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ORIGINAL ARTICLE

Vertical structure of small eukaryotes in three lakes that differ by their trophic status: a quantitative approach

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In lakes, the diversity of eukaryotic picoplankton has been recently studied by the analysis of 18S ribosomal RNA gene sequences; however, quantitative data are rare. In this study, the vertical structure and abundance of the small eukaryotic size fraction (0.2–5 μm) were investigated in three lakes by tyramide signal amplification–fluorescent *in situ* hybridization targeting six phylogenetic groups: Chlorophyta, Haptophyta, Cercozoa, LKM11, Perkinsozoa and fungi. The groups targeted in this study are found in all lakes; however, both the abundance and structure of small eukaryotes are dependent on the system's productivity and depth. These data highlighted the presence of Chlorophyta contributing on an average to 19.3%, 14.7% and 41.2% of total small eukaryotes in lakes Bourget, Aydat and Pavin, respectively. This study also revealed the unexpected importance of Haptophyta, reaching 62.8% of eukaryotes in the euphotic zone of Lake Bourget. The high proportions of these pigmented cells highlight the underestimation of these groups by PCR-based methods. The presence of pigmented Chlorophyta in the deepest zones of the lakes suggests a mixotrophic behaviour of these taxa. We also confirmed the presence of putative parasites such as Perkinsozoa (5.1% of small eukaryotes in Lake Pavin and Bourget) and, with lower abundances, fungi (targeted by the MY1574 probe). Cells targeted by LKM11 probes represented the second group of abundance within heterotrophs. Open questions regarding the functional roles of the targeted groups arise from this study, especially regarding parasitism and mixotrophy, which are interactions poorly taken into account in planktonic food web models.

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Introduction

Although small eukaryotes are less numerous than their prokaryotic counterparts, they have a major role in biogeochemical cycles, especially in global carbon cycling in marine environments (Li, 1994; Liu *et al.*, 2009) and in microbial food web processes (Caron *et al.*, 1999). Despite their ecological importance, small eukaryotes (<5 μm) have remained poorly described because of their small size and lack of morphological characteristics. However, in the last few years, because of the development of molecular techniques and new cultivation approaches, researchers have started to perform diversity and spatial distribution analyses of marine and, to a lesser extent, of freshwater small eukaryotes (for example, see López-Garcia *et al.*, 2001; Massana *et al.*, 2004; Lefranc *et al.*, 2005; Not *et al.*, 2008, Lepère *et al.*, 2008). These studies, based on 18S ribosomal RNA (rRNA) gene sequence analyses, revealed considerable diversity and the existence of novel groups of sequences unrelated to cultured and sequenced organisms. Surveys of picoplanktonic protists, by sequencing the 18S rRNA gene in different environmental settings, have shown similar diversity patterns (Massana and Pedrós Alió, 2008; Worden and Not, 2008), with dominance of non-photosynthetic groups, including tiny parasites (Guillou *et al.*, 2008; Lepère *et al.*, 2008) and grazers (Massana *et al.,* 2006). In most conventional studies of marine plankton, these organisms were usually lumped in a black box labelled 'small heterotrophic

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flagellates'. In contrast to the 18S rRNA gene clone libraries data, epifluorescence microscopy typically reveals a dominance of photosynthetic or mixotrophic cells over heterotrophic cells in the ocean (ca 80% vs 20%, respectively; Jurgens and Massana, 2008). Undeniably, there is a lack of congruity between microscopic and molecular analyses. This highlighted the fact that sequences deposited in databases may give a significantly biased view of diversity, especially with regard to pigmented cells. Alternative approaches that help to assess the diversity of pigmented populations in more detail have recently been developed. These include the construction of clone libraries from flow cytometry-sorted populations (Shi *et al.*, 2009), studies specifically targeting plastid genes (Fuller *et al.*, 2006; Lepère *et al.*, 2009) and the use of taxon-specific primers (Viprey *et al.*, 2008). However, PCR biases and potential issues with this approach remain.

Fluorescent *in situ* hybridization coupled with tyramide signal amplification (TSA–FISH) seems to be a good alternative, as it does not require any PCR amplification step. However, it is important to note that great care needs to be taken with regard to the specificity of probes, to avoid false-positive or -negative results, which lead to under or overestimation of the number of cells theoretically targeted. Even though most of the data (sequences from environmental samples) were obtained in oceanic systems, several recent studies conducted in lacustrine environments allowed us to obtain a reasonable amount of sequences (Lefranc *et al.*, 2005; Richards *et al.*, 2005; Lefèvre *et al.*, 2008; Lepère *et al.*, 2007, 2008). These data allowed the design of specific oligonucleotide probes for FISH analyses to investigate the extent of the seasonal distribution and abundance of these uncultured protists in a lacustrine euphotic zone (Mangot *et al.*, 2009). These first quantitative data brought new insight into the diversity of lacustrine small eukaryotes, especially by highlighting the diversity within heterotrophic eukaryotic assemblages, and the relative importance of pigmented cells. However, we clearly need to investigate the structure and *in situ* abundances of eukaryotic groups more extensively, which have been revealed by recent studies to be important in lakes.

In this study, the diversity and vertical distribution of small eukaryotes (0.2–5 μm) were examined by TSA–FISH in three lakes that differ mainly by their trophic status (oligomesotrophic, mesotrophic and eutrophic). We report here the quantification of these small eukaryotes using both newly designed probes (Mangot *et al.*, 2009) targeting Cercozoa, Perkinsozoa, LKM11 and also previously published probes targeting Haptophyta, Chlorophyta and fungi (Simon *et al.*, 2000; Baschien *et al.*, 2008).

Materials and methods

Study site and sampling

The study was conducted in three lakes: the oligomesotrophic Lake Pavin (Puy de Dôme, France; 45°29′45″ N, 2°53′18″ E), the mesotrophic Lake Bourget (Haute Savoie, France; 45°43′55″ N, 5°52′06″ E) and the eutrophic Lake Aydat (Puy de Dôme, France; 45°39′50″ N, 2°59′04″ E). Lake Pavin, located at an altitude of 1197 m, is a typical crater mountain lake (meromictic) with a maximum depth of 92 m. Lake Aydat was formed when a lava flow dammed the small river Veyre. It is a dimictic lake with a maximum depth of 15 m, situated at an altitude of 825 m. Lake Bourget is located in eastern France on the edge of the Alps (altitude 231.5 m). It is a warm, monomictic lake and constitutes France's largest natural reservoir with a maximum depth of 145.4 m (mean depth of 81 m). The main physical and chemical characteristics of these three lakes are reported in Table 1.

We chose to carry out TSA–FISH analysis during the thermal stratification of the lake to detect possible vertical contrasts in the structure of eukaryotic assemblage. Samples were collected at different depths on 4 July 2006, 26 June 2006 and 5 July 2006 for lakes Pavin, Bourget and Aydat, respectively. Sampling was carried out at a permanent station located at the deepest zone of the water column. Water samples (from 15 to 100 ml depending on the lake) were prefiltered through 5 μm-pore-sized polycarbonate filters (Millipore, Molsheim, France) at a very low vacuum to prevent cell damage and kept in 150-ml plastic bottles for less than 2 h during transport until processing in the laboratory for microbial collection.

Table 1 Main physical-chemical characteristics of the different lakes sampled

	Coordinates	*Trophic status*	*Maximum depth*	*NO_3-N ($mg\,N\,l^{-1}$)*[a]	*NH_4-N ($mg\,N\,l^{-1}$)*[a]	*PO_4-P ($mg\,P\,l^{-1}$)*[a]	*Water clarity*[a]
Bourget	45°43′55″ N, 5°52′06″ E	Mesotrophic	145.4	0.57	0.002	0.01	7.2
Pavin	45°29′45″ N 2°53′18″ E	Oligomesotrophic	95	0.15	0.035	0.02	6.9
Aydat	45°39′50″ N, 2°59′04″ E	Eutrophic	15	0.21	0.009	0.02	1.9

[a]annual means integrating all water column.

Table 2 Oligonucleotide probes used in this study

Probes	*Sequence (5′–3′) of probes*	*Specificity*	*References*
EUK1209R	GGGCATCACAGACCTG	Eukaryota	Giovannoni *et al.* (1988)
CERC_02	AATACGAGCACCCCCAAC	Cercozoa	Mangot *et al.* (2009)
NCHLO01	GCTCCACTCCTGGTGGTG	NonChlorophyceae	Simon *et al.* (1995)
CHLO01	GCTCCACGCCTGGTGGTG	Most Chlorophyta/some nonChlorophyta	Simon *et al.* (1995)
CHLO02	CTTCGAGCCCCCAACTTT	Chlorophyta	Simon *et al.* (2000)
PRYM02	GGAATACGAGTGCCCCTGAC	Haptophyta	Simon *et al.* (2000)
LKM11_01	TACTGTCACTACCTCGCC	LKM11	Mangot *et al.* (2009)
LKM11_02	TGGTCCTCAAACCAAC	LKM11	Mangot *et al.* (2009)
MY1574	TCCTCGTTGAAGAGC	Fungi (Eumycota)	Baschien *et al.* (2008)
PERKIN_01	GAGGATGCCTCGGTCAA	Perkinsozoa	Mangot *et al.* (2009)
PERKIN_02	GCCAAACATTG TACTGCG	Perkinsozoa	Mangot *et al.* (2009)

The filtrate obtained was fixed with 4% (final concentration) formaldehyde and incubated at 4 °C for 1 h. Fixed cells were then collected on 0.2 μm-pore polycarbonate filters (Millipore; pressure <150 mbar). These cells (size fraction 0.2–5 μm) were subjected to TSA–FISH counts and 4'-6-diamidino-2-phenylindole (DAPI) staining. The filters were preserved by dehydration in an ethanol series (50%, 80% and 100% for 3 min each) and stored at −20 °C in the dark until analysis.

Water temperature and dissolved oxygen concentration were determined with a multiparameter probe (YSI GRANT 3800, Grant Instrument, Cambridge, UK). Chlorophyll *a* concentrations were obtained by spectrophotometry, as described previously by Strickland and Parsons (1972).

Counts of bacteria and eukaryotic groups (size fraction <5 μm) using TSA–FISH

Probe design, TSA–FISH and DAPI staining. Probes were designed with the Probe_Design option of the ARB program package (http://www.arb-home.de) as described in Mangot *et al.* (2009). They were tested in a gradient of formamide (0–50%) in hybridization buffer at constant temperature (35 °C). According to observations, 40% formamide gave the best results of hybridization and was therefore used for routine counting. To screen for any specific probes, these new probes were tested on lacustrine eukaryotic cultures (Chlorophyceae, Chrysophyceae, Cryptophyceae, Cyanophyceae, Bacillariophyceae, Cercozoa and Dinophyceae). When cultivated organisms were not available for positive testing (Perkinsozoa, LKM11), probes were validated using only bioinformatics tools and negative controls.

TSA–FISH was carried out exactly as described in Lepère *et al.* (2008), following the hybridization conditions initially described by Not *et al.* (2002). Hybridized cells were visualized under blue light (490/515 nm) with a Nikon Eclipse TE200 epifluorescence microscope (Nikon France, Champigny sur marne, France) fitted with a mercury light source at ×100 magnification. For each sample, at least 50 randomly chosen microscopic fields were analysed and counted manually (on an average, a minimum of 50 cells were counted). These specific probes targeting six phylogenetic groups of freshwater eukaryotes are described in Mangot *et al.* (2009) and are listed in Table 2. Coupled with TSA–FISH, filters were stained by DAPI (5 μg ml^{-1} final concentration) as described in Masquelier and Vaulot (2008) allowing eukaryote and bacteria count. Under UV light (350/461 nm), the eukaryotic cell nucleus appeared as a separate organelle, whereas prokaryotic organisms appeared as cells uniformly stained without visible nucleus. These counts allowed the direct comparison of the number of eukaryotic cells stained by DAPI and those targeted by the mix of EUK1209R, CHLO01 and NCHLO01 probes.

Statistical analysis. To investigate the relationships between the vertical distribution of small eukaryotes measured by TSA–FISH and physico-chemical and biological parameters, canonical correspondence analysis (CCA) was used (Ter Braak, 1986). Variables taken into account to explain the distribution of small eukaryotes included temperature, dissolved oxygen, depth, bacterial abundance and chlorophyll *a*. These statistics were computed with R software using the Vegan package for CCA and related methods (http://www.cran.r-project.org/).

Results and discussion

Main physico-chemical and biological characteristics of the three lakes

Temperature profiles confirm that our sampling period corresponded to the thermal stratification of lakes, with a relatively constant temperature in the hypolimnion. The hypolimnion zone starts from 8 m, 25 m and 7 m for Pavin, Bourget and Aydat lakes, respectively (Figure 1). The transparency values (Secchi depths) for the three lakes were 3.0 m, 7.3 m and 1.9 m for lake Pavin, Bourget and Aydat, respectively. Secchi depth (Z_s) measurements were used to estimate the euphotic depth (Z_{eu}), according to the relationship $Z_{eu} = 2.42 \times Z_s$ (Wetzel and Likens, 1995). The euphotic zone extended over

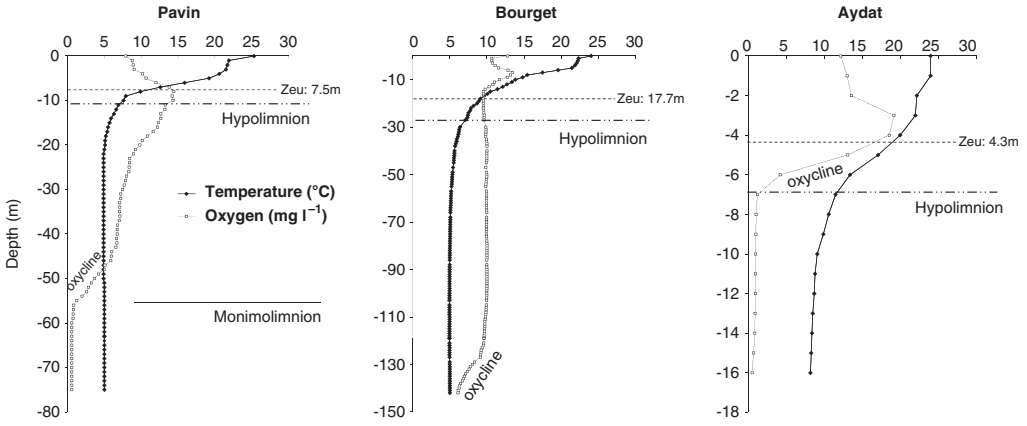

Figure 1 Vertical profiles of temperature (°C) and oxygen concentration (mg l^{-1}). The dotted line corresponds to the limit of the hypolimnion. Zeu corresponds to the limit of euphotic zone.

one-third of the mixolimnion in lake Pavin and Aydat (Zeu: 7.3 m and 4.6 m, respectively), whereas in Lake Bourget, it reached 30 m depth, extending to the limit of the hypolimnion. Dissolved oxygen concentration ranged in the epilimnion on an average from 11 mg l^{-1} in Lake Bourget to 13.5 mg l^{-1} in lakes Pavin and Aydat. A clear depletion in oxygen characterized the deepest points sampled in Lake Aydat and in the monimolimnion of Lake Pavin (Figure 1).

For all lakes, maximal total bacterial abundances are found in the euphotic zone, especially in Lake Aydat, with a maximum of 10.7×10^6 cells ml^{-1} at 5 m (Figure 2). Bacterial concentration ranged from 0.58 to 6.1×10^6 cells ml^{-1} (average: 2.8×10^6) and from 0.76 to 4.18×10^6 cells ml^{-1} (average: 1.9×10^6) in lakes Pavin and Bourget, respectively. Mean chlorophyll *a* values (average: 6.99 µg l^{-1} (4.1–11.2 µg l^{-1})) were higher in Lake Aydat. The highest values were reached below the euphotic zone in Lake Aydat. No fluctuation of chlorophyll *a* concentration was recorded in Lake Pavin (average: 0.4 µg l^{-1}), whereas in Lake Bourget, a peak was recorded at 10 m depth (9.9 µg l^{-1}), close to the thermocline (Figure 2).

Abundance, diversity and vertical distribution of small eukaryotes (<5 µm-size fraction)

The abundance of small eukaryotes (targeted by the mix of EUK1209R, CHLO01 and NCHLO01 probes) varied on an average from 5285 cells ml^{-1} (1414–9428 cells ml^{-1}, Pavin) to 31593 cells ml^{-1} (16452–42832 cells ml^{-1}, Aydat; Figure 2). A clear gradient of densities is observed from the top to the bottom in Lake Aydat, whereas rather stable abundances are recorded along the water column in Lake Pavin. A significant peak of abundance is recorded at 10 m depth in Lake Bourget. In the hypolimnion, the abundance supported average cell numbers of 3159 cells ml^{-1} and 25 353 cells ml^{-1} for lakes Pavin and Aydat, respectively. These values are comparable to those obtained previously in ocean or lacustrine environments and are higher in eutrophic conditions (Lepère *et al.*, 2008; Not *et al.*, 2008; Mangot *et al.*, 2009).

To estimate the percentage of cells targeted by the mix of probes EUK1209R, CHLO01 and NCHLO01, we compared these quantifications of small eukaryotes with those obtained with DAPI staining. On an average, 84% of total small eukaryotes positive by DAPI staining were detected with the mix. Moreover, a significant correlation could be drawn between the results obtained by both methods ($r = 0.98$, $P < 0.05$). In lakes Pavin and Bourget, the proportions of small eukaryotes identified by our probes compared with total eukaryotes (targeted by the mix of probes EUK1209R, CHLO01 and NCHLO01) are high, especially in the upper layers (epilimnion and metalimnion), in which the rates reached 72% and 76%, respectively; the lowest rates are obtained in the deepest sampling points. In the eutrophic system (Aydat), the proportions of eukaryotes targeted by specific probes are lower than those obtained in Bourget and Pavin (average of targeted cells: 25.5% of eukaryotes detected by the mix of probes), suggesting that a majority of small eukaryotes belong to other phylogenetic groups, such as ciliates, cryptophytes, chrysophytes, which are groups previously detected in this planktonic size class (Richards *et al.*, 2005; Lepère *et al.*, 2008). As a consequence, it seems that (i) the assemblages of small eukaryotes in mixoliminion in the eutrophic system are different from those observed in the oligo and oligo-mesotrophic lakes; (ii) the deepest point of the water columns, close to the benthic zone, is composed of a specific assemblage.

Pigmented small eukaryotes: unexpected importance of Chlorophyta and Haptophyta

Previous studies conducted in lakes using the cloning–sequencing method with primers targeting gene coding for 18S rRNA highlighted that

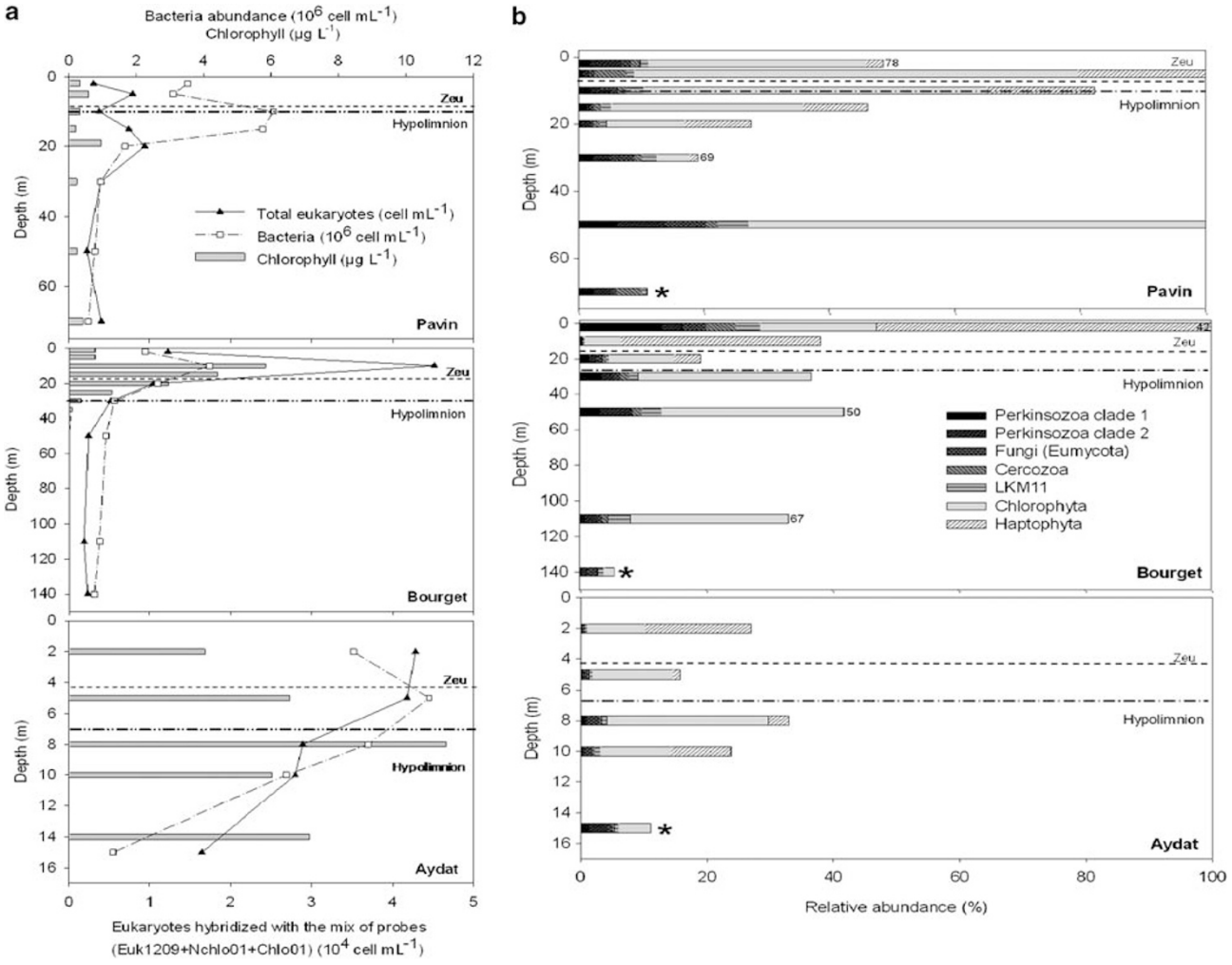

Figure 2 (**a**) Abundances of small eukaryotes targeted by the mix of probes (Euk1209 + NCLO01 + CHLO01; cells ml^{-1}; ▲), bacteria (10^6 cells ml^{-1}) and chlorophyll concentration (µg l^{-1}; grey horizontal bar). (**b**) Relative abundance of cells targeted by the oligonucleotide probes Chlorophyceae, Prymnesiophyceae, fungi, LKM11, Perkinsozoa clade 1, Perkinsozoa clade 2, Cercozoa (%: proportion of the mix (Euk1209 + NCLO01 + CHLO01)). * Depth at which Prymnesiophyceae (Haptophyta) were not counted. Numbers correspond to hybridization percentage when the mix (Euk1209 + NCLO01 + CHLO01) allowed targeting <80% of the DAPI (lowest values indicate a lower efficiency of FISH staining). Zeu corresponds to the euphotic zone.

Chlorophyta and Haptophyta sequences were always present at a very low proportion in clone libraries (Richards *et al.*, 2005; Chen *et al.*, 2009; Lefèvre *et al.*, 2008; Lepère *et al.*, 2008). In Lake Bourget, only one Haptophyta and five Chlorophyta sequences were retrieved in libraries constituted from epilimnic samples (Lepère *et al.*, 2008). However, using FISH (CHLO02 probe), Mangot *et al.* (2009) showed during a 1-year study that Chlorophyta were well represented, accounting for 17.9% of small eukaryotes in Lake Bourget. Similarly, Chen *et al.* (2009) observed, by morphological observations, that some pigmented taxa, such as *Tetraedron* sp., were abundant in the Meiliang Bay, but were not detected by molecular analysis. All these data suggest that 18S rRNA gene-based studies may underestimate pigmented cells in samples, and because of their limitations, molecular approaches based on PCR may not reflect the real diversity. Our quantitative approach by the TSA–FISH method (Figure 3) confirmed these potential PCR biases, revealing the relative importance of Chlorophyta and Haptophyta in all three lakes studied.

The highest abundances are recorded in the eutrophic Lake Aydat, with a maximum of 7857 cells ml^{-1} at 8 m depth and a mean abundance of 6213 cells ml^{-1}; however, on an average, proportions of Chlorophyta are highest in the mesotrophic lake (Bourget), contributing to 19.3% of total eukaryotes. In the three lakes, the highest abundances were observed in the first 10 metres, but not close to the surface (maximal abundances at 5 m, 10 m and 8 m for Pavin, Bourget and Aydat, respectively). Statistical analysis such as CCA allow to link environmental variables to a diversity data set. CCAs globally reveal that the presence of Chlorophyta is linked to temperature and bacterial abundance (Figure 4); however, in the case of Lake

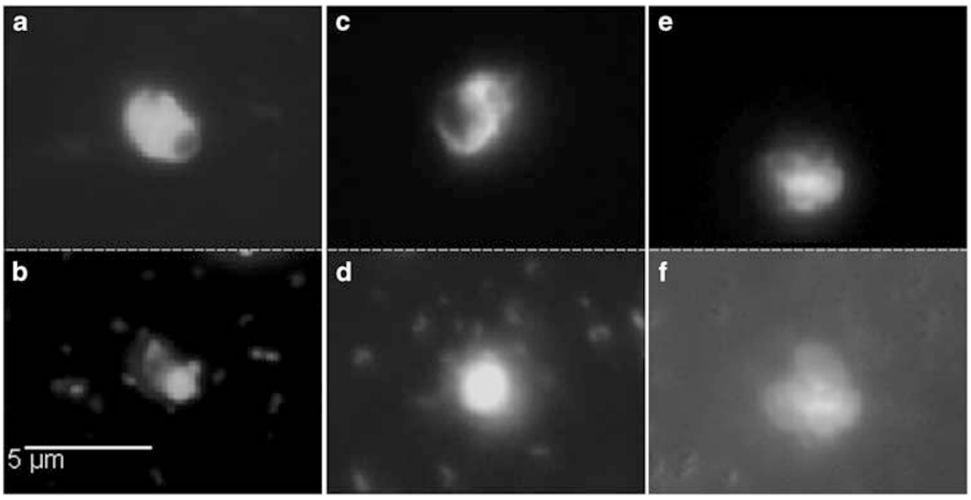

Figure 3 Epifluorescence micrographs of Perkinsozoa (**a**), Cercozoa (**c**) and Haptophyta (**e**) cells targeted by PERKIN_01, CERC_02 and PRYM02 probes. (**b**), (**d**) and (**f**) show the overlay of the same cell observed under UV (showing blue nucleus after DAPI staining). Scale bar is always 5 µm.

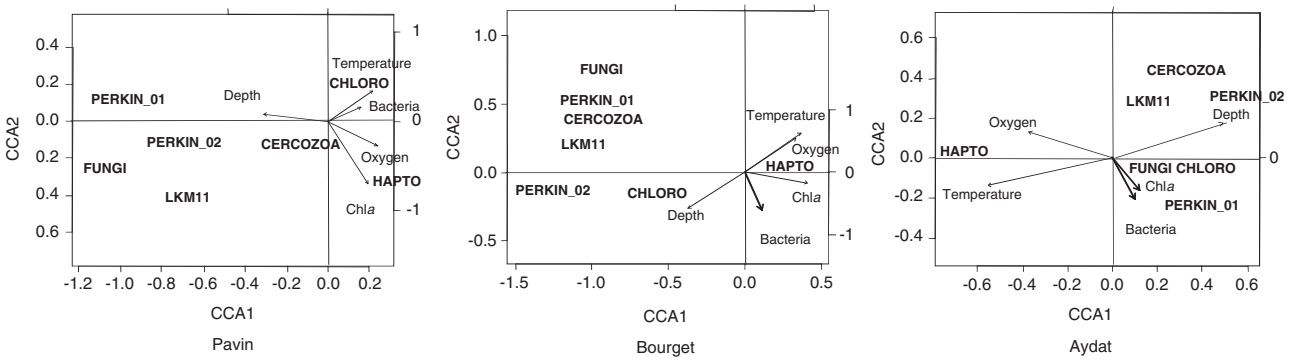

Figure 4 Canonical correspondence analysis (CCA) plot realized with cell abundances detected by the TSA–FISH method. PERKIN_01, PERKIN_02, HAPTO and CHLORO correspond to Perkinsozoa probe 1, Perkinsozoa probe 2, Haptophyta and Chlorophyta probes respectively. CHl *a* means chlorophyll *a*.

Bourget, a clear association is also observed with depth, because of a significant abundance of Chlorophyta at 20 m, 30 m and 50 m depth. Surprisingly, Chlorophyta are present all along the water column, even in the deepest points out of the euphotic zone; we counted, for example, up to 950 cells ml^{-1} in the deepest sampling points of Lake Pavin. The presence of pigmented cells, traditionally considered as photoautotrophs, in the deepest zone of lakes clearly suggests a mixotrophic behaviour of these taxa. Chlorophyta sequences found in these lakes were mostly affiliated to Chlamydomonadales (Lepère *et al.*, 2008). Recent experiments by Tittel *et al.* (2009) showed that heterotrophy occurred in Chlamydomonas to exploit dissolved organic carbon by osmotrophy. Moreover, Ukeles and Rose (1976) showed the mixotrophy of various strains of chlorophytes a long time ago. The importance of mixotrophy in the smallest size fraction of planktonic food web functioning should probably be reconsidered in view of these results. The dominance of Chlamydomonales within small Chlorophyta in lakes highlights a difference in the composition of small pigmented eukaryotes between lakes and oceans. Indeed, the main pigmented class identified in the ocean is the Prasinophyceae, and so far no sequences of Prasinophyceae have been detected in lakes.

Our results also showed the presence of members of prymnesiophytes (Haptophyta) in the three lakes. Although Haptophyta are well known in marine systems (Moon-van der Staay *et al.*, 2000; Iglesias-Rodriguez *et al.*, 2002), especially because of their ability to form toxic blooms (Gjøsæter *et al.*, 2000; Baker *et al.*, 2007), only a dozen Haptophyta species have been previously described from freshwater or terrestrial habitats (John *et al.*, 2002). However, some haptophyte blooms have been previously recorded in different lacustrine systems (Nicholls *et al.*, 1982; Hansen *et al.*, 1994), suggesting an importance of this group in other freshwater ecosystems.

CCA plots revealed an opposite distribution between Chlorophyta and Haptophyta groups (Figure 4). Haptophyta seems to be confined to surface waters, 0–20 m, especially in Lake Bourget (Figure 4), but they can also be detected below the photic zone in lakes Pavin and Aydat. On an average, Haptophyta contribution was less important than that of chlorophytes in all the lakes studied, and seems to decrease with the trophic level. The lowest values of relative abundance for this group were obtained in the eutrophic Lake Aydat, accounting for 7.7% of total eukaryotes. No Haptophyta $<2\,\mu m$ was detected; they were mostly found in the size fraction between $2\,\mu m$ and $5\,\mu m$ (results not shown). This is an interesting result, as described species of Prymnesiophyceae are generally considered to be larger cells (Vaulot *et al.*, 2008). However, recently, this group was recognized as a major component of the eukaryotic picoplankton in marine water and particularly in oligotrophic waters (Liu *et al.*, 2009). Liu *et al.* (2009) showed that the phylogenetic position of these tiny Haptophyta implies that they are photophagotrophic, in agreement with the recent discovery of dominant bacterivory by small eukaryotic phytoplanktons in oceans (Zubkov and Tarran, 2008). According to Zubkov and Tarran (2008), small algae ($<5\,\mu m$) carry out 40–95% of the bacterivory in the euphotic layer of the temperate North Atlantic Ocean in summer, suggesting the global significance of mixotrophy. This finding reveals that even the smallest algae have less dependence on dissolved inorganic nutrients than previously thought, obtaining a quarter of their biomass from bacterivory. Moreover, phagotrophy in photosynthetic Haptophyta was well described in genus *Chrysochromulina* (Legrand *et al.*, 2001), a genus observed in various lakes (Temponeras *et al.*, 2000). Mixotrophy may then provide a competitive advantage over both purely phototrophic microalgae (including cyanobacteria) and non-pigmented protists (Stickney *et al.*, 2000; Domaizon *et al.*, 2003; Troost *et al.*, 2005; Kamjunke *et al.*, 2007) in oligotrophic systems and/or in situation of phosphorous depletion (epilimnion in summer stratification). Even though we need further investigations to define the importance of this process, some of the Haptophyta revealed in these lakes could be bacterivorous.

Among pigmented cells, cryptophytes are also known for their mixotrophic behaviour, and could be present in the small size fraction of eukaryotes; for example, Mangot *et al.* (2009) showed by the TSA–FISH method that cryptophytes accounted on an average for 9.5% of the total small eukaryotes ($<5\,\mu m$) for over 1 year in Lake Bourget (0–20 m). Cryptophytes were not counted by TSA–FISH in this study, but some cryptophytes, most of them bigger than $5\,\mu m$, were detected in our samples (data not shown).

This study showed that, although pigmented small eukaryote contribution varied along vertical profiles and lakes, generally, pigmented cells, Haptophyta and Chlorophyta (on an average 77% of the community targeted by the eight probes) dominated over colourless cells (fungi, LKM11, Cercozoa and Perkinsozoa). Therefore, the hypothesis put forward by Lepère *et al.* (2008), in a study conducted on Lake Bourget about the predominance of heterotrophic organisms (65% of OTUs (Operational taxonomic Units), 85% of clones in 18S rRNA gene clone libraries) in the $<5\,\mu m$ eukaryote assemblage, does not seem to be validated by the quantitative results reported here.

Of course our data, which present a punctual picture of the small eukaryotes structure, have to be considered in view of the seasonal changes in small eukaryote structure. Even though such data (TSA–FISH counts) are still rare, clear modifications were recently observed in Lake Pavin and Lake Bourget (Lepère *et al.*, 2006; Mangot *et al.*, 2009). Mangot *et al.* (2009) considered the layers 0–20 m in Lake Bourget, and showed that, although the largest phototroph abundance was recorded in July, small heterotrophs, for instance, fungi, peak in autumn and winter. It is more than likely that pigmented small eukaryotes account for the greatest part of the community during summer, whereas heterotrophs are more important during the rest of the year. In addition, previous studies revealed the importance of other heterotrophic small eukaryotes in lakes, such as Chrysophyceae and ciliates, which are known to be an essential component of the small planktonic community but not taken into account in this study (Massana *et al.*, 2004; Boenigk *et al.*, 2005; Lovejoy *et al.*, 2006).

Non-pigmented small eukaryotes: the opened black box. The cloning–sequencing approach has recently allowed the opening of the black box of unidentified small heterotrophic eukaryotes and raised the important question of the taxonomic composition and functional roles of this planktonic assemblage (Guillou *et al.*, 2008; Lefèvre *et al.*, 2008; Lepère *et al.*, 2006, 2008; Massana *et al.*, 2009). These molecular results provide knowledge on their distribution and specific role in aquatic systems and highlight the potential importance of the process usually left out of aquatic trophic food web functioning as parasitism. The quantitative approach applied in this study shows that the targeted non-pigmented eukaryotes (probes CERC_02; LKM11_01 and _02; MY1574; PERKIN_01 and 02) represented from 100 cells ml^{-1} (Bourget, 140 m depth) to 1327 cells ml^{-1} (Aydat, 8 m). Although the highest abundances of non-pigmented eukaryotes were recorded in Lake Aydat (average abundances: 533 cells ml^{-1}, 410 cells ml^{-1} and 1034 cells ml^{-1}, in Pavin, Bourget and Aydat, respectively), these heterotrophs also represented the lowest relative abundance in this eutrophic lake. Their contribution increased with depth up to 7% of total hybridized cells. Similarly, in Lake Pavin, colourless small eukaryotes contributed on an average to 13.5% of the total signal, with

a maximum signal of 32.6% at 50 m. Colourless cells represented an important fraction of small eukaryotes in surface waters in Lake Bourget, especially at 2 m, constituting 32% of the total eukaryotes. Thus, the variations of abundances and/or proportions of these heterotrophs are not clear, along with the vertical profile of each lake, but changes are significant according to the trophic status.

Fungi generally represented $<2\%$ (116 cells ml^{-1}, vertical mean) of the total small eukaryotes, with the exception of Lake Pavin in which fungi contribute for 7% of the total hybridization at 50 m. In this lake, the CCA plot showed the distribution of fungi to be related to depth. Despite their low proportions, fungi had the highest abundance in Lake Aydat, with a maximum at 8 m (517 cells ml^{-1}), in association with an increase in chlorophyll concentrations. The fungi characterized in these ecosystems were mostly chytrids, affiliated to the chytridial *Rhizophydium* and *Nowakowskiella* clades (Lefranc *et al.*, 2005; Lefèvre *et al.*, 2008; Lepère *et al.*, 2008), a parasitic group known to be often host specific and highly infectious. They are able to be parasitic to numerous algal species (diatoms, dinoflagellates, Chrysophyceae, Chlorophyceae) at high infection rates (over 90% of host cells). Recently, Rasconi *et al.* (2009) provided quantitative preliminary data on infectious chytrids within phytoplankton communities in two contrasting lakes. Abundance of fungi was lower in the mesotrophic lake, representing on an average 1.6% of total eukaryotes in the 0–20 m layer. However, Mangot *et al.* (2009) showed that fungal abundance was largely related to diatom dynamics, especially *Melosira varians,* and to a lesser extent to the dynamics of Chlorophyta and Chrysophyceae in Lake Bourget. In this lake, these authors reported relative abundances varying from 2.3% to 27.1% of total small eukaryotes in the epilimnion.

The LKM11 group was detected in all lakes, with an average of 195 cells ml^{-1} (3.3% of total small eukaryotes); it represented the second group of abundance within the heterotrophs. Globally, the highest abundance along vertical profiles was found in the meta- or hypo-limnion, even though they were not specifically found in the anoxic zone as suggested by Lara *et al.* (2009). In Lake Bourget, they could represent up to 51% of the small heterotrophs targeted in the zone close to the benthos (110 m). The LKM11 group is a non-cultivated set of eukaryote organisms first described by Van Hannen *et al.* (1999). Although their functional role is still unknown, these organisms seem to be associated with the decomposition of phytoplanktonic organisms (microalgae and cyanobacteria), and could therefore contribute to the decomposition of organic compounds in oligotrophic and oligo-mesotrophic systems. Recently, Lara *et al.* (2009) reported that the environmental clade LKM11 and Rozella form the deepest branching clade of fungi and highlighted the hypothesis that the two groups might be composed to a large extent (if not entirely) of parasites. Even if it would be premature to draw any conclusion on the lifestyle and ecology of these organisms on the basis of only environmental sequences, a number of open questions arose about LKM11 ecological role and phylogenetic evolution.

Among putative parasitic groups, Perkinsozoa is one that has been recently detected in freshwater (Lefranc *et al.*, 2005; Lepère *et al.*, 2008). The highest abundance of Perkinsozoa was found in the eutrophic Lake Aydat, with a mean abundance of 309 cells ml^{-1} (clades 1 and 2), and reached a maximum in the deepest zone (699 cells ml^{-1}). In contrast, Lake Bourget and Pavin showed a lower abundance of this group, on an average 178 cells ml^{-1} for both lakes and with the greatest abundance in the epilimnion. As confirmed by the CCA plot (Figure 4), clade 1 (PERKIN_01) dominated generally in the epilimnion, whereas clade 2 (PERKIN_02) showed highest values in the hypolimnion. An exception was observed for Lake Pavin in which clade 2 dominated over clade 1 only at 2 m and 5 m, respectively. Although a high proportion of Perkinsozoa sequences was found in lakes Aydat and Bourget (Lepère *et al.*, 2008), the relative abundances recorded for these two putative parasites were in general less than expected, representing on an average 5% of the total targeted small eukaryotes. However, Perkinsozoa is the dominant group within heterotrophic cells targeted by our probes. In addition, Mangot *et al.* (2009) showed that abundance of Perkinsozoa clades 1 and 2 could represent up to 31.6% of the total targeted small eukaryotes in July in Lake Bourget (0–20 m layers). Perkinsozoa phylum, which is assumed to be entirely parasitic (Dungan and Reece, 2006), has mainly been studied in marine environments. Only Brugerolle (2002, 2003) has described the presence of an algal-parasitic Perkinsozoa in a freshwater environment (*Cryptophagus subtilis* renamed *Rastimonas subtilis*). Lacustrine sequences branched with high bootstrap values with *Perkinsus marinus* (Mangot *et al.*, 2010). In seawater environments, *Perkinsus marinus* is a parasite of bivalve (Cáceres-Martínez *et al.*, 2008). The *Perkinsozoa* group also includes various protist parasites, and especially *Parvilucifera infectans*, which is known to infect different dinoflagellate genera (Park *et al.*, 2004). Mangot *et al.* (2009), showed that the dynamics of Perkinsozoa clade 1 was linked to the dynamics of dinoflagellates *Peridinium* and *Ceratium,* suggesting that lacustrine Perkinsozoa may mirror marine Perkinsozoa. Fungi (affiliated to chytrids) and Perkinsozoa produced free-motile flagellate zoospores of 2–7 μm diameter without any distinctive morphological characteristics (Burreson *et al.*, 2005). This could explain why they have never been observed in studies based on classical microscopic approaches, suggesting that the zoosporic form of these organisms could have been miscounted as phagotrophic flagellates in previous studies.

The abundances of these heterotrophs (Perkinsozoa, LKM11 and fungi) are not irrelevant with regard to heterotrophic nano-flagellate abundances in lakes; for example, in Lake Pavin, Perkinsozoa, LKM11 and fungi abundances range from 0.3 to 0.6×10^3 cells ml^{-1} (vertical profile), whereas heterotrophic nano-flagellate abundances range from 0 to 4×10^3 cells ml^{-1} (spring, 0–90 m) (Colombet *et al.*, 2006).

Our data, along with the ones published by Mangot *et al.* (2009), suggested the quantitative importance of putative parasites identified by the 18S rRNA gene, previously ignored by non-molecular methods. In addition, results obtained with molecular and TSA–FISH approaches showed differences with oceanic ecosystems. Indeed, parasite groups also seem to be important in marine environments, but most of them are affiliated with Syndiniales (Guillou *et al.*, 2008) and are able to colonize all the marine habitats investigated so far, from oceanic surface waters to sediments. The exclusively marine lifestyle of this order as a whole, including sequences from uncultured organisms, is confirmed by the fact that their 18S rRNA gene sequences have not been retrieved from terrestrial or freshwater PCR surveys, but rather come solely from marine ecosystems.

Similar to Perkinsozoa and fungi, Cercozoa have probably been classified as unidentified flagellates in many studies because of their general lack of distinct morphological features. Cercozoa were present throughout the water column of the three studied lakes, with the exception of the deepest zone (140 m) of Lake Bourget. The latter showed the lowest abundances of Cercozoa (average 41 cells ml^{-1}). The highest values were recorded in the oligo-mesotrophic Lake Pavin, and reached a maximum at 5 m, contributing to 5% of the total small eukaryotes (394 cells ml^{-1}). A 2-year-long survey conducted on Lake Pavin revealed the importance of Cercozoa especially at 5 m (Lepère *et al.*, 2006). Moreover, the presence of Cercozoa in Lake Pavin was associated with diatoms dynamics (Lepère *et al.*, 2006). Microscopic observation in this study showed diatoms to be more abundant in Lake Pavin compared with the other lakes (data not shown). Free-living Cercozoa are known to feed on bacteria, fungi, algae and even other protozoa; some, although proportionally rarer, are considered as parasites of phytoplankton. One such example is the *Cryothecomonas* genus, which is capable of parasiting diatoms (Kühn *et al.*, 2000).

In conclusion, this study is the first to describe the vertical variations in both the total abundance and the main small eukaryotic groups in lacustrine systems. Our results suggesting a structuring impact linked to trophic level and depth are consistent with the recent conclusions reported by Chen *et al.* (2009). It appears here a specificity of small eukaryote structure in the eutrophic lake and in the deepest zones close to the sediments. However, it is necessary to take into account the fact that the eutrophic lake also presents the lowest depth, with consequently a different functioning from lakes Bourget and Pavin clearly stratified and not mixed every year down to the bottom.

We show that, although 18S clone libraries allowed us to discover a high diversity within the small heterotrophic eukaryotes, the FISH method highlighted the quantitative importance of some pigmented cells, such as Haptophyta in the euphotic zone of the three studied lakes. Significant data regarding picohaptophytes exist on marine systems, in which they are known to contribute significantly to the primary production (Jardillier *et al.*, 2010). However, such data are scarce in lakes, emphasizing the originality of our results. Several questions now arise with regard to the global distribution of these small pigmented cells (biogeography, marine vs freshwater systems) and their physiology and ecological function (photoautotophy, mixotrophy). In addition, the presence of chlorophytes in the deepest zone of these lakes also suggests that mixotrophy could be an important process in the functioning of the lacustrine ecosystem. Globally, it seems useful now to obtain more data (sequences) to describe the molecular diversity of the freshwater pigmented picoplankton. We also confirmed the presence of putative parasites always grouped in the CCA plot on one axis and linked with depth. However, further investigations are needed to assess their ecological relevance and their interaction with other planktonic communities.

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