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Diversity of cyanobacteria and heterotrophic bacteria in cyanobacterial blooms in Lake Joutikas, Finland

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ABSTRACT: Water samples were collected in August 2001 and 2002 from the eutrophic Lake Joutikas during cyanobacterial blooms. DNA and RNA were isolated from size fractionated samples and the diversity of the bacteria present in each fraction was studied by PCR amplification of partial 16S rRNA and denaturing gradient gel electrophoresis (DGGE) fingerprinting. Major bands from the gels were sequenced for further identification. Cyanobacteria were also identified and counted under the microscope. *Anabaena/Aphanizomenon* were the most abundant cyanobacteria in both years, although the dominant species was different each year. When comparing the 2 techniques, equal numbers of abundant *Anabaena/Aphanizomenon* morphotypes were detected by microscopy and phylotypes by DGGE. The genera *Microcystis* and *Synechococcus* appeared more abundant in the DGGE analysis than under the microscope. In the heterotrophic bacterial community variation was observed between the bloom samples from the 2 years. *Verrucomicrobia* was the most abundant group in both years in both DNA- and RNA-derived profiles. Otherwise the patterns based on DNA- and RNA-derived DGGE-profiles differed, especially in 2002. The presence of *Actinobacteria* and *Chloroflexi* was less pronounced in RNA-based than in DNA-based analysis. This indicates that their relative biomass was smaller than estimated by DNA-analysis. It might also indicate that they were metabolically inactive. In contrast, in 2002, the CFB group (*Cytophaga-Flavobacterium-Bacteroides*) and δ-*Proteobacteria* were more prominent in the RNA-based than in the DNA-based profiles. Thus they probably formed a substantial fraction of biomass and/or were active members in the blooms.

KEY WORDS: Cyanobacteria · Heterotrophic bacteria · DGGE · DNA · RNA · Diversity

INTRODUCTION

Toxic cyanobacterial blooms, i.e. mass occurrences, are a worldwide problem for water quality, especially in eutrophic lakes. The diversity of the co-existing heterotrophic bacteria in the bloom and their effects on human and animal health as well as their possible interactions with cyanobacteria are poorly known. In general, bacterial community composition depends on predation and the organic material available. Different bacterial communities have been shown to develop on the cyanobacterial detritus and green algal detritus (van Hannen et al. 1999a,b). It has been suggested that nitrogen and products from carbon dioxide fixation are transferred from cyanobacteria to attached bacteria in a bloom (Paerl 1984) and that the activity of associated bacteria responds to cyanobacterial exudates (Wang & Priscu 1994). Endotoxins are a group of toxins that are components of the cell wall of cyanobacteria and gram-negative bacteria (Kuiper-Goodman et al. 1999). High endotoxin concentrations occasionally detected in cyanobacterial blooms might be produced by co-existing gram-negative bacteria (Rapala et al. 2002). However, only a little is known about the co-existing

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bacteria in cyanobacterial blooms whereas freshwater bacterial communities in general have been studied extensively (e.g. Hiorns et al. 1997, Konopka et al. 1999, Crump et al. 1999, Casamayor et al. 2000, 2002, Glöckner et al. 2000, Lindström & Leskinen 2002, Zwart et al. 2002).

In spite of improved culturing techniques, most microorganisms in aquatic ecosystems still remain uncultured (Zwart et al. 2002). Therefore, molecular biology techniques such as denaturing gradient gel electrophoresis (DGGE) using the 16S rRNA as a molecular marker are commonly used for diversity studies. In DGGE, DNA fragments of the same length can be separated on the basis of melting domain structure and nucleotide composition (Muyzer & Smalla 1998). In studies of aquatic bacterial communities both 16S rRNA gene and reverse transcribed rRNA have been used in the DGGE analysis. Several authors have used RNA-derived DGGE patterns to infer the potentially active bacterial population (Teske et al. 1996, Bernard et al. 2000, Schäfer et al. 2001, Winter et al. 2001, Troussellier et al. 2002). Although active cells tend to have more RNA than inactive ones, the RNA content varies greatly among species (Flärdh et al. 1992, Lee & Kemp 1994, Binder & Liu 1998, Fegatella et al. 1998). This makes the use of rRNA as an indicator of activity problematic in natural mixed-species communities. The content of rRNA has also been found to correlate with the cell volume (Kemp et al. 1993, Binder & Liu 1998) and thus RNA-based DGGE analysis could alternatively be interpreted to reveal those bacteria that form a substantial fraction of biomass in the system.

Little is known about the bacterial communities associated with cyanobacteria in freshwater lakes. We wanted to find out which cyanobacteria and heterotrophic bacteria are present in the cyanobacterial blooms. We isolated both DNA and RNA from the water and bloom samples collected in 2 years and subjected them to DGGE analysis and sequencing of 16S rRNA fragments. We also compared the cyanobacterial DGGE patterns to the results of traditional microscopic examination of cyanobacteria.

Table 1. Description of Lake Joutikas and physical and chemical characteristics of water. nd: not determined

Variable	Date	
	Aug 22, 2001	Aug 20, 2002
Surface area (km^2)	0.11	
Mean depth (m)	2.5	
Maximum depth (m)	3.8	
Total phosphorus (mg P l^{-1})	nd	63
Total nitrogen (mg N l^{-1})	nd	1700
Chlorophyll *a* (mg l^{-1})	160	71
NH_4-N (mg l^{-1})	93	110
Water temperature (°C)	20.0	21.3
Oxygen (mg l^{-1})	nd	4.6
Conductivity (mS m^{-1})	nd	8.5
Water colour (mg Pt l^{-1})	nd	60
pH	7.2	7.3
Secchi depth (m)	0.7	0.5
Microcystin (µg l^{-1})	0.67	14

MATERIALS AND METHODS

Study site and sampling of bacteria. Water samples were taken from Lake Joutikas on August 23, 2001, and August 22, 2002. Lake Joutikas is a eutrophic lake situated in Southern Finland. It is a small and shallow lake (Table 1) where heavy cyanobacterial blooms have been a common phenomenon for many summers. Water samples for nucleic acid extractions were collected by a tube sampler from a depth of 0 to 0.3 m. Five µm filters (Osmonics, Polycarbonate, 47 mm) were used to collect cyanobacteria, and 1 µm (Osmonics, Polycarbonate, 47 mm) and 0.2 µm filters (Pall corporation, Supor-200, 47 mm) to collect the rest of the bacteria. Filtrations were performed by the lakeside within 30 min of sampling. Separate bloom samples from the surface water (approximately 1 cm layer) were also taken. Both the filters and the bloom samples were placed into 2 ml polypropylene tubes (Nalgene) and frozen immediately in liquid nitrogen. In the laboratory, samples for RNA isolation were stored at –80°C and samples for DNA isolation at –20°C. In addition, from samples collected by the Uusimaa Regional Environment Centre on August 22, 2001, and August 20, 2002, toxin concentrations were analyzed, cyanobacteria were identified under the microscope, the cells of the different cyanobacterial genera were counted and the cell counts converted to biomasses using the database of the Finnish Environment Institute (unpubl.). Physical and chemical characteristics of water were analyzed by the Uusimaa Regional Environment Centre (Table 1).

Nucleic acid extraction. DNA and RNA were extracted by bead beating and the CTAB (cetyltrimethyl-ammonium bromide) method with FastPrep tubes and FastPrepFP120 bead beating system (Bio 101). Frozen filters were transferred to the FastPrep tubes (lysis matrix A for DNA and lysis matrix C for RNA) containing 1 ml of cold lysis buffer (100 mM Tris-HCl, pH 8, 1.5% SDS, 10 mM EDTA, 1% deoxycholate, 1% Igepal-CA630, 5 mM thiourea, 10 mM dithiothreitol). The cells were disrupted mechanically with the FastPrepFP120 bead beater at 5.0 m s^{-1} for 30 s. After homogenisation the tubes were placed on ice for 5 min and then centrifuged for 1 min at 15 300 × *g*. The supernatant (1 ml) was divided between two 2 ml tubes and 225 µl of 5 M NaCl and 170 µl of 10%

CTAB in 0.7 M NaCl were added and mixed. The tubes were incubated for 20 min at 65°C. An equal volume of chloroform was added, the tubes were mixed and centrifuged at 10 600 × *g* for 10 min. DNA and RNA samples from 5 µm filters in 2002 were treated 2 or 3 times with 10% CTAB to reduce the amount of polysaccharides. The upper phase was collected in aliquots of 450 µl into new 1.5 ml Eppendorf tubes. The samples were precipitated with ice-cold 96% ethanol and dissolved in 50 µl H_2O.

DNA extracts were further purified by either a Prep-A-Gene purification kit (Bio-Rad) (year 2001) or a NucleoTrap purification kit (Macherey-Nagel) (year 2002). Aliquots of RNA samples were treated with RNase-free DNase (Promega) for 30 min at 37°C. DNase was inactivated and removed by phenol-chloroform extraction followed by ethanol precipitation. The quality of DNA was checked in 1.5% agarose gel and the total RNA in 1.2% agarose formaldehyde gels.

Reverse transcription of RNA. RNA was converted to cDNA in reverse transcription. The 20 µl reaction volume contained 7.5 µl of RNA, 1.5 µl of random nonamers, 500 µM dNTPs, 2 µl of 10× M-MuLV buffer, 200 U of M-MuLV reverse transcriptase (Finnzymes) and 7 µl of DEPC-water. The absence of contaminating DNA in RNA samples was tested by performing the PCR reaction without reverse transcription step (RT negative control). Extracted total nucleic acids (including DNA) were amplified for positive control and sterile water for negative control of PCR. After PCR, aliquots of the RT products together with positive and negative controls were analysed by electrophoresis in 1.5% agarose gel.

PCR amplification of 16S rRNA fragments. Eubacterial 16S rRNA gene primers F-968-GC and R-1401 (Nübel et al. 1996) were used to amplify PCR products for DGGE. PCR reactions were performed in a volume of 25 µl containing 2 µl of template DNA or cDNA, 200 nM of primers F-968-GC and R-1401, 200 µM dNTP solution, 2.5 µl of 10× reaction buffer, 1 M Betaine (Sigma), 0.3 U of DyNAzyme™ II DNA Polymerase (Finnzymes) and sterile water up to 25 µl. The PCR program consisted of a denaturing step of 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 2 min. A final extension step of 72°C for 10 min was then performed. The size and purity of the amplification products were checked in 1.5% agarose gel. Two parallel PCR reactions from each sample were prepared and combined after amplification by ethanol precipitation and dissolved in 25 µl of water. The combined PCR products were loaded into the DGGE gel.

DGGE analysis. Approximately 400 bp long DNA- and RNA-derived amplification products were analyzed in parallel in DGGE gels. DGGE buffers and gels were prepared according to the manufacturer's instructions for the Dcode™ Universal Mutation Detection system (Bio-Rad). A peristaltic pump system together with the Gradient Maker (Amersham Pharmacia Biotech) was used for casting of the gels. PCR products were separated in 6% polyacrylamide gels in 0.5 × TAE (20 mM Tris-acetate, 10 mM acetate, 0.5 mM Na_2EDTA) with a denaturing gradient from 35 to 55% denaturants (urea and formamide). DGGE gels were polymerised over night. Electrophoresis was performed for 4.5 h at a constant voltage of 150 V and a temperature of 60°C in the Dcode™ apparatus containing a magnetic stirrer bar. After electrophoresis, the gels were stained for 40 min with Gelstar nucleic acid stain (BioWhittaker Molecular Applications) and recorded under UV-light using Kodak 1D image analysis software.

Standards for the DGGE gels were prepared by running PCR amplified DNA samples isolated from cyanobacterial cultures or lake samples in DGGE and excising 5 intense DGGE bands at different levels from the gel so that the majority of the bands in the Lake Joutikas samples were located between the positions of the shortest and furthest migrating standard bands. A mixture of these PCR products was used as a standard in each DGGE gel. Three standard lanes were included on every gel.

The DNA- and RNA-derived samples were analysed side by side in DGGE gels. The presence and intensity of bands in DGGE gels was transformed into numerical data by GelCompar 4.1 software (Applied Maths BVBA). The software measured an optical density profile through each DGGE lane (sample), and calculated the band positions and relative (%) intensity of each band against the whole lane (100%).

Sequencing of PCR products. Prominent DNA bands were excised from the DGGE gels with a sterile scalpel and eluted from the gel slices by sterile water at 4°C overnight. These eluted products were reamplified and the PCR products were separated again in DGGE. Separated products were reamplified with primers F-968, without the GC-clamp, and R-1401 and purified by Microcon PCR purification tubes (Millipore). The purified products were sequenced with the Big Dye cycle sequencing ready reaction kit (Applied Biosystems) according to the manufacturer's instructions using the reverse primer R-1401. Sequencing reactions were analysed by an Applied Biosystems 310 genetic analyser. More than 100 bands were sequenced to get all the main bands at different positions on the gel sequenced at least once. Finally, 33 different good quality sequences were obtained.

Analysis of data. A BLAST search (Altschul et al. 1997) at the GenBank database was performed for the 16S rRNA sequences retrieved from the lake samples. Our sequences were aligned with the sequences of the highest similarity using the ARB software package

(version; Linux Beta 030822, O. Strunk and W. Ludwig, Technische Universität München [www.arb-home.de]). The full-length sequences were added to the ARB database by using the maximum parsimony tool. Shorter sequences (<600 bases, including the sequences of this study and the Changjiang River sequences) were added to the tree with the ARB parsimony tool, which allows the addition of short sequences to existing phylogenetic trees without changing global tree topologies. Alignments were checked and corrected manually. Dendrograms for each phylogenetic group were obtained by using the Phylip DNAPARS parsimony tool included in the ARB software.

RESULTS

The sequence similarities for the 33 different sequences obtained from the DGGE bands are shown in Table 2. The phylogenetic analysis of the sequences gave 9 distinct clusters: *Actinobacteria*, CFB (*Cytophaga-Flavobacterium-Bacteroides*), *Chloroflexi* (formerly called green nonsulfur bacteria), *Verrucomicrobia*, α-, β-, γ- and δ-*Proteobacteria* and *Cyanobacteria* (Fig. 1A–F). Most of the sequences had nearest relatives among uncultured species sequenced from freshwater environments in other studies. In the BLAST search the DGGE bands of cyanobacteria showed 98 to 100% similarity to several *Anabaena* or *Microcystis* species. Only the names that were listed first are shown in Table 2.

Based on microscopic analysis of the cyanobacterial community genera *Anabaena* and *Aphanizomenon* were the most prevalent genera of the bloom in both years (Fig. 2). The total biomass of *Anabaena* was about 3-fold in 2002 (17.9 mg l^{-1}) compared to 2001 (5.9 mg l^{-1}). For these genera the microscopy and sequencing results of DGGE bands corresponded well. In 2001, 4 different *Anabaena/Aphanizomenon* morphotypes were prevalent in the bloom according to microscopic identification (*Anabaena fusca*, *Anabaena macrospora*, *Aphanizomenon flexuosum*, and *Aphanizomenon gracile*) and also 4 strong bands representing phylotypes of *Anabaena/Aphanizomenon* were detected from the DGGE gels (bands 2, 3, 4 and 7). In 2002 only one dominant *Anabaena* morphotype (*A. macrospora*) was identified under the microscope and accordingly only one intense band (7) was seen in the DGGE gel.

The biomass of *Microcystis* was 9-fold in 2001 compared to the year 2002 (Fig. 2). Accordingly, in the DGGE gel, 2 intense bands showing a similarity to *Microcystis* (5 and 6) were seen in 2001 but in 2002 they were weak (Fig. 3). Yet, their relative intensity among cyanobacterial bands obtained from the 5 µm filter was rather high (Fig. 3). In DGGE, 2 intense *Synechococcus* bands (8 and 9) were seen in 2002, one less intense one (9) was seen in 2001 (Fig. 3). As with *Microcystis*, the relative biomass of *Synechococcus* detected by microscopy was much lower than indicated by DGGE (Fig. 2). Determined by microscopy, *Woronichinia* was abundant in both years and *Aphanocapsa* in 2001, but no sequenced band showed a similarity to these genera. For all cyanobacteria the DNA- and RNA-derived DGGE patterns looked very similar (Fig. 3). The microcystin concentration in the surface sample was 0.67 µg l^{-1} in 2001 and 14 µg l^{-1} in 2002 (Table 1). Thus, the cyanobacterial community was more toxic in 2002.

The structure of the heterotrophic bacterial community in the Lake Joutikas samples based on DGGE fingerprinting and sequence analysis was somewhat different in 2001 and 2002, but the main bacterial groups remained the same (Figs. 3 & 4). The most abundant bacterial groups in both years were *Verrucomicrobia*, *Actinobacteria* and *Chloroflexi*. *Verrucomicrobia* was the most abundant group in both years in both DNA- and RNA-derived profiles. The presence of *Actinobacteria* and *Chloroflexi* was less pronounced in RNA- than in DNA-derived analysis, especially in 2002. γ-*Proteobacteria* (bands 20 and 21 in Fig. 3) was an additional abundant group in 2001. Sequences related to α-*Proteobacteria* (bands 17 and 18) and β-*Proteobacteria* (band 19) were present as minor groups on DGGE profiles in 2001. δ-*Proteobacteria* (band 22), 2 *Actinobacteria*-related bands (28 and 29) and CFB (band 25) were new sequence types which appeared in 2002. The number of *Actinobacteria*-related DNA-derived bands was higher in 2002 than 2001 (Fig. 3, 0.2 µm filter), but in general, more bands were observed in 2001 than in 2002 indicating a more diverse community in 2001.

For certain phylotypes, the intensities of DNA- and RNA-derived bands differed clearly (Fig. 4). Bands representing *Actinobacteria* (bands 26, 27, 28, 29 and 30), *Chloroflexi* (band 23) and one phylotype of *Verrucomicrobia* (band 13) were intense in DNA-derived DGGE profiles but faint in RNA-derived DGGE profiles, indicating that these phylotypes represented numerically abundant taxa that, however, did not contribute substantially to the biomass and were perhaps inactive. On the other hand, certain representatives of *Aphanizomenon* sp. (band 10), *Verrucomicrobia* (band 12), CFB (band 25), and eukaryotic chloroplasts (band 33) had relatively strong bands in the RNA-derived profiles compared to the DNA-derived profiles. These phylotypes probably contributed to the biomass more than their numbers estimated by DNA-analysis would have indicated.

Table 2. Sequence similarities of the excised DGGE bands from Lake Joutikas in 2001 and 2002

No. in DGGE gel (Fig. 3)	Original code for DGGE bands	Accession no.	Similarity (%)	Organism	Accession no.	Taxonomic description	Source
1	Jo50, 51, 68, 79, 98-01	AJ620824	99	*Anabaena solitaria*	AJ293105	*Cyanobacteria*	Lake Karpjärvi, Finland
2	Jo1, 24-01	AJ620825	99	*Anabaena flos-aquae* NRC525-17	AF247597	*Cyanobacteria*	Buffalo Pound Lake, Canada
3	Jo2, 25-01	AJ620826	98	*Anabaena flos-aquae* NRC525-17	AF247597	*Cyanobacteria*	Buffalo Pound Lake, Canada
4	Jo3, 65-01	AJ620827	98	*Anabaena flos-aquae* NRC525-17	AF247597	*Cyanobacteria*	Buffalo Pound Lake, Canada
5	Jo27, 42, 53-01	AJ620828	100	*Microcystis flos-aquae* UWOCC C3	AF139329	*Cyanobacteria*	United States
6	Jo78-01	AJ620829	98	*Microcystis aeruginosa* NIES89	U03403	*Cyanobacteria*	Lake Kawaguchi, Japan
7	Jo38, 44-02	AJ620830	100	*Anabaena spiroides*	AJ293116	*Cyanobacteria*	Lake Aydat, France
8	Jo46-02	AJ620831	98	*Synechococcus* sp. TAG	AF448066	*Cyanobacteria*	
9	Jo3-02	AJ620832	99	*Synechococcus* sp. BO8807	AF317074	*Cyanobacteria*	Lake Constance, Central Europe
10	Jo 126-01	AJ620833	100	*Aphanizomenon flos-aquae*	AY038035	*Cyanobacteria*	Brielse Meer, The Netherlands
11	Jo11, 32, 33, 74, 122, 152, 169, 172-01 Jo47, 53-02	AJ620834	98	Uncultured bacterium FukuN106	AJ289983	*Verrucomicrobia*	Lake Fuchskuhle, Germany
12	Jo95, 123, 135, 150-01	AJ620835	94	Uncultured bacterium FukuN106	AJ289983	*Verrucomicrobia*	Lake Fuchskuhle, Germany
13	Jo10, 35, 56-02	AJ620836	99	Uncultured bacterium FukuN106	AJ289983	*Verrucomicrobia*	Lake Fuchskuhle, Germany
14	Jo7, 118, 128, 146-01	AJ620837	97	Uncultured bacterium FukuS27	AJ290012	*Verrucomicrobia*	Lake Fuchskuhle, Germany
15	Jo119, 147-01	AJ620838	100	Uncultured bacterium CR99-2-04	AF429190	*Verrucomicrobia*	Changjiang River, China
16	Jo60-01, Jo27-02	AJ620839	99	Unidentified eubacterium LD19	AF009974	*Verrucomicrobia*	Lake Loosdrecht, The Netherlands
17	Jo151-01	AJ620840	93	Uncultured α-proteobacterium MB13G10	AY033319	α-*Proteobacteria*	Monterey Bay, CA, USA
18	Jo23, 76, 158, 162-01	AJ621043	96	*Caulobacter* sp.	AJ227766	α-*Proteobacteria*	
19	Jo115-01	AJ620841	89	Uncultured beta proteobacterium clone 44a-U1-9	AY082479	β-*Proteobacteria*	Biofilm of acid mine drainage system
20	Jo62, 97-01	AJ620842	93	*Thiocapsa roseopersicina* 1711	AF113000	γ-*Proteobacteria*	Prevost lagoon, France
21	Jo127, 170-01	AJ620843	96	Uncultured bacterium LO13.6	AF358020	γ-*Proteobacteria*	Peat soil, UK
22	Jo39-02	AJ620844	97	Uncultured bacterium mle1-27	AF280857	δ-*Proteobacteria*	Wastewater bioreactor
23	Jo61, 87, 136-01	AJ620845	100	Uncultured bacterium clone CR99-24-49	AF429159	*Chloroflexi*	Changjiang River, China
24	Jo84-01	AJ620846	99	Uncultured bacterium clone CR99-35-18	AF428976	*Chloroflexi*	Changjiang River, China
25	Jo24-02	AJ620847	100	Uncultured bacterium clone CR98-35-74	AF428956	*CFB*	Changjiang River, China
26	Jo88-01, Jo32-02	AJ620848	97	Gram-positive bacterium SOGA22	AJ244799	*Actinobacteria*	Cuttlefish glands, English Channel, France
27	Jo153-01	AJ620849	100	Uncultured bacterium CR99-2-31	AF429217	*Actinobacteria*	Changjiang River, China
28	Jo51-02	AJ620850	100	Uncultured bacterium clone HT2E3	AJ290054	*Actinobacteria*	Lake Fuchskuhle, Germany
29	Jo41-02, 52-02	AJ620851	100	Uncultured bacterium GKS2-103	AJ290024	*Actinobacteria*	Lake Gossenkoellesee, Austria
30	Jo125-01	AJ620852	100	Uncultured bacterium CR99-2-40	AF429226	*Actinobacteria*	Changjiang River, China
31	Jo70-01, 117-01	AJ620853	95	Unidentified cryptomonad OM283	U70724	Cryptophyta Chloroplast	Cape Hatteras, NC, USA
32	Jo31-02	AJ620854	96	*Rhodomonas abbreviata*	AF545627	Cryptophyta Chloroplast	
33	Jo145-01	AJ620855	92	Chloroplast *Guillardia theta*	AF041468	Cryptophyta Chloroplast	

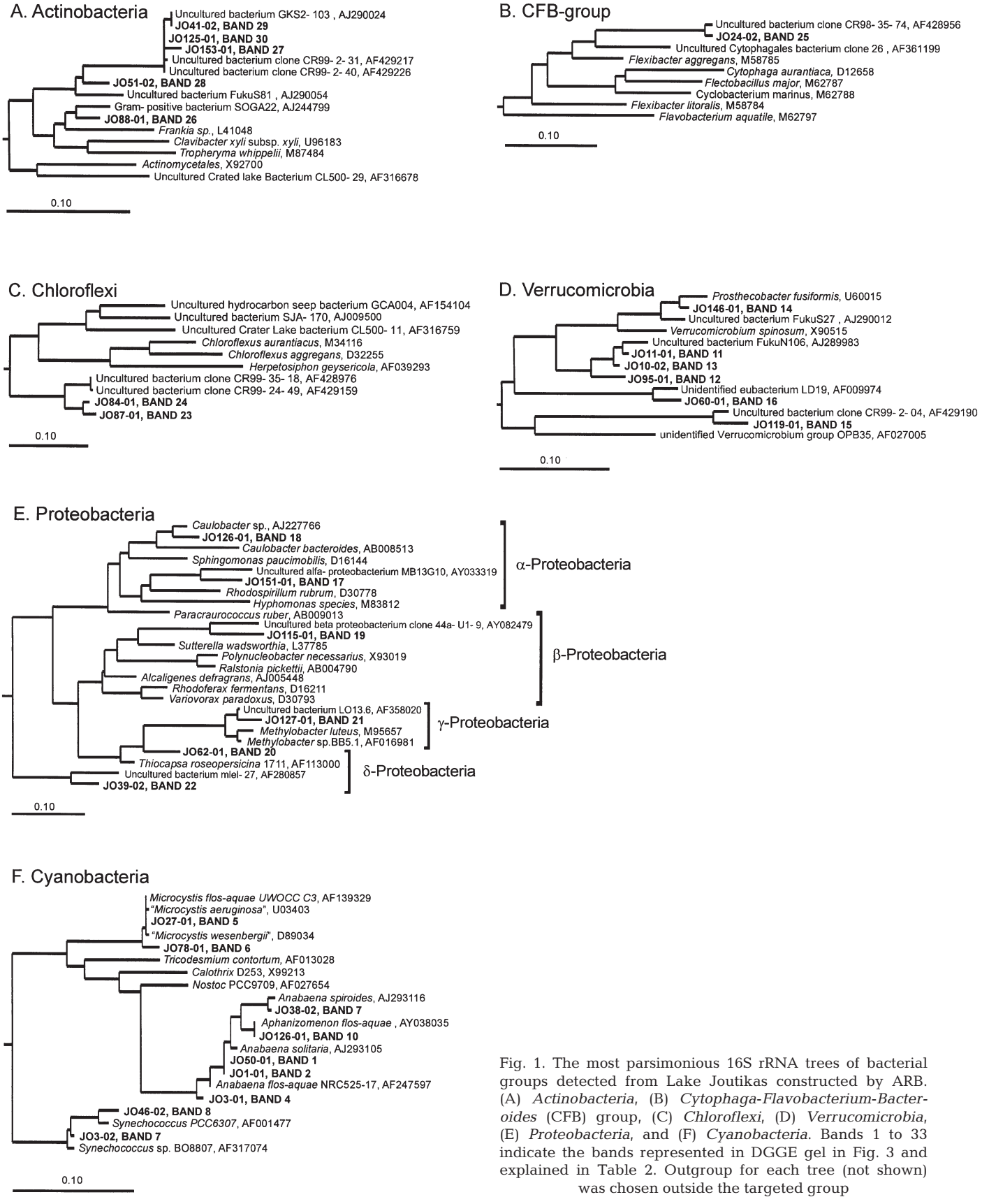

Fig. 1. The most parsimonious 16S rRNA trees of bacterial groups detected from Lake Joutikas constructed by ARB. (A) *Actinobacteria*, (B) *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, (C) *Chloroflexi*, (D) *Verrucomicrobia*, (E) *Proteobacteria*, and (F) *Cyanobacteria*. Bands 1 to 33 indicate the bands represented in DGGE gel in Fig. 3 and explained in Table 2. Outgroup for each tree (not shown) was chosen outside the targeted group

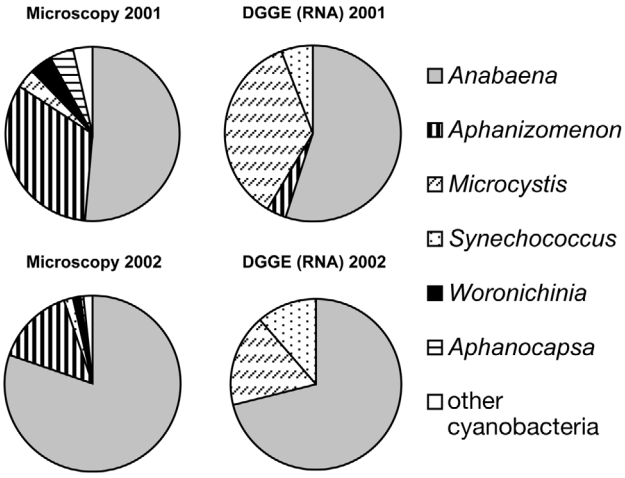

Fig. 2. Relative composition of the cyanobacterial community in blooms in 2001 and 2002 as determined by microscopy (biomasses, µg l^{-1}) and sequencing of DGGE phylotypes (relative intensities of sequenced bands in RNA-derived DGGE gels)

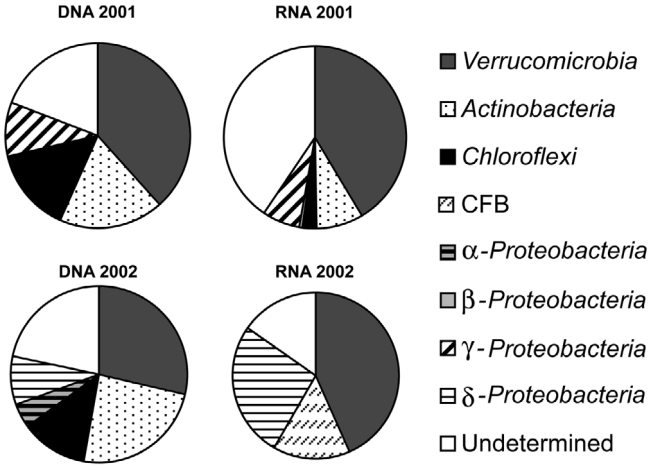

Fig. 4. Relative composition of the heterotrophic bacterial community in blooms in 2001 and 2002 as determined by intensities of bands from DNA- and RNA-derived DGGE gels. Bands from the filters with pore sizes 1 and 0.2 µm were used in the diagrams

DISCUSSION

We studied the diversity of cyanobacteria and heterotrophic bacteria present in the cyanobacterial blooms in the eutrophic Lake Joutikas. Different cyanobacterial species dominated the bloom in the studied years but the dominant cyanobacterial genera were the same (*Anabaena/Aphanizomenon*). The taxonomy of cyanobacteria was previously based on morphological features, which makes comparison of microscopic and sequencing results difficult. Currently, however, cyanobacterial taxonomy is under revision to accommodate phylogenetic relationships. The genera *Anabaena* and *Aphanizomenon* have very similar 16S rRNA gene sequences (Castenholz 2001, Gugger et al. 2002). Seven *Anabaena/Aphanizomenon* morphotypes were detected under the microscope and 4 of them were very abundant. Six (bands 1, 2, 3, 4, 7 and 10) different genotypes were detected from DGGE gels that belonged to the genera *Anabaena/Aphanizomenon.* The same closest relative (*Anabaena flos-aquae*) was retrieved for bands 2, 3 and 4 from GenBank even if the bands migrated at different levels in the gel. It is possible that these bands were from different subspecies of genera *Anabaena/Aphanizomenon* or different copies of the 16S rRNA gene in one organism (Iteman et al. 2002). *Anabaena* is known as the most important toxin-producing genus in Finnish lakes (Sivonen et al. 1990). In our study, in 2002 the cyanobacterial population was dominated by *Anabaena* and the detected microcystin level was higher than in 2001.

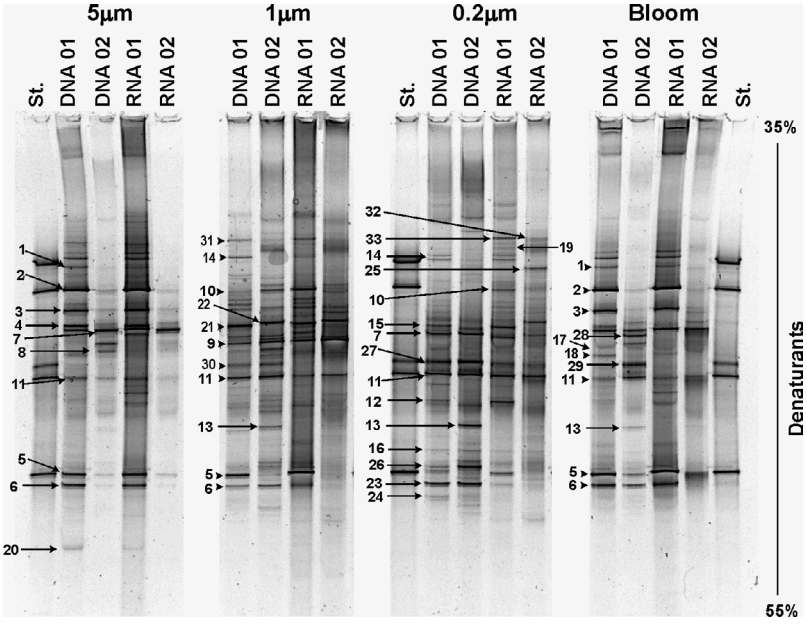

Fig. 3. DGGE patterns of DNA- and RNA-derived 16S rRNA products amplified from Lake Joutikas samples from years 2001 and 2002. Samples were from filters with pore size 5, 1 and 0.2 µm and non-filtered bloom sample. For bands numbered 1–33 sequence similarities with GenBank sequences are shown in Table 2

The genera *Anabaena/Aphanizomenon* had a greater biomass than genus *Microcystis*, although their band intensities in DGGE were similar in the 2001 samples. This might be due to different DNA-cell biomass ratios in the 2 genera with different cell morphologies or due to different amplification efficiency in PCR (Wintzingerode et al. 1997). In this study, the cyanobacterial genera that formed only a small part of the biomass under the microscope were not detected by DGGE or their bands were too faint to be excised for sequencing. Microscopy and sequencing results of DGGE bands have also previously been found to correspond with each other so that organisms present at higher cell concentrations were recovered from the sequenced DGGE bands, but not the ones with lower cell concentrations (Casamayor et al. 2000, 2002). Roughly, DGGE cannot detect populations whose abundance is less than 1% of the total cell count (Muyzer et al. 1993).

In Lake Joutikas the main heterotrophic bacterial divisions present in the blooms on both sampling dates were *Verrucomicrobia*, *Actinobacteria* and *Chloroflexi*. The same bacterial divisions have been found in other freshwater environments (e.g. Hiorns et al. 1997, Crump et al. 1999, Konopka et al. 1999, Casamayor et al. 2000, 2002, Glöckner et al. 2000, Lindström & Leskinen 2002, Zwart et al. 2002). The *Cytophaga-Flavobacterium-Bacteroides* group (CFB), a very common bacterial division in freshwaters, was found in RNA-derived gels in 2002. α-, β-, and γ-*Proteobacteria* were detected in 2001 but not in 2002. In 2002 δ-*Proteobacteria* were present instead.

Of our sequences representing *Verruromicrobia*, Jo11-01, Jo10-02 and Jo95-01 were related to the sequences from the group FukuN18 (Zwart et al. 2002), sequence Jo60-01 to group LD19 and sequence Jo119-01 to CL120-10. All *Verrucomicrobia* found in this study have also been commonly found in other Finnish lakes with various trophic statuses (Haukka et al. unpubl. results). Our sequences Jo41-02, Jo125-01 and Jo153-01, representing *Actinobacteria*, were related to the sequences from group ACK-M1 (Zwart et al. 2002) as well as to the sequences from other Finnish lakes (Haukka et al. unpubl. results). The actinobacterial band 26, which appeared in a lower position in the gel than the other *Actinobacteria*, had a sequence further related to the other *Actinobacteria* sequences found in Lake Joutikas. Two of our sequences belonged to the phototrophic division *Chloroflexi*, formerly called green non-sulphur bacteria (Björnsson et al. 2002). Sequences belonging to the organisms in this division have previously been found in contrasting environments, e.g. at 500 m depth in the ultra-oligotrophic Crater Lake (Urbach et al. 2001), in the chemocline of the meromictic alpine Lake Cadagno (Bosshard et al. 2000) and in wastewater treatment plants (Björnsson et al. 2002). Our sequences were very similar to the partial sequences (600 bp) obtained from the Changjiang River or the adjacent lakes (CR99-35-18 and CR99-24-49 by Sekiguchi et al. (2002), but they did not seem to be closely related to any full-length *Chloroflexi* sequences.

The divisions *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia* and the CFB group have previously been found in the cyanobacteria-dominated Lakes Loosdrecht and IJssel in the Netherlands (Zwart et al. 1998, 2002) and in the Adirondack Mountain lakes in the eastern USA, which contained a low number of cyanobacteria (Hiorns et al. 1997). A similar bacterial community composition has also been found in other lakes, reservoirs and rivers (Crump et al. 1999, Glöckner et al. 2000, Trusova & Gladyshev 2002, Sekiguchi et al. 2002, O'Sullivan et al. 2002).

Members of the CFB group are very common freshwater bacteria (Kenzaka et al. 1998, Crump et al. 1999, Sekiguchi et al. 2002, O'Sullivan et al. 2002) and they are known to play an important role in the turnover of organic matter (Cottrell & Kirchman 2000). *Cytophaga* sp., belonging to CFB, is known for its ability to produce exopolysaccharide slime (Larkin 1989), which could explain the large amount of slime in our samples in 2002. Studies of various freshwater microbial communities have shown that the β-proteobacterial group often comprises a large proportion of freshwater bacterial communities (Hiorns et al. 1997, Pernthaler et al. 1998, Glöckner et al. 2000), but it was rare in Lake Joutikas.

A primer set of F-968 and R-1401 (Nübel et al. 1996) for the eubacterial 16S rRNA gene region was used in this study while in many other DGGE studies of freshwater (e.g. Casamayor et al. 2000, 2002, Jaspers et al. 2001, J. Pernthaler et al. 2001, Kisand & Wikner 2003) a primer set of F341 and R907 (Muyzer et al. 1995) has been used. The number of CFB and β-*Proteobacteria*-related groups that we found from Lake Joutikas were lower than previously found elsewhere in freshwaters by the primer set of F341 and R907. Therefore we checked the sequences of our primers in the ARB database and confirmed their theoretical ability also to amplify CFB and β-proteobacterial sequences. We have also studied less eutrophic Finnish lakes using DGGE with the primers F-968 and R-1401. In the meso-eutrophic Lake Vesijärvi, the meso-oligotrophic Lake Ahvenlammi and the humic Lake Sammalisto *Actinobacteria* were the most prominent members followed by *Verrucomicrobia* (our unpubl. results). In all cases the α-, β-, γ- and δ-*Proteobacteria*, CFB-group and other divisions were detected sporadically. The possible effect of the choice of the primer set in the community composition detected by DGGE is not known.

In many studies the analysis of RNA-derived DGGE bands has been used to identify the possibly active populations of different environments (Teske et al. 1996, Griffiths et al. 2000, Schäfer et al. 2001, Norris et al. 2002, Troussellier et al. 2002). Indeed, several studies support the use of cellular RNA or the ribosome content, as an indicator of *in situ* growth rate of a chosen bacterial population in natural assemblages (Poulsen et al. 1993, Binder & Liu 1998, Worden & Binder 2003). However, while the copy number of rRNA gene operons per chromosome is typically under 10 in environmental bacteria (Klappenbach et al. 2000), the number of ribosomes can vary greatly among different species. A marine oligotrophic ultramicrobacterium was found to reach a maximum of 2000 ribosomes per cell in mid-log phase but even 200 ribosomes per cell were sufficient for maximum rates of growth (Fegatella et al. 1998). On the contrary, marine *Vibrio* cells were found to contain 20 000 to 35 000 ribosomes at the onset, and 8000 ribosomes after 4 d of starvation (Flärdh et al. 1992). Additionally, the number of ribosomes varies between the different phases of the cell cycle depending on e.g. the growth strategy of the species (A. Pernthaler et al. 2001) and temperature (Lee & Kemp 1994). However, in general, it seems that the ribosome content is correlated with the cell size, which in turn is related to biomass (Kemp et al. 1993, Binder & Liu 1998). Thus the RNA-based DGGE analysis might give a better picture than the DNA-based DGGE analysis on those bacteria that form a substantial fraction of biomass in the studied community. In our case, DNA- and RNA-derived DGGE bands of the same samples were different. Typically the actinobacterial bands were strong in DNA- and weak in RNA-derived gels, which would indicate that even if they are numerous their biomass is relatively low in the community. This is supported by the recent results that *Actinobacteria* can numerically constitute even more than a half of the bacteria in freshwater picoplankton, but they are small in size (Sekar et al. 2003). In addition, their growth strategy might be comparable to such strains as the marine oligotrophic ultramicrobacterium *Sphingomonas* sp., which was shown to contain a low number of ribosomes even when it is active (Fegatella et al. 1998). However, severe biases have been reported in the use of PCR-based methods and therefore quantitative comparison of the results should be made cautiously (Wintzingerode et al. 1997).

Substrate availability, weather conditions such as temperature and light, and predation influence the composition of cyanobacterial and heterotrophic bacterial communities in aquatic environments (Muylaert et al. 2002). The water temperature in Lake Joutikas was over 20°C on both of our sampling dates and during most of the summer. High temperatures together with high phosphorous levels were the probable factors that favoured the growth of cyanobacteria. Cyanobacteria as a source of organic material have been found to lead to a different bacterial community composition than green algae (van Hannen et al. 1999a). The highest abundances of heterotrophic bacteria have previously been observed during and after the cyanobacterial bloom in freshwater (Mayer et al. 1997, Bouvy et al. 2001). The increase in the number of bacteria is probably due to the release of easily utilisable dissolved organic matter from cyanobacterial exudates during the bloom and after its breakdown. Temporal changes in the bacterial community may occur in freshwater in a short period of time (Höfle et al. 1999, Jaspers et al. 2001, Van der Gucht et al. 2001). Complex interactions exist between phytoplankton, bacteria (Riemann et al. 2000), viruses (van Hannen et al. 1999b), protozoa (J. Pernthaler et al. 2001) and zooplankton (Höfle et al. 1999) in aquatic environments that all affect to the composition of the cyanobacterial and other bacterial communities.

We were interested in the diversity of cyanobacteria and heterotrophic bacteria occurring together in cyanobacterial blooms. We found typical freshwater bacteria in the blooms. Our results suggest that the heterotrophic bacterial community of the lake is present in a bloom regardless of the autotrophic bacterial phylotypes forming the bloom. Future studies should investigate whether certain heterotrophic bacteria have more specific interactions with cyanobacteria and whether the cyanobacterial toxins have an effect on the diversity and activity of the heterotrophic bacteria.

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