Phosphatase activity in the sea

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Abstract

Phosphatase is a key-enzyme in the marine environment, although life in the sea is normally not P limited. Expression of phosphatase in algae is generally regulated by the prevailing external concentration of inorganic phosphate, but the internal N:P ratio may also play a role. For bacteria, additional mechanisms like their C and N demands may be important. This is suggested by high phosphatase activities occasionally measured in eutrophic or deep water in the presence of relatively high phosphate concentrations. The distribution of phosphatase activity among the particulate and the dissolved fractions is highly variable. In particular, the dissolved fraction can contribute considerably to the total phosphatase activity (up to 70%), which differs from the pattern of other hydrolytic ectoenzymes. Parts of this fraction may originate from marine protozoa. The contribution of bacteria and phytoplankton to the particle-associated fraction of phosphatase is extremely variable, depending on P-availability, the dominant organisms, water depth and environmental factors. Community analysis revealed that bacteria attached to marine snow and N_2-fixing cyanobacteria were frequently strong producers of phosphatase. Field studies carried out on a great variety of marine regions suggest that phosphatase activity is generally a good indicator of the P status of phytoplankton. Several heat-stable or heat-labile phosphatases, isolated from marine organisms living in extreme or other environments have been recommended for biotechnological applications.

Abbreviations: AMP – adenosine monophosphate; APA – alkaline phosphatase activity; APase – alkaline phosphatase; DOC – dissolved organic carbon; DOP – dissolved organic phosphorus; G6P – glucose-6-phosphate; 3-o-MFP – 3-o-methylfluorescein-phosphate; MUF (or MU) – 4-methylumbelliferone; MUF-P – 4-methylumbelliferyl-phosphate; P_i – inorganic phosphate; PME – phosphomonoesters; PMEase – phosphomonoesterase; pNP – p-nitrophenol; pNPP – p-nitrophenyl-phosphate; POC – particulate organic carbon; SRP – soluble reactive phosphorus; 5′-PNase – 5′-nucleotide phosphodiesterase

Introduction

This review deals with the extracellular alkaline phosphatases of marine organisms which play a major role in the 'microbial loop' (Azam et al., 1987). In addition to phytoplankton, phototrophic and heterotrophic prokaryotes, protozoa are also included, though the digestive enzymes of these organisms are *a priori* not truly extracellular. However, after the digestive process, the enzymes are released into the environment and contribute to the pool of 'freely dissolved' enzymes in the sea. The phosphatases of macroalgae and metazoa are not considered. Nevertheless, it is worth mentioning that many studies on marine phosphatases concern animals and that the phosphatases of higher animals are a good indicator of physiological fitness (e.g. Singh & Green, 1986; Lan et al., 1995).

Extracellular phosphatases are a significant component of most marine algae and bacteria and play a prominent role in the recycling of organic P and in

the avoidance of P limitation in the sea. Studies on phosphatase in the sea are relatively rare in comparison to the limnetic environment. This may be due to the fact that N, rather than P, is normally the growth-limiting factor for phototrophic organisms in the sea (e.g. Tyrrell, 1999). However, the number of reports on P limitation in coastal and even in offshore regions is increasing. P limitation in the coastal environment may be caused by a stronger seasonal reduction of P compared to N in the river effluents (e.g. Labry et al., 2002). Some major offshore examples are represented by areas in the Mediterranean Sea (Krom et al., 1991; Thingstad & Rassoulzadegan, 1995; Zohary & Robarts, 1998), the Sargasso Sea and the Gulf Stream (Cotner et al., 1997; Rivkin & Anderson, 1997). For regions in the subtropical North Pacific long term selection for N_2-fixing organisms has been hypothesised to be responsible for increasingly P-limited conditions (Benitez-Nelson & Karl, 2002). Nevertheless, the matter of P limitation and phosphatase regulation in the sea is not yet clear and may be different for bacteria and phytoplankton.

According to the definition by Priest (1984), extracellular enzymes are principally located outside the cell membrane. Chróst (1991) distinguishes between ectoenzymes, which are located outside the cell membrane, but still in contact with the cell and extracellular enzymes which are no longer in contact with their producers. 'Phosphorus regeneration by the hydrolysis of P_i from organic or other complex P compounds, soluble or particulate, in which the hydrolysed P_i is released outside the cell' was defined as microbial ecto-phosphohydrolase activity by Ammerman (1991).

Generally, phosphatases are represented by a whole 'bunch of enzymes' (Boavida, 1990), characterised by different half-saturation constants, temperature and pH optima. This suggests that their role in the ecology and chemistry of the marine environment is more complicated than may be expected from the simple hydrolytic reactions which they catalyse in nature.

General properties of marine extracellular phosphatases

Phosphatases seem to be inducible catabolic ectoenzymes, as are most other hydrolytic ectoenzymes of aquatic micro-organisms. Early studies (cited in Perry, 1972) have demonstrated that alkaline phosphatase is highly specific for the monophosphate ester bond, but does not exhibit specificity for the organic moiety. These enzymes generally show Michaelis-Menten kinetics. Phosphatases of microplankton (>90 μm) may not follow Michaelis-Menten kinetics as pointed out by Gambin et al. (1999), particularly for the phosphatases secreted by Cypris larvae of the genus *Balanus*. There are only few indications of the phosphatase kinetics of marine micro-organisms in the literature: Perry (1972) reported a K_m for the phosphatase of a tropical diatom of 0.12 μM PO_4^{3-}. In natural waters of the Mediterranean Sea $K_t + S_n$ values averaged 0.008 μM PO_4^{3-} (Zohary & Robarts, 1998) and ranged in the Gironde river plume between 0.002 to 0.038 μM PO_4^{3-} and 0.07 to 0.09 μM PO_4^{3-} for the bacterial fraction (0.2–1 μm) and the algal fraction (> 1 μm), respectively (Labry et al., 2002). The concentration of substrate saturation for phosphatases of *Trichodesmium* was determined to be about 50 μM by Nausch (1997). APase was inhibited at phosphate concentrations of 15 μg l^{-1} P – PO_4^{3-} (in the Plussee, Germany, Chróst 1991). The characteristics of 5'-nucleotidase (5'-PNase) are clearly different from APase (Ammerman & Azam, 1991). 5'-PNase was not inhibited by low concentrations of P_i and showed a strict substrate specificity. A value of 0.041 μM PO_4^{3-} was determined for $K_t + S_n$ of 5'-PNase in coastal marine waters, which is in the range of alkaline phosphatase. Generally, the substrate specificity of phosphatase is little explored. However, there seems to be a difference between small and large molecular dissolved organic P in the way that addition of the latter had little effect on phosphatase (Huang et al., 1999).

Experiments on size fractionation of particles in seawater have demonstrated that phosphatase activity is present in the bacterial size fractions (0.2–3 μm) and in algae size fractions (3–150 μm). It can also occur in high amounts in the free dissolved state (particle free fraction <0.2 μm) (Hoppe, 1986; Table 1) as confirmed by Li et al. (1998), who found 42–74% of total APA in the dissolved fraction. In this case, 50–71% of the particulate APA was associated with picoplankton. In contrast, the other hydrolytic ectoenzymes tested were mainly represented by bacteria (free-living or attached to detritus).

Several authors indicate that the regulation of phosphatases of phytoplankton is mediated by the external P_i concentration (Sakshaug et al., 1984; Chróst & Overbeck, 1987; Paasche & Erga, 1988; Uchida, 1992) or, additionally, by the internal N:P ratio (Myklestad & Sakshaug, 1983) (Fig. 1). Paasche

Table 1. Distribution of phosphatase activity combined with particle size classes in the brackish water of Kiel Fjord (western Baltic Sea) in late summer. Values are % total activity of water sample. <0.2 μm represents dissolved enzymes, 0.2–1 μm and 1–3 μm represent the bacterial size classes and 3–8 μm together with 8–150 μm contain algae, protozoa and detritus. After fractionation the water sample was incubated with increasing amounts of substrate analogue MUF-P. The contribution of dissolved enzymes to total enzyme activity increases with increasing substrate concentration, while the contribution of bacteria (0.2–1 μm) increases at low substrate concentrations. No activity was detected in the largest size class, but this is unusual

Size class (μm)	Phosphatase activity (% total)				
	< 0.2	0.2–1	1–3	3–8	8–150
Substrate conc. (μM)					
0.1	33.3	66.6	0	0	0
0.5	53.8	38.5	0	7.7	0
50	68.8	18.8	0	12.3	0
100	68.1	20.7	0	11.2	0
1000	75.7	9.9	12.2	2.1	0

& Erga (1988) pointed out that phosphatase activity may also depend on the P-demand of the organisms in question and on the season of observation (Huang & Hong, 1998). For example, studies on P/N-limitation patterns in the Oslo-Fjord revealed that N-limitation occurred during the spring bloom of diatoms and P limitation during dinoflagellate dominance in the late summer period (Paasche & Erga, 1988). In another Norwegian fjord Gade & Gustavson (1999) found that inhibition of phosphatase by UV-B radiation may enhance P limitation. Further, for marine macrophytes, allometric factors such as the ratio of surface area to volume ratios must also be considered (Hernández et al., 1999). On the other hand, the mechanism of regulation of bacterial phosphatases remains 'obscure' (Siuda & Güde, 1994) and seems to be linked to the C cycle. Such a link was also hypothesized by Benitez-Nelson & Buesseler (1999) who derived from their ^{32}P and ^{33}P measurements 'that bacteria and picoplankton were hydrolysing specific DOP components for their carbon and possibly nitrogen content'. These observations suggest that extracellular phosphatase activity serves multiple ecological functions by supplying the pools of P and available organic C (and possibly also N) simultaneously. Anticipating C limitation of bacteria this property of phosphatase may be of special interest in substrate-depleted marine regions. However, as emphasised by Carlsson & Graneli (1993), not only the availability of substrates is important for phosphatase generation but also their chemical composition. These authors detected an increase of APA
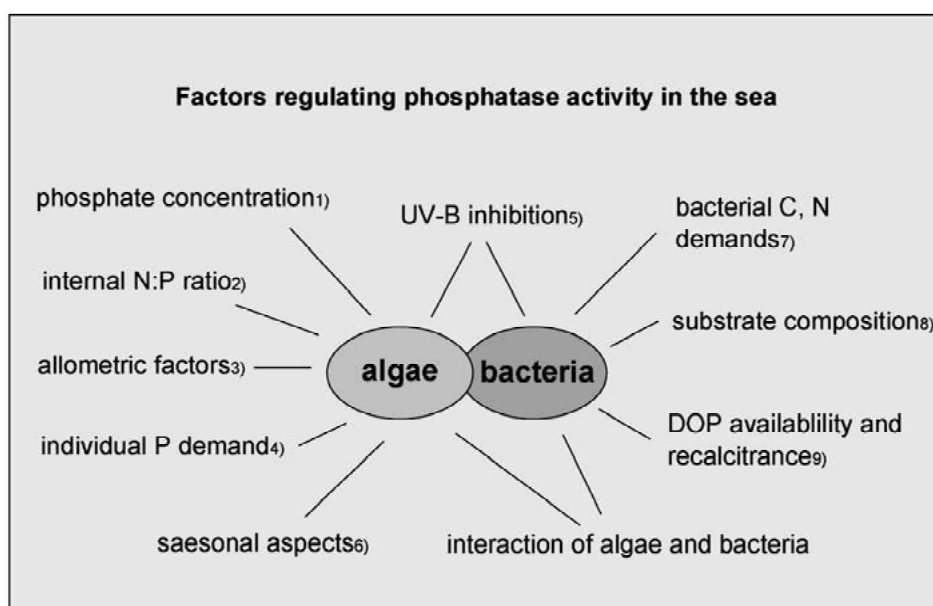

Figure 1. Important factors regulating phosphatase activity in the sea, as reported in text. (1) e.g. Sakshaug et al., 1984; (2) Myklestad & Sakshaug, 1983; (3) Hernández et al., 1999; (4) Paasche & Erga, 1988; (5) Gade & Gustavson, 1999; (6) Huang & Hong, 1998; (7) Siuda & Güde, 1994; Benitez-Nelson & Buesseler, 1999; (8) Carlsson & Graneli, 1993; (9) Thingstad & Rassoulzadegan, 1995.

in the presence of humic acid supplements, which increased the availability of N sources and consequently the need for P. Thingstad & Rassoulzadegan (1995) derived from their investigation of P-limited Mediterranean zones that imported P_i could be transformed to recalcitrant DOP within the food web, which would decrease the pool of biologically available P and C as well. Another very special mechanism or regulation was found by Espeland & Wetzel (2001) who observed that in coastal wetland biofilms photosynthetically induced pH changes triggered alkaline phosphatase while the activity of other enzymes was reduced.

Methods, substrates and tracers

The biological part of the biogeochemical P-cycle in nature is less complicated than that of the N or S cycles and can be studied by the application of relatively simple methods. Generally, the hydrolysis of natural and artificial substrates by phosphatases is characterised by the following equations (Obst, 1995):

(1) R_1-phosphate + H_2O → phosphatase → R_1-OH + H_3PO_4, valid for natural P-compounds,

(2) methylumbelliferyl phosphate + H_2O → phosphatase → methylumbelliferone + H_3PO_4, valid for fluorogenic MUF-substrates, and

(3) 4-nitrophenyl phosphate + H_2O → phosphatase → 4-nitrophenol + H_3PO_4, valid for colorimetric NPP-substrates.

The optimal pH values for acid and alkaline phosphatases are 4–6 and 8.5–9.5, respectively. The most common phosphate generating ectoenzymes measured in the sea are alkaline phosphatase, phosphodiesterase and 5′-P-nucleotidase. Synthetic substrate analogues used for the estimation of the *in situ* activity of alkaline or acid phosphatase are P-derivates of phenolphthalein, phenol, α- or β-naphthol, 5-bromo-4-chloro-3-indole (BCI) (Manafi et al., 1991), *p*-NP, MUF (Pettersson, 1980; Hoppe, 1983) and 3-o-methylfluorescein (Perry, 1972; Kobori & Taga, 1979). In the case of phosphodiesterase, bis-*p*-NPP (Chróst & Krambeck, 1986) and in the case of 5′-PNase, $[\gamma\text{-}^{32}P]$-ATP (Ammerman & Azam, 1991) are used. The nitrophenyl compounds are frequently not sensitive enough for phosphatase measurements in the open sea, but they can be used in polluted/eutrophic inshore waters, in sediments and in experiments with isolated plants, animals and particles concentrated by filtration. To select for enzymes which are (a) dissolved in the water, (b) combined with free-living bacterial cells, (c) associated with algal cells (together with enzymes of bacteria attached to particles) or (d) with larger particles, water samples may be fractionated by pre-filtration through 0.2 or 3 μm Nuclepore filters and 20-μm net, respectively.

If phosphatase activity falls below the detection limit of the selected tracer, for instance in the deep sea, particles may be harvested on filters and re-suspended in natural water from the site prior to incubation with the model substrate (Koike & Nagata, 1998). Phosphatase activities of organisms in sediments can be determined by slurring the sediment with sterile seawater and subsequent incubation with the selected tracer substrate. The use of sublimate ($HgCl_2$) has been recommended as a preservative and control in phosphatase experiments with sea water by Christian & Karl (1995). Freezing is recommended for the storage of sediment samples. In this case, the measurement of fluorescence is performed after thawing in the supernatant of the centrifuged sediments (Köster et al., 1997). However, it has to be taken into consideration that lysis of cells and subsequent release of intracellular enzymes may occur.

A variety of naturally occurring combined molecules have been used in phosphatase experiments concerning substrate inhibition or phosphate generation, e.g. glucose-6-phosphate, glucose-1-phosphate, phosphogluconic acid, phytic acid (inositol hexaphosphate, Rivkin & Swift, 1980), ATP, AMP, ADP, CMP, RNA, DNA, glycerol-3-phosphate (3-phosphoglycerate), phospholipids (e.g. lecithin-type compounds), phosphoglycolate (Waghmode, 1985), phosphoamide (Nicolopoulou et al., 1994) or fructose-1,6-diphosphate (Overbeck, 1991). A very interesting new approach enabling the detection of the phosphatase potentials of individual (phytoplankton) cells was recently introduced into the marine microbiological field (Dyhrman & Palenik, 1997; Gonzalez-Gil et al., 1998; Long et al., 1998). Theses authors used the green fluorescing ELF-97-phosphate (Molecular Probes) as a substrate analogue for phosphatase. The fluorescent constituent of this water soluble compound precipitates after hydrolysis around the active cells. Fluorescence can then be detected by visual microscopic observation or flow cytometry. ELF-97-phosphate was applied by Jochem (2000) for probing the physiological state of phytoplankton at the single cell level. Recently, Dyhrman & Palenik (2001) have developed an alternative antibody-based method for monitoring the presence of phosphatase activity in single cells of the dinoflagellate *Prorocentrum mini-*

mum. The percentage of antibody-labelled cells correlated significantly with that of the cells labelled by ELF-97. Staining dyes for the localisation of phosphatase activity such as BCI-P – successfully used with larger tissues – are frequently not sensitive enough with bacteria and small cells of algae.

A special question in the designing of methods for phosphatase activity measurement concerns the application of buffers. The decision about the use or non-use of buffers depends on the aim of the experiment. From the biochemical viewpoint, buffering of an enzymatic reaction (preferably at the optimal pH) is necessary to standardise and optimise the enzymatic process. When working with seawater or even brackish water this does not appear to be necessary, because the medium is already buffered, though frequently not at the optimal pH. If a truly ecological experiment is desired, where the results of phosphatase measurement are to be compared or correlated with the results obtained for other biological variables, buffering should be avoided. This is especially required if the estimated rates of phosphatase activity are to be integrated in calculations of the dynamics and the budget of P under natural conditions.

Phosphatases of marine eukaryotes

Phosphatase activity of phytoplankton

Phytoplanktonic organisms can be strong producers of extracellular phosphatases, which provides a mechanism of independence from bacterial re-mineralisation (Rivkin & Swift, 1980). Nevertheless, their growth can be limited by the availability of P_i. Investigating the properties of unialgal blooms of *Skeletonema costatum* in a Norwegian fjord, Myklestad & Sakshaug (1983) proposed the ratio of phosphatase activity to cellular P content as a sensitive indicator of P-limited growth. Phosphatase activity was generally low at cellular N/P ratios below 14 (the 'balance point' for the N/P ratio of *Skeletonema costatum* was around 11–12) while higher ratios induced elevated phosphatase activity. Likewise, Sala et al. (2001) suggested to use the ratio between phosphatase and aminopeptidase activity for evaluating nutrient limitation of microbial communities *in situ*. Li et al. (2000) found that carbohydrate accumulated in two marine diatoms (*Skeletonema costatum* and *Nitzsschia closterium*) more strongly under P than under N limitation. Activity of P-ase was low under N limitation.

Testing the properties of APase extracted from axenic cultures of the marine dinoflagellate *Prorocentrum micans* (thus containing extracellular as well as intracellular phosphatases), Uchida (1992) observed a competitive inhibition of the enzyme by orthophosphate. APA increased when the cells were cultured under P-depleted conditions. Furthermore, the enzyme activity of cells grown with β-glycerophosphate was higher than with orthophosphate, although it was low compared to the phosphatase activity of P-depleted cells. Testing four marine dinoflagellates (species of *Amphidinium, Ceratium, Prorocentrum* and *Scrippsiella*) Sakshaug et al. (1984) revealed an adaptation mechanism of phosphatase induction. The organisms showed 'virtually nil alkaline phosphatase activity in nutrient saturated cells but readily demonstrable activity in all species when P-deficient'. Accordingly, Vargo & Shanley (1985) observed a two orders of magnitude increase in the phosphatase activity of natural populations of the red-tide dinoflagellate *Ptychodiscus brevis* after addition of ammonium, which certainly created P limitation for the algae. Clearly multi-phasic enzyme kinetics were detected for the phosphomonoesterase of the oceanic dinoflagellate *Pyrocystis noctiluca,* which was characterised by three different K_m values at substrate concentrations between 0.1 and 222 μM pNPP (Rivkin & Swift, 1980). APA was regulated by P_i and exhibited a diurnal maximum and a nocturnal minimum of activity. Surprisingly, the uptake of the P_i which was generated by glucose-6-P or β-glycerol-P hydrolysis was 9 and 14 times, respectively, smaller than its production.

Extracellular phosphatase activities of marine protozoa

Special attention should be paid to the phosphatases of marine protozoa (e.g. flagellates, ciliates), because these enzymes may contribute substantially to the fairly unexplored pool of 'free dissolved' phosphatases in the sea, which represents a mixture of enzymes of different origin. However, information is scarce. Phagotrophy of protozoa may be an important mechanism of P_i regeneration in marine environments (Ammerman, 1991) due to subsequent release of P_i and phosphatase. However, little is known about the ecological significance of this process. Enzymatic properties, including phosphatase (it was not clear, whether extracellular or intracellular enzymes were studied) of potentially pathogenic amoe-

bae (*Acanthamoeba* sp.) from the sediments of marine sewage dumping sites, were investigated by Sawyer et al. (1987). Morphologically similar species could be distinguished by the diversity of their enzymatic faculties. Nagata & Kirchman (1992) studied the release of macromolecular organic compounds by heterotrophic marine nanoflagellates *(Paraphysomonas imperforata)* fed by ^{3}H-labelled bacteria. Among a great variety of products, the digestive enzyme acid phosphatase was released into the surrounding medium, which could not be degraded by the co-occurring protease. Certainly such enzymes contribute to the fairly unexplored pool of free dissolved phosphatases in the sea.

Phosphatase properties of marine prokaryotes

Several marine bacteria isolated from animal and plant tissues and from seawater have been investigated for their phosphatase properties. Nausch (1997) transferred colonies of the N_2-fixing *Trichodesmium* sp. from the Atlantic Ocean and the Caribbean into sterile seawater and compared their phosphatase activities with their other enzymatic properties. The turnover rate ($\% \, h^{-1}$) of the natural phosphatase substrates was much higher than that of any other tested enzyme substrate. Similarly, Yentsch et al. (1972) observed very strong phosphatase activity combined with *Trichodesmium* (quoted as *Oscillatoria) erythraeum* colonies isolated from nutrient-depleted waters of the Strait of Florida. Stihl et al. (2001) recorded in the Red Sea higher phosphatase activity per unit chlorophyll of the rare puff and bow-tie colonies of *Trichodesmium* compared with tuft colonies. Investigating the growth characteristics of cultured *Nodularia spumigena*, Huber & Hamel (1985) found a very strong increase of phosphatase activity during the stationary phase. Though growth rates were not affected by P supply, the onset of the stationary phase was much earlier in P-deficient than in P-sufficient cultures.

Many allochthonous organisms introduced into the marine environment are subjected to starvation. In a study of metabolic changes of *Escherichia coli* during starvation in seawater, Nicolopoulou et al. (1994) registered a decrease in alkaline and acid phosphatase as well as phosphoamidase in contrast to increasing lipase and leucine arylamidase activities. Strategies of survival of *Escherichia coli* in seawater were investigated by Gauthier et al. (1990). Cells with high APA induced by pre-incubation with polyphosphate survived for longer periods in seawater than cells with low or no activity. A survival mechanism was also derived by Kobori et al. (1979) from their results of bacterial phosphatase activity in coastal and oceanic water of the Pacific. Testing the constitutive phosphatase properties of 319 bacterial cultures isolated from eutrophic bays and the open ocean they found that 63% of the offshore bacteria but only 11–55% of the bay bacteria possessed phosphatase. *Pseudomonas* species dominated in offshore regions while *Vibrios* were most abundant in the bays. Forty-four marine bacteria isolated from aggregates and the surrounding water were tested for their cell-specific enzymatic properties by Martinez et al. (1996). Cell-specific APA ranged widely (0.7 – 410 amol P $cell^{-1} \, h^{-1}$), as was the case for other hydrolytic ectoenzymes. The authors concluded that shifts in the dominance between species of natural bacterial communities could strongly influence the patterns of dissolved polymer and particle decomposition in seawater. Significant differences between the enzyme activities of free-living and attached bacteria were not found.

Mechanisms of phosphatase activity in a Gram-negative bacterium isolated from the surface water off the Southern California coast were investigated by Martinez & Azam (1993). The application of cold osmotic shock revealed that approximately 63% of P-ase was 'shockable', indicating that the major part of this enzyme was located in the periplasmic space. It was anticipated that the enzyme was (probably) not associated with the cytoplasmic membrane, which may explain its tendency to occur in the free dissolved state.

Community phosphatase activities

It is commonly accepted that dissolved organic carbon (DOC) released by the exudation of phytoplankton limits bacterial growth in the photic zone of the sea. However, phosphate limitation of oceanic bacterioplankton at stations in the Gulf Stream and the Sargasso Sea has recently been reported by Rivkin & Anderson (1997). P_i-amended water samples of these regions showed 5–6-fold higher bacterial growth rates than the non-amended controls, 'suggesting that ambient concentrations of labile DOC were sufficient to sustain vigorous growth'. The reason for this finding remains unclear because phosphatase activity was not measured. Anticipating a rapid recycling of phosphate from organic P-compounds by phosphatases as it usually occurs, only an unusually high C:N:P ra-

tio of the prevailing DOC and POC can be assumed. Copin-Montegut & Copin-Montegut (1983) have reviewed in different marine regions (Atlantic, Indian, Antarctic oceans, Mediterranean Sea) that the deviation (increase) of the C:P ratio of particulate organic matter from the Redfield ratio was much stronger than that of the C:N ratio.

In the euphotic mixed layer of the ocean phosphatase activity may originate from algae, free-living bacteria, bacteria attached to particles and protozoa. Investigating the hydrolytic dissolution of marine aggregates such as larvacean houses, diatom flocs and faecal pellets Smith et al. (1992) measured a very strong accumulation of phosphatase activity by the attached bacterial community in comparison to the ambient water, which was in most cases much stronger than for protease, chitinase, chitobiase and glucosidase. This means that settling particles could well serve as export vehicles for phosphatase into deeper water layers. Phosphatase activity (together with protease, laminarinase and xylanase) in marine snow from Howe Sound (British Columbia) was even higher than in the underlying sediments (Amy et al., 1987). High phosphatase activity rates of bacteria attached to particles were accompanied by a relatively higher P_i uptake of attached bacteria in comparison to free-living bacteria (Paerl & Merkel, 1982). Phosphatase activity in sea ice of the antarctic Weddell Sea during midwinter was investigated by Helmke and Weyland (1995). Melted, heavily colonised ice cores incubated at 1 °C exhibited much higher V_m values and hydrolysis rates of phosphatase than the water beneath the ice. In contrast to protease activity, phosphatase activity did not correlate with bacterial viable counts in the vertical profile of the ice indicating other sources of origin. Surprisingly, the temperature optimum of these phosphatases was 50 °C, while the optimum of the other tested enzymes (peptidase, α- and β-glucosidase) was 30 °C.

The interactions between corals and bacteria in the mucus of the coral *Colpophyllia natans* were investigated by Santavy et al. (1994) at Key Largo, Florida. If the coral was under stress by infection (black band disease), it produced more mucus and the activities of the bacteria were generally enhanced. Phosphatase activity was especially affected and recommended as a tool for the prediction of coral decline. Lysis of bacteria (e.g. *Vibrio* spp.) by marine phage can strongly affect the phosphatase activity of the non-infected bacterioplankton. This was observed by Middelboe et al. (1996) in P-limited cultures, where the APA increased by 89% in comparison to control cultures without phage. The uptake of DOC also increased by 72%.

Regional investigations of phosphatase activity carried out in the Lena River delta and the adjacent Laptev Sea by Saliot et al. (1996) showed that hydrolysis rates were low in the river, but increased considerably in the brackish water of the river plume. In the inner Oslofjord Paasche & Erga (1988) detected N limitation of the first diatom bloom in spring, while P limitation occurred in the dinoflagellate dominated plankton during summer and autumn, which was deduced from increases in APA during 24-h incubation experiments. It was assumed that P limitation during the summer might have resulted from recent efforts of local sewage purification. Chlorophyll-specific APase rates in the surface waters of the English Channel were investigated by Davies and Smith (1988). Activity ranged from 0.004 to 0.57 nM PO_4^{3-} μg^{-1} chlorophyll *a*. In the eutrophic Tokyo Bay measured phosphatase activities were strongly influenced by hydrography (stratification and water mixing periods) (Taga & Kobori, 1978). Furthermore, Taga and Kobori showed positive relationships of phosphatase activity with viable bacterial counts, chlorophyll *a*, particulate DNA and also with inorganic and total P concentrations. Thus the phosphatases of the bacteria were not repressed at the high inorganic P levels in the bay. Phosphatase was generally recommended as an indicator of the degree of eutrophication. A good correlation between the depth distribution of APA and *Synechococcus* was found in the northern Red Sea (Li et al., 1998). 'Based on the regulation of APA synthesis, its secretion and stability' the authors proposed 'the use of APase for a (partial) assessment of the P-status of marine planktonic communities.'

The influence of tidal conditions on phosphatase activity and other bacterial variables was studied by Hoppe et al. (1996) in the Lagoon of Aveiro (Portugal). Phosphatase activity was strongly enhanced during low tide (being 2.4 times that of high tide values) but this increase was low in comparison to the increase of protease activity (6.1 times higher at low than high tide). Phosphatase activity reflected quite well the mixing of lagoon water with seawater during the tide (calculated from salinity), while values of protease could only be explained by anticipating a strong stimulation during low tide. Investigating changes of microbial activities in the gradient of eutrophication of the Schlei Fjord (Western Baltic Sea), Hoppe (1983) found a very strong increase in phosphatase activity in the hypereutrophic inner part of the fjord compared to

Table 2. Phosphatase activities (water: [nM PO$_4^{3-}$ h^{-1}], sediment: [nmole PO$_4^{3-}$ cm^{-3} h^{-1}]) in marine environments, together with two comparisons from lake environments

Environment	Condition	Substrate	P-ase activity	Reference
Central North Pacific	subtropical, surface	3-o-MFP	< 0.48–7.8	Perry (1972)
Baltic Sea, Schlei Fjord	hypertrophic, spring	MUF-P	196–390	Hoppe (1983)
Baltic Sea, Kiel Fjord	mesotrophic, annual range	MUF-P	4–160	Hoppe (1986)
Baltic Sea, Kiel Bay	oligotrophic, annual range		2–7	
Southern California Bight	surface	MUF-P	< 0.4–66.7	Smith et al. (1992)
Antarctica, Weddell Sea	water beneath sea-ice	MUF-P	0.08–0.9	Helmke & Weyland (1995)
	inside sea-ice		0.08–8.1	
Arctic, Lena-river delta	brackish water	MUF-P	0.7–27	Saliot et al. (1996)
Central Pacific	only particles, surface	MUF-P	0.21–1.49	Koike & Nagata (1997)
	and 1000–4000 m		0.03–0.35	
Red Sea	oligotrophic	p-NP-P	40–150	Li et al. (1998)
Baltic Sea, river plume	annual range	MUF-P	~5–550	Nausch (1998)
Baltic Sea, river plume	winter	MUF-P	5.7	Nausch et al. (1998)
	summer		183–270	
	autumn		9.8–31.2	
Eastern Mediterranean	P-limited, surface	MUF-P	0.01 – 0.24	Zohary & Robarts (1998)
Indian Ocean	SW-Monsoon: surface	MUF-P	0.1–6.5	Hoppe & Ullrich (1999)
	and 800–2000 m		0.4–4.6	
Mississippi plume	trophic gradient	MUF-P	26–505	Ammerman & Glover (2000)
North Sea, sediment	intertidal mudflats	MUF-P	15.1–115.3	Coolen & Overmann (2000)
eutrophic lake	Diatom bloom	MUF-P	30–220	Middelboe et al. (1995)
hypertrophic reservoir		MUF-P	192–1685	Kwag et al. (1995)

the mesotrophic outer part. Despite the highly abundant Pi, phosphatase was even more enhanced than any other of the measured enzymes (peptidase, chitinase, α- and β-glucosidase). Since nitrate was not depleted and ammonia was abundant in considerable amounts it is suggested that the bacterial part of the community used phosphatase as a tool to overcome C-limitation.

Evaluating the pattern of APA in gradients of salinity and eutrophication in the Pomeranian Bight (southern Baltic Sea), Nausch (1998) determined a threshold concentration of 0.2 μM P$_i$, below which specific APA of algal cells increased. At P$_i$ concentrations between 0.2 μM and 1 μM a linear correlation between APA and the phytoplankton biomass was observed. Intensive studies on APA and P regeneration were also carried out by Ammerman (1991) in the estuarine and coastal environments of the Southern California Bight, the Chesapeake Bay and the estuary of the Hudson River. Generally, the results revealed that 'much of the SRP (soluble reactive P) was not biologically available due to complex formation with iron or other chemical processes'. Biologically available phosphate – which may be only 10% of the

SRP concentration – has to be supplemented by additional P$_i$ inputs via hydrolysis of DOP by APase and 5′-PNase to supply the measured P$_i$ demands of phytoplankton and bacteria.

A very special environment with respect to APA is represented by pelagic chemoclines (oxic/sulfidic gradients). APA increases considerably at the oxic-sulfidic interface, coinciding with total bacteria, bacterial growth and other enzyme activities (observations from Gotland deep, Baltic Sea) (author, unpublished data). Particularly in shallow chemoclines, where bacterial photosynthesis occurs, APA can be very high as measured in the Norwegian fjord Saelenvann (author, unpublished data) and also in meromictic lakes (Bañeras et al., 1999), suggesting that P may be a limiting factor for photosynthetic bacteria. Experiments showed that phosphatase was less affected by sulfide than other hydrolytic ectoenzymes in both sea and fresh water.

Studies on the distribution and properties of phosphatases in marine sediments are rare. Sediments in gradients of eutrophication in coastal lagoons were investigated by Köster et al. (1997) in the Baltic

Sea and by Sabil et al. (1994) in the Lagoon of Venice. Köster et al. found that phosphatase activity was markedly reduced in anoxic and deeper layers of muddy sediments. On the other hand, laboratory experiments under simulated marine conditions have shown that particularly phytic acid is rapidly degraded under anaerobic conditions (Suzumura & Kamatani, 1995). In coastal sediments of the Indian Ocean during the Monsoon periods, Venkateswaran & Natarajan (1983) found an inhibition of phosphatase when P_i was present in 'excessive' amounts. In contrast, Kobori & Taga (1979) observed a positive relationship between the phosphatase activity and the concentrations of total and inorganic P in sediments from deep regions (5900 – 7700 m) of the Pacific Ocean, where 'excessive' amounts of phosphate were not present. A curious pattern of phosphatase activity was detected by Coolen & Overmann (2000) in the 124,000-year-old sapropel layers below the sediment surface in the deep anoxic Urania Basin (eastern Mediterranean Sea). Despite the assumption that organic matter in these 'old' sediment layers should be highly refractory, high potential phosphatase and aminopeptidase activities were measured, indicating the presence of metabolically active bacteria which utilized at least 'part of the sub-fossil kerogen'.

Some measurements of P-ase activity in different marine environments are summarised in Table 2. Generally, P-ase activities cover a very wide range, but increase drastically in regions with high degrees of eutrophication. Such regions are characterised by high abundance of bacteria and phytoplankton and relatively low concentrations of P_i, if thermal and/or salinity stratification occur during the growth season. Thus the high demand of P_i for growth and energy requirements may trigger P-ase induction. The very high values of P-ase activity sometimes observed in limnetic environments are apparently not reached in the marine domain.

Phosphatase activity in deep regions of the sea

Very little is known about the phosphatases in the aphotic meso-pelagic and bathy-pelagic zones of the ocean. On the other hand, just the meso-pelagic zone (or at least its upper layer) is most important for the recycling of P into the euphotic surface layer during winter deep water mixing periods and in upwelling areas. Extracellular phosphatases prevailing in the deep sea can only originate from (a) the bacteria living there, (b) the microbiota of sinking particles, (c) enzymes adsorbed to sinking particles and (d) the phosphatase load of deep currents. Harvesting the particles of 1000–4000 m deep water samples of the Central Pacific Ocean, Koike & Nagata (1998) found that the phosphatase activity per unit of water in the deep was generally about half that of the surface water. In contrast, the activity of the other measured enzymes such as α- and β-glucosidase decreased to zero. The authors attributed this observation to an export of phosphatase activity from the surface down to the deep sea by fast sinking particles.

Preliminary investigations in the deep Sogne Fjord (Norway) (author, unpublished) revealed similarly contrasting patterns of phosphatase activity and the activities of the other measured hydrolytic enzymes. Phosphatase activities maintained the high values which were measured in the surface layer down to 1200 m, while the other enzyme activities (protease, glucosidases, chitinase) showed a considerable decrease at depth. Comparable observations were also made in the meso-pelagic zone of the Indian Ocean along a S–N transect during the S–W. monsoon period (Hoppe & Ullrich, 1999). In most of the depth profiles phosphatase at depth increased considerably in comparison to the surface values by factors up to 7, while the activity of other hydrolytic enzymes (except occasionally peptidase) decreased to zero. Cell-specific phosphatase activities of bacteria, calculated on the basis of the community phosphatase activity and the total bacterial direct counts, turned out to be up to 30 times higher in deep waters than at the surface. Of course, it has to be taken into consideration that probably only part of the phosphatase activity was directly associated with the cells while the other part was dissolved in the water. Phosphatase activities co-varied frequently with the increasing P_i concentrations in the meso-pelagic zone. Prevailing P_i concentrations of up to 3.5 μM are normally expected to inhibit phosphatase synthesis. However, the functions of free dissolved phosphatases and those associated with the bacteria size class of particles may be different (Table 1; Hoppe, 1986). While the contribution of dissolved phosphatases to total phosphatase activity increased with increasing substrate availability it was the contrary with respect to the bacteria-associated phosphatases. This suggests that the enzyme kinetics of these types of enzymes are different, which may be due – at least partially – to the association of free enzymes with colloidal materials. The 'slow' dissolved enzymes are possibly less affected by high

P_i-concentrations. In contrast to the observations reported above, phosphatase activities in the NW Mediterranean Sea decreased drastically at depths below 100 m (Tamburini et al., 2002). However, if integrated over depth, 'potential hydrolytic fluxes within the productive surface layer (10–200 m), through the twilight zone (200–1000 m) and through the deep water mass (1000–2000 m) were roughly in the same order of magnitude'. Furthermore, it turned out that samples maintained under *in situ* pressure developed 2.3 times higher phosphatase activity rates than the decompressed parallels. Investigating the same Mediterranean regions, van Wambeke et al. (2002) observed a shift of bacterial limitation from P to C (by substrate addition) at a depth where soluble reactive P was still undectectable (approximately 50 m). Generally, phosphatase activity in deep water seems to be influenced by the organic matter production in the euphotic zone and the sedimenting portion of these materials, regardless of the prevailing P_i concentrations in the deep.

Special applications of (marine) phosphatase in biotechnology

Highly sensitive phosphatase tools are increasingly used in molecular-biological and immunological approaches (Scanlan & Wilson, 1999). Phosphatase conjugated immunoglobulin B antibodies were used by Cary et al. (1993) for the identification and localisation of bacterial endosymbionts in hydrothermal vent organisms. Wright et al. (1996) used a highly specific APase-labelled DNA probe for the identification of the human pathogen *Vibrio vulnificus* in environmental samples from the Chesapeake Bay. A rapid end-labelling of nucleic acids by T4 polynucleotide kinase was achieved by the use of a heat-labile phosphatase for de-phosphorylation and subsequent heat-inactivation (Kobori et al., 1984). The heat-labile enzyme was isolated from an Antarctic marine bacterium. The monomeric enzyme was characterised by a molecular mass of 68 000 and a temperature optimum of 25 °C. After a treatment for 10 min at 55 °C it was irreversibly inactivated. Another heat-labile monomeric phosphatase was isolated from a marine *Vibrio* sp. by Hauksson et al. (2000). This enzyme was inactivated with a half-life of 6 min at 40 °C. A relatively heat-stable phosphatase with extremely high activity at temperatures from 20 to 50 °C was isolated from a marine bacterium (*Deleya marina*) living in

the coelomic fluid of the mussel *Crenomytilus grayanus* (Ivanova et al., 1994). The optimal temperature for the alkaline phosphatase of the euarcheon *Pyrococcus abyssi* isolated from a deep-sea hydrothermal vent was even 70 °C with a half life of 5 h at 105 °C (Zappa et al., 2001). Phosphatase induction may also play a role in crude oil degradation (De Souza et al., 1996). This property was combined with two marine bacterial isolates characterised by very high capacities of oil degradation. Very strong effects of a genetically engineered marine bacterium (GEM) (*Achromobacter* sp.) with enhanced APA on phosphate concentration and phytoplankton biomass development in microcosm experiments were reported by Sobecky et al., (1996). Surprisingly, the GEM could establish a numerically stable population within 2 to 3 weeks of incubation in seawater and maintain a phosphate accumulation above control levels for longer than one month. An APase was isolated from the marine dinoflagellate *Prorocentrum minimum* and described by Dyhrman & Palenik (1997). This enzyme-protein molecule could be a target for developing an antibody probe for use in environmental phosphatase research. Recently, procaryotic cellular serine/threonine protein phosphatases have been introduced as sensitive indicators of toxins such as e.g. okadaic acid, microcystins or nodularins, produced by marine dinoflagellates and cyanobacteria (e.g. Shi et al., 1999).

Outlook

The ecological function and significance of phosphatases of phytoplankton and bacteria is different. Algae produce their phosphatases to escape P limitation, so phosphatase is repressed at high phosphate concentrations. The organic part of the P-compounds which is liberated from the combined molecules by algal phosphatases is normally not taken up by the algae themselves, but may serve as an additional substrate supply (C, N) for heterotrophic bacteria. Exceptionally, under certain conditions, algae may also use amino acids by enzymatic oxidation (Pantoja & Lee, 1994). For bacteria the mechanism of regulation appears more complex. Bacteria possess phosphatases, which was clearly documented by the strong hydrolysis of fluorogenic model substrates by bacterial pure cultures (Kim & Hoppe, 1986). Whether or not they take up phosphate from the environment may depend on their growth status and internal P recycling. Bacteria should compete with algae for P in P-limited
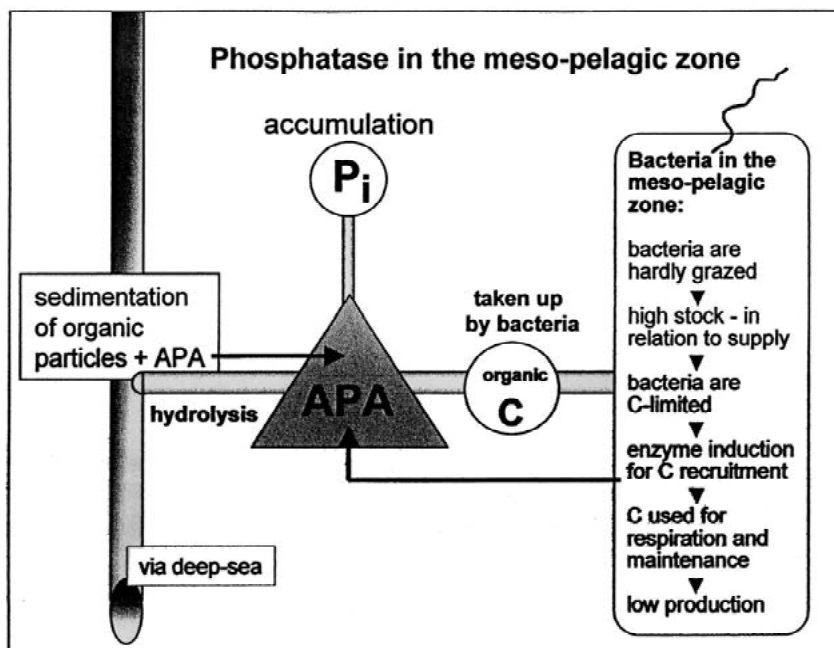
Figure 2. Conceptual model of the function of phosphatase in deep oceanic water. Bacteria in the deep are C-limited for two reasons: (1) Inorganic P and N are abundant. (2) Bacteria are hardly grazed in the deep, therefore they establish a high standing stock (in relation to their poor nutrition) and need most (if not all) of the available C to supply their demands for respiration and maintenance. Bacteria in deep water are C limited and new production of bacterial biomass can only occur if additional organic C becomes available. This is mediated by the generation of organic C from combined molecules by extracellular enzymes. The occasionally observed strong increase of phosphatase activity in deep water suggests that the functional role of phosphatase changes in deep water. Not the generated P_i is important for the bacteria, but the C-content of the combined molecules. Consequently, P_i accumulates slowly in the water and the C-compound is incorporated (after Hoppe & Ullrich, 1999). Dissolved phosphatase and phosphatas exported from the surface by sedimentation of particles may support this process.

situations, because their C:N:P ratios are lower than those of algae (Istvanovics et al., 1990). However, bacteria seem to have the ability to alter the C:N:P ratios of their biomass (Tezuka, 1990). Furthermore, bacteria have the possibility to incorporate non-hydrolysed organic P compounds, such as glucose-6-phosphate or glycerol-3-phosphate. This seems to be realised by low affinity enzymes which operate efficiently at high substrate concentrations (Argast & Boos, 1980; Heath & Edinger, 1990). Further, bacteria have the possibility to select P or N from high-ratio C:N:P substrates. If heterotrophic bacteria produce phosphatase in eutrophic regions or deep water where P_i is abundant the 'second' function of phosphatases, the regeneration of utilisable organic C and N sources (Siuda & Güde, 1994; Benitez-Nelson & Buesseler, 1999; Hoppe & Ullrich, 1999), should be paid more attention: phosphatase activity can be very marked and rapid and so – after hydrolysis – the organic moiety of P compounds can contribute considerably to bacterial substrate supply. A conceptional model of the general functions of

phosphatase in the aphotic deep water is presented in Figure 2.

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