APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 2005, p. 5935–5942 Vol. 71, No. 10
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.10.5935–5942.2005

Genetic Diversity of Small Eukaryotes in Lakes Differing by Their Trophic Status

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Received 1 December 2004/Accepted 10 May 2005

Small eukaryotes, cells with a diameter of less than 5 μm, are fundamental components of lacustrine planktonic systems. In this study, small-eukaryote diversity was determined by sequencing cloned 18S rRNA genes in three libraries from lakes of differing trophic status in the Massif Central, France: the oligotrophic Lake Godivelle, the oligomesotrophic Lake Pavin, and the eutrophic Lake Aydat. This analysis shows that the least diversified library was in the eutrophic lake (12 operational taxonomic units [OTUs]) and the most diversified was in the oligomesotrophic lake (26 OTUs). Certain groups were present in at least two ecosystems, while the others were specific to one lake on the sampling date. Cryptophyta, Chrysophyceae, and the strictly heterotrophic eukaryotes, Ciliophora and fungi, were identified in the three libraries. Among the small eukaryotes found only in two lakes, Choanoflagellida and environmental sequences (LKM11) were not detected in the eutrophic system whereas Cercozoa were confined to the oligomesotrophic and eutrophic lakes. Three OTUs, linked to the Perkinsozoa, were detected only in the Aydat library, where they represented 60% of the clones of the library. Chlorophyta and Haptophyta lineages were represented by a single clone and were present only in Godivelle and Pavin, respectively. Of the 127 clones studied, classical pigmented organisms (autotrophs and mixotrophs) represented only a low proportion regardless of the library's origin. This study shows that the small-eukaryote community composition may differ as a function of trophic status; certain lineages could be detected only in a single ecosystem.

Picoeukaryotes are probably the most abundant eukaryotes on earth. They are found in all lakes and oceans at densities from 10^2 to 10^4 cells/ml (8, 32). They constitute essential components of the microbial food web and play significant roles in the geochemical cycle (5, 8, 50).

It is difficult to characterize these organisms by simple observation with optical microscopy, and cultivation methods do not allow all the organisms to grow. Natural assemblages can be studied, without cultivation, by using chromatographic methods, high-performance liquid chromatography, and gas chromatography (4, 44). Pigment and/or fatty acid analysis can provide some information on the structure and dynamics of the phototrophic and/or heterotrophic behavior of planktonic organisms, but the phylogenetic information supplied by these methods is limited.

In the last decade, the introduction of molecular techniques into microbial ecology has greatly increased our knowledge by identifying the smallest aquatic microorganisms and, more particularly, prokaryotes. Within *Eubacteria* at least 13 novel divisions have been catalogued, and certain clusters, such as SAR11, appear to be significant components of the marine bacterioplankton (21, 25). Novel archaeal lineages without any known cultured organisms have also been recognized (13, 20).

Despite the power of molecular ecology techniques, these methods have not been as widely used for microeukaryotes as for prokaryotes. Several recent studies have analyzed the diversity of small eukaryotes (<3 or 5 μm), sampled in different oceanic ecosystems, by gene cloning and sequencing of rRNA genes and have shown high phylogenetic diversity (15, 34, 39). Moon-van der Staay et al. (39) identified a wide variety of lineages mainly affiliated with photosynthetic classes. They retrieved sequences not clearly assigned to any known organisms. The study carried out in deep Antarctic waters by López-García et al. (34), under conditions considered inhospitable, showed the presence of many new lineages affiliated with nonphotosynthetic groups including two new distinct alveolate groups, which represented 65 to 76% of the clones analyzed. According to Moon-van der Staay et al. (39), the analysis of picoeukaryotic diversity in the surface waters of the Mediterranean, North Atlantic, and Antarctic regions demonstrated the presence of many photosynthetic and heterotrophic lineages. A large proportion of clones belonged to novel lineages including, novel stramenopiles and novel alveolates.

It should be emphasized that these studies on small aquatic eukaryote diversity were conducted in marine ecosystems. Thus, little is known about the diversity of this planktonic community in lake systems, despite the large numbers of pigmented organisms that participate in primary production (1, 51) and colorless cells that are generally considered grazers of prokaryotic and eukaryotic cells (11). These organisms are able to use dissolved organic matter directly through the phagotrophic process (49).

In this study the diversity of small eukaryotes (0.2 to 5 μm) was examined by cloning and sequencing eukaryotic rRNA genes in three lakes differing by their trophic status (oligotrophic, oligomesotrophic, and eutrophic). The aim of the study was to determine (i) the structure of small eukaryotes in lacustrine systems and (ii) whether or not the composition of the small eukaryote community is dependent on the system's productivity. As far as we know, this work is the first descrip-

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TABLE 1. Main characteristics of the different lakes sampled

Lake	Trophic status	Coordinates	Maximum depth (m)	Sampling date (day/mo/yr)	Temp (°C)	Oxygen concn (mg/liter)	Chl *a* concn (μg/liter)
Godivelle	Oligotrophic	45°23′N, 2°55′W	55	17/07/02	14.9	18	0.02
Pavin	Oligomesotrophic	45°30′N, 2°53′W	95	02/07/02	15	9.9	1.9
Aydat	Eutrophic	45°39′N, 2°59′W	15	06/08/02	25.5	7.4	12.2

tion of small-eukaryote diversity in lakes by using molecular techniques.

MATERIALS AND METHODS

Study site and sampling. The study was conducted in three lakes (Massif Central, France): the oligotrophic Lake Godivelle (lac d'en haut), the oligomesotrophic Lake Pavin, and the eutrophic Lake Aydat (Table 1). The circular Lake Godivelle, situated at an altitude of 1,239 m with a maximum depth of 44 m, occupies a volcanic explosion crater. Lake Pavin, situated at an altitude of 1,197 m, is a typical crater mountain lake with a maximum depth of 92 m. Aydat Lake 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 (46). Mean chlorophyll *a* (Chl *a*) concentrations were <1, 2, and 12 μg/liter in Godivelle, Pavin and Aydat lakes, respectively. Average total phosphorus concentrations (in micrograms of P per liter) were 4 in Lake Godivelle, 10 in Lake Pavin, and 35 in Lake Aydat (46). The temperatures and dissolved oxygen and Chl *a* concentrations measured at the sampling date (summer 2002) are reported in Table 1.

One sample per lake was collected in the euphotic zone with a Van Dorn bottle at the deepest point in the three lakes. Water samples (from 70 to 120 ml depending on the lake) were prefiltered through 5-μm-pore-size polycarbonate prefilters (Millipore) at a very low vacuum to prevent cell damage (pressure, <2 kPa) and kept in 150-ml plastic bottles for less than 2 h during transport until processing in the laboratory for microbial collection. The microbial biomass was collected on 0.2-μm-pore-size (pressure, <10 kPa) polycarbonate filters (Millipore) and stored at −80°C until nucleic acid was extracted.

Nucleic acid extraction. The filters were covered with TE buffer (1× Tris and EDTA) and a lysosyme solution (final concentration, 250 $\mu g \cdot ml^{-1}$) and were incubated at 37°C for 30 min. Then sodium dodecyl sulfate (10%) and proteinase K (final concentration, 100 $\mu g \cdot ml^{-1}$) were added, and the filters were incubated at 37°C for at least 60 min. A cetyltrimethylammonium bromide (CTAB) solution (final concentration, 1% in a 0.7 M NaCl solution) was added, and samples were incubated at 65°C for 10 min. Nucleic acids were extracted with chloroform-isoamyl alcohol (24:1); the aqueous phase containing nucleic acids was kept and purified by adding phenol-chloroform-isoamyl alcohol (25:24:1). After isopropanol (0.6 volume) addition, the nucleic acids were precipitated at −20°C for 12 h. After centrifugation, the DNA pellet was ethanol rinsed and resuspended in 50 μl of TE buffer. The DNA yield was quantified by a fluorescence assay (DNA quantification kit; Sigma), and nucleic acid extracts were stored at −20°C until analysis.

Eukaryotic rRNA genetic libraries. Eukaryotic 18S rRNA genes were amplified with eukaryote-specific primers Ek-1F (CTGGTTGATCCTTGCCAG) and Ek-1520r (CYGCAGGTTCACCTAC) (33). The PCR mixture (50 μl) contained about 10 ng of environmental DNA, 200 μM of each deoxynucleoside triphosphate, 2 mM $MgCl_2$, 10 pmol of each primer, 1.5 U of *Taq* DNA polymerase (Eurobio), and the PCR buffer supplied with the enzyme. Reactions were carried out in an automated thermocycler (MJ Research PTC 200-cycler) with the following cycle: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min, 30 s; and a final extension at 72°C for 10 min. Several PCR products (at least four 50-μl samples) were pooled, precipitated with ethanol-sodium acetate, and resuspended in 50 μl of sterile water.

These PCR products were used to construct one clone library for each of the three lakes (Godivelle, Pavin, and Aydat) by using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's recommendations.

Fingerprint analysis and rRNA gene sequencing. Around 50 clones from each library were randomly picked from different plates. The presence of the 18S rRNA gene insert in positive colonies was checked by PCR amplification using flanking vector primers (M13f and M13r). Amplicons of the expected size were subsequently digested with the restriction enzyme HaeIII, and the resulting restriction fragment length polymorphism (RFLP) products were separated by electrophoresis in a 2.5% low-melting-point agarose gel (NuSieve) at 60 mV for about 3 h. Clones from the same library (i.e., lake) that produced the same RFLP pattern were grouped together and considered members of the same operational taxonomic unit (OTU). Thereafter, the OTUs from the three libraries were checked by terminal RFLP (T-RFLP) analysis. 18S rRNA genes from clones were amplified as described above, except that the fluorescently labeled forward primer 1f-FAM (6-carboxyfluorescein) (labeled at the 5′ end with fluorescent sequencing dye [MWG Biotech, Germany]) was used. PCR products were purified using the Qiaquick PCR purification kit (QIAGEN), visualized on 1% agarose gels, and quantified (DNA quantification kit; Sigma). Enzymatic digestions were performed separately for each restriction enzyme used by incubating 100 ng of PCR products with 20 U of MspI and RsaI (Sigma) at 37°C overnight. The samples were desalted with Microcon columns (Amicon; Millipore). The terminal restriction fragments (T-RFs) were separated on an automated sequencer (ABI 3700), and T-RF sizes were determined using Genescan analytical software.

At least one clone of each OTU was selected for sequencing. Double-stranded plasmid DNAs from selected clones were extracted with a QIAprep Spin Miniprep kit (QIAGEN). Euk-1F and an internal primer (Ek-555f [AGTCTGGTG CCAGCAGCCGC] or Ek-NSF573 [CGCGGTAATTCCAGCTCCA]) were used for partial sequencing, and a vector primer and an internal primer were used for complete sequencing. Nineteen OTUs were totally sequenced. Sequencing reactions were performed by MWG (http//www.mwg-biotech.com).

Phylogenetic analysis. To determine the first phylogenetic affiliation, each sequence was compared with sequences available in databases using BLAST from the National Center for Biotechnology Information and the Ribosomal Database Project (2, 37). The sequences were aligned with complete sequences of an ARB database using the latter's automatic alignment tool (www.arb-home.de) (36). The resulting alignments were checked and corrected manually, considering the secondary structure of the rRNA molecule. Sequences were inserted into an optimized tree according to the maximum parsimony criteria without allowing any changes to the existing tree topology (ARB software). The resulting tree was pruned to retain the closest relatives, sequences representative of eukaryotic evolution, and our clones. The sequences were screened for potential chimeric structures by using Chimera check from Ribosomal Database project II and by performing fractional treeing on the 5′ and 3′ ends of the sequenced DNA fragments. One obviously chimeric sequence was discarded from the analysis.

Rarefaction analysis was performed using Analytic Rarefaction software (version 1.3) (www.uga.edu/~strata/software/Software.html), based on the analytic solution presented by Raup (43) and Tipper (55).

Nucleotide sequence accession numbers. Nucleotide sequences determined in this study have been deposed in GenBank under accession numbers AY642693 to AY642748.

RESULTS AND DISCUSSION

The objective of this work was to study the taxonomic composition of the community of small eukaryotes along a gradient of eutrophication in lacustrine environments never before described by molecular techniques. We analyzed three clone libraries from three lakes differing in their trophic statuses: oligotrophic, oligomesotrophic, and eutrophic.

Methodological aspects. The water was prefiltered through a 5-μm-pore-size filter to take into account the small eukaryotic cells observable by standard epifluorescence microscopy, but whose taxonomic characterization is often impossible, and which represent a large proportion of the microorganisms in

the lakes (9, 52, 53). Moreover, the use of this fraction (0.2 to 5 μm) makes it easier to compare the results of this study with those obtained in other ecosystems (e.g., references 15 and 34), which also used the same prefiltration. The organisms targeted in this way by molecular techniques correspond to small eukaryotes with a maximum size in the region of 5 μm and not to the definition of picoplankton in the strict meaning of the term. It is well known that whatever the aquatic ecosystem, prefiltration allows some cells that are larger than their nominal pore sizes to pass through and can lead to the retention of smaller cells if the filters are clogged (10, 15). As emphasized by Díez et al. (15), the approach used to collect small eukaryotes is very important. Using epifluorescence microscopy after primulin coloration (see the protocol in reference 52), we observed the abundance of small eukaryotes (diameter, <5 μm) in the nonfiltered and filtered fractions in several samples. We detected a slight decrease in total abundance (10 to 15%) but no modification of diversity inferred by morphological inspection. However, both water filtration and DNA amplification (58) can bias the characterization of small plankton.

The diversity in the clone libraries is underestimated by RFLP patterns generated using a single restriction enzyme (16, 47). Thus, different RFLP patterns can correspond to the same sequences, or identical RFLP patterns can correspond to different OTUs. To limit this bias, we also used T-RFLP analysis, a highly reproducible and robust technique that yields high-quality fingerprints consisting of fragments of precise sizes (42). Thus, in some rare cases (three), some clones (A54 and P1.31, P1.25 and PG5.34, and P34.10 and PG5.31) with the same RFLP patterns showed different T-RFLP profiles. Furthermore, since the sequences were not similar, these were considered to be different. Analysis of each library highlighted 12 (eutrophic lake), 18 (oligotrophic lake), and 26 (oligomesotrophic lake) different OTUs. No OTUs occurred in all three lakes (Table 2). The diversity rarefaction curves (Fig. 1) obtained from the clones of Lake Aydat and, to a lesser degree, from Lake Godivelle tend to reach a plateau, in contrast to the curve for Lake Pavin. More specifically, with the clone library from Lake Aydat, the curve clearly shows saturation of diversity, allowing us to deduce that, in this case, this library is certainly representative of the composition of small eukaryotic plankton for the period studied, whereas the library of Lake Pavin represents only the most abundant clones. Thus, the highest diversity was observed in the oligomesotrophic lake (Lake Pavin).

More exactly, certain groups were present in at least two ecosystems, while the others were specific to one lake on the sampling date (Table 2; Fig. 2).

Lineages present in at least two lakes. Cryptophyta were represented by OTUs from the three libraries that mostly stemmed from the oligotrophic (three OTUs) and oligomesotrophic (seven OTUs) ecosystems. These OTUs formed three clearly distinct clades (G5.11, P1.27, P1.30, and P34.3; A54, P1.31, P1.25, and PG5.34; P34.10 and PG5.31) (Fig. 2). The presence of this lineage in the smallest planktonic fraction agrees with previous results obtained from lacustrine ecosystems (26, 49) and from lakes studied in the geographic area where Cryptophyta predominate among the pigmented organisms (9, 52, 53). Chrysophyceae, which include autotrophic, mixotrophic, and heterotrophic taxa (3, 23), were represented by 10 OTUs (A34, A43, A1, P1.35, PG5.22, A42, P34.48, P34.45, P34.28, and PG5.3) from three libraries. More specifically, sequences A1, P1.35, and A42, on the one hand, and sequences P34.28 and PG5.3, on the other, are associated with strictly heterotrophic flagellates: *Paraphysomonas* and *Oikomonas*. A43 and A34 belong to the genus *Poterioochromonas*, a mixotrophic flagellate. These three genera belong to clades including phagotrophic organisms or organisms at least capable of phagotrophy (3). Clones P34.45 and PG5.22, on the one hand, and clone G5.2, on the other, have a different but special position among the Stramenopiles (Fig. 2). They have the strongest similarity with heterotrophic species but are not clearly associated in a clade with known organisms. The Stramenopiles include initially heterotrophic organisms that have acquired a chloroplast during their evolution (31). In our analysis, clone G5.2 is clearly positioned in the tree before the acquisition of chloroplasts. Therefore, within the Stramenopiles, we identified two sequences associated with purely heterotrophic lineages: clone G5.2, related to the Hyphochytriomycetes, and clone P34.6, included in the lineage of Bicosoecida and with low similarity to *Cafeteria roenbergensis* (88%). Among the strictly heterotrophic small eukaryotes, both Ciliophora and fungi were identified in the three libraries. Three OTUs out of six in the Ciliophora lineage have the highest similarity with the genus *Oxytricha*, already identified in the marine environment in the picoplanktonic fraction by Díez et al. (15). With the exception of one OTU (A44), fungi were identified in the oligotrophic and mesotrophic lakes (PG5.12, P34.43, P34.27, P1.36, G5.10, and G5.16). These OTUs were affiliated with the lineage of Chytrids, known as parasites, for example, of green algae (35) and diatoms (6) in lacustrine ecosystems. The fungi of these ecosystems could participate in regulating planktonic populations by parasitism.

Among the small eukaryotes found only in two lakes, Choanoflagellida and environmental sequences (LKM11) were not detected in the eutrophic system whereas Cercozoa were confined to the oligomesotrophic and eutrophic lakes. Among these lineages, Cercozoa have the highest diversity (six OTUs), and with the exception of one sequence, these OTUs are not closely related to any in the database (≤88%) (Table 2). The Cercozoa are a complex group of eukaryotes, encompassing a wide variety of organisms and including some of the most abundant nonphotosynthetic amoebae, flagellates, and plasmodiophorid plant pathogens known. The morphological, ecological, and genetic diversity of the Cercozoa is enormous (27), and they are present in many different environments (47). In our libraries, five OTUs out of six were affiliated with the genus *Cercomonas*, with a low similarity on average (87%). These cells, which are extremely common in almost all types of soil (4 to 10 μm), are highly metabolic with strong amoeboid properties. The second cercozoan genus is affiliated with *Heteromita globosa* (one OTU in the Lake Pavin library), whose globular cells are approximately 4 to 6 μm long (18). The clade LKM11, grouping sequences G5.3, P34.42, PG5.28, and P34.11, is clearly differentiated on the tree, while at the same time it is associated with fungi. These sequences, first defined in the study by Van Hannen et al. (56), are affiliated with noncultivable eukaryotes taken from a lacustrine ecosystem. These organisms, associated with the decomposition of algae and

TABLE 2. Number of clones belonging to each OTU in genetic libraries and phylogenetic affiliations of the representative clones sequenced

Taxon	OTU	Closest relative	Similarity (%)	No. of clones in library of Lake:		
				Godivelle	Pavin	Aydat
Chlorophyta	PG5.14	*Mychonastes homosphaera*	99	1		
Haptophyceae	P34.19	*Chrysochromulina throndsenii*	97		2	
Cryptophyta	P34.10	*Storeatula major*	89		5	
	PG5.31	*Storeatula major*	88	1		
	P1.25	*Chroomonas* sp.	89		1	
	PG5.34	*Chroomonas* sp.	86	1		
	P1.31	*Chroomonas* sp.	88		1	
	A54	*Chroomonas* sp.	89			3
	G5.11	*Geminigera cryophila*	98	1		
	P1.30	*Geminigera cryophila*	98		1	
	P34.3	*Geminigera cryophila*	98		1	
	P1.27	*Geminigera cryophila*	96		1	
	P1.40	*Geminigera cryophila* sp2	95		1	
Chrysophyceae	P34.48	*Hibberdia magna*	94		1	
	A34	*Poterioochroomonas malhamensis*	94			1
	A43	*Poterioochroomonas malhamensis*	97			2
	P34.28	*Oikomonas mutabilis*	92		1	
	PG5.3	*Oikomonas mutabilis*	91	1		
	A1	*Paraphysomonas butcheri*	98			1
	A42	*Paraphysomonas foraminifera*	97			4
	P1.35	*Paraphysomonas bandaiensis*	97		1	
	PG5.22	*Paraphysomonas foraminifera*	96	2		
	P34.45	*Spumella elongata*	95		1	
Bicosoecida	P34.6	*Cafeteria roenbergensis*	88		1	
Hyphochytriomycetes	G5.2	*Rhizidiomyces apophysatus*	92	1		
Dinophyceae	G5.1	*Prorocentrum mexicanum*	94	2		
	PG5.8	*Prorocentrum mexicanum*	93	3		
	G5.7	*Gymnodinium beii*	93	1		
Ciliophora	P34.38	*Glaucoma chattoni*	97		1	
	PG5.26	*Oxytricha nova*	91	4		
	PG5.20	*Oxytricha nova*	90	1		
	A27	*Oxytricha nova*	91			1
	P34.44	*Prorodon teres*	81		1	
	P1.24	*Prorodon teres*	90		7	
Perkinsozoa	A20	*Perkinsus marinus*	87			18
	A48	*Perkinsus marinus*	86			9
	A31	*Perkinsus marinus*	90			1
Cercozoa	P1.23	*Cercomonas* sp.	86		2	
	A50	*Cercomonas* sp.	86			4
	P1.18	*Cercomonas* sp.	95		1	
	P34.13	*Cercomonas* sp.	87		1	
	A51	*Cercomonas* sp.	79			1
	P34.14	*Heteromita globosa*	88		1	
Choanoflagellida	P1.39	*Diaphanoeca grandis*	89		1	
	PG5.16	*Diaphanoeca grandis*	90	1		
Fungi	PG5.12	*Spizellomyces acuminatus*	93	1		
	A44	*Spizellomyces acuminatus*	90			2
	P34.27	*Spizellomyces acuminatus*	92		1	
	P1.36	*Spizellomyces acuminatus*	90		1	
	G5.10	*Spizellomyces acuminatus*	87	1		
	G5.16	*Spizellomyces acuminatus*	83	13		
	P34.43	*Spizellomyces acuminatus*	93		1	
Environmental sequences	P34.42	Unidentified eukaryote LKM11	92		1	
	P34.11	Unidentified eukaryote LKM11	87		2	
	PG5.28	Unidentified eukaryote LKM11	84	5		
	G5.3	Unidentified eukaryote LKM11	85	1		

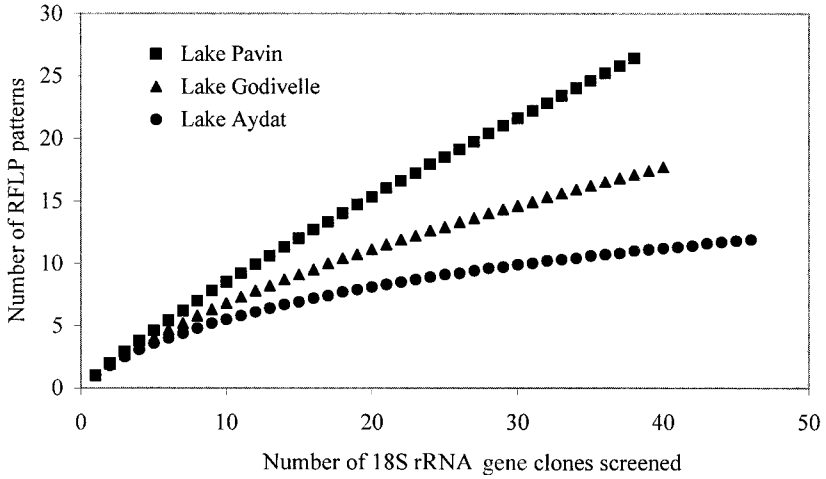

FIG. 1. Rarefaction curves determined for the different RFLP patterns of 18S rRNA gene clones. The number of different RFLP patterns was determined after digestion with the restriction endonuclease HaeIII.

cyanobacteria (56), could participate in the decomposition of detritus in the oligotrophic and oligomesotrophic systems.

Lineages specific to one lake. Some taxa seem to be specific to the ecosystem studied. Thus, three OTUs, linked to the new phylum of Perkinsozoa, which includes the marine parasites *Perkinsus* and *Parvilucifera* (41), were detected only in the Aydat library (A31, A48, and A20). They represent about 60% of the Aydat library and are affiliated with low similarities (86 to 90%) to *Perkinsus marinu*s. This result raises the possibility that the small eukaryotes of this lake played an important role in controlling algal populations during the study period. Among the pigmented organisms, the Haptophyta, Dinoflagellata, and Chlorophyta lineages were found only in one ecosystem. The sequence associated with Haptophyta is strongly affiliated with the genus *Chrysochromulina* (Table 2), a phagotrophic phytoflagellate (29). Three OTUs (G5.7, PG5.8, and G5.1) from the Godivelle library (oligotrophic) are affiliated with the Dinoflagellata, which include heterotrophic and autotrophic taxa, whose evolution may be linked to tertiary endosymbiosis (28), and more particularly with the autotrophic flagellates Gymnodiniales and Prorocentrales. Their detection in this planktonic fraction may be due to filtration problems or else to the existence of unidentified small Dinoflagellata. The low levels of similarity calculated (93 and 94%) with known Dinoflagellata and the fact that these flagellates have already been identified in the picoplanktonic fraction of marine ecosystems by the same molecular techniques (15, 39) could confirm the presence in this smallest fraction of a potentially new genotype belonging to this lineage.

Small-eukaryote diversity in relation to trophic status. The phylogenetic position of a clone enables hypotheses as to whether an organism is pigmented or colorless to be made (Table 3) (3, 14, 15, 29). For all three lacustrine libraries, we observed that 30 clones were considered to be pigmented (autotrophic or mixotrophic) whereas 94 were affiliated with heterotrophic lineages (Table 3) (the 2 OTUs P34.45 and PG5.22 belonging to Stramenopiles have been classified as indeterminate). The clones classified as heterotrophic accounted for more than 60% of the clones studied, whatever the library. This strong proportion of heterotrophs is in agreement with the results of counts by epifluorescence microscopy, which show that the pigmented organisms generally account for only a low proportion of small eukaryotes in lakes of this area (9, 52, 53). It may be noted that Chlorophyta are represented by only one sequence, of which there is only one example in the Godivelle library, whereas this lineage is reputed to be widespread in marine and lacustrine ecosystems (26). Fatty acid analysis performed for the 0.2- to 5-μm fraction of Lakes Godivelle and Pavin shows that Chlorophyceae may be present in the oligotrophic lake (unpublished data). The sampling period, as well as DNA extraction and amplification problems specific to this lineage, could explain their low presence. Thus, the use of bead beating or freeze-thaw cycles (48) could improve DNA extraction. However, these methodological considerations are probably insufficient for explaining the results obtained in this study; studies conducted in the marine environment using similar extraction and amplification techniques have detected many sequences affiliated with green algae (15, 39).

Some lineages were present in all ecosystems. However, this study shows that the diversity of small-eukaryote communities varies from lake to lake, with some lineages being present only in a given lake and absent from others. For example, Perkinsozoa were found only in the Aydat library, while Cercozoa were never detected in Lake Godivelle. Another factor that has to be taken into account is the relative importance of clones. Thus, the Godivelle library was dominated by fungi (31% of the clones) and the Aydat library by Perkinsozoa (60%). It is difficult to characterize these organisms by simple observation with optical microscopy, and these variations are not always detectable. For example, *Poterioochromonas*, which is a flagellate with loricae, is often difficult to identify because the loricae are inconspicuous when examined by light microscopy. Cells can also escape from their loricae and are then indistinguishable from *Ochromonas* spp. (14, 17). *Paraphysomonas* is characterized by siliceous scales on the cell surface, but these scales cannot be easily seen with light microscopy (7). Thus, several taxa identified here have certain morphological similarities and were probably not identified. However, as in

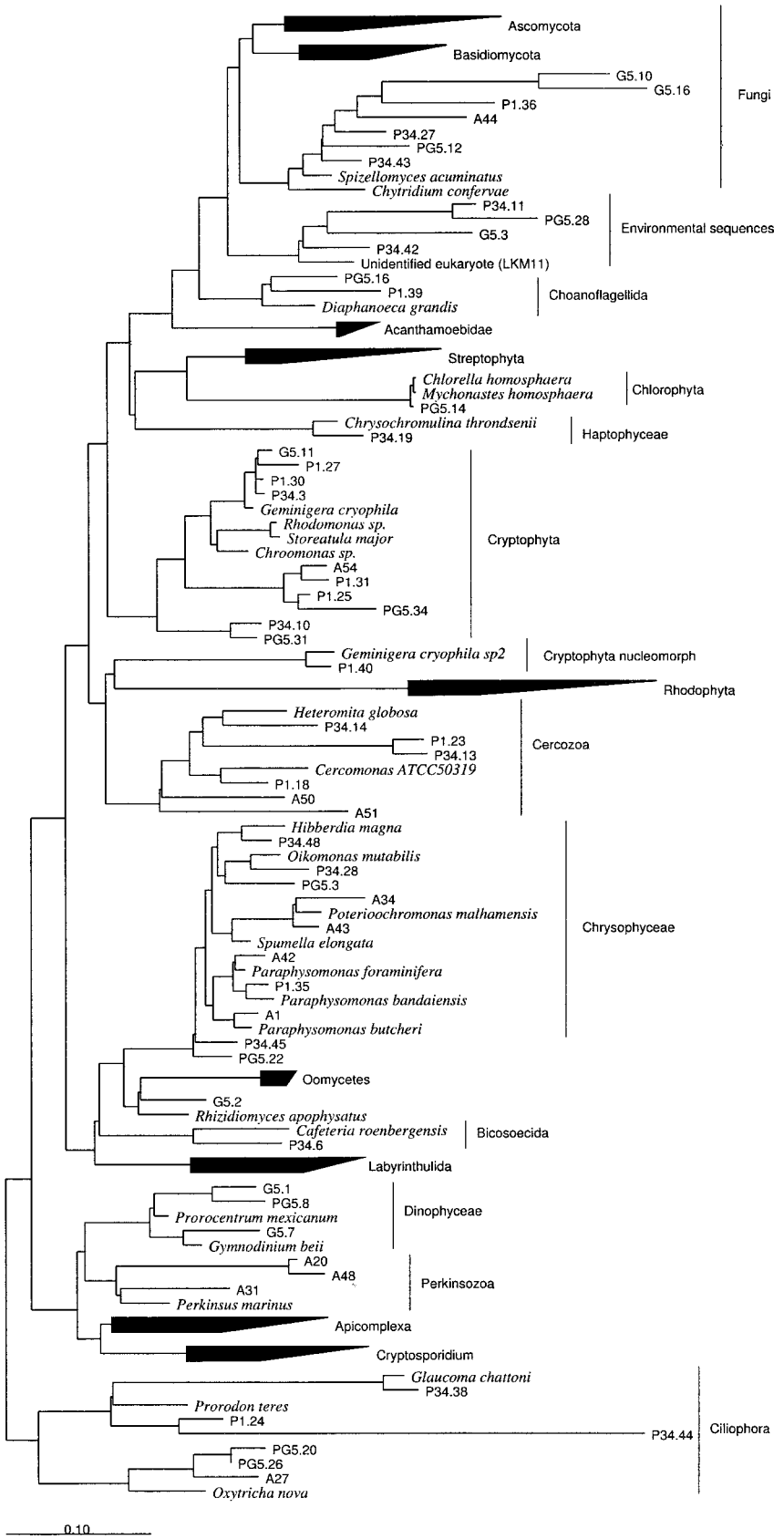

FIG. 2. Phylogenetic tree of eukaryotic small-subunit rRNA genes showing the positions of environmental clones. The tree was constructed using the ARB software package.

TABLE 3. Diversity of pigmented versus nonpigmented small eukaryotes in clone libraries from lakes differing in trophic status

Taxon	No. of OTUs (clones) in Lake:		
	Godivelle (oligotrophic)	Pavin (oligomesotrophic)	Aydat (eutrophic)
Overall total	18 (41)	26 (39)	12 (47)
Pigmented			
Chlorophyta	1	0	0
Haptophyceae	0	1	0
Cryptophyta	3	7	1
Chrysophyceae	0	1	2
Dinoflagellata	3	0	0
Total	7 (10)	9 (14)	3 (6)
Colorless			
Fungi	3	3	1
Environmental samples	2	2	0
Choanoflagellates	1	1	0
Cercozoa	0	4	2
Chrysophyceae	1	2	2
Bicosoecida	0	1	0
Hyphochytriomycetes	1	0	0
Ciliophora	2	3	1
Perkinsozoa	0	0	3
Total	10 (29)	16 (24)	9 (41)
Indeterminate			
Stramenopiles	1	1	0
Total	1 (2)	1 (1)	0 (0)

most studies conducted in the marine environment using cloning-sequencing techniques (15, 34, 38, 39, 60), these data were obtained from a single sample collected from one point on a single date, and therefore seasonal variations (9, 52, 53) are not taken into account. Moreover, according to Finlay (19), free-living microbial eukaryotes, such as ciliates, are probably sufficiently abundant to have a worldwide distribution. This view, for example, expects that microbial eukaryotes such as Ascomycetes will prove to be nearly ubiquitous geographically, whereas the distribution of these organisms tends to show some geographical differentiation (22). On the other hand, recent reports demonstrate that limited dispersal is also possible for some prokaryotes (59). Finally, according to Coleman (12), Finlay's assumption may be acceptable only for marine protistans and it would be less likely to apply to freshwater microbial eukaryotes. Many studies have also shown that the population composition of free-living microbial eukaryotes, such as microalgae and ciliates, varies in relation to the trophic status (29, 45). It is therefore possible that the variations in lacustrine small-eukaryote community composition can be partially explained by this factor. The OTU distribution (Fig. 1 and Table 3) may be related to the distributions determined in aquatic environments for algae, the bacteria belonging to the *Cytophaga-Flavobacterium-Bacteroides* group (24), and other organisms. Thus, it was possible to demonstrate under experimental conditions that algal diversity follows a hump-shaped progression along a eutrophication gradient and that this progression results from a compromise between competition, predation, accessibility of nutrient resources, and many other ecological processes (30, 54). The development of small eukaryotes could be limited by nutrient resources in an oligotrophic environment and by predation and competition in a eutrophic environment. As in marine environments (57), the diversity of heterotrophic organisms (35 OTUs) may be higher than that of autotrophic organisms (19 OTUs) in the smallest planktonic fraction (Table 3). More specifically, on studying the libraries independently, it can be seen that the ratio between colorless OTUs and pigmented OTUs increased with the trophic status and was highest in the eutrophic ecosystem. However, the latter ecosystem differs from the other two by its low diversity and a library strongly dominated by Perkinsozoa. To a certain extent, this trend appears to be of the same type as that described by Vaulot et al. (57), who showed that this ratio is higher in coastal than in oceanic environments.

Generally, this study showed that the clones in these ecosystems tend to form specific clades, even when related to a clearly defined phylogenetic group; moreover, many similarities remain lower than 90%. This undoubtedly results from the fact that despite their ecological importance, few studies have dealt with the small eukaryotes or picoeukaryotes of aquatic ecosystems, and thus, few 18S rRNA gene sequences are available. The few studies that do exist concern marine pelagic environments and very specific marine environments such as hydrothermal sediments (33). The clones in this study are not affiliated with the new Alveolata (groups I and II) (40) or Stramenopiles (15) lineages. Chrysophyceae appear to dominate in this latter clade, and they are an essential and ubiquitous component of plankton in freshwater environments. However they are rarely dominant in marine environments. It should also be noted that in this study, in contrast to studies in marine environments, diatoms were absent from this planktonic fraction (0.2 to 5 μm). These results are in agreement with those obtained by microscopy counts, which very rarely detect any small diatoms such as Cyclotella in the lakes studied. Clones belonging to fungi also accounted for a considerable proportion, but these have not been detected or represent only a low percentage of the marine clone libraries (15, 34).

This study shows that as with other planktonic communities, the small eukaryotes of lacustrine ecosystems described by molecular techniques tend to show some differences from those of oceanic systems and vary as a function of trophic level. On the other hand, phylogenetic determination highlights the presence of original clades such as Perkinsozoa in the eutrophic lake or chytrids in the two other lakes. The abundance of potentially parasitic organisms probably plays a significant role in controlling the population dynamics of protists in aquatic systems. However, their quantitative and functional importance remains to be determined.

REFERENCES

1. **Agawin, N. S. R., C. M. Duarte, and S. Agustí.** 2000. Nutriment and temperature control of the contribution of picoplankton to phytoplankton biomass and production. Limnol. Oceanogr. **45:**591–600.
2. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Andersen, R. A., Y. Van de Peer, D. Potter, J. P. Sexton, M. Kawachic, and T. LaJeunesse.** 1999. Phylogenetic analysis of the SSU rRNA from members of the Chrysophyceae. Protist **150:**71–84.
4. **Ansotegui, A., A. Sarobe, J. M. Trigueros, I. Urrutxurtu, and E. Orive.** 2003. Size distribution of algal pigments and phytoplankton assemblages in a coastal-estuarine environment: contribution of small eukaryotic algae. J. Plankton Res. **25:**341–355.
5. **Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer, and F. Thingstad.**

1983. The ecological role of water column microbes in the sea. Mar. Ecol. Prog. Ser. **10:**257–263.
6. **Canter, H. M., and G. H. Jaworski.** 1981. The effect of light and darkness upon infection of *Asterionella formosa* Hassall by the chytrid *Rhizophydium planktonicum* Canter emend. Ann. Bot. **47:**13–30.
7. **Caron, D. A., R. J. Gast, E. L. Lim, and M. R. Dennet.** 1999. Protistan community structure: molecular approaches for answering ecological questions. Hydrobiologia **401:**215–227.
8. **Caron, D. A., E. R. Peele, E. L. Lim, and M. R. Dennett.** 1999. Picoplankton and nanoplankton and their trophic coupling in the surface waters of the Sargasso Sea south of Bermuda. Limnol. Oceanogr. **44:**259–272.
9. **Carrias, J. F., C. Amblard, and G. Bourdier.** 1996. Protistan bacterivory in an oligomesotrophic lake: importance of attached ciliates and flagellates. Microb. Ecol. **31:**249–268.
10. **Carrias, J. F., A. Thouvenot, C. Amblard, and T. Sime-Ngando.** 2001. Dynamics and growth estimates of planktonic protists during spring in Lake Pavin, France. Aquat. Microb. Ecol. **24:**163–174.
11. **Carrick, H. J., G. L. Fahnenstiel, E. F. Stoermer, and R. G. Wetzel.** 1991. Observations on the importance of the zooplankton-protozoan trophic link. Limnol. Oceanogr. **36:**1335–1345.
12. **Coleman, A. W.** 2000., The significance of a coincidence between evolutionary landmarks found in mating affinity and a DNA sequence. Protist **151:**1–9.
13. **Delong, E. F.** 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. USA **89:**5685–5689.
14. **De Puytorac, P., J. Grain, and J. P. Mignot.** 1987. Précis de Protistologie. Editions Boubée and Fondation Polignac, Paris, France.
15. **Díez, B., C. Pedrós-Alió, and R. Massana.** 2001. Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. Appl. Environ. Microbiol. **67:**2932–2941.
16. **Dunbar, J., S. Takala, S. M. Barns, J. A. Davis, and C. R. Kuske.** 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rDNA gene cloning. Appl. Environ. Microbiol. **65:**1662–1669.
17. **Edgar, S. M., and R. A. Andersen.** 2003. The systematic of *Ochromonas* (Chrysophyceae). J. Phycol. **39:**14–19.
18. **Ekelund, F., N. Daugbjerg, and L. Fredslund.** 2004. Phylogeny of *Heteromita*, *Cercomonas* and *Thaumatomonas* based on SSU rDNA sequences, including the description of *Neocercomonas jutlandica* sp. nov., gen. nov. Eur. J. Protistol. **40:**119–135.
19. **Finlay, B. J.** 2002. Global dispersal of free-living microbial eukaryote species. Science **296:**1061–1063.
20. **Fuhrmann, J. A., K. MacKallum, and A. A. Davis.** 1992. Novel major archaebacterial group from marine plankton. Nature **356:**148–149.
21. **Giovanonni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso sea bacterioplankton. Nature **345:**60–63.
22. **Green, J. L., A. J. Holmes., M. Westoby, I. Oliver, D. Briscoe, M. Dangerfield, M. Gillings, and A. J. Beattie.** 2004. Spatial scaling of microbial eukaryote diversity. Nature **432:**747–750.
23. **Hahn, M. W., and M. G. Höfle.** 2001. Grazing of protozoa and its effect on populations of aquatic bacteria. FEMS Microbiol. Ecol. **35:**113–121.
24. **Horner-Devine, M. C., M. A. Leibold, V. H. Smith, and B. J. M. Bohannan.** 2003. Bacterial diversity patterns along a gradient of primary productivity. Ecol. Lett. **6:**613–622.
25. **Hugenholtz, P., B. M. Goebel, and N. R. Pace.** 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. **180:**4765–4774.
26. **Johnson, P. W., and J. M. Sieburth.** 1982. *In situ* morphology and occurrence of eucaryotic phototrophs of bacterial size in the picoplancton of estuarine and oceanic waters. J. Phycol. **8:**312–327.
27. **Keeling, P. J.** 2001.; Foraminifera and Cercozoa are related in actin phylogeny: two orphans find a home? Mol. Biol. Evol. **18:**1551–1557.
28. **Kuvardina, O. N., B. S. Leander, V. V. Aleshin, A. P. Myl'nikov, P. J. Keeling, and T. G. Simdyanov.** 2002. The phylogeny of colpodellids (Eukaryota, Alveolata) using small subunit rRNA genes suggests they are the free-living ancestors of apicomplexans. J. Eukaryot. Microbiol. **49:**498–504.
29. **Laybourn-Parry, J.** 1992. Protozoan plankton ecology. Chapman and Hall, London, United Kingdom.
30. **Leibold, M. A.** 1999. Biodiversity and nutrient enrichment in pond planktonic communities. Evol. Ecol. Res. **1:**73–95.
31. **Leipe, D. D., S. M. Tong, C. L. Goggin, S. B. Slemenda, N. J. Pieniazek, and M. L. Sogin.** 1996. 16S-like rDNA sequences from *Developayella elegans*, *Labyrinthuloides haliotidis*, and *Proteromonas lacertae* confirm that the stramenopiles are a primarily heterotrophic group. Eur. J. Protistol. **32:**449–458.
32. **Li, W. K. W., D. V. Subba Rao, W. G. Harrison, J. C. Smith, J. J. Cullen, B. Irwin, and T. Platt.** 1994. Autotrophic picoplankton in the tropical ocean. Science **219:**292–295.
33. **López-García, P., H. Philippe, F. Galle, and D. Moreira.** 2003. Autochthonous eukaryotic diversity in hydrothermal sediment and experimental microcolonizers at the Mid-Atlantic Ridge. Proc. Natl. Acad. Sci. USA **100:**697–702.
34. **López-García, P., F. Rodríguez-Valera, C. Pedrós-Alió, and D. Moreira.** 2001. Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature **409:**603–607.
35. **Lopez-Llorca, L. V., and P. Hernandez.** 1996. Infection of the green alga *Oocystis lacustris* Chod with the Chytrid fungus *Diplochytridium deltanum* (Masters) Karling. An SEM study. Micron **27:**355–358.
36. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
37. **Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese.** 1997. The RDP (ribosomal database project). Nucleic Acids Res. **25:**109–111.
38. **Massana, R., L. Guillou, B. Díez, and C. Pedros-Alio.** 2002. Unveiling the organisms behind novel eukaryotic ribosomal DNA sequences from the ocean. Appl. Environ. Microbiol. **68:**4554–4558.
39. **Moon-van der Staay, S. Y., R. De Wachter, and D. Vaulot.** 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature **409:**607–610.
40. **Moreira, D., and P. López-García.** 2002. The molecular ecology of microbial eukaryotes unveils a hidden world. Trends Microbiol. **10:**31–38.
41. **Noren, F., O. Moestrup, and A. S. Rehnstam-Holm.** 1999. *Parvilucifera infectans* Noren et Moestrup gen. et sp. nov. (Perkinsozoa phylum nov.): a parasitic flagellate capable of killing toxic microalgae. Eur. J. Protistol. **35:**233–254.
42. **Osborn, A. M., E. R. B. Moore, and K. N. Timmis.** 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ. Microbiol. **2:**39–50.
43. **Raup, D. M.** 1975. Taxonomic diversity estimation using rarefaction. Paleobiology **1:**333–342.
44. **Reuss, N., and L. K. Poulsen.** 2002. Evaluation of fatty acids as biomarkers for a natural plankton community. A field study of a spring bloom and a post-bloom period off West Greenland. Mar. Biol. **141:**423–434.
45. **Reynols, C. S.** 1984. Phytoplankton periodicity: the interaction of form, function and environmental variability. Freshwat. Biol. **14:**111–142.
46. **Rioual, P.** 2002. Limnological characteristics of 25 lakes of the French Massif Central. Ann. Limnol. **38:**311–327.
47. **Romari, K., and D. Vaulot.** 2004. Composition and temporal variability of picoeukaryote communities at a coastal site of the English Channel from 18S rDNA sequences. Limnol. Oceanogr. **49:**784–798.
48. **Savin, M. C., J. L. Martin, M. Legresley, M. Giewat, and J. Rooney-Varga.** 2004. Plankton diversity in the Bay of Fundy as measured by morphological and molecular methods. Microb. Ecol. **48:**51–65.
49. **Sherr, E. B.** 1988. Direct use of high molecular weight polysaccharides by heterotrophic flagellates. Nature **335:**1225–1227.
50. **Stockner, J. G., and N. J. Antia.** 1986. Algal picoplankton from marine and freshwater ecosystems: a multidisciplinary perspective. Can. J. Fish. Aquat. Sci. **43:**2472–2503.
51. **Stockner, J. G., and K. S. Shortreed.** 1989. Algal picoplankton production and contribution to food-webs in oligotrophic British Columbia lakes. Hydrobiologia **173:**151–166.
52. **Thouvenot, A., D. Debroas, M. Richardot, L. B. Jugnia, and J. Dévaux.** 2000. A study of changes between years in the structure of plankton community in a newly-flooded reservoir. Arch. Hydrobiol. **149:**131–152.
53. **Thouvenot, A., M. Richardot, D. Debroas, and J. Dévaux.** 1999. Bacterivory of metazooplankton, ciliates and flagellates in a newly flooded reservoir. J. Plankton Res. **21:**1659–1679.
54. **Tilman, D.** 1982. Resource competition and community structure. Princeton University Press, Princeton, N.J.
55. **Tipper, J. C.** 1979. Rarefaction and rarefiction—the use and abuse of a method in paleontology. Paleobiology **5:**423–434.
56. **Van Hannen, E. J., W. Mooij, M. P. van Agterveld, H. J. Gons, and H. J. Laanbroek.** 1999. Detritus-dependent development of the microbial community in an experimental system: qualitative analysis by denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **65:**2478–2484.
57. **Vaulot, D., K. Romari, and F. Not.** 2002. Are autotrophs less diverse than heterotrophs in marine picoplankton? Trends Microbiol. **10:**266–267.
58. **Von Wintzingerode, F., U. B. Goebel, and E. Stackebrandt.** 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. **21:**213–229.
59. **Whitaker, R. I., D. W. Grogan, and J. W. Taylor.** 2003. Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science **301:**976–978.
60. **Yuan, J., M. Chen, S. Peng, H. Zhou, Y. Chen, and L. Qu.** 2004. Genetic diversity of small eukaryotes from the coastal waters of Nansha islands in China. FEMS Microbiol. Lett. **240:**163–170.