Phosphate uptake proteins as markers for the nutrient status of freshwater cyanobacteria
Dignum, M.
Chapter 5

Alkaline Phosphatase induced fluorescence adds a nutrient status selection parameter to flow cytometric analysis of freshwater phytoplankton.

Marco Dignum\textsuperscript{1}, Hans L. Hoogveld\textsuperscript{2}, Hans C. P. Matthijs\textsuperscript{1}, Hendrikus J. Laanbroek\textsuperscript{2}, and Roel Pel\textsuperscript{2}

\textsuperscript{1} Aquatic Microbiology/Institute for Biodiversity and Ecosystem Dynamics/University of Amsterdam, Nieuwe Achtergracht 127, NL-1018WS Amsterdam, The Netherlands
\textsuperscript{2} Centre for Limnology/Netherlands Institute of Ecology/Royal Netherlands Academy of Arts and Sciences, Rijksstraatweg 6, NL-3631AC Nieuwersluis, The Netherlands
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Abstract

Alkaline phosphatase (AP) activity provides a suitable indicator for the nutrient status of phytoplankton. The novel alkaline phosphatase substrate, ELF-97 phosphate, yields intensely green fluorescent precipitates upon enzymatic dephosphorylation, and therefore traces AP activity back to its producer. Both fluorescence microscopy and flow cytometry allowed detection of cells with AP induced fluorescence with UV excitation. Several, but not all tested cyanobacteria showed AP induced fluorescence after staining with the ELF substrate. The presence of endogenous fluorescent pigments in photoautotrophic organisms allowed flow cytometric distinction (blue excitation) of different clusters in the phytoplankton community in Lake Loosdrecht (the Netherlands): eukaryotic algae (diatoms and green algae), chlorophyll $a$ and $b$ containing but phycobilin-less cyanobacteria (predominantly Prochlorothrix hollandica), and phycocyanin-containing cyanobacteria (predominantly Planktolyngbya limnetica). AP induced fluorescence adds an extra, nutrient status related parameter in flow cytometry (UV excitation). The dominant cyanobacterium in Lake Loosdrecht (the Netherlands), *P. limnetica*, possesses a derepressible AP, whereas in the second most abundant strain, *P. hollandica*, AP is absent. Within the population of *P. limnetica* variable sensitivity towards ELF-97 staining was found, resulting in distinct groups with different nutrient status.

*Key words:* Alkaline phosphatase, fluorescence, flow cytometry, nutrient status, ELF
Introduction

Enzymatic analyses to detect alkaline phosphatase (AP) activity provide information about the nutrient status with respect to surplus/lack of inorganic phosphate (P$_i$) in phytoplankton. Although bulk AP activity has been widely used as a means of diagnosing P$_i$-deficiency, an important flaw in its use as a P$_i$-deficiency indicator is the uncertainty in the origin of the enzymes. In many older studies, AP activities are assumed to be of algal origin and used as indicator for P$_i$-deficiency without any proof of their origin (Jansson et al. 1988). In search for suitable indicators for the phytoplankton nutrient status that trace molecular markers back to the cells that produced them, several reviews have been published in recent years (Palenik and Woods 1998; La Roche et al. 1999; Scanlan and Wilson 1999; Jochem 2000; Beardall et al 2001). Molecular methods such as fluorescent in situ hybridisation or immuno-fluorescence require many reaction steps. A biochemical indicator like the enzymatic assay approach is very straightforward and convenient, and comprises only one reaction step and short staining time compared to immuno-labelling.

Many cultured algal species show increased AP activity when they are subjected to a lack of P$_i$. For example, AP activity was induced 5-25 times in nine cultured species, among which Chlorella pyrenoidosa, Scenedesmus dimorpha, Microcystis aeruginosa and Aphanizomenon flos-aqua, the latter also showing constitutive AP activity (Fitzgerald and Nelson 1966). Induction of AP at low P$_i$-concentrations was also described for Anacystis nidulans, later renamed as Synechococcus sp. (Ihlenfeldt and Gibson 1975). Both Oscillatoria sp. and Anabaena sp. show no significant constitutive levels of AP activity, and induce the enzyme in P$_i$-deficient conditions (Marco and Orús 1988). This is in agreement with data for Anabaena flos-aqua, A. variabilis, A. spiroides and A. cilicrica, but AP activity was not induced O. spiroides and O. prolifera (Marco and Orús 1988). AP activity thus seems ubiquitous among many, but not all algae.

Flow cytometry is a valuable tool in aquatic microbiology due to its speed in determining numbers and size of microorganisms (e.g. Vives-Regó et al. 2000). Additional biochemical and physiological information is provided by the detection of fluorescent molecules present in the cells. The objective of this study was to develop a simple, direct method to detect the cellular response of freshwater phytoplankton to phosphate deficiency in a flow cytometer. The presence of endogenous fluorescent pigments in photoautotrophic organisms on the one hand allows distinction of different populations, but on the other hand limits the choice of probes. Members of the ELF-97 TM substrate family (Huang et al. 1993; Haugland 1995) have suitable excitation and emission wavelengths. ELF-97 phosphate yields intensely green fluorescent precipitates upon enzymatic dephosphorylation, and
therefore traces AP activity back to its producer. Application of this substrate to phytoplankton and its use in flow cytometry was first published by González-Gil et al. (1998), and was recently described in a review by Jochem (2000). The first application to freshwater phytoplankton was recently published (Rengefors et al. 2001), but did not involve flow cytometry. In the present work we have studied AP activity in several strains of cyanobacteria, and tested the applicability of the ELF-97 method in flow cytometry with samples from a highly eutrophied lake in the Netherlands, Lake Loosdrecht, which is dominated by filamentous cyanobacteria.

**Experimental procedures**

**Growth conditions**

Batch cultures were growing exponentially until the onset of phosphate deficiency. To arrive at these conditions we diluted (1:10) pre-cultures from complete BG-11 medium (Rippka et al. 1979), containing 175 μM P, into BG-11 medium without phosphate. This medium was substituted with 175 μM KNO₃ to replace potassium normally provided as K₂HPO₄. Incubation time was 5-7 days. Control (nutrient replete) cultures were growing in the same conditions with complete BG-11 medium. Field samples were taken from Lake Loosdrecht, a shallow eutrophic lake in the Netherlands (52°11' N, 5°3'E; area 9.8 km²; mean depth 1.85 m; P_total = 40-60 μg.L⁻¹). Due to prolonged external nutrient loading, this lake is dominated by filamentous cyanobacteria and has a low transparency (Secchi-disc depth about 0.5 m).

**Alkaline Phosphatase assays**

The classical assay (Bessey et al. 1946) for AP activity with the substrate para-Nitrophenyl-phosphate (pNPP) was adapted for measurement at 405 nm in a microplate reader (VERSAmax, Molecular Devices, USA). To 160 μl of culture we added 40 μl of 18 mM p-NPP (Sigma 104 phosphatase substrate), in 1 M Tris-HCl buffer with 10 mM MgCl₂, pH 8.0. The AP activity was expressed per optical density unit at 730 nm. The novel alkaline phosphatase substrate 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (ELF-97™, Molecular Probes; Huang et al. 1993; Haugland 1995) was applied to several freshwater cyanobacteria. Cells were centrifuged for 15 minutes at a maximum of 1000x g and resuspended into the buffer provided with the endogenous phosphatase detection kit. Because the salt concentration was very high in the buffer (3M NaCl), the following alternative buffers were tested: 1/5 dilution of the provided buffer in water or 1/10 dilution in 30 mM HEPES pH 8, 1 mM MgCl₂, 0.1 mM ZnCl₂. The optimal reaction time was empirically determined to be 30 minutes at room temperature.
**Fluorescence Microscopy**

Visual inspection of the progress of the ELF-97 hydrolysis was carried out on a Zeiss standard RA instrument with UV epifluorescence furnishing: excitation filter (bandpass 415 ± 25 nm), dichromatic mirror (FT 460), and suppression filter (longpass 475 nm). Samples were centrifuged for 5 minutes at 1000 x g and resuspended in 10-25 µl of mounting buffer provided with the endogenous AP detection kit (Molecular Probes) for temporary storage. Fluorescence microscopic images were taken on a Leica DIALUX 20 EB microscope (Leitz Wetzlar, Germany) fitted with a Plomopak filtercube with excitation filter (bandpass 390 ± 35 nm), dichromatic mirror (RKP 455), and suppression filter (longpass 470 nm). The high intensity of the green fluorescent ELF-97 signal permitted the use of background light (clear field) to distinguish non-stained cells. The microscope was equipped with a Sony (Japan) DXC-950P 3CCD colour video camera and CMA-D2 camera adaptor.

**Flow Cytometry**

Samples were filtered over a 70 µm plankton filter to prevent obstruction of the flow cytometer. Some samples were mildly fixed with final concentrations of 0.01% w/v para-formaldehyde and 0.1% w/v glutaraldehyde. Flow cytometric analyses were carried out on an Epics Elite instrument (Coulter, Hialeah, Florida, USA), equipped with a gated amplifier facility for measuring with two lasers successively. The water-cooled Argon-Krypton lasers (Coherent 70C) were set on 80 mW output of 488 nm wavelength and 60 mW output on multiline UV (350.7-356.4 nm). The trigger was set on Chlorophyll fluorescence emission peak. Forward scatter light was filtered with a 488 ± 10 nm bandpass filter before hitting the photodiode. The 90° light beam first hit a 45° 488 nm dichroic longpass filter. The reflected light from this 488 DL was filtered through a 488 ± 10 nm bandpass filter and measured with a photomultiplier as side scatter. After the 488 DL the light passed through a 488 nm blocking filter, and was separated using a 45° 550 nm longpass filter. A 525 ± 20 nm bandpass filter was used in front of the PMT for measuring the green fluorescence of ELF-97 alcohol excited by the UV laser. The remaining light was separated using 45° 660 nm dichroic longpass filter to measure orange fluorescence of phycocyanin (bandpass 637 ± 10 nm) and red fluorescence of chlorophyll (bandpass 675 ± 20 nm). The 525 nm UV pre-amplification was set to 1.0, and the threshold for CHLpk10 was adjusted to suppress the extra background noise resulting from higher amplification. All variables were measured on a four-decade logarithmic scale of the integrated signal. WinMDI version 2.8 software (J. Trotter, 1999) was used to create Figs. 3, 5, 6, and 8 offline.
**Figure 1.** Reaction scheme for enzymatic conversion of ELF-97 substrate (water soluble and weakly blue fluorescent with UV excitation) to ELF-97 alcohol, which precipitates into micro-crystals that are strongly green fluorescent with UV excitation.

**Figure 2.** Fluorescence microscopic images (UV excitation) of cells with alkaline phosphatase related fluorescence after ELF-97 staining of nutrient replete and P$_1$-depleted batch cultures of *Anabaena variabilis* (A and B respectively) and *Synechococcus elongatus* PCC 7942 (B and D respectively), and alkaline phosphatase related fluorescence after ELF-97 staining of a sample from Lake Loosdrecht (E).
Results

Enzymatic dephosphorylation of the ELF-97 alkaline phosphatase substrate (Fig.1) yields highly green fluorescent micro-crystals of ELF-alcohol at the site of enzymatic activity. The high intensity of the ELF-97 signal permitted the use of background light (clear field microscopy) to distinguish non-stained cells. The staining gives a cell-bound fluorescent signal, referred to as AP induced fluorescence, examples of which are shown in clear field/fluorescence microscopic images of *Anabaena variabilis* (Fig. 2A,B), *Synechococcus elongatus* PCC 7942 (Fig. 2C,D), and a typical sample from lake Loosdrecht (Fig. 2E). Green fluorescent micro-crystals had a low abundance in cells from batch cultures of *A. variabilis* and *S. elongatus* with excess P<sub>i</sub> (Fig. 2A,C), but were much more abundant in P<sub>i</sub>-depleted cells (Fig. 2B,D). In the lake water sample, filamentous cyanobacteria with various amounts of AP induced fluorescence were seen (Fig 2E), among which *Planktolyngbya limnetica* (synonym Oscillatoria cf. limnetica, may include several closely related strains, see Komárková-Legnerová and Cronberg 1992), *Prochlorothrix hollandica* (Burger-Wiersma et al. 1989), *Planktothrix agardhii*, *Limnothrix redekei*, *Aphanizomenon flos-aquae*, and *Anabaena* spp. Several eukaryotic species showed AP induced fluorescence among which we visually identified the green algae *Pediastrum* spp.,

**Figure 3.** Histograms showing the number of events with 525 nm green fluorescence (UV excitation) in samples from batch cultures of model cyanobacteria *Anabaena variabilis* (A and B), *Synechococcus elongatus* PCC 7942 (C and D), and *Synechocystis* sp. strain PCC 6803 (E and F). ELF-stained samples have a grey background, and control treatments (same treatment as ELF-stained cells, without the ELF-97 substrate) are shown in overlay. A, C, and E: Nutrient replete cultures. B, D, and F: P<sub>i</sub>-depleted cultures. Discrete intensity levels of 525 nm emission are marked: low (M1), intermediate (M2), and high (M3) 525 nm fluorescence intensity respectively.
and *Scenedesmus* spp., and the diatoms *Diatoma elongatum*, *Cyclotella* spp., and *Navicula* spp. (not shown).

Using the UV laser facility on the flow cytometer to excite the cell-bound ELF product, fluorescence emission at 525 nm was detected in single filaments of *A. variabilis* (Fig. 3A,B), and in single cells of *S. elongatus* (Fig. 3C,D) and *Synechocystis* sp. PCC 6803 (Fig. 3E,F). Cyanobacterial strains relevant to lake Loosdrecht were subjected to the same assay. The presently dominant cyanobacterial strain in the lake Loosdrecht is *Planktolyngbya limnetica*. The chlorophyll *a* and *b* containing, phycobilin-less cyanobacterium *Prochlorothrix hollandica* (Burger-Wiersma et al. 1986) is the second most abundant species.

Relatively rare filamentous cyanobacteria in lake Loosdrecht are *Limnothrix redekei*, *Planktothrix agardhii*, *Aphanizomenon flos-aquae*, and *Anabaena* spp. Filamentous colonies from batch cultures of *P. limnetica* strain MR1 (Fig. 4A, B), which was isolated at the Centre for Limnology, *P. hollandica* strain PCC 9006 (Fig. 4C, D), and *P. agardhii* (Fig. 4E, F) show different levels of 525 nm emission intensity. Most cells from batch cultures of *Aphanizomenon flos-aquae 1401/7* showed lysis after ELF-staining, which biased the results, and the ELF-staining of this strain is therefore not shown. In some strains, a fraction of the cells had increased levels of green autofluorescence, which was not due to ELF-staining (e.g. Fig. 3D, F and Fig. 4A, E, F). *S. elongatus* and *P. limnetica* showed three distinct peaks with different levels of 525 nm emission intensities (Fig. 3D and Fig. 4B respectively).
Figure 5. Histograms showing the number of events with 525 nm green fluorescence (UV excitation) in samples from Lake Loosdrecht on two dates. ELF-stained samples have a grey background, and control treatments (same treatment as ELF-stained cells, without the ELF-97 substrate) are shown in overlay. A: sample from 15-10-2001. B: sample from 10-12-2001. Discrete intensity levels of 525 nm emission are marked: low (M1), intermediate (M2), and high (M3) 525 nm fluorescence intensity respectively.

Lake samples also showed a partition into three discrete intensity levels of 525 nm emission (Fig. 5), therefore we classified these peaks as low, intermediate and high 525 nm fluorescence intensity respectively. Quantification of the relative numbers of particles with increased (intermediate and high) levels of 525 nm fluorescence after subtraction of the percentage of particles in control treatments without ELF-substrate, referred to as AP induced fluorescence, is presented in Table 1, along with AP activity measurements with the classical para-Nitrophenyl phosphate (pNPP) assay. P$_1$-depleted batch cultures of *S. elongatus* showed high activity in the pNPP assay as well as AP induced fluorescence with ELF-97 substrate.

Surprisingly, *Synechocystis* sp. PCC 6803 showed high activity in the pNPP assay, but no ELF-signal. *A. variabilis* shows quantitative induction of ELF-signal in batch cultures. Batch cultures of *P. limnetica* strain MR1 showed intermediate and high AP induced fluorescence in flow cytometry. A fraction from the filaments from nutrient replete batch cultures of *P. hollandica* strain PCC 9006 showed intermediate 525 nm fluorescence after incubation with ELF-97 phosphate. This signal did not increase in P$_1$-depleting conditions. Visual inspection by fluorescence microscopy revealed no visible ELF-signal, and the pNPP assay produced no statistical confidence for this species (Table 1). Batch cultures of *P. agardhii* showed high 525 nm fluorescence in nutrient replete cultures, which was even increased in P$_1$-depleted cultures. The AP induced fluorescence in batch cultures of *S. elongatus* and *P. limnetica* were susceptible to inhibition by high concentrations of P$_1$. When cells grown in lack of P$_1$ were incubated with 400 μM of P$_1$ for 5 minutes before staining with ELF-97-phosphate, the ELF-signal in the high 525 nm intensity peak was halved, and after incubation with 2 mM of P$_1$ or higher concentrations, none of the cells had any high ELF-signal (results not shown).
Table 1. Inventory of phytoplankton cultures with excess phosphate (+P<sub>i</sub>) and lack of phosphate (-P<sub>i</sub>), tested for Alkaline Phosphatase activity with ELF-97 phosphate (ELF) and para-Nitrophenylphosphate* (pNPP) as substrates.

<table>
<thead>
<tr>
<th>Species</th>
<th>ELF-signal (% particles-% in control)</th>
<th>pNPP (A&lt;sub&gt;405&lt;/sub&gt;.min&lt;sup&gt;-1&lt;/sup&gt;/A&lt;sub&gt;730&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>high</td>
</tr>
<tr>
<td><em>Anabaena variabilis</em></td>
<td>3.8</td>
<td>9.1</td>
</tr>
<tr>
<td><em>Planktolyngbya limnetica MR1</em></td>
<td>3.8</td>
<td>0.6</td>
</tr>
<tr>
<td><em>Planktothrix agardhii</em></td>
<td>0.2</td>
<td>6.2</td>
</tr>
<tr>
<td><em>Prochlorothrix hollandica PCC 9006</em></td>
<td>10.3</td>
<td>0.6</td>
</tr>
<tr>
<td><em>Synechococcus elongatus PCC 7942</em></td>
<td>45.1</td>
<td>1.8</td>
</tr>
<tr>
<td><em>Synechocystis</em> sp. strain PCC 6803</td>
<td>1.6</td>
<td>-0.1</td>
</tr>
</tbody>
</table>

* For the AP activity with pNPP, 95% confidence intervals are given with degrees of freedom in brackets.

** AP activities for Anabaena variabilis and Synechocystis sp. PCC 6803 were not measured with the pNPP substrate in this experiment, but showed clear derepression in other measurements. AP activities for Synechocystis sp. PCC 6803 are discussed in chapter 2 and 3 of this thesis, results for Anabaena variabilis did not have the same level of statistical evaluation.
To optimise the staining procedure, samples of lake Loosdrecht water were incubated with ELF-97 substrate and sampled in a time series. The experiment demonstrates that there was hardly any staining development within the first 5 minutes (Fig. 6A). After 10 minutes, more than 20% of the cyanobacteria in the sample had an intermediate 525 nm intensity, while about 5% show high 525 nm fluorescence. After this time, the fraction of cells with high intensity gradually increased, at the expense of the intermediate 525 nm fluorescence fraction. The reaction reached saturation after 30 minutes (Fig. 6A).

A plot of the mean relative fluorescence (relative to the base peak of non-stained cells, expressed in relative fluorescence units at 525 nm, rfu_{525nm} per cell) in time shows linear increase of the fluorescence intensity in the different fractions (Fig. 6B). After 30 minutes of reaction, the mean intensity in the group with intermediate 525 nm fluorescence was about 100 times higher than that in the group with low 525 nm fluorescence. The mean intensity in the group with high 525 nm fluorescence was about 900 times higher than that in the group with low 525 nm fluorescence. Because the salt concentration was very high in the buffer (3M NaCl), cells were liable to break when subjected to the low salt concentrations and high pressure differences in the sheath fluid of the flow cytometer.

When the reaction was carried out with a 1 in 5 dilution of the provided buffer in water, staining was even better than with undiluted buffer (results not shown), but with 1 in 10 dilution no AP induced fluorescence was detected. However, 1/10 dilution in 30 mM HEPES pH 8 with 1 mM MgCl$_2$ and 0.1 mM ZnCl$_2$ gave a strong ELF signal (also not shown).
Flow cytometric analysis of freshwater samples from lake Loosdrecht (the Netherlands) with blue (488 nm) excitation, allows separation of phytoplankton according to differences in photosynthetic pigmentation. In a bivariate plot of chlorophyll $a$ (675 nm) vs. phycocyanin (640 nm) fluorescence (Fig. 7A), the phytoplankton community separated into clusters of eukaryotic algae, chlorophyll $a$ and $b$ containing but phycobilin-less cyanobacteria (prochlorophytes), phycobilin-containing cyanobacteria, and detritus. Treatment with ELF-97 phosphate caused the clusters in the 675 nm vs. 640 nm plot to draw towards each other (Fig. 7B). The cyanobacterial cluster shifted to lower 640 nm intensity as well as lower 675 nm intensity. The prochlorophyte cluster shifted only in 675 nm, resulting in overlap of the clusters. Higher amplification of both 640 and 675 nm photomultiplier tubes allowed the clusters to be separated again (Fig. 7C). In the ELF-stained samples an extra cluster appeared parallel to the cluster of cyanobacteria.

**Figure 7.** Two-dimensional histograms (dot plots) of red chlorophyll $a$ fluorescence (y-axis, 675 nm) vs. orange phycocyanin fluorescence (x-axis, 640 nm) with blue excitation at 488 nm (samples from 15-10-2001). A: Sample directly injected into flow cytometer, showing good separation of clusters of eukaryotic algae (I), prochlorophytes, predominantly *P. hollandica* (II), phycocyanin-containing cyanobacteria, predominantly *P. limnetica* (III), and detritus, predominantly dead phytoplankton cells (IV). B: After ELF-staining the clusters move towards each other with voltage of photomultiplier tube set at 1000V. C: Voltage of photomultiplier tubes set at 1500V gives separation again. The arrow indicates an additional cluster of cyanobacteria that appears only after ELF-97 staining.

The three groups of different 525 nm intensities are separately represented in two-dimensional chlorophyll vs. phycocyanin fluorescence plots (Fig. 8). The low 525 nm intensity group contains all clusters, whereas the intermediate 525 nm intensity group is relatively enriched in cyanobacteria, and contains much less detritus. The high 525 nm intensity group mainly contains eukaryotic algae and cyanobacteria, and is low in prochlorophytes and detritus. Particles in the anomalous cluster of cyanobacteria have a very strong ELF-signal (Fig. 8C). We have some morphological indications, for example the presence of heterocysts, that this might be *Aphanizomenon flos-aquae*. 
Summation of the number of particles in the intermediate and high 525 nm intensity groups gave the following relative quantities after subtraction of the numbers in the control treatment: of all particles, about 11.5% had AP induced fluorescence; of the eukaryotic algae (about 1% of all particles), about 39% had AP induced fluorescence; of the *P. hollandica* cluster (about 10% of all particles) about 13% had AP induced fluorescence; of the cluster of phycocyanin-containing cyanobacteria, including the ‘*Aphanizomenon’ cluster (about 22% of all particles), about 27% had AP induced fluorescence, and in the cluster of detritus (about 60% of all particles), about 4% had AP induced fluorescence.

**Figure 8.** Cluster analysis in two-dimensional histograms (dot plots) of red chlorophyll *α* fluorescence (y-axis, 675 nm) vs. orange phycocyanin fluorescence (x-axis, 640 nm) with blue excitation 488 nm (samples from 15-10-2001). A: Particles with low green fluorescence (gate M1 in Fig. 5B). B: Particles with intermediate alkaline phosphatase induced fluorescence (gate M2 in Fig. 5B). C: Particles with high alkaline phosphatase induced fluorescence (gate M3 in Fig. 5B). Clusters that contain eukaryotic algae (I), *P. hollandica* (II), *P. limnetica* (III), and detritus (IV), and an additional cluster of cyanobacteria (arrow) are indicated.

**Discussion**

The ELF-97 AP substrate provides a readily applicable method to determine the P-sensing status of freshwater phytoplankton, expanding the use of UV excitation in flow cytometry. Flow cytometry gives quantitative measurements in terms of relative fluorescence, if these measurements are taken after saturation of fluorescence accumulation or at a specific time point (Jochem 2000). No cells with ELF-97 signal were visible within the first 5 minutes of incubation. Fluorescence intensity increased linearly over time and did not reach a maximum within the experimental period (1 hr). These results indicate that the rate-limiting step for the formation of ELF-97 alcohol is not the enzymatic conversion, but rather a transport process, such as diffusion of the rather large substrate across the outer cell layers.
In the product information provided by the manufacturer a staining time of 30 seconds is recommended (Haugland 2001). According to our experiments the partition of cells with ELF-97 signal reached a steady level after 30 minutes, corresponding to the staining time found in literature (González-Gil et al. 1998; Dyhrman and Palenik 1999). The observation that the increase in fluorescence intensity continued, while the division of cells into distinct intensity groups stabilized, suggests a biological origin of this division. The ELF-signal was therefore expressed in percentage of particles in defined groups or clusters. It is interesting to note that the ELF-signal was not evenly distributed over the cells, but was localized in spots. This punctated labelling pattern was previously described (González-Gil et al. 1998; Dyhrman and Palenik 1999), and can be explained by nucleation of micro-crystals; at the site where the first crystallization nuclei form, crystallization of more ELF-97 alcohol would take place in a confined space. Lack of staining in 1:10 diluted reaction buffer was probably due to the dependence of the enzymatic activity on the presence of zinc and magnesium. Providing these cofactors in HEPES buffer did not give satisfactory results, probably due to the lack of other, unknown factors provided in the reaction buffer of the endogenous phosphatase detection kit. The ELF-signal was susceptible to inhibition by prior addition of millimolar concentrations of P₁. P₁ is a strong competitive inhibitor of AP activity (Healey 1973; Siuida and Chróst 1987; O'Brien and Herschlag 2001). Nutrient replete batch cultures of the tested cyanobacteria showed low levels of AP activity, due to a constitutive level of AP expression. These results indicate that the ELF-signal originates from P₁-inhibitable enzymes, probably only AP.

Presence of endogenous fluorescent pigments in photoautotrophic organisms allowed flow cytometric distinction (at blue excitation) of different clusters in the phytoplankton community in Lake Loosdrecht (the Netherlands), consisting of eukaryotic algae (diatoms and green algae), chlorophyll a and b containing but phycobilin-less cyanobacteria (predominantly *Prochlorothrix hollandica*), and phycoerythrin-containing cyanobacteria (predominantly *Planktolyngbya limnetica*). The fluorescent signal varies linearly with the length of filamentous cyanobacteria (Hofstraat et al. 1991), and the amount of both pigments does not vary independently. This resulted in long-stretched clusters of cyanobacteria in bivariate 675 nm vs. 640 nm plots (Fig. 7 and 8). *P. hollandica*, which contains mainly chlorophyll a, little chlorophyll b, and no phycoerythrin, had a normal distribution in these plots. The cluster of eukaryotic algae represents low numbers of many different species, and is therefore amorphic. ELF-97 phosphate was shown to add a parameter to flow cytometric analysis of phytoplankton; its spectral characteristics do not interfere with the autofluorescence of these photosynthetic organisms. The staining procedure had an adverse effect on the separation of the clusters, which was countered by increasing the voltage of the photomultiplier tubes.
Cyanobacterial filaments were weakened towards mechanical strain after the ELF-97 staining procedure, probably due to the high concentration of NaCl (3M) in the detection buffer provided with the endogenous AP detection kit. The staining, especially after longer storage time (hours) and after washing in sheath fluid, caused filaments to break during flow cytometry. Omission of washing steps and restraint of centrifugal force to 1000x g did not prevent the filaments from breaking. Use of ethanol to dehydrate the cells, as recommended in the product protocol (see also Rengefors et al 2001), resulted in changed properties of the photosynthetic pigments. Mild fixation with low concentrations of glutaraldehyde and formaldehyde gave good preservation of filaments after ELF stain, and did not influence photosystems fluorescence. In contrast to the findings of Rengefors et al. (2001) these fixation conditions did not hinder AP induced fluorescence. AP induced fluorescence depended on P-concentration in batch cultures; the tested organisms showed a variable sensitivity towards ELF-97 staining. The dominant cyanobacterium in Lake Loosdrecht (the Netherlands), *P. limnetica*, was shown to possess a derepressible AP, whereas in the second most abundant strain, *P. hollandica*, AP induced fluorescence is absent. In spite of the discrepancy in the staining of *Synechocystis* PCC 6803, we believe that AP induced fluorescence reflects species-specific variation in the adaptive strategies towards P-deficiency. Furthermore, we found heterogeneous sensitivity towards ELF-97 staining within the population of cyanobacteria in the lake, resulting in discrete groups with apparent different nutrient status. This heterogeneity cannot be attributed to the existence of multiple closely related strains, as pure cultures of *S. elongatus, P. limnetica*, and *P. agardhii* also showed discrete intensity peaks. In a recent study using flow cytometric detection of endogenous AP in mammalian cells (Telford et al. 2001), similar subsets with varying levels of AP induced fluorescence were found in the context of cell cycle analysis. The intra-specific variation of AP induced fluorescence in filamentous cyanobacteria poses an interesting physiological problem, which we will address to in chapter 6.