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**NOTE**

# ALKALINE PHOSPHATASE ACTIVITY AS A FUNCTION OF INTERNAL PHOSPHORUS CONCENTRATION IN FRESHWATER PHYTOPLANKTON[1]

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**Single-cell alkaline phosphatase (AP) activity is being increasingly used to characterize phosphorus (P) status of individual species of phytoplankton. As phytoplankton growth rates depend more directly on the internal rather than external P concentrations, we determine the AP activity in the two species of freshwater phytoplankton, *Scenedesmus quadricauda* (Turpin) Bréb. and *Asterionella formosa* Hassall, as a function of internal P concentration. AP activity strongly correlated with cellular P, increasing almost linearly with decreasing cellular P in both species. The dynamics of initial responses of AP activity to P limitation, as well as the final levels of AP activity, when cellular P approached minimum quotas, differed in two species. After P addition, cellular P concentrations increased rapidly, but AP activity remained high for several days. The lag in AP activity down-regulation following an increase in cellular P made it difficult to infer current P status of cells under dynamic P conditions.**

*Key index words:* **alkaline phosphatase; cellular P quota; chlorophyll; diatoms; green algae; phosphorus limitation; phytoplankton**

*Abbreviations:* **AP, alkaline phosphatase; ELF, enzyme labeled fluorescence; PP, particulate phosphorus; $Q_{minP}$, minimum cellular phosphorus concentration**

Phosphorus is a major limiting nutrient in many aquatic ecosystems, especially in temperate freshwater lakes (Hecky and Kilham 1988). Phytoplankton primary productivity, biomass, and community composition strongly depend on phosphorus availability. Therefore, it is important to determine the degree of P limitation in phytoplankton. Under P-limiting conditions (low inorganic P concentrations), phytoplankton synthesize the enzyme alkaline phosphatase (AP; EC 3.1.3.1), which hydrolyzes a phosphate group from diverse organic molecules (Cembella et al. 1984). The intensity of AP expression is commonly used as an indicator of P deficiency in algae (Healey and Hendzel 1979, Cotner and Wetzel 1992). Recently, labeling an AP-based enzyme labeled fluorescence (ELF) assay has allowed single-cell detection of AP activity and species-specific characterization of P stress in phytoplankton (Gonzalez-Gil et al. 1998, Nedoma et al. 2003). Previous studies determined cell-specific AP activity as a function of external P concentration for diverse freshwater and marine phytoplankton and found a significant negative correlation between the AP expression and external P concentration (Dyhrman and Palenik 1999, Dyhrman et al. 2002, Rengefors et al. 2003). Thus, cell-specific AP activity is being increasingly used to infer the degree of P limitation in different species of phytoplankton in nature (Dyhrman and Palenik 1999, Rengefors et al. 2001, 2003). However, phytoplankton growth depends more directly on the internal rather than external P concentration, since cells are able to accumulate and store P beyond immediate metabolic needs in a process termed ''luxury uptake'' (Droop 1973, Morel 1987, Grover 1991a). Species differ in their ability to store and utilize P (Grover 1991b), so that at the same ambient P concentrations, species exhibit different degrees of P limitation. Estimating the internal P concentrations in different species of phytoplankton in natural communities would provide information on the degree of limitation in individual species. Can we use AP activity of individual species to infer the internal P status of cells? Could the AP activity be a useful proxy for internal P concentration? Answers to these questions will allow us to rapidly characterize nutrient status and degree of growth limitation by P for each species in phytoplankton communities in nature, as it is impractical to measure cellular P of individual species directly in natural communities. The estimates of internal P concentration (cellular quota) could then be used to predict growth rates

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of individual species according to the Droop model (Droop 1973, Morel 1987, Grover 1991b). To our knowledge, no studies have explicitly tested how species-specific AP activity depends on intracellular P, rather than on external P concentration in different species.

Here we investigated the relationship between AP activity and internal P concentration in two common species of freshwater phytoplankton, the green alga *S. quadricauda* and the diatom *A. formosa* Hassl. to determine whether and when the intensity of AP expression can be used to infer intracellular P concentrations in different species. We also investigated the relaxation of AP expression as a function of internal P concentration in both species after the addition of P.

The culture CPCC 605 *A. formosa* was obtained as UTCC 605 *A. formosa* from the University of Toronto Culture Collection (now known as the Canadian Phycological Culture Centre at the University of Toronto). CPCC 605 and UTEX 614 *S. quadricauda* cultures grown in regular WC medium (Guillard 1975), then transferred to P-free WC medium, and grown in batch regime for >40 d at 20°C under 14:10 light:dark (L:D) cycle at irradiance of 50 μmol quanta $\cdot m^{-2} \cdot s^{-1}$ measured in flasks using a QSL light meter (Biospherical Inc., San Diego, CA, USA). Each species was grown in duplicate cultures. Cellular P content, chl *a* concentration, and AP activity were monitored periodically. After cells reached their minimum internal P concentration ($Q_{min}$), fresh medium containing 50 μM P was added, and the above measured physiological responses to P addition were assessed at days 2 and 6 after the addition of P. As the main aim of the experiment was to establish a relationship between the AP activity and cellular P concentration, but not the detailed dynamics of AP after P addition, we did not sample the experiment more frequently after the addition of P.

Particulate P (PP) was measured using the persulfate digestion method (Wetzel and Likens 1995, Lampman et al. 2001); each time-series sample from each duplicate culture was run in triplicate. Cellular P concentration was calculated by normalizing PP by cell density (Grover 1991a). Cell density was determined by microscopic counting in a haemocytometer (Leica epifluorescent microscope, Leica Microsystems GmbH, Wetzlar, Germany; Brightline haemocytometer, Hausser Scientific, Horsham, PA, USA). Samples were filtered onto GF/C filters (Whatman, Maidstone, UK) (in triplicate), and chl *a* concentration was measured using a calibrated Turner Designs fluorometer (TD-700, Turner Designs, Sunnyvale, CA, USA) after a 24 h extraction in 90% acetone (triplicates) (Neale et al. 1998, Litchman et al. 2002). Because the preliminary experiments showed that phosphate concentration in P-free medium with algae inoculated was below the detection limit (1–2 μg P $\cdot L^{-1}$, using the standard ammonium molybdate method) for the duration of the incubations, we did not quantify it in this experiment.

Cell sizes (lengths and widths of 25 or 30 random cells) were measured in duplicate cultures for each species using the image analysis software (Image-ProPlus, Media Cybernetics Inc., Bethesda, MD, USA) in the beginning and at the end of the growth in P-free medium. Cell volumes were calculated using geometric figure approximations (prolate spheroid for *S. quadricauda* and a rectangular box for *A. formosa*, Hillebrand et al. 1999). Cell volumes under P-replete and P-starved conditions were compared using the nonparametric Wilcoxon–Mann–Whitney test.

We fitted an exponential decay function $Q_P = Q_{minP} + be^{-ct}$, where $Q_P$ is the cellular P concentration, to the time series of P concentration under P limitation to estimate the rate of cellular P decline (time constant *c*) and the minimum cellular P concentration $Q_{min}$.

*AP labeling.* To detect cell-specific AP expression, we used a modified method of labeling AP with ELF-97 substrate reagent (ELF-97 endogenous phosphatase detection kit [E-6601] from Molecular Probes Inc., Carlsbad, CA, USA) described in Rengefors et al. (2003). Aliquots of cultures (1–2 mL) were concentrated by centrifuging in microcentrifuge tubes, supernatant was aspirated, and 1.5 mL of 70% ethanol was added. Samples were incubated for 1 h at room temperature and then centrifuged at 1,500*g* for 5 min. Supernatant was discarded, and the pellet was resuspended in 100 μL of ELF substrate in PBS buffer (20-fold dilution) and incubated in the dark at room temperature for 2 h.

Enzyme labeled fluorescence labeling was quantified under a Leica epifluorescence microscope with a DAPI filter (Rengefors et al. 2003). Total cell

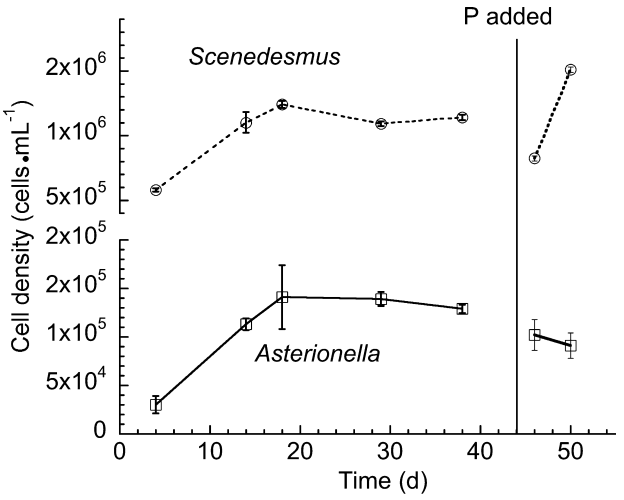


FIG. 1. Cell densities of the two species, *Asterionella formosa* and *Scenedesmus quadricauda,* during the experiment (in P-free medium and after P addition). Time of the P addition (day 44) is shown with a vertical line. Error bars are standard errors of the duplicate cultures.

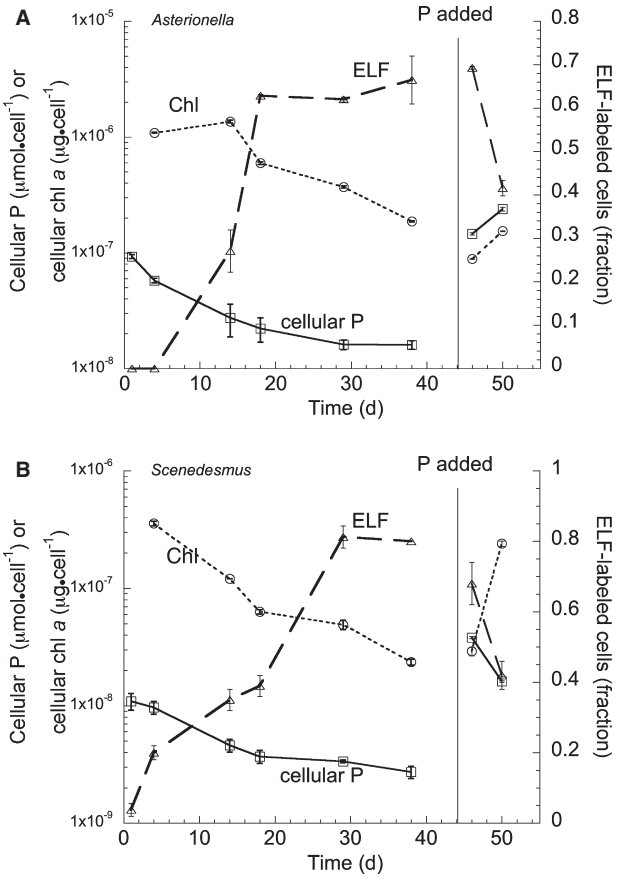


FIG. 2. Dynamics of cellular P concentration, chl *a*, and alkaline phosphatase (AP) activity (expressed as ELF-labeled cells as a fraction of total cells) in the two species placed in P-free medium and after addition of P (day 44). Error bars are standard errors of the duplicate cultures. (A) *Asterionella formosa*; (B) *Scenedesmus quadricauda*. ELF, enzyme labeled fluorescence.

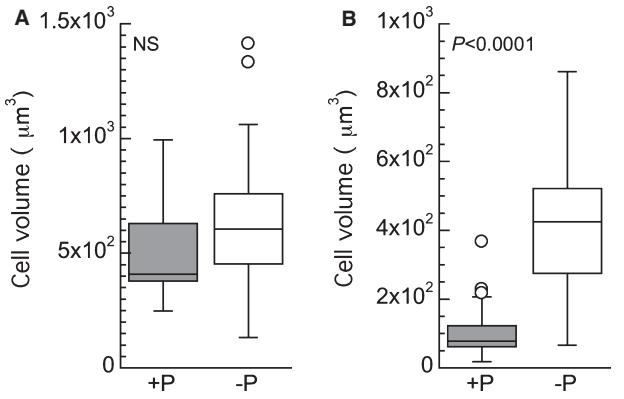


FIG. 3. Box plots of cell volumes of two species under P-replete and P-limited conditions. Duplicate cultures were measured for each species. P-limited cells approached their minimum P quotas ($Q_{minP}$). The nonparametric Wilcoxon–Mann–Whitney test was used to compare cell volumes (significance level $P < 0.05$). (A) *Asterionella formosa*; (B) *Scenedesmus quadricauda*. NS, not significant.

number of *A. formosa* was also estimated using phase-contrast optics (×400). Quantification of ELF fluorescence in *S. quadricauda* required a higher magnification (×1,000) with immersion oil. Cell images were captured at a fixed exposure using a RETIGA 1300*R* camera (Qimaging Corp., Surrey, Canada). AP expression was quantified by counting at least 100 cells for each sample, recording the number of cells with ELF fluorescence, and calculating the proportion of cells exhibiting AP activity.

Cell densities first increased after transfer to P-free medium and then stabilized until P was added (Fig. 1). Cellular P and chl *a* concentration declined in both species with time in P-free medium (Fig. 2). Cellular chl *a* concentrations dropped ~10-fold, and cellular P declined 4- or 6-fold in *S. quadricauda* and *A. formosa*, respectively. A significant decrease in cellular chl *a* concentration under P-limitation is often observed in marine and freshwater algae (e.g., Geider et al. 1998, Litchman et al. 2003). Cellular P concentrations declined nonlinearly with time, faster in *A. formosa*, reaching their minimum quotas ($Q_{minP}$) after at least 20 d in P-free medium, indicating significant storage capacity for P (Table 1 and Fig. 2). The minimum quota values obtained for the two species are comparable to the previously published values (Gotham and Rhee 1981, Rhee and Gotham 1981, Smith and Kalff 1982). AP activity increased with decreasing cellular P (Fig. 2). More than 80% of *S. quadricauda* cells and almost 70% of *A. formosa* cells exhibited AP activity when cellular P approached their respective minimum quotas ($Q_{minP}$) (Fig. 2). The initial responses to P limitation differed in the two species: *A. formosa* did not exhibit any detectable AP activity for the first 4 d after being placed in P-free medium, although its cellular P quota was declining. *S. quadricauda* responded to P-limitation faster, by increasing AP activity within the first 4 d (Fig. 2). Under P limitation, mean cell volumes increased significantly only in *S. quadricauda* (Fig. 3).

TABLE 1. Parameters of the exponential decline in cellular P in *Asterionella formosa* and *Scenedesmus quadricauda* in P-free medium. Equation $Q_P = Q_{minP} + be^{-ct}$ was fitted to the data, where $Q_P$ is the cellular P concentration at time $t$, $Q_{minP}$ is the minimum cellular P content (minimum quota), and $b$ and $c$ are constants.

| Parameter | *A. formosa* | *S. quadricauda* |
|---|---|---|
| $Q_{minP}$ (µmol P · cell$^{-1}$) | $1.73 \times 10^{-8} \pm 1.89 \times 10^{-9}$ | $2.50 \times 10^{-9} \pm 5.80 \times 10^{-10}$ |
| $b$ (µmol P · cell$^{-1}$) | $8.88 \times 10^{-8} \pm 4.34 \times 10^{-9}$ | $9.71 \times 10^{-9} \pm 6.86 \times 10^{-10}$ |
| $c$ (d$^{-1}$) | 0.18 ± 0.02 | 0.10 ± 0.02 |
| $R^2$ | 0.995 | 0.985 |

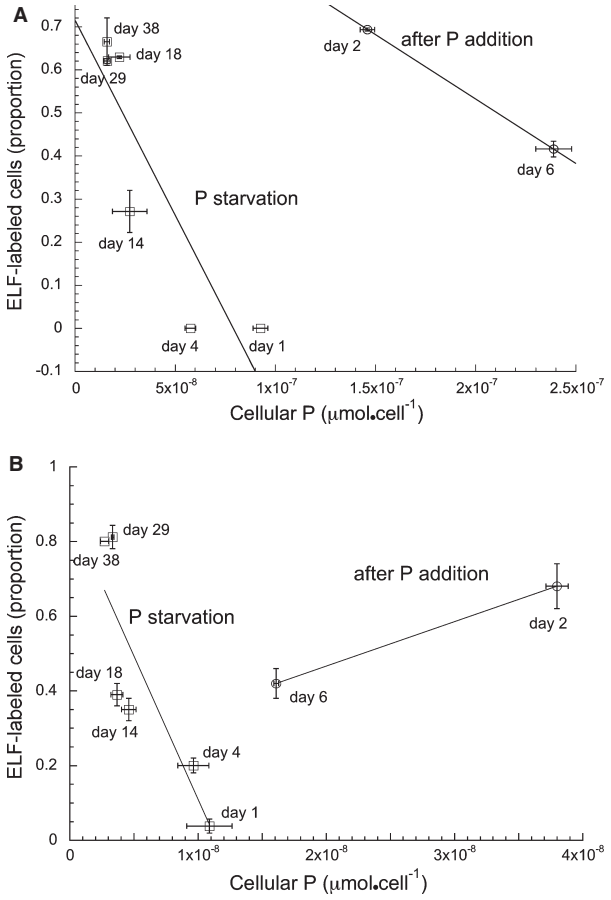


FIG. 4. Relationship between alkaline phosphatase (AP) activity (ELF-labeled cells) and cellular P concentration during P-starvation and after P addition in two species of freshwater algae. (A) *Asterionella formosa*; (B) *Scenedesmus quadricauda*. ELF, enzyme labeled fluorescence.

After P addition, cell densities declined due to dilution with P-rich medium and then increased in *S. quadricauda*, indicating a faster response to enrichment, compared to *A. formosa*, whose cell densities did not increase even after 6 d following the addition of P (Fig. 1). Cellular P concentration increased in *A. formosa* and increased initially (measured on day 2 after P addition) and then decreased in *S. quadricauda* (day 6 after P addition) (Fig. 2), likely due to the dilution of cellular P concentration by growth (higher growth rate response to P addition was observed in *S. quadricauda*, Fig. 1). Cellular chl concentration increased after P addition in both species but increased faster in *S. quadricauda* (Fig. 2). The observed faster physiological responses of *S. quadricauda* to P addition agree well with its general ecological strategy as an *r*-strategist associated with high nutrient conditions (Reynolds 1984).

During the P starvation phase, AP expression increased almost linearly with decreasing cellular P content in both species (Fig. 4). After P addition, the AP expression declined in both species, but because the internal P dynamics differed between species, the dependence of AP activity on cellular P also differed between the two species (Fig. 4). A decrease in AP expression occurred much slower than the change in cellular P concentration (Fig. 4). This lag in the AP response to P addition resulted in the same AP activity associated with several-fold different internal P concentrations (Fig. 4). In the starvation phase, the 70% or 80% of cells in each culture with detectable AP activity corresponded to the internal P concentrations close to species' respective minimum P quotas ($Q_{\mathrm{minP}}$), while after P addition, similarly high AP activity corresponded to internal P concentrations almost 10-fold greater than their $Q_{\mathrm{minP}}$ (Fig. 4). The lag in the response of AP activity to increased P concentration could be due to the stable nature of this enzyme, where despite the down-regulation of the gene encoding the AP, previously synthesized AP produces residual ELF staining.

Previous studies reported a considerable variation in the expression of AP activity in different species from the same environment and at the same external P concentration (Rengefors et al. 2003). We hypothesize that this variability may in part be due to a species-specific dependence of AP expression on the internal P concentration. For example, when internal P concentrations approached their respective minimum quotas, the percent of cells with detectable AP expression differed in *A. formosa* and *S. quadricauda* (<70% and >80%, respectively). Moreover, under the same external P concentrations, temperature, and light conditions, the two species also differed in how fast they approached their minimum quotas, which can also contribute to the observed variation in AP expression in different taxa in nature (Rengefors et al. 2003, Dyhrman and Ruttenberg 2006). The internal P concentration can vary among species even at the same external P concentrations (Shuter 1978), and different species may have a different relationship between internal P concentration and synthesis of AP.

In summary, cell-specific AP expression apparently depended on the internal P concentration, increasing with declining cellular P concentration, which suggests that it reflects the internal P status of cells. However, because there was a lag in the AP response (decrease in activity) to increased cellular P concentrations, the same AP expression in the population, expressed as a fraction of cells with AP activity, may correspond to very different internal P concentrations, depending on the previous P conditions (Fig. 4). This may limit the application of the single-cell quantification of AP activity to characterize P status of cells under dynamic P regimes. Other, more direct measures of P stress, such as up- and down-regulation of genes encoding for AP and other P-stress enzymes may be more applicable.

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