J. Phycol. **34,** 206–213 (1998)

REVEALING GENETIC DIVERSITY OF EUKARYOTIC MICROORGANISMS IN AQUATIC ENVIRONMENTS BY DENATURING GRADIENT GEL ELECTROPHORESIS[1]

Erik J. van Hannen,[2] Miranda P. van Agterveld, Herman J. Gons, and Hendrikus J. Laanbroek

Netherlands Institute of Ecology, Centre for Limnology, Rijksstraatweg 6, 3631 AC Nieuwersluis, The Netherlands

ABSTRACT

A new Eucarya-specific 18S rDNA primer set was constructed and tested using denaturing gradient gel electrophoresis to analyze the genetic diversity of eukaryotic microorganisms in aquatic environments. All eukaryal lines of descent exhibited four or fewer nucleotide mismatches in the forward primer sequence, except for the Microspora line of descent. The reverse primer annealed to a more conserved region with fewer than two nucleotide mismatches. Genomic DNA from test organisms with different numbers of nucleotide mismatches were amplified to test primer specificity. Relatively low annealing temperatures allowed the amplification of sequences with up to four nucleotide mismatches while still maintaining specificity for the eukaryal domain. Denaturing gradient gel electrophoresis was used to separate similarly sized PCR products of environmental samples, and the obtained banding patterns were converted to a binary format for statistical comparisons. Cluster analysis of these patterns showed similar results to a cluster analysis based on environmental variables. This approach provides an analytical tool to study the population structure and molecular ecology of eukaryotic microbial communities inhabiting aquatic environments.

Key index words: 18S rRNA; aquatic environments; community structure; DGGE; Eucarya; genetic diversity; nucleotide mismatches; Southern blot hybridization

Abbreviations: DGGE, denaturing gradient gel electrophoresis; PCR, polymerase chain reaction; RDP, Ribosomal Database Project; SSU rRNA, small-subunit ribosomal RNA; UPGMA, unweighted pair group method using arithmetic averages

The application of molecular biological techniques to solve problems in microbiology has changed our view of the diversity and evolution of microbial life. Phylogenetic trees based on the small-subunit ribosomal RNA (SSU rRNA) gene have revealed the existence of three major branches (domains) of life: the Archaea, the Bacteria, and the Eucarya (Woese 1987, Woese et al. 1990). In general, molecular retrieval approaches are simple and straightforward: by extracting nucleic acids from the environment and subsequently amplifying the SSU rRNA gene, the diversity of microbial communities and the phylogeny of species can be studied without the need of cultivating the microorganisms (Amann et al. 1995).

Recently, denaturing gradient gel electrophoresis (DGGE) has been used to profile the genetic diversity of complex microbial communities (Muyzer et al. 1993). This technique is based on the melting behavior of different DNA sequences in a polyacrylamide gel prepared with an increasing concentration of denaturing substances (Fisher and Lerman 1979). Because the melting characteristics of helical DNA molecules in the gel depends on nucleotide composition, fragments of the same length can be separated based on nucleotide sequence. DGGE has been used successfully to analyze microbial genetic diversity in the natural environment (Muyzer and de Waal 1994, Muyzer et al. 1995, Wawer and Muyzer 1995, Ferris et al. 1996, Santegoeds et al. 1996, Teske et al. 1996, Ferris et al. 1997, Ferris and Ward 1997, Kowalchuk et al. 1997b) but has only recently be applied to eukaryotic cells in a study of fungal infections in root systems (Kowalchuk et al. 1997a).

Although earlier studies used universal primers to amplify DNA from natural samples (Giovannoni et al. 1990, Schmidt et al. 1991), there is now a growing tendency to limit investigations to bacterial and archaeal species. Only a few studies have measured eukaryal diversity in nature using molecular techniques (Lim et al. 1993, Simon et al. 1995, Lange et al. 1996), despite the potential for the presence of evolutionarily interesting but not yet detected eukaryotic microorganisms, especially in anaerobic environments (Pace 1997).

Here we describe a set of new PCR primers for the amplification of a fragment of the eukaryotic 18S rDNA molecule and the subsequent separation of a mixture of these fragments by DGGE. To test the new primer set in the natural environment, water samples collected from different locations within a freshwater lagoon system were analyzed by DGGE, and the genetic data were related to more classical environmental variables such as dissolved organic matter and algal pigment concentration.

MATERIALS AND METHODS

Algal cultures. Pure cultures of phytoplankton species (Table 1) were grown in 0.5-L Erlenmeyer flasks in modified Guillard medium (Van Hannen and Gons 1997) at room temperature for 2 weeks. Algal material was harvested by filtering a 50-mL aliquot of the growing culture onto a 0.2-μm nitrocellulose membrane filter (25 mm diameter; Schleicher & Schuell, Dassel, Germany). The filter was cut in half with a sterile surgical knife, and both halves were stored at −80° C in 2-mL screw-capped tubes (Sarstedt, Nümbrecht, Germany) until nucleic acids were extracted.

Protozoal cultures. Both protozoal species were cultured in the modified Guillard medium at room temperature, and additional yeast extract was added to the *Bodo caudatus* culture to a final

[1] Received 30 July 1997. Accepted 12 December 1997.

[2] Author for reprint requests; e-mail vanhannen@cl.nioo.knaw.nl.

TABLE 1. *Eukaryotic and bacterial species used in this study to test the PCR primer set. The phylogenetic placement of each species that appears in the Ribosomal Database Project is shown in the first column. Mismatches, number of mismatches with the forward primer; nsa, no sequence available.*

Taxonomy	Species	Mismatches	Source
Chlorophyta	*Ankistrodesmus falcatus* (Corda)	nsa	Strain E01, Centre for Limnology
	Chlorella vulgaris (Beyerinck)	0	Strain E03, Centre for Limnology
	Scenedesmus obliquus (Turp et Kuetzing)	0	Strain E07, Centre for Limnology
	Chlamydomonas sp. (Ehrenberg)	0	Strain E11, Centre for Limnology
Bacillariophyta	*Stephanodiscus hantzschii* (Grunow)	nsa	Strain E10, Centre for Limnology
	Nitzschia palea (Kützing)	1	Strain E09, Centre for Limnology
Cryptophyta	*Cryptomonas* sp. (Ehrenberg)	0	Strain E12, Centre for Limnology
Chordata	*Homo sapiens*	2	Provided by Helga Steffens, Medisch Spectrum Twente, Enschede, The Netherlands
Diplomonadida	*Giardia lamblia* (Kunstler)	3	David Horner, Natural History Museum, London, U.K.
Kinetoplastida	*Bodo caudatus* (Ehrenberg)	4	An Vos, Research Institute for Agrobiology and Soil Fertility, Haren, The Netherlands
Euglenida	*Euglena gracilis* (Klebs)	4	Ellen van Donk, Wageningen Agricultural University, Wageningen, The Netherlands
Gram-positive bacteria	*Lactococcus lactis lactis* (Schleifer)	8	Strain B1, Netherlands Institute of Dairy Research, Eden, The Netherlands
	Micrococcus luteus (Cohn)	9	DSM 20030
	Streptomyces coelicolor (Waksman et Henrici)	9	DSM 40233
	Rhodococcus erythropolis (Gray et Thornton)	9	DSM 43188
Proteobacteria, γ subdivision	*Acinetobacter* sp. (Beijerinck)	11	Strain BD413, K. Hellingwerf, University of Amsterdam, Amsterdam, The Netherlands
	Escherichia coli (Castellani et Chalmers)	11	Strain b01, Centre for Limnology

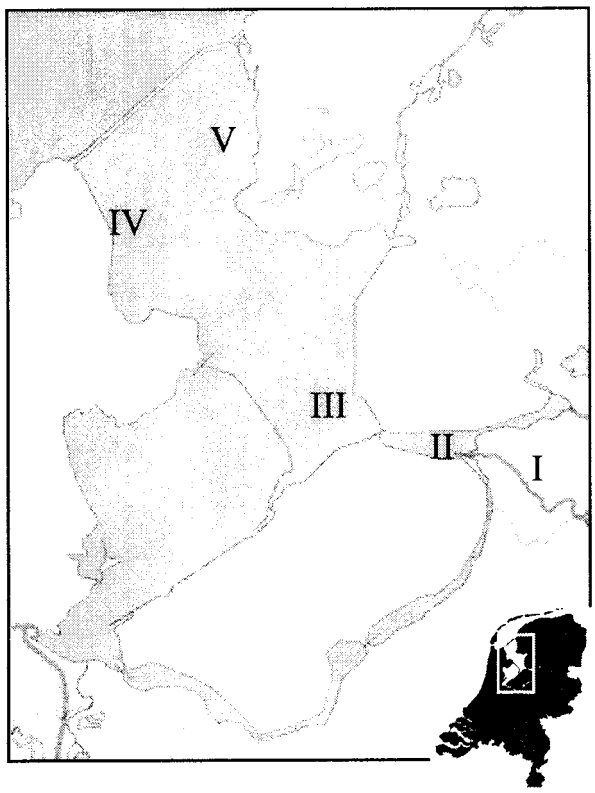

FIG. 1. Map showing the location of the sampling sites within the Dutch freshwater lagoon system. I, River IJssel; II, Lake Ketelmeer; III, southern water body; IV, northwestern water body; and V, northeastern water body of the Lake IJsselmeer.

concentration of 1 mg·L^{-1}. Biomass was collected as described above for the algal cultures.

Bacterial cultures. A culture of *Lactococcus lactis lactis* was grown at 30° C on TGV media (containing 10 g tryptone, 3 g meat extract, 5 g yeast extract, 20 g glucose, 2 mL Tween 80, 20 g K_2HPO_4, 15 g agar, and 40 mL filtered tomato juice per liter; pH 8). Cultures of *Acinetobacter* sp. (growth temperature: 30° C), *Escherichia coli* (37° C), and *Micrococcus luteus* (30° C) were grown in a medium containing 5 g peptone, 3 g meat extract, and 15 g agar per liter. Cultures of *Streptomyces coelicolor* (28° C) and *Rhodococcus erytropolis* (28° C) were grown in a medium containing 4 g glucose, 4 g yeast extract, 10 g malt extract, 2 g $CaCO_3$, and 12 g agar per liter. Bacterial biomass was collected as described above for the algal cultures.

Study sites and sample collection. Water samples were collected in September 1996 from the River IJssel, Lake Ketelmeer, and at three different locations in Lake IJsselmeer, The Netherlands (Fig. 1). Sampling sites within this freshwater lagoon system were chosen for the different hydrological characteristics. The River IJssel (sampling site I) discharges 10% to 25% of the River Rhine water into Lake Ketelmeer (sampling site II: 35 km^{-2} surface area; 3 m mean depth). The southern site (sampling site III) in Lake IJsselmeer (1190 km^2 surface area; 4.5 m mean depth) is close to the outlet of Lake Ketelmeer. The northwestern (sampling site IV) and northeastern (site V) sampling sites in Lake IJsselmeer are separated from the southern site by a distance of ca. 30 km. Water retention time of Lake Ketelmeer and Lake IJsselmeer are 1 week and 4.8 months, respectively (Berger and Sweers 1988). Two-thirds of the Lake IJsselmeer water enters through the River IJssel and Lake Ketelmeer. The remaining part originates from land drainage and precipitation (Berger 1987). Water column samples were collected using a Friedlinger sampling device. Samples were transported to the laboratory on ice and stored at 4° C until total particulate matter was collected by filtration within 1 day of sampling, as described above for the algal cultures.

Chemical parameters. Chlorophyll *a* and phaeophytin pigment concentrations were measured according to Moed and Hallegraef (1978). Particulate inorganic matter (PIM) and particulate organic matter (POM) were measured after filtration on GF-F filters (Whatman, Kent, United Kingdom) as the residue and loss on ignition at 500° C, respectively.

Nucleic acid extraction. To lyse the cells, 0.5 g of zirconium beads (0.1 mm diameter), 0.5 mL TE buffer (10 mM Tris, pH 7.6, 1

TABLE 2. *Eukaryotic oligonucleotide sequences used in this study. Numbering according to the corresponding nucleotide position. Both probes are situated on insertions in the SSU rDNA gene that is amplified by the primers.*

Type	5′ sequence	Nucleotide position
Forward primer with GC-rich clamp	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC[a] TCTGTGATGCCCTTAGATGTTCTGGG	1427–1453[b]
Reverse primer	GCGGTGTGTACAAAGGGCAGGG	1616–1637[b]
Kinetoplastida-specific probe	GACACGCGCACTACAATGTCAGTGAGAACA	1737–1766[c]
Euglena gracilis-specific probe	TCGACATGCCCACTCCGGTGGGC	1973–1995[d]

[a] GC-rich clamp.
[b] *Saccharomyces cervisae* rRNA.
[c] *Bodo caudatus* rRNA.
[d] *Euglena gracilis* rRNA.

mM EDTA) and 0.5 mL buffered phenol (pH 7–8) were added to the tubes containing the filter and shaken vigorously (5000 rpm) on a Mini Beadbeater (Biospec products, Bartlesville, Oklahoma) for 2 min with intermittent cooling on ice. The tubes were then centrifuged for 5 min at 10,000 × *g* and the upper (aqueous) phase was collected and extracted twice with phenol-chloroform-isoamylalcohol (25:24:1 v/v/v). The DNA in the aqueous phase was then precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5) and two volumes of 96% (v/v) ethanol and centrifuging for 30 min at 14,000 × *g*. The DNA was then dissolved in TE buffer, incubated in the presence of RNAse A (2 mg·mL^{-1}) at 70° C for 10 min and purified on a Wizard column (Promega, Madison, Wisconsin) according to the manufacturer's recommendations.

Oligonucleotide primer design. Eukaryotic PCR primers (Table 2) were designed to satisfy the following requirements: (1) the primer site must be located in a region that exhibits a minimum number of base mismatches with the eukaryal domain, (2) the gene sequence amplified by the PCR primers must contain a variable region to maximize DGGE resolution, and (3) the amplified region must contain less than 400 bp, since large PCR products are not clearly separated by the denaturing gel. All available eukaryotic SSU rRNA gene sequences from the SSU rRNA database (Van de Peer et al. 1997) and the Ribosomal Database Project (Maidak et al. 1997) were compared using the DCSE software (De Rijk and De Wachter 1993). The eukaryotic PCR primers designed for this study appear in Table 2.

Polymerase chain reaction. PCR amplifications were performed in 50-μL volumes containing approximately 100 ng of template DNA, Expand HF buffer (Boehringer Mannheim, Mannheim, Germany), 1.5 mM $MgCl_2$, 0.5 μM of each primer, 200 μM of each deoxynucleotide, 400 ng bovine serum albumin, and 1.3 U Expand High Fidelity DNA polymerase (Boehringer Mannheim). PCR cycling was performed using a Perkin Elmer 480 thermocycler. The temperature program was as follows: 1 denaturing step at 94° C for 5 min followed by 25 cycles of 94° C for 0.5 min, 52° C for 1 min, and 68° C for 1.5 min, then a final extension step of 68° C for 10 min. Genomic DNA from the pure cultures that appear in Table 1 was used to verify the amplification of 18S rRNA sequences with known numbers of nucleotide mismatches. In addition, a mixture containing 10 ng each of the 10 eukaryotic genomes (Table 1, except *Giardia lamblia*) was made prior to PCR amplification. As a control, bacterial genomic DNA was added to PCR mixtures at three different template concentrations (7500, 750, 75 ng) and subjected to a PCR with 25 cycles as described above. Annealing temperatures were estimated using the %GC method of the Oligo software (National Biosciences, Inc., Plymouth, Massachusetts). Concentrations of genomic DNA were estimated by separation on 0.8% agarose gels, staining with ethidium bromide (described below) and analyzing digitized images using ImageQuant software (Molecular Dynamics Ltd., Kemsing, United Kingdom).

Denaturing gradient gel electrophoresis. DGGE was performed essentially as described by Muyzer et al. (1993). Briefly, similarly sized PCR products were separated on a 1.5-mm-thick, vertical gel containing 8% (w/v) polyacrylamide (37.5:1 acrylamide:bisacrylamide) and a linear gradient of the denaturants urea and formamide, increasing from 30% at the top of the gel to 55% at the bottom. Here, 100% denaturant is defined as 7 M urea and 40% v/v formamide. Ten microliters of the single-template PCR products, 50 μL of the mixed-template PCR product, and equal concentrations of PCR products from the environmental DNA samples were applied to individual lanes in the gel. Electrophoresis was performed in a buffer containing 40 mM Tris, 40 mM acetic acid, and 1 mM EDTA, pH 7.6 (0.5× TAE), and 75 V were applied to the submerged gel for 16 h. Nucleic acids were visualized by staining for 1 h in 0.5× TAE buffer containing 0.5 mg·L^{-1} ethidium bromide, followed by destaining for 5 min in demineralized water and photographing the gel using a CCD camera (The Imager, Appligene, Illkirch, France). Digitized images were inverted using the software Photostyler (Aldus Corporation, Seattle, Washington). Contrast and gray balance of the entire image were adjusted to reduce background. To facilitate comparison of the natural samples, the DGGE banding patterns were converted to a binary matrix. The presence or absence of a nucleic acid band at the same height in each lane was marked with a 1 or 0, respectively. Gel images were enlarged two times to facilitate band detection.

Oligonucleotide probe hybridization. Oligonucleotide probes for the Kinetoplastida group and for *Euglena gracilis* were designed using SSU rRNA gene sequences from the SSU rRNA database and the DCSE program as described above. Probes were situated on insertions specific for the Kinetoplastida group and *E. gracilis* and therefore can be expected to be specific for these groups. Both probes, which appear in Table 2, were covalently linked to a biotin moiety at the 5′ end. To hybridize these probes, the DGGE gel was blotted for two hours onto a positively charged Nylon membrane (Boehringer Mannheim) using the Trans-Blot SD system (BIO-RAD). The membrane was prehybridized in 10 mL 2× SSPE buffer (0.02 M Na_2HPO_4, 0.36 M NaCl, 2 mM EDTA) + 0.1% sodiumdodecylsulfate (SDS) for 5 min. After adding 40 pmol of the probe, the membrane was hybridized overnight at 65° C. The membrane was then washed twice with 2× SSPE + 0.1% SDS for 10 min at 65° C. After washing, the membrane was incubated in 10 mL of 2× SSPE + 0.5% SDS + 2.5 μL streptavidin-peroxidase conjugate (Boehringer Mannheim) at 42° C for 45 min. The membrane was then washed for 10 min in 2× SSPE + 0.5% SDS, 2× SSPE + 0.1% SDS, and 2× SSPE at 42° C. To visualize the probe, the membrane was incubated in Peroxidase Blotting Substrate (Boehringer Mannheim) for 1 min and the photographic film (X-omat AR, Kodak, Rochester, New York) was exposed for 0.5 h.

Statistical analysis. Environmental parameters (Table 3) were scaled to standard deviation units to enable the comparison of the different variables according to the equation:

$$X_i' = \frac{X_i - \overline{X}}{\mathrm{SD}} \qquad (1)$$

where X_i' is the scaled parameter X_p and $\overline{X}$ represents the arithmetic mean. Scaled parameters were then used to calculate a Euclidean distance matrix. The binary (01) data generated from the DGGE patterns were used to calculate a distance matrix after Nei and Li (1979) according to the equation:

TABLE 3. *Frequency of eukaryotic sequences from the Ribosomal Database Project (RDP) with different numbers of mismatches in the forward primer. Only the taxa that may appear in aquatic environments are shown.*

Taxonomy	Frequency of sequences containing the following number of mismatches						Total	Taxon description
	0	1	2	3	4	>4		
Chlorophyceae	39	—	—	—	—	—	39	Phytoplankton
Charophyceae	8	—	—	—	—	—	8	Phytoplankton
Haptophyceae	1	—	—	—	—	—	1	Phytoplankton
Rhodophyceae	3	1	5	—	—	—	9	Phytoplankton
Chromophyceae	4	18	—	—	—	—	22	Phytoplankton
Dinophyceae	6	6	—	—	—	—	12	Phytoplankton
Cryptophyta	2	1	—	—	—	—	3	Autotrophic/heterotrophic flagellates
Chlorarachniophyceae	4	—	—	—	—	—	4	Autotrophic flagellates
Euglenida	—	—	—	—	1	—	1	Mixotrophic flagellates
Kinetoplastida	—	—	—	—	15	—	15	Heterotrophic flagellates
Choanoflagellida	2	—	—	—	—	—	2	Heterotrophic flagellates
Ciliophora	—	9	3	1	15	—	25	Ciliates
Hartmannellidae	1	—	—	—	—	—	1	Amoeboid protists
Acanthamoebidae	2	—	—	—	—	—	2	Amoeboid protists
Phreatamoebids	—	—	—	1	—	—	1	Amoeboid protists
Heterolobosea	—	—	1	3	—	—	4	Amoeboid protists
Eumycota	86	30	1	—	—	—	117	Fungi
Myxomycetes	—	—	1	—	—	—	1	Acellular slime molds
Diplomonadida	—	—	—	5	—	—	5	Mostly parasitic protists
Apicomplexa	6	23	1	—	11	—	41	Parasitic protists
Haplosporida	—	—	1	—	—	—	1	Parasitic protists
Parabasalidea	—	—	—	—	1	—	1	Parasitic protists
Microsporidia	—	—	—	—	—	10	10	Parasitic protists
Entamoebidae	—	—	—	—	3	—	3	Parasitic amoeboid protists
Dictyostelida	—	—	—	—	1	—	1	Parasitic amoeboid protists

$$D_i = 1 - \frac{2N_{AB}}{(N_A + N_B)} \quad (2)$$

where D_i is the distance between the patterns detected in lanes A and B, N_{AB} is the number of bands shared by both lane A and lane B, N_A is the number of bands in lane A, and N_B is the number of bands in lane B. Both distance matrices were then used in an unweighted pair group method using arithmetic averages (UPGMA) cluster analysis (e.g. Manly 1994) using the NEIGHBOR program of the PHYLIP software package (Felsenstein 1989). To minimize the effect of ties (i.e. some datasets can derive more than one topology) as described by Backeljau et al. (1996), we chose subreplicates and randomized the input order. Trees were constructed using the Treemap software (Page 1994).

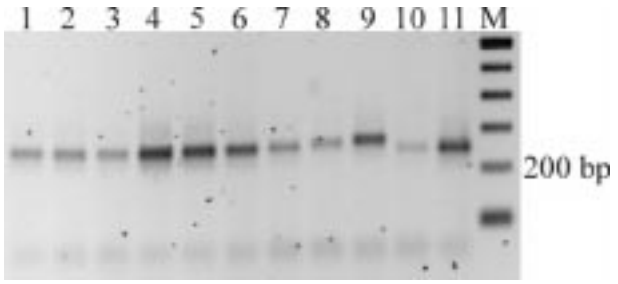

FIG. 2. Negative image of an ethidium-bromide-stained agarose gel with the amplified rDNA product (~210 bp without the GC-clamp). 1, *Ankistrodesmus falcatus* (no sequence available); 2, *Chlorella vulgaris* (no mismatches); 3, *Scenedesmus obliquus* (no mismatches); 4, *Chlamydomonas* sp. (no mismatches); 5, *Nitzschia palea* (1 mismatch); 6, *Stephanodiscus hantzschii* (no sequence available); 7, *Cryptomonas* sp. (no mismatches); 8, *Euglena gracilis* (four mismatches); 9, *Bodo caudatus* (four mismatches); 10, *Homo sapiens* (two mismatches); 11, mixture (of DNA in lanes 1–10); M, marker (100-bp ladder).

RESULTS

Polymerase chain reaction. Two PCR primers were constructed that selectively amplified a part of the 18S rRNA gene of ca. 210 bp. Although the primer regions were highly conserved in most eukaryal lines of descent, some more deeply divergent taxa showed significant numbers of mismatches (>4) in the forward primer sequence. The reverse primer sequence targeted a more conserved region and had a maximum of two nucleotide mismatches in the Microspora line of descent. In Table 3, all eukaryotic sequences that appear in the RDP database from microorganisms that can occur in aquatic habitats were listed according to number of mismatches with the forward primer sequence.

Because of the high number of nucleotide mismatches with the forward primer sequence in the archaeal and bacterial domains (eight or more mismatches), we were able to lower the annealing temperature of the PCR reaction from a calculated 63° to 52° C without amplifying noneukaryotic rDNA. By using an annealing temperature of 52° C, it was possible to amplify 18S rRNA gene sequences with up to four mismatches in the forward primer sequence (Fig. 2), although *G. lamblia* DNA (three mismatches) was not amplified using these primers. Six PCR reactions containing different bacterial DNA as template were negative for all template concentrations (data not shown).

Denaturing gradient gel electrophoresis. PCR products of the same size but with different nucleotide se-

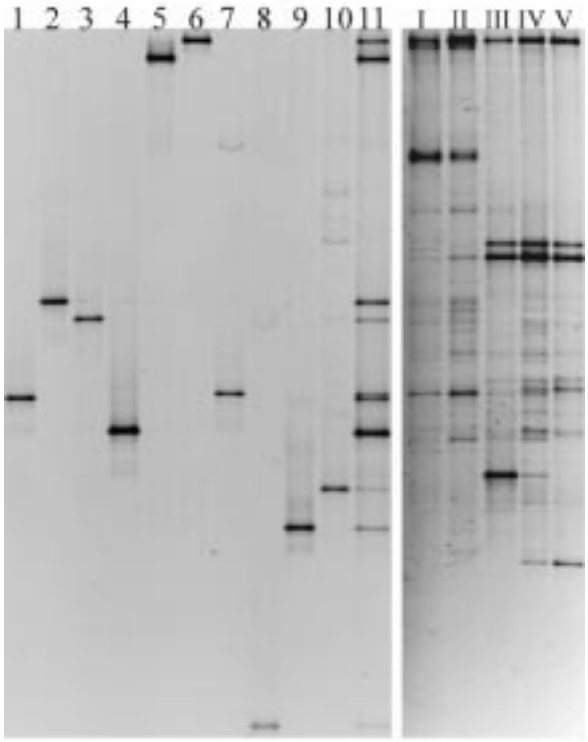

FIG. 3. Negative image of an ethidium-bromide-stained 30%–55% DGGE gel. 1, *Ankistrodesmus falcatus* (no sequence available); 2, *Chlorella vulgaris* (no mismatches); 3, *Scenedesmus obliquus* (no mismatches); 4, *Chlamydomonas* sp. (no mismatches); 5, *Nitzschia palea* (1 mismatch); 6, *Stephanodiscus hantzschii* (no sequence available); 7, *Cryptomonas* sp. (no mismatches); 8, *Euglena gracilis* (four mismatches); 9, *Bodo caudatus* (four mismatches); 10, *Homo sapiens* (two mismatches); 11, mixture of 1–10; I, River IJssel; II, Lake Ketelmeer; III, southern water body; IV, northwestern water body; V, northeastern water body of Lake IJsselmeer.

quences were clearly separated on a DGGE gel (Fig. 3). The four *Chlorophyceae* species (lanes 1 to 4) were readily separated, as were the two *Bacillariophyceae* species (lanes 5 and 6). In lane 11, the mixture of all 10 species could be resolved and recognized by comparison to their individual bands in lanes 1–10. Lanes I–V show the DGGE pattern of the natural samples and exhibit 12, 17, 15, 20, and 14 bands, respectively. In total, 26 different bands could be recognized in the PCR products of the natural freshwater samples.

Southern blot hybridization. To confirm the PCR amplification of template DNA with up to four mismatches in the forward primer sequence, biotin-labeled probes against Kinetoplastida and *E. gracilis* were constructed. This would exclude the possibility of amplifying DNA from a contaminating species with fewer mismatches or misidentifying the two species. The results of the Southern blot hybridization with these probes are shown in Figure 4. Both probes were specific under the conditions used in this study and bound only to the *B. caudatus* (Kinetoplastida, Fig. 4B) or the *E. gracilis* bands (Fig. 4C).

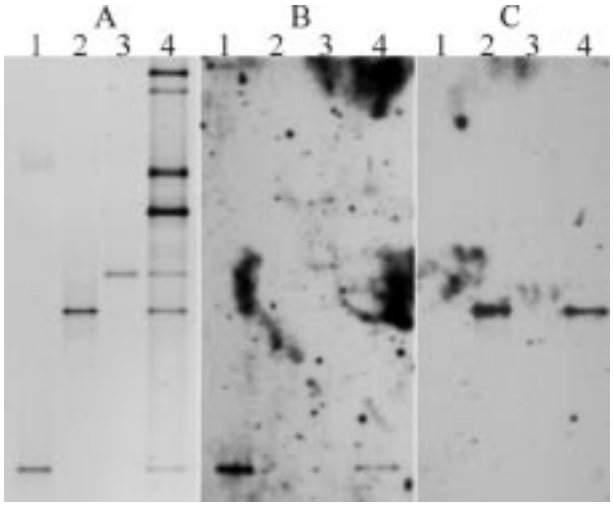

FIG. 4. Results of the Southern blot hybridization with the Kinetoplastida- and *Euglena gracilis*-targeting oligonucleotide probes. Panel A, negative image of a part of the ethidium-bromide-stained DGGE gel that appears in Figure 4; panel B, hybridization of the same gel with the *Euglena gracilis*-specific probe; panel C, hybridization of the same gel with the Kinetoplastida-specific probe. 1, *Euglena gracilis*; 2, *Bodo caudatus*; 3, *Homo sapiens*; 4, mixture.

Genetic versus environmental clustering. Based on the environmental variables POM, DOM, [Chl *a*] and [Phaeo] (Table 4), the River IJssel in September 1996 clustered separately from the Lake Ketelmeer and Lake IJsselmeer samples (Fig. 5, left panel). The two northern sample sites (IV and V) appeared to be identical in the cluster analysis and were clearly separated from the southern sample site of Lake IJsselmeer (III) and Lake Ketelmeer. The genetic diversity showed a slightly different pattern (Fig. 5, right panel). In this analysis, River IJssel and Lake Ketelmeer clustered together and were separated from the Lake IJsselmeer samples. Within Lake IJsselmeer, genetic analysis revealed a separation between the two northern and the southern sample sites.

DISCUSSION

The use of molecular techniques to estimate the diversity of uncultivated microorganisms in natural environments is well established in microbial ecology. Although the number of studies focusing on bacterial diversity is rapidly growing, less attention has been

TABLE 4. *Chemical parameters measured on the five sampling locations. PIM, particulate inorganic matter; POM, particulate organic matter; chl* a, *chlorophyll* a*; Phaeo, phaeopigment.*

Location	Description	PIM ($mg \cdot L^{-1}$)	POM ($mg \cdot L^{-1}$)	Chl *a* ($\mu g \cdot L^{-1}$)	Phaeo ($\mu g \cdot L^{-1}$)
I	River IJssel	10.4	3.2	3.3	5.6
II	Lake Ketelmeer	36.8	8.9	14.1	12.6
III	Lake IJsselmeer S	44.2	15.5	51.1	22
IV	Lake IJsselmeer NW	20.1	20.1	101.4	30.2
V	Lake IJsselmeer NE	26.5	27.6	76.2	27.4

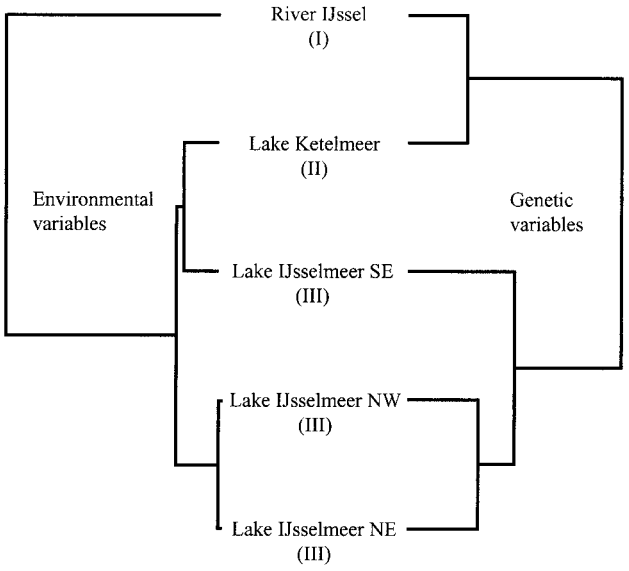

FIG. 5. Comparison of the UPGMA trees calculated using environmental parameters (left panel) and genetic data (right panel).

given to the microorganisms belonging to the domain Eucarya. In this study we developed and tested a PCR primer set to amplify a small part of the eukaryotic SSU rRNA gene. The primers were designed to anneal with highly conserved regions of the gene to allow amplification of most of the lines of descent within the eukaryal domain that can occur in aquatic habitats. Because DGGE separation requires small PCR products (<400 bp), primers were designed to amplify a small (ca. 210 bp) and variable region of the SSU rRNA gene.

Although molecular techniques may circumvent cultivation-based problems of underestimating microbial diversity, these techniques may introduce their own errors. Differential DNA extraction from cells will underestimate the abundance of microorganisms with rigid cell walls (Ogram et al. 1988, Johnson 1991). Sequences with a high GC content (Reysenbach et al. 1992) and sequences with primer sequence mismatches may result in preferential amplification of more readily amplified sequences. Genome size and *rrn* gene copy number also influence amplification efficiency (Farrelly et al. 1992) and may severely bias amplification of community DNA, since eukaryotic species may contain up to several thousands of copies of the rRNA gene (Appels et al. 1980, Long and Dawid 1980). Correction for these errors requires knowledge about the species or sequences. Because most RNA sequences derived from natural environments are from uncultivated species, estimations of the abundance of these organisms based on amplified rDNA sequences are likely to be biased and should be cautiously interpreted.

Initially, we tested existing universal eukaryotic primers designated 18e, 18j, and 18h (Hillis and Dixon 1991). Three combinations of these primers yielded PCR products of ca. 400 (18e and 18h), 800 (18j and 18h) and 1200 bp (18e and 18j). Because none of these primer pairs yielded acceptable separations on the denaturing gradient gel, we decided to construct a new universal eukaryotic primer set that would yield a PCR product with approximately the same length as the primers used by Muyzer et al. (1993) and would satisfy all the other requirements for successful DGGE separation. Because the eukaryal domain exhibits significant differences in SSU rRNA gene sequence from both the archaeal and bacterial domains, it was possible to construct a primer set that selectively amplified most eukaryal representatives tested. However, sequence variation within the eukaryal domain would not allow construction of primers without nucleotide mismatches. For instance, the more deeply diverging Microspora line of descent exhibited more than four mismatches with the forward primer sequence.

To test primer specificity, we used DNA from organisms with known numbers of mismatches in the primer region rather than testing a large number of related species. Less stringent PCR conditions allowed the amplification of sequences with up to four nucleotide mismatches. This observation was confirmed by Southern blot hybridization (Fig. 4) using *B. caudatus* and *E. gracilis* oligonucleotide probes. We were unable to amplify *G. lamblia* DNA, which contained three mismatches. This may have been due to the high GC content (74.7%) of the DNA from this species, which can inhibit PCR amplification. The positions of the mismatches were randomly distributed throughout the priming site, even within a given taxon. Mismatches were never located at the 3′ end of the priming site. Besides the relatively low annealing temperature, this may also explain our success amplifying sequences with four mismatches since mismatches at the 3′ end will strongly influence polymerase binding and subsequent functioning.

If we assume a range of zero to two mismatches in the forward primer sequence to be amplified by the primer set, we can speculate on the specificity of these primers. All phytoplankton-related sequences fall within this range as do all flagellated protist sequences except for the groups Euglenida and Kinetoplastida. Amplification of these two groups with four mismatches is possible as shown by the Southern blot hybridization. Because all Kinetoplastida sequences in the RDP database displayed the same mismatches as exhibited by the tested *B. caudatus* species, we anticipate all members of this taxa to be detected by the primer set. Of the 25 Ciliophora-related sequences in the RDP database, 15 exhibit four mismatches in the forward primer sequence. However, this number is slightly biased due to the 13 *Tetrahymena* sequences that exhibited four mismatches. Some of the members of the Ciliophora can be amplified by the primers but those belonging to the Tetrahymenina should be tested before amplification is assured. The amoeboid protists within Hartmannel-

lidae and Acanthamoebidae groups showed no mismatches and will most likely be detected by the primers. The other two amoeba groups (Phreatamoebids and Heterolobosea) exhibit two to three mismatches. Amplification of these sequences may be possible based on our amplification success of sequences with up to four mismatches. All fungal sequences fall within the range of two mismatches and are expected to be detected by the primers. Most of the sequences belonging to parasitic protists exhibit three to four mismatches except some of the Apicomplexa-related sequences. Amplification of these groups are doubtful. Although most of these parasitic protists can be found in aquatic habitats, it is not likely that these strict parasites will be encountered as free living cells in high numbers. We thus conclude that this primer set will detect the majority of the dominant eukaryotic microorganisms occupying aquatic habitats.

When equal amounts of template DNA were amplified in individual PCR reactions using this primer set, we observed little effect of the number of template mismatches on PCR product concentration. In fact, some lanes containing amplification products of sequences without mismatches (lanes 2, 3, and 7; Fig. 2) appear to contain less PCR product than lanes with two and four mismatches (lanes 6 and 9, respectively). One explanation for this discrepancy could be that inhibiting substances in the PCR reaction have limited the yield. Plant polysaccharides are known inhibitors of the DNA polymerase used during PCR (Demeke and Adams 1992), and a varying polysaccharide content of the different phytoplankton species can influence PCR yield. Although no bias was observed when individual templates were used, those sequences with fewer mismatches were preferentially amplified when the test DNA was added to the PCR in equal amounts (Fig. 3, lane 11). Consequently, the use of band intensity as a measure of abundance may result in an underestimation of the abundance of genotypes with mismatches. We therefore used only presence or absence of genotypes to compare the DGGE patterns.

To test the primers on a mixture of unknown sequences, we analyzed the genetic composition of a freshwater lagoon system. The DGGE patterns of these natural samples showed great variation in band numbers, position and band intensity (Fig. 3, lanes I–V). To compare the banding pattern from the different environmental samples, the presence or absence of bands in each lane was converted to a binary matrix and subjected to cluster analysis. The resulting UPGMA tree is compared to a similar tree constructed from more standard water quality parameters at each of the sampling sites in Figure 5. If comparable water bodies contain similar eukaryotic microbial communities, then we would expect the branching order of both trees to be similar. Indeed, sites with similar environmental parameters displayed similar DGGE banding patterns. The genetic clustering of the River IJssel with Lake Ketelmeer can be explained by the very short retention time of the river water in Lake Ketelmeer (about 1 week). Within 1 week, major changes in the community composition of this highly turbid water are not likely to occur. As water entered the large Lake IJsselmeer (5 months mean water retention time), shifts in community composition were detected by DGGE. The division between the southern and the northern parts of Lake IJsselmeer is consistent with *a priori* considerations of the chemical gradients within the lake. Clustering based on a limited number of chemical variables showed a somewhat different pattern than the genetic clustering. These differences may be explained by stochastic elements, such as the extensive wind resuspension of sediments that occurred just before sample collection. Due to this resuspension, the particulate inorganic matter concentrations in Lake Ketelmeer and Lake IJsselmeer were similar. If more than four chemical variables had been used in the UPGMA clustering, Lake Ketelmeer may have clustered with the River IJssel rather than the southern sampling site of Lake IJsselmeer.

Unlike bacteria, small eukaryotes do exhibit enough discriminative morphological features to aid in their identification. However, complete identification of small eukaryotic cells such as protists is time consuming and can only be achieved by experts. Molecular techniques are relatively new tools in microbial ecology that greatly assist the identification of morphologically similar microorganisms. DGGE can easily be used to monitor genetic diversity on a large scale. Subsequent direct sequencing of the PCR fragments separated on DGGE can provide further information about the identity of the genotypes. We found that the amplified region (ca. 210 bp) contains sufficient information to establish identification to the phylum level (unpubl.). By using Southern blot hybridization with oligonucleotide probes, genotypes can be identified to the species level. For example, the genus-specific *Phaeocystis* probe (PHAEO01) constructed by Lange et al. (1996) is situated on the rDNA fragment amplified by this primer set.

Although molecular techniques may introduce errors that are difficult to control, careful interpretation can add valuable information to our limited knowledge about the ecology of eukaryotic microorganisms. Construction of fluorescent oligonucleotide probes from sequences obtained through molecular techniques can be used to estimate the abundance of naturally occurring microorganisms. Extensively used in bacterial ecology, information gained by DGGE may also contribute to our understanding of the diversity of eukaryotic microorganisms.

We gratefully thank Stephen Nold and Gabriël Zwart for critical reading of the manuscript and helpful suggestions. Special thanks are due to Klaus Schwenk, for valuable suggestions on DGGE pattern analysis, and Jeannine Ebert and Hans Hoogveld for measuring the chemical variables. This is publication 2371, Centre for Limnology, Netherlands Institute of Ecology.

Amann, R. I., Ludwig, W. & Schleifer, K-H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59:143–69.

Appels, R., Gerlach, W. L., Dennis, E. S., Swift, H. & Peacock, W. J. 1980. Molecular and chromosomal organization of DNA sequences coding for the ribosomal RNAs in cereals. *Chromosoma* 78:293–311.

Backeljau, T., Debruyn, L., Dewolf, H., Jordaens, K., Vandongen, S. & Winnepenninckx, B. 1996. Multiple UPGMA and neighbor-joining trees and the performance of some computer packages. *Mol. Biol. Evol.* 13:309–13.

Berger, C. 1987. Habitat en ecologie van *Oscillatoria agardhii* Gomont. Een limnologische studie van ondiepe hypertrofe meren (Drontermeer, Veluwemeer, Wolderwijd en Eemmeer; IJsselmeer; Lauwersmeer) van 1971–1981. Ph.D. thesis, University of Groningen, The Netherlands, 233 pp.

Berger, C. & Sweers, H. E. 1988. The IJsselmeer and its phytoplankton—with special attention to the suitability of the lake as a habitat for *Oscillatoria agardhii* Gom. *J. Plankton Res.* 10: 579–99.

Demeke, T. & Adams, R. P. 1992. The effects of plant polysaccharides and buffer additives on PCR. *BioTechniques* 12:332–34.

De Rijk, P. & De Wachter, R. 1993. DCSE, an interactive tool for sequence alignment and secondary structure research. *Comput. Appl. Biosci.* 9:735–40.

Farrelly, V., Rainey, F. A. & Stackebrandt, E. 1992. Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl. Environ. Microbiol.* 61:2798–801.

Felsenstein, J. 1989. PHYLIP—phylogeny inference package. *Cladistics* 5:164–6.

Ferris, M. J., Muyzer, G. & Ward, D. M. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl. Environ. Microbiol.* 62:340–6.

Ferris, M. J., Nold, S. C., Revsbech, N. P. & Ward, D. M. 1997. Population structure and physiological changes within a hot spring microbial community following disturbance. *Appl. Environ. Microbiol.* 63:1367–74.

Ferris, M. J. & Ward, D. M. 1997. Seasonal distribution of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 63:1375–81.

Fisher, S. G. & Lerman, L. S. 1979. Length-independent separation of DNA restriction fragments in two dimensional gel electrophoresis. *Cell* 16:191–200.

Giovannoni, S. J., DeLong, E. F., Schmidt, T. M. & Pace, N. R. 1990. Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. *Appl. Environ. Microbiol.* 56: 2572–5.

Hillis, D. M. & Dixon, M. T. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Q. Rev. Biol.* 66:411–53.

Johnson, J. L. 1991. Isolation and purification of nucleic acids. *In* Stackebrandt, E. & Goodfellow, M. [Eds.] *Nucleic Acid Techniques in Bacterial Systematics.* John Wiley & Sons Ltd., West-Sussex, United Kingdom, pp. 1–19.

Kowalchuk, G .A., Gerards, S. & Woldendorp, J. W. 1997a. Detection and characterisation of fungal infections of Ammophila arenaria (marram grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18S rDNA. *Appl. Environ. Microbiol.* 63:3858–65.

Kowalchuk, G. A., Stephen, J. R., de Boer, W., Prosser, J. I., Embley, T. M. & Woldendorp, J. W. 1997b. Analysis of ammonia-oxidising bacteria of the β subdivision of the class *Proteobacteria* in sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Appl. Environ. Microbiol.* 63:1489–97.

Lange, M., Guillou, L., Vaulot, D., Simon, N., Amann, R. I., Ludwig, W. & Medlin, L. K. 1996. Identification of the class Prymnesiophyceae and the genus Phaeocystis with ribosomal RNA-targeted nucleic acid probes detected by flow cytometry. *J. Phycol.* 32:858–68.

Lim, E. L., Amaral, L. A., Caron, D. A. & DeLong, E. F. 1993. Application of rRNA-based probes for observing marine nanoplanktonic protists. *Appl. Environ. Microbiol.* 59:1647–55.

Long, E. O. & Dawid, I. B. 1980. Repeated genes in Eukaryotes. *Annu. Rev. Biochem.* 49:727–64.

Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J. & Woese, C. R. 1997. The RDP (Ribosomal Database Project). *Nucleic Acids Res.* 25:109–11.

Manly, B. F. J. 1994. *Multivariate Statistical Methods. A Primer,* 2nd ed. Chapman & Hall, London, 215 pp.

Moed, J. R. & Hallegraeff, G. M. 1978. Some problems in the estimation of chlorophyll-a and phaeopigments from pre- and post-acidification spectrophotometric measurements. *Int. Rev. Ges. Hydrobiol.* 63:787–800.

Muyzer, G. & de Waal, E. C. 1994. Determination of the genetic diversity of microbial communities using DGGE analysis of PCR-amplified 16S rDNA. *In* Stal, L. J. & Caumette, P. [Eds.] *Microbial Mats: Structure, Development and Environmental Significance.* Springer-Verlag, Heidelberg, Germany, pp. 207–14.

Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16s rRNA. *Appl. Environ. Microbiol.* 59:695–700.

Muyzer, G., Teske, A., Wirsen, C. O. & Jannasch, H. W. 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16s rDNA fragments. *Arch. Microbiol.* 164:165–72.

Nei, M. & Li, W. H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* 76:5269–73.

Ogram, A. V., Sayler, G. S. & Barkay, T. T. 1988. The extraction and purification of microbial DNA from sediments. *J. Microbiol. Methods* 7:57–66.

Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. *Science* 276:734–40.

Page, R. D. M. 1994. Parallel phylogenies: reconstructing the history of host–parasite assemblages. *Cladistics.* 10:155–73.

Reysenbach, A., Giver, L. J., Wickham, G. S. & Pace, N. R. 1992. Differential amplification of rRNA genes by polymerase chain reaction. *Appl. Environ. Microbiol.* 58:3417–18.

Santegoeds, C. M., Nold, S. C. & Ward, D. M. 1996. Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from a hot spring cyanobacterial mat. *Appl. Environ. Microbiol.* 62:3922–28.

Schmidt, T. M., Delong, E. F. & Pace, N. R. 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *J. Bacteriol.* 173:4371–78.

Simon, N., Lebot, N., Marie, D., Partensky, F. & Vaulot, D. 1995. Fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes to identify small phytoplankton by flow cytometry. *Appl. Environ. Microbiol.* 61:2506–13.

Teske, A., Wawer, C., Muyzer, G. & Ramsing, N. B. 1996. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Appl. Environ. Microbiol.* 62:1405–15.

Van de Peer, Y., Jansen, J., De Rijk, P. & De Wachter, R. 1997. Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Res.* 24:111–16.

Van Hannen, E. J. & Gons, H. J. 1997. UV-C induced lysis and detritus production of *Oscillatoria limnetica* in a two stage continuous flow system. *J. Plankton Res.* 19:723–33.

Wawer, C. & Muyzer, G. 1995. Genetic diversity of *Desulfovibrio* spp. in environmental samples analyzed by denaturing gradient gel electrophoresis of [*nife*] hydrogenase gene fragments. *Appl. Environ. Microbiol.* 61:2203–10.

Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* 15:221–71.

Woese, C. R., Kandler, O. & Wheelis, M. L. 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Natl. Acad. Sci. USA* 87:4576–79.