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Flow cytometric detection of phosphatase activity combined with ^{13}C-CO_2 tracer-based growth rate assessment in phytoplankton populations from a shallow lake

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ABSTRACT: To determine nutrient availability and growth rates of phytoplankton on a population level, improvement of discriminative power in fluorescence-activated cell sorting is required. We have combined fluorescence of the endogenous photosynthetic pigments chlorophyll *a* and phycocyanin with a phosphate deficiency (P-deficiency) related stain (enzyme-labelled fluorescence, ELF-97) that yields green fluorescent precipitates at the site of phosphatase activity, referred to as ELF alcohol (ELFA) fluorescence, to sort phytoplankton from Lake Loosdrecht (The Netherlands). Stable isotope labelling with ^{13}C-enriched CO_2 enabled assessment of specific growth rates of sorted populations by pyrolytic methylation-gas chromatography and in-line compound specific isotope-ratio mass spectrometry. The dominant population in the lake, the filamentous cyanobacterium *Limnothrix* sp., was growing under P-deficiency in spring, but the availability of phosphate increased in summer. Continuous flow of phosphate-rich medium into a laboratory-scale enclosure of lake water resulted in washout of the cells with ELFA fluorescence, and increased growth rates. In addition, this study revealed population heterogeneity within the cluster of phycocyanin-containing cyanobacteria. ELFA fluorescence thus reflects the level of P-deficiency of freshwater cyanobacteria and microalgae, but is modulated by metabolic activity of cells within the population.

KEY WORDS: Alkaline phosphatase · Enzyme-labelled fluorescence · Filamentous cyanobacteria · Fluorescence-activated cell sorting · Population-specific growth rate · Phytoplankton · Stable isotope labelling

INTRODUCTION

Phototrophic microorganisms (phytoplankton), especially cyanobacteria, present a nuisance when abundant in lakes. Many shallow, eutrophic lakes are dominated by filamentous cyanobacteria. Population dynamics, physiological status, and growth rates of these aquatic microorganisms are therefore important indicators for water quality. Molecular genetic techniques and analysis of protein patterns expand the knowledge of diversity and dynamics in phytoplankton communities in lakes (e.g. Lyra et al. 1997, Zehr & Voytek 1999), but do not provide information on a cellular level. Flow cytometry (FCM) enables detection and sorting of individual cells by light scattering characteristics and autofluorescence of photosynthetic pigments.

FCM analysis of fluorescence emission intensities at 675 and 640 nm, originating from 488 nm blue excitation of chlorophylls and phycobilisomes respectively, reveals separate clusters of green algae and cyanobacteria (Hofstraat et al. 1991, Vives-Rego et al. 2000,

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Becker et al. 2002). The addition of fluorescent markers to the cells adds new parameters for FCM analysis, allowing determination of the physiological state and nutrient availability on an individual cell level (Jochem 2000, Beardall et al. 2001).

Natural isotopic variability due to fractionation-inducing biochemical reactions retains information about the physiological state of phytoplankton (sub)-populations (e.g. Popp et al. 1998, Pel et al. 2003). By linking fluorescence-activated cell sorting (FACS) and isotope-ratio mass spectrometry through in-line pyrolytic methylation (pyrolytic gas-chromatography-isotope ratio mass spectrometry, Py-GC-IRMS), the cells can be probed for their population-specific $\delta^{13}C$ signature (Pel et al. 2004a). This novel method allows assessment of population-specific growth rates from phytoplankton cells that are labelled with ^{13}C-CO_2 (Pel et al. 2003, 2004b).

Primary production mainly depends on the availability of light, carbon, and nutrients. 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 & Kilham 1988, Correll 1999). Phosphatase activity allows access to organically bound P-compounds; many phytoplankton species synthesize alkaline phosphatases (AP) only when they lack directly available P, i.e. orthophosphate (P_i) (Siuda & Chróst 1987, Jansson et al. 1988). The presence and activity of these enzymes thus gives information about the nutrient status of the cells. The AP activity, expressed per unit of biomass, has long been used to define severe, mild and absence of growth limitation by P in natural and cultured phytoplankton species (Healey & Hendzel 1975). 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, with rapidly increasing AP activity at low growth rates (Smith & Kalff 1981, Siuda & Chróst 1987). A similar response curve exists for the relation between AP activity and cellular P in phytoplankton biomass (Gage & Gorham 1985), between AP activity and P_i concentration in eutrophic lakes in general (Siuda & Chróst 1987), and between enzyme activity and P_i concentration for *Synechococcus* sp. PCC 7942 specifically (Schreiter et al. 2001).

AP activity may thus indicate the onset of P-deficiency in phytoplankton. Bulk AP activity, however, is not an ideal indicator for several reasons (Graziano et al. 1996). First, the enzyme activity follows different patterns in various organisms; in some it is constitutive or even completely absent. Second, heterotrophic bacteria may be responsible for a significant fraction of the measured activity. Third, 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 objections to bulk AP activity measurements are largely met by use of a novel phosphatase substrate, a member of the enzyme-labelled fluorescence substrate family (ELF-97^{TM}) that yields highly fluorescent precipitates of ELF alcohol (ELFA) at the site of enzymatic activity (Huang et al. 1993, Haugland 1995). ELF phosphate (ELFP) has been used for the detection of endogenous phosphatases in complex mixtures of marine phytoplankton by FCM (González-Gil et al. 1998) and fluorescence microscopy (Dyhrman & Palenik 1999), and is also applicable to freshwater phytoplankton (Rengefors et al. 2001, Nedoma et al. 2003, Rengefors et al. 2003, Dignum et al. 2004). In this study we apply ELFP in FCM to assess the P_i-sensing status of individual cells and trichomes in the phytoplankton community of Lake Loosdrecht (The Netherlands) and link this information to ^{13}C-CO_2 tracer-based growth rates derived from FCM-sorted fractions of selected populations.

MATERIALS AND METHODS

Study site. Lake Loosdrecht, a shallow eutrophic lake in the Netherlands (52° 11′ N, 5° 3′ E; area: 9.8 km^2; mean depth: 1.9 m; P_{total} = 40 to 60 µg l^{-1}; N_{total} = 1.4 to 1.9 mg l^{-1}), is part of a system of interconnected lakes originating from industrial peat mining (for details see Hofstra & Van Liere 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). Due to prolonged external nutrient loading, the lake has changed from mesotrophic conditions (clear water dominated by submerged plants), to highly eutrophic conditions (water with low transparency, dominated by filamentous cyanobacteria). Lake Loosdrecht is a typical example of a lake for which restorative measures (70% reduction in total P-load since 1984; Dignum 2003) have not yet resulted in a return of the preferred clear water situation with submerged macrophytes. In this study, water samples were collected at weekly intervals (April to December 2001) off the West-End harbour jetty in Lake Loosdrecht. Various filamentous cyanobacteria are present in the lake. The currently most abundant strain was formerly referred to as *Oscillatoria* cf. *limnetica* (Van Tongeren et al. 1992), but is morphologically identical to *Planktolyngbya limnetica* (Komárek & Čáslavská 1991, Komárková-Legnerová & Cronberg 1992). Recent taxonomic revisions, however, have placed an iso-

late from this group (Isolate MR1) in the Limnothrix/Pseudoanabaena group (Suda et al. 2002). 16S-rRNA and DGGE analyses have revealed several closely related strains belonging to this group in Lake Loosdrecht (Zwart et al. 2004). For the sake of simplicity, we will refer to members of this group as *Limnothrix* sp. The second most abundant population is *Prochlorothrix hollandica* (Burger-Wiersma et al. 1986). This cyanobacterium has no phycobilins, but possesses chlorophyll *b* (chl *b*) (Matthijs et al. 1994). Measurements of phosphate concentrations in the lake water were carried out using the colorimetric method of Murphy & Riley (1962) according to the American Society for Testing and Materials (ASTM) standard test method D515-78 for phosphorus in water.

Laboratory-scale enclosure (LSE). Water from Lake Loosdrecht (130 l; March 27, 2001) was incubated in a LSE (Rijkeboer et al. 1990), which had a temperature of 16°C, and a light regime of 230 µmol photons m^{-2} s^{-1} photosynthetically available radiation (PAR) just below the surface with a light:dark cycle of 16:8 h (high-intensity daylight lamp: HMI 1200 W, Osram). The LSE was continuously mixed, and pH and dissolved oxygen concentration were kept below 9 and below 120% of saturation, respectively. A continuous flow of mineral medium was added at a rate of 240 ml h^{-1}. A solution with 1.7 mmol l^{-1} P_i (K_2HPO_4) was added at a rate of 10 ml h^{-1}. In addition, a solution containing 1.11 mg l^{-1} $FeCl_2 \cdot 4H_2O$ and 1.33 mg l^{-1} Na_2EDTA was added at a rate of 10 ml h^{-1}. The total medium dilution rate was 0.05 d^{-1}.

Enzyme-labelled fluorescence (ELF). The labelling procedure with the phosphatase substrate 2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (ELF-97™ phosphate; ELFP) was carried out as previously described (Dignum et al. 2004). 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 (volume: 14 ml) were centrifuged for 15 min at 2000 × *g*. The cell pellets were resuspended into 50 µl of the buffer provided by the manufacturer (Molecular Probes), containing a 1:20 dilution of ELFP substrate. After 30 min incubation at room temperature (20°C) in the dark, the reaction was stopped by 100× dilution in flow cytometer sheath fluid (1% Isoton Beckman-Coulter; see Dignum et al. 2004). Control samples were treated in the same procedure, without the ELFP substrate.

Microscopy. Visual inspection of the green fluorescent signal from the product, ELF-97 alcohol (ELFA) was carried out on a Zeiss standard RA fluorescence microscope with UV epifluorescence furnishing: excitation filter (bandpass 366 ± 25 nm), dichromatic mirror (FT 420), and suppression filter (longpass 418 nm). Trichome volumes were calculated from trichome length and width, as determined by digitising tablet (Hoogveld & Moed 1993). Quantitative counts were carried out on a Zeiss microscope with 2 ml counting chamber, using the inverted method according to the Utermöhl-principle (Utermöhl 1958).

Flow cytometry. FCM analyses and FACS were carried out on an Epics Elite instrument (Coulter), using the settings previously described (Dignum et al. 2004). Excitation (Ex.) wavelengths were 488 and 350.7 to 356.4 nm (multiline UV). Emission (Em.) was measured for ELFA (525 ± 20 nm), phycocyanin (637 ± 10 nm) and chlorophyll (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 4-decade logarithmic scale of the integrated signal. Settings for FACS were: frequency 21.3 to 21.4 kHz, drive 65%, deflection amplitude 48 to 50%, drop delay 21.0 to 25.9 drops sorted with coincidence abort on. After FACS, the identity of the fractions was confirmed microscopically. Dotplots and histograms (Fig. 1) were created with WinMDI version 2.8 (copyright J. Trotter 1999; http://facs.scripps.edu/software.html).

Population-specific ^{13}C-CO_2 tracer-based growth rate assessment. Samples from the LSE and lake water were incubated in a cylindrical ^{13}C-labelling vessel (1.4 l; diameter 0.1 m). Temperature and light conditions (irradiation and light:dark cycle) were chosen to resemble the conditions measured in the field, and are specified in Table 1 (see also Pel et al. 2004b). Light fluctuations that cells experience due to their circulation in the lake water column were simulated by switching the lamp (halogen 7 IPR lamp, Mazda 1500 W) on and off at 15 min intervals during the light period. The samples were continuously stirred at 10 rpm to prevent particulate material from settling. Dissolved inorganic carbon (DIC) in the original water was supplemented with 6 mg ^{13}C-$NaHCO_3$ (99 atom% ^{13}C) to an enrichment grade of 1500‰. After 0, 6, 24, 30 and 48 h of labelling, 50 ml subsamples were stained with ELFP and analysed by FCM. Biomass from the major phytoplankton groups was separated by FACS (8×10^4 to 3×10^5 cells/trichomes, collected by centrifugation for 12 min at 15 000 × *g*) and applied to Py-GC-IRMS analysis according to Pel et al. (2004a). Population-specific, diel-averaged growth rates (d^{-1}) were calculated from the rate of ^{13}C-CO_2 incorporation into mono- or polyunsaturated fatty acids (Pel et al. 2003), calibrated from continuous cultures (Pel et al. 2004b). For cyanobacteria and green algae (poly) unsaturated C_{18} fatty acids were analysed, whilst $C_{14:1}$ and $C_{16:1}$ fatty acids were analysed for *Prochlorothrix hollandica* and diatoms, respectively.

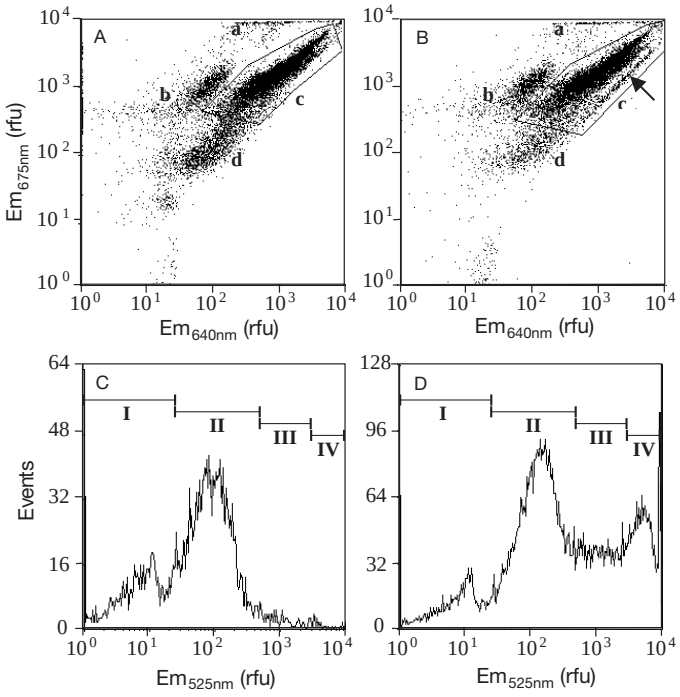

Fig. 1. Flow cytometric analysis of endogenous pigments (chlorophyll and phycocyanin) and enzyme-labelled fluorescence alcohol (ELFA) fluorescence in (A,C) unstained control samples and (B,D) ELF-stained samples from Lake Loosdrecht (April 23, 2002). (A,B) Bivariate plots of chlorophyll *a* fluorescence (emission [Em.] 675 nm; excitation [Ex.] 488 nm) and phycocyanin (PC) fluorescence (Em. 640 nm; Ex. 488 nm) showing clusters of micro-algae (Cluster a), PC-lacking cyanobacteria (Cluster b), PC-containing cyanobacteria (Cluster c), and detritus (Cluster d). Note that an additional cluster of cyanobacteria appears next to the normal cluster, indicated by the arrow. (C,D) Histograms showing ELFA fluorescence (Em. 525 nm; UV-excitation) in the cluster of PC-containing cyanobacteria encircled in (B). Subsets of cyanobacteria with different Em. 525 nm intensities are depicted: low 525 nm fluorescence (I), increased 525 nm autofluorescence (II), intermediate ELFA fluorescence (III), and high ELFA fluorescence (IV). rfu: relative fluorescence units

RESULTS

Seasonal variation of P_i-availability and phytoplankton growth rates in Lake Loosdrecht

Bivariate plots of chlorophyll *a* (chl *a*) fluorescence (Em. 675 nm; Ex. 488 nm) against phycocyanin (PC) fluorescence (Em. 640 nm; Ex. 488 nm), as presented in Fig. 1A, reveal more or less separate clusters of eukaryotic phytoplankton (Cluster a, consisting of green algae and diatoms), PC-lacking cyanobacteria (Cluster b), PC-containing cyanobacteria (Cluster c), and a cluster containing detritus (Cluster d). To assess the availability of P_i for individual phytoplankton, samples from Lake Loosdrecht were stained with ELFP. Green fluorescence (Em. 525 nm; Ex. 353 nm) of ELF-stained and control samples was plotted in histograms (e.g. Fig. 1D and C respectively). These histograms revealed several subsets with different emission intensities, which we named Subsets I to IV. Subsets III and IV showed increased fluorescent emission from ELFA formation compared to control experiments without ELFP added. On average, the fluorescence 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. The percentage of events in Subsets III and IV, minus the percentage of events in the same intensity regions in control experiments, represents the percentage of particles with phosphatase-dependent fluorescence, referred to as ELFA fluorescence. After ELF-staining, an additional cluster of cyanobacteria appeared next to the normal cluster of PC-containing cyanobacteria (marked with an arrow in Fig. 1B). Trichomes in this cluster were morphologically identified

Table 1. *Limnothrix* sp. population. Incubation conditions and growth rates (d^{-1}) in spring and summer 2001. Growth rates were estimated from ^{13}C-CO_2 incorporation in cellular (poly)unsaturated C_{18} fatty acids. Incident light intensity in incubation vessel, based on average field conditions over 5 d prior to incubation. ELFA: enzyme-labelled fluorescence alcohol

Date (d/mo)	Week	Water temp. (°C)	Incident light ($\mu E\ m^{-2}\ s^{-1}$)	Light cycle light:dark (h)	Growth rate (d^{-1})	Trichome numbers (10^3 n ml^{-1})	Particles with ELFA (%)
26/03	13	7	47	13.5:10.5	0.02	69	29
25/04	17	12	140	14:10	0.024	138	
02/05	18	14	125	14:10	0.05	122	
07/05	19	14	125	15:9	0.06	106	50
14/05	20	19	200	15.5:8.5	0.14	165	31
21/05	21	16	157	16:8	0.07	185	
28/05	22	17	200	16:8	0.06	177	
12/06	24	17	190	16.5:7.5	0.07	166	60
04/07	27	22	190	16.5:7.5	0.11	145	35
21/08	34	22	130	14:10	0.08	149	28
28/08	35	23	140	14:10	0.065	205	23

as *Aphanizomenon* sp., comprised up to 5% of the total fraction of PC-containing cyanobacteria, and showed extremely abundant ELFA fluorescence.

Fig. 2A shows the seasonal changes in population densities of PC-containing cyanobacteria (predominantly *Limnothrix* sp.), PC-lacking cyanobacteria (*Prochlorothrix hollandica*), and eukaryotic phytoplankton (diatoms and green algae), as determined by FCM. The dominant population of the filamentous cyanobacterium *Limnothrix* sp. in Lake Loosdrecht grew to a maximum of about 2×10^5 trichomes ml^{-1} in the growth season of 2001. The pronounced seasonal phytoplankton growth curves seen in Fig. 2A were not seen in other biomass indicators, such as chl *a* concentrations (not shown). Total-P concentrations varied between 45 and 85 µg P l^{-1} (not shown). Dissolved P_i levels were below the detection limit for spectrometric analysis throughout the year (less than 10 µg P l^{-1}; not shown). The number of particles with ELFA fluorescence was determined for each cluster in the period March to December 2001 (Fig. 2B–D). Population-specific growth rates of the *Limnothrix* sp. population were assessed from the incorporation rate of ^{13}C-tracer into C_{18} (poly)unsaturated fatty acids over 24 h, by enrichment of lake water with ^{13}C-carbonate in a ^{13}C-labelling vessel on several dates in spring and summer 2001 (Table 1).

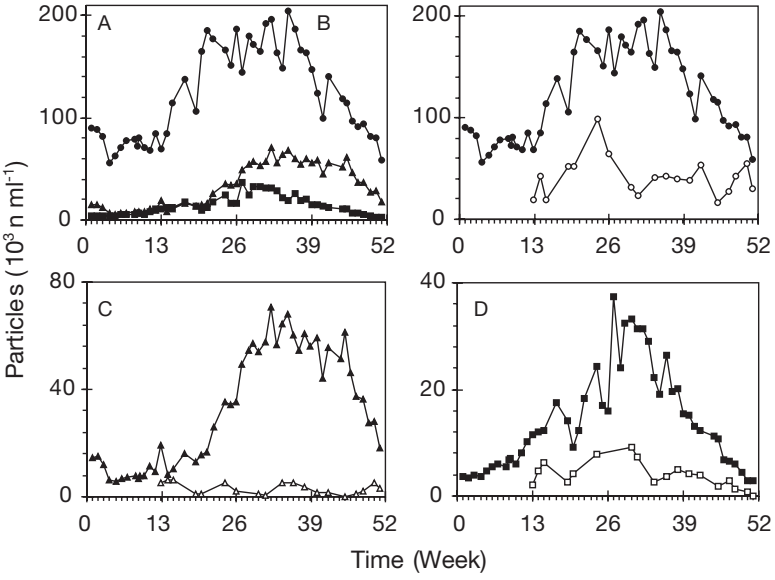

Fig. 2. Seasonal variation of cell densities and ELFA fluorescence in phytoplankton from Lake Loosdrecht in 2001. Week 1 is the first week of the year. (A) Flow cytometric counts (n) of phycocyanin (PC)-containing cyanobacteria, comprising predominantly *Limnothrix* sp. (●), the PC-lacking cyanobacterium *Prochlorothrix hollandica* (▲), and micro-algae, comprising both diatoms and green algae (■). (B,C,D) Total number (closed symbols) and number with ELFA fluorescence (measured from Week 13; open symbols) of (B) PC-containing cyanobacteria, (C) PC-lacking cyanobacteria, and (D) eukaryotic micro-algae

The number of PC-containing cyanobacteria, predominantly *Limnothrix* sp., increased from Week 13 onwards (Fig. 2B), although the growth rate was still low (0.02 d^{-1}; Table 1). After a relative collapse of the population in Week 19, the growth rate was very high in Week 20 (0.14 d^{-1}), concurrent with a high temperature. The population slowly decreased between Weeks 21 and 24 (growth rate 0.07 d^{-1}), while the number of trichomes with ELFA fluorescence quickly increased. The growth rate was relatively high again in Week 27 (0.11 d^{-1}), which was also warm. In that period a dip in trichome density occurred and the percentage of trichomes with ELFA fluorescence quickly decreased. In Weeks 34 and 35, which had high temperatures, the growth rate was about 0.07 d^{-1} again. The number of *Limnothrix* sp. trichomes with ELFA fluorescence was stable and low in this period. A very low number of *Prochlorothrix hollandica* trichomes showed increased 525 nm fluorescence (Fig. 2C). This species has very low non-inducible phosphatase activity (Dignum et al. 2004). Eukaryotic phytoplankton showed a variable fraction of cells with ELFA fluorescence (Fig. 2D).

Laboratory-scale enclosure (LSE)

The effect of added phosphate to the phytoplankton community in the lake water was studied by incubating a large volume of lake water (130 l; March 27, 2001) under light conditions that resemble the natural irradiance in spring. Previous work had established that under the conditions used, the P_i-loading rate (3 µmol P l^{-1} d^{-1}) exceeded the capacity of the particles for P_i-uptake after 3 d (Rijkeboer et al. 1990). In these conditions the population density increased during about 14 d (Figs. 3A & 4A). A marked decrease in length of filamentous cyanobacteria was observed during the first 3 d, resulting in a decreasing biomass volume (Fig. 3A). Samples from the LSE (1.4 l) were incubated with ^{13}C-bicarbonate at the beginning of the experiment (Day 0), in the growth phase (Day 7), and at the end of the growth phase (Day 14). Particles in the various clusters were sorted by FACS according to their content of fluorescent pigments, and growth rates were assessed from incorporation rates of ^{13}C-labeled CO_2 into population-specific fatty acids (Table 2; see also Pel et al. 2004b). Although FCM cannot distinguish diatoms and green algae (Cluster a), differences in the fatty acid profiles of these 2 groups (Pel et al. 2004a)

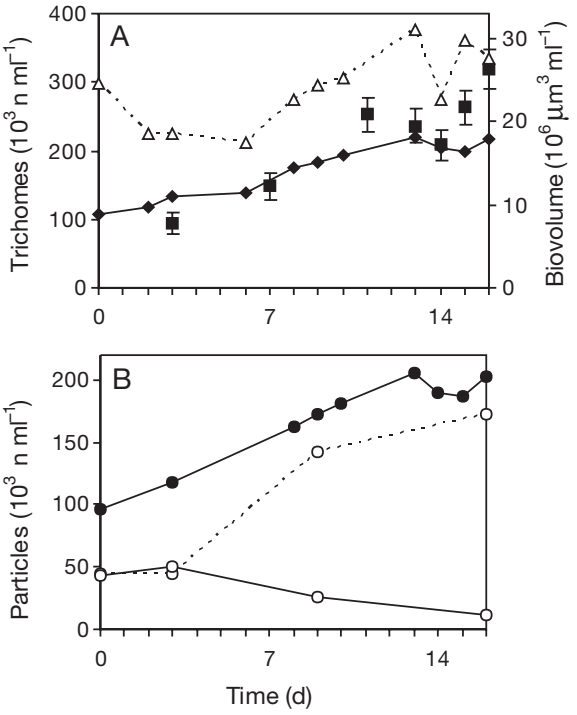

Fig. 3. Cyanobacteria in a laboratory-scale enclosure (LSE) of Lake Loosdrecht water, incubated on March 27, 2001. (A) Microscopic counts (n) (■, SE), flow cytometric counts (◆), and biomass volume (△) of filamentous cyanobacteria (with and without phycocyanin, PC). (B) Flow cytometric determination of the number (n) of PC-containing cyanobacteria (predominantly *Limnothrix* sp.) with ELFA fluorescence in the LSE. Total number of PC-containing cyanobacteria (—). ELF-stained samples (○) are separated into particles with low green fluorescence emission (---) and particles with ELFA fluorescence (—)

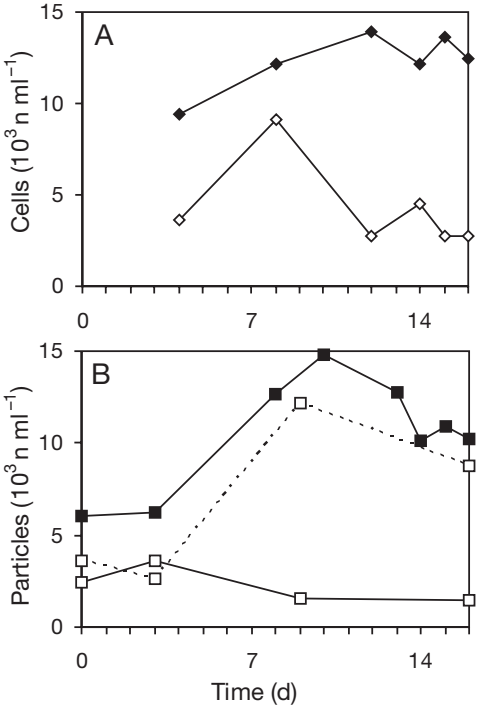

Fig. 4. Micro-algae in a laboratory-scale enclosure (LSE) of Lake Loosdrecht water, incubated on March 27, 2001. (A) Microscopic counts (n) of diatoms (◆), and green algae (◇). (B) Flow cytometric determination of the number (n) of micro-algae (diatoms and green algae together) with ELFA fluorescence in ELF-stained samples from the LSE. Total number of cells (■). ELF-stained samples (□) are separated into cells with low green fluorescence emission (---) and particles with ELFA fluorescence (—)

allowed separate estimation of their growth rates (Pel et al. 2003, 2004b). In the field sample (Day 0) the ^{13}C-CO_2 tracer-based growth rates were similar to the dilution rate of the LSE (0.05 d^{-1}). In the growth phase (Day 7) the growth rates were much higher than found in the lake. At the end of the growth phase (Day 14) the growth rate had decreased again for all groups, except the PC-containing cyanobacteria (Cluster c). These 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 Days 0, 3, 9, and 16 were stained with the ELFP substrate. The number of PC-containing cyanobacteria that showed ELFA fluorescence during the LSE experiment is shown in Fig. 3B. About 45% of these trichomes had ELFA fluorescence on Day 0, and about 43% on Day 3. The number of trichomes with ELFA fluorescence gradually decreased to about 15% on Day 9, and to about 5.5% on Day 16. The decrease of the number of PC-containing cyanobacteria with ELFA fluorescence is about 0.06 d^{-1}, which is close to the dilution rate (0.05 d^{-1}). At the same time, the number of trichomes with low intensity 525 nm fluorescence increased rapidly to a maximum between Days 3 and 13. In this period the growth rate, corrected for the dilution rate, was about 0.14 d^{-1}, calculated from the FCM growth curve (Fig. 3), and about 0.24 d^{-1} calculated from the microscopic counts (Fig. 3A). *Prochlorothrix hollandica* (Cluster b) is not shown, because it has no inducible phosphatase activity (Dignum et al. 2004). Microscopic counts of eukaryotic phytoplankton (Fig. 4A) showed that the net growth of green algae slowed down at an early stage (between Days 9 and 13). ^{13}C-CO_2 tracer-based 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 ELFA fluorescence on Day 0, increasing to 58% on Day 3, and then decreasing to 11% on Day 9 and 14% on Day 16 (Fig. 4B). The total number of eukaryotic phytoplankton reached a maximum around Day 10, and then declined (Fig. 4B).

Table 2. Growth rates (d^{-1}) of phytoplankton populations in a laboratory-scale enclosure separated by flow cytometry, as estimated from ^{13}C-CO_2 incorporation in cellular fatty acids

Cluster	Day 0	Day 7	Day 14
Cyanobacteria	0.02	0.36	0.40
Prochlorothrix hollandica	0.01	0.24	0.17
Diatoms	0.08	0.85	0.30
Green algae	0.07	0.51	0.15

Characteristics of subsets from the *Limnothrix* sp. group

ELFA fluorescence histograms revealed that staining was not uniform for all trichomes in the *Limnothrix* sp. group. We studied the characteristics of subsets by applying FACS to collect the following fractions from a ^{13}C-labelling vessel in April 2002 in triplicate: the total cyanobacterial cluster (Cluster c) consisting mainly of *Limnothrix* sp. trichomes (64% of all particles in the samples, and 83% of the live phytoplankton; Fig. 1B), a subset of cyanobacteria with low 525 nm fluorescence (Fig. 1D; I), a subset with increased 525 nm autofluorescence (Fig. 1D; II), a subset with intermediate ELFA fluorescence (Fig. 1D; III), and a subset with high ELFA fluorescence (Fig. 1D; IV). The increased 525 nm autofluorescence was caused by the use of 0.1% (w/v) glutaraldehyde (in combination with 0.01% (w/v) formaldehyde) as a fixative (authors' unpubl. results). Fixation was necessary in this experiment to prevent filaments from breaking during FACS. Several parameters were recorded for each subset (Table 3). To compare the P_i-sensing status and photosynthetic capacity, we inspected bivariate plots of chl *a* fluorescence versus ELFA fluorescence (not shown). Subset I with low 525 nm fluorescence had low 675 nm fluorescence (Table 3). The trichomes in this subset had a much lower average volume, and the estimated growth rate was lower than that of the other subsets. Subset II (525 nm Em. intensity 15 times that of Subset I) was most abundant. These trichomes also had lower than average 675 nm fluorescence, were of medium volume, and their growth rate was slightly lower than that of the total group. The fraction of particles with ELFA fluorescence was 34.8%. Subset III (525 nm Em. intensity 150 times that of Subset I) had higher chl *a* fluorescence than the average, slightly larger mean volume, and a growth rate comparable to the total group. Subset IV (525 nm Em. intensity 700 times that of Subset I) had the highest chl *a* fluorescence, filament volume comparable to the total group, and the growth rate was slightly higher than the total group.

DISCUSSION

In this contribution to the study of population-specific growth of freshwater phytoplankton, we have combined 3 recent developments in flow cytometry. This new combination of methods allows us to assess the availability of P for phototrophic growth on a population level in lakes where phosphate concentrations are very low. First, 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 to 70 µm diameter of the sort droplets (Pel et al. 2003, 2004a). Second, the ELFP substrate provided a readily applicable method to determine the P_i-sensing status of freshwater phytoplankton, resulting in ELFA fluorescence that added a physiology-related parameter for UV excitation in

Table 3. Characteristics of cyanobacterial subsets in a ^{13}C-CO_2-labelling vessel (1.4 l) with water from Lake Loosdrecht (April 22, 2002). Mean values from duplicate experiments are given with standard deviations in brackets. Subset I: cyanobacteria with low green fluorescence; Subset II: cyanobacteria with fixation induced green autofluorescence; Subset III: cyanobacteria with intermediate ELFA fluorescence; and Subset IV: cyanobacteria with high ELFA fluorescence. The last column (Total) represents the complete PC-containing cyanobacterial cluster. FCM: flow cytometry; rfu: relative fluorescence units

FCM variable	I	II	III	IV	Total
rfu 525 nm	0.83 (0.03)	12.3 (1.2)	126 (1.3)	569 (2.5)	99.3 (3.8)
rfu 675 nm	101 (1.6)	149 (1.7)	197 (0.3)	226 (3.3)	160 (1.1)
% per subset[a]	8.8 (0.9)	48.7 (0.9)	20.7 (0.3)	18.3 (1.8)	100
% in control samples[a]	22.3 (1.7)	70.6 (1.1)	3.5 (0.6)	0.7 (0.1)	100
Trichome volume (mm^3)[b]	127 (13)	285 (21)	314 (29)	278 (21)	268 (49)
Growth rate (d^{-1})[c]	0.053 (0.016)	0.072 (0.016)	0.086 (0.012)	0.093 (0.002)	0.089 (0.007)

[a]Percentages of the total number of particles in the *Limnothrix* sp.cluster

[b]Calculated from average length and width, determined according to Hoogveld & Moed (1993)

[c]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

FCM (González-Gil et al. 1998, Dignum et al. 2004). Third, labelling of the phytoplankton with ^{13}C-CO_2 enabled assessment of specific growth rates of sorted populations by a combination of FACS and Py-GC-IRMS (Pel et al. 2003, 2004b).

P_i-availability for phytoplankton in Lake Loosdrecht

Filamentous cyanobacteria are either a minor component of the phytoplankton community in Dutch shallow lakes, or they are strongly dominant (Scheffer et al. 1997). Cyanobacteria are generally good competitors for both light and P_i. High abundance of filamentous cyanobacteria occurs predominantly under conditions of mutual shading in dense phytoplankton communities (Jensen et al. 1994, Scheffer et al. 1997). Restoration of the trophic status of eutrophied shallow lakes by reducing the P-loading rate can be a slow process, because several feedback mechanisms exist that keep the water turbid (Gons et al. 1991, Havens & Schelske 2001). Although dissolved reactive P-concentrations in Lake Loosdrecht have become very low in recent years, internal P-supplies prevent a decrease in P-availability (Riegman & Mur 1986, Dignum 2003). P_i is liberated from the sediment by mineralization and wind resuspension (Marsden 1989), and from suspended particulate organic matter (mainly detritus; Rijkeboer et al. 1991). The availability of organic phosphate is indicated by AP activity (Boström et al. 1988). However, not all phytoplankton species have an inducible AP. The 2 major populations of filamentous cyanobacteria in Lake Loosdrecht have different adaptation strategies toward low phosphate concentrations and low light conditions. The dominant species, *Limnothrix* sp., has an inducible AP, and has PC as an accessory photosynthetic pigment. The second most abundant population in the lake, *Prochlorothrix hollandica*, has no inducible AP (Dignum et al. 2004) and no PC (Matthijs et al. 1994). Instead, this species relies on phosphate storage inside the cells (Ducobu 1998), and contains some chl *b*. The uptake strategy of this species probably depends on pulses of P_i caused by wind resuspension of sediment, in which P_i is liberated by mineralization.

We have shown that in spring 2001 about 50% of the individual trichomes of the PC-containing cyanobacteria in Lake Loosdrecht had phosphatase activity, detected as ELFA fluorescence. In a previous study, we established that in P_i-depleted batch cultures of *Limnothrix* sp. Isolate MR1 also about 50% of the trichomes had ELFA fluorescence (Dignum et al. 2004). A marked increase of biomass in the LSE with Lake Loosdrecht water with continuously added phosphate supports the view that these results indicate P_i-limited growth: the percentage of ELFA-labelled particles in the *Limnothrix* sp. cluster declined with the P addition and increasing growth rate of these cyanobacteria in the LSE. Trichomes with ELFA fluorescence disappeared from the LSE at a rate that was similar to the dilution rate. It is likely that no new AP was produced after 3 d in the LSE with P_i added. Presumably, cells that contain the enzyme were washed out, and newly formed cells did not induce AP, because their growth was not limited by P_i-availability anymore. Numbers of cyanobacterial trichomes and algal cells in the LSE did not increase at the rate estimated from ^{13}C-CO_2 incorporation. The *Limnothrix* sp. population even showed a high ^{13}C-CO_2 tracer-based growth rate on Day 14, when trichome numbers and biomass volume were decreasing. This observation implies significant loss factors; viral lysis may have been responsible for the apparent losses in the LSE (Gons et al. 2002), but other factors cannot be ruled out. In contrast, the growth rates of the other phytoplankton populations had declined on Day 14, which may have been due to shading by the increased *Limnothrix sp.* population, or enhanced grazing by micro-zooplankton.

In the *Limnothrix* sp. population in the lake, the fast increase in number of trichomes with ELFA fluorescence in Weeks 21 to 24 (Fig. 2B) indicates P_i-depletion. The decrease in trichome density in this period indicates that loss factors surpassed the growth rate. This effect was more dramatic in Week 27, when a high growth rate concurred with a fast decrease in trichome density (Table 1). A decrease in the percentage of trichomes with ELFA fluorescence in this period (Table 1) indicates that more P_i was available. This may have been due to an increased mineralization rate in the sediment, following the higher summer temperatures. However, the high growth rates (>0.1 d^{-1}) were also directly influenced by the temperature increase in the lake water. The lack of biomass increase in this period may be attributed to loss factors, such as grazing, automortality or viral/bacterial lysis (Rashidan & Bird 2001, Veldhuis et al. 2001, Gons et al. 2002). In Weeks 34 and 35, the number of trichomes with ELFA fluorescence was stable and low (Fig. 2B), indicating sufficient P_i-availability. Even though temperatures were high in this period and P_i-availability was sufficient, growth rates were below 0.1 d^{-1} (Table 1). Probably, light-limitation occurred. It is interesting to note that growth of the *Prochlorothrix hollandica* population concurred with this period of lower ELF fluorescence in *Limnothrix* sp. This population may benefit from the liberated P_i that could not be used by the *Limnothrix* sp. population, and may also be less liable to the loss factors that limit the *Limnothrix* sp. population.

Heterogeneity within the *Limnothrix* sp. population

ELF staining has revealed heterogeneity within the *Limnothrix* sp. cluster; ELFA fluorescence divided the cluster into subsets with different emission intensities. An additional cluster of PC-containing cyanobacteria (arrow in Fig. 1B) was morphologically identified as *Aphanizomenon* sp. This species shows very high intensities of ELFA fluorescence; the enhanced 640 nm fluorescence separating this cluster from the main one is probably due to spill-over from ELFA fluorescence. The heterogeneity within the *Limnothrix* sp. cluster could not be explained by the presence of other cyanobacteria in this cluster, as they are only a minor fraction. The *Limnothrix* sp. group itself is known to include several closely related species with trichomes of highly variable length and width (Zwart et al. 2004). Differences in the thickness of the cells' outer layers and subsequent longer diffusion time may have caused uneven staining among cells (see also Rengefors et al. 2003). 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 active cells may express the most urgent need for nutrients and show the first signs of limitation. Other, less active cells may express less AP. A subset of extremely limited cells may not be capable of responding to their environment with the synthesis of new enzymes. The results from the ^{13}C-labelling experiment support this view; both chl *a* fluorescence and growth rates increased with increasing ELFA fluorescence. An interesting parallel was found in a recent study using FCM 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. Support for this view comes from a segregated P_i-starvation response model for *Escherichia coli* (Van Dien & 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. This observation complicates the interpretation of results from the ELF method. It is not sure if a decrease in ELFA fluorescence is caused by an alleviation of the P-limitation, or conversely, by an extreme P-limitation hampering de novo synthesis or accessibility of AP. Therefore, additional information is needed, notably about cell cycle phase (e.g. Veldhuis et al. 2001). Parpais et al. (1996) have reported a remarkable, irreversible arrest of the marine cyanobacterium *Prochlorococcus* sp. 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 provide additional information about the nutrient status of phytoplankton (Parpais et al. 1996).

In conclusion, the ELF-97 method, combined with FACS and ^{13}C-CO_2 tracer-based growth rate estimations, offers insight into the population dynamics in relation to the nutrient status of freshwater phytoplankton, by tracing the AP activity back to the individual cells that produced it. Rapid recycling of P in the lake, aided by the synthesis of phosphatases that convert organic P into P_i, and adsorption of P_i to detritus (Gons et al. 1991, Rijkeboer et al. 1991), may explain why the *Limnothrix* sp. population in Lake Loosdrecht is resistant to ecological restoration measures. However, only a relatively small fraction of the *Limnothrix* sp. population shows the ELFA signal, which raises questions about the interpretation of the results. Future research should therefore include detailed studies into variability in the life histories of the individual trichomes, and into the derepression behaviour of AP and the loss/disappearance of AP activity from *Limnothrix* sp. cells, e.g. by using continuous cultures providing stringent control of P_i availability.

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