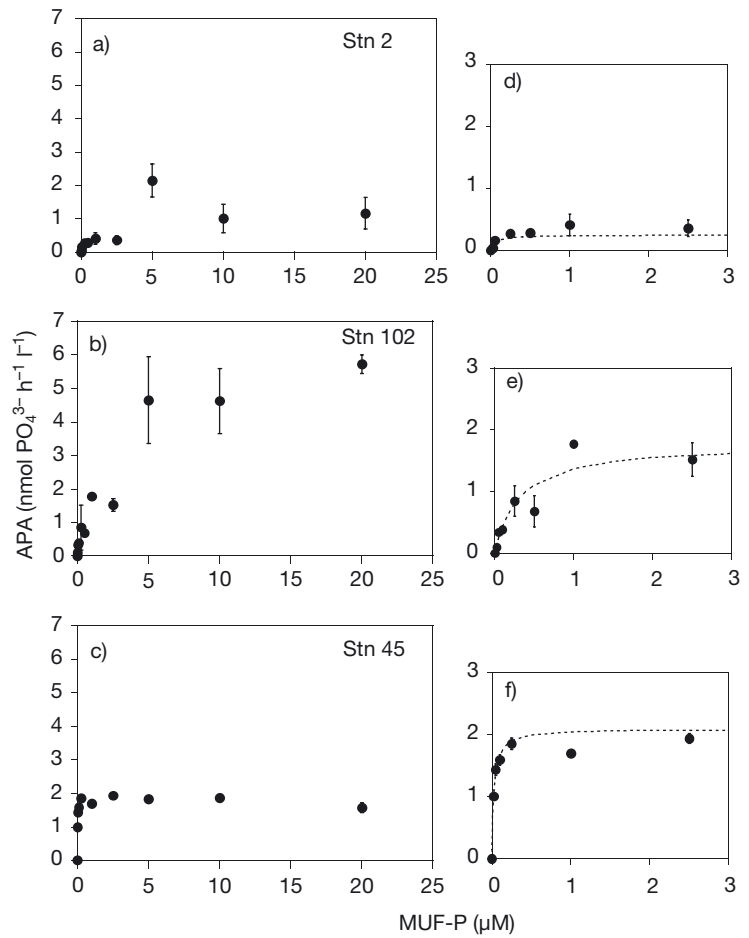

Fig. 3. Alkaline phosphatase activity (APA) as a function of substrate additions of methyl-umbelliferyl phosphate (MUF-P). (a) Stn 2; (b) Stn 102; (c) Stn 45. (d) to (f) are details of (a) to (c), respectively, at lower substrate concentrations. Dashed lines represent the fit by the Li parameterisation technique

sionally the whole-community V_m may be underestimated. Although we are concerned about that, ambient natural substrates for APA are usually at very low concentrations in marine environments (~50 to 150 nM, Karl & Yanagi 1997). This, together with the aforementioned better goodness-of-fit of the Li equa-

Table 1. Nutricline depth (Z_{nut}; upper limit of the nutricline layer), chl maximum depth (Z_{CM}), integrated chl (from 0 to 150 m), and phosphate concentration at the chl maximum level, at the stations where kinetics analyses were carried out

Stn	Z_{nut} (m)	Z_{CM} (m)	Chl (mg m^{-2})	PO$_4^{3-}$ (µM)
2	94	100	24	0.03
102	77	72	31	0.07
45	5	48	35	0.20

Table 2. Kinetic parameters (mean, SE in parentheses) estimated by the Li equation (Eq. 3). V_m is the maximum velocity, K is the half saturation constant, S_n is the ambient concentration of substrates, and T is the turnover time

Stn	V_m (nM h^{-1})	$K + S_n$ (nM)	T (h)
2	0.25 (0.08)	56 (30)	224 (140)
102	1.8 (0.58)	312 (126)	176 (90)
45	2.1 (0.09)	27 (3)	13 (1)

tion (see 'Materials and methods'), endorses the use of the Li-model for estimating kinetic parameters as the more appropriate.

The open water station (Stn 2) showed much lower values of V_m than the others (Table 2), which showed no differences in V_m between them (0.25, 1.8 and 2 nM h^{-1}, for Stns 2, 102 and 45, respectively). In contrast to that, $K + S_n$ at Stn 102 was an order of magnitude higher than at Stns 2 and 45. The biological background for such higher values of $K + S_n$ in the former may be either an increase in available substrates (increase in S_n), or changes in the types of alkaline phosphatases (change in K due to, e.g. changes in composition of natural assemblages).

The different kinetic parameters obtained at the 3 stations support the 3 types of kinetics of nutrient uptake stated by Azam & Ammerman (1984) based on experimental evidence. They suggested that if natural substrate concentrations are very low in seawater, microorganisms will develop very high-affinity systems for efficient acquisition of these substrates, and low $K + S_n$ and V_m would be expected. It is consistent with the oligotrophic character of Stn 2, where low values of both parameters were found.

However, if microorganisms were adapted to high microenvironmental substrate concentrations, one would predict high $K + S_n$ and high V_m, and this could be what it is happening at Stn 102, which showed high values of both parameters.

At Stn 45, located in the cyclonic eddy near the upwelling area, a combination of low $K + S_n$ and high V_m was obtained. This kinetic behaviour would provide metabolic flexibility in a heterogeneous and fluctuating environment, as reported by Ammerman & Azam (1984).

There was a clear oligotrophic-eutrophic decreasing gradient in turnover time values (224, 176 and 13 h for Stns 2, 102 and 45, respectively).

APA inhibition by phosphate addition

Inhibition experiments were carried out at 3 stations located in a straight line that arose from the African shelf to Fuerteventura (Fig. 1). Stn 131, located in the upwelling area, presented a strong eutrophic character, with very high chl values extending from surface down to 25 m depth, and low values of salinity in the whole water column (Fig. 4). At Stn 131, the nutricline was found up to 50 m shallower, and integrated chl was much higher than in the other 2 stations (Table 3). Stn 137 was located in the channel, and was affected by an upwelling filament, as derived from the salinity decrease in surface (Fig. 4) and as reported by Barton et al. (unpubl.). Chl showed relatively high values from 25 m depth (below the pycnocline), and reached a deep maximum around 60 m depth (Fig. 4). Stn 141 was situated near Fuerteventura Island and showed relatively constant values of salinity in the whole water column, and a deep chl maximum at ca. 75 m depth, at the base of the pycnocline (Fig. 4).

The response of APA to the addition of phosphate was different between the stations (Fig. 5). In the eutrophic station (Stn 131), APA was slightly affected by the amount of Pi added. Below the CM, APA showed a ca. 20% decrease at Pi addition >0.5 µM, while at surface (coinciding with the chl maximum) no clear inhibition occurred.

In the channel station (Stn 137), inhibition was stronger than in the eutrophic station. APA at the CM was not affected by Pi additions less than 0.25 µM, but
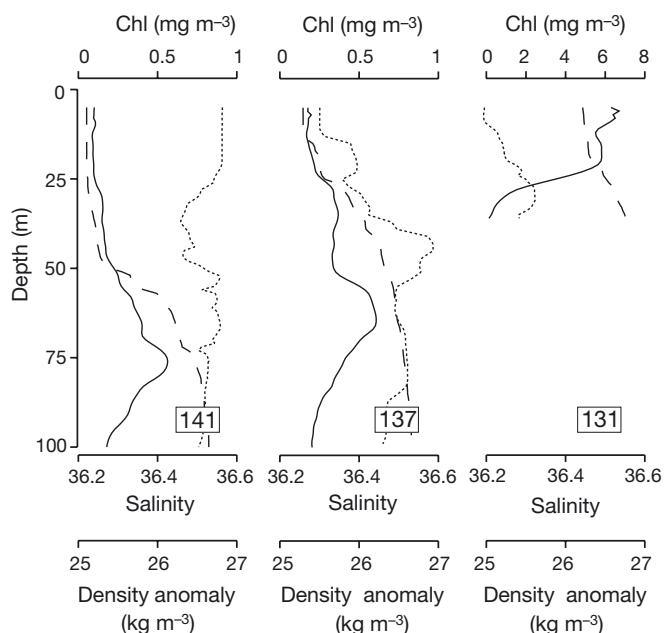

Fig. 4. Vertical profiles of chl (solid line), density anomaly (broken line) and salinity (dashed line) at stations of dissolved inorganic phosphorus (Pi) addition experiments: Stn 141 (Fuerteventura), Stn 137 (channel) and Stn 131 (upwelling)

Table 3. Nutricline depth (Z_{nut}), chl maximum depth (Z_{CM}) and integrated chl (from 0 to 150 m) at the stations where Pi addition experiments were carried out

Stn	Z_{nut} (m)	Z_{CM} (m)	Chl (mg m^{-2})
131	3	6	132[a]
137	60	64	38
141	50	75	28

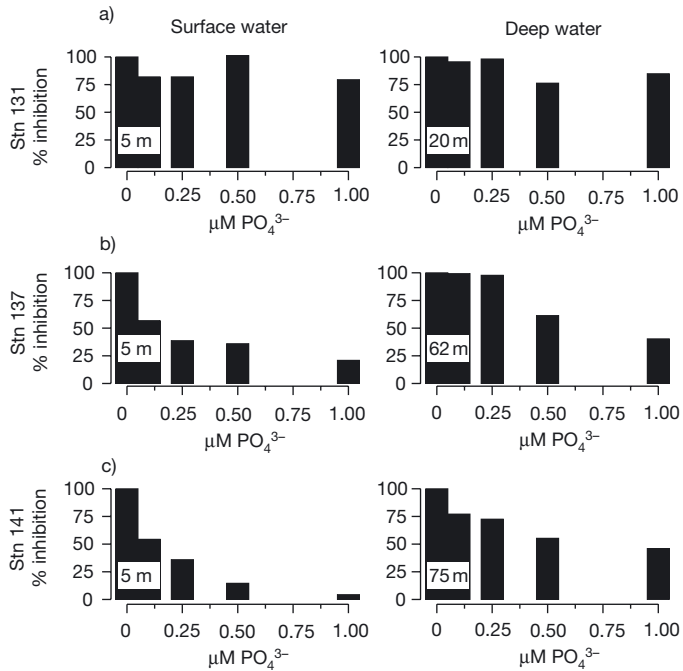[a]Integrated down to the cast depth (35 m)

Fig. 5. % inhibition of alkaline phosphatase activity (APA) by dissolved inorganic phosphorus (Pi) additions. (a) Stn 131 (up-welling), (b) Stn 137 (channel), (c) Stn 141 (Fuerteventura). Left panels: surface samples; right panels: deep samples (chl maximum [CM] of Stns 137 and 141); numbers in boxes are depths of sampling

Inhibition of APA was therefore higher at a higher oligotrophic level, and was stronger at the surface than at the CM.

To study the reason for the different responses in inhibition experiments, we analysed in detail the characteristics of the 3 stations (Table 4).

Alkaline phosphatase activity increased towards inshore-upwelled waters. This trend also held for proteins, chl, PP and bacterial abundance. Overall, Pi concentration at the surface level was near the detection limit, whereas at the CM level it was also low, but increased towards the upwelling station. Nevertheless, total phosphorus concentration presented 4- to 6-fold higher values in the upwelling station at both surface and CM level, and the supply of phosphorus by vertical flux across the nutricline was 1 order of magnitude higher in the upwelling. Hence, low Pi values in the euphotic zone of the eutrophic station seem to be linked to surplus stored P (luxury consumption) in the microbial cells.

The increase in APA towards the more eutrophic stations was small compared to the increase in proteins, resulting in much higher APA per protein under oligotrophic conditions than in the upwelling (Table 4). When normalised by particulate phosphorus (APA/PP), the values increased steadily following the eutrophy-oligotrophy gradient.

it decreased steadily down to 50% of control when 1 µM Pi was added. At the surface, APA was drastically affected by the addition of Pi. With only 0.1 µM Pi added, APA was almost 50% inhibited, and as much as 80% inhibited with 1 µM Pi added.

At the station near Fuerteventura (Stn 141), inhibition was also strong. At the CM, APA was inhibited by ca. 25 and 50% at 0.1 and 0.5 µM Pi addition, respectively. At the surface, as in Stn 137, APA dropped to ca. 50% of its initial value, at only 0.1 µM Pi added, and then inhibition was even more drastic and activity was 96% inhibited at 1 µM Pi addition.

DISCUSSION

Kinetic parameters

In general, values of kinetic parameters found in the literature are higher than those found in this study. V_m was up to 1 to 2 orders of magnitude higher in Lake Plußsee (Chróst & Overbeck 1987), in the NW Mediterranean (Thingstad et al. 1998) and in the Gulf of Mexico (Ammerman & Glover 2000). Nevertheless, Cotner et al. (1997) reported values of V_m in the same order as ours in the Sargasso Sea (1.39 to 2.70 nM h^{-1}), and Hoppe &

Table 4. Values of selected biological variables in the stations where dissolved inorganic phosphorus addition experiments were carried out. APA: alkaline phosphatase activity (nM h^{-1}), Prt: proteins (µg l^{-1}), chl: chlorophyll (µg l^{-1}), BA: bacterial abundance (cell $ml^{-1} \times 10^6$), Pi: dissolved inorganic phosphorus (µM), TP: total phosphorus (µM), PP: particulate phosphorus (nM), APA:Prt ratio (nmol mg^{-1} h^{-1}), APA:PP (nmol $µg^{-1}$ h^{-1}) ratio, Pi flux: diffusive Pi fluxes across the nutricline (mmol P m^{-2} h^{-1})

	Stn	Depth (m)	APA	Prt	Chl	BA	Pi	TP	PP	APA:Prt	APA:PP	Pi flux
Upwelling	131	5 (CM)	0.36	643	6.58	2.34	0.04	0.40	379	0.57	1.0	0.032
		20	0.29	587	7.26	1.58	0.16	0.50	117	0.50	2.5	
Channel	137	5	0.18	73	0.19	1.59	0.04	0.09	51	2.40	3.4	0.005
		62 (CM)	0.10	47	0.46	1.20	0.06	0.10	37	2.16	2.8	
Fuerteventura	141	5	0.07	53	0.15	1.11	0.04	0.06	22	1.30	3.1	0.004
		75 (CM)	0.08	44	0.53	1.12	0.04	0.06	18	1.94	4.8	

Ullrich (1999) also obtained values close to ours in the euphotic zone of the Indian Ocean. A recent study carried out along a trans-Mediterranean transect, with a station located in the North African upwelling (Van Wambecke et al. 2002), reported values of V_m (0.62 to 12.6 and 1.1 nM h^{-1} in the upwelling station) and $K + S_n$ (0.076 to 0.206 and 0.848 µM in the upwelling station) similar to the ones reported in our study.

Measurements of APA kinetics in seawater are scarce. Moreover, unfortunately, direct comparisons with literature values are not always possible, since substrates used to measure APA are not always the

Table 5. Kinetics of alkaline phosphatase activity (APA) in natural waters. Substrate used for APA measurement and range of substrate concentration for kinetic experiments; V_m: maximum velocity; K': $K + S_n$ (see Table 2 legend for definitions); and sample fraction, in case of fractionated assays. p-NPP: p-nitrophenyl phosphate; MUF-P: 4-methylumbelliferyl phosphate; MFP: 3-methylfluorescein phosphate; ^{32}P-G6P: ^{32}P-glucose-6-phosphate

Concentration range (µM)	V_m (nM h^{-1})	K' (µM)	Fraction	Site	Source
p-NPP					
50–800	1000–20 000	150–420	Total (i.e. unfiltered water)	Freshwater systems	Heath (1986)
250–2000	–	–	Total	East Twin Lake	Boavida & Heath (1988)
–	–	700–900	Particulate (0.25–90 µm)	Mediterranean littoral	Gambin et al. (1999)
10–2100	60–4560	1–50	Total	Donghu Lake (China)	Yiyong & Xinyu (1997)
MUF-P					
0.5–40	60	–	Total	Baltic Sea	Hoppe (1983)
10–200	7–430	11.8–59.6	Total	Lake Plußsee	Chrost & Overbeck (1987)
–	221–393	0.52–2.61	Total	Lake Constance	Siuda & Güde (1994)
–	212–319	0.15–0.23	Total	Lake Schleinsee	
0–5	37	0.23	Bacterial (0.2–1 µm)	Lake Herrensee (oligotrophic)	Hantke et al. (1996)
0–5	84	0.23	Algal (>1 µm)		
0–5	153	0.85	Dissolved (<0.2 µm)		
0–5	66	0.15	Bacterial (0.2−1 µm)	Lake Bräuhaussee (mesotrophic)	
0–5	79	0.44	Algal (>1 µm)		
0–5	135	0.77	Dissolved (<0.2 µm)		
0–5	71	0.14	Bacterial (0.2−1 µm)	Lake Thaler See (eutrophic)	
0–5	293	0.40	Algal (>1 µm)		
0–5	248	0.47	Dissolved (<0.2 µm)		
100, 150[a]	0.25–1.49	–	Total	Central Pacific	Koike & Nagata (1997)
–	1.39–2.70	–	Total	Sargasso Sea	Cotner et al. (1997)
0.005–0.2	23–33	0.20–0.21	Total	French Mediterranean coast	Thingstad et al. (1998)
250[a]	2–3	–	Total	Indian Ocean	Hoppe & Ullrich (1999)
0.01–10	200–500	0.2–0.3	Total	Gulf of Mexico	Ammerman & Glover (2000)
1[a]	3.4–119	–	Total	Florida Bay	Cotner et al. (2000)
0.025–1	0.62–12.6	0.076–0.21	Total	Mediterranean Sea	Van Wambeke et al. (2002)
0.025–1	1.1	0.84	Total	North African upwelling	
0.025–25	0.25–2.1	0.027–0.31	Total	NE Atlantic	This study
MFP					
–	40	0.31	Total	Chesapeake Bay	Taft et al. (1977)
0–3	260	0.48	Total	Lake Ontario	Pick (1987)
0–1	6–171	0.095–0.69	Total	Sandsfjord (western Norway)	Thingstad et al. (1993)
0–2	22	0.42	Particulate (>0.45 µm)	Lake Minnesota	Rose & Axler (1998)
	31	0.63	Dissolved (<0.45 µm)		
[32]P-G6P					
–	84	0.086	Bacterial (0.2–3 µm)	East Twin Lake	Hernández et al. (1996)
	456	0.38	Algal (>3 µm)		

[a]One concentration method (i.e. APA estimation at a single saturating substrate concentration), K' data not available

Table 6. Comparison between kinetic parameters (mean; SE in parentheses) estimated by the Li-parameterisation (Eq. 3) and the Michaelis-Menten (M-M) equation (Eq. 1). M-M fit was performed by KaleidaGraph software

Stn	$V_{m\,(Li)}$ (nM h^{-1})	$V_{m\,(M\text{-}M)}$ (nM h^{-1})	$K'_{(Li)}$ (nM)	$K_{m\,(M\text{-}M)}$ (nM)
2	0.25 (0.08)	1.22 (0.31)	56 (30)	1138 (1262)
102	1.8 (0.58)	4.77 (0.65)	312 (126)	1850 (1055)
45	2.1 (0.09)	1.80 (0.06)	27 (3)	17 (4)

Table 7. Kinetic parameters (mean; SE in parentheses) obtained from Wright-Hobbie plots. S_a is the range of substrate added, V_m is the maximum velocity, K' is the sum $K + S_n$ (see Table 2 legend for definitions)

Stn	S_a (µM)	V_m (nM h^{-1})	K' (nM)
2	0–0.5	0.36 (0.07)	117 (86)
	1–25	1.09 (0.24)	421 (196)
102	0–2.5	1.72 (0.19)	379 (97)
	2.5–25	6.49 (1.27)	3410 (606)
45	0–25	1.71 (0.05)	40 (23)

same, the range of substrate concentrations often differ, and sometimes data from size-fractionated assays instead of from the whole sample are shown (Table 5). Generally, when p-NPP (p-nitrophenyl phosphate instead of MUF-P substrate is used, kinetic parameters reported in the literature reach much higher values (from 1 to 2 orders of magnitude), but it also depends largely on the range of substrates used for the assays. Garcia et al. (1997) used equal concentrations of both p-NPP and MUF-P (3 to 1000 µM) to study phosphatase kinetics in a *Phaeodactylum tricornutum* culture, and obtained high values of V_m and K' for both substrates, although K' for p-NPP was slightly higher (8.2 and 3.1 µM for p-NPP and MUF-P, respectively). Chrôst & Overbeck (1987) also reported very high K' values (11.8 to 59.6 µM) in Lake Plußsee using MUF-P as substrate (Table 5). These values were up to 3 orders of magnitude higher than those reported in most of the studies that have been carried out with MUF-P, but these high K' values are probably related to the higher range of substrate concentrations used in both works. As we mentioned above (see 'Materials and methods'), for calculation of V_m and K' in this study we decided to use the Li-parameterisation that gives a better fit at low substrate concentrations, which are likely to be encountered in the area of study. Nevertheless, if we calculate them using a direct non-linear fit to the M-M equation (Eq. 1, see 'Materials and methods'), results obtained are quite different (Table 6). Except for Stn 45, where results are in the same range as those obtained by Li-parameterisation, in the other 2 stations estimates of V_m and K' are much higher using the M-M equation. $V_{m\,(M\text{-}M)}$ values are close to the asymptotes towards which V tends, at high values of substrate (Fig. 3a–c), whereas $K_{(M\text{-}M)}$ in Stns 2 and 102 reached values up to 1 or 2 orders of magnitude higher than those estimated by the Li parameterisation, and even higher than most of the data reported by other authors (Table 5).

Wright-Hobbie linearisation (Eq. 4, see 'Materials and methods') was also performed to study how kinetic patterns varied over the substrate range used. The resultant non-linear plots in the 2 oligotrophic stations

(Stns 2 and 102) revealed a multiphasic pattern in the kinetic curve, while the eutrophic station exhibited a single kinetic pattern over the range of MUF-P considered (data not shown). Kinetic parameters computed from these plots are shown in Table 7. Considering only the lower range of substrate concentration, V_m estimates obtained by Wright-Hobbie linearisation are very similar to those obtained by the Li model, whereas K' estimates are in the same order of magnitude but generally higher. However, in the upper range of MUF-P concentration, kinetic parameters are in the same order of magnitude of those estimated with the Michaelis-Menten equation.

The coexistence of high-affinity and low-affinity systems either in individual populations or due to heterogeneous natural assemblages, which give rise to different values of V_m and K' depending on the range of substrate considered, possibly reflects adaptation to varied substrate concentration regimes (Azam & Hodson 1981). Garcia et al. (1997) also obtained multiphasic kinetics of APA for both p-NPP and MUF-P in their study with *Phaeodactylum tricornutum*.

Therefore, in order to study the contribution of AP to phosphate regeneration in aquatic environments, the fitting technique is important, and it is also important to carry out kinetic experiments over the range of naturally occurring substrate concentrations, because otherwise hydrolysis rates may be underestimated. In this sense Hantke et al. (1996) pointed out that Heath (1986) and Boavida & Heath (1988) concluded that AP only satisfied 1% of P planktonic demand as a consequence of the high substrate concentrations used in their study, which resulted in very high K', whereas AP would provide the main part of P for phosphate uptake in Heath's (1986) study using K's in the same order of magnitude as those obtained by Hantke et al. (1996). These K's are in the same range that those obtained in all the studies reflected in Table 5, in which kinetics were performed at lower levels of substrate.

There is evidence in the bibliography of distinct patterns of ectoenzyme kinetics in different trophic environments (Hoppe 1983, Rath et al. 1993, Hantke et

al. 1996). Consistent with the results obtained in this study (i.e. lower V_m at the most oligotrophic station), Hoppe (1983) concluded that V_m decreased more or less regularly as one approached the open sea in a study in 2 eutrophied Baltic Sea fjords and the adjacent offshore waters. However Van Wambeke et al. (2002) obtained greater values of V_m towards more oligotrophic waters.

Phytoplankton-associated APA has been used as an indicator of phosphorus limitation because it is synthesised at low levels of P availability (Petterson 1980), repressed when P becomes available (Perry 1972, Elser & Kimell 1986), and it has been shown to be inversely related to extracellular and intracellular P concentrations. Nevertheless, in natural systems it is difficult to distinguish among phosphatases produced by other organisms such as zooplankton (Boavida & Heath 1984, Jansson et al. 1988) or bacterioplankton (Cembella et al. 1984, Jansson et al. 1988), and there may also be a background level of constitutive enzymes. Therefore, results of using APA as a P-deficiency index in ecological studies have not always been satisfactory (Pick 1987, Jamet et al. 1997).

However, evidence of APA being repressed under high Pi concentrations in aquatic ecosystems in the literature is frequent. Chrôst & Overbeck (1987), in lake Plußsee, observed that APA significantly decreased when the ambient phosphate concentrations were higher than 0.5 µM. Moreover, Ammerman & Azam (1991) reported that AP activity was usually low and sometimes unmeasurable in the P-rich waters around New York, and Nausch (1998), in the Baltic Sea, observed a significant decrease in APA at ambient phosphate concentrations higher than 1 µM.

Together, these observations lead to workers' attempts to use APA as a P-deficient index.

It is therefore somewhat surprising that we have obtained the highest hydrolysis rates (the lowest turnover times) and the lowest $K + S_n$ at the most eutrophic station, where the highest Pi concentrations were found and, therefore, the lowest hydrolysis and lowest MUF-substrate affinity were expected.

Nevertheless, Hoppe (1983) also found a very strong increase in APA towards the eutrophic part of a fjord, and Hantke et al. (1996) also reported an increase in hydrolysis rates with higher eutrophic level. Likewise, Kobori & Taga (1979) measured high APA at high inorganic phosphorus levels in Tokyo Bay.

High hydrolysis rates in eutrophic habitats may be explained by larger abundance of organisms in these areas, which may lead to much shorter turnover times.

But why was APA highly efficient in the eutrophic station when Pi concentrations were likely sufficient for the plankton requirement? Chrôst & Overbeck (1987) suggested that bacteria might produce APA to hydro-

lyse organophosphoric compounds, but probably utilised the organic moiety as an organic carbon source.

Moreover, it has been observed that bacterial AP has higher substrate affinity (lower K') than algal AP (Chrôst & Overbeck 1987, Hantke et al. 1996, Hernández et al. 1996), and Chrôst & Overbeck (1987) found that, while algal $K + S_n$ increased at increasing additions of Pi, bacterial $K + S_n$ remained near unchanged (i.e. bacterial phosphatases were not Pi-inhibited). That suggests that the low $K + S_n$ values found at the eutrophic station may be related to bacteria instead of to phytoplankton.

Effect of phosphate in APA

It is well documented that the synthesis of many ectoenzymes produced by aquatic microorganisms is inhibited (or even repressed) by the end product that is derived from the substrate and accumulates in the cell or in the surrounding environment.

There are several studies of inhibition of APA by the addition of phosphate, or the regulatory function of phosphate on APA.

Rivkin & Swift (1979), in a study with the oceanic dinoflagellate *Pyrocystis noctiluca*, obtained notable reduction in enzyme activity after the addition of 0.05 µM PO_4^{3-} (25% reduction), and at 3 µM PO_4^{3-} added APA was undetectable within 2 h.

In culture studies with a red tide dinoflagellate, Vargo & Shanley (1985) found that after 30 min of incubation with 20 µM Pi, APA had dropped to 28% of its initial value, whereas Pick (1987) found that phosphate additions greater than those observed under natural conditions in Lake Ontario (1 µM) were needed to inhibit APA to 50% of its initial level within 18 h. Rose & Axler (1998) in lake superior have several data sets showing APA reductions of >50% after 3 to 4 d in response to additions of 0.32 µM. Moreover, Ammerman & Azam (1991) showed that APA was 75% inhibited by 0.1 mM Pi.

From these studies, it may be concluded that addition of Pi to seawater causes a loss of APA and a repression of APA synthesis at certain Pi concentrations.

In this study, APA inhibition by Pi became stronger towards surface and oligotrophic waters.

In upwelling areas, the continuous pumping of phosphate into the euphotic zone promotes nutrient luxury consumption by phytoplankton cells that store P in an inorganic form (surplus P) (Becerra 2001). It has been observed that the amount of surplus P stored in algal cells is inversely proportional to the specific activity of APA of the algal size fraction (Gage & Gorham 1985, Chrôst & Overbeck 1987). Thus, both the surplus P and

the higher phosphate concentration in the upwelling waters could explain the lower APA:protein and APA:PP ratios in the eutrophic station. The higher hydrolysis rates in the nutrient-rich station could therefore be a result of a larger abundance of organisms (i.e. many cells with low activity per cell).

However, we cannot reject the possibility that a few organisms were not Pi-inhibited, presenting high activity per cell. Kuo & Blumenthal (1961) demonstrated that phosphate-repressible APA is not a common property of bacteria. As phytoplankton APA is likely repressed or deactivated in Pi-rich waters, the APA we encountered in the upwelling was probably related to the bacterial fraction.

The near lack of inhibition in the upwelling station, compared to the oligotrophic ones, supports the hypothesis of APA being of bacterial origin in the upwelled waters. Another fact that supports this suggestion is the low $K + S_n$ obtained in the kinetic experiment carried out at a nutrient-rich station, as we mentioned above.

The determination of APA in seawater involves the measurement of both induced and constitutive enzymes. Due to the repressible properties of APA by Pi, constitutive enzymes may be especially important in Pi-rich habitats (Forsberg & Cheng 1980). However, Wilkins (1972) demonstrated that bacterial APA was induced in excess of Pi by starvation for nucleotides. Hence, bacterial APA in the upwelling station may be either constitutive or produced in response to insufficient easily assimilable organic carbon, or both.

Linear regression of APA versus bacterial biomass in eutrophic waters showed that bacterial biomass explained a high percentage of the APA observed ($r^2 =$ 0.71, p = 0.002, n = 10, S-plus software), while chl concentration was not correlated with APA (r^2 = 0.18, p = 0.2).

Sobecky et al. (1996) studied the impact of the introduction in natural assemblages of an engineered bacterium that constitutively expressed high levels of alkaline phosphatase. The overproduction of APA affected the rate of phosphate formation, which resulted in higher phosphate concentrations in the medium. This led to an increase in phytoplankton biomass (up to 14-fold) in both the oligotrophic and more eutrophic waters tested.

Generally, the ambient pool of labile DOC has a higher concentration in eutrophic than in oligotrophic systems (Søndergaard & Middelboe 1995). Thingstad et al. (1997) modelled the interactions of bacteria, phytoplankton and bacterivorous protozoa to study the balance between DOC production and consumption along a gradient from oligotrophy to eutrophy. One solution of the model led them to suggest that a shift to C-limited bacteria at a higher eutrophic level would depend on which processes increase most with enrichment, DOC production by phytoplankton or bacterial consumption.

Most DOP compounds must be dephosphorylated before the C moiety can be taken up into the cell (Bengis-Garber & Kushner 1982, Ammerman & Azam 1991, Hernández et al. 1996), and hydrolysis is usually the rate-limiting step for the incorporation of organic substrates (Hoppe & Ullrich 1999). Some authors have made experiments of competitive inhibition of APA by various organic substrates (Pick 1987, Cotner & Wetzel 1991, Hernández et al. 1996). However, these substrates are almost always phosphorylated and, thus, it is difficult to assess whether there will be inhibition of APA by addition of the organic moiety, which could highlight whether bacteria uses their AP to obtain easily assimilable carbon. Nevertheless, Cotner & Wetzel (1991) observed inhibition on hydrolysis of ATP (by APA and 5-PN) after addition of adenine.

In a study on the responses of bacterioplankton and phytoplankton to organic carbon and inorganic nutrient additions in contrasting oceanic ecosystems, Caron et al. (2000) found that in the nutrient-rich waters of Georges Bank, bacterial production increased in response to glucose additions, while in the nutrient poor waters of the Sargasso Sea, bacterial production increased with inorganic nutrient additions, and showed no response to glucose additions. Kirchman et al. (2000) also found that glucose amendments stimulated bacteria production by as much as 10-fold in the California upwelling regime. This seems to be evidence for the occurrence of C-limited bacteria in nutrient-rich waters.

Recently, Van Wambeke et al. (2002) also found evidence of stimulation of bacterial production by organic carbon addition (glucose) to samples from the North African upwelling, whereas at stations located in the oligotrophic Eastern Mediterranean, glucose amendments had no effect on bacterial production.

Greater inhibition by phosphate additions in the oligotrophic stations probably reflects the fact that phytoplankton APA increased towards oligotrophy. At the CM level, inhibition was always lower, probably because CM was usually occurring in the upper part of the nutricline, where ambient phosphate concentration and, thus, intracellular phosphorus were higher.

The general trend emerging from the aforementioned studies and the present work is that C-deficient bacteria in eutrophic waters present high-affinity APA to get easily assimilable carbon and, thus, this APA is not affected by high concentrations of phosphate, whereas in oligotrophic waters AP is involved mainly in supplying phosphorus for phytoplankton production, and therefore inhibited by phosphate amendments.

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