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Flow cytometry sorting of freshwater phytoplankton

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Abstract We used the flow sorting capacities of a bench-top FACSCalibur flow cytometer to analyze the phytoplankton community of four different aquatic ecosystems. We show that despite the high optical, mechanistic, and hydrodynamic stress for the cells while sorted, most of the targeted populations could be isolated and grew in mixed culture media subsequent to sorting. Forty-five phytoplankton taxa were isolated, including green algae (29 species), cyanobacteria (eight), diatoms (seven), and cryptomonads (one). The isolation success average was high since 80% of the total sorted populations grew successfully and 47% constituted monocultures. It is noteworthy, however, that some groups could not be isolated, as for example colonial cyanobacteria, chrysophytes, euglenophytes, desmids, or dinoflagellates, and some species such as *Cryptomonas* sp. were very sensitive to the sorting process. It is proposed that flow cytometric analysis of freshwater phytoplankton might be a relevant tool for water managers and could be applied in some specific cases, such as early monitoring of blooming taxa or basic bio-monitorings of key species. The higher isolation average obtained from the flow sorting can also be powerful for the physiological or molecular study of some taxa after their cultivation.

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Introduction

During the last two decades, flow cytometry (FCM) has been recognized as a powerful tool for the study of phytoplankton ecology, especially for spatial and seasonal trends (Peperzak et al. 2000). Due to the light scattering and the multi-color fluorescence emission (due to the chlorophylls and the phycobilins) of the cells, mixed populations in a sample can be detected and discriminated (Chisholm et al. 1988; Olson et al. 1985, 1991; Yentsch and Horan 1989). FCM is especially suited for the study of tiny organisms such as the picophytoplankton (Crosbie et al. 2003a; Ivanikova et al. 2007; Sarmento et al. 2006, 2008), which is difficult to observe and count by other techniques (typically microscopy) because of their very small size (<5 μm; Vaulot et al. 1989). This technology was first used in marine ecosystems in the mid-1980s (Olson et al. 1985) to quantify picocyanobacteria and the smallest eukaryotic algae which had been largely ignored in previous studies (Chisholm et al. 1988; Courties et al. 1994; Weisse 1993). It also allowed the discovery of the most abundant oceanic photosynthetic organism, i.e., *Prochlorococcus* (Chisholm et al. 1988). Several marine picophytoplankters such as *Prochlorococcus* (Chisholm et al. 1988; Dandonneau et al. 2006; Jacquet et al. 2001; Marie et al. 1997; Partensky et al. 1999), PE-rich *Synechococcus* (Jacquet et al. 1998; Li and Dickie 2001; Partensky et al. 1996; Vaulot and Marie 1999), or again the smallest eukaryotic phytoplankter, *Ostreococcus* (Courties et al. 1994), have been identified and studied extensively using FCM. In freshwater ecosystems, however, it took longer before FCM was used to study

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population dynamics of autotrophic picoplankton (Crosbie et al. 2003b) or the whole microbial community including phytoplankton, bacteria, and viruses (e.g., Goddard et al. 2005; Personnic et al. 2009). Regarding the larger phytoplankton, FCM has not really been used to study populations in natural systems, especially in freshwater environments (Crosbie et al. 2003b; Tijdens et al. 2008; Toepel et al. 2004, 2005).

In order to obtain reliable information on ecophysiological features of phytoplankton species, it is necessary to isolate them physically from their natural environment and culture them separately (Reckermann 2000). In that goal, a variety of methods has been applied such as the isolation by pipetting (Hoshaw and Rosowski 1973), isolation on Petri dish (Koch 1881), or again the serial dilution technique (Throndsen 1995). With the development of FCM and associated functions such as sorting, a new alternative and attractive isolation method has emerged more recently compared to the methods cited above. In fact, the sorting module, which allows a physical separation of the cells from a mixed assemblage, has become a regular option for many instruments. However, our feeling is that it has only been poorly used, especially to study natural phytoplanktonic population dynamics in freshwater. As pointed out by Reckermann (2000) while reviewing flow sorting in aquatic ecology, possible applications are numerous. Briefly, Sensen et al. (1993) sorted different flagellated algae (such as *Cyanophora paradoxa*, *Haematococcus lacustris*, *Scherffelia dubia*) using FCM to obtain thereafter clonal cultures. Li (1994) could measure primary productivity of different groups after sorting. Dubelaar and Jonker (2000) showed that isolations using FCM sorting could be used to test fluorescent probes and molecular techniques for the analysis of phytoplankton. Pel et al. (2004a) reported the link of flow cytometric cell sorting and compound-specific ^{13}C analysis to determine population-specific isotopic signatures and growth rates in cyanobacteria-dominated African lakes. More recently, this method has been proposed to be of special interest for isolating potentially toxigenic cyanobacteria, such as *Planktothrix rubescens*, with the goal to analyze toxins production under different environmental conditions (Jacquet, unpublished; Oberhaus et al. 2007). Finally, FCM sorting might be useful to isolate some new species in a lake for instance, previously identified by microscopy, when no more information is available and molecular biology assays are required (Vives-Rego et al. 2000).

It is noteworthy, however, that flow sorting may also display important drawbacks. Typically, it is likely that such a technology may not be allowed to isolate all species identified by traditional microscopical analysis. Cells might be affected or destroyed by fluid acceleration, electrical, or mechanistic shock as well as by optical stress. We should also keep in mind that size limitation is likely in many flow cytometers with upper limits of typically 30 to 150 μm (Dubelaar and Jonker 2000). Thus, particles that exceed this size would not be sorted and isolated. In addition, considering that small cells are more abundant than larger ones, it is often easier to isolate the former than the latter (Veldhuis and Kraay 2000). FCM is not suited for rare events. Finally, numerous species form chains (in particular diatoms or cyanobacteria) or produce colonies and this can lead to some mistakes during the sorting step if they are not separated with the acceleration of the fluid and counted as single entities.

In the present study, we show that it is of great interest to know what kind of organisms is hidden behind the dot plots shown on the screen of the flow cytometer computer. For different freshwater ecosystems, we tested a flow cytometric method in order to sort and culture the major algal taxa. In parallel, microscopic observations were carried out to identify the species isolated from the different clusters. Potential applications are discussed.

Materials and methods

Samples were taken from three natural peri-alpine lakes (Geneva, Bourget, and Annecy) located at the border of the French Alps and one artificial reservoir (Marne, also referred to as Lac du Der) located in the Champagne region, 200 km east of Paris. The three peri-alpine lakes have different trophic status. Briefly, Lake Geneva passed between 1960 and 1980 from an oligotrophic to a eutrophic status. Programs engaged in the early 1970s for water quality restoration became fruitful from the mid-1980s (Anneville et al. 2002), and this lake is now considered as mesotrophic, according to OECD criteria (OECD 1982). Lake Bourget has experienced water eutrophication between 1950 and 1980. Water quality restoration programs began at the end of the 1970s and early 1980s, which led to an important reduction of the phosphorus concentration for the last 20 years (from 120 to ~20 μg L^{-1}). However, a bloom of the filamentous and toxic cyanobacterium *P. rubescens* has been constantly detected since 1996 during summers and autumns (Briand et al. 2005; Jacquet et al. 2005). Lake Annecy has been protected from the wastewater discharge by a peripheral sewer collecting secondary network since 1961. Nutrient concentrations in this lake are particularly low so that it is often referred as the "cleanest" lake of France (Masson et al. 2001; Nicoud and Manalt 2001).

Each peri-alpine lake was sampled at a single station which corresponds to the reference station for the water quality survey of these ecosystems. In the Reservoir Marne, two stations were explored since, at that time, no reference station had been identified (Rolland et al. 2009). The first

one (553) is a closed nautical basin submitted to nutrient pollution from a little river and consequently prone to phytoplankton blooms that can represent a potential problem for ecosystem functioning and public health. The second station (501) receives water from two feeding channels derived from two rivers, and this area presents high phytoplankton and phosphorus concentrations. Reservoir Marne is also a mesotrophic ecosystem when considered as a whole, but the two stations analyzed are eutrophic.

This study was conducted between February and June 2005 in Lakes Geneva, Bourget, and Annecy and between May and September 2006 in Reservoir Marne. Samples were obtained using a Van Dorn bottle at different discrete depths between 0 and 20 m, i.e., from surface down to the bottom layer of the euphotic zone. All samples were brought back to the laboratory within only a few hours under cool and dark conditions and processed immediately.

Flow cytometry analysis and cell sorting

A FACSCalibur (Becton Dickinson) flow cytometer equipped with an air-cooled argon laser (15 mW power, 488 nm excitation), a red-emitting diode (635 nm excitation), and four filters for fluorescence emission was used. Following the method of Marie et al. (2000), previously diluted 1-μm diameter yellow-green fluorescent beads (Molecular Probes Inc.; 2 μL of stock solution in 2 mL of milliQ water) were added to 2 mL bulk samples for later signal normalization. Forward scatter (FSC) indicative of the cell size and shape, side scatter (SSC) indicative of cell granularity, size and refractive index, green fluorescence from phycobilin (515–545 nm, FL1), orange fluorescence from phycoerythrin (PE; 564–606 nm, FL2), and red fluorescence from chlorophyll *a* (Chl*a*; >650 nm, FL3) were recorded for each cell. Analyses were run for 3 min for the smallest forms (picocyanobacteria) and for up to 8 min for the largest forms (filamentous cyanobacteria and eukaryotes) at the highest single flow rate of 95–100 μL min^{-1} of our machine and by changing the flow cytometer setup for each size class to detect the whole community. For small cells, parameter settings were as follows: FSC (E01), SSC (400), FL1 (500), FL2 (500), and FL3 (500), whereas for large cells, the settings were FSC (E00), SSC (300), FL1 (350), FL2 (300), and FL3 (300). Abundance of each population was calculated according to the formulae: $N = (n \times 1,000)/q \times t$, where q is the flow rate (microliter per minute), t is the duration (minutes) of the acquisition, n is the number of events counted by the flow cytometer, and N is the number of cells per milliliter. Data were collected in listmode files and analyzed using CYTOWIN (Vaulot 1989). Subsequently, the cytometer sorting function was used to separate and characterize the different populations detected.

The FCM sorting function allowed us to separate physically homogeneous populations from the heterogeneous mixture of cells, based upon the specific light scattering and fluorescent characteristics of each cell. In this way, every cluster was regarded as an individual phytoplankton population and the "exclusion mode" of the FCM sorting function was used. This means that the sort occurred only when a target cell was identified—there were no non-target cells in the sort envelope—leading to the highest purity made possible by the device of the sorted population. Then, every cluster was isolated and cultured. For this purpose, the sheath fluid generally used (i.e., milliQ water, FACSFlow™ or filtered natural water) was replaced by a mixture of nine different culture media, in equal proportion, available in the laboratory (MWC, L-C, BB, BG-11, ASM1, DV, JAWORSKI, Z+Si+Vitamin, Z) in order to promote non-limiting growth conditions for any sorted species. Consequently, each sorted population was incubated in the same mixture of culture medium.

Culture conditions

Each population of interest observed in both setup previously established was sorted in a 50-mL Falcon tube and transferred to a 250-mL Nunclon bottle. Bottles were placed in a culture chamber with white (DiaLux) light intensities of 50–55 μmol photons m^{-2} s^{-1} in 16:8 h light/dark conditions at a constant temperature of 18°C. To maintain the populations isolated, the culture medium was regularly changed, and once they were developed (generally 3 weeks later), the samples were observed using a light-inverted microscope (Zeiss Axiovert 135) for taxonomic identification. In addition, epifluorescence microscopy (Leitz-Wetzar, Dialux 20), equipped with a filter set for blue light excitation, was used for picocyanobacterial observation.

Results

A total of 175 isolations were carried out in the four hydrosystems using the FCM sorting module. In the perialpine lakes, 130 samples were sorted: 36 came from Lake Geneva, 49 from Lake Bourget, and 45 from Lake Annecy. In the Reservoir Marne, 45 samples were sorted: 23 issued from station 553 and 22 from station 501. The higher isolation success average (monospecific cultures, see Table 1) was obtained in the samples from Lake Annecy (58%), followed by those from Lakes Bourget (54%) and Geneva (45%), and those from stations 553 (37%) and 501 in the Reservoir Marne (26%).

The major algal taxa isolated and then identified by microscopy in the four hydrosystems as well as the different pigmentation fluorescence used to classify them

Table 1 Classification of the different species isolated from the four hydrosystems using FCM sorting

Accessory pigment		Systematic groups	Species	Hydrosystems			
Phycocyanin	Phycoerythrin			Reservoir Marne	Lake Geneva	Lake Bourget	Lake Annecy
−	−	Bacillariophyte	*Asterionella Formosa* Hassal		X	X	X
			Diatoma tenuis Agardh		X	X	
			Fragilaria crotonensis[b] Kitton			X	X
			Navicula sp. Bory de St. Vincent 1822	X			
			Nitzschia sp. Hassal 1845	X			
			Stephanodiscus minutulus[a] (Kützing) Cleve and Möller		X	X	
+[b]	−[b]	Cyanobacteria	*Anabaena solitaria* Klebahn	X			
			Aphanizomenon flos-aquae[a] Ralfs			X	
			Planktothrix agardhii[a] (Gomont) Anagnostidis and Komárek	X			
			Planktothrix rubescens[a] (DeCandolle ex Gomont) Anagnotidis and Komárek			X	
			Pseudanabaena acicularis[a] *(Nygaard) Anagnotidis and Komárek*			X	X
			Pseudanabaena catenata Lauterborn		X		
			Pseudanabaena galeata[a] *Böcher*	X			
			Pseudanabaena limnetica[a] (Lemmermann) Komárek	X	X	X	X
			Synechococcus sp.[a] Nägeli 1849	X	X	X	X
−	+	Cryptophyte	*Cryptomonas* sp.[a] Ehrenberg 1838	X			
−	−	Chlorophyte	*Ankyra* sp. Fott 1957	X			
			Chlamydomonas sp. Ehrenberg 1833	X			X
			Chlorella vulgaris[a] Beijerinck	X	X	X	X
			Choricystis minor[a] (Skuja) Fott	X	X	X	
			Coelastrum microporum Nägeli	X			
			Crucigenia sp. Morren 1830			X	
			Crucigeniella rectangularis[a] (Nägeli) Komárek	X			
			Didymocystis bicellularis (Chodat) Komárek	X			
			Hyaloraphidium contortum Pascher and Koršikov	X			
			Monoraphidium arcuatum (Koršikov) Hindak	X			
			Monoraphidium circinale Nygaard	X			
			Monoraphidium contortum[a] (Thuret) Komárkova.-Legnerová		X		
			Monoraphidium convolutum (Corda) Komárkova.-Legnerová	X			
			Monoraphidium dybowskii (Woloszynska) Hindak and Komárkova.-Legnerová	X			
			Monoraphidium griffithii (Berkeley) Komárkova.-Legnerová	X			
			Monoraphidium kormakovae[a] Nygaard				X
			Monoraphidium minutum (Nägeli) Komárkova.-Legnerová	X			
			Monoraphidium tortile (W. and G.S. West) Komárkova.-Legnerová	X			
			Mougeotia gracillima[a] (Hassal) Wittrock		X		
			Mougeotia sp.[a] Agardh 1824	X			
			Scenedesmus acutus Meyen	X			
			Scenedesmus bicaudatus Deducenko	X			

[a] Species isolated and maintained as a monoculture

[b] Except for *Planktothrix rubescens*, *Pseudanabaena catenata*, *P. limnetica*, *P. acicularis*, and *Synechococcus* sp. whose cells contain more phycoerythrin than phycocyanin

from FCM parameters are listed in Table 1. Forty-five phytoplankton species have been isolated from the four systems. Most of them were chlorophytes (29 taxa), nine taxa were classified in the cyanobacterial group, six belonged to the bacillariophytes (i.e., diatoms), and finally the cryptophytes were represented by the single genus *Cryptomonas* sp. It can be noted that no chrysophytes or euglenophytes as well as desmids or dinoflagellates were obtained in the different isolations while they were present in microscopic counts (data not shown).

Seasonal dynamics of *Synechococcus* spp.

Picocyanobacteria were always observed at all stations. As this community can be very diverse genetically (Crosbie et al. 2003c), this group should be called *Synechococcus* spp. and thereafter in the manuscript we only refer to them as *Synechococcus*. Unique signatures of *Synechococcus* were observed in each of the five stations. In Lakes Geneva (Fig. 1a–d) and Bourget (Fig. 1e–h), it was highly abundant in June (47×10^3 and 8×10^3 cells mL^{-1}, respectively). Among the three alpine lakes, the higher abundances over the period of this study were found in Lake Annecy (Fig. 1i–l), where densities reached 37×10^3 cells mL^{-1}. Very interestingly and by contrast to the other lakes investigated, signatures of *Synechococcus* in this lake were elongated. In stations 501 (Fig. 1m–p) and 553 (q–t) of the Reservoir Marne, *Synechococcus* signatures were smaller (more compact) in May, August, and September and corresponded to small abundances whereas they were thicker in July and were related to a higher abundance during this period, reaching densities up to 60×10^3 cells mL^{-1}.

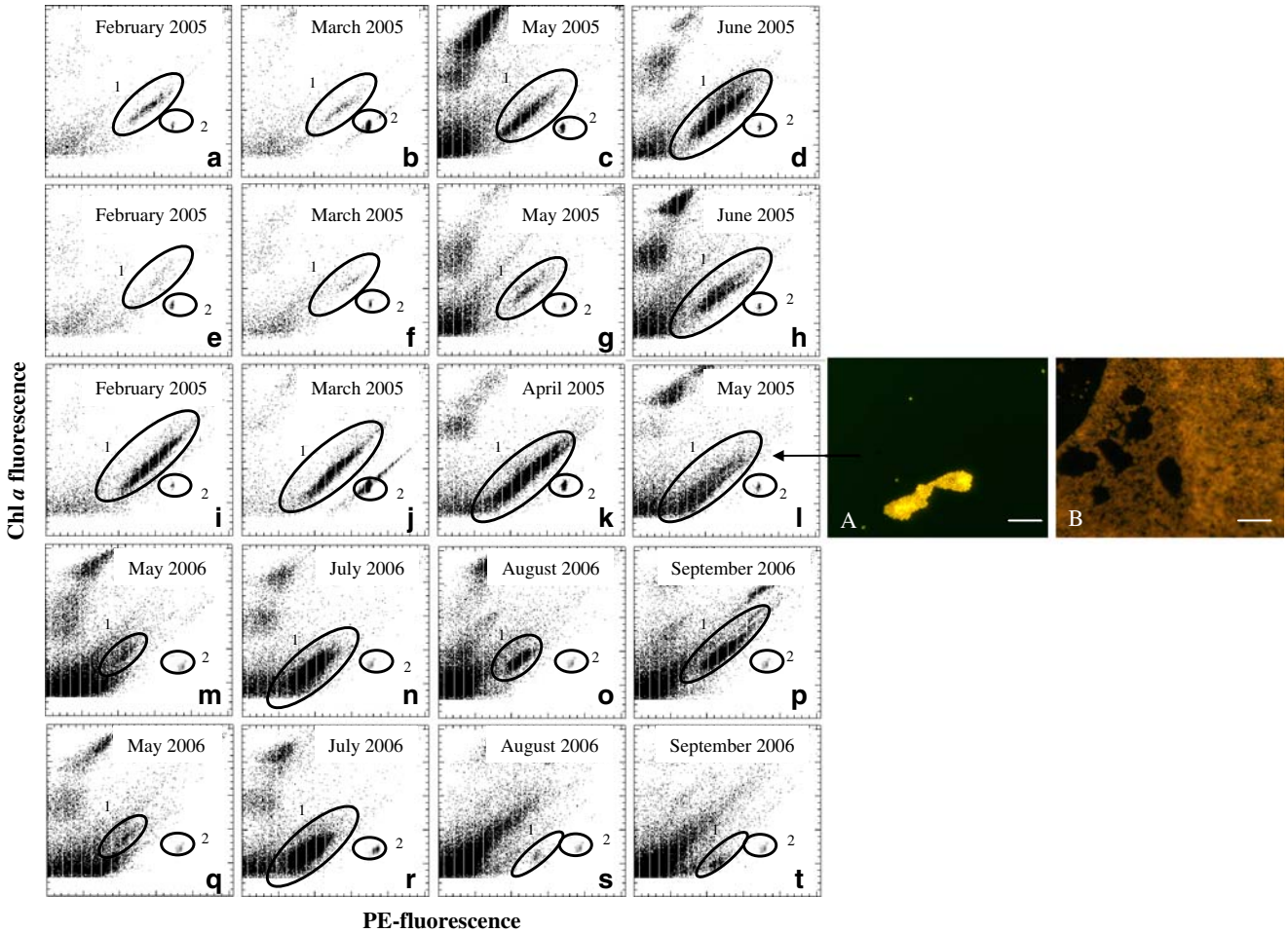

Fig. 1 PE vs. Chl*a* fluorescence cytograms showing the temporal evolution of *Synechococcus* spp. in Lakes Geneva (**a**–**d**), Bourget (**e**–**h**), and Annecy (**i**–**l**) and stations 501 (**m**–**p**) and 553 (**q**–**t**) from the Reservoir Marne. (*1 Synechococcus* spp., *2* 1-μm beads). *In the picture*: *Synechococcus* microcolonies before (*A*) and after (*B*) sorting and culturing (Scale bar=10 μm). Note that most populations were unicellular forms

Seasonal dynamics of other phytoplankton populations or groups

The seasonal distribution of other species was observed even though many of the populations isolated were mixed. Thus, information can be highlighted about some clusters that did not contain exactly the same species from one date to another but had the same fluorescence pattern because they belonged to the same taxonomic class. As an example, for station 501 (Fig. 2a–d), the first cluster of interest (a mixed green algal community) presented an elevated chlorophyll *a* fluorescence, information about potential high chlorophyll *a* content, and a low or absence of PE fluorescence because of a lack of this pigment in those algae. The cluster had more or less the same signature (related to beads fluorescence) along the sampling season but the microscopic determination clearly revealed some differences in its composition. In fact, while in May and July, this cluster was mainly composed of *Chlorella vulgaris*, *Hyaloraphidium contortum*, and several species of *Monoraphidium* spp., there was a new mixed community in August composed essentially of species of *Scenedesmus*. Finally, several *Monoraphidium* species found in May and July returned in September. The abundance of this cluster decreased between May and July (3 to 2×10^3 cells mL^{-1}), then there was a high increase in August (22×10^3 cells

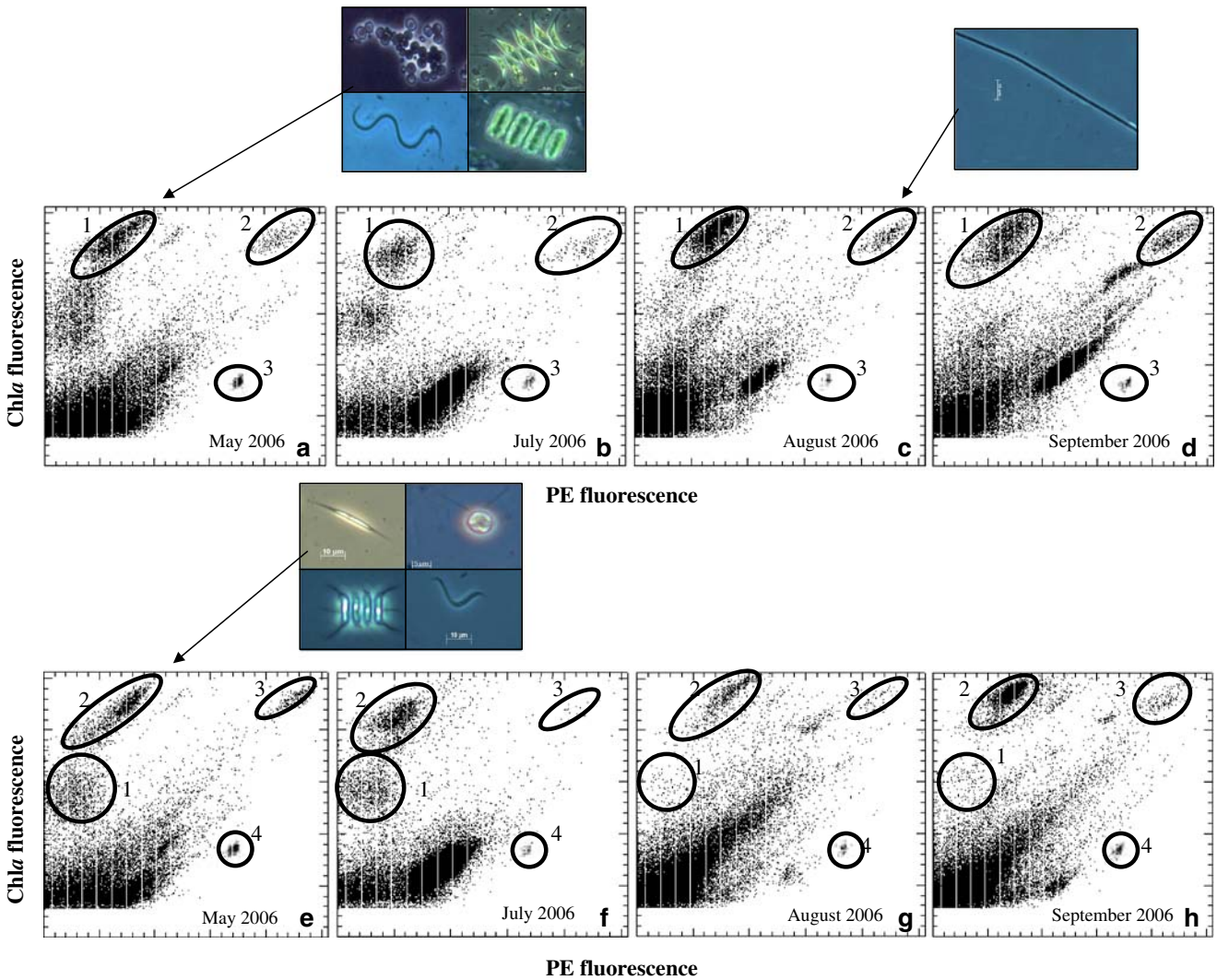

Fig. 2 PE vs. Chl*a* fluorescence cytograms showing the seasonal evolution of the phytoplankton composition (**a–d**) for two clusters isolated from station 501 of the Reservoir Marne (*1* mixed green algae community, *2 Pseudanabaena limnetica* (picture of a filament), *3* beads) and **e–h** for three clusters isolated from station 553 of the Reservoir Marne (*1 Chlorella vulgaris*, *2* mixed green algae community, *3* mixed green algae community, *4* beads). Pictures of the mixed green algae communities with *Chlorella vulgaris*, *Scenedesmus acuminatus*, *Hyaloraphidium contortum*, *S. linearis* (upper left, from up left to right down) and with *Ankyra lanceolata*, *Chlamydomonas* sp., *Scenedesmus spinosus*, *Monoraphidium* sp. (middle, from up left to right down)

mL^{-1}), and finally it turned lower again in September (4×10^3 cells mL^{-1}). A second cluster we could discriminate was the cyanobacterium *Pseudanabaena limnetica.* The position of the cluster 2 underlined high Chl*a* and PE fluorescence, probably as an indication of elevated pigment cell concentrations. This second cluster presented the same shape along the sampling season but particles seemed to form more or less dense clouds of dots, revealing the variation in abundance of this species. We observed that the number of cells decreased between May and July (from 0.5 to 0.4×10^3 cells mL^{-1}), then there was a strong increase in the abundance of this species (2×10^3 cells mL^{-1} in August) and finally a slight decrease in September (0.9×10^3 cells mL^{-1}).

Station 553 displayed more or less the same patterns as previously described for station 501 (Fig. 2e–h) but populations were less distinct. For instance, *C. vulgaris* (cluster 1) was clearly identified as an abundant cluster in May and July (3.9 and 3.5×10^3 cells mL^{-1}), but the population decreased in August and September (0.07 and 0.04×10^4 cells mL^{-1}). The second cluster was identified as a mixed green algal community that changed along the sampling season. It was first composed of *Ankyra lanceolata* and several species of *Monoraphidium* spp. (3×10^3 cells mL^{-1}). In July, *A. lanceolata* disappeared to favor the growth of green flagellates (4×10^3 cells mL^{-1}) and, as for station 501, August was characterized by the appearance of several *Scenedesmus* species (2×10^3 cells mL^{-1}). Additionally, the phytoplanktonic community was essentially composed of little flagellates, *Chlamydomonas*-like, and *Scenedesmus* species (3×10^3 cells mL^{-1}) in September. Finally, the third cluster was mainly composed of large cells in May (0.6×10^3 cells mL^{-1}) as well as in September (0.3×10^3 cells mL^{-1}). These populations were supposed to be composed of phycoerythrin-rich species, but after isolation and 3 weeks of culturing, we identified green algae species under the microscope. Unfortunately, we failed sorting and culturing this population in July and August.

The specific composition in a cluster seemed to be more or less the same along the season for a single station but clusters having the same fluorescence in cytograms from different stations were not characterized by the same species. This was typically the case for the second cluster of station 501 (filamentous cyanobacteria) and the third cluster of station 553 (mixed green algae population).

"Long-term" monitoring was not always possible because some specific clusters isolated were present at a particular time but not at the others. This was particularly true for *Cryptomonas* sp., a phycoerythrin-rich species whose abundance reached 0.8×10^3 cells mL^{-1}, that was only isolated in September in station 501 because it was not present before.

Specific determination of phytoplankton

Different clusters observed in the cytograms Chl*a* vs. PE fluorescence were clearly identified as characteristics for some species such as the filamentous phycoerythrin-rich cyanobacteria *Pseudanabaena limnetica* and *P. catenata* or the chlorophyte *Mougeotia gracillima* in Lake Geneva (Fig. 3). In Lake Bourget, we could identify clusters of the phycoerythrin-rich *P. rubescens*, *Pseudanabaena acicularis* and *P. limnetica* (Jacquet et al. 2005; Ivanikova 2006), the phycocyanin-rich cyanobacteria *Aphanizomenon flos-aquae* (Benedetti et al. 2004), and some diatoms. Note that the presence of the cluster represented by *A. flos-aquae* in the zone of phycoerythrin-rich algae was more difficult to explain but this was observed at several occasions. All these species were also the dominant taxa (in terms of density) in the natural samples observed in the microscope (data not shown).

In the Reservoir Marne, the clusters of the phycoerythrin-rich *P. limnetica* and the cryptophyte *Cryptomonas* sp. were also identified. The FCM signatures of the selected species were clearer at station 501 than at station 553 which confirmed the nutrient-enriched character of station 501. Moreover, these two species were abundant in the microscopic observations during September and probably facilitated the isolation.

Finally, looking at the cultures 3 weeks after the isolation, *Cryptomonas* sp. seemed to be healthy and motile favoring the subculturing into the specific Jaworski's medium for Cryptophytes (Jaworski et al. 1981) and the survival of this species.

Figure 3 shows that several species of filamentous cyanobacteria, both PE- and PC-rich, were easily identified in the Chl*a* vs. PE fluorescence plot, and to a lower extent, some chlorophytes and diatoms. Obviously, several signatures of these two last groups were best identified to the species level in the forward scatter vs. Chl*a* fluorescence cytograms (Fig. 4). Small species such as *C. vulgaris* presented a low FSC scatter and Chl*a* fluorescence, corresponding to probable low cell size and chlorophyll *a* cell content, respectively. In contrast, larger species such as *M. gracillima* and *Fragilaria crotonensis* showed high FSC scatter and high Chl*a* fluorescence. In this cytogram, some species belonging to different classes but with similar size and shape as is the case of *Diatoma tenuis* and *M. gracillima* which can measure between 40 and 70 μm in length and have a parallelepiped shape were also identified. In the same figure, we could also observe that some diatoms such as *F. crotonensis* and *Asterionella formosa* are in the same cluster. This result is not surprising because both elongated diatoms are found in the same size range (60–105 μm in length), and they also have the same pigmentation. In Fig. 4, several aspects are

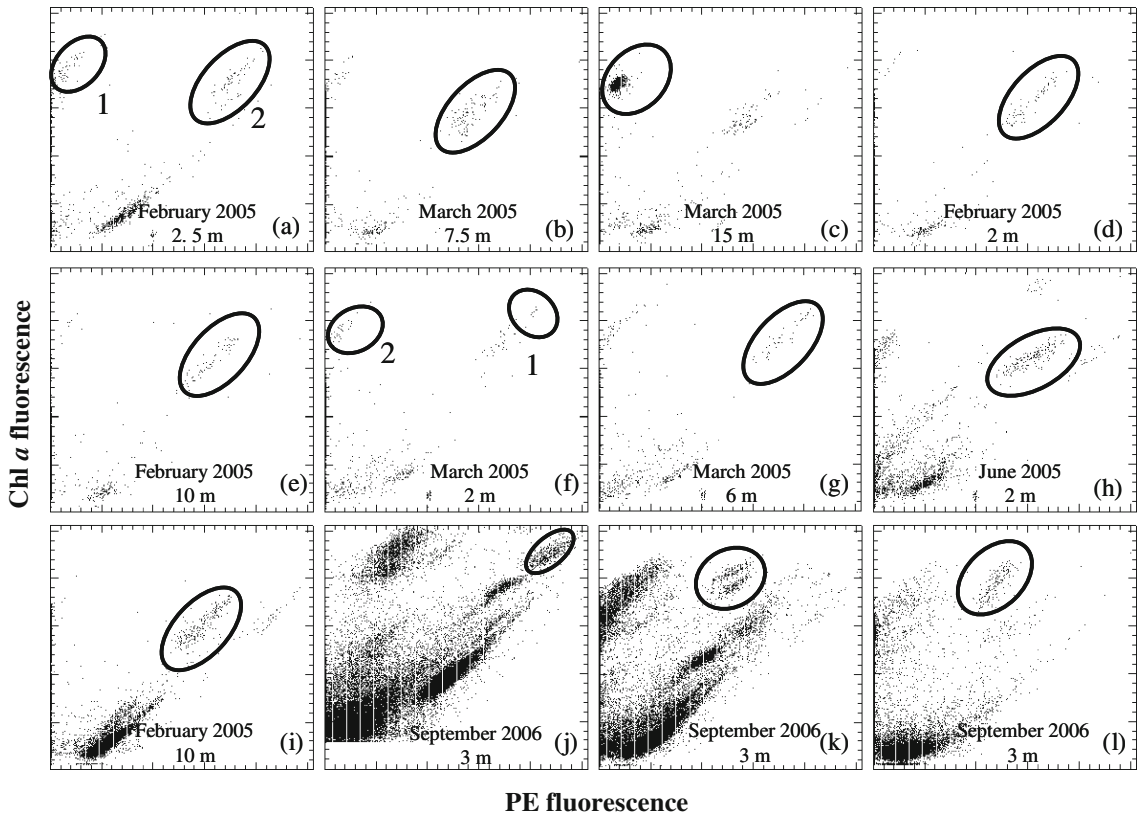

Fig. 3 PE vs. Chl*a* fluorescence cytograms showing typical signatures of phytoplankton species in the four hydrosystems. Lake Geneva: **a** *1 Mougeotia gracillima*, *2 Pseudanabaena catenata* and *P. limnetica*, **b** *Pseudanabaena limnetica*, **c** *Mougeotia gracillima*; Lake Bourget: **d** *Planktothrix rubescens*, **e** *Pseudanabaena limnetica*, **f** *1 Planktothrix rubescens*, *2* mixed diatoms (*Stephanodiscus minutulus*, *Asterionella formosa*, *Fragilaria crotonensis*), **g** *Pseudanabaena acicularis*, **h** *Aphanizomenon flos-aquae*; Lake Annecy: **i** *Pseudanabaena acicularis*; Reservoir Marne (station 501): **j** *Pseudanabaena limnetica*, **k** *Cryptomonas* sp.; Reservoir Marne (station 553): **l** *Cryptomonas* sp.

highlighted: elongated diatoms are placed in the upper part of the cytogram. Then, the signatures of the small chlorophytes *Monoraphidium contortum* and *C. vulgaris* have a specific shape and localization in the lower part of the cytogram. This was observed in several sampling dates and would allow studying the seasonal evolution of these species in the future.

Discussion

Flow cytometry and sorting have proved to be a powerful technique with many potential applications in aquatic sciences (Collier and Campbell 1999; Jochem 2000; Reckermann 2000; Yentsch et al. 1983). However, they have been mainly applied in the context of the marine field research whereas limnetic ecosystems have comparatively received less attention. Crosbie (2002) reported from a search in *Current Contents* from 1993 to 2002 (by using a battery of keywords such as flow cytometry, picocyanobacteria, picoplankton, picoeukaryotes, ocean, sea, marine, lake, river, freshwater, limnology, reservoirs, limnetic or inland waters) 151 references for "oceanography" and only 16 for "limnology". Such a ratio between "marine" and "freshwater" was even higher in 2008 while making a rapid search on ISI Web of Science from 1992 to nowadays, and using keywords such as flow cytometry OR flow cytomet* OR flow sorting AND freshwater OR marine (not shown). Here, we report one of the rare studies where FCM sorting was applied to a variety of freshwater ecosystems and can be proposed as a powerful monitoring tool.

Success of isolations

The success average of the phytoplankton isolations in the four hydrosystems was very dissimilar, being higher in the peri-alpine lakes, particularly in the oligotrophic Lake Annecy and less successful in the two eutrophic stations of the Reservoir Marne. At first sight, this result could be closely related with the trophic status of the systems, where more species coming from oligo- to mesotrophic waters could be isolated and grown on artificial media while species from rich systems were not. Was our culture medium not rich enough to maintain the species isolated

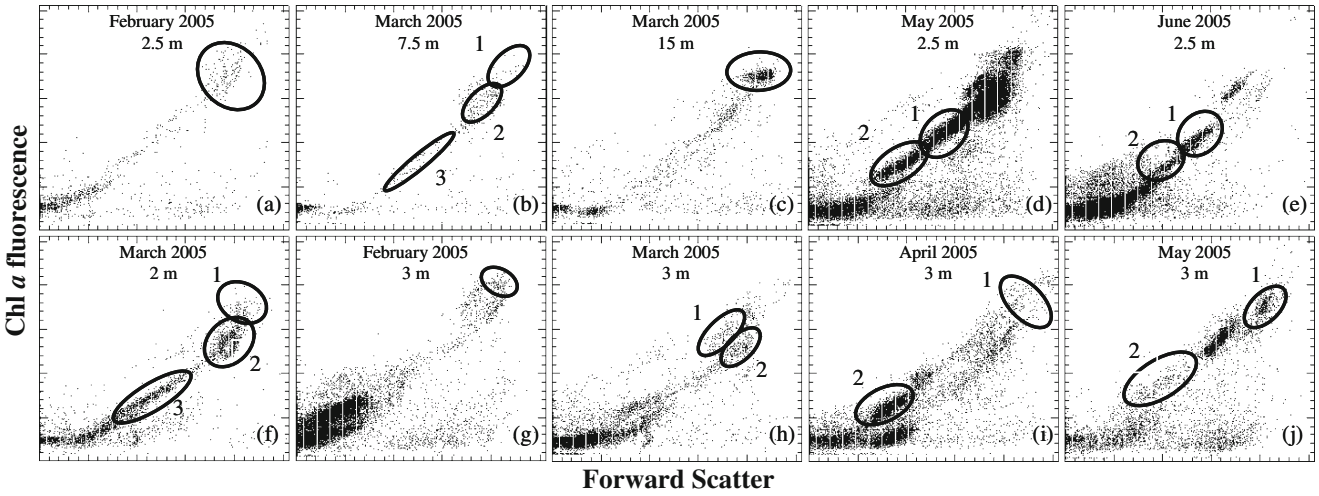

Fig. 4 Forward scatter vs. Chl*a* fluorescence cytograms showing typical signatures of phytoplankton species in the three peri-alpine lakes. Lake Geneva: **a** *Mougeotia gracillima* and *Diatoma tenuis*; **b** *1 Scenedesmus* sp. and *Schroederia setigera*, *2 Stephanodiscus minutulus*, *3 Chlorella vulgaris*; **c** *Mougeotia gracillima*; **d**, **e** *1 Monoraphidium contortum*, *2 Chlorella vulgaris*; Lake Bourget: **f** *1 Asterionella formosa*, *2 Fragilaria crotonensis*, *3 Chlorella vulgaris*; Lake Annecy: **g** *Fragilaria crotonensis*; **h** *1 Monoraphidium komarkovae*, *2 Asterionella formosa* and *Fragilaria crotonensis*; **i** *1 Asterionella formosa* and *Fragilaria crotonensis*, *2 Chlorella vulgaris*; **j** *1 Fragilaria crotonensis*, *2 Chlamydomonas* sp.

from the Reservoir Marne? We assume that this was probably not the case regarding the medium recipes. Another explanation was that a higher number of isolations were carried out in the peri-alpine lakes, and as a consequence, it was possible that we simply obtained a higher success average. However, in our study, 80% of the total sorted cultures grew successfully and 47% were monocultures. In addition, the cultures grew after only 3 weeks following the isolation.

Among possible reasons for the high success average obtained after population sorting, the good quality of the culture medium was a good explanation. In fact, such a mixture was very efficient to guarantee the growth of all isolated phytoplanktonic groups within the first 3 weeks without any visible nutrient-limited condition. It is noteworthy, however, that, after this period, a lot of cultures started to lyse. As an example, the diatom *F. crotonensis* formed chains after 3 weeks, and by using the same mixture of nutritive media in the following days, the culture decayed progressively. The only way of maintaining the population was to change gradually the mixture by a more specific culture medium as this is generally done for classical algal culturing (Grobbelaar 2004).

Applications

Seasonal evolution of Synechococcus spp.

The cluster of PE-rich *Synechococcus* was clearly identified in the four hydrosystems. This picocyanobacterial group is characterized by a specific Chl*a* vs. PE fluorescence FCM signature commonly found in most aquatic ecosystems, making its spatio-temporal monitoring possible (Crosbie et al. 2003b; Jacquet et al. 1998; Li and Dickie 2001; Partensky et al. 1996; Vaulot and Marie 1999). The presence of picocyanobacteria was important in the four ecosystems, being a persistent and abundant component of the oligotrophic Lake Annecy throughout the study, while in the two other peri-alpine lakes, this community was better represented in late spring–early summer when the thermal stratification/clear water phase took place (Anneville and Leboulanger 2001; Jacquet et al. 2005). In the Reservoir Marne, the picocyanobacteria had its maximum abundance with thermal stratification between the end of June and the end of July. In agreement with the literature, picocyanobacteria are present in lakes of different trophies but they are likely to be the dominant group all year long in oligotrophic systems whereas they took advantage of the low nutrient concentrations found in surface waters in spring and summer in mesotrophic lakes (Callieri 2008; Weisse 1993). Several authors have suggested that small phytoplankton cells do best in resource-poor habitats. This phenomenon has been linked to the greater surface area to volume ratio of small organisms promoting efficient uptake of nutrients, which gives them a competitive advantage over larger organism when nutrients are scarce (Drakare et al. 2003; Raven 1998). Thus, this community may be of particular interest to monitor the trophic status evolution of freshwater ecosystems, and FCM is clearly the best tool to obtain precise counts and non-time-consuming short-term dynamics of this “canary in the coal mine” (Schallenberg and Burns 2001; Weisse and Mindl 2002).

Following the same idea, FCM signatures of *Synechococcus* in Lake Annecy were characterized by a long "tail" which had been reported in other lakes as a consequence of high proportions of colonial forms of these picocyanobacteria (Collier 2000). The presence of these colonial forms or aggregates was confirmed by epifluorescence microscopy (Fig. 1 A, B) and this was attributed to oligotrophic conditions or nutrient depletion during the maximal thermal stratification in summer as a strategy for more efficient nutrient recycling (Passoni and Callieri 2000; Schallenberg and Burns 2001; Stockner and Shortreed 1991). Stockner (1991) also suggested that this strategy could be an anti-predator mechanism. Our data suggest that the high concentration of picocyanobacteria aggregates observed in Lake Annecy is a consequence of the low nutrient concentrations of this lake. Once sorted, such colonies were generally disrupted so that it is likely that studying the proportion of colonial vs. single cells of this community will still require epifluorescence microscopy or more powerful FCM.

Seasonal evolution of other phytoplankton groups

Identification of the seasonal evolution and succession of the different phytoplankton groups identified in the Reservoir Marne was not easy since most of the reservoir is empty from October to February, and consequently, sampling cannot be performed along the year. Although sorting experiments were thus conducted only from late spring to early autumn, a lot of information can be drawn from the results obtained from the phytoplankton composition and abundance inside the different clusters. The first cluster identified in station 501 and the second cluster isolated in station 553 (Fig. 3) contained species that changed from 1 month to another. These species belonged to the chlorophytes that mainly contain chlorophyll *a* and *b* pigments and lack other accessory pigments which explains their position in the FL3 (Chl*a* fluorescence) axis (Nozaki 2003; Wetzel 2001). *Monoraphidium* spp. were observed in May, July, and September in station 501 as well as in May in station 553. For both stations, several species of *Scenedesmus* spp. were found in August and also in September for station 553. In addition, *A. lanceolata* was isolated in May in the second cluster of station 553 (Fig. 3) and a filamentous cyanobacterium, *P. limnetica*, was isolated from station 501. This last species is optically defined as a pale blue-green algae whose phycocyanin (Ivanikova 2006; Komárek and Anagnostidis 2005) is responsible for an olive blue-green color. Moreover, the species *Cryptomonas* sp. has an elevated PE fluorescence and has been isolated in September from station 501. Finally, in station 553, the third cluster was composed of large cells that exhibited a high FL2 fluorescence associated with phycoerythrin. Unfortunately, after 3 weeks of culturing, green algae were largely present in the flasks. Thus, it is possible that we isolated cells with a large quantity of phycoerythrin but the culture was probably "contaminated" with green algae that rapidly outcompeted these phycoerythrin-rich species as it is explained for *Chlorella* (see below). It is also possible that the mixed medium was not strongly adapted to the phycoerythrin-rich species and allowed green algae to take advantage of these conditions.

One more aspect that can be highlighted is the difficulty to foresee specific monitoring if the species change along time within a same cluster despite a regular fluorescence pattern. In fact, communities referred to as the "mixed green algae" were, on one hand, composed of different species and, on the other hand, the diversity changes among seasons. On the contrary, clusters containing *C. vulgaris* or *P. limnetica* could be prone to an annual monitoring as these species maintained from one date to another and information about the abundance of these species could be obtained.

Specific identification of algae

Even if several authors have pointed out the limitations of flow cytometry to discriminate phytoplankton species (Collier 2000; Dubelaar and Jonker 2000), the isolation of the most abundant species from the four systems studied (excepted for the chrysophytes and colonial cyanobacteria) was possible using the flow cytometry sorting option. Since the clusters of several taxa were here well-discriminated (*Synechoccoccus* sp., *P. limnetica*, *C. vulgaris*, *M. contortum*, *M. gracillima*, *P. rubescens,* etc.), numerous studies could be carried out in the future using this simple methodology. One of the largest interests is the study of the seasonal trends of the main phytoplankton species as had been shown by several authors before (Hofstraat et al. 1994; Rutten et al. 2005) without the time-consuming microscopic analysis. However, we should keep in mind that the diversity of algae found in the field is larger than that possibly isolated using FCM sorting. In any case, if the analysis of major functional groups of phytoplankton needs to be routinely done, this methodology offers obviously higher frequency analysis in both time and space (Dubelaar and Jonker 2000). Another advantage of the sorting method is the possibility to isolate specific clusters (species) from an algal mixture in order to study their physiological, ecological, or genetic features (Jochem 2000), and in the case of potentially toxic species (i.e., *P. rubescens*), to detect quickly their presence and take preventive measures.

Early warning

The accurate identification of harmful algal clusters using FCM represents an important tool for detecting the low-

level occurrence of toxic species, giving early warning of the probability of bloom development (Dubelaar and Jonker 2000). As an example described by Rutten et al. (2005), routine analyses using FCM allowed them to identify a bloom of the harmful species *Phaeocystis* spp. in Dutch coastal waters. As a result of improved monitoring, the sluice gate was closed to prevent the inflow of this haptophyte in a contiguous lake. In our study, several clusters of potentially toxic filamentous cyanobacteria were clearly identified, as is the case of the phycoerythin-rich *P. rubescens* and in a lesser extent the phycocyanin-rich *A. flos-aquae* in Lake Bourget. The first can produce hepatotoxins and forms blooms in this lake since 1996, at least during summer and autumn periods (Jacquet et al. 2005), and the second one is a potential producer of the neurotoxin anatoxin-a (Rapala et al. 1993). In this way, potentially toxic species can be quickly detected and enumerated by flow cytometric routine analysis to determine if their concentrations represent a public health risk. This could be applied to *P. rubescens* dynamics in Lake Bourget (Jacquet, unpublished). Furthermore, flow cytometer sorting allows the effective isolation of the harmful algae and their culture with high success average to carry out physiological or molecular studies, which provide rapid and sensitive diagnoses for the presence of toxic and toxigenic cyanobacteria (Ouellette and Wilhelm 2003).

Limitations for the isolation of specific groups

We were not able to cultivate colonial cyanobacteria such as *Aphanocapsa* or chrysophytes such as *Dinobryon*, which were, however, frequently observed by microscopy whatever the lake considered. In the case of the colonial cyanobacteria, shear forces from the flow cytometer may have caused the breaking of the cell aggregates (Rutten et al. 2005), making difficult its development after sorting. In addition, Pel et al. (2004b) mentioned that colonial phytoplankton does not well support cell sorting. In the case of the delicate siliceous wall of *Dinobryon*, several hypotheses could be proposed. Chrysophytes are characterized and dominated by yellow-brown xanthophyll and carotenoid pigments that hide the green color of chlorophyll *a* (Wetzel 2001). Thus, it is likely that the FASCalibur was not calibrated to easily detect such kind of pigments in contrast to phycobilin, phycoerythrin, chlorophyll *a*, and phycocyanin if using the optional fourth fluorescence. In addition, the FACSCalibur flow cytometer is not suited to sort particles whose size exceeds 130 μm. *Dinobryon* and *Aphanocapsa* colonies can reach 130 and 300 μm, respectively, so that it may have been possible that the diameter of the flow sorting tube was not large enough to allow particle absorption and selection. Cryptophytes also were very difficult to isolate in the peri-alpine lakes while this group at time could be relatively abundant. Isolation was possible from samples of the Reservoir Marne. Actually, deformation of cell membranes is frequently observed in cryptophyte cells after preservation with Lugol's fixative (Rutten et al. 2005). Probably, membranes of cryptophytes are also very sensitive to the sorting process, more especially to the mechanical stream capture. This could result in a high loss of cells' viability after sorting and maybe the reason why we did not succeed in the isolation of this group in some occasions. Such a hypothesis was checked before (in the sample) and after selection and sorting of PE-rich cells by using direct cell observation under the microscope. We observed indeed some cells of *Cryptomonas* to be very damaged. We also think that this membrane integrity loss could be linked to nutrient concentration and osmotic stress, since replacing the media by filtered lake water may conduct to a better cell recovery (Rimet, personal communication). In the future, this aspect should probably deserve more attention. Indeed, an isolation technique that could be more appropriate for cryptophytes is the application of serial dilutions (Landry and Hassett 1982), consisting in adding a certain volume of an enriched sample to a lesser one until isolation of a single cell be done. The streak plating, which is another classical microalgal isolation technique, is probably more suitable for small species (<10 μm, typically nano- rather than microalgae) or algae that grow well on a substrate (Stein 1973).

Finally, we can point out that the FACSCalibur is not really designed to make precise cell sorting. In fact, this flow cytometer presents an optional sorting module but it is not a sorter as a whole. Consequently, the inability to track and monitor some of the dominant populations in the water system is more than a significant limitation. It is likely that using an advanced cell sorting function might be useful and more appropriate although we obtained a large success. To cite some examples, the FACSVantage (Becton Dickinson) or related device (FACSAria of the Influx) would probably provide a better cell sorting frequency because of increased speed, accuracy, and reliability and allowing sorting cells larger than previously mentioned with the FACSCalibur. Moreover, up to 16 cell populations can be identified using gates of various sizes and shapes, grouped in logical combinations and overlaps with no performance loss of sort or analysis rates (Mollet et al. 2008).

Sample contamination

Some of the samples were contaminated by "contaminant" species such as *Chorella vulgaris* or *Choricystis minor*. While analyzing the different cultures after a few days using FCM and/or microscopy, some cultures were indeed observed to be made of one dominant species (the one we selected) and some cells of *Chlorella*. Rapidly, such samples

were only made of the non-desired chlorophyte. This replacement phenomenon has already been observed during an experiment dealing with competition for light between some phytoplankton species (Huisman et al. 1999). They found that in a steady-state monoculture of *Scenedesmus* inoculated by a small number of *Chlorella*, the latter was able to invade. Conversely, *Scenedesmus* was not able to invade a steady-state culture of *Chlorella*. Moreover, in an experiment where four species were inoculated together, *Chlorella* became the final dominant species after a few days. This confirms that *Chlorella* has a critical advantage over other phytoplankton species in culture. Even more, it can outcompete cyanobacteria under low light conditions. The dominance of chlorophytes on cyanobacteria has also been identified in other culture conditions. For example, it has been shown that *Prochlorothrix* competitively displaced *Planktothrix* under both a constant and a pulse phosphorus supply (Ducobu et al. 1999). Moreover, another invasion experiment between two species from the same taxonomic class has highlighted that *F. crotonensis* could invade *Tabellaria fenestrata* cultures, but that *Tabellaria* could not invade *Fragilaria* cultures under phosphate-limited conditions (Tilman and Sterner 1984). Thus, depending on environmental conditions, some species can take advantage and dominate the medium. What could explain the dominance of *Chlorella* or *Choricystis* in our cultures was clearly the non-limiting nutrient conditions offered to this small size group. It has been demonstrated indeed that for a single-celled organism the surface is a critical interface between the organism/cell and its environment. Exchange of materials often occurs through the process of diffusion in which dissolved molecules or other particles move from areas of higher concentration to areas of lower concentration (although some exchange is mediated by cellular mechanisms). This type of exchange is a passive process and, as a result, imposes constraints upon the size of a single-celled organism. Materials must be able to reach all parts of a cell quickly and, when volume is too large relative to surface area, diffusion cannot occur at sufficiently high rates to ensure this (Schmidt-Nielson 1984). With this in mind, it is likely that *Chlorella* took advantage of its high surface to volume ratio and dominates species whose ratio is lower (Drakare et al. 2003). Finally, light was also likely to favor a green alga such as *Chlorella* since it was not limiting both in terms of quality and quantity.

In conclusion, as for medical sciences where FCM monitoring represents a valuable survey analytical tool or, more recently, in phytoplankton marine ecology when deployed on moored systems (e.g., Thyssen et al. 2008), we showed here that FCM could be applied to limnetic survey after simple species identification using the sorting function. In fact, this technique could be applied in some specific cases, for example, the monitoring of a blooming species, the study of the physiological state of some species, or the impact of chemical contaminants (pesticides, metals) on species growth rate. The high time-consuming microscopical determination and counting of phytoplankton limit sampling frequencies below an acceptable level for the monitoring of dynamic ecosystems such as lakes or reservoirs. Although having a limited discrimination power, we showed that simple (and relatively low cost) benchtop FCM may allow the analysis of large numbers of samples and, after taxonomic identification of selected sorted cells by microscopy, can constitute a basic tool for (further) key species bio-monitorings. For this purpose, flow cytometers should not be limited to research laboratories and water managers could be trained for the use of such a technology. As pointed out by Jochem (2000), we strongly believe that "flow cytometry is still in its infancy", especially as regards to phytoplanktonic survey in freshwater ecosystems.

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