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Species-specific alkaline phosphatase activity in freshwater spring phytoplankton: Application of a novel method

K. RENGEFORS[2], K. PETTERSSON[1], T. BLENCKNER[1] AND D. M. ANDERSON
BIOLOGY DEPARTMENT, MAIL STOP #32, WOODS HOLE OCEANOGRAPHIC INSTITUTION, WOODS HOLE, MA 02543–1049, USA AND [1]ERKEN LABORATORY, DEPARTMENT OF LIMNOLOGY, EVOLUTIONARY BIOLOGY CENTER, UPPSALA UNIVERSITY, NORR MALMA 4200, SE-76173 NORRTÄLJE, SWEDEN

[2]PRESENT ADDRESS: LIMNOLOGY, DEPARTMENT OF ECOLOGY, ECOLOGY BUILDING, SE-22362 LUND, SWEDEN

For the first time, in situ alkaline phosphatase activity (APA) was studied at the species level in a natural spring community of freshwater phytoplankton. This was achieved by utilizing a substrate called enzyme labeled fluorescence (ELF), which forms fluorescent precipitates at the site of APA. Samples collected from Lake Erken, Sweden, were assayed with ELF and a number of species belonging to Bacillariophyceae, Chlorophyceae and Dinophyceae were labeled. Within these species we found temporal changes in the percentage of the populations that were ELF labeled. Our results demonstrate that species are not all utilizing AP at the same time, and that within one species, individual cells express different levels of APA. Comparisons between ELF activity and bulk community APA measurements, using standard methods, showed cases both of agreement and disagreement. In this study, we were unable to determine whether some of the fragile flagellates were producing AP. These cells were destroyed in the ELF-labeling process. Overall, we found that the ELF method provided new information on APA in freshwater phytoplankton, and that species-specific measurements are possible, but not for all classes of phytoplankton.

INTRODUCTION

Phosphorus limitation in phytoplankton has been studied using a variety of methods including nutrient incubation experiments and direct measurements of phosphorus uptake, cellular phosphorus content, nutrient ratios, and/or the presence of alkaline phosphatase activity (APA) (Vincent, 1981). Of these, alkaline phosphatase (AP) is the only biochemical indicator of phosphorus limitation in phytoplankton, in the laboratory (Kuenzler, 1965) as well as in the field (Berman, 1970; Pettersson, 1980; Vincent, 1981). Furthermore, most alkaline phosphatases appear to be inducible, in contrast to acid constitutive phosphatases (Cembella *et al.*, 1984). Alkaline phosphatases can be found on the outer surface of the cell where they cleave a variety of phosphate monoesters in the surrounding water (Kuenzler, 1965), thereby allowing phytoplankton to utilize organic phosphates when inorganic phosphate is no longer available (Kuenzler and Perras, 1965).

The main limitation of the APA bioassay is that it provides no information on which algal taxa or specific populations are utilizing AP. This is because APA detection has traditionally been performed on bulk natural water using soluble colorimetric (such as p-nitrophenyl phosphate) or fluorogenic substrates (such as methylumbelliferyl phosphate). These assays do not differentiate between AP produced by different algal taxa, between groups of planktonic organisms, or between dissolved and cell-bound AP. Differential filtration can provide information on the distribution of AP as a function of particle size, and thus permit a crude separation between phytoplankton and bacteria (Jansson, 1976; Pettersson *et al.*, 1993; Hantke *et al.*, 1996a,b).

A novel method called ELF (Enzyme Labeled Fluorescence) (González-Gil *et al.*, 1998a) allows for *in situ* fluorescent labeling of alkaline phosphatases, thereby overcoming several of the problems with bulk APA assays. The ELF assay is based on the formation of a fluorescent product, which is very bright and photostable, and thereby permits prolonged observation and storage. A colorless substrate, ELF (Molecular Probes, Inc., OR), forms an insoluble, yellow-green fluorescent product when it reacts with alkaline phosphatase (Huang *et al.*, 1992). The precipitation occurs at the site of enzymatic activity, e.g. on the cell surface, and the product can easily be viewed with epifluorescence microscopy. Thus, it is possible to visually determine which species contain AP, and to measure the relative quantity of the AP present (flow cytometry). The ELF method offers great potential for localizing which species produce AP in natural water samples, but thus far the procedure has not been tested on freshwater species in cultures or in the field.

González-Gil *et al.* showed that ELF precipitates formed in several marine dinoflagellates and one marine prymnesiophyte culture when these were grown under phosphorus-deficient conditions (González-Gil *et al.*, 1998a,b). These results were confirmed by standard measurements of APA. When the cultures were grown in phosphorus-replete medium, the cells exhibited no ELF activity, indicating that the assay detected repressible alkaline phosphatases rather than constitutive acid phosphatases.

The objective of this study was to apply the novel ELF bioassay to a multi-species natural freshwater community in order to determine which phytoplankton species are phosphorus limited during a spring succession. ELF labeling was compared with soluble bulk APA measurements of the whole community, in order to compare and contrast the results of the two methods. We decided to apply the ELF assay in the dimictic Lake Erken, Sweden, during spring, when the changes in nutrient concentrations and species composition are large.

METHOD

Study site

Lake Erken is a moderately eutrophic lake (total P ~25 µg P l^{-1}, total N ~700 µg N l^{-1}) situated in Southeastern Sweden (Pettersson, 1985). Due to carbonate-rich soils, the lake is characterized by high conductivity and alkalinity. The lake is normally ice-covered from December until April, with an onset of the summer stratification at the end of May or beginning of June.

The species composition of phytoplankton, as well as their nutrient status, have been monitored intensively in several studies, including Boström and Pettersson (Boström and Pettersson, 1977) and Pettersson (Pettersson, 1980, 1985). These studies demonstrated that APA is a good indicator of phosphorus deficiency, and that severe phosphorus limitation occurs after the spring bloom, as indicated by all deficiency indicators tested.

Analyses of nutrients and bulk alkaline phosphatase activity (APA)

Water samples were taken at different frequencies: every two days during the phytoplankton spring bloom, and once a week in the post spring peak period. The water was sampled in the morning with a tube sampler to obtain a 0–8 m depth integrated in the deepest part of the lake. Measurements of soluble reactive phosphorus (SRP) (Murphy and Riley, 1962), particulate phosphorus (PP), total phosphorus (TP) (Menzel and Corvin, 1965), nitrate (Wood *et al.*, 1967), ammonia (Svensson and Anfält, 1982), silicate (Mullin and Riley, 1955) and chlorophyll *a* (Parsons and Strickland, 1963) were made immediately after sampling. Nitrate, ammonia and SRP were analyzed using flow injection analysis (FIA) after filtering through a GF/F (~0.2 µm) filter. Dissolved organic phosphorus (DOP) was calculated by subtracting PP and SRP from TP. Dissolved oxygen, temperature, and pH were measured in the field at the time of sampling.

Alkaline phosphatase activity was measured according to the method of Pettersson (Pettersson, 1980). Methyl umbelliferyl phosphate, MUP, (10^{-4} mol l^{-1}) in 0.1 mol l^{-1} Tris-buffer pH 8.3 was used. Lake water samples (4 ml; in triplicate) were temperature-stabilized in a water-bath of 20°C for 10 min, and thereafter 0.5 ml MUP solution was added to all samples. Fluorescence was measured with a Turner TD-700 filter-fluorometer immediately, and then subsequently with intervals adjusted to the activity in the sample (at least four measurements within 2 h or less).

Phytoplankton samples

Phytoplankton was sampled weekly during spring (from April 13 until June 3, 1998) in the deepest part of the lake. A depth-integrated sample was taken with a tube sampler (2 m in length) in proportion to the volume of each layer, from the surface down to 8 m, and was mixed well in a bucket. A 100 ml subsample was taken out and preserved with Lugol's solution. A single estimate count of 10–25 ml was made for each sampling occasion using settling chambers. The samples were viewed with a Zeiss inverted microscope. The entire area of the chamber was counted at a 100× magnification for larger species, while two diagonals were counted at 400× magnification for small species (≤15 µm). Average cell volumes were calculated using formulae given by Blomqvist and Herlitz (Blomqvist and Herlitz, 1996). From this information, biovolume and then carbon biomass were calculated using the conversion formula $V (\mu l^3) * 10^{-6} = \text{Biomass} (\mu g\ C)$.

Insoluble ELF- assay

Lake water was subsampled and then split into three 150 ml aliquots. Each aliquot was centrifuged for 5 min at

6000 ***g***. The supernatant fluid was then aspirated off and the pellet transferred to a microfuge tube. After this step, the protocol of González-Gil *et al.* (González-Gil *et al.*, 1998a) for determination of ELF (Enzyme Labeled Fluorescence) activity was followed. The cell suspension was incubated for 30 min in 70% ethanol. The tubes were centrifuged (5 min, at 6000 ***g***) and the supernatant fluid removed by aspiration. A solution of 100 μl ELF reagent and ELF Detection Butter (1:20 v/v) was added, and the cells were incubated for another 30 min in the dark at room temperature. The incubation was followed by four washes (centrifugation) using 0.2 μm filtered 0.1 mol l^{-1} phosphate-buffered saline (PBS) to remove residual substrate. Samples were stored in the microfuge tubes with 0.1 mol l^{-1} PBS in the dark, at 4°C. Negative controls with only ELF Detection Buffer and no ELF substrate added were also prepared.

For viewing, the supernatant fluid was aspirated away and the settled sample was resuspended. One drop was placed on a microscope slide and was observed under a Nikon epifluorescence microscope with a DAPI (4′, 6′-diamidino-2-phenyl-indole) filter set (excitation 365 ± 8 nm; emission >420 nm) using a 100 W mercury lamp. Molecular Probes Inc. recommend the DAPI filter as ELF has its maximum excitation center at about 350 nm, with an emission at 530 nm. All the cells in the sample were counted in standard light (Tungsten) and Mercury light simultaneously, with Tungsten light switched off repeatedly to check for ELF activity. Cells were divided into two groups on the basis of presence or absence of ELF precipitates.

Methodological adaptations

As discussed below, fragile small flagellates were lost while processing phytoplankton for the ELF assay. In order to improve the method, several adaptations were attempted. First, a change to a filtration rather than a centrifugation method was tested. Samples were filtered through 2 μm polycarbonate filters (Nucleopore, Whatman, Millipore, Poretics) using gravity filtration, as these are translucent and permit viewing with the microscope.

To improve the stability of the fragile flagellates, natural samples as well as phosphorus-limited cultures [a marine dinoflagellate *Amphidinium* sp. (AMPHI) and a marine haptophyte *Isochrysis galbana* (TISO)] were preserved with Ütermohl's solution, glutaraldehyde (final concentration 0.1, 0.5, 1, 2%), formaldehyde (final concentration 2%), histochoice (final concentration 1X, 2X) or ethanol (final concentration 67%).

RESULTS

Spring phytoplankton species succession

The beginning of this study coincided with the peak of the spring bloom (March 31) which then declined quickly until it was completely gone by April 20. Diatoms, particularly the small *Stephanodiscus* cf. *hantzschii* v. *pusillus* (diameter ~6 μm), dominated both numerically and in terms of biomass (μg C l^{-1}) during this period (Table I, Figure 1). The spring bloom was followed by a transition period (end of April to mid-May) of a mixed

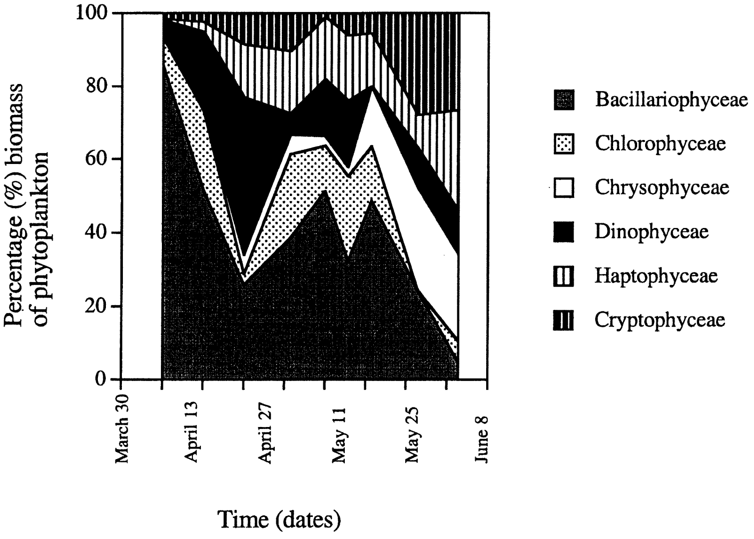

Fig. 1. Seasonal changes in phytoplankton biomass in Lake Erken. Phytoplankton divided into six different classes and shown as percentage of the total biomass. Biomass based on microscopic counts and calculations to carbon biomass (μg C l^{-1}).

Table I: Dominant phytoplankton in biomass (μg C l^{-1}) and number of individuals (cells l^{-1}) during different periods of the spring development, as defined by species composition

Phytoplankton taxa	Biomass (µg C l^{-1})	No. of individuals (Cells l^{-1})
Period A. Bacillariophyceae (March 31–April 20)		
Asterionella formosa	6.3 – 3.7	$1.1 \times 10^5 - 6.3 \times 10^4$
Chrysochromulina parva	0.60 – 1.1	$3.7 \times 10^5 - 6.7 \times 10^5$
Rhodomonas lacustris	1.3 – 0.14	$6.3 \times 10^5 - 7.0 \times 10^4$
Stephanodiscus cf. *hantzschii* v. *pusillus*	120 – 16	$5.0 \times 10^6 - 2.1 \times 10^6$
Stephanodiscus cf. *rotula*	12.0 – 0.07	$1.7 \times 10^4 - 1.0 \times 10^2$
Period B. Bacillario-/Chryso-/Dino-/ Haptophyceae (April 27–May19)		
Asterionella formosa	5.0 – 8.6	$8.0 \times 10^4 - 1.5 \times 10^5$
Chrysochromulina parva	3.9 – 2.8	$2.4 \times 10^6 - 2.4 \times 10^6$
Ochromonadaceae spp.	0.42 – 1.3	$1.1 \times 10^5 - 9.0 \times 10^4$
Stephanodiscus cf. *hantzschii* v. *pusillus*	0.82 – 0.68	$1.0 \times 10^5 - 1.9 \times 10^5$
Woloszynskia ordinata	7.8 – 0	$7.0 \times 10^4 - 0$
Period C. Chryso-/Crypto-/Haptophyceae (May 27–June 3)		
Chrysochromulina parva	1.1 – 7.0	$3.4 \times 10^6 - 4.3 \times 10^6$
Cryptomonas sp.	1.9 – 4.1	$1.1 \times 10^4 - 2.3 \times 10^4$
Ochromonadaceae spp.	2.8 – 5.0	$3.6 \times 10^5 - 1.4 \times 10^6$
Rhodomonas lacustris	0.36 – 1.91	$1.3 \times 10^5 - 9.4 \times 10^5$

phytoplankton assembly, with diatoms, dinoflagellates and haptophytes contributing to the phytoplankton biomass. The haptophyte *Chrysochromulina parva* was more abundant than any other species and often dominated the biomass as well. Later on (end of May until early June), the phytoplankton assembly was characterized by the disappearance of diatoms and a dominance of flagellates. The flagellates included Chrysophyceae, Cryptophyceae and Haptophyceae, with *C. parva* still being the most numerous species.

Phytoplankton biomass

Chlorophyll *a* (Chl *a*) values peaked at 31 µg l^{-1} on March 31 (Figure 2). After April 2, Chl *a* decreased to 1–3 µg l^{-1}, from April 20 onwards. Corresponding carbon biomass, calculated from phytoplankton counts, peaked at 239 µg C l^{-1} on April 13 and then dropped to below 50 µg C l^{-1} from April 20 onwards.

Nutrients and physical parameters

Dissolved nutrient levels were high at the beginning of winter, prior to the start of this study, with SRP at 15–20 µg P l^{-1}, nitrate at 100 µg NO_3-N l^{-1}, ammonia at 40 µg NH_4-N l^{-1} and silicate at 300–400 µg l^{-1} (data not shown). The dissolved inorganic nutrients all decreased rapidly as the spring bloom developed. By April 2, SRP, nitrate and ammonia were close to the detection limit and silicate was at 77 µg l^{-1} (Figure 3A, B). All inorganic nutrients except SRP increased again in late April/early May. Total P decreased from a peak on March 31, but increased again somewhat in May (Figure 3B). Particulate P also decreased from a high level of 20 µg P l^{-1} in the winter to about 5 µg P l^{-1} at the end of the spring bloom. Particulate P stayed around 8 µg P l^{-1} during the remainder of the study. DOP followed the pattern of total P and decreased from 17 µg P l^{-1} at the beginning of the study to below the detection limit at the beginning of May, only to increase again towards the end of May.

Throughout the study the water column was circulating, beginning at temperatures of 1°C increasing to 13°C by the end of May (data not shown). The water column did not stratify until June 30 when surface temperatures rose to 17°C. During the entire spring period, the pH of the water varied between 8.1 and 8.6, and the dissolved oxygen in the upper 8 m was never lower than 85% saturation.

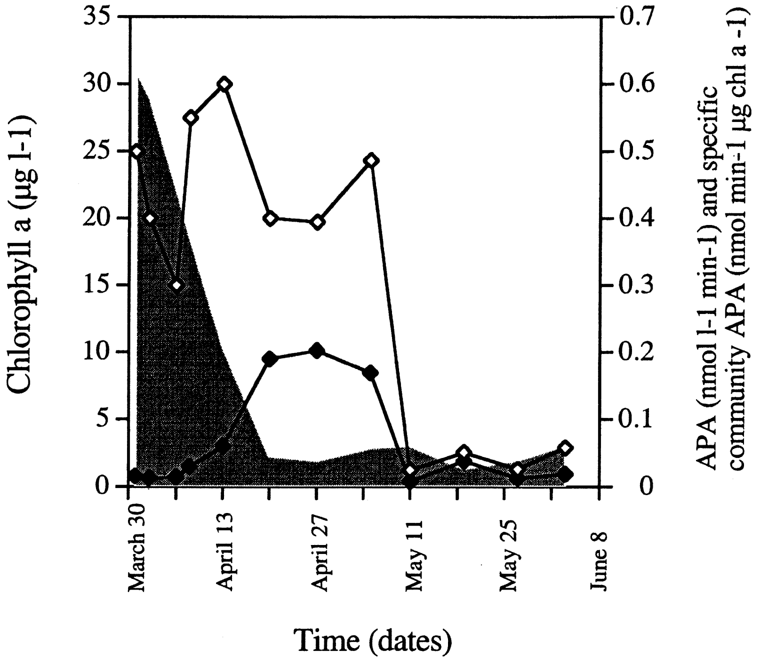

Fig. 2. Alkaline phosphorus activity (APA) displayed along with chlorophyll *a* measurements during spring in Lake Erken. Bulk APA (nmol l^{-1} min^{-1}) shown by open diamonds (◇). Specific community APA, i.e. APA per Chl *a* (nmol min^{-1} µg Chl *a*$^{-1}$) illustrated as filled diamonds (◆), chlorophyll *a* (µg l^{-1}) shown as shaded gray area.

Alkaline phosphatase activity

Bulk community alkaline phosphatase activity (APA) was moderate to low throughout most of the spring season, with values ranging between 0.3 and 0.6 nmol l^{-1} min^{-1} up until May 5, after which it dropped to 0.06 nmol l^{-1} min^{-1} and below (Figure 2). Specific community AP activity, i.e. the ratio between APA and biomass (Chl *a*) was low on the first sampling dates, ranging between 0.01 and 0.02 nmol l^{-1} min^{-1} µg Chl *a*$^{-1}$ (Figure 2). A peak of 0.2 nmol l^{-1} min^{-1} µg Chl *a*$^{-1}$ occurred on and around April 27. By May 11, the specific APA dropped nearly an order of magnitude (Figure 2), and remained low thereafter.

ELF activity

Throughout the study, ELF activity was observed mainly in diatoms and dinoflagellates, but also in chlorophytes. Most ELF activity was found in populations of *Stephanodiscus* cf. *hantzschii* v. *hantzscii* and *Gymnodinium* spp. Other species/genera which showed ELF activity were the dinoflagellate *Peridinium aciculiferum*, and the diatoms *Aulacoseira* spp. and *Fragillaria crotonensis* (Figure 4). Only some individuals within a taxon displayed ELF activity, varying from a small fraction up to 75% of a given species.

During the peak and crash of the spring diatom bloom, little or no ELF activity was observed in the dominating diatom species. However, up to 50% of the population of *Gymnodinium* spp. was observed with AP activity during this time. After the crash of *Stephanodiscus* cf. *hantzschii* v. *pusillus*, up to 30% of the population of the larger *Stephanodiscus* cf. *hantzschii* v. *hantzscii* (diameter ~20 µm) showed ELF activity. The highest proportion of ELF-labeled cells within a species was observed at the end of May and beginning of June, when up to 75% of the *Gymnodinium* spp. population and 20–60% of the *Stephanodiscus* cf. *hantzschii* v. *hantzscii* showed ELF activity.

Other species/genera which either only occasionally showed ELF activity, or were too few in numbers for any statistical certainty, are listed in Table II along with the more numerous species with ELF activity. In addition to diatoms and dinoflagellates, the taxa with observed ELF activity were mainly chlorophytes. The phytoplankton species dominating the biomass (Table I) rarely or never showed ELF activity (Figure 4). For example, within *Stephanodiscus* cf. *hantzschii* v. *pusillus*, *Stephanodiscus* cf. *rotula*

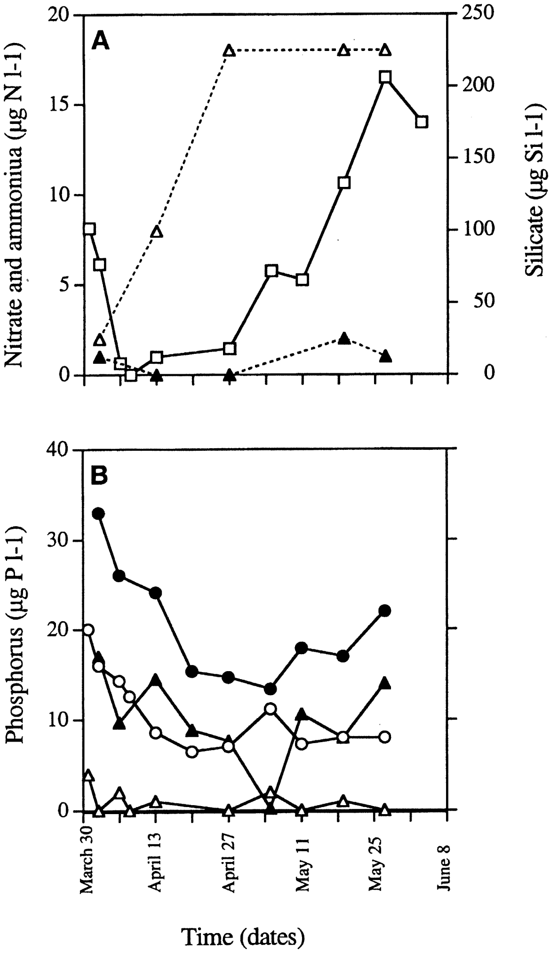

Fig. 3. (**A**) Inorganic nitrogen and silicate in Lake Erken water (0–8 m integrated) from March 30 through June 3, shown as µg l^{-1}. Nitrate is illustrated by filled triangles (▲), ammonia by open triangles (△) and silicate by open squares (□). (**B**) Phosphorus (µg P l^{-1}) in Lake Erken water (0–8 m integrated) from March 30 through June 3. Total phosphorus as filled circles (●), particulate phosphorus as open circles (○), DOP as filled triangles (▲) and SRP as open triangles (△).

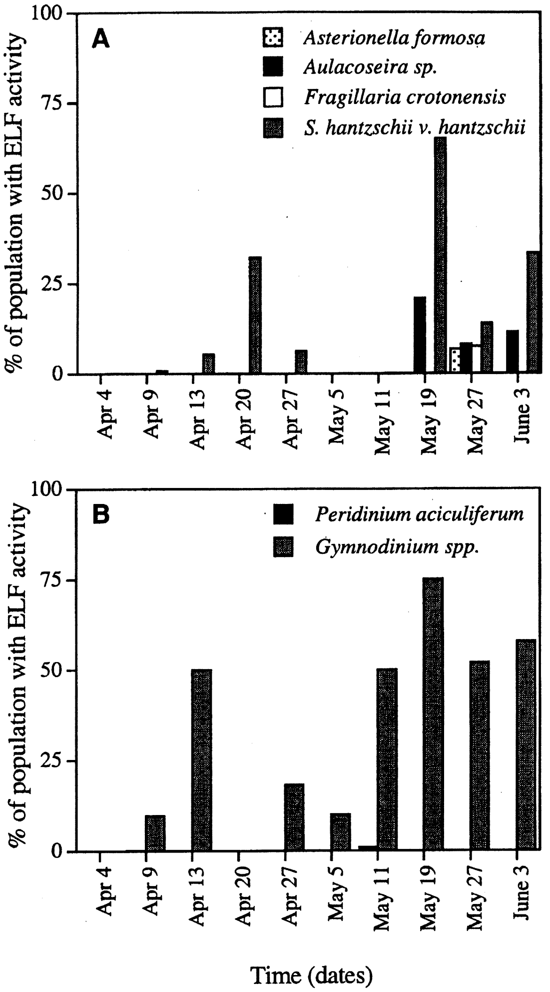

Fig. 4. Percentage of algal populations with ELF activity, as observed in different phytoplankton species/genera, at different dates during the spring season (April 4 to June 3, 1998) in Lake Erken. Panel **A** shows four diatom species and panel **B** shows the two dinoflagellate genera with ELF activity.

and *Asterionella formosa*, only occasional cells displayed AP activity. *Woloszynskia ordinata* cells were never observed with ELF. Dominating small fragile flagellates (Chryso-, Crypto- and Haptophyceae) were not encountered/identified in the samples that had been processed for ELF.

Methodological adaptations

Staining on filters resulted in a low and patchy yield of ELF activity in the cultures as well as natural samples processed. No differences were observed between the different brands of polycarbonate filters (data not shown).

The most efficient fixative in retaining cell shape after filtration was glutaraldehyde at a final concentration of 2% (Table III). At 1.0% final concentration, cells (such as *Rhodomonas* sp.) had shrunk but were recognizable, while at 0.5 and 0.1% glutaraldehyde, *Rhodomonas* cells were crushed beyond recognition following filtration. Formaldehyde did not preserve the cell shape as well as glutaraldehyde. Histochoice and ethanol could not stabilize the cell structure at all. Fixation with Ütermohl's solution also resulted in loss of fragile cells.

Table II: Species/genera observed with ELF activity in three classes of phytoplankton

Class	Species/Genera
Bacillariophyceae	*Aulacoseira* spp.*
	*Asterionella formosa**
	*Fragillaria crotonensis**
	Stephanodiscus cf. *hantzschii* v. *pusillus**
	Stephanodiscus cf. *hantzschii* v. *hantzschii*
	Stephanodiscus cf. *rotula*
Dinophyceae	*Ceratium hirundinella*
	Gymnodinium spp.*
	*Peridinium aciculiferum**
	Peridinium cinctum
Chlorophyceae	*Oocystis* sp.
	Pandorina sp.

Numerous species (100 cells or more counted per sample), indicated by *, are also shown in Figure 4, which illustrates changes in ELF activity during the season.

In combination with most fixatives, the ELF labeling generally failed. ELF labeling did occur with ethanol, but not with any of the other fixatives (Table III) for all classes of algae in the field samples. In only one case, the marine haptophyte culture *Isochrysis*, was some ELF activity retained when preserved with either formaldehyde or glutaraldehyde.

DISCUSSION

For the first time, *in situ* alkaline phosphatase activity was studied at the species level in a natural spring community of freshwater phytoplankton. Until now, this has not been possible, since the standard soluble AP bioassays only measure AP activity for the entire planktonic community or size-fractionated parts. The new technique, which utilizes a substrate called ELF, has, prior to this study, only been tested in culture, and then for marine phytoplankton (González-Gil *et al.*, 1998a,b). Our studies have shown that several freshwater species belonging to different classes of phytoplankton can be labeled with ELF, while some other species do not withstand the processing necessary to stain the cells.

ELF applied to natural communities

With the ELF technique it was possible to determine which specific species within a phytoplankton community were or were not producing alkaline phosphatase. A number of species belonging to Bacillariophyceae, Chlorophyceae, and Dinophyceae were observed with ELF activity.

One of the advantages of the ELF technique was immediately apparent—the species-specific labeling revealed the different nutritional status of various taxa. Our observations demonstrated that in a natural community of phytoplankton, only some of the species are contributing to the production of AP. This agrees well with the general perception that different species have different phosphorus requirements and uptake abilities. We believe that the presence or absence of ELF reveals species-specific differences in the conditions that induce APA, rather than that the ELF method is not a general indicator of AP activity. Nevertheless, it is possible that if a particular species does not express AP, or if its AP does not

Table III: Test of stability of cell shape of fragile flagellates with ELF labeling after fixation

Fixative concentration	Final shape	Retention of cell labeling	Result of ELF
Glutaraldehyde	0.1	poor	unsatisfactory
	0.5	poor	unsatisfactory
	1.0	satisfactory	unsatisfactory
	2.0	excellent	unsatisfactory
Formaldehyde	2.0	satisfactory	unsatisfactory
Histochoice	1X	poor	unsatisfactory
	2X	poor	unsatisfactory
Utermöhler's	–	poor	unsatisfactory
Ethanol	67%	poor	excellent

become labeled with ELF, it could be experiencing P limitation that would be undetectable by current methods. These are issues that are under investigation in a separate project.

We also encountered differences in ELF activity within populations. Sometimes, only a small fraction of a population was observed with ELF-labeled AP activity, while at other times, up to 75% of a population was labeled (Figure 4). These results indicate that individuals within one population may experience different conditions, depending both on the microenvironment surrounding them, and on their individual internal P status.

The present study indicates that ELF activity is an indicator of repressible AP in the field as well as in the laboratory (González-Gil *et al.*, 1998a,b). Our results illustrate that AP was inducible/repressible since cells within a taxum were not constantly labeled/unlabeled with ELF. For instance, the diatom *Aulacoseira* was present in the water column throughout the study, but only showed ELF activity at the end of May. Another species, the dinoflagellate *P. aciculiferum*, only showed ELF activity during its final phase of decline, before disappearing completely from the water column. During this whole period, SRP was very low, but apparently the internal cellular stores of P were not depleted until May.

In this study, we were unable to determine whether or not the flagellates *C. parva*, *Cryptomonas* spp., *R. lacustris* or any of the species of the Ochromonadaceae family were producing AP. These species were not present in the phytoplankton samples that had been labeled with ELF (see section on Methodological aspects).

Nutrient limitation in Lake Erken: APA versus ELF

The spring diatom bloom in Lake Erken was not P limited, as indicated by both the ELF assay and the bulk specific community APA. Little or no ELF activity was observed in the cells of the dominating *Stephanodiscus* cf. *hantzschii* v. *pusillus* during the spring bloom, consistent with the specific community APA, which was low during the spring peak. The values of the specific community APA were about 10-fold lower than the values recorded in previous studies in Lake Erken (Pettersson, 1980). Nevertheless, both studies showed that the collapse of the spring bloom was not caused by phosphate limitation. The rapid drop of silicate between March 30 and April 7, which coincided with the crash of the bloom, suggests that this nutrient was a major factor in the decline of the spring diatom bloom.

After the spring diatom bloom, there was a short period (April 20 to May 5) during which specific community APA indicated P limitation. Specific community APA increased, amounting to 0.2 nmol min^{-1} Chl a^{-1} (Figure 2). This increase corresponded with a decrease in particulate P, SRP and DOP (Figure 3B). Surprisingly, the peak of specific APA was not supported by the ELF data. Two of the species (*Asterionella formosa* and *Woloszynskia ordinata*) which dominated the biomass at the time showed no ELF activity, even though *A. formosa* was observed with ELF at other times. Furthermore, *Cryptomonas* spp. and *C. parva* also contributed substantially to the phytoplankton biomass, but neither of these could be identified/observed with the microscope after labeling with ELF and may have been responsible for the observed APA. The relatively high APA may also be due to other organisms, such as bacteria or zooplankton (Jansson, 1976; Pettersson *et al.*, 1993; Hantke *et al.*, 1996a,b). Another source of APA may be extracellular AP produced by lysing phytoplankton, bacteria or zooplankton. Only by sequential filtration would it have been possible to separate the dissolved AP and bacterial-bound AP from the bulk APA measurements.

During the month of May at the end of spring, the highest percentages of ELF activity were observed, suggesting P limitation in several species. These species consisted mainly of diatoms and dinoflagellates. Bulk community APA and SRP also indicated P limitation in May (Figures 2 and 3B). However, no corresponding increase was observed in specific community APA (per Chl *a*). This can be explained by the fact that the species with ELF activity were not dominating the phytoplankton biomass (contribution maximum 20% of biomass), whereas those that were dominant (*Cryptomonas* sp., *C. parva*, Ochromonadaceae) had either lower inorganic P requirements or had reverted to mixotrophic nutrition. Studies by Caron *et al.* (Caron *et al.*, 1993) and Nygaard and Tobiesen (Nygaard and Tobiesen, 1993) have shown that mixotrophy can be important in supplying chrysophytes with phosphorus.

Methodological aspects: applicability and limitations

Although ELF is a useful tool for detecting phosphorus limitation in Bacillario-, Chloro- and Dinophyceae, it provided no information for the chryso-, crypto- and haptophytes. These cells are small and fragile and were apparently lost or destroyed beyond recognition during the ELF labeling procedure. The cause of this loss was the centrifugation steps required for concentrating the algae and washing the stain. Tests with monocultures of large and less fragile Chrysophytes (*Mallomonas* sp. and *Dinobryon divergens*) showed that these were labeled with ELF when grown in P-limiting conditions (unpublished data). Although extensive attempts were made to improve the ELF method (for fragile flagellates) using gentle gravity filtration and fixatives, none was successful. We found that the method cannot be easily adapted for detecting AP in small fragile algae.

Conclusions

To conclude, we found that the ELF method does work with freshwater phytoplankton, and in natural samples, but with some limitations. The ELF labeling indicated that the spring bloom in Lake Erken was not P limited, as was confirmed with low community specific APA. Furthermore, ELF showed that some species were P-limited in late May, even though community specific APA was low. The technique thus made it possible to identify species with P deficiency during a period when other phytoplankton had ample supplies of phosphorus. ELF thereby gives a much more detailed view of P deficiency within the phytoplankton community than traditional bulk APA measurements only, and demonstrates that bulk community APA measurements can be misleading, unless there is a monoculture dominance of a single species. Despite the shortcomings of the method, we consider ELF to be a useful extension of the traditional soluble APA assays. Due to the advantages and limitations of both methods, future field studies should utilize both ELF and bulk APA to obtain a complete picture of the APA of phytoplankton.

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