Detecting the phosphate status of phytoplankton by enzyme-labelled fluorescence and flow cytometry

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Abstract

The novel phosphatase substrate, ELF-97 phosphate, yields intensely green fluorescent precipitates of ELF-97 alcohol (ELFA) upon enzymatic dephosphorylation, and thereby traces phosphatase activity back to its producer. In this study, we show that ELFA fluorescence is a useful tool in flow cytometric analysis of natural phytoplankton populations. Presence of endogenous fluorescent pigments allowed flow cytometric distinction of clusters in the phytoplankton community in Lake Loosdrecht (The Netherlands): Eukaryotes (diatoms and green algae), chlorophyll *a* and *b* containing but phycobilin-less cyanobacteria (*Prochlorothrix hollandica*), and phycocyanin-containing cyanobacteria (predominantly *Limnothrix* sp.). Several, but not all tested cyanobacteria showed ELFA fluorescence. The dominant *Limnothrix* sp. possesses a derepressible phosphatase, whereas the second most abundant strain, *P. hollandica*, did not have phosphatase activity. Within both natural and cultured populations of *Limnothrix* sp. we found discernible levels of ELFA fluorescence, indicating the presence of subpopulations with different physiological characteristics.
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1. 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, it has an important flaw in its use as a P_i-deficiency indicator, that is the uncertainty of 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 with-out any proof of their origin [1]. The search for suitable indicators for the phytoplankton nutrient status, that trace molecular markers back to the cells that produced them, has been covered in several reviews [2–6]. 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 a short staining time compared to immuno-labelling.

Many cultured phytoplankton 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 are *Chlorella pyrenoidosa*, *Scenedesmus dimorpha*, *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* [7]. Induction of AP at low P_i-concentrations was also described for *Anacystis*

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nidulans, later renamed as *Synechococcus* sp. [8]. Both *Oscillatoria* sp. and *Anabaena* sp. show no significant constitutive levels of AP activity, and induce the enzyme in P_i-deficient conditions [9]. This is in agreement with data for *Anabaena flos-aquae*, *Anabaena variabilis*, *Anabaena spiroides* and *Anabaena cilidrica*, but AP activity was not induced in *Oscillatoria spiroides* and *Oscillatoria prolifera* [9]. AP activity thus seems to be under control of P_i-availability for many, but not for all phytoplankton species.

Flow cytometry is a valuable tool in aquatic microbiology due to its speed in determining numbers and size of microorganisms (e.g. [10]). Additional biochemical and physiological information is provided by the detection of fluorescent pigment 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 enzyme-labelled fluorescence (ELF) substrate family [11,12] have suitable excitation and emission wavelengths. The ELF-97[TM] phosphate (ELFP) substrate yields intensely green fluorescent precipitates of ELF-97[TM] alcohol (ELFA) upon enzymatic dephosphorylation and therefore traces phosphatase activity back to its producer. Application of this substrate to phytoplankton and its use in flow cytometry was first published by Gonzàlez-Gil et al. [13]. Applications to freshwater phytoplankton were recently published [14–16], but did not involve flow cytometry. In the present work, we have studied phosphatase activity in several strains of cyanobacteria, and tested the applicability of the ELF-method in flow cytometry with samples from a highly eutrophied lake in The Netherlands, Lake Loosdrecht, which is dominated by filamentous cyanobacteria.

2. Materials and methods

2.1. Growth conditions

Axenic cultures of *Synechococcus elongatus* PCC 7942, *Synechocystis* sp. PCC 6803, and *Prochlorothrix hollandica* PCC 9006 were obtained from Pasteur Culture Collection (Paris, France). Cultures of *Planktothrix agardhii* (CCAP), *Aphanizomenon flos-aquae* 1401/7 (CCAP), *A. variabilis* ATCC 29413, and *Limnothrix* sp. isolate MR1 (isolated at The Netherlands Institute for Ecology-Centre for Limnology) were not free of bacterial contaminants. Batch cultures were growing exponentially until the onset of phosphate deficiency. To arrive at these conditions we diluted pre-cultures (1:10) from complete BG-11 medium [17], containing 175 μM

P_i, into BG-11 medium without phosphate. This medium was substituted with 175 μM KNO_3 to replace potassium normally provided as K_2HPO_4. The period of incubation 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 km^2; mean depth 1.85 m; total phosphate 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 0.4 m).

2.2. Alkaline phosphatase assays

The classical assay [18] for AP activity with the substrate *p*-nitrophenyl phosphate (*p*-NPP) was adapted for measurement at 405 nm in a microplate reader (VERSAmax, Molecular Devices, USA) to reduce the interference with the chlorophyll *a* absorption at 430 nm. 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 $MgCl_2$, pH 8.0. The AP activity was related to biomass via expression per optical density unit at 730 nm. The novel alkaline phosphatase substrate 2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (ELF-97[TM], Molecular Probes; [11,12]) was applied to several freshwater cyanobacteria. In contrast to the product protocol, we omitted the ethanol fixation in sample preparation, to prevent bleaching of pigments from the cells. Instead, cells were used fresh in most cases. Fixation of cells with 0.1% (w/v) glutaraldehyde and 0.01% (w/v) formaldehyde (final concentrations) was used to prevent cell lysis where indicated. Samples (vol. 14 ml) were centrifuged for 15 min at a maximum of 2000*g*. The cell pellets were resuspended into 50 μl of the buffer provided by the manufacturer of the endogenous phosphatase detection kit. The content of known components is 30 mM TEA, 3 M NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and a proprietary non-salt component (Molecular Probes®), with an ELFP substrate to buffer dilution of 1:20 (the ELFP substrate concentration is not specified by the manufacturer). The optimal reaction time was empirically determined to be 30 min at room temperature (20 °C); the reaction was stopped by 100 times dilution in flow cytometer sheath fluid (1:100 dilution of Isoton II, Coulter; 7.9 g L^{-1} NaCl, 0.4 g L^{-1} KCl, 1.9 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} NaH_2PO_4, 0.4 g L^{-1} Na_2EDTA and 0.3 g L^{-1} NaF).

2.3. Fluorescence microscopy

Visual inspection of the progress of the ELFP hydrolysis was carried out on a Zeiss standard RA fluorescence microscope with UV epifluorescence furnishing: excitation filter (bandpass 366 ± 25 nm), di-

chromatic mirror (FT 420), and suppression filter (longpass 418 nm). After reaction with the ELFP substrate, samples were centrifuged for 5 min at 1000g and resuspended in 10–25 μl of mounting buffer provided with the endogenous phosphatase 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 Ploemopak 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 ELFA 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.

2.4. Flow cytometry

All samples were filtered over a 70-μm plankton filter before staining, to prevent obstruction of the flow cytometer. Flow cytometric analyses were carried out on an Epics Elite instrument (Coulter, Hialeah, FL), 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), respectively. The trigger was set on chlorophyll fluorescence emission peak at 10 V. 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 filter was filtered through a 488 ± 10 nm bandpass filter and measured with a photomultiplier tube as side scatter. After the 488-nm dichroic longpass filter, 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 photomultiplier tube for measuring the green fluorescence of ELFA 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 detection threshold for the peak signal of chlorophyll 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 (copyright J. Trotter, 1999; http://facs.scripps.edu) was used to create Figs. 2–4, 6 and 7 offline. The identity of particles in the phytoplankton clusters observed in flow cytometry was confirmed by microscopy of the corresponding fractions as collected by fluorescence-activated cell sorting. Settings for flow cytometric cell sorting

were: frequency 21.3–21.4 kHz, drive 65%, deflection amplitude 48–50%, and drop delay 21.0–25.9, three drops sorted with coincidence abort on.

3. Results

3.1. Analysis of ELF-stained cells from laboratory culture and natural resources by microscopy and flow cytometry

Enzymatic dephosphorylation of the ELFP phosphatase substrate yielded highly green fluorescent microcrystals of ELFA at the site of enzymatic activity. The staining gave a cell-bound fluorescent signal, referred to as ELFA fluorescence, which seemed to be associated with the periphery of the cells. Examples of ELFA fluorescence are shown in microscopic images of *A. variabilis* (Figs. 1(a) and (b)), *S. elongatus* PCC 7942 (Figs. 1(c) and (d)), and a typical sample from Lake Loosdrecht (Fig. 1(e)). The high intensity of the ELFA signal permitted double exposure with UV light and clear field to simultaneously visualise ELFA fluorescence and cell morphology. Green fluorescent ELFA micro-crystals appeared in low abundance in cells from batch cultures of *A. variabilis* and *S. elongatus* with excess P_i (Figs. 1(a) and (c)), and were much more abundant in P_i-depleted cells (Figs. 1(b) and (d)). In the lake water sample, filamentous cyanobacteria with various amounts of ELFA fluorescence were seen (Fig. 1(e)). Several eukaryotic species showed ELFA fluorescence among which we visually identified the green algae *Pediastrum* spp. 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 ELFA product, fluorescence emission at 525 nm was detected in single filaments of *A. variabilis* (Figs. 2(a) and (b)), and in single cells of *S. elongatus* (Figs. 2(c) and (d)), but not in *Synechocystis* sp. PCC 6803 (Figs. 2(e) and (f)). Cyanobacterial strains relevant to Lake Loosdrecht were subjected to the same assay. The dominant cyanobacterial strain in the Lake Loosdrecht was *Limnothrix* sp. (previously identified as *Oscillatoria* cf. *limnetica* or *Planktolyngbya limnetica*; the population may include several closely related strains, see [19,20]). The chlorophyll *a*- and *b*-containing, phycobilin-less cyanobacterium *P. hollandica*, which was discovered in this lake [21,22], was the second most abundant species. Relatively rare filamentous cyanobacteria in Lake Loosdrecht were *Limnothrix redekei*, *Planktothrix agardhii*, *Aphanizomenon flos-aquae*, and *Anabaena* spp. Filamentous colonies from batch cultures of *Limnothrix* sp. isolate MR1 (Figs. 3(a) and (b)), *P. hollandica* strain PCC 9006 (Figs. 3(c) and (d)), and *P. agardhii* (Figs. 3(e) and (f)) showed different levels of 525-nm emission intensity. Most cells from batch cul-
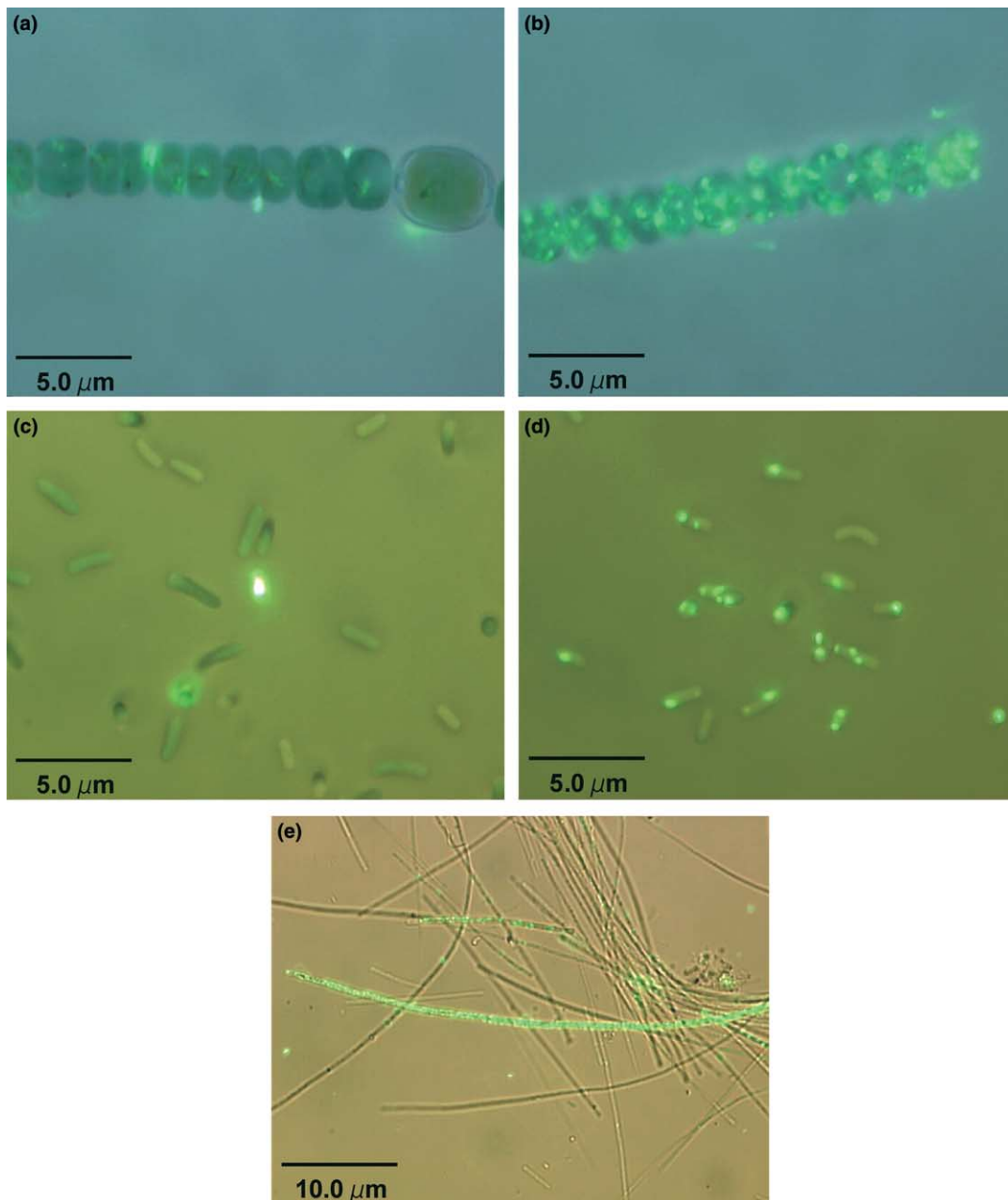
Fig. 1. Fluorescence microscopic images (UV excitation) of cells with ELFA fluorescence. Nutrient replete and P_i-depleted batch cultures of *Anabaena variabilis* (a,b) and *Synechococcus elongatus* PCC 7942 (c,d), and a sample from Lake Loosdrecht (e), were stained with ELFP phosphatase substrate. Flow cytometric analyses of these samples are presented in Figs. 2 and 4.

tures 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.

Synechococcus elongatus and *Limnothrix* sp. showed three distinct peaks with different levels of 525-nm emission intensities (Figs. 2(d) and 3(b), respectively). In some strains, a fraction of the cells had increased levels of green autofluorescence, which was not due to ELF-staining (e.g., Figs. 2(d) and (f) and 3(a), (e) and (f)). Division of ELFA fluorescence into three discrete domains, though at present purely empirical, proved

valuable in our analysis of lake samples. These often also showed a partition into three intensity levels of 525-nm emission (Fig. 4(b)); 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 ELFA fluorescence, after subtraction of the percentage of particles in control treatments without ELFP substrate, is presented in Table 1, along with AP activity measurements with the classical *p*-nitrophenyl phosphate (*p*-NPP) assay. P_i-depleted batch cultures of
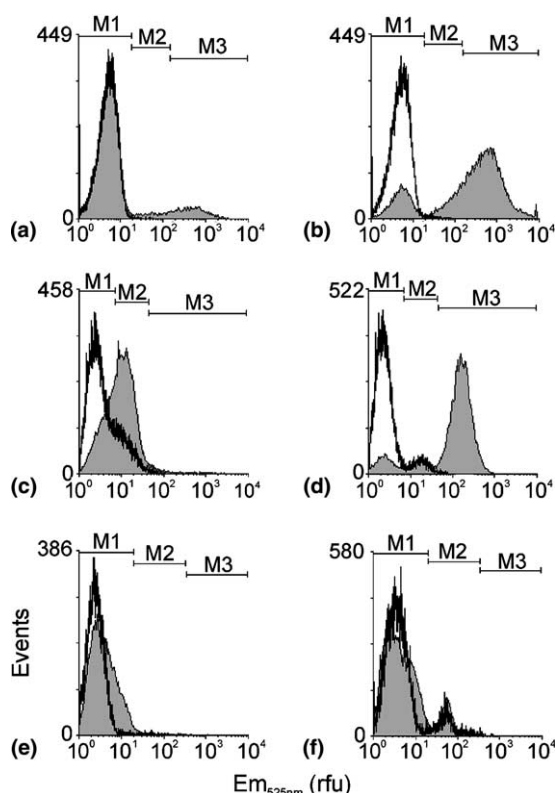
Fig. 2. Cytograms showing the number of events with 525-nm green fluorescence (UV excitation) in batch cultures of model cyanobacteria: *Anabaena variabilis* (a,b), *Synechococcus elongatus* PCC 7942 (c,d), and *Synechocystis* sp. PCC 6803 (e,f). ELF-stained samples have a grey background and control treatments (same treatment as ELF-stained cells, without the ELFP substrate) are shown in overlay. (a,c,e) Nutrient replete cultures and (b,d,f) P_i-depleted cultures. Discrete intensity levels of 525-nm emission are marked: low (M1), intermediate (M2), and high (M3) 525-nm fluorescence intensity, respectively.
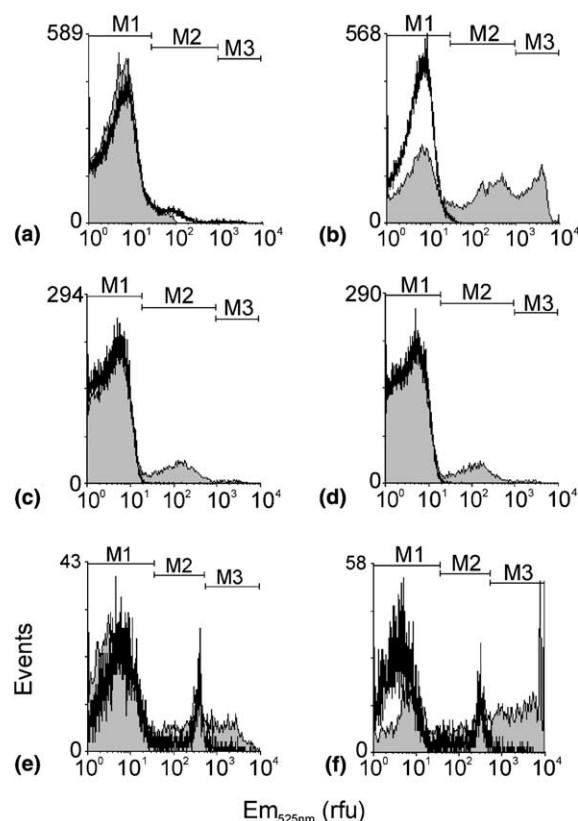

Fig. 3. Cytograms showing the number of events with 525-nm green fluorescence (UV excitation) in batch cultures of filamentous cyanobacteria relevant for Lake Loosdrecht: *Limnothrix* sp. isolate MR1 (a,b), *Prochlorothrix hollandica* PCC 9006 (c,d), and *Planktothrix agardhii* (e,f). ELF-stained samples have a grey background and control treatments are shown in overlay. (a,c,e) Nutrient replete cultures and (b,d,f) P_i-depleted cultures. Discrete intensity levels of 525-nm emission are marked: low (M1), intermediate (M2), and high (M3) 525-nm fluorescence intensity, respectively.

S. elongatus showed high activity in the *p*-NPP assay as well as ELFA fluorescence with ELFP substrate. P_i-depleted batch cultures of *Limnothrix* sp. isolate MR1 showed intermediate activity in the *p*-NPP assay, and both intermediate and high ELFA fluorescence in flow cytometry. A fraction of the filaments from nutrient replete batch cultures of *P. hollandica* PCC 9006 showed intermediate 525-nm fluorescence after incubation with ELFP. This signal did not increase in P_i-depleting conditions. Visual inspection by fluorescence microscopy revealed no visible ELFA signal and the *p*-NPP 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 increased in P_i-depleted cultures. However, such an increase was not observed with the *p*-NPP assay.

The formation of ELFA in batch cultures of *S. elongatus* and *Limnothrix* sp. was susceptible to inhibition by high concentrations of P_i. When cells grown in lack of P_i were incubated with 400 μM of P_i for 5 min before incubation with ELFP, the ELFA signal in the
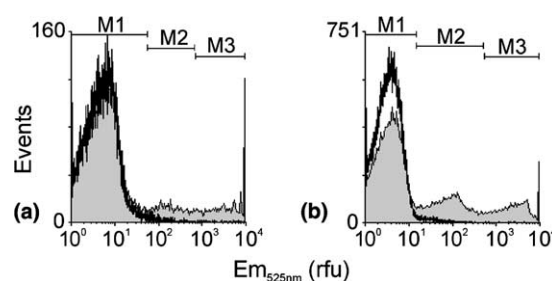

Fig. 4. Cytograms showing the number of events with 525-nm green fluorescence (UV excitation) in samples from Lake Loosdrecht on two dates. ELF-stained samples have a grey background and control treatments are shown in overlay. (a) A sample from 15-10-2001 and (b) a sample from 10-12-2001. Discrete intensity levels of 525-nm emission are marked: low (M1), intermediate (M2), and high (M3) 525-nm fluorescence intensity, respectively.

high 525-nm intensity peak was halved, and after incubation with 2 mM P_i or higher concentrations for 5 min, none of the cells had any high ELFA signal (results not shown).

Table 1
Inventory of phytoplankton cultures with excess phosphate ($+P_i$) and lack of phosphate ($-P_i$), tested for alkaline phosphatase activity with ELF-97 phosphate (ELFA signal) and *p*-nitrophenyl phosphate[a] (*p*-NPP) as substrates

Species	ELFA signal (% particles–% in control)				*p*-NPP (A_{405} min^{-1} A_{730}^{-1})[a]	
	$+P_i$		$-P_i$		$+P_i$	$-P_i$
	Intermediate	High	Intermediate	High		
Limnothrix sp. isolate MR1	3.8	0.6	25.8	16.8	8.8 ± 3.7 (2)	95 ± 7 (15)
Planktothrix agardhii	0.2	6.2	−0.8	23.8	31 ± 35 (5)	23 ± 50 (3)
Prochlorothrix hollandica PCC 9006	10.3	0.6	8.4	0.4	40 ± 42 (7)	38 ± 68 (3)
Synechococcus elongatus PCC 7942	45.1	1.8	−0.7	79.0	61 ± 4 (10)	387 ± 19 (13)

Staining time of 30 min was sufficient to get stable ratios between intermediate and high levels.
[a] For the AP activity with *p*-NPP, 95% confidence intervals are given with degrees of freedom in brackets.

3.2. Optimisation of ELF-staining for lake samples

The ethanol fixation step from the original protocol was omitted, because this treatment extracts the photosynthetic pigments that allow distinction of phytoplankton groups. Without fixation, ELF-staining was possible, but filamentous cyanobacteria were weakened towards mechanical strain after the ELF-staining procedure. After longer storage time (hours) and after washing in sheath fluid, the staining caused trichomes to break, especially during cell-sorting. This effect was probably due to the high concentration of salt in the detection buffer provided with the ELF-97 endogenous AP detection kit (3 M NaCl; Molecular Probes, personal communication). Omission of washing steps and restraint of centrifugal force to a maximum of 1000*g* did not prevent the filaments from breaking. Mild fixation with low concentrations of glutaraldehyde and formaldehyde gave good preservation of cells and their pigments after ELF-staining. However, this fixation gave rise to some background green fluorescence (unpublished results).

To optimise the staining procedure, we incubated samples of Lake Loosdrecht water with ELFP substrate and sampled in a time series. The experiment demonstrated that there was hardly any staining development within the first 5 min (Fig. 5(a)). After 10 min, more than 20% of the cyanobacteria in the sample had an intermediate 525 nm intensity, while about 5% showed 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 min (Fig. 5(a)). 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$_{525 nm}$ per cell) in time showed linear increase of the fluorescence intensity in the intermediate and high fractions (Fig. 5(b)). After 30 min 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
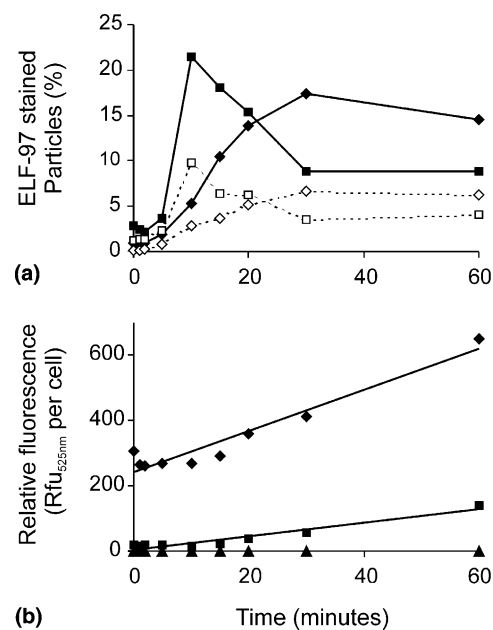

Fig. 5. Time series of ELF-staining of a sample from Lake Loosdrecht. (a) Percentages of cells in the clusters with high and intermediate fluorescence intensity at 525 nm. The total number of particles is shown by open symbols (dashed lines); particles in the cyanobacterial cluster are shown by closed symbols (solid lines). Diamonds represent high 525-nm fluorescence intensity, and squares represent intermediate 525-nm fluorescence intensity. (b) Mean relative fluorescence in different intensity clusters. Triangles, squares, and diamonds represent the clusters with low, intermediate, and high 525-nm fluorescence intensity, respectively. Linear fits are shown in solid lines.

fluorescence was about 900 times higher than that in the group with low 525-nm fluorescence.

3.3. Flow cytometric analysis of ELF-stained natural phytoplankton

Flow cytometric analysis of freshwater samples from Lake Loosdrecht with blue (488 nm) excitation allowed separation of phytoplankton according to differences in photosynthetic pigmentation. In a bivariate plot of chlorophyll *a* (675 nm) vs. phycocyanin (640 nm) fluorescence (Fig. 6(a)), the phytoplankton community separated into clusters of Eukaryotes (diatoms and

green algae), chlorophyll *a* and *b* containing but phy-cobilin-less cyanobacteria (*P. hollandica*), phycobilin-containing cyanobacteria, and detritus (predominantly dead phytoplankton cells). Treatment with ELFP caused the clusters in the 675-nm vs. 640-nm plot to draw towards each other (Fig. 6(b)). The cyanobacterial cluster shifted to lower 640-nm intensity as well as lower 675-nm intensity. The *P. hollandica* 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. 6(c)). In the ELF-stained samples an extra cluster appeared parallel to the cluster of cyanobacteria (Fig. 6(c)).

The three groups of different 525-nm intensities, as indicated by Fig. 4(a), are separately represented in two-dimensional chlorophyll vs. phycocyanin fluorescence plots (Fig. 7). The low 525-nm intensity group contained all clusters, whereas the intermediate 525-nm intensity group was relatively enriched in cyanobacteria, and

contains much less detritus. The high 525-nm intensity group mainly contained algae and cyanobacteria, and was low in *P. hollandica* and detritus. Particles in the anomalous cluster of cyanobacteria had a very strong ELFA signal (Fig. 7(c)) and morphologically resembled *Aphanizomenon flos-aquae*. The percentages of particles with ELFA fluorescence in these phytoplankton groups and the detritus cluster, calculated by summation of the number of particles in the intermediate and high 525-nm intensity groups and subtraction of the numbers in the control treatment, are shown in Table 2.

4. Discussion

4.1. Optimisation of ELF-staining

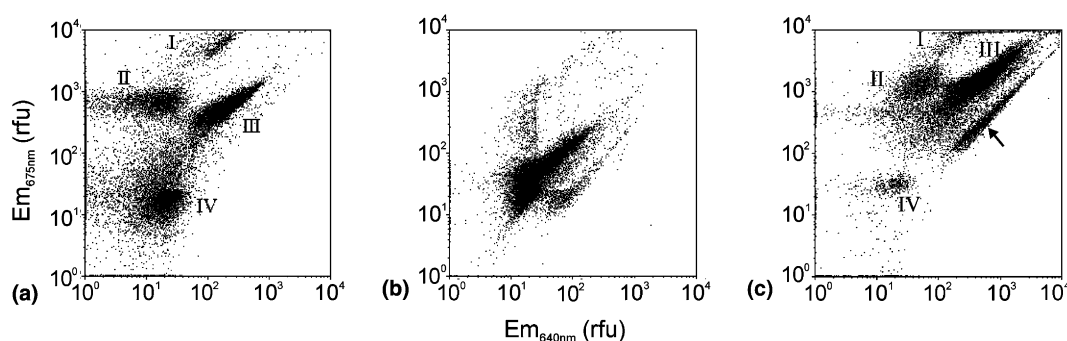The ELFP substrate provides a readily applicable method to determine the P_i-sensing status of freshwater

Fig. 6. Two-dimensional cytograms (dotplots) of red chlorophyll *a* fluorescence (*y*-axis, 675 nm) vs. orange phycocyanin fluorescence (*x*-axis, 640 nm) with blue excitation of 488 nm (samples from 15-10-2001). (a) Sample directly injected into flow cytometer, showing good separation of clusters of algae (I), *P. hollandica* (II), phycocyanin-containing cyanobacteria (predominantly *Limnothrix* sp.; III), and detritus (predominantly dead phyto-plankton cells; IV). (b) After ELF-staining the clusters move towards each other with voltage of photomultiplier tubes set at 1000 V. (c) Voltage of photomultiplier tubes set at 1500 V gives separation again. The arrow indicates an additional cluster of cyanobacteria that appears only after ELF-staining.
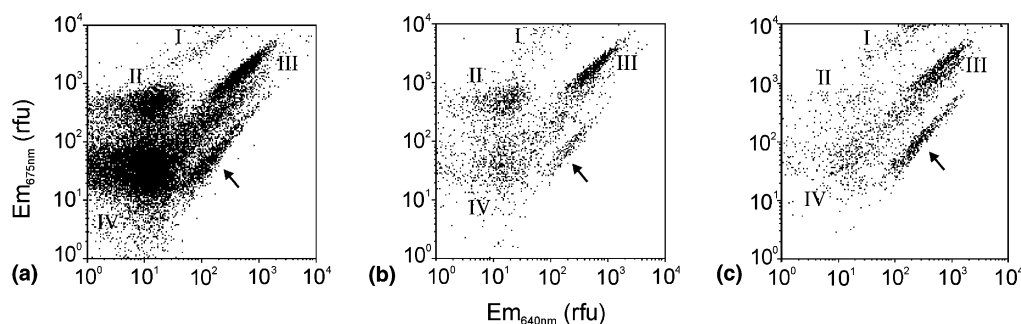

Fig. 7. Cluster analysis in two-dimensional cytograms (dotplots) of red chlorophyll *a* fluorescence (*y*-axis, 675 nm) vs. orange phycocyanin fluo-rescence (*x*-axis, 640 nm) with blue excitation of 488 nm (samples from 15-10-2001). (a) Particles with low green fluorescence (gate M1 in Fig. 4(a)). (b) Particles with intermediate alkaline phosphatase induced fluorescence (gate M2 in Fig. 4(a)). (c) Particles with high alkaline phosphatase-induced fluorescence (gate M3 in Fig. 4(a)). Clusters that contain algae (I), *P. hollandica* (II), *Limnothrix* sp. (III), and detritus (IV), and an additional cluster of cyanobacteria (arrow) are indicated.

Table 2
Percentage of cells with ELFA fluorescence in a sample from Lake Loosdrecht, separated into various phytoplankton clusters

	Percentage from total number of particles	Percentage with ELFA fluorescence
Total	100	12
Detritus	60	4
Phycocyanin-containing cyanobacteria (predominantly *Limnothrix* sp.)	22	27
Phycocyanin-less cyanobacteria (*P. hollandica*)	10	13
Algae (eukaryotes)	1	39

In the first column, the size of the cluster in given as percentage of the total number of particles. The second column gives the percentage of particles with ELFA fluorescence (number of particles with intermediate and high 525-nm fluorescence in ELF-stained samples minus the number of particles in these intensity groups in control samples).

phytoplankton, expanding the use of UV excitation in flow cytometry to study phytoplankton. Flow cytometry gives quantitative measurements in terms of relative fluorescence emission; the measurements have to be taken after accumulation of fluorogenic compounds has been stopped at a specific time point [5]. The product information provided by the manufacturer recommends a staining time of 30 s [23]. However, lag-times in ELF-staining of minutes up to hours were recently reported [16]. In our experiments, staining hardly took place within the first 5 min of incubation. After this initial lag-time, fluorescence intensity increased linearly over time and did not reach a maximum within the experimental period (1 h). The partition of cells with ELFA signal reached a steady level after 30 min, corresponding to the staining time found in earlier work [13,24]. These results indicate that the rate-limiting step for the formation of ELFA was not the enzymatic conversion, but rather a transport process, for example diffusion of the rather large substrate across the outer cell layers. Fixation with ethanol, as recommended in the product protocol, was not possible in our flow cytometric application, as bleaching of the cells due to pigment extraction prevents the simultaneous measurement of autofluorescence to distinguish phytoplankton groups. In contrast to the findings of Rengefors et al. [14], we found that mild fixation with a combination of glutaraldehyde and formaldehyde did not hinder phosphatase activity and development of ELFA particles, although an increased background of green fluorescence was observed. This fixation gave good preservation of cells and their pigments after ELF-staining.

The observation that the increase in fluorescence intensity continued, while the division of cells into distinct intensity groups stabilised, suggests a biological origin of this division. The ELFA signal was therefore expressed in percentage of particles in defined groups or clusters. It is interesting to note that the ELFA signal was not evenly distributed over the cells, but was localised in spots. This punctuated labelling pattern was previously described [13,24] and can be explained by nucleation of micro-crystals of ELFA at the site of en-

zymatic activity. Zinc and magnesium cofactors are required for the ELF-assay, as substitution of the buffer provided with the endogenous phosphatase kit by other buffers did not produce results unless these cofactors were present (results not shown). The ELFA signal was susceptible to inhibition by prior addition of millimolar concentrations of P_i (results not shown). These findings show that the ELFA signal originates from remarkably stable, P_i-inhibitable, zinc- and magnesium-dependent enzymes. These are well-known characteristics of extracellular, alkaline phosphatases [25–27]. Further indications to this point come from the work of Block and Grossman [28], showing that the inducible phosphatase in *Synechococcus* (formerly *A. nidulans*) is homologous to the well-characterised alkaline phosphatase of *Escherichia coli*, and furthermore, that it is located in the periplasm, or loosely associated to the cell wall.

4.2. Flow cytometric phytoplankton separation

Presence of endogenous fluorescent pigments in photoautotrophic organisms allowed flow cytometric distinction (with blue light excitation) of different clusters in the phytoplankton community in Lake Loosdrecht, consisting of Eukaryotes (diatoms and green algae), chlorophyll *a* and *b* containing but phycobilin-less cyanobacteria (*P. hollandica*), and phycocyanin-containing cyanobacteria (predominantly *Limnothrix* sp.). The fluorescent signal varies linearly with the length of filamentous cyanobacteria [29] 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 (Figs. 6 and 7). *P. hollandica*, which contains mainly chlorophyll *a*, little chlorophyll *b*, and no phycocyanin, had less stretched clusters in these plots. The cluster of Eukaryotes represents low numbers of many different species and is therefore amorphic. Interference of the spectral characteristics of ELFA with the autofluorescence of these photosynthetic organisms could be compensated by increasing the voltage of the photomultiplier tubes (Figs. 6(b) and (c)).

4.3. Differences in response to lack of phosphate

ELFA fluorescence depended on P_i-concentration in batch cultures; the tested organisms showed a variable sensitivity towards ELF-staining. Nutrient replete batch cultures of the tested cyanobacteria showed low levels of AP activity, due to a constitutive level of AP expression. The dominant cyanobacterium in Lake Loosdrecht, *Limnothrix* sp., was shown to possess a derepressible AP, whereas in the second most abundant strain, *P. hollandica*, ELFA fluorescence is absent. We believe that ELFA fluorescence reflects species-specific variation in the adaptive strategies towards P_i-deficiency. Furthermore, we found heterogeneous sensitivity towards ELF-staining within the *Limnothrix* population in the lake (Fig. 7). This heterogeneity is probably not a result of the existence of multiple closely related strains, as pure cultures of *Limnothrix* sp. isolate MR1 (Fig. 3(a)), as well as *S. elongatus* (Fig. 2(b)) also showed discrete subsets with discernable fluorescent intensity under P_i-depleted conditions. In a study using flow cytometric detection of endogenous AP in mammalian cells [30], similar subsets with discernable levels of ELFA fluorescence were found in the context of cell cycle analysis. The intra-specific variation of ELFA fluorescence in filamentous cyanobacteria is an interesting physiological phenomenon, which we will address in future work.

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