Phosphate uptake proteins as markers for the nutrient status of freshwater cyanobacteria
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Chapter 6

Localized fluorescent labelling of alkaline phosphatase activity in phytoplankton cells reflects their in situ growth potential

Marco Dignum¹, Hans L. Hoogveld², Virgilio Floris², Herman J. Gons², Hans C. P. Matthijs¹, and Roel Pel²

¹ Aquatic Microbiology/Institute for Biodiversity and Ecosystem Dynamics/University of Amsterdam, Nieuwe Achtergracht 127, NL-1018WS Amsterdam, the Netherlands
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

To determine nutrient availability and growth rates on a population level, improvement of discriminative power in fluorescence-activated cell sorting (FACS) is required. We have combined fluorescence of the endogenous photosynthetic pigments chlorophyll $a$ and phycocyanin with a phosphate deficiency related stain (ELF-97), referred to as alkaline phosphatase (AP) induced fluorescence, to sort phytoplankton from Lake Loosdrecht (The Netherlands). Stable isotope labelling of the phytoplankton with $^{13}$C-enriched CO$_2$ enabled assessment of specific gross growth rates of sorted phototrophic populations by pyrolytic methylation-gas chromatography and in-line compound specific isotope-ratio mass spectrometry (py-GC-IRMS). The dominant population in the lake, *Planktolyngbya limnetica*, was growing under P-limitation in spring, but the P-availability increased in summer, possibly due to increased mineralization. Continuous addition of phosphate to a laboratory scale enclosure of lake water resulted in washout of the cells with AP-induced fluorescence, and increased growth rates. In addition, this study revealed population heterogeneity within the cluster of phycocyanin-containing cyanobacteria. AP-induced fluorescence thus reflects the level of P-deficiency and growth potential of freshwater cyanobacteria and eukaryotic algae, but is modulated by the vitality of subsets in the population.

*Key words:* Alkaline phosphatase induced fluorescence, fluorescence-activated cell sorting, isotope-ratio mass spectrometry, in situ growth rate, phytoplankton
Introduction

Flow cytometry enables detection and sorting of individual phytoplankton cells by autofluorescence of photosynthetic pigments. Flow cytometric analysis of fluorescence emission intensities at 675 and 640 nm originating from 488 nm blue excitation of chlorophylls and phycobilisomes reveals separate clusters of green algae and cyanobacteria (Hofstraat et al. 1991; Vives-Rego et al. 2000; Becker et al. 2002). Molecular genetic techniques and analysis of protein patterns expand the knowledge of diversity and dynamics in communities of filamentous cyanobacteria in lakes (Lya et al. 1997; Zehr and Voytek 1999). Furthermore, natural isotopic variability due to fractionation-inducing biochemical reactions (Brenna 2001) retains information about the physiological state of phototrophic (sub-)populations (e.g. Popp et al. 1998). By linking fluorescence-activated cell sorting (FACS) and isotope-ratio mass spectrometry through in-line pyrolytic methylation, the cells can be probed for their population-specific $\delta^{13}C$ signature. This novel method allows assessment of growth rates of selected species of phytoplankton in situ, if the cells are labelled with $^{13}C$-carbonate (Pel et al., 2002). Population dynamics can thus be studied in detail. Currently, efforts have focused on determining nutrient availability on an individual cell level (Jochem 2000; Beardall et al. 2001), to improve the discriminative power of flow cytometry by including a physiology-related optical attribute (this thesis, chapter 5).

Cyanobacteria present a nuisance when abundant, and their growth potential often depends on the availability of phosphorus (P). Because it has no atmospheric source for replenishment, P is the most likely of the macronutrients to become the growth limiting factor in natural freshwater conditions, and its availability in lakes can change dynamically (Schindler 1977; Hecky and Kilham 1988; Correll 1999). Alkaline Phosphatase (AP) activity increases the spectrum of P-compounds available for transport and assimilation (Siuda and Chróst 1987). AP enzymes are synthesized in many microorganisms only when they lack directly available P, orthophosphate (P$_i$). The presence and activity of these enzymes thus represents a useful aspect of the phosphate sensing-status of the cells. Similarity in AP activity, expressed per unit of ATP in particulate matter, among several natural and cultured phytoplankton species was sufficient to allow definition of AP activity values characteristic of severe, mild and no limitation (Healey and Hendzel 1979). In natural samples AP activity correlated with other signs of P shortage. For example, AP activity reached equilibria in continuous flow cultures of natural phytoplankton communities, showing a hyperbolic relationship between equilibrium AP activity and growth rate (Smith and Kalff 1981). Such an inverse relationship, with rapidly increasing AP activity at low growth rates, is probably due to variation in responsiveness between populations when P is the limiting factor (Siuda and Chróst...
AP activity linked to phytoplankton growth

A similar response curve exists for the relation between AP activity and cellular P in the phytoplankton biomass of Minnesota lakes (Gage and Gorham 1985), between AP activity and $P_i$ concentration in eutrophic lakes in general (Siuda and Chrost 1987), and between enzyme activity and $P_i$ concentration for *Synechococcus* sp. PCC 7942 specifically (Schreiter et al. 2001).

AP activity seems useful as an indicator for the onset of P-deficiency. Bulk AP activity, however, is not an ideal indicator for several reasons (Graziano et al. 1996). Firstly, the enzyme activity follows different patterns in various organisms, in some it is even constitutive or completely absent. Secondly, heterotrophic bacteria are often responsible for a significant fraction of the measured activity. Thirdly, measured rates are relative and do not always co-vary with other indicators of P-deficiency. Usually most of the AP activity is confined to the periphery of cells and only a small fraction is excreted. The use of a novel substrate that yields highly fluorescent precipitates at the site of enzymatic activity (Huang et al. 1993; Haugland 1995), largely meets the objections against bulk AP activity measurements. The enzyme-labelled fluorescence (ELF) phosphatase substrate, member of the ELF-97™ substrate family, is suitable for application to the detection of endogenous AP in complex mixtures of phytoplankton in a flow cytometer (González-Gil et al. 1998; Dyhrman and Palenik 1999; Jochem 2000), and is also applicable to freshwater phytoplankton (Rengefors et al. 2001). In this study we assess the $P_i$ sensing-status of individual cells and colonies in the phytoplankton community of Lake Loosdrecht (the Netherlands) with a flow cytometer and link this information to in situ growth potentials of selected population clusters.

**Experimental procedures**

**Study site**

Lake Loosdrecht, a shallow eutrophic lake in the Netherlands (52°11'N, 5°3'E; area 9.8 km²; mean depth 1.85 m; $P_{\text{total}} = 40-60$ µg.L$^{-1}$; $N_{\text{total}} = 1.4-1.9$ mg.L$^{-1}$), is part of a system of interconnected lakes originating from industrial peat mining (for details, see Hofstra and Van Lier 1992). It is generally completely wind-mixed, and has a very low transparency (Secchi-disc depth almost always <0.5 m; Gons et al. 1992). Since 1932 water from one of the adjacent polders was pumped through the Loosdrecht lakes to supply drinking water for the city of Amsterdam. The water was supplemented from the nearby River Vecht, which was highly polluted with organic waste and nutrients. Due to this prolonged external nutrient loading, the lakes have changed from mesotrophic clear water dominated by submerged plants, to highly eutrophic water with low transparency dominated by filamentous cyanobacteria. In 1984 remedial measures were taken to reduce the external P loading rate. Most importantly, the inlet of water was replaced by chemically
“dephosphoryzed” water from the Amsterdam-Rhine Canal (Hofstra and Van Lier 1992). In this study, water samples were collected at weekly intervals (April-December 2001) from a single sampling point off the West-End harbour jetty in Lake Loosdrecht. In Lake Loosdrecht various filamentous cyanobacteria are present. The currently most abundant strain was formerly called Oscillatoria cf. limnetica, but later proved to belong the Pseudanabaena group. 16S-rRNA and DGGE analyses have revealed multiple closely related strains belonging to this group in Lake Loosdrecht (Zwart, personal communication), morphologically identical with Planktolyngbya limnetica (Komárek and ?aslavska, 1991; Komárková-Legnerová and Cronberg 1992). The second most abundant population is Prochlorothrix hollandica (Burger-Wiersma et al. 1986). This organism has no phycobilins, and has little chlorophyll b (Matthijs et al. 1994).

**Culture conditions**

Laboratory scale enclosures (Rijkeboer et al. 1990) filled with water from Lake Loosdrecht (130 L) had a temperature of 16 °C, and a light regime of 230 μmol photons.m⁻².sec⁻¹ photosynthetically available radiation (PAR) just below the surface with a light:dark cycle of 16:8 h. The cultures were continuously mixed and aerated. A continuous flow of mineral medium was added at a rate of 240 ml.h⁻¹. A solution with 1.7 mM P (K₂HPO₄) was added at a rate of 10 ml.h⁻¹. In addition, a solution containing 1.11mg.l⁻¹ FeCl₂·4H₂O and 1.33 mg.l⁻¹ Na₂EDTA was added at a rate of 10 ml.h⁻¹. The total medium dilution rate was 0.05 d⁻¹. Subcultures from the LSE or lake water samples were incubated in ¹³C-labeling-enclosures (1.4 l; optical depth 0.1 m) under the following conditions: temperature 16 °C, pH 8, light regime 47 μmol photons.m⁻².sec⁻¹ (PAR); light:dark cycle 16:8 h (halogen 7 IPR lamp, Mazda 1500 W), and no fresh medium supply. Dissolved inorganic carbon (DIC) in the subcultures was supplemented with 6 mg ¹³C-NaHCO₃ (99 atom% ¹³C) to an enrichment grade of 1500‰. In situ population-specific growth rates of phototrophs were estimated from the rate of ¹³C-CO₂ incorporation in mono- or polyunsaturated fatty acids over 24 hours according to Pel et al.2002a (see also below).

**Enzyme labelled fluorescence (ELF)**
The alkaline phosphatase substrate 2-(5'-chloro-2'- phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (ELF-97™) was part of an endogenous phosphatase detection kit (Molecular Probes USA). Cells were centrifuged for 15 minutes at a maximum of 1000x g and resuspended into the buffer provided with the kit. To prevent cell lysis during cell sorting, washing steps were omitted. The samples were kept on ice instead and diluted straight into 1% isoton for insertion into the flow cytometer. Visual inspection of the ELF-97 signal 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).
Flow Cytometry

Samples were filtered over a 70 μm plankton filter to prevent obstruction of the flow cytometer. Part of the samples were lightly fixed (final concentrations 0.01% w/v para-formaldehyde and 0.1% w/v glutaraldehyde) to give better preservation of filaments during and after their handling in ELF-staining and subsequent cell sorting, while fully retaining autofluorescence. Flow cytometric analyses and FACS were done on an Epics Elite instrument (Coulter, Hialeah, Florida, USA), equipped with a gated amplifier facility for measuring with two lasers successively. Two 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). To get a good separation of clusters, the photo-multiplier tubes were set to PMT640 = 1450 V and PMT675 = 1300 V. The 525 nm UV pre-amplification was set to 1.0, and the threshold for CHLpkl0 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. Histograms (Fig. 4) were created with WinMDI version 2.8 (J. Trotter, 1999). Settings for sorting were: frequency 21.3-21.4 KHz, drive 65%, deflection amplitude 48-50%, drop delay 21.0-25.9, 3 drops sorted, coincidence abort on. After flow cytometric sorting, the identity of the phytoplankton fractions was confirmed microscopically.

Isotope-ratio mass spectrometry and population-specific 13C-labeling

Phototrophs collected by flow sorting were applied to py-GC-IRMS analysis in the following way (Pel et al. 2002a+b). Cells were pelleted by centrifugation (12 minutes 12000 rpm). Using a μl-syringe, total amounts of 8x10^4 to 3x10^5 cells/filaments were deposited onto ferromagnetic wires (Curie-point 480 °C) together with trimethylphenylammonium hydroxide in methanol as derivatising agent. Pyrolysis was performed using a micro-volume Curie-point reactor (FOM-4LX) developed by FOM Amolf (Amsterdam, The Netherlands). Loaded wires were allowed to dry at room temperature under reduced pressure and continuous rotation. A quantitative release of cellular fatty acids by in situ methylation was achieved by a 3-s pyrolysis time (Dworzanski et al. 1990). Volatilised methylated fatty acid con-
stituents were swept splitless into a capillary gas chromatograph coupled to a Finnigan Delta-S isotope ratio monitoring mass spectrometer via a Finnigan type II combustion interface (py-GC-IRMS). Carbon isotopic composition is reported in δ notation: $\delta^{13}C$ in parts per thousand ($\%$) = \left[ \frac{^{13}C/^{12}C_{\text{sample}}}{^{13}C/^{12}C_{\text{reference}}} - 1 \right] \times 10^3 \text{ (Pel et al., 1997)}. Diel-averaged growth rates (d$^{-1}$) were calculated from the rate of $^{13}$C-CO$_2$ incorporation into population-specific fatty acids according to Pel et al. 2002$^{a+b}$: poly-unsaturated C$_{18}$ were used for cyanobacteria and green algae, C$_{14:1}$ and C$_{16:1}$ for P. hollandica and diatoms, respectively.

Results

Laboratory Scale Enclosure (LSE)
A large volume of lake water (130L) was incubated under light conditions that resemble the natural springtime irradiance. The temperature was 16°C, which was high compared to the in situ lake temperature at the time of sampling (± 6°C). At saturating P-loading rate (96.8 µg P l$^{-1}$ day$^{-1}$) the P$_i$ input exceeded the capacity of the particles for P$_i$-uptake after 3 days (Rijkeboer et al. 1990). These conditions allowed the phytoplankton populations to increase in density during about 14 days (Fig. 1A and Fig. 2A). A marked decrease in length of filamentous cyanobacteria was observed during the first three days, resulting in a decreasing biomass volume (Fig. 1A). Subcultures (1.4L) were incubated with $^{13}$C-bicarbonate at the beginning of the experiment (Day 0), in the growth phase (Day 7), and in the declining phase (Day 14). Incorporation rates of $^{13}$C-labeled CO$_2$ into membrane fatty acids correlate to the growth rates of the cells. Phytoplankton clusters were sorted by flow cytometry, according to their content of fluorescent pigments. In bivariate histograms (e.g. Fig. 4A and B) plotting chlorophyll a (Chl-a) fluorescence (Em. 675 nm; Ex. 488 nm) against phycocyanin fluorescence (Em. 640 nm; Ex. 488 nm). The cyanobacteria can be separated into a phycocyanin-containing cluster (predominantly P. limnetica) and a phycocyanin-less cluster (predominantly P. hollandica). Although the type of flow cytometer used in this study cannot distinguish diatoms and green algae, differences in the fatty acid profiles of these two groups (Pel et al. 2002$^{a+b}$) allowed separate estimation of their growth rates (Table 1). In the field sample (Day 0) the growth rates of all phytoplankton groups were about equal to the dilution rate of the LSE. In the growth phase (Day 7) the growth rates were much higher. Hereafter (Day 14) the growth rate was markedly lower for all groups, except the phycocyanin-containing cyanobacteria. Cyanobacteria were dominant at the start of the experiment, and during the experiment they increased more in biomass than the other groups. Samples taken from the LSE at Day 0, 3, 10, and 16 were stained with the ELF-97 AP substrate.
Figure 1. Phycocyanin-containing cyanobacteria in a laboratory scale enclosure (LSE) of Lake Loosdrecht water. A: Microscopic counts (closed squares with 95% confidence intervals), flow cytometric counts (closed diamonds with solid line), and biomass volume (open triangles with broken line). B: Flow cytometric determination of the number of filaments with AP-induced fluorescence in ELF-stained samples from the LSE. The total number of phycocyanin-containing cyanobacteria is presented in closed circles (solid line). ELF-stained samples (open circles) are separated into particles with low green fluorescence emission (broken line) and particles with AP-induced fluorescence (solid line).
Figure 2. Eukaryotic algae in a laboratory scale enclosure (LSE) of Lake Loosdrecht water. A: Microscopic counts of diatoms (closed diamonds with 95% confidence intervals), and green algae (open diamonds with 95% confidence intervals). B: Flow cytometric determination of the number of eukaryotic algae (diatoms and green algae together) with AP-induced fluorescence in ELF-stained samples from the LSE. The total number of cells is presented in closed squares (solid line). ELF-stained samples (open squares) are separated into cells with low green fluorescence emission (broken line) and particles with AP-induced fluorescence (solid line).
The number of cyanobacteria that showed intense green fluorescence (525 nm) after incubation with the ELF-97 phosphatase substrate and with UV excitation, compared to control samples (referred to as AP-induced fluorescence), during the LSE experiment is shown in Fig. 1B. About 45% of the cyanobacteria had AP-induced fluorescence on Day 0, and about 43% on Day 3. The number of cells with AP-induced fluorescence gradually decreased to about 15% on Day 10, and about 5.5% on Day 16. The decrease of the number of cells with AP-induced fluorescence is about 0.06 day\(^{-1}\), which is close to the dilution rate (0.05 day\(^{-1}\)). At the same time, the number of cells with low 525 nm fluorescence increased rapidly to a maximum between Day 3 and 13. The growth rate was about 0.09 day\(^{-1}\), calculated from the FCM growth curve (Fig 1A and B), and 0.19 calculated from the microscopic counts (Fig. 1A). These values were calculated from the increase between day 3 and day 13, and not corrected for the dilution rate. *P. hollandica* did not have any AP-induced fluorescence (results not shown). Microscopic counts of eukaryotic algae (Fig. 2A) showed that the growth of green algae slowed down at an early stage (between Day 9 and 13). Growth rates of diatoms and green algae were very high on Day 7, but had decreased on Day 14. Of the total (diatoms and green algae), about 40% had AP-induced fluorescence on Day 0, increasing to 58% on Day 3, and then decreasing to 11% on Day 10 and 14% on Day 16 (Fig. 2B). The total number of eukaryotic algae reached a maximum around Day 10, and then declined (Fig. 2B).

### Table 1. Growth rates (d\(^{-1}\)) of phytoplankton growth rates of phytoplankton populations in a laboratory scale enclosure separated by flow cytometry, as estimated from \(^{13}\)C-CO\(_2\) incorporation in cellular fatty acids.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanobacteria</td>
<td>0.02</td>
<td>0.36</td>
<td>0.40</td>
</tr>
<tr>
<td><em>P. hollandica</em></td>
<td>0.01</td>
<td>0.24</td>
<td>0.17</td>
</tr>
<tr>
<td>Diatoms</td>
<td>0.08</td>
<td>0.85</td>
<td>0.30</td>
</tr>
<tr>
<td>Green algae</td>
<td>0.07</td>
<td>0.51</td>
<td>0.15</td>
</tr>
</tbody>
</table>

**Seasonal variation of AP-induced fluorescence in Lake Loosdrecht**

In the period March to December 2001 measurements of AP linked fluorescence were done with water samples from Lake Loosdrecht. Fig. 3A shows the seasonal population densities of phycocyanin-containing cyanobacteria (predominantly *P. limnetica*), phycocyanin-less cyanobacteria (predominantly *P. hollandica*), and eukaryotic algae (both diatoms and green algae), as determined by flow cytometry. In histograms plotting green fluorescence (UV excitation, emission at 525 nm; e.g. Fig. 4C and D), two distinct subsets with different emission intensities were observed after ELF-staining (Fig. 4D), as compared to non-treated control samples (Fig. 4C).
On average, the mean intensity of particles in subset III with intermediate 525 nm emission intensity was about 45 times higher than that in subset I with lowest 525 nm emission intensity. The average intensity of particles in subset IV with high 525 nm fluorescence was about 600 times higher than that in subset I. Between 4 to 25% of all particles, and 3 to 30% of phycocyanin-containing cyanobacteria had a high intensity of 525 nm fluorescence throughout the year.

Figure 3. Seasonal variation of AP-induced fluorescence in phytoplankton from Lake Loosdrecht in 2001. A: growth curves of phycocyanin-containing cyanobacteria, comprising predominantly *Planktolyngbya limnetica* (circles), phycocyanin-less cyanobacteria, comprising predominantly *Prochlorothrix hollandica* (triangles), and eukaryotic algae, comprising both diatoms and green algae (squares). B, C, and D: Total number (closed symbols) and number with AP-induced fluorescence (open symbols) of phycocyanin-containing cyanobacteria (B), phycocyanin-less cyanobacteria (C), and eukaryotic algae (D). E: Number of particles in an anomalous cluster of diazotrophic filamentous cyanobacteria (probably *Aphanizomenon* spp.) with very high AP-induced fluorescence emission intensity.
The number of events in subsets III and IV were added up, and minus the number of events in these intensity regions in control experiments, this represents the number of particles with AP-induced fluorescence. The fraction of cyanobacteria that showed AP linked fluorescence was higher in spring and early summer, but decreased in late summer. The number of cyanobacteria with AP-induced fluorescence remained stable in autumn, while the number of cyanobacteria without AP-induced fluorescence decreased (Fig. 3B). A very low fraction of Prochlorothrix hollandica showed increased 525 nm fluorescence (Fig. 3C). Eukaryotic algae showed a variable fraction of cells with AP linked fluorescence (Fig. 3D). After ELF-staining an anomalous cluster of cyanobacteria appeared next to the normal cluster (marked with an arrow in Fig. 4B). Filaments in this cluster were morphologically identified as Aphanizomenon spp., comprised up to 5% of the total fraction of phycocyanin-containing cyanobacteria, and showed extremely abundant AP-induced fluorescence. The cell-numbers in this cluster fluctuated strongly in summer (Fig. 3E).

In situ specific growth rates of subsets of cyanobacteria

A laboratory enclosure (1.4L) of lake water was incubated with $^{13}$C-NaHCO$_3^-$ (99 atom% $^{13}$C) to an enrichment grade of 1500%. In situ specific growth rates were estimated from the uptake of dissolved inorganic carbon and the rate of incorporation in C$_{18}$-polyunsaturated fatty acids over 24 hours. The following fractions were collected by FACS: the total cyanobacterial cluster consisting mainly of P. limnetica filaments (64% of all particles in the samples, and 83% of the live phytoplankton; Fig. 4B), a subset of cyanobacteria with low 525 nm fluorescence (Fig. 4D; I), a subset with increased 525 nm autofluorescence (Fig. 4D; II), a subset with intermediate AP-induced fluorescence (Fig. 4D; III), and a subset with high AP-induced fluorescence (Fig. 4D; IV). Several parameters were recorded for all of these subsets (Table 2). To compare the P$_i$-sensing status (AP-induced fluorescence) and vitality of the cells (Chl-$a$ fluorescence), we inspected bivariate plots of Chl-$a$ fluorescence (Ex. 488 nm, Em.675 nm) vs. AP-induced fluorescence (Ex.353 nm, Em.525 nm). Subset I with low 525 nm fluorescence had low 675 nm fluorescence (Table 2). The filaments in this subset had a much lower mean filament volume, and the estimated growth rate was lower than that of the other subsets. Subset II (emission intensity 15 times that of subset I) was most abundant. These filaments also had lower than median 675 nm fluorescence, were of medium volume, and their growth rate was slightly lower than the median for these subsets. The fraction of cyanobacteria with AP-induced fluorescence was 34.8%. Subset III (emission intensity 150 times that of subset I) had higher Chl-$a$ fluorescence than the median, slightly larger filament volume, and a median growth rate. Subset IV (emission intensity 700 times that of subset I) had the highest Chl-$a$ fluorescence,
average filament volume, and a slightly but not significantly higher growth rate than average.

Figure 4. Histograms of unstained control samples (A, C) and ELF-stained samples (B, D) from Lake Loosdrecht (23-04-2002). A and B: Bivariate plots of chlorophyll $a$ fluorescence (Em. 675 nm; Ex. 488 nm) and phycocyanin fluorescence (Em. 640 nm; Ex. 488 nm) showing clusters of eukaryotic algae (Fig. 4A: a), phycocyanin-less cyanobacteria (Fig. 4A: b), phycocyanin-containing cyanobacteria (Fig. 4A: c), and detritus (Fig. 4A: d). The total cluster of cyanobacteria that was sorted and described in Table 2 is shown in Fig. 4B. Note that an anomalous cluster of cyanobacteria appears next to the normal cluster, indicated by the arrow. C and D: Histograms showing AP-induced fluorescence (Em. 525 nm; UV-excitation). Subsets of cyanobacteria with different 525 nm emission intensities are depicted in Fig 4D: low 525 nm fluorescence (I), increased 525 nm autofluorescence (II), intermediate AP-induced fluorescence (III), and high AP-induced fluorescence (IV).
Table 2. Characteristics of cyanobacterial subsets in a $^{13}$C-labeled laboratory enclosure of water from Lake Loosdrecht (22/04/2002). Subset I: cyanobacteria with low green fluorescence, subset II: cyanobacteria with fixation induced green autofluorescence, subset III: cyanobacteria with intermediate AP-induced fluorescence, and subset IV: cyanobacteria with high AP-induced fluorescence. The last column represents the complete phycocyanin-containing cyanobacterial cluster.

<table>
<thead>
<tr>
<th>FCM variable</th>
<th>Subset</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rfu 525 nm</td>
<td></td>
<td>0.83 (0.03)</td>
<td>12.3 (1.2)</td>
<td>126 (1.3)</td>
<td>569 (2.5)</td>
<td>99.3 (3.8)</td>
</tr>
<tr>
<td>Rfu 675 nm</td>
<td></td>
<td>101 (1.6)</td>
<td>149 (1.7)</td>
<td>197 (0.3)</td>
<td>226 (3.3)</td>
<td>160 (1.1)</td>
</tr>
<tr>
<td>% per subset</td>
<td></td>
<td>8.8 (0.9)</td>
<td>48.7 (0.9)</td>
<td>20.7 (0.3)</td>
<td>18.3 (1.8)</td>
<td>100</td>
</tr>
<tr>
<td>% in control samples</td>
<td></td>
<td>22.3 (1.7)</td>
<td>70.6 (1.1)</td>
<td>3.5 (0.6)</td>
<td>0.7 (0.1)</td>
<td>100</td>
</tr>
<tr>
<td>Filament volume (µm$^3$)</td>
<td></td>
<td>127 (13)</td>
<td>285 (21)</td>
<td>314 (29)</td>
<td>278 (21)</td>
<td>268 (49)</td>
</tr>
<tr>
<td>Growth rate (d$^{-1}$)$^b$</td>
<td></td>
<td>0.053 (0.016)</td>
<td>0.072 (0.016)</td>
<td>0.086 (0.012)</td>
<td>0.093 (0.002)</td>
<td>0.089 (0.007)</td>
</tr>
</tbody>
</table>

$^a$Mean values from duplicate experiments are given with standard deviations in brackets.

$^b$Diel-averaged growth rates estimated from $^{13}$C-CO$_2$ incorporation in (poly)unsaturated C$_{18}$ fatty acids of ELF-stained cyanobacterial subpopulations retrieved by flow cytometric cell sorting.
Discussion

In this contribution we have combined three novel developments in aquatic microbial ecology to study population specific growth of freshwater phytoplankton, fully employing the gating operability of the flow cytometer. Firstly, fine-tuning of the droplet delay settings of the flow cytometer allowed FACS of filamentous cyanobacteria, despite the fact that trichome lengths by far exceeded the 60-70 μm diameter of the sort droplets (Pel et al. 2002a). Secondly, the ELF-97 AP substrate provided a readily applicable method to determine the P sensing status of freshwater phytoplankton, resulting in AP-induced fluorescence that added a physiology-related parameter for UV excitation in FACS (this thesis, chapter 5). Thirdly, labelling of the phytoplankton with the stable isotope $^{13}$C enabled assessment of specific gross growth rates of sorted phototrophic populations by a combination of FACS and pyrolysis-GC-IRMS (Pel et al. 2002a).

A marked increase in spring phytoplankton biomass from Lake Loosdrecht in a laboratory scale enclosure with continuously added phosphate strongly suggested that P$_i$ was limiting the growth in the lake at that time of year. About half of the phycocyanin-containing cyanobacteria in the lake showed AP-induced fluorescence. The filaments with AP-induced fluorescence disappeared at a rate that was similar to the dilution rate of the LSE. AP is stable for days if not weeks (Jansson et al. 1988); therefore it is likely that no new AP was produced after three days in the LSE with P$_i$ added. Presumably, cells that contain the enzyme were washed out, and newly formed cells did not show any AP-induced fluorescence, because their growth was not limited by P-availability. The numbers of cells/filaments in the LSE did not increase at the rate estimated from $^{13}$C-incorporation. The dominant cyanobacterial strain, *P. limnetica*, even showed a high estimated growth rate on day 14, when the increase of filament numbers and biomass volume had already subsided. This implies significant losses, possibly by viral lysis (Gons et al. 2002). In contrast, the growth rates of the other phytoplankton populations had declined on day 14, probably due to shading by the increased *P. limnetica* population, and in the case of diatoms by silicon-deficiency.

The *P. limnetica* population in Lake Loosdrecht grew to a maximum of about $2 \times 10^5$ filaments.ml$^{-1}$ in the summer of 2001. In spring the number of filaments with AP-induced fluorescence kept pace with the overall biomass increase, indicating P-limited growth. When the population size stabilized in summer, the number of filaments with AP-induced fluorescence decreased, indicating that the P-limitation was relaxing. This may have been due to an increased mineralization rate in the sediment, following the higher summer temperatures. ELF staining allowed
flow cytometric enumeration of an additional population of cyanobacteria, morphologically identified as *Aphanizomenon flos-aqua*, which has a low and fluctuating abundance. The *P. hollandica* population grew to a maximum of about 7.10⁴ filaments.ml⁻¹. This strain has a low background of green fluorescence, not due to derepressible AP (this thesis, chapter 5). The eukaryotic algae in the lake reached a maximum about 3.10⁴ cells.ml⁻¹. The proportion of cells with AP-induced fluorescence did not show much seasonal variation. A further indication that AP may be very stable, and function in the degradation and reuse of cellular detritus came from microscopic observations of the green algae *Scenedesmus* sp. and the diatom *Monoraphidium* sp. showing AP-induced fluorescence in ‘empty’ cell walls (results not shown). The detritus fraction showed AP-induced fluorescence (not shown), of which the origin was not established, but must be either heterotrophic bacteria or derived from phytoplankton. Part of the samples were fixed with low concentrations of glutaraldehyde and formaldehyde to avert breakage of the filaments during cell sorting (this thesis, chapter 5). In some of the fixed samples increased levels of green autofluorescence were associated with the filaments, which was absent in samples after control treatment. A potential source of green background fluorescence might have been flavonoids released from lysed cells; fixation of cells could link released compounds from broken cells to the outside of intact cells, explaining why the increased background of green fluorescence in fixed samples could not be washed away. Fluorescence microscopic inspection revealed that another source of background fluorescence came from amorphous light-green and blue-green fluorescent particles (size about 10 μm). Presumably this originated from fulvic acids adsorbed to particulate organic matter, e.g. peat, which emit light in the 525 nm region after UV excitation, and are especially present in degraded peat land (Kalbitz et al. 2000).

ELF staining revealed heterogeneity within the *P. limnetica* cluster; AP-induced fluorescence divided the cluster in discrete subsets with different emission intensities. This could not be explained by the presence of other cyanobacteria in this cluster, although the *P. limnetica* group itself may include several closely related species with trichomes of highly variable length and width (see Komárková-Legnerová and Cronberg 1992). Differences in the thickness of the cells’ outer layers and subsequent longer diffusion time may have caused uneven staining among cells. Another explanation, however, is that population heterogeneity arises from the cells being in different growth phases. In this view, some of the cells are actively growing while others are in a resting state. The most vital cells may express the most urgent need for nutrients, and show the first signs of limitation. Other, less vital cells may express less AP. A subset of extremely limited cells may not be vital enough to respond to their environment with the synthesis of new
enzymes. The results from the $^{13}$C-labeling experiment support this view; both Chl-$a$ fluorescence and estimated growth rates increased with increasing AP-induced fluorescence. An interesting parallel was found in a recent study using flow cytometric detection of endogenous AP in mammalian cells (Telford et al. 2001). Similar subsets with varying levels of ELF-97 signal were detected, and explained in the context of cell cycle-associated protein expression. In an immuno-fluorescent method to detect cell-surface exposed epitopes of protein F on *Pseudomonas aeruginosa* by flow cytometry, the percentage of positively stained cells was also lower than expected (Hughes et al. 1996). The explanation, offered also by these authors, was that the cell-surface marker protein was being surface accessible to various degrees during the cell cycle. Support for this view comes from a segregated $P_i$-starvation response model for *Escherichia coli* (Van Dien and Keasling 1999), which accounts for culture heterogeneity, and states that only a fraction of the cells may have their AP derepressed at a particular time. Furthermore, Parpiais et al. (1996) have reported a remarkable, irreversible arrest of the marine cyanobacterium *Prochlorococcus* spp. in the DNA replication (S) phase, when subjected to P-starvation. Moreover, these authors suggested that examination of the cell cycle of natural populations could be used to determine whether they are limited by P (Parpiais et al. 1996).

Attempts to restore the trophic status of a lake by reducing the external P loading rate do not always succeed, primarily because a decrease in P concentrations is prevented by internal supplies of P (Marsden 1989). Lake Loosdrecht is a typical example of a lake for which restorative measures (implemented in 1984) have not yet resulted in a return of the preferred clear water situation with submerged macrophytes. The response of a lake community may show considerable resilience to recovery, resulting in a discontinuous response of filamentous cyanobacteria to changes in the total P concentration (Mur et al. 1989). In Dutch shallow lakes the filamentous cyanobacteria are either a minor component of the phytoplankton community, or they are strongly dominant (Scheffer et al. 1997). High abundance of these cyanobacteria occurs predominantly under shady conditions, whereas no correlation was found with total P concentration (Jensen et al. 1994; Scheffer et al. 1997). Thus, the low depth averaged light rather than high nutrient availability in eutrophic lakes leads to dominance. These low light conditions have been promoted by increased (eukaryotic) algal growth and frequent resuspension of the sediment, and the cyanobacteria also promote shady conditions themselves. Normally, vertical light attenuation increases with the P level. When cyanobacteria dominate, the attenuation will be higher at the same nutrient concentration (Mur et al. 1989). When, starting from a low total P level in a lake not dominated by filamentous cyanobacteria, the nutrient loading is slowly increased, light attenuation will grad-
ually increase too. Above a critical total P value, a sudden increase in turbidity occurs, because cyanobacteria become dominant. If the total P concentration is reduced again, the cyanobacteria will stay dominant until a much lower critical nutrient is reached, and the system returns to a less turbid state dominated by other algae. It can be inferred from this pattern that filamentous cyanobacteria are much more persistent in lakes that have a high background turbidity (Scheffer et al. 1997), as is the case for Lake Loosdrecht. Rapid recycling of P in the lake, aided by the synthesis of AP to convert organic P into P_i; and adsorption of P_i; to detritus (Rijkeboer et al. 1991; Gons et al. 1991), may explain part of the resilience of cyanobacteria to restoration by reduction of the P load. The ELF-97 method, combined with FACS and growth rate estimations from py-GC-IRMS offers insight in the population dynamics in relation to the nutrient status of freshwater phytoplankton, by tracing the AP activity back to the individuals that produced it.

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