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THE ROLE OF EXTRACELLULAR PHOSPHATASES IN AQUATIC ENVIRONMENTS

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Resumo

A hidrólise enzimática da matéria orgânica é um processo central no ciclo dos nutrientes em ambientes aquáticos, permitindo que os nutrientes retidos na fração particulada e orgânica dissolvida se tornem disponíveis aos microorganismos aquáticos. Neste artigo, é apresentada uma revisão bibliográfica sobre o papel das fosfatases extracelulares nos ambientes aquáticos. Especial ênfase será destinada à dinâmica das fosfatases extracelulares em ecossistemas aquáticos brasileiros. Fosfatases extracelulares são enzimas que catalizam a hidrólise do fósforo orgânico em fosfato inorgânico. Tais enzimas são produzidas principalmente por algas, bactérias e zooplâncton, e podem desempenhar importante papel na estruturação das comunidades planctônicas em ecossistemas aquáticos. Entretanto, ainda não é muito bem conhecido o grau de importância das fosfatases no fornecimento de fosfato inorgânico para o plâncton. A quantidade e qualidade dos compostos orgânicos dissolvidos são fatores chave que controlam a atividade das fosfatases nas águas naturais. Além disso, a síntese das fosfatases é reprimida por altas concentrações de fosfato inorgânico, enquanto sua repressão é anulada na presença de baixos níveis de fosfato. Tais características constituem a base teórica para o uso das fosfatases como um indicador da deficiência de fósforo em algas cultivadas e em comunidades fitoplanctônicas naturais, embora algumas restrições para esta abordagem têm sido sugeridas. Neste levantamento, métodos disponíveis para medir a atividade das fosfatases e sua atividade nos sedimentos e em comunidades epifíticas, também são brevemente discutidas.

Palavras-chave: fosfatases extracelulares, fósforo, plâncton, epifiton, ecossistema aquático.

Abstract

Enzymatic hydrolysis of organic matter is a major process in nutrient cycling in aquatic environments, allowing nutrients retained in the particulate and dissolved organic fractions to become available to aquatic microorganisms. In this paper, a literature review about the role of extracellular phosphatases in aquatic environments is presented. Special emphasis will be given to the dynamics of extracellular phosphatase activity in Brazilian ecosystems. Extracellular phosphatases catalyze the hydrolysis of organic phosphorus compounds to inorganic phosphate. These enzymes are produced mainly by algae, bacteria and zooplankton, and may play an important role in structuring plankton communities in aquatic ecosystems. However, it is not well known to which extent phosphatase activity is important to provide inorganic phosphate to plankton. The quantity and quality of dissolved organic compounds are key factors that control phosphatase activity in natural waters. Moreover, phosphatase synthesis is repressed by high concentrations of inorganic phosphate and it is derepressed when phosphate reaches low concentrations in the medium. These characteristics constitute the theoretical basis for the use of extracellular phosphatase activity as a measure of phosphorus deficiency of algae in culture and in the natural phytoplankton community, although some restrictions to this approach have been suggested. In this paper, methods available to measure the activity of phosphatases, and their activity in sediments and in epiphytic communities are briefly discussed.

Key-words: extracellular phosphatases, phosphorus, plankton, epiphyton, aquatic ecosystem.

Introduction

Microbial cells are able to take up only small (low molecular weight) molecules for their metabolic activities. Large molecules cannot penetrate cell membranes and be directly utilized by microorganisms. However, the organic matter (OM) found in aquatic ecosystems is mainly (<95%) composed of polymeric, high molecular weight compounds (Thurman, 1985). In order to become available for microbial cells, these substances have to undergo preliminary transformation involving enzymatic depolymerization and hydrolysis.

Primary production (phytoplankton) and bacterial production, which are the bases of food webs in pelagic systems, are dependent on nutrient availability, such as nitrogen and phosphorus. Bacteria also need sources of readily utilizable dissolved organic carbon. Therefore, the maintenance of higher trophic levels depends on the efficiency of OM mineralization. Extracellular enzymes produced by microorganisms have an important role in this process, because they are regarded as the major responsible factor for the degradation of polymeric substrates via enzymatic hydrolysis (Münster, 1991).

Although the factors controlling enzymatic degradation of OM are crucial to the understanding of the mechanisms for detritus transformation and utilization in aquatic systems, this approach has been largely neglected in the ecological studies of Brazilian aquatic environments. Few studies conducted on Brazilian systems have been found to include the quantification of extracellular phosphatase activity, such as: Setaro & Melack (1984), about the responses of natural phytoplankton to experimental additions of nutrients; Rugani (1992), about the dynamics of phosphatases in a floodplain lake; Panosso & Pinto (1997), Panosso & Esteves (1999) and Panosso & Esteves (2000) about the dynamics of extracellular phosphatases in two coastal lagoons.

This article aims to provide a review of the role of extracellular phosphatases in aquatic environments in general and in Brazilian ecosystems in particular. The approaches focused in this review are: the biochemical characteristics of extracellular phosphatases; the regulation of enzyme synthesis and how the environment influences the synthesis and activity of extracellular phosphatases; which organisms produce extracellular phosphatases; the role of these enzymes for community structure and the use of phosphatase activity as an indicator of trophic status in aquatic systems; the role of phosphatases in nutrient regeneration; phosphatases in the periphyton and sediment; methods used for measurement of phosphatases activity in water. By pointing out research needs in the field of phosphorus mineralization through phosphatase activity, this review aims to encourage Brazilian ecologists to intensify the development of studies concerning this approach in Brazilian aquatic ecosystems.

Definitions of extracellular enzymes and phosphatases

According to Wetzel (1991) extracellular enzymes or exoenzymes are enzymes that hydrolyse substrates outside the cell, no matter whether these enzymes are membrane-bound or free in medium. Hydrolytic enzymes catalyze the cleavage of covalent bonds such as C-O (esters and glycosides), C-N (proteins and peptides), and O-P (phosphates).

Phosphatases (or phosphomonoesterases) catalyze the hydrolysis of a variety of phosphomonoesters, liberating inorganic phosphate according to the reaction (Siuda, 1984; Jansson *et al.*, 1988):



OR is the organic fraction of the phosphomonoester molecule.

According to the pH at which phosphatases show maximum activity, they are classified as alkaline (pH between 7,6 - 10,0) or acid (pH 2,6 - 6,8). Siuda (1984) pointed out that the synthesis of acid phosphatases, contrary to alkaline phosphatases, is in general not repressed by orthophosphate. Because most of the studies about phosphatases in natural environments have been conducted in alkaline waters, alkaline phosphatases are the enzymes most intensively evaluated.

Alkaline phosphatases are dimeric molecules (constituted by two subunits) of molecular weight about 160,000 daltons (Chróst, 1991). Two zinc atoms are present in each subunit. The first atom, which is essential to the structural integrity of the enzyme, is tightly bound to the molecule. The second zinc atom is less tightly bound and it is involved in the catalytic process (Crofton, 1982). Magnesium ions stimulate phosphatase activity by binding to an effector site on each subunit (Linden *et al.*, 1977). Extracellular alkaline phosphatases are involved in a variety of metabolic processes besides the hydrolysis of organic phosphorus, such as the transport of sodium, potassium, phosphate and tiamine across the cell membrane (McComb *et al.*, 1979).

Regulation of extracellular phosphatases

Exoenzyme synthesis is regulated mainly by the presence of substrates and end products of the enzymatic reaction (Jansson *et al.*, 1988). The substrates and end products of the catalysis promoted by extracellular phosphatases are the phosphomonoesters and orthophosphate, respectively.

Enzymes are classified according to the factors that control their synthesis. However, there is no uniformity in the use of this terminology in Aquatic Ecology. In this work, the definitions used by Chróst (1990) and Cembella *et al.* (1984) are applied: constitutive enzymes are the ones synthesized independently of an activation (they are produced continuously in the cell); inducible enzymes have their synthesis started (induction) only in the presence of their respective substrates.

Enzyme synthesis may also be controlled by the end product of the catalysis. Synthesis may be repressed when the end product derived from the substrate accumulates in the cell or in the surrounding medium. These enzymes are named repressible. On the other hand, enzymes that are synthesized independently of product concentration are named irrepressible. Repressible enzymes are derepressed when the concentration of the repressor (in general the end product of the catalyzes) in the medium is decreased.

Information on the control of phosphatases synthesis in aquatic systems is contradictory. Jansson *et al.* (1988) mentioned that "induction, where phosphatases activity is enhanced by addition of substrate, seems rather uncommon (or not investigated)". However, Chróst (1990) suggested that most extracellular enzymes produced by aquatic microorganisms are inducible and few of them are constitutive (such as some amylases and bacterial proteases). Chróst (1990) argued that a constant production of extracellular enzymes would represent an unnecessary energy lost in environments where organic substrates are not constantly available. According to Jansson *et al.* (1988) probably a background activity of a constitutive nature exists in most organisms and most lake waters.

Synthesis of extracellular phosphatases of microorganisms is enhanced in phosphate depleted medium, indicating enzymatic derepression. This has been shown to occur in natural environments as well as in laboratory cultures (Siuda, 1984; Chróst & Overbeck, 1987; Siuda & Chróst, 1987; Chróst, 1994). Repression and derepression of phosphatase synthesis were first shown *in vitro* for *Escherichia coli* by A. Torriani-Gorini in 1958 (Lugtenberg, 1987). Although virtually all alkaline phosphatases seem to be repressible by inorganic phosphate (Cembella *et al.*, 1984), some microorganisms grown in laboratory cultures (Antia & Watt, 1965; Boavida & Heath, 1986) and natural populations (Vargo & Shanley, 1985), do not have their phosphatase synthesis totally controlled by repression and derepression mechanisms.

Chróst & Overbeck (1987) suggested that synthesis of extracellular phosphatases in natural communities is not directly controlled by the concentration of inorganic phosphorus. Instead, repression of phosphatase synthesis is regulated by the intracellular content of phosphorus in microorganisms, which obviously depends on the ambient concentration of inorganic phosphorus. For instance, Chróst & Overbeck (1987) found that extracellular phosphatase synthesis was derepressed in a natural plankton community when the intracellular polyphosphate content decreased to below ca. 15% of the total particulate phosphorus and dissolved inorganic phosphate concentration was low (below 0.32 μM). Panosso & Pinto (1998) and Panosso & Esteves (1999) found no correlation between concentrations of inorganic phosphate and phosphatase activity in some Brazilian coastal lagoons (located in Rio de Janeiro State, at 41°46'W and 22°21'S), probably because phosphate concentration was below the threshold level (0.3 μM) to repress phosphatase synthesis.

In another Brazilian freshwater system, the Infern o Lagoon (a floodplain lake located at 47°50'W and 21°36'S), Rugani (1992) concluded that algae extracellular phosphatase activity increased when inorganic phosphate reached limiting levels, whereas bacterial phosphatases did not respond to variations in phosphate concentrations in the water.

Thus, are phosphatases in aquatic systems inducible or constitutive? Is their synthesis irrepressible or is it controlled by repression mechanisms? The results described above indicate a large variability of the patterns of extracellular phosphatase synthesis. This is possibly caused by the large variability in the chemical composition of natural waters. It is likely that the control of exoenzyme synthesis in natural populations should vary according to changes in environmental factors such as concentration of dissolved organic phosphorus (substrate) and inorganic phosphate (end product of the catalysis).

In fact, microbial populations may be able to adapt the control of their enzyme systems to environmental conditions. For example, synthesis of β -galactosidase (responsible for the hydrolysis of galactoses) is inducible in *Escherichia coli* (Demain, 1990). However, a constitutive mutant population is selected by growing *E. coli* cultures in lactose-depleted medium. For this mutant population the synthesis of β -galactosidase is not shut off when the substrate is absent, allowing the utilization of very low concentrations of substrate (Demain, 1990). The selection of constitutive mutants has been shown only for laboratory cultures and the occurrence of such process in natural environments has not yet been investigated.

Another example of adaptation of exoenzyme regulation to environmental conditions was reported by Torriani-Gorini (1987). The production of extracellular alkaline phosphatases is repressed in *E. coli* under phosphate-replete conditions (Lugtenberg, 1987). Mutant cells that do not have the synthesis of extracellular alkaline phosphatases repressed by inorganic phosphate are selected when the culture is grown in phosphate-replete medium with β -glycerol-phosphate as carbon source (Torriani-Gorini, 1987). In this case, hydrolysis of β -glycerol-phosphate is a means to obtain carbon (and not phosphate) by the microbial cells. In natural waters, Chr st (1994) and Siuda & G de (1994) suggested that high phosphatase activity found in phosphate-sufficient lakes is connected to the hydrolysis of organic phosphates used as a carbon source.

The quality of the dissolved organic matter is also likely to affect the production of exoenzymes in microorganisms. For instance, Cotner & Wetzel (1991) and Scholz & Marxen (1996) concluded that spatial and temporal variations in the composition of the organic matter in natural waters promoted the production of different isoenzymes (with distinct kinetics) of bacterial extracellular phosphatases. These examples point out that concentration and quality of dissolved organic matter and inorganic ions are key factors for the dynamics of exoenzymes in aquatic systems.

The control of extracellular phosphatase activity

The mechanisms of enzymatic induction and repression discussed above are related to the control of enzyme *synthesis*. Exoenzymes that are synthesized have their *activity* also regulated by environmental factors. The interactions of phosphatases with the end product of the catalysis (inorganic phosphate), and with other ions and organic molecules (such as humic substances) are important for the control of phosphatase activity in aquatic ecosystems.

Inorganic phosphate inhibits the activity of alkaline phosphatases in natural environments through the process of competitive inhibition (Chróst, 1990). This is because the end product of the catalysis competes with the substrates for the active sites in the enzyme molecules, decreasing the affinity of the enzyme for the substrate (Hoppe, 1983).

Polyphenols associated with humic substances may promote a decrease in phosphatase activity by means of noncompetitive inhibition (Wetzel, 1991). In this process, the inhibitor do not bind to the active site of the enzyme molecule, but to another site. Studies on the humic substance-enzyme interactions are relevant because between 30 and 80% of the dissolved organic carbon in lakes is contained in humic compounds (Thurman, 1985).

Polyphenols are major components (between 40 e 80%) of the humic substances (Wetzel, 1991) and have high affinity to proteins, such as enzymes (Beart *et al.*, 1985). Extracellular phosphatase activity was shown to be decreased by polyphenols, originated from littoral vegetation (Serrano & Boon, 1991). The content of humic substances was suggested to influence phosphatase activity in some Brazilian coastal lagoons (Panosso & Esteves, 1999). Phosphatase activity was higher in the Imboassica Lagoon (lower humic content) than in the Cabiúnas Lagoon (higher humic content). The inhibition of phosphatase activity by polyphenols may promote a decrease in metabolic processes such as photosynthesis, especially if the system is phosphorus limited (Wetzel, 1991; Kim & Wetzel, 1993).

Several ions, such as cadmium, copper, mercury, nickel, tungsten, and molybdenum inhibit phosphatase activity (Flint & Hopton, 1976, 1977; Whitton *et al.*, 1990). Although magnesium and zinc are activators for alkaline phosphatases (McComb *et al.*, 1979), at high concentrations they may inhibit their activities (Whitton *et al.*, 1990).

The effects of ions and phenolic compounds on phosphatase activity seem to depend on their concentrations in the medium. For example, polyphenols may complex with inhibitory ions, decreasing their availability. Consequently, the chances for the occurrence of binding between these inhibitory ions and enzymes are decreased and enzymatic activity may be enhanced (Serrano & Boon, 1991). On the other hand, polyphenols can complex with ions that are essential for the molecular structures of the phosphatases (e.g. magnesium). In this case, decreasing magnesium availability can promote a decrease in enzymatic activity.

Wetzel (1991) argued that the formation of polyphenolic-enzyme complexes prevents the hydrolysis of exoenzymes by proteases or their degradation by other processes. These complexes allow storage in a suppressed but chemically active state. Enzymes can be further transported and reactivated in other parts of the aquatic system (Wetzel, 1991). Reactivation of enzymes associated to humic compounds may occur after the separation of these complexes through photolysis promoted by the ultraviolet radiation (Stewart & Wetzel, 1981; Wetzel, 1993). However, this hypothesis has not been tested yet. Shallow aquatic systems, with a large littoral zone and large input of humic compounds, characteristics commonly found in Brazilian lakes, are especially suitable to test such hypothesis.

Origin of phosphatases in plankton

Bacteria, phytoplankton and zooplankton are the major producers of extracellular phosphatases in aquatic environments (Jansson *et al.*, 1988). The origin of phosphatases is a relevant issue because the ability to utilize organic compounds as a source of inorganic phosphates may be an important nutritional strategy for organisms living in a phosphorus-depleted environment (Chróst, 1990). Bacteria have been shown to be the main responsible organism for the extracellular phosphatase activity in freshwater (Francko, 1983; Chróst *et al.*, 1989; Berman *et al.*, 1990; Francko, 1991; Jamet *et al.*, 1997). However, the dominance of phytoplankton phosphatases has been found in several aquatic ecosystems (see review by Jansson *et al.* (1988); Sala & Güde, 1996). The relative importance of phytoplankton and bacterioplankton as producers of extracellular phosphatases has been shown to vary among Brazilian aquatic systems. For instance, Rugani (1992) found that algal contribution (50%) to the total phosphatase activity in a floodplain lake was higher than the contribution of bacteria (20%). Panosso & Esteves (1999) showed that phosphatase activity was correlated with phytoplankton biomass in the Imboassica Lagoon, but it was correlated with bacteria biomass in the Cabiúnas Lagoon.

The ability of phosphatase synthesis in the phytoplankton is species-dependent. High phosphatase activity has been found in species of Chrysophyceae (Olsson, 1983), in the genus *Oscillatoria* (Feuillade *et al.*, 1990), in cultures of *Chlorella vulgaris* (Moraes, 1988) and in blooms of the cyanobacteria *Nodularia spumigena* (Huber & Hamel, 1985). In a Brazilian coastal lagoon, peaks of phosphatase activity were found connected to high densities of *Chaetoceros* spp., *Campylodiscus* spp. and a colonial species of Chroococcales (Panosso & Pinto, 1998). However, the ability of synthesis of extracellular phosphatases by these species requires confirmation through laboratory studies using axenic cultures or cell-specific phosphatase assays, such as the one described by González-Gil *et al.* (1998).

Phosphatases originated from the zooplankton were detected by Stevens & Parr (1977), Wynne & Gophen (1981), Boavida & Heath (1984) and Hantke *et al.*

(1996). However, it is not known to what extent zooplankton phosphatases contribute to the overall activity in the natural waters.

Phosphatases may be released to the medium through excretion by intact plankton cells or from dying and disintegrating cells (Jansson *et al.*, 1988). These "free" enzymes become dissolved in water and often comprise a substantial part of the total phosphatase activity (between 14 and 70%; Pettersson, 1980; Chróst *et al.*, 1984; Chróst *et al.*, 1989).

The relative contribution of phytoplankton, bacteria and dissolved enzymes to the total phosphatase activity in aquatic systems seems to depend on a variety of factors, such as the presence (and physiological state) of phytoplankton blooms (Chróst *et al.*, 1989), the taxonomic composition of the phytoplankton (Berman *et al.* 1990), the availability of phosphate and iron (Francko, 1986), and the presence of humic substances (Wetzel, 1981).

Phosphatase activity as an indicator of phosphorus-deficiency in the phytoplankton and trophic condition of lakes

Kuenzler & Perras (1965) and Fitzgerald & Nelson (1966) showed that extracellular alkaline phosphatase activity in the water increases when phytoplankton growth is limited by inorganic phosphate. These works formed the basis for the use of alkaline phosphatase activity as a measure of phosphorus-deficiency in the phytoplankton.

However, several studies have shown that phosphatase activity may be a poor indicator of phosphorus-deficiency in natural phytoplankton communities (Cembella *et al.*, 1984; Boavida & Heath, 1984; Dodds, 1995). The evidence behind these conclusions are as follows: phosphatase synthesis is not always repressed by inorganic phosphate, which means that phosphatase activity may be detected even in phosphorus-sufficient conditions; other sources of phosphatases, such as zooplankton (Boavida & Heath, 1984), bacteria (Chróst & Overbeck, 1987) and allochthonous enzymes (Stevens & Parr, 1977) may influence the quantification of phytoplankton phosphatases. Thus, in order to evaluate the nutritional state of the phytoplankton, Jansson *et al.* (1988) suggested the utilization of different indices combined: phosphatase activity, intracellular content of phosphorus, and intracellular ratios between nitrogen and phosphorus.

For instance, Setaro & Melack (1984) concluded that phytoplankton growth in Lake Calado (an Amazon floodplain lake, located at 3°15'S and 60°34'W) was predominantly limited by nitrogen availability at the beginning of the falling water level period. Their conclusions were based on different physiological evidences, such as the rapid uptake of ammonium, ammonium enhancement of dark ¹⁴C uptake, lack of orthophosphate uptake in short-term essays and low phosphatase activity (9 nmol pNP.µg⁻¹ Chlorophyll-a.min⁻¹).

Phosphatase activity has also been suggested as an indicator of the trophic condition (oligotrophy or eutrophy) of aquatic ecosystems (Jones, 1972). This author found a positive correlation between alkaline phosphatase activity and total phosphorus, and between phosphatase activity and microbial biomass in several lakes. In these lakes, the concentrations of inorganic phosphate remained below the levels necessary to promote repression and inhibition of extracellular phosphatases. Therefore, enhancement of phosphatase activity was associated to the increase of the microbial biomass, which is connected to the degree of eutrophication. The increase of phosphatase activity under eutrophic conditions was also found by Rath *et al.* (1993) and Hantke *et al.* (1996), although the cell specific activity was higher in the oligotrophic environments. On the other hand, examples where low phosphatase activity was detected in eutrophic systems have also been reported in the literature (e.g. Boavida & Marques, 1995). Therefore, phosphatase activity is not an ubiquitous indicator of trophic condition of aquatic environments.

Phosphorus turnover time as a means to quantify the effect of disturbance on phosphorus mineralization

Ecosystem disturbances caused by anthropogenic activities may promote a decrease in nutrient recycling and an increase in nutrient turnover time (Odum, 1985). Based on this concept, Panosso & Esteves (2000) hypothesized that spatial and temporal variations in phosphorus turnover time in the plankton may be used to detect effects of disturbances in plankton communities. These authors established an index of potential phosphorus turnover time (PPTT) as an attempt to quantify the effect of disturbances on phosphorus recycling within the plankton community. This index is calculated as the ratio between the total sestonic phosphorus and extracellular phosphatase activity, assuming that the hydrolysis of dissolved organic compounds through phosphatases is a major source of inorganic phosphorus for the plankton (Panosso & Esteves, 2000).

The PPTT was calculated for the Imboassica Lagoon (Rio de Janeiro State, Brazil) before and after the opening of the sandbar which isolates this lagoon from the sea, a common type of anthropogenic disturbance in coastal lagoons. This disturbance caused a shift in the mechanism controlling primary producers in the Imboassica Lagoon, yielding increasing phytoplankton biomass and longer PPTT, at least in the most affected sites in the lagoon (Panosso & Esteves, 2000). In the case studied for the Imboassica Lagoon, the PPTT index showed that the disturbance caused by artificial sandbar opening decreased phosphorus recycling. However, the generality of this index needs to be tested for other ecosystems and other types of disturbances.

The role of extracellular phosphatases on phosphorus mineralization

The dissolved organic matter in aquatic environments is a mixture of allochthonous and autochthonous organic compounds of high and low molecular weight

(Lampert & Sommer, 1997). The major part of this organic pool is composed by organic acids, carbohydrates, aminoacids, proteins, nucleic acids, lipids, phosphoric esters and humic substances (Münster & Chróst, 1990; Münster & Albrecht, 1994). Because organic molecules may be the most abundant form of soluble phosphorus in the water (Chróst & Overbeck, 1987), the primary production in many aquatic systems may be dependent on the regeneration of inorganic phosphate (Lampert & Sommer, 1997).

In their review article, Jansson *et al.* (1988) concluded that the hydrolysis of organic compounds through phosphatase activity provides a significant fraction of phosphate taken up by plankton microorganisms. However, contradictory findings were reported by Boavida & Heath (1988) and Siuda & Güde (1994). They showed that hydrolysis of organic molecules through phosphatase activity did not represent a significant source of phosphate for planktonic populations in lakes in North America. Heath (1986) found that less than 1% of the phosphate requirement by the plankton was provided through this mechanism, at least in some North American lakes. The importance of phosphate regeneration through hydrolysis catalyzed by the phosphatases seems to depend on substrate concentration in the water (Heath & Cooke, 1975; Cotner & Heath, 1988). Because these concentrations are often low in continental waters (Solorzano, 1978; Chróst *et al.*, 1984), the importance of the phosphatases for plankton nutrition may be not significant.

The relative importance of extracellular phosphatases for phosphorus regeneration in the water also depends on the qualitative composition of the organic matter. In the presence of a large pool of phosphomonoesters, the phosphatases would probably be significant, as discussed above. However, other types of organic molecules require different enzymes to be hydrolyzed. Inorganic phosphate may be also originated from nucleotides, for instance, which are hydrolyzed by bacterial exoenzyme 5'-nucleotidase (Ammerman & Azam, 1985; 1991a). This enzyme is not inhibited by inorganic phosphate. Therefore, in environments where the phosphate concentration is high enough to inhibit phosphatase activity, 5'-nucleotidase may be more important for phosphorus mineralization.

The transformations of dissolved organic matter by the exposure to UV light or sunlight may be another significant mechanism involved in the release of inorganic phosphate from the organic fraction (Francko & Heath, 1979; Yiyong, 1996). Francko (1986, 1990) proposed a model which recognizes that phosphatase activity and UV photoreduction are major orthophosphate regenerative mechanisms, which can co-occur in lakes. Francko (1986, 1990) hypothesized that enzymatic regeneration of phosphate may be most important in clearwater or eutrophic lakes, whereas UV-promoted release of phosphate should be more significant in oligotrophic but polyhumic lakes. It is also possible that lakes may be arranged in a continuum, from systems exhibiting one predominant mechanism to those in which features of more than one mechanism co-occur (Francko, 1986, 1990). However, this hypothesis has not been definitively confirmed.

An attempt to evaluate whether the hydrolysis of organic molecules through phosphatase activity satisfies planktonic phosphate demand in some Brazilian aquatic environments was made by Panosso & Esteves (2000). These authors concluded that inorganic phosphate regenerated through extracellular phosphatases may supply a significant part of the phosphate required by plankton communities in the Imboassica lagoon, but not in the Cabiúnas Lagoon. Other mechanisms of phosphorus mineralization, such as photodegradation, might be more important than extracellular phosphatases in the Cabiúnas Lagoon, which has a higher humic content than the Imboassica Lagoon.

Overall, it seems that it is not correct to assume that the hydrolysis of organic compounds by means of extracellular phosphatases is always the most significant mechanism of phosphate regeneration in natural waters. Other regenerative mechanisms, such as the participation of other enzyme systems and abiotic mechanisms, should be taken into account.

Methods for measuring extracellular phosphatases activity

The methods commonly applied for detection of phosphatase activity in natural environments and phytoplankton cultures are based on the hydrolysis of artificial substrates (added to the samples) by the enzyme bound to the cells and dissolved in the water. After the incubation of the samples in the presence of an artificial substrate (organic phosphorus), its hydrolysis is measured through the increase of the product (orthophosphate) per time and volume units. However, the concentration of orthophosphate released is often too low to be easily detected. Therefore, the substrates used in the enzymatic assays are those that originate colored or fluorescent organic compounds after hydrolysis. The amount of these compounds released after the enzyme-substrate reaction are stoichiometrically equal to the amount of orthophosphate released (Siuda, 1984). Such organic products are quantified by means of colorimetric or fluorimetric methods, respectively.

The most widely applied colorimetric method is based on the use of the p-nitrophenyl phosphate (pNPP) as a substrate (Bessey *et al.*, 1946; Fitzgerald & Nelson, 1966; Reichardt *et al.*, 1967). The pNPP is hydrolyzed by phosphatases releasing p-nitrophenol (pNP), with a maximum absorption at 400-418 nm. Methods that apply fluorogenic substrates were described by Perry (1972), Pettersson & Jansson (1978) and Pettersson (1979). Among the fluorogenic substrates, the most widely used is the 4-methylumbelliferyl phosphate (MUF-P), whose hydrolysis originates a fluorescent product, the 4-Methylumbelliferone (MU) (Pettersson & Jansson, 1978).

The absolute values of phosphatase activity for a given sample differ according to the substrate employed in the enzymatic assay. For instance, Berman *et al.* (1990) found phosphatase activities reaching $0.93 \mu\text{gPO}_4 \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ and $0.14 \mu\text{gPO}_4 \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ when they used pNPP and MUF-P, respectively. These discrepancies are due to differences in the affinity of the enzymes by distinct substrates (Berman *et al.*,

1990). However, results of phosphatase activities obtained by means of both substrates have similar patterns when different samples are compared. According to Berman *et al.* (1990), fluorogenic substrates are preferable compared to colorimetric ones when low phosphatases activities in the samples are expected. However, fluorogenic substrates originate higher variability in the results of a given sample.

Methods based on radiolabelled natural substrates have recently been applied in studies about extracellular enzymes in natural communities. For instance, Hernández *et al.* (1996) investigated the regeneration and utilization of phosphorus by microorganisms in natural environments using glucose-6-phosphate radiolabelled with ^{32}P as substrate. The advantage of radiolabelled substrates is that organic compounds naturally found in aquatic systems may be employed in the enzymatic assay.

A method to detect extracellular phosphatase activity connected to single cells in a given sample was recently developed by González-Gil *et al.* (1998). This method has the potential to indicate which cells or which species in a natural community are responding to phosphorus limitation through the synthesis of extracellular phosphatases. This approach represents an advantage comparing to the traditional methods, which do not discriminate the origin of phosphatases in the sample.

The method described by González-Gil *et al.* (1998) is based on the use of a colorless molecule commonly called ELF (Enzyme-Labeled Fluorescence) substrate or reagent (Molecular Probes Inc., OR, USA). The unreacted ELF substrate is soluble and does not have high fluorescence. When reacted with the extracellular phosphatases, the phosphate is liberated and an insoluble yellow-green product precipitates rapidly at the site of the enzymatic activity (González-Gil *et al.*, 1998). The samples are further observed under fluorescence microscopy using a long-pass Hoechst/DAPI (4',6-diamino-2-phenyl-indole) filter set (excitation 365 ± 8 nm and emission >420 nm). The presence of the yellow-green spots on the cells indicates the sites where the reaction phosphatase-substrate took place.

The role of enzymes in the food webs

According to the classical models of food webs the organic matter is transferred to phytoplankton directly to zooplankton and then to the higher trophic levels, functioning in a linear path (phytoplankton-zooplankton-fish) (Pomeroy, 1974).

Several investigations about food webs conducted in the 70's and 80's showed that there are important connections between dissolved organic matter-bacteria-protozoa, which originated the concept of "microbial loop" (Azam *et al.*, 1983). This concept, added to the conventional food web model, established that part of the carbon fixed during the photosynthesis (5 to 50%) is excreted by phytoplankton and subsequently taken up by bacteria. Bacteria is preyed by heterotrophic protozoa (flagellates and ciliates), which are consumed by macrozooplankton (e.g. crustaceans). Then, part of the energy returns to the classical food web (to higher trophic levels) through fish predation, for example.

The microbial loop model emphasizes the role of bacteria and bacterivorous organisms in organic matter transfer and mineralization. However, these processes strongly depend on the organic matter transformations mediated by microbial enzymes. The enzymatic hydrolysis of high molecular weight compounds is the initial and obligatory hydrolytic step of microbial nutrient regeneration (Hoppe, 1991). Therefore, it is suggested that the microbial loop model (Azam *et al.*, 1983) be amended to include the mineralization of dissolved organic matter by microbial extracellular enzymes (Hoppe, 1991).

Phosphatase activity in other compartments of the aquatic systems *Epiphytic communities*

Among the studies about enzymes in aquatic environments, major attention has been devoted to enzymatic activities related to plankton communities. However, substantial extracellular enzymatic activity has been detected in epiphytic communities associated to aquatic macrophytes (Burkholder & Wetzel, 1990; Chappell & Goulder, 1994a), stones (Chappell & Goulder, 1992, 1994b) and artificial substrates (Sinsabaugh & Linkins, 1988; Burkholder & Wetzel, 1990; Jones & Lock, 1993).

Variations in phosphatase activity in epiphytic communities is connected to the type of the colonized substrate (Sinsabaugh & Linkins, 1988; Chappell & Goulder, 1994a), water characteristics, such as pH, temperature, concentration of microelements, and attached biomass (Chappell & Goulder, 1994b) and seasonal changes (Sinsabaugh & Linkins, 1988).

Epiphytic communities may be responsible for half of the total primary production in lakes (Wetzel, 1983). Therefore, it would not be surprising that this high metabolic activity is also followed by high activities of extracellular enzymes. A comparison of enzyme activities between plankton and epiphyton is not always possible due to differences in the methodological approach used in the enzymatic assays, and also due to the difficulties in normalizing the results to comparable units.

A comparison between exoenzymatic activities (phosphatases, β -D-glucosidase, β -D-galactosidase e β -D-xylosidase) connected to epiphytic communities and in the surrounding pond water was made by Chappell & Goulder (1994a). They concluded that the enzymatic activity detected in 1 cm² of shoot surface of *Phragmites australis* corresponded approximately to the extracellular activity found in 2.5 liters of surrounding pond water. Therefore, in aquatic environments densely colonized by aquatic macrophytes, as it is the case of many Brazilian lakes and reservoirs, the total extracellular enzymatic activity in the epiphyton may be much higher than in the plankton.

An attempt to measure epiphytic phosphatases activity in a Brazilian coastal lagoon was made by R. Panosso (unpublished data). Pieces (size ca. 16 cm²) of sub-

merged leaves of *Typha domingensis* were collected in the Imboassica Lagoon and transported to the laboratory in polycarbonate bottles with lagoon water. No conspicuous loss of epiphytic biomass was observed during collection and handling. Epiphyton was gently scraped off from the leaves. Thirty-five ml of sterile distilled water were used to wash the leaves during the epiphyton removal procedure in order to loosen residual biomass attached to the plant. Aliquots of the epiphyton diluted in 35 ml of water were used for the phosphatase assay. Extracellular phosphatase activity was assayed using p-NPP as substrate, following the procedure described by Panosso & Esteves (1999). Extracellular phosphatase activity in the epiphytic community associated with leaves of *T. domingensis* from the Imboassica lagoon was found to be $9.6 \text{ nmol.cm}^{-2}.\text{h}^{-1}$. This value is similar to the phosphatase activity in the epiphyton associated with *Phragmites australis* and *Elodea canadensis* (average 13.3 and $5.33 \text{ nmol.cm}^{-2}.\text{h}^{-1}$, respectively) reported by Chappell & Goulder (1994a).

Sediment

Phosphatase activity has been studied in marine (e.g. Kobori & Taga, 1979; Meyer-Reil, 1991), lagoon (e.g. Degobbis *et al.*, 1984), lake (e.g. Holdren & Armstrong, 1980; Newman & Reddy, 1992) and stream sediments (e.g. Marxsen & Schmidt, 1993; Scholz & Marxsen, 1996). The enzymatic hydrolysis of organic phosphorus may be a limiting step for microbial production in the sediment, and may contribute with a large part of the phosphorus flux in this compartment (Marxsen & Witzel, 1991; Scholz & Marxsen, 1996).

Moreover, phosphatases associated to sediments may contribute to the total activity in the water column by means of resuspension of the superficial layers of the sediment (Newman & Reddy, 1992). These events deserve special attention in the quantification of phosphorus hydrolysis in the water column of shallow lakes highly exposed to wind, features commonly found in Brazilian lakes.

Research Perspectives

One major limitation for the use of extracellular phosphatases activity to evaluate the quantitative importance of enzymatic hydrolysis in phosphate regeneration is that the actual rate of organic phosphorus hydrolysis and the concentration of substrates in natural environments are often not known. A good approximation of the actual rate of release of phosphate from naturally occurring substrates requires the calculation of kinetic parameters of extracellular phosphatases present in the water (e.g. Chróst, 1991; Panosso & Esteves, 1999), and the determination of phosphomonoester concentrations (e.g. Boavida, 1991). Although the determination of kinetic parameters is time-consuming, this approach should be encouraged in order to promote a better understanding of the role of phosphatase activity in phosphorus mineralization.

The mobilization of phosphate from dissolved organic matter may be promoted by other enzyme systems besides phosphatases, such as 5'-nucleotidase

(Ammerman & Azam, 1991a, 1991b), and by abiotic mechanisms such as photoreduction by ultraviolet radiation (Francko, 1986, 1990). Nutrient recycling by zooplankton and fish has also been found to be a significant source of phosphate in pelagic areas (Reimertsen *et al.*, 1986; Brabrand *et al.*, 1990). The relative importance of these mechanisms for phosphate regeneration is unclear and constitute a relevant approach in aquatic ecology.

The knowledge about the origin of extracellular phosphatases in natural communities could be greatly improved through the use of the ELF method (González-Gil *et al.*, 1998) in studies of enzymes in aquatic systems. Approaches applied so far for investigating this question are based on enzymatic assays with size fractionated samples derived from differential filtration (Franko, 1983) and with algal monocultures. However, the resolution of differential filtration for detecting phosphatases is not satisfactory because results of the enzymatic assays reveal the activities associated to different size classes, and not to particular populations. Moreover, differential filtration may produce biased results (Münster, 1991; Panosso, 1998). Enzymatic assays using laboratory axenic cultures reveal the ability of extracellular phosphatase synthesis of particular species, but do not clarify in which environmental conditions this ability is expressed. The use of methods to detect phosphatases activity connected to single cells, such as the ELF method, would help the understanding of the role of microorganisms ability to mineralize organic phosphorus in structuring plankton communities. Additionally, other sources of phosphatases so far neglected in aquatic ecology, such as fungi, should be investigated.

Finally, studies about extracellular phosphatase activity in sediment and epiphytic communities are scarce and deserve special attention from aquatic ecologists. These compartments may contribute to a large part of phosphorus mineralization in lakes, especially in shallow systems with abundant littoral vegetation. This type of environment is abundant among the Brazilian inland water bodies, but the dynamics of phosphatase activity in their sediment and epiphytes is not known.

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