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Experimental investigation of taxon-specific response of alkaline phosphatase activity in natural freshwater phytoplankton

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Abstract

It is widely accepted that alkaline phosphatase activity (APA) is an efficient indicator of phosphate limitation in freshwater phytoplankton communities. In this study, we investigated whether the response in APA to phosphate limitation differs among the taxa in a mixed phytoplankton assemblage. We used the new enzyme-labeled fluorescence (ELF) technique, which allows microscopic detection of phosphate limitation in individual cells of multiple species. The most prominent findings of this study were that alkaline phosphatase (AP) was induced in many, but not all taxa and that different taxa, as well as different cells within a single taxon, experienced different degrees of phosphate stress under the same environmental conditions. Our approach was to manipulate the limiting nutrient in a natural freshwater phytoplankton community by incubating lake water in the laboratory. We induced nitrogen (N) or phosphate limitation through additions of inorganic nutrients. Both the ELF assay and bulk APA indicated that the lake phytoplankton were not phosphate limited at the start of the experiment. During the experiment, several chlorophyte taxa (e.g., *Eudorina* and an unidentified solitary spiny coccoid) were driven to phosphate limitation when inorganic N was added, as evidenced by a higher percentage of ELF-labeled cells relative to controls, whereas other chlorophyte taxa such as *Actinastrum* and *Dictyosphaerium* were not phosphate stressed under these conditions. In the phosphate-limited treatments, little or no ELF labeling was observed in any cyanobacterial taxa. Furthermore, all taxa observed after the ELF labeling procedure (>10-μm fraction) were labeled with ELF at least on one occasion, demonstrating the wide applicability of the ELF method. By using ELF labeling in tandem with bulk APA, the resolution and analysis of phosphate limitation was increased, allowing the identification of specific phosphate-stressed taxa.

A common dilemma when studying nutrient limitation in phytoplankton is that a choice must be made between working with natural communities at the total phytoplankton biomass level and working at the species level using laboratory cultures. Because of methodological limitations, it is difficult to do studies at the species or genus level in natural waters. In order to bridge this gap, we here utilize the new technique enzyme-labeled fluorescence (ELF) for detecting phosphate limitation in individual species within a natural freshwater community. Traditionally, methods for detecting the nutrient status of a natural phytoplankton community operate on the total algal biomass and are unable to resolve responses at the species level. Laboratory culture experiments, on the other hand, lend insight into species-level adaptations but provide little information on how the species would react as part of a natural community, in situ.

Alkaline phosphatases (APs) are inducible enzymes, generally located on the outer surface of the cell (Cembella et al. 1984), which cleave organically bound phosphorus from

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phosphomonoesters. These enzymes allow algae to utilize dissolved organic phosphorus (P) when orthophosphate is no longer available in the water or is present at low enough levels for phytoplankton to perceive stress (Kuenzler 1965; Kuenzler and Perras 1965). The alkaline phosphatase activity (APA) assay is commonly used as an indicator of phosphorus deficiency in phytoplankton (Healey and Hendzel 1979, 1980; Pettersson 1980; Vincent 1981). When tested on laboratory cultures and in the field, this assay typically correlates well with other P deficiency indicators, such as particulate P/carbon (C), particulate nitrogen (N)/P, P uptake kinetics, and particulate carbohydrate/C ratios (Healey and Hendzel 1979).

A disadvantage of the APA bioassay and other available physiological indicators of P deficiency is that they are an average of the response of the whole community in a water sample. APA is measured fluorometrically (Perry 1972) or colorimetrically (Horwitz et al. 1966) on bulk lake or seawater samples. The only means of separation between classes of planktonic organisms is through differential size filtration, which does not clearly separate different taxa. Consequently, this assay provides no information on the nutrient status of the individual taxa involved.

In contrast, the ELF assay permits detection of APs at the single cell level (González-Gil et al. 1998*a*). The ELF-97 substrate (Molecular Probes) reacts with cell surface–bound AP and forms a fluorescent precipitate at the site of APA. It is then possible to visually observe ELF-labeled AP sites in individual cells with epifluorescence microscopy. With the ELF method, AP detection can be made in individual taxa within a mixed community. The ELF assay has successfully been tested on some marine dinoflagellate species both in the laboratory (González-Gil et al. 1998*a*) and in the field (Dyhrman and Palenik 1999). Rengefors et al. (2001), in contrast to the previous studies, investigated the ELF labeling of an entire community of freshwater phytoplankton during and after a spring bloom.

In the present study, we investigated whether APA response to phosphate limitation differs among taxa within a natural freshwater phytoplankton community. Previous experiments using bulk APA analysis have not been able to provide the information necessary to resolve this matter. We tested the hypothesis of differential response in APA by using the ELF assay in a nutrient addition incubation experiment in laboratory mesocosms. Under these controlled conditions, our objectives were to compare results from ELF labeling to bulk APA measurements and to evaluate whether the taxa observed showed AP activity when driven to phosphate limitation. The experimental setup also permitted us to determine whether inorganic N or phosphate was limiting the phytoplankton biomass at different sites in a freshwater lake.

Methods

The nutrient enrichment experiment was performed on water from Ashumet Pond (41°30′N, 70°35′W), Massachusetts. The pond is considered mesotrophic but with a trend toward a eutrophic state because of groundwater N and P contamination from an abandoned sewage treatment facility within the Massachusetts Military Reservation (AFCEE 2000).

Integrated surface water (0–1.7 m) samples were collected from two different sites in Ashumet Pond on 30 August 1999: Fisherman's Cove (FC) and Deep Basin (DB). Water was also collected from 4 m depth at Deep Basin (DB4). Prior to initiation of the experiment, the water was filtered through a 300-μm sieve to remove large zooplankton grazers and then was split into 5-liter transparent polycarbonate flasks. Three treatments of 5 liters each were incubated in triplicate in the laboratory at 23°C, at a light level of 400 μmol quanta m^{-2} s^{-1}, using a 16 : 8 light : dark (LD) cycle. The treatments were Control (no nutrient additions), PO_4 (addition of orthophosphate to a final concentration of 5 μmol L^{-1}), and dissolved inorganic nitrogen (DIN; addition of NH_4^+ and NO_3^- to a final concentration of 10 μmol L^{-1} each). The final nutrient concentrations were set to be in excess of natural levels in a mesotrophic lake. Nutrient spikes were added at the start of the experiment (Day 1) and repeated on Day 3. The experiment was terminated on Day 5 because preliminary experiments had shown that at least 3 d were needed to detect changes in bulk APA and ELF.

A 500-ml aliquot of lake water was collected and sieved through a 10-μm mesh to concentrate the sample. Cells smaller than 10 μm in diameter were mainly small chrysophytes, which are destroyed during the ELF staining procedure (Rengefors et al. 2001). The concentrated sample was then processed for ELF staining by first centrifuging for 10 min at ~4,000 × g. The supernatant was aspirated, and the pellet (~1 ml) was transferred to a 1.5-ml microfuge tube. Afterwards, the protocol of González-Gil et al. (1998*a*) for ELF labeling was followed. Briefly, the pellet was incubated for 30 min with 1 ml of 70% ethanol. The tubes were then centrifuged (5 min, at 5,500 × g), and the supernatant was removed by aspiration. A solution of ELF reagent and ELF detection buffer (100 μl, both supplied with the ELF kit) at a 1 : 20 (v/v) concentration was added to the pellet, 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, pH 7.8) to remove residual substrate. Samples were stored in the microfuge tubes with 0.1 mol L^{-1} PBS in the dark at 4°C up to 1 month (samples can be stored at least 3 months, Rengefors unpubl. data) until the samples could be viewed microscopically.

For viewing, most of the supernatant was aspirated (after gravity settling), and the concentrated sample was resuspended in the residual volume. One drop was placed on a microscope slide and was observed under a Zeiss epifluorescence microscope with a DAPI (4′,6′-diamidino-2-phenyl-indole) filter set (ELF has a maximum emission centered at 520 nm when excited at 350 nm) using a 100-W mercury lamp. All the cells in the sample were counted with standard illumination (tungsten) alternated with mercury excitation to check for ELF activity. Enumerated cells were divided into two groups on the basis of presence or absence of ELF precipitates. Percent ELF for a given taxon was determined as the fraction of fluorescently labeled cells relative to the total number of cells counted.

Fifty-milliliter aliquots were preserved with Lugol's solution for phytoplankton counts at the beginning (triplicates from the field) and the end of the experiment (for each replicate of the different treatments). Triplicate treatments were combined, and 5–10-ml subsamples were enumerated in settling chambers using a Zeiss IM35 inverted microscope. Large taxa were counted at ×125 magnification in the entire chamber, whereas small taxa (<10 μm) were counted along one diameter at ×500 magnification. Biomass (μg C L^{-1}) was then determined by volumetric calculations, which were converted to biomass using C-factors (Blomqvist and Herlitz 1996).

Aliquots of 100 ml from each sample replicate were stored immediately at −30°C until analyzed (2–4 and 6–8 months later) for bulk APA. Long-term freezing and refreezing and rethawing had no effect on bulk APA (Haupert 2000). Bulk alkaline phosphatase activity (APA) was determined by hydrolyzing a fluorogenic substrate, 4-methylumbelliferyl phosphate (MUF-P), into the fluorescent 4-methylumbelliferone (MUF) species. Sample splits (0.300 ml per replicate) were spiked with a range of substrate concentrations (0.5, 0.75, 1.0, 3.0, 7.0, 10.0 μmol L^{-1} MUF), and hydrolysis was determined by measuring the temporal increase in spiked sample fluorescence using an Applied Biosystems Cytofluor 4000TC, a fluorescence multiwell plate reader (Haupert 2000). The samples were run at the in situ pH of the pond water/incubated samples. APA was measured in triplicate for each concentration of substrate at a temperature-controlled setting of 30°C. Each sample was run with a set of triplicate MUF standards (0, 10, 50, 100, 200, and 400 nmol L^{-1}) made in the same sample matrix. Calibration curves were generated and used to calculate hydrolysis rates for each substrate concentration. The subsequent hydrolysis rates, or reaction velocities versus initial MUF-P concentration, were then used to determine the substrate-saturated enzyme activity (V_{max}) for the sample employing Michaelis–Menten enzyme kinetics. V_{max} values were determined using Lineweaver-Burke plots (Voet and Voet 1995).

Water from each replicate was filtered (cellulose acetate filter, 0.8-μm pore size) for nutrient analyses (phosphate, nitrate, ammonium) on both Day 2 and Day 5, as well as prior to the start of the experiment. Chlorophyll *a* (Chl *a*) analyses were performed on lake water at the start of the experiment and on pooled triplicates for each of the treatments on Day 5. Chl *a* was analyzed using acetone extraction and fluorometric determination after acidification (Standard Methods 1992).

Results of ELF analyses are presented as percentage of the total population of each taxon that was labeled with ELF. Statistical analyses were made using SigmaStat, performing a two-way analysis of variance on taxa that were normally distributed. Tukey's test was then used as a pairwise multiple comparison procedure. For other taxa, a nonparametric one-way analysis was performed on each treatment. Dunn's method was used for pairwise multiple comparison. Subsequently, a Mann–Whitney rank sum test was performed on each pair. Bulk APA data were also analyzed using a two-way analysis of variance followed by Fisher's test using StatView 5.0.

Table 1. Compilation of the taxa encountered in lake water from Ashumet Pond (in situ or after incubation in the laboratory) after undergoing the ELF labeling procedure (>10-μm fraction). The table indicates whether ELF-labeled AP has been detected in the taxa observed.

Class	Genus/taxon	ELF activity
Bacillariophyceae	*Aulacoseira* spp.	Yes
	Rhizosolenia spp.	Yes
Chlorophyceae	*Actinastrum* sp.	Yes
	Closteriopsis spp.	Yes
	Coelastrum spp.	Yes
	Dictyosphaerium spp.	Yes
	Eudorina spp.	Yes
	Micratinium sp.	Yes
	Oocystis sp.	Yes
	Pediastrum spp.	Yes
	Scenedesmus spp.	Yes
	Sphaerocystis sp.	Yes
	SSGS (spiny solitary green coccoid)	Yes
	Greens in gelatinous sheath	Yes
Chrysophyceae	*Dinobryon* spp.	Yes
Cyanophyceae	*Anabaena* spp.	Extremely rare
	Planktolyngbya sp., *Planktothrix* sp.	Extremely rare
Dinophyceae	*Peridinium* spp.	Yes
	Gymnodinium sp.	Yes
Euglenophyceae	*Trachelomonas* spp.	Yes

Results and discussion

Alkaline phosphatase activity response differs among taxa—The single-cell AP labeling method, ELF, proved to be a widely useful diagnostic tool to study taxon-specific phosphate limitation in this natural phytoplankton community, as it labeled a range of taxa spanning several phytoplankton classes (Table 1). Figure 1 illustrates how fluorescent ELF precipitates are viewed in the microscope on three different taxa. All taxa observed in the ELF-labeled samples (>10-μm fraction) were labeled with ELF at least once, although the percentage of labeled cells among populations varied. The observed taxa included representatives of Bacillariophyceae, Chlorophyceae, Chrysophyceae, Dinophyceae, Euglenophyceae, and Cyanobacteria (Table 1). Small (<10 μm) chrysophytes and cryptophytes, constituting ~10% of the biomass, were lost in the labeling procedure.

At the start of the experiment, the in situ phytoplankton community in Ashumet Pond was dominated by Cyanophyceae, which made up the major part of the biomass (40–50%) at all sites based on calculated biomass (μg C L^{-1}) (Fig. 2). Chlorophytes (20–30%) and chrysophytes (10–15%) made up most of the remainder (Fig. 2). *Planktolyngbya* sp. and *Planktothrix* sp. dominated the cyanobacterial biomass at all sites, whereas the chlorophyte biomass was dominated by a solitary spiny green coccoid (SSGC). Chrysophyte biomass was composed of various small flagellates of the Ochromonadaceae family and, to a much lesser extent, of various species of *Dinobryon*. Diatoms were not important in the biomass (3–9%) and comprised mainly

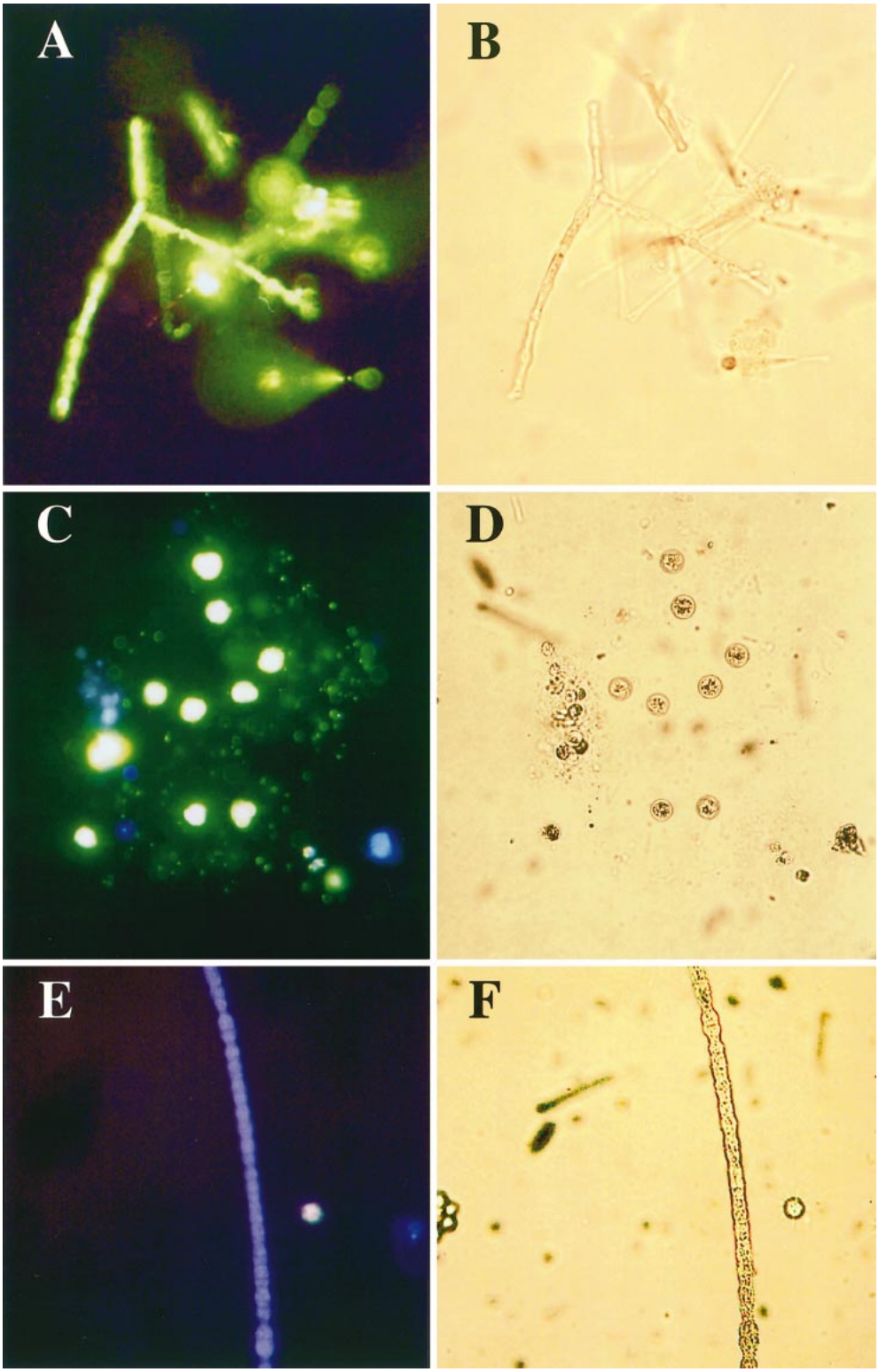

Fig. 1. ELF labeling of AP in natural phytoplankton. Photographs show phytoplankton illuminated with mercury light using a DAPI filter set (left panels) and in standard tungsten light (right panels). Note the bright green fluorescence in diatoms (A) and green algae (C), as well as the lack of labeling in the cyanobacteria (E). (A) ELF-labeled diatoms. (B) Diatoms in tungsten light. (C) ELF-labeled green algae. (D) Green algae in tungsten light. (E) ELF-treated cyanobacteria that remained unlabeled, suggesting the absence of cell surface AP. (F) Filamentous cyanobacteria in tungsten light. Data in panels A and B are for field samples from Ashumet Pond, whereas panels C–E are for DIN treatments on Day 5.

small centrics and *Aulacoseira* sp. Dinoflagellate biomass was also small (5–7%) and comprised a number of *Peridinium* and *Gymnodinium* species (Fig. 2).

By adding inorganic nitrogen, phosphate limitation could be induced in several taxa, resulting in changes in the extent of ELF labeling. Statistical analyses of ELF activity were made only on taxa that dominated in the ELF-processed samples, which allowed for counts of 100 or more cells per replicate. Several of the dominant green algae showed a very strong increase in ELF labeling when incubated with inorganic nitrogen additions (Fig. 3; Table 2), suggesting that they were driven to phosphate stress. For example, SSGC, *Eudorina* sp., and an unidentified colonial green alga had a significantly higher (>75% above initial values) percentage of ELF-labeled cells in the nitrogen addition treatment ($P < 0.05$ Mann–Whitney rank sum test or Tukey's test) (Table 2). *Coelastrum* sp. also had an elevated percentage of ELF (20%) compared to the initial values, although not significantly different from the phosphate treatment. The highest Chl *a* values were observed in the treatments with inorganic nitrogen additions (Fig. 4), demonstrating that algal growth had taken place. Moreover, the increases observed in biomass were primarily due to an increase of chlorophytes (Fig. 2), particularly SSGC (Table 2).

Nitrogen addition did not lead to increased AP production in all taxa, for example, in *Dictyosphaerium* and *Actinastrum* (Chlorococcales). Nevertheless, these taxa are capable of synthesizing/producing AP that can be detected with the ELF assay, as shown by a few ELF-labeled cells encountered after 5 d incubation (Fig. 3; Table 2). *Dictyosphaerium* and *Actinastrum* were therefore most likely not phosphate stressed. The lack of phosphate stress could be due to previous luxury consumption of P, innate low P requirements, or both. In this case, it would appear that 5 d were not enough to deplete internal phosphorus stores of these taxa. Compared to SSGC, the sparsely labeled chlorophyte taxa have small cells (~50 μm^3 compared to ~400 μm^3), which could explain their smaller P demand, more efficient P uptake, or both. It is also true that no growth was observed in these taxa (Table 2). An alternative explanation for the lack of ELF-labeled cells, then, is that these taxa were not growing and therefore were not experiencing phosphate stress.

Among the nondominant taxa, only a few showed ELF labeling at the beginning of the experiment, but nearly all were labeled after the 5-d incubation with added DIN. These taxa included the green algae *Pediastrum* spp., *Micratinium* sp., *Oocystis* sp., *Closteriopsis* sp., and *Scenedesmus* spp. (Table 1). Other algae labeled with ELF were a small centric diatom (diameter = 5 μm), *Rhizosolenia* sp. (diatom), *Di-*

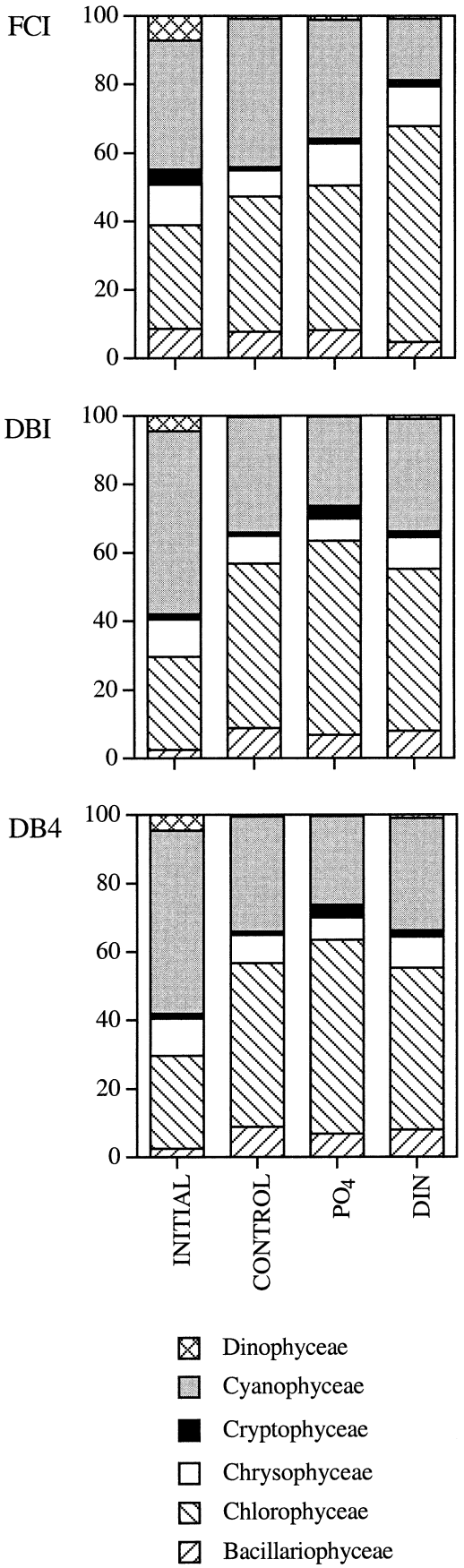

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Fig. 2. Percent biomass of the different phytoplankton classes in the initial field samples and after 5 d incubation. Biomass was calculated from microscopic measurement into biovolume and then converted ($\mu g\ C\ L^{-1}$). The three panels show the three sites: Fisherman's Cove integrated (FCI), Deep Basin integrated (DBI), and DB4. Phytoplankton is subdivided into six major classes. Note that chlorophytes increased in relation to total biomass in all the DIN treatments.

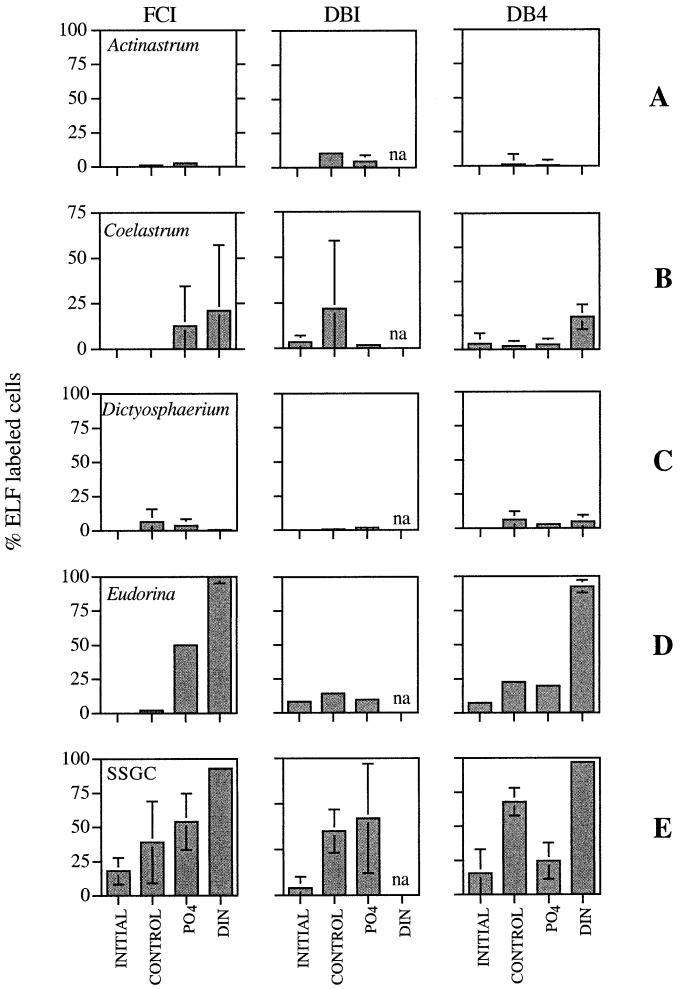

Fig. 3. Results of the ELF assay on five different taxa in the initial field sample and after 5 d incubation. Note the differences in response among the taxa. *Eudorina* and SSGC have low levels of ELF in initial samples from at least one of the three locations. *Actinastrum* and *Dictyosphaerium* do not exhibit any ELF activity in initial samples from any of the three locations. ELF labeling of *Eudorina* and SSGC increases to 75% or more in DIN treatments, indicating that these taxa have been driven to a state of phosphate stress. In contrast, *Actinastrum* and *Dictyosphaerium* are apparently not driven to phosphate stress in the DIN treatment, as indicated by the low percent ELF-labeled cells. On the *y*-axis, the panels show the percentage of ELF-labeled cells within phytoplankton populations at the three sites, FCI, DBI, and DB4. Each row of panels shows one taxon: *Actinastrum* sp., *Coelastrum* sp., *Dictyosphaerium* sp., *Eudorina* sp., and SSGC (=spiny solitary green coccoid). na, data not available.

nobryon spp. (Chrysophyceae), and *Trachelomonas* spp. (Euglenophyceae) (Table 1).

The extent of ELF labeling was limited in cyanobacteria, for which only occasional filaments were found with ELF precipitates. Our incubation experiment results suggest that cyanobacteria were growth limited (Table 2; Fig. 2), possibly because of light limitation. The simplest explanation for lack of ELF-labeled cyanobacteria is, therefore, that because they were not growing, they were not forced into a state of phosphorus limitation. Other explanations are possible, however. The cyanobacteria present in the lake at the time were *Anabaena* spp., *Planktothrix* spp., and *Planktolyngbya* sp., all with filamentous organization. Filamentous cyanobacteria are known for their luxury consumption of phosphate, which they store as polyphosphates (Kualev and Vagabov 1983) or sugar phosphates (Thompson et al. 1994). As a result, they possess the ability to survive and grow in nutrient-depleted water (Reynolds 1984; Istvánovics et al. 1993) and, hence, might require long periods of phosphate stress before inducing AP production. These two factors—lack of growth during the experiment and the possibility of luxury phosphate stores—are plausible explanations for lack of ELF labeling of cyanobacteria. However, neither can be confirmed or resolved with the present data. Another factor that we have considered is that the gelatinous sheath of these cyanobacteria could have interfered with ELF labeling (*see* penultimate paragraph).

The dinoflagellates were an exception to the general lack of AP observed in the phytoplankton at the beginning of the experiment. There were several species of Peridiniales in the initial lake water samples (Fig. 2), and the majority of these were heavily labeled with ELF precipitates. Because of the incubation conditions (small containers in the laboratory), most dinoflagellates had disappeared by Day 5, although remaining cells were ELF-labeled in all treatments. Because dinoflagellates are poor competitors for phosphate (Pollingher 1988) compared to chloro- and cyanophytes, they might utilize AP in order to satisfy their P demand, even when phosphate is available. Alternatively, it is possible that the dinoflagellates were senescent, and their APA reflected a last desperate measure to remain in the plankton. In support of this last explanation, we note that in Lake Erken, Sweden, dinoflagellates first started producing AP when only a few senescent cells remained in the water (Rengefors et al. 2001). For dinoflagellates, there is also a possibility of constitutive phosphatases (as suggested by Dyhrman and Palenik 1999). However, the other phytoplankton classes represented in this study did not exhibit any APA in the initial samples, demonstrating that APA is not constitutive in these nondinoflagellate taxa.

Our results suggest that AP is produced in most naturally occurring taxa, although some experiments have shown that certain algal cultures (e.g., *Pseudoanabaena catenata*) do not produce AP in the laboratory (Kuenzler 1965; Kuenzler and Perras 1965; Healey and Hendzel 1979). Furthermore, we have shown that the ELF substrate reacts with the AP on a wide range of cell types and organization: coccoids, monads, filaments, and colonies.

ELF labeling of phosphate-stressed phytoplankton: Manifestation of intra- and intertaxon variability—Different taxa, and different cells within a single taxon, experienced different degrees of phosphate stress under the same environmental conditions. For example, although almost 100% of the *Eudorina* sp. colonies were ELF labeled in the treatments with nitrogen additions (Fig. 3D), other green algae such as *Dictyosphaerium* sp. (also colonial) had only a few percent of the population labeled (Fig. 3C). Also, 10–25% of the SSGC population was labeled at the start of the experiment (Fig. 3E), indicating that part of the population was

Table 2. Alkaline phosphatase activity as indicated by ELF labeling in dominant phytoplankton taxa at three sites: FCI, DBI, and DB4. Results are recorded as percent ELF (i.e., the percentage of the total number of cells counted within a taxon that are ELF labeled). Mean values are given ($n = 3$). Statistical comparison was made between sites and treatments. There are no statistical differences between sites.

		ELF (%)*				Growth† (change in biomass, *n*-fold increase or decrease)		
Class/taxon	Site	Initial	Control	PO_4	DIN	Control	PO_4	DIN
Chlorophyceae								
Actinastrum sp.	FCI	0	1.1	0.4	0	0	1	0
	DBI	0	1.1	2.6	NA	−1	0	0
	DB4	0	10	4.5	0	0	0	0
Coelastrum sp.	FCI	0	0	13	21	0	0	1
	DBI	3.3	22	1.7	NA	0	0	0
	DB4	4.3	2.5	3.7	24	1	1	1
Dictyosphaerium sp.	FCI	0	6.5	3.7	0.33	0	0	0
	DBI	0	0.67	1.7	NA	0	0	0
	DB4	0	6.2	2.8	4.8	1	1	0
Colonial green sp.‡	FCI	0	3.2	30§	90§	0	1	1
	DBI	0	34	NC	NA	0	0	0
	DB4	0	23	11§	82§	0	1	1
Eudorina sp.‡	FCI	0	2.3	50§	100§	NA	NA	NA
	DBI	8.3	14	9.7	NA			
	DB4	7.6	22	20§	92§			
SSGC‡	FCI	18	39	54§	93§	2	12	5
	DBI	4	35	42	NA	5	4	10
	DB4	16	68	25§	97§	2	3	4
Cyanophyceae								
Anabaena spp.	FCI	0	0	0	0	0	0	0
	DBI	0	0	0	NA	0	0	0
	DB4	0	0	0	0	1	1	1
Planktolyngbya sp. and *Planktothrix* sp.	FCI	0	0	0.0033	0	0	0	0
	DBI	0	0	0	NA	1	0	0
	DB4	0	0	0	0	0	0	0

DB4, Deep Basin (4 m); DBI, Deep Basin integrated (0–1.7 m); ELF, enzyme-labeled fluorescence; FCI, Fisherman's Cove integrated (0–1.7 m); NA, data not available; NC, no cells present in sample; SSGC, spiny solitary green coccoid.

* Data are shown for initial conditions (in the field prior to start of the experiment), as well as at the end of the experiment (after 5 d incubation), for three treatments: Control (no nutrient additions), PO_4 (final concentration, 5 μmol L^{-1} orthophosphate), DIN (final concentration total, 20 μmol L^{-1} of nitrate and ammonium).

† Growth is illustrated as *n*-fold change (negative or positive) from original biomass, calculated from cell counts. Only >1-fold changes are considered reliable indicators of growth.

‡ Significant differences between treatments as analyzed by two-way ANOVA for SSGC and by Kruskal–Wallis (nonparametric) for other taxa.

§ Significant treatment effects for pairwise comparisons of PO_4 against DIN by Tukey's test (parametric, $P < 0.05$) for SSGC and by Mann–Whitney rank sum test (nonparametric) for other taxa.

experiencing phosphate stress in the lake. In contrast, most other taxa shown in Fig. 3 were not experiencing phosphate stress in situ, as indicated by little or no ELF activity in initial samples. These findings show that individual cells within the same population do not all have the same physiological status. A possible explanation for the observed variability includes heterogeneity in the particular microclimate, as well as individual differences in cell age, life cycle stage, growth rate, and genetic make-up. Temporal variations in the percentage of ELF-labeled cells within a single species have been observed previously in field studies of a marine (Dyhrman and Palenik 1999) and a freshwater system (Rengefors et al. 2001). Our study is distinct from these previous studies in that the marine study (Dyhrman and Palenik 1999) focused only on one species (*Prorocentrum minimum*), whereas we focused on the entire phytoplankton population (>10-μm fraction). Furthermore, our manipulation of nutrient levels using the nutrient addition methodology allowed us to force the in situ population to a state of phosphate stress, in contrast to the other freshwater study (Rengefors et al. 2001), which was a nonexperimental field study. Because close to 100% ELF labeling is achieved in laboratory cultures when cells are grown in phosphate-deficient media (K. Rengefors pers. obs.; González-Gil et al. 1998*a*), we can rule out that the variation in percentage of ELF labeling observed in the field is due to labeling inefficiency.

Comparison between bulk APA and the ELF assay—Bulk APA measurements and ELF labeling of dominant taxa generally followed the same trend. At the start of the experiment, little or no bulk APA was observed in the lake at any of the stations (Fig. 4), which is consistent with the absence or low levels of ELF-labeled cells in the in situ samples at the start of the experiment. Likewise, after 5 d, bulk APA was generally higher in the DIN treatments than for all other treatments ($P < 0.05$, two-way ANOVA, Fisher's test; Fig.

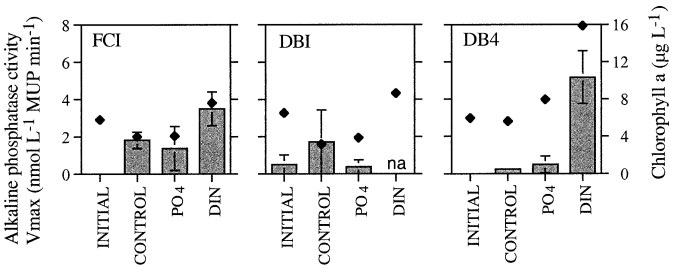

Fig. 4. Bulk alkaline phosphatase activity (APA), shown in shaded bars, of the initial field sample and at the end of the 5-d incubation for the three sites: Fisherman's Cove integrated (FCI, 0–1.7 m), Deep Basin integrated (DBI, 0–1.7 m), and DB4 (4 m). Chl *a* (μg L^{-1}) is reported as filled diamonds for each treatment and initial in situ levels. Note the low initial bulk APA, indicating that Ashumet Pond was not phosphate limited at the start of the experiment. Bulk APA was, as predicted, significantly higher ($P < 0.05$, two-way ANOVA) in the DIN treatments compared to the other treatments. Bulk APA is reported as V_{max} (nmol L^{-1} MUP min^{-1}). Error bars denote standard deviations, $n = 3$. Where no error bars are shown, SD = 0. No replicates were available for initial samples. na, data not available.

4), in correspondence with the increased ELF activity in most taxa by Day 5 (Table 2; Fig. 3). However, although bulk APA clearly reveals phosphate stress in the general population in the DIN treatment, it is completely incapable of providing information on how different taxa might be responding to the nutrient environment. When limited to bulk APA data alone, the assumption commonly drawn from such data is that the phytoplankton, in general, are phosphate stressed. The ELF assay, in contrast, reveals specific information on the nutrient status of distinct taxa, such as the observation we have made in our study that chlorophytes, but not cyanobacteria, were phosphate stressed (Table 2). At times, there were also contradictions between bulk APA and ELF activity. For example, bulk APA was low in situ (initial sample); nevertheless, most dinoflagellates had high ELF activity. However, the biomass of dinoflagellates was low; thus, their APA had no effect on the bulk APA. Another contradiction between bulk APA and ELF was in the PO_4 treatment, where bulk APA was low, whereas SSGC (the dominant phytoplankton species) had a relatively high percentage of ELF-labeled cells. Because ELF activity was much lower in the initial samples, constitutive AP cannot explain these contradictory data. There was sufficient phosphate in the water ($\geq$10 μmol L^{-1}) at the end of the experiment to exclude the possibility of phosphate limitation in the PO_4 treatment. One plausible explanation for the presence of APA in SSGC under these conditions is a lag in the repression of APA in SSGC. Nevertheless, with the data at hand, we cannot test this explanation, so these results cannot be definitively explained.

Thus, our experimental results show that ELF labeling enables additional resolution that bulk bioassays alone cannot achieve. Although detailed information on APA at the species level is accessible through the use of the ELF assay, it is at present a qualitative and not a quantitative assay. The combination of the two bioassays therefore provides the most complete and detailed information and will make possible a more shaded and physiologically more sound picture of the ecology of natural phytoplankton communities. However, the potential remains to develop a system to quantify ELF in the field using epifluorescent microscopy along with an image analysis system. A highly sensitive digital color camera would be required because less sensitive black and white systems cannot detect different degrees of ELF labeling (Rengefors unpubl. data).

The ELF method: A useful tool to detect inducible APA in natural phytoplankton—Our data clearly indicate that APA is inducible, and not constitutive. Initial samples exhibited no ELF activity, whereas high levels were observed in many taxa by Day 5 in the DIN treatments, supporting the contention that APA is a good indicator of phosphate stress. Although the presence of constitutive APA would compromise our ability to use ELF as an index of phosphate stress, the data presently available seem to indicate that potential constitutive APA might be limited to dinoflagellates. The dinoflagellates observed in our study were ELF labeled already at the start of the experiment; thus, whether or not they were constitutive could not be resolved with our experimental setup. On the other hand, the laboratory studies by González-Gil et al. (1998*a*,*b*) showed that ELF labeling corresponded to inducible APA in the marine dinoflagellate and prymnesiophyte species investigated.

The potential presence of attached bacteria could also cause uncertainty in the interpretation of algal ELF results. Bacterial AP can be stained with ELF (Carlsson and Caron 2001; K. Rengefors pers. obs.) in phosphate-limited algal cultures. However, bacterial cells are typically few and are morphologically distinct from AP precipitates on the algal cell wall, and it is therefore easy to visually distinguish attached bacterial cells from true algal cell surface APA.

Interestingly, the cyanobacteria in this experiment were only rarely labeled with ELF. Recent data from laboratory studies (Holz and Rengefors unpubl. data) on a cultured colonial cyanobacteria surrounded by a gelatinous sheath (*Gloeocapsa* sp.) show that cyanobacteria can and do become ELF labeled under phosphate-stressed conditions. Furthermore, Dyhrman et al. (2002) showed that the filamentous marine cyanobacteria, *Trichodesmium* spp., were ELF labeled when phosphate stressed. Thus, it is likely that the cyanobacteria in our incubation experiment were not phosphate limited. Also, we did observe some labeled cyanobacteria (Table 1), suggesting that inhibition of ELF labeling is not problematic and that another explanation for lack of labeling of these cells must be sought (*see* first section of *Results and discussion*). However at present, it cannot be ruled out that the gelatinous sheaths of, for example, *Planktolyngbya* sp., one of the cyanobacteria species present in our samples, prevent effective ELF labeling. Therefore, further studies using laboratory cultures are necessary to resolve this issue.

The ELF method can be used to analyze AP induction in individual taxa in a mixed natural community. The ELF assay can be applied to an array of different species, including both eukaryotes and cyanobacteria. In this study with the ELF method, we could detect increased alkaline phosphatase activity in a number of taxa as a response to phosphate lim-

itation. We found that the manifestation of phosphate stress varies both among and within taxa when exposed to the same environmental conditions. The ELF assay provides more information than bulk APA alone; thus, we argue that these two methods should be used together. Further investigations are needed to remove any doubt that cyanobacteria are successfully labeled with ELF when phosphate stressed and to study the significance of constitutive phosphatases in certain species. Finally, the phytoplankton community in Ashumet Pond at the time of the nutrient addition experiment was not phosphate limited, and the green algae, but not the cyanobacteria, were N limited. The ELF method shows promise in providing nuanced insight into the physiology of phytoplankton in response to changing nutrient environments, something that the traditional bulk APA assay, when used in isolation, is unable to do. When used in tandem, these two methods can provide an assessment of inducible APA in a mixed natural phytoplankton community from the total population level down to the individual taxa, or even the individual cell, level.

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