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Phosphatases; origin, characteristics and function in lakes

Mats Jansson, Håkan Olsson & Kurt Pettersson
Institute of Limnology, Box 557, 751 22 Uppsala, Sweden

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Abstract

Phosphatases catalyze the liberation of orthophosphate from organic phosphorus compounds. The total phosphatase activity in lake water results from a mixture of phosphatases localized on the cell surfaces of algae and bacteria and from dissolved enzymes supplied by autolysis or excretion from algae, bacteria and zooplankton. External lake water phosphatases usually have pH optima in the alkaline region. Acid phosphatases generally seem to be active in the internal cell metabolism. The synthesis of external alkaline phosphatases is often repressed at high phosphate concentrations and derepressed at low phosphate concentrations. Phosphatase activity has therefore been used as a phosphorus deficiency indicator in algae and in natural plankton populations. The possibilities for this interpretation of phosphatase activity in lake water are limited, however, and this is discussed. The *in situ* hydrolysis capacity, i.e. the rate by which orthophosphate is released from natural substrates, is unknown. However, we advocate that this process is important and that the rate of substrate supply, rather than phosphatase activity, limits the enzymatic phosphate regeneration.

Introduction

Phosphatases are enzymes which promote the degradation of complex phosphorus compounds into orthophosphate and an organic moiety and are thus believed to have an essential function in the nutrient dynamics of lakes. The occurrence of enzymatic breakdown of organic phosphorus in lake water was first suggested by Steiner (1938) but more intensive studies on phosphatases and their function in aquatic systems did not begin until around 1960. During recent decades the development of improved analytical techniques and growing interest in phosphorus turnover in aquatic systems have led a large number of ecologists and physiologists to study phosphatases in freshwater environments.

In this article we give a brief review of the function, characteristics and role of phosphatases in lakes. We focus on what we consider to be relevant aspects of the ecological significance of phosphatases and refer the reader with a deeper interest in physiological implications of phosphatases to, e.g., the excellent book of McComb *et al.* (1979).

The phosphatase concept

The term phosphatase is commonly used for enzymes which catalyze the hydrolysis of esters and anhydrides of phosphoric acid (Feder, 1973). Most often, 'phosphatase' is used synonymously with phosphomonoesterases, also called phosphomonoesterhydrolases, which are a group of enzymes which can catalyze the hydrolysis of a

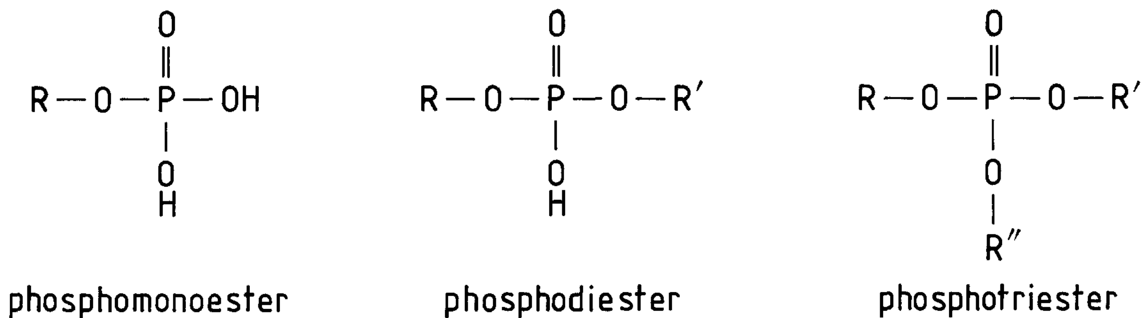

Fig. 1. General formulas of different types of phosphate esters. R represents the organic part of the molecule.

rich variety of phosphomonoesters. Similar but functionally different enzymes are the phosphodiesterases (comprising the nucleases). General formulas for the substrates of the different types of phosphoesterases are shown in Fig. 1.

Phosphatases have also been suggested to be involved in metabolic processes other than hydrolysis, such as transport of substances across membranes and synthesis of new organic phosphates (transphosphorylation) (McComb *et al.*, 1979). In the following, we will concentrate on the catalytic breakdown of phosphomonoesters, which is by far the most studied phosphatase-process in lake waters. For reasons of simplicity the short term phosphatase will be used.

Reaction mechanism

The reaction mechanism for phosphatase catalysed hydrolysis of phosphate esters (Fig. 2) is divided into four steps:

1. Non-covalent binding of the substrate to the enzyme.
2. Alcohol release from the complex and orthophosphate becomes covalently bound to the enzyme forming a phosphoryl-enzyme compound.
3. Conversion of the phosphoryl-enzyme compound, through uptake of water, to a non-covalent complex.
4. Release of orthophosphate and regeneration of free enzyme.

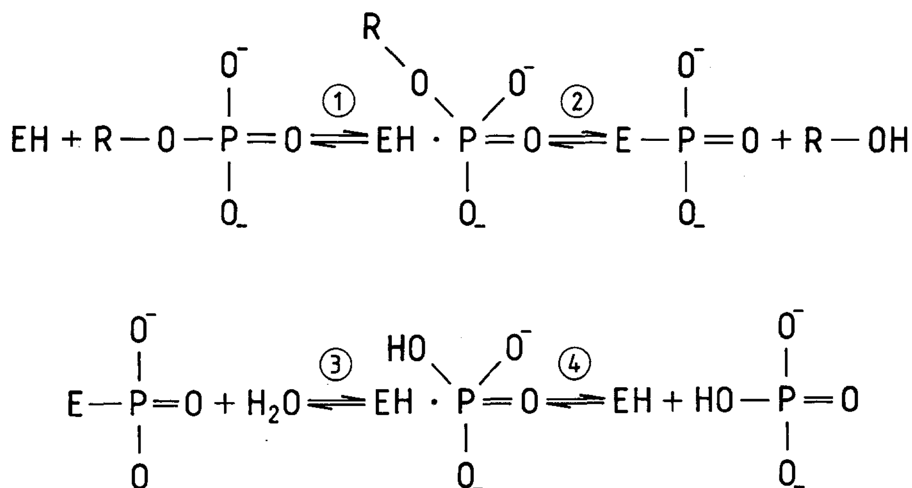

Fig. 2. Reaction-scheme for the enzyme catalysed hydrolysis of phosphate esters as described by McComb *et al.* 1979. See text for more information about the reaction-steps.

Any of the steps 2-3 can be rate-limiting for the overall reaction (McComb *et al.*, 1979).

The phosphatase activity will primarily depend on the type and concentration of the substrate and the enzyme. Other factors affecting phosphatase activity are temperature, ionic strength, pH and metal ions (McComb *et al.*, 1979). The effects of ionic strength and metal ions depend on the type of enzyme, pH of the medium and types of ions involved. Alkaline phosphatases have been characterized as metallo-enzymes with an essential metal ion, most often Zn^{2+}, taking part in the reaction at the active site of the enzyme. Addition of divalent cations to the reaction medium can increase the phosphatase activity. Possible mechanisms of this activation are discussed by Spiro (1973).

Measurements of phosphatase activity

Phosphatase activity is assayed by hydrolysis of a suitable artificial substrate and detection of the increase over time of the organic product or orthophosphate. Usually the maximal potential activity is desired as an estimate of the amount of phosphatases in the sample. For this purpose the substrate concentration should be high enough to allow the reaction to proceed at maximum velocity.

Phosphatase activity can be measured on water samples without any pre-treatment. The measured activity then represents the activity of phosphatases dissolved in the water and enzymes associated with living cells and dead particles. Differential filtration allows the determination of activity associated with particles of different size. The activity can be measured in filtrates giving the particulate activity as the difference between total and dissolved activity. The filter can also be homogenized in buffer or in filtered and/or autoclaved water for subsequent determination of activity of retained material.

Analysis of internal cellbound phosphatases is possible after sonical (Reichardt, 1971, 1973; Rivkin & Swift, 1980; Klotz, 1985b), mechanical (Yentsch *et al.*, 1972; Lin, 1977), chemical (McComb *et al.*, 1979) or enzymatic (Ihlenfeldt & Gibson, 1975) lysing of cell preparations. However, intracellular phosphatases might become inactive if they are released from their original matrix and exposed to a new environment. Talpasayi (1962), for example, found that living cells of algae exhibited higher acid phosphatase activity than damaged cells.

The phosphatase activity measured in routine assays cannot be used for predictions of *in situ* hydrolytic activity. The most obvious reasons are that:

a. Substrate concentrations are many times lower in nature than those used in phosphatase analyses. Consequently the natural rate of hydrolysis will be significantly lower.
b. Standardized temperature and pH are often not realistic compared with those in lake waters.
c. The structure of natural substrates is more or less unknown. Although the activity of phosphatases is restricted only to the P-O bond of the organic phosphate the affinity for the substrate can differ depending on the structure of the organic part of the molecule. The possible representativeness of the artificial substrate in this respect is unknown.

Problems and possibilities to estimate *in situ* hydrolysis are discussed under a separate heading later in this chapter.

Measurements of potential phosphatase activities in lake water often require high sensitivity of the assay to keep the incubation time short and to avoid preservative agents or concentrations of enzymes. The most sensitive assays utilize substrates which give a fluorescent product with low detection limit. Of the six substrates tested by Pettersson & Jansson (1978) (methylumbelliferylphosphate, methylfluoresceinphosphate, p-nitrophenylphosphate, glycerophosphate, phenolphtaleinphosphate and 1-naphtolphosphate) the fluorogenic methylumbelliferylphosphate (MUP) was recommended for phosphatase activity measurements in lake waters, because it offered the most sensitive method and gave low background fluorescence. MFP, 3-o-methylfluoresceinphosphate is another frequently used

fluorogenic substrate (Perry, 1972; Jansson, 1976, 1977; Sproule & Kalff, 1978; Kobori & Taga, 1979a,b; Healey & Hendzel, 1979, 1980; Smith & Kalff, 1981; Vincent, 1981; Wetzel, 1981; Stewart & Wetzel, 1982a,b; Francko & Wetzel, 1982; Francko, 1983, 1984a,b; Currie & Kalff, 1984; Bothwell, 1985). However, this substrate has a high background fluorescence which necessitates use of a low substrate concentration which might be in conflict with the need to have the reaction proceed at substrate saturation. Hitherto the most common substrate in freshwater studies has been p-NPP, p-nitrophenylphosphate, where the reaction product p-nitrophenol is detected colorimetrically (Reichardt *et al.*, 1967; Berman, 1969, 1970; Reichardt, 1971, 1973; Jones, 1972a,b; Heath & Cooke, 1975; Verstraete *et al.*, 1976; Stevens & Parr, 1977; Wynne, 1977, 1981; Sayler *et al.*, 1979; Fuhs *et al.*, 1982; Tiwari & Mishra, 1982; Livingstone *et al.*, 1983; Livingstone & Witton, 1984; Matavulj *et al.*, 1984; Chrost *et al.*, 1984; Halemejko & Chrost, 1984; Klotz, 1985a,b). The substrate p-NPP offers a reliable determination when working with high phosphatase concentrations as in algal cultures or if long incubation times can be tolerated. However, the fluorogenic substrates are superior for phosphatase measurements in most lake waters, since the possibility to detect low amounts of the organic product allows the measurements to be completed within one hour. A short incubation time is recommended to avoid contamination, bacterial growth, the use of preservatives and cell-lysis during the measurements.

Origin of phosphatases

As far as investigated all components of the biota in aquatic environments produce phosphatases but the quantitatively most important contributors to the overall phosphatase activity appear to be bacteria, phytoplankton and zooplankton.

Bacteria

Bacteria certainly express phosphatase activity. Much of what is known about the function and physiology of phosphatases is derived from studies on *Escherichia coli* (see McComb *et al.*, 1979), and phosphatase activity has been shown in bacterial strains isolated from lake water (Jones, 1972a; Chrost *et al.*, 1984; Halemejko & Chrost, 1984). However, few ecological studies have been made on aquatic bacterial phosphatases. Reichardt (1973) showed that myxobacteria in Mindelsee produced large amounts of phosphatases when isolated in the laboratory, but he was unable to verify their importance *in situ*. Similarly, Jones (1972a) found that between 13 and 58% of viable bacteria (isolated on agar plates) from Lake Windermere and Esthwaite Water produced phosphatases but concluded that although the phosphatase activity in the lakes covariated with both bacterial and algal biomass it was impossible to determine their origin.

Investigations concerning the origin of lake phosphatases often apply indirect observations such as the presence (Reichardt *et al.*, 1967; Jones, 1972a) or the absence (Pettersson, 1980) of correlations between phosphatase activity and bacterial numbers. Chrost *et al.* (1984) arbitrarily defined phosphatases which passed a 3 μm filter but were retained on a 0.45 μm filter as 'bacterial' while those retained on 3 μm membranes were regarded as originating from living algae and bacteria associated with detritus. With this method they found that bacterial phosphatases were dominant in deep water layers of Lake Glebokie, Poland.

However, a more rigorous analytical approach, including correlations and size fractionations, should be applied to determine the relative abundance of algal and bacterial phosphatases. Thus, Stewart & Wetzel (1982a) separated phosphatase and ^{14}C-labelled (autotrophic uptake) seston by centrifugation and proposed that non-algal sestonic phosphatases could be a major component of the particulate associated phosphatase pool. Furthermore, they suggested that part of the 'dissolved enzymes', passing 0.45 or 0.50 μm

membranes could in fact be bound to bacteria. If the results of Stewart & Wetzel are generally valid, then bacterial phosphatases may be more common than presently believed.

More specific studies on aquatic bacterial phosphatases have been made with marine species. Unfortunately (in this context) these are more dedicated to physiological features than ecological implications. From studies of marine pseudomonads (Thomson & MacLeod, 1974a,b; Hassan & Pratt, 1977) it appears that phosphatases which hydrolyze external substrates in these bacteria are located in the periplasmatic space i.e. the part of the cell surface between the inner and outer layer of the cell envelope. Although demonstrated in, e.g., culture strains of *Micrococcus* (Glew & Heath, 1971), excretion of phosphatases from living cells seems to be less common in bacteria than, e.g., in algae. Corpe & Winters (1972) studied hydrolytic enzymes from different marine pseudomonads and found excretion of some enzymes but not phosphatases. In a recent paper, Kobori & Taga (1980) stated that they were the first to report release of extracellular phosphatases from actively growing marine bacteria (a pseudomonad, isolated from 200 m depth in the North Pacific Ocean). Kobori *et al.* (1979) found that bacteria in coastal water produced repressible phosphatases, whereas a major part of phosphatase producing bacteria in oceanic water had constitutive enzymes with high activity.

Bacterial enzymes should be dominant in the sediments but few investigations on phosphatases in sediments are reported. Reichardt (1978) found a positive correlation between alkaline phosphatase producing bacteria and alkaline phosphatase activity in the uppermost layer of the sediment of Lake of Constance (Obersee), and good correlations between phosphatase activity and viable bacterial cell counts in sediments. Jansson (1975) found considerable activity (compared with lake water) and pronounced vertical patchiness of phosphatases in pore-water of surface sediments in subarctic lakes but it was not possible to separate bacterial contribution from that of benthic algae. Pettersson (unpubl.) detected phosphatase activity in the surface sediments of Lake Erken. The activity increased with water depth and was correlated with organic content, indicating bacterial production.

Algae

Synthesis of phosphatases with external function has been frequently demonstrated in cultured algae (Overbeck, 1962; Kuenzler, 1965; Kuenzler & Perras, 1965; Healey, 1973; Healey & Hendzel, 1979; Wynne, 1981; Smith & Kalff, 1981). Phosphatases are located on the cell surface (Brandes & Elston, 1956) or in cell membranes (Kuenzler & Perras, 1965, Møller *et al.*, 1975) and the release of extracellular phosphatases in cultures is frequently reported (Healey, 1973; Aaronson & Patni, 1976; Patni *et al.*, 1977; Aaronson, 1971; Walther & Fries, 1976; Healey & Hendzel, 1979; Wynne, 1981).

Phosphatase activity has been found in all major groups and numerous species of algae and it is more difficult (if at all possible) to find an algae that does not synthesize phosphatases than one that does. However, as for bacteria, conclusions about the role of algae for the enzymatic regeneration of phosphate are based mainly on indirect evidence, mostly relationships between phytoplankton biomass and phosphatase activity.

Pettersson (1980) thus concluded that phytoplankton was the source of phosphatase activity in Lake Erken since the bacterial biomass was negligible and since spatial and temporal variations in phosphatase activity were well correlated with changes in chlorophyll concentration. Heath & Cooke (1975) found high phosphatase activity co-occurring with *Aphanizomenon* blooms and also that enzymes and algae were retained on filters of the same porosity. From these results they concluded that *Aphanizomenon* was the dominant producer of phosphatases in East Twin Lake during summer. Jansson (1975) arrived at a similar conclusion from studies in subarctic lakes where size fractionation with filters of the porosities 0.20, 0.45, 1.00, 3.00 and 5.00 μm showed that phosphatases and chlorophyll were nearly always retained on the same filters. Olsson (1983)

found a clear connection between phosphatase activity and dominance of small chrysomonads in acidic lakes.

Phosphatase activities are often assumed to be of algal origin and used as indicators of phosphorus deficiency without any proof of their origin. Although the general understanding that algal phosphatases are the most important in epilimnetic waters probably is correct, it should be stressed that a limited number of studies offer substantial proof of this statement.

Zooplankton

Excretion of phosphatases from zooplankton has been reported by Steiner (1938) (mixed net sample), Rigler (1961) (*Daphnia magna*), Jansson (1976) (*Bosmina obtisurostris, Holopedium gibberum, Cyclops scutifer*), Wynne & Gophen (1981) (*Mesocyclops leuckarti*) and Boavida & Heath (1984) (*Daphnia magna*). Characterization of phosphatases excreted by zooplankton using gel filtration (Jansson, 1976) gel electrophoresis (Wynne & Gophen, 1981) and anion exchange chromatography (Boavida & Heath, 1984) indicate that enzymes released from zooplankton are indeed produced by the animals themselves and not by components of their food.

The zooplankton phosphatases can contribute considerably to the overall phosphatase activity. Thus, Jansson (1976) found that approximately 50% of the dissolved phosphatase activity in Lake Hymenjaure (25% of total activity) was identical with phosphatases released by zooplankton. Boavida *et al.* (1984) have also suggested that diurnal variations in the phosphatase activity in East Twin Lake may be explained by cladoceran migration.

Dissolved phosphatases

'Free' phosphatases are generally defined as enzymes which pass 0.45 μm membrane filters. Such enzymes are nearly always found in lakes and often contribute a substantial part of the total phosphatase activity. Table 1 offers a survey of the occurrence of dissolved phosphatase activity in filtered or cell-free samples from lakes, algal cultures and zooplankton. The essential characteristics of dissolved phosphatases do not seem to differ from those of seston bound phosphatases (Glew & Heath, 1971; Jansson *et al.*, 1981).

Dissolved enzymes are supplied by excretion from growing algae and zooplankton but perhaps less so by bacteria (see above). Another important source should be losses from dying and disintegrating cells (Overbeck & Babenzien, 1964; Reichardt *et al.*, 1967; Berman, 1970). It is not clear how, or to what extent, active excretion of phosphatases takes place and whether it is more beneficial to release these enzymes or to localize them on the external cell surface.

Few attempts have been made to establish the origin of dissolved enzymes in lake waters and no clear picture of the contribution from different sources exists. It should also be pointed out that the operational definition 'enzymes which pass a 0.45 μm filter', does not exclude the part of dissolved enzymes associated with small planktonic organisms. Nonetheless, qualitative methods using biochemical separation techniques to characterize phosphatases from defined parts of seston and the dissolved pool should offer possible means of tracing the origin of dissolved phosphatases (Jansson 1976; Stewart & Wetzel, 1982a; Boavida & Heath, 1984).

Characteristics of phosphatases

Alkaline and acid phosphatases

Phosphatases typically have maximum hydrolyzing capacity at different pH values; hence the common division into alkaline and acid phosphatases. Alkaline phosphatases have pH optimum well above 7, not seldom between 9 and 10, while acid phosphatases are those which express the highest activity below pH 7, generally between pH 4 and pH 6. However, the fact that different enzymes have different pH optima does not mean that they are totally inactive at, or do not tolerate, other pH values.

Table 1. Free 'dissolved' phosphatase activities detected in samples from lakes, ponds, algal cultures and due to zooplankton excretion.

Type of sample (L = lake water A = algal cult. Z = zoo-plankton) P = pond	Type of phosphatases assayed (Ac = acid N = neutral Al = alkaline)	Pore-size if filter was used (μm) (– = not specified) (centr. = centrifugation)	% of total phosphatase activity	References
L	Ac	0.20	ca. 50	Jansson *et al.*, 1981
L/A	Ac, Al	0.22	–	Reichardt, 1971
L	Ac, Al	0.45	ca. 10–90	Halemejko & Chrost, 1984
L/Z	N	0.20	25	Jansson, 1976
L	N	0.22	–	Jones, 1972a,b
L	N	0.45	ca. 30	Chrost *et al.*, 1984
L	Al	0.20	15–70	Pettersson, 1980
L	Al	0.22	<6	Francko, 1983
L	Al	0.22	–	Tiwari & Mishra, 1982
L	Al	0.45	0–57 $\bar{x}$ = 16	Berman, 1970
L	Al	0.45	20–98 $\bar{x}$ = 48	Francko, 1984b
L	Al	0.45	4–82 $\bar{x}$ = 16	Gunatilaka, 1984
L	Al	0.45	5–50 $\bar{x}$ = 26	Healey & Hendzel, 1979
L	Al	0.50	often >50	Wetzel, 1981
L	Al	0.60	7–25	Reichardt *et al.*, 1967
L	Al	centr.	14–61	Stewart & Wetzel, 1982a
P	Ac, Al	–	–	Overbeck, 1964
P	Al	0.45	11–28	Berman & Moses, 1972
A	Ac	centr.	29–65	Aaronson & Patni, 1976
A	Ac	centr.	–	Antia & Watt, 1964
A	Ac, Al	0.45	–	Wynne, 1981
A	Ac, Al	centr.	(>cellular)	Aaronson, 1971
A	Ac, Al	centr.	–	Patni *et al.*, 1977
A	N	0.45	often <50	Huber *et al.*, 1983
A	Al	0.45	10–50	Healey, 1973
A	Al	0.45	4–20	Healey, 1985
A	Al	0.45	5–80	Healey & Hendzel, 1979
A	Al	0.45	–	Lin, 1977
A	Al	–	0 – ca. 10	Kuenzler & Perras, 1965
A	Al	–	0–63	Walther & Fries, 1976
Z	Ac, Al	0.45	–	Boavida & Heath, 1984
Z	N	–	–	Rigler, 1961

Both alkaline and acid phosphatases have been found as external and internal enzymes in algae and bacteria (Siuda, 1984) and both types can be excreted by zooplankton (Wynne & Gophen, 1981). The alkaline phosphatases with external function are by far the most studied type in aquatic ecosystems. Conditions which favour activity of acid phosphatases are not common in most lakes. Therefore acid phosphatases are believed to be less important for *in situ* phosphorus mineralization. It is also a fact that algae and bacteria growing in alkaline environments generally produce more alkaline than acid phosphatases with external function (Kuenzler & Perras, 1965; Aaronson & Patni, 1976; Cembella *et al.*, 1984).

However, in many respects alkaline and acid phosphatases share essential characteristics (see e.g. Feder, 1973). Both types are also broad in specificity against different substrates, i.e. their

activity is restricted only to the P-O bond on the phosphomonoesters. However, differences have been reported. Alkaline phosphatases often require divalent metal ions for their activity and are inhibited by chelators such as EDTA, while acid phosphatases are frequently specifically inhibited by fluoride (Cembella *et al.*, 1984).

In a thorough characterization of phosphatases in the acidified Lake Gårdsjön (Jansson *et al.*, 1981) four different acid phosphatases (MW-criteria) were found. They were all produced by plankton, mainly small species ($< 5\ \mu m$), they clearly had an external function, were inhibited by orthophosphate and had K_m-values similar to many alkaline phosphatases. It was proposed that these enzymes represented an adaptation to acid conditions and had the same function as typical alkaline phosphatases in lakes with higher pH.

Acid and alkaline phosphatases show essential differences concerning their location in the cell and the mode of their synthesis. Acid phosphatases are often found inside algal cells rather than in contact with the surrounding medium (Møller *et al.*, 1975; Wynne, 1977; Schmitter & Jurkiewicz, 1981; Siuda, 1984). Furthermore, in contrast to alkaline phosphatases the synthesis of these internal acid phosphatases are generally not repressed by orthophosphate (Kuenzler & Perras, 1965; Wynne, 1977).

It is therefore possible that acid phosphatases are constitutive (see below) enzymes produced mainly to serve the internal phosphorus metabolism while alkaline phosphatases have external functions and a synthesis which depend on the ambient phosphorus nutrition.

Control of synthesis and inhibition by phosphate

The synthesis of enzymes is influenced most by the substrate supply or reaction products. Inducible enzymes are those where synthesis starts in the presence of suitable substrates. Constitutive enzymes are produced independently of an activator, i.e. they are more or less constantly synthesized in the cell.

Repression occurs when a compound, often the end product of the enzyme-catalyzed reaction, turns off the enzyme synthesis. Consequently, derepression is the onset of enzyme production after the depletion of the repressor. High levels of inorganic phosphate often repress phosphatase synthesis (Cembella *et al.*, 1984).

Inhibition is when a compound reacts with the enzyme itself and stops its activity. Orthophosphate is a common inhibitor which competes with phosphate esters for the active sites on the phosphatases; an example of competitive end product inhibition. This terminology can be confusing and the literature contains several examples where induction, for example, is used synonomously with derepression.

Most of the types of regulation mechanisms are reported from different aquatic organisms (Table 2). Induction, where phosphatase activity is enhanced by addition of substrate, seems rather uncommon (or not investigated). Aaronson & Patni (1976) demonstrated that the secretion of acid phosphatases in *Ochromonas danica* increased after addition of glucose-1-phosphate and glucose-6-phosphate. Constitutive enzymes have been reported from both bacteria and algae and probably a background activity of a constitutive nature exists in most organisms and most lake waters. Algal acid phosphatases seem to be mostly constitutive while algal alkaline phosphatases are mostly repressible. As mentioned previously, acid phosphatases are mostly located internally while alkaline phosphatases generally have external functions in algae. It is logical that enzymes with the function of supplying the algae with phosphate from outside the cells are regulated by the phosphate supply. Several studies have shown that an internal phosphate pool regulates the synthesis of repressible phosphatases (Fitzgerald & Nelson, 1966; Lien & Knutsen, 1973; Wynne, 1977; Pettersson, 1980, 1985; Elgavish *et al.*, 1982). When the pool is filled, synthesis of enzymes is shut down. Derepression occurs after the depletion of the pool.

Phosphate inhibition and repressible bacterial enzymes are probably more frequent (cf. McComb *et al.*, 1979) than appears from Table 2.

Table 2. Regulation of phosphatase synthesis and phosphate inhibition of phosphatase activity in different aquatic algae and bacteria.

Type of phosphatase	Induction	Mode of synthesis		Inhibition by phosphate	
		Constitutive	Repression	Yes	No
Bacterial					
acid	–	–	–	–	–
alkaline	–	Marine pseudomonads (Hassan & Pratt, 1977, Thomson & MacLeod, 1974a)	Marine pseudomonads 4 species (Hassan & Pratt, 1977)	–	Marine pseudomonads (Kobori & Taga, 1980)
Algal					
acid	[1]*Ochromonas danica* (Aaronson & Patni, 1976)	Several marine sp. (Kuenzler & Perras, 1965) *Melosira granulata* (Healey & Hendzel, 1979) *Chlamydomonas reinhardtii* (Lien & Knudsen, 1973)	*Euglena gracilis* (Price, 1969) *Gymnodinium nelsonii* (Kuenzler & Perras, 1965) *Synura uvella* (Healey & Hendzel, 1979) *Chlamydomonas reinhardti* (Patni *et al.*, 1977)	*Chlamydomonas acidophila* (Boavida & Heath, 1984) *Peridinium cinctum* (Wynne, 1977)	*Peridinium cinctum* (Wynne, 1977)
alkaline	–	–	Several algal species (Kuenzler & Perras, 1965) Several freshwater sp. (Fitzgerald & Nelson, 1966; Healey & Hendzel, 1979) *Chlamydomonas reinhardti* (Patni *et al.*, 1977)	*Chlamydomonas acidophila* (Boavida & Health, 1984) *Schroederia setigera* (Jansson, 1976)	–

[1] Substrate supply increased the secretion of phosphatases. Whether this was due to increased synthesis was not reported.

In this respect Table 2 certainly reflects the lack of relevant investigations rather than the real situation. The data in Table 2 are almost entirely obtained with cultured organisms or isolated enzymes. Several field investigations have been performed which support the hypothesis that alkaline phosphatases to a large extent are repressible and produced mainly during periods of pronounced phosphorus deficiency (see section of 'Alkaline phosphatase activity as a phosphorus deficiency indicator').

Phosphatases excreted by zooplankton seem to be less inhibited by phosphate than algal phosphatases (Jansson, 1976; Boavida & Heath, 1984).

Stability of phosphatases

Dissolved phosphatases are functional for relatively long periods under 'sterile' conditions in the laboratory. Alkaline phosphatases, when incubated with chloroform-saturated water, have been found to decline in activity less than 20% over ten days (Berman, 1970) and 30% over seven days (Kobori & Taga, 1979a). Jansson *et al.* (1981) found that dissolved acid phosphatases from Lake Gårdsjön kept their activity unchanged for twenty days and after 69 days still 10% of the activity remained (chloroform-treated water). Similar results were obtained when sterile filtered water was incubated, which indicates that the chloroform itself had no direct effect on the function of the enzyme. Reichardt *et al.* (1967) reported that phosphatases of the Pluss-See lost half of their activity within 3 days, and Halemejko & Chrost (1984) reported that free phosphatases retained 100% activity in the environment for 4 days after their release. However, Halemejko & Chrost did not present the methods used to obtain these results.

In laboratory studies, Rivkin & Swift (1980) found phosphatases from *Pyrosystis nocticula* to be stable with a low rate of turnover. On the other hand, Lien & Knutsen (1973) found phosphatases from *Chlamydomonas reihardti* to be unstable.

It is unlikely that the long functional time of phosphatases obtained in the absence of living plankton is applicable to lake ecosystems. Olsson (1983) noted a half-life of 40 days for acid phosphatases under sterile conditions. However, the observed activity in the lake decreased by 50% within two weeks. Based on field observations, Pettersson (1980) concluded that the turnover time of dissolved phosphatases in Lake Erken was only a few hours. The mechanism for the inactivation or breakdown of phosphatases *in situ*, however, remains to be clarified.

Substrate affinity

The tendency for phosphatases to combine with and hydrolyze their substrates is given by the K_m-value (Michaelis-Menten constant). K_m is the substrate concentration at which the reaction proceeds at half its maximum speed. A low K_m means that the enzyme has a high affinity to the substrate. However, the use of the Michaelis-Menten equation in a mixture of enzymes which all hydrolyze the same substrate (as in the phosphatase assay) is, from a theoretical standpoint, not correct. The function obtained can represent either the enzyme with the lowest K_m and highest saturation level or two or more enzymes, e.g. one with low K_m and low saturation level and one with higher saturation level. However, for comparison it is practical to characterize a group of enzymes by calculating a Michaelis-Menten equation.

K_m varies with substrate structure. For lake water phosphatases this variation is small (Pettersson & Jansson, 1978), which may be explained by the fact that these phosphatases are not substrate specific. Other factors which significantly influence K_m are pH and temperature. Comparison of K_m-values from different studies in fresh waters is therefore of less value due to great variation in incubation conditions. Usually K_m-values for alkaline and acid phosphatases between 10^{-6} and 10^{-4} M are reported.

An interesting observation was made by Pettersson (1980) who found that the K_m of the phosphatases in Lake Erken varied by one order

of magnitude during a year, with the lowest values in situations with pronounced phosphorus deficiency. He concluded that phytoplankton adapted themselves to low phosphorus supply not only by increasing their enzyme production but also by producing enzymes with improved ability to use low substrate concentrations.

Temperature dependence

Generally phosphatases have Q_{10} values between 1.5 and 3 and temperature optima well above the maximum temperature of natural waters; most often between 30 and 60 °C. Huber & Kidby (1984a) presented data from several investigations on natural waters and algal cultures showing phosphatase activity maxima mostly in the range 25–50 °C. Although enzymes from individual species may differ considerably in their temperature dependence, the temperature/activity relationship in the mixed populations of organisms found in lakes tends to be rather uniform (Healey & Hendzel, 1979). Healey & Hendzel found a similar relationship for three different lake samples, all showing temperature maxima at 35–40 °C.

Alkaline phosphatase activity as a phosphorus-deficiency indicator

In 1965 Kuenzler & Perras stated that many species of marine algae produce alkaline phosphatases when they become phosphorus-deficient, or formulated conversely, the production is repressed in phosphorus-sufficient algae. Fitzgerald & Nelson (1966) presented similar results for freshwater algae and reported as much as 25 times more alkaline phosphatase activity in phosphorus-limited algae. These two papers formulated the basis for the use of alkaline phosphatase activity as an indicator for phosphorus deficiency of algae in culture and in mixed populations from lake water. Healey (1978) confirmed that many algal species respond sufficiently similarly to phosphorus deficiency to permit measurements of phosphatase activity on natural mixed populations.

Theoretically, *the production rate* of derepressible phosphatases should give the best measurement of phosphorus deficiency. The only attempt, so far, to estimate the production of phosphatases in a lake was made by Olsson (1983). The method was based upon calculations of the difference between the import and the export of potential phosphatase activity. The activity changes obtained in this manner were compared with the measured activity variations in the lake. This approach is applicable only if the losses of active phosphatases *in situ* can be estimated. It is also a time-consuming method which needs detection of short-term variations.

In practice, the potential phosphatase activity and its variations has been used as an indicator of phosphorus deficiency. Several investigations, describing an inverse relationship between alkaline phosphatase activity and phosphorus in aquatic ecosystem, have motivated this approach. An inverse function has been shown to exist between the following variables:

1. Alkaline phosphatase activity and phosphate concentration (Jansson, 1975; Pettersson, 1980, 1985; Francko, 1984a; Chrost *et al.*, 1984).
2. Extracellular alkaline phosphatase activity and phosphate concentration (Reichardt, 1971).
3. Alkaline phosphatase activity and total phosphorus concentration (Berman, 1970; Smith & Kalff, 1981).
4. Alkaline phosphatase activity and total cellular phosphorus (Oláh & Toth, 1978; Gage, 1978; Pettersson, 1985).
5. Alkaline phosphatase activity and cellular surplus phosphorus (Fitzgerald & Nelson, 1966; Pettersson, 1980, 1985).
6. Alkaline phosphatase activity and phosphate uptake rate (Sproule & Kalff, 1978).
7. Cellular alkaline phosphatase activity and total cellular phosphorus (Wynne, 1977).

In each instance, low levels of alkaline phosphatase activity were associated with high con-

centrations of phosphorus. High levels of alkaline phosphatase activity were detected only when the concentrations of phosphorus were extremely low. It must be stressed that the potential phosphatase activity (when used as a deficiency indicator) should be measured at or near the saturation concentration of the substrate to give an estimate equivalent to the amount of active enzymes.

The potential phosphatase activity should be related to the biomass of the phosphatase producing organisms giving the specific potential phosphatase activity. Several biomass estimators have been used in investigations reported in the literature, but the most common are particulate organic matter (POM), particulate carbon, adenosine triphosphate and chlorophyll *a* (Pettersson, 1980).

Gage (1978) suggested the use of POM as the most reliable biomass estimator, while Pettersson (1980) proposed that the general picture was not affected by the choice of biomass estimator although chlorophyll *a* might vary due to interspecific variations as well as to environmental conditions. In situations with large amounts of detritus, ATP might be preferable in order to estimate the living biomass (Pettersson, 1980). Olsson (1983) related the phosphatase activity to the biomass ($mm^3 l^{-1}$) of small *Chrysophyceae*, since these algae were the main producers of phosphatases in the investigated lakes.

Critical specific phosphatase levels for different degrees of phosphorus starvation have been suggested by different authors (Table 3).

Derepression of alkaline phosphatase activity is often associated with low total cellular phosphorus concentration or low polyphosphate concentration of the cells. Both specific phosphatase activity and total cell phosphorus or polyphosphate concentration are in themselves deficiency indicators. However, they are not independent of each other since the repression – derepression of alkaline phosphatase is controlled by certain cell phosphorus fractions, probably polyphosphate (Cembella *et al.*, 1984).

Olsen *et al.* (1983) found that the derepression of the synthesis of alkaline phosphatases in chemostat cultures of *Chlamydomonas reinhardtii* started below an internal phosphorus level of about 6–7 μgP mgC^{-1} (3–3.5 μg $mgPOM^{-1}$). Fitzgerald & Nelson (1966) concluded that a surplus phosphorus (polyphosphate) level of 0.8 μgP $mgPOM^{-1}$ or less indicated a likelihood for derepression of phosphatase production. This was confirmed by Healey (1973) for *Anabaena variabilis*. Pettersson (1980, 1985) reported values in the interval 0.2–1.0 μg surplus-P $mgPOM^{-1}$ for spring phytoplankton in Lake Erken with high alkaline phosphatase activities and a threshold value of about 0.5 μg surplus-P $mgPOM^{-1}$ was determined from a set of data from several years.

The K_m-value of the alkaline phosphatases can be used as an indicator for phosphorus deficiency. The Michaelis constant of the phosphatases present in Lake Erken during the spring thus decreased from 2.4 $\mu mol\, l^{-1}$ in April to 0.28 $\mu mol\, l^{-1}$ in the middle of May when the availability of phosphorus was very low

Table 3. Specific phosphatase activity levels suggested to indicate different degrees of phosphorus starvation in planktonic communities.

	Levels of phosphatase activity ($nmolPO_4$ $mgPOM^{-1}$ h^{-1})			Comment
	Constitutive	Critical P-starvation	Severe P-starvation	
References:				
Gage & Gorham (1985)	<24	>48	>125	lake water plankton
Healey & Hendzel (1979)	<20	20–100	>100	algal cultures
Pettersson (1980)	3–12	33–42	75	lake water plankton
Pettersson (1985)	12–24	–	70–210	lake water plankton

(Pettersson, 1980). Reichardt & Overbeck (1969) reported an adaptation of the Michaelis constant of free dissolved phosphatases in the eutrophic Pluss-See to the amount of 'substrate phosphorus'. However, the explanation might just as well have been a low supply of phosphate to algae, as suggested for Lake Erken, since a low K_m was associated with low concentration of inorganic phosphate and 'substrate phosphorus' was equal to total phosphorus minus inorganic phosphate. Probably only a minor part of the 'substrate phosphorus' was made up of phosphomonoesters.

On the whole, very little information is available about the Michaelis constant of phosphatases in aquatic systems and its relation to availability of phosphorus.

Factors interfering with the use of phosphatase activity as a deficiency indicator

The usefulness of phosphatases as indicators of phosphorus deficiency has been questioned by Cembella *et al.* (1984). They concluded that 'the current practice of using assays of alkaline phosphatase as bioindicators of the nutritional status in natural phytoplankton populations is probably reckless and fraught with undesirable complications'. The main reasons leading to this conclusion were that bacterial phosphatases and phosphatases originating from lysed and aged cells could significantly influence the phosphatase activity detected. Significant contributions from zooplankton (Jansson, 1976; Boavida & Heath, 1984) are an additional related complication.

Apart from uncertainties about the internal (within lake) origin, confusion may also be caused by external input of enzymes. Stevens & Parr (1977) reported a high input of phosphatases to Lough Neagh, which severely decreased the value of the assay as an indicator of phosphorus deficiency. Dissolved phosphatases often contribute significantly to the total phosphatase activity. In addition to the difficulties in tracing their origin, they can be active for a long time (days to weeks) (Jansson *et al.*, 1981; Olsson, 1983). This means that phosphatases produced in a certain situation may remain active at another time and place. Another problem is that the constitutive activity which is not influenced by phosphorus starvation can be highly variable among species. Fitzgerald & Nelson (1966) thus found a tenfold variation between different fresh-water algae, with the highest values in blue-green algae.

Increased phosphatase activities can also be induced by other conditions than low phosphorus supply. Wilkins (1972) found that alkaline phosphatases of *Escherichia coli* were induced by starvation for pyrimidines or for guanine and not by lowering of the internal phosphate pool. Francko & Wetzel (1982) found a positive correlation between particulate cyclic AMP (cAMP) and specific activity of alkaline phosphatases for certain phytoplanktonic associations in two trophically dissimilar lakes. However, no correlation was found between dissolved cAMP and alkaline phosphatase activity. According to Francko (1984b), the alkaline phosphatase activity measured in lake water samples can be influenced by abundant dissolved nucleotides. Small additions (0.2–10 $nmol\,l^{-1}$) of cAMP caused increases (up to 525%) or decreases (up to 58%) in the measured phosphatase activity. In Lawrence Lake and Wintergreen Lake, Francko & Wetzel (1982) determined dissolved cAMP in the range 16–324 $pmol\,l^{-1}$).

Diurnal variations of phosphatases have been demonstrated (Reichardt, 1971; Rivkin & Swift, 1979; Wynne, 1981; Chrost *et al.*, 1984; Huber & Kidby, 1984b; Huber & Hamel, 1985). To some extent the diurnal variations have been related to changes in the nutritional status of algae. Nevertheless, the diurnal variations stress the time of sampling as a factor influencing the phosphatase activity.

Conclusion

Since the phosphatases in the aquatic environment can be of different origin and age and the measured activity can be temporarily influenced by other factors than nutritional status of the abundant microorganisms, phosphatase activity

should not be utilized as a phosphorus deficiency indicator in natural plankton assemblages without consideration of the most obvious uncertainties. As a minimum it is recommended that the origin of the phosphatases giving the measured activity is established. Other phosphorus deficiency indicators such as phosphorus content and N/P ratio of cells should be included in the study.

Enzymatic regeneration of phosphate *in situ*

The facts that phosphatases are synthesized in large amounts in situations of phosphorus deficiency and that the end product of the phosphatase catalyzed reaction – orthophosphate – is readily assimilated by algae, provide a good basis for the hypothesis that phosphatases are essential for phosphate regeneration and planktonic phosphorus nutrition in lakes.

This hypothesis has motivated many limnological phosphatase studies and it is suprising that so few investigations have tested its validity. To a large extent this probably depends on methodological problems. These comprise the determination of orthophosphate enzymatically regenerated from naturally occurring substrates, while at the same time other biological or abiotic phosphorus transformations are prevented. Ideally, this should be accomplished without changing the structure of the phosphatase substrates or the rate by which they are supplied.

Theoretically, *in situ* hydrolyzation rates could be estimated if the concentration of the artificial substrate in the assay is kept so low that it imitates natural substrate levels. However, apart from the difficulty to predict natural substrate concentrations (see below), the low substrate concentrations which must be used conflict with analytical accuracy (Taft *et al.*, 1977). Furthermore, it is doubtful whether the phosphatases have the same affinity for artificial as they have for natural substrates.

Because of the difficulties in quantifying the enzymatic regeneration of phosphate *in situ*, doubts have been raised on the ecological significance of phosphatases. Rigler (1961) considered phosphatases unimportant for phosphorus mineralization in lakes. However, Riglers (1961) conclusion was based on the observation that no increase in orthophosphate concentration was detected in filtered water, in spite of high phosphatase activity. Similar results were later reported by, e.g., Berman & Moses (1972) and Pettersson (1980).

On the other hand, Berman (1970), Berman & Moses (1972) and Kobori & Taga (1979a) found that considerable amounts of phosphate were generated when phosphatases were incubated with unfiltered water saturated with chloroform. Chloroform kills the organisms without affecting the phosphatase activity (Berman, 1969). From the hydrolysis rates obtained in this way, Berman (1970) calculated that the enzymatic phosphate release was sufficient to meet the phosphorus demand of phytoplankton in Lake Kinneret.

Jansson (1977) obtained pronounced phosphate liberation when lake water phosphatases were added to autoclaved water. Moreover, by adding phosphatases to ^{32}P-labelled seston, incubating and subsequently characterizing dissolved compounds by gel filtration, Jansson (1977) was able to demonstrate that phosphatases released orthophosphate from dead seston. No other dissolved phosphate than orthophosphate appeared during the experiment.

The interpretations of these results are obvious. Lake water seldom contains more than minute amounts of easily degradable organic phosphates in dissolved form. Particulate matter is a necessary source of phosphatase substrates. Phosphatases release orthophosphate either directly from dead seston or from dissolved substrates supplied from living or dead particulate matter. It is difficult to conclude anything about the dynamics of this process since most experiments include chloroform or autoclaving, affecting both structure and supply of degradable compounds. The results of Jansson (1977) and the difficulty in finding dissolved phosphomonoesters in lake water (Rigler, 1964; Berman & Moses, 1972; Pettersson, 1980), suggest that the phosphatases can hydrolyse substrates faster than they are supplied, i.e. the enzymatic recirculation of phos-

phorus is limited by the rate of substrate supply rather than by enzyme activity.

Indications of rapid hydrolysis *in situ* were given by Heath & Cooke (1975) who found high concentrations of phosphomonoesters in East Twin Lake during parts of the year. The peak concentration was almost 60 μg esterified P l^{-1} (an extremely high value) which was almost entirely depleted in less than a week simultaneously with an increase in phosphatase activity.

Currie & Kalff (1984) reported that algae in mixed algal/bacterial cultures efficiently utilized dissolved organic phosphorus excreted by plankton. According to the findings of Kuenzler & Perras (1965) this uptake should have been preceded by phosphatase mediated release of orthophosphate. From studies in an eutrophic lake, Halemejko & Chrost (1984) concluded that bacteria were responsible for the mineralization of phytoplankton and that phosphatases, either associated with microorganisms or free, could be important in regeneration of organic phosphorus compounds.

Other indirect evidence of the important role of phosphatases was found by Jansson (1981) in the study of phosphatases in the acidic Lake Gårdsjön. The acid phosphatase activity in the lake was extremely high; approximately ten times the alkaline phosphatase activity in other Swedish lakes. It was found that the large amounts of dissolved aluminium in this acidified lake combined with the phosphate group on phosphate esters, thereby blocking these from enzymatic attack. The blocking of the substrate was competitive and could be overcome by an increase of the phosphatase concentration; hence the high activity in the lake. It was also possible to experimentally enhance the planktonic phosphatase production by increasing the aluminium concentration. This adaptation by plankton was interpreted so that access to orthophosphate via enzymatic regeneration was crucial for their growth.

In summary, based on our own observations and those of others, we feel confident that phosphatases efficiently hydrolyze naturally occurring organic phosphates and that the phosphorus turnover in biota depends on this process. However, further studies should be undertaken in order to quantify the rates by which phosphate esters are released from living and dead particulate material and enzymatically degraded in aquatic environments.

Concluding discussion

Most phosphatase studies in lakes have been made assuming that phosphatase activity can be used to characterize a phosphorus deficient phytoplankton community or because phosphatases are believed to be important for the recirculation of organically bound phosphatases. The theoretical base for these assumptions was formed already in the sixties by results of, e.g. Kuenzler & Perras (1965), Fitzgerald & Nelson (1966) and Berman (1970). In none of these aspects has research during the last fifteen years been very successful. Both assumptions still have to be scientifically verified.

The difficulties in transforming, to lake ecosystems, the results obtained with cultured algae where the phosphatase activity is often well correlated with the degree of phosphorus starvation, are mainly due to the complexity of natural environments. The background activity of phosphatases, not influenced by phosphorus nutrition, is most likely highly variable due to significant contributions from other sources than algae; such as bacteria, zooplankton and sometimes tributaries. Variable constitutive phosphatase production in different species increases this complexity.

Additional problems are different substrate affinity, pH-optimum and stability of phosphatases in natural communities in connection with difficulties to find relevant estimators of biomass for specific activity calculations. Since few successful attempts have been made to penetrate or solve these problems no useful generalizations are at hand today which could serve as guidelines for phosphorus deficiency characterization by phosphatase activity measurements.

At present, the use of phosphatase activity as a deficiency indicator in lakes can be recommended only if the many pitfalls are considered. Preferably, simultaneous recording of other reactions of phosphorus deficiency should be made.

The other aspect of phosphatase activity in lakes, i.e. the significance of enzymatic phosphate regeneration relative to other mineralization processes, is even less clear. It is at the same time a more interesting and perhaps also more important field of future investigation. It should be more urgent to investigate a process which may significantly favour the nutrition of organisms than simply to characterize a phosphorus deficient community. At least it would be valuable to know whether or not this process is of ecological importance.

In this paper we have advocated that phosphatases efficiently hydrolyze naturally occurring organic phosphates, which therefore seldom accumulate in lake water. We suggest that one of the difficulties in finding evidence for this process is that in lake water (especially when oligotrophic) the phosphatases hydrolyze substrates immediately after or simultaneously with their exposure to the enzymes.

With this proposal we indirectly stress the necessity of studying not only the phosphatases but also the structure, formation, stability and turnover time of their possible substrates. In other words, with a nutrient dynamical approach more effort should be spent in studying the connection between the enzymes and their substrates than between the enzymes and the organisms which produce them.

This does not imply that the latter aspect should be neglected. On the contrary, we feel that much of the present confusion concerning the role of phosphatases in lakes stems from the fact that to little is known about the origin of phosphatases in lakes.

Most likely the role of phosphatases in lakes would become clearer if we knew when, where and how bacteria, algae and zooplankton contribute to the overall activity. Particularly the role of bacterial phosphatases needs more investigation.

Enzymatic phosphorus transformation in the sediment is another unexploited field of research. Phosphatases could be one of several important mechanisms by which organic phosphorus is recycled to lake water. Sediment should offer an ideal environment for studies of bacterial enzymes as well as the interaction between enzymes and substrates.

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