Seasonal dynamics and phylogenetic diversity of free-living and particle-associated bacterial communities in four lakes in northeastern Germany

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ABSTRACT: The phylogenetic diversity and seasonal dynamics of free-living and particle-associated bacterial communities were investigated in the epilimnion of 4 lakes of the Mecklenburg Lake District, northeastern Germany. All lakes differed in their limnological features, ranging from oligotrophic to eutrophic and dystrophic. Bacterial community structure and seasonal dynamics were analyzed by denaturing gradient gel electrophoresis (DGGE) and clone libraries of 16S rRNA gene fragments. Communities of free-living and particle-associated bacteria greatly differed among the lakes. In addition, significant differences occurred between both bacterial fractions within each lake. Seasonal changes were more pronounced in free-living than in particle-associated bacterial communities. Non-metric multidimensional scaling (NMDS) analyses revealed several strong correlations between bacterial communities (both free-living and particle-associated) and environmental variables such as pH, dissolved organic carbon (DOC), phytoplankton biomasses, and primary production. Phylogenetically, all cloned and sequenced 16S rRNA gene fragments belonged to already known freshwater clusters. Clone libraries of free-living bacteria were dominated by sequences of *Actinobacteria*, *Bacteroidetes*, and *Betaproteobacteria*, whereas those of particle-associated bacteria predominantly consisted of *Cyanobacteria* and *Bacteroidetes* sequences. Other freshwater phyla such as *Alpha-* and *Gammaproteobacteria*, *Verrucomicrobia*, *Planctomycetes*, and members of Candidate Division OP10 were found in low proportions. These differences may indicate an adaptation of distinct bacterioplankton communities to the respective environmental conditions of each lake.

KEY WORDS: Bacterioplankton communities · Phylogenetic diversity · Seasonal dynamics · Freshwater bacteria · DGGE · Clone libraries

INTRODUCTION

Heterotrophic bacteria are known to play a key role in biogeochemical processes and are responsible for the break-down of organic matter and the remineralisation of nutrients (Cotner & Biddanda 2002). Until the late 1980s, aquatic bacteria were considered as an entity or 'black box' without any further differentiation of their taxonomic composition. An important first step towards understanding the role of aquatic bacterial communities is the determination of the phylogenetic diversity of respective bacterioplankton communities (Cottrell & Kirchman 2000a). The introduction of mole-cular methods to aquatic microbial ecology has facilitated the study of bacterioplankton community structure (Morris et al. 2002). Several studies have recently characterized bacterial community composition and dynamics of different aquatic habitats by culture-independent methods such as denaturing gradient gel electrophoresis (DGGE) or sequence analyses of 16S rRNA gene fragments (e.g. Rappé et al. 2000, Trusova & Gladyshev 2002, Van der Gucht et al. 2005). However, there are fewer studies on freshwater than on marine bacterial communities. Although bacterial communities in freshwater are generally dominated by *Betaproteobacteria*, *Actinobacteria*, and members of

Bacteroidetes, pronounced differences and variations are found between free-living and particle-associated bacterial communities (Weiss et al. 1996, Riemann & Winding 2001, Schweitzer et al. 2001, Selje & Simon 2003). Regardless, no comprehensive seasonal study of free-living and particle-associated bacterioplankton communities of different freshwater habitats exists yet.

Although many studies focus on the composition of freshwater bacterioplankton communities, surprisingly few examine the temporal and seasonal dynamics of bacterial communities in detail (Pinhassi & Hagström 2000, Zwisler et al. 2003, Kent et al. 2004). Thus, seasonal and spatial dynamics of complex freshwater bacterioplankton communities are still poorly understood. Nevertheless, it has been shown that changes in environmental conditions significantly influence bacterial community structure throughout distinct seasons and periods (e.g. Langenheder & Jürgens 2001, Crump et al. 2003, Kent et al. 2004). Various environmental variables, such as dissolved organic carbon (DOC), grazing by heterotrophic nanoflagellates, or phytoplankton biomass have been found to affect freshwater bacterial communities (Methé & Zehr 1999, Pernthaler et al. 2001, Muylaert et al. 2002).

In this study we compared the seasonal dynamics and phylogenetic diversity of free-living and particle-associated bacterial communities of 4 selected lakes of the Mecklenburg Lake District, northeastern Germany, to assess (1) whether both bacterial fractions consistently differ from each other, (2) whether there are pronounced seasonal and spatial patterns in community structure of both fractions, and (3) whether observed patterns could be linked to environmental variables.

MATERIALS AND METHODS

Study sites and sample collection. Four lakes of different limnological features were selected for this study. All lakes were located in the same geographical region, the Mecklenburg Lake District (northeastern Germany), which was formed after the last ice age (Weichselian Stage). Oligotrophic Lake Stechlin and mesotrophic Lake Breiter Luzin are among the deepest lakes in this area (68.5 and 58.5 m, respectively) and are both characterized by high hypolimnetic oxygen concentrations (up to 60% O_2 saturation). Lake Stechlin is situated in the middle of a mixed forest composed mainly of beech and pine trees, whereas the catchment area of Lake Breiter Luzin consists of natural forests and farm land.

In comparison, eutrophic Lake Tiefwaren is strongly influenced by anthropogenic activities. Extensive agriculture and industry in the late 1980s resulted in a

hypertrophic state, but a restoration approach by a combination of aluminate and calcium hydroxide precipitation between 2001 and 2005 (Koschel et al. 2006) yielded its present eutrophic state.

The relatively small Lake Grosse Fuchskuhle is situated in a mixed forest and was artificially divided into 4 compartments (SW, NW, NE, SE) by large plastic curtains for biomanipulation experiments in the 1990s (Kasprzak 1993, Koschel 1995). Owing to large differences in their limnology (Bittl & Babenzien 1996, Hehmann et al. 2001), the NE and SW compartments were selected for this study. The dystrophic SW compartment is the most acidic compartment of the lake (pH 4.7), because it is strongly influenced by the high input of humic matter from the adjacent bog area. The mesotrophic NE compartment receives less humic acids and is hence more neutral (pH 6.5). A summary of the most important physicochemical and biological variables of all studied lakes is shown in Table 1.

All lakes were sampled monthly between April 2003 and March 2004, except during ice coverage in December (Lake Breiter Luzin), January (all lakes), and February (Lake Grosse Fuchskuhle). Composite water samples representing the epilimnion were collected with a Ruttner sampler at the deepest point of each lake. Based on thermal stratification, sub-samples were taken from 0, 5, and 10 m depth (April to May and October to March) or 0 and 5 m depth (June to September) in Lake Stechlin, Lake Breiter Luzin, and Lake Tiefwaren, respectively, and mixed in sterile glass flasks in equal volumes. Epilimnetic samples of the 2 compartments of Lake Grosse Fuchskuhle were collected as mixed samples from 0 and 2 m depth (April and October to March) or as surface samples (May to September), owing to the relatively shallow epilimnion. All water samples were transported to the laboratory in dark cooling boxes and processed 2 to 4 h after sampling.

DNA extraction and PCR amplification of 16S rRNA gene fragments. Particle-associated bacteria were retained by filtering 150 to 300 ml of sample from each assay onto a 5.0 µm Nuclepore membrane. Free-living bacteria were collected by filtering 100 to 150 ml of the 5.0 µm filtrate onto a 0.2 µm Nuclepore membrane. Extraction of genomic DNA was performed using a standard protocol with phenol/chloroform/isoamylalcohol, SDS, polyvinylpyrrolidone, and zirconium beads (Allgaier & Grossart 2006).

For DGGE analysis, a 550 bp fragment of the 16S rRNA gene was amplified using the primer pair 341f and 907r (5′-CCTACGGGAGGCAGCAG-3′ and 5′-CCGTCAATTCMTTTGAGTTT-3′, respectively; Muyzer et al. 1998). At the 5′-end of primer 341f, an additional 40 bp GC-rich nucleotide sequence (GC-clamp) was added to stabilize migration of the DNA

Table 1. Limnological characteristics of sampled lakes. pH, PO_4-Phosphate (PO_4-P), dissolved organic carbon (DOC), bacterial number, primary production, and bacterial production data are average values of epilimnetic samples from Apr–Nov 2003 and Mar 2004; trophic status was determined following guideline EUR 14563 EN of the Commission of the European Communities (Premazzi & Chiaudani 1992)

Parameter	Lake Stechlin[a] (ST)	Lake Grosse Fuchskuhle[b]		Lake Breiter Luzin[c] (BL)	Lake Tiefwaren[d] (TW)
		NE basin (FNE)	SW basin (FSW)		
Geographic position	53° 10′ N, 13° 02′ E	53° 06′ N, 13° 02′ E		53° 20′ N, 13° 28′ E	53° 31′ N, 12° 42′ E
Max. depth (m)	69.5	5.6		58.5	24
Surface area (km^2)	4.3	0.02		3.57	1.4
Catchment area (km^2)	26.0	0.005		14.0	17.5
Stratification (2003)	Jun–Oct	May–Sep	May–Sep	Jun–Oct	Jun–Sep
Secchi depth (m)	6.5–10.5	1.0–2.2	0.9–1.5	1.6–3.8	3.1–8.9
Trophic status	oligotrophic	eutrophic	dystrophic	mesotrophic	eutrophic
pH	8.5 ± 0.2	6.5 ± 0.6	4.7 ± 0.2	8.5 ± 0.2	8.3 ± 0.2
PO_4-P ($\mu g\ l^{-1}$)	2 ± 0.9	5 ± 2	7 ± 2	3 ± 4	2 ± 1
DOC ($mg\ l^{-1}$)	4.3 ± 1.1	10.3 ± 1.3	24.8 ± 5.1	5.8 ± 0.6	10.6 ± 5.3
Bacterial number ($10^6\ ml^{-1}$)	1.35 ± 0.58	2.74 ± 1.0	1.93 ± 0.69	1.93 ± 0.49	2.21 ± 0.47
Primary production ($\mu g\ C\ l^{-1}\ d^{-1}$)	31 ± 11	182 ± 156	512 ± 863	103 ± 44	114 ± 99
Bacterial production ($\mu g\ C\ l^{-1}\ d^{-1}$)	22 ± 15	39 ± 21	63 ± 101	51 ± 58	35 ± 37

Characteristics of catchment area are [a]mixed forest, [b]mixed forest and bog area, [c]forest and agriculture, [d]forest and small town

fragment in DGGE (Muyzer et al. 1993). The PCR reaction mixture contained 2 to 5 µl template DNA, each primer at a concentration of 200 nM, each deoxyribonucleoside triphosphate at a concentration of 250 µM, 2 mM $MgCl_2$, 5 µl of 10× PCR reaction buffer, and 0.5 U of *BioTaq* Red DNA polymerase (Bioline) in a total volume of 50 µl. PCR amplification was performed with a Gradient Cycler PT-200 (MJ Research) using the following conditions: initial denaturation at 95°C (3 min) followed by 30 cycles of denaturation at 95°C (1 min), annealing at 55°C (1 min), and extension at 72°C (2 min). A final extension at 72°C for 10 min completed the reaction.

For clone libraries, the almost complete 16S rRNA gene was amplified using the primer pair 8f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-GGYTACCTTGTTACGACTT-3′) specific to the domain Bacteria (Muyzer et al. 1995). PCR conditions were as previously described (Allgaier & Grossart 2006).

DGGE analysis of PCR products. PCR products were analyzed using the Ingeny PhorU DGGE-System (Ingeny) according to the protocol of Brinkhoff & Muyzer (1997). DGGE was performed in a 7% (v/v) polyacrylamide gel with a denaturing gradient of 40 to 70% urea and formamide. To obtain identical gradients among different DGGE gels, an automatic pump with a constant flow of 5 ml min⁻¹ was used. For better comparability of different DGGE profiles, PCR products were quantified on agarose gels using a quantitative DNA ladder (Low DNA Mass Ladder, Invitrogen), and similar amounts of DNA were loaded onto DGGE

gels. All DGGEs were run in 1 × TAE (tris acetate EDTA) electrophoresis buffer (40 mM Tris-HCl [pH 8.3], 20 mM acetic acid, 1 mM EDTA) for 20 h at a constant voltage of 100 V and constant temperature of 60°C. Gels were stained with 1 × SYBR Gold (Molecular Probes) and documented using an AlphaImager 2200 Transilluminator (Biozym). A mixture of DNA from 3 isolates derived from the studied lakes was used as an external standard to compare different DGGE gels.

Analyses of DGGE profiles and multivariate statistics. Bacterial communities of the 4 studied lakes were analyzed on different DGGE gels. Banding patterns were then compared among gels using the software GelCompar II version 3.5 (Applied Maths). The external standard was used to standardize the DGGE profiles of single gels and to perform correct and reliable band matching among different gels. We applied a 5 to 15% background subtraction depending on the signal-to-noise ratio of the gels. Band-based binary presence/absence tables were calculated and single bands of each DGGE profile were scored using the minimum profiling tool of GelCompar II with a cut-off value of 5%. Thus, all bands of at least 5% of the density of the darkest band were accounted as present. Position tolerances of 0.40% (free-living bacteria) and 0.48% (particle-associated bacteria) were applied to determine whether single bands from different gels were identical. Presence/absence tables were imported into the ordination software PC-ORD version 4.0 (MJM Software Design). We used non-metric multidimensional scaling (NMDS) ordinations instead of con-

strained ordination techniques (e.g. canonical correspondence analysis [CCA]) to avoid distortions originating from the non-normal distribution of our species data obtained from DGGE profiles (McCune & Grace 2002). NMDS uses only rank order information of a similarity matrix of samples rather than the original data matrix. In a first step, NMDS searches for the best model describing differences in bacterial community structure; in a second step, limnological variables are fitted independently to this model by means of multiple regression criteria. In contrast, constrained ordination techniques directly search for the most suitable model describing relationships between community data and environmental variables, accounting for both data sets at the same time.

Primary NMDS analyses of all gels were performed by a standard set-up using relativised Sørensen's distance measures, random starting coordinates, a stepdown from 6 to 1 dimensions, an instability criterion of 0.0001, 300 iterations to reach stability, 50 runs with real data sets, and 100 runs with the Monte Carlo permutation test (Mehner et al. 2005). The final run was performed with the optimum number of dimensions (2 to 5 dimensions) and the appropriate configurations as starting coordinates. Varimax rotation was applied to find corresponding groups and sample units. For the identification of particular environmental variables accounting for changes in bacterial community composition within the epilimnion of the lakes, Pearson's product moment correlations of limnological variables and DGGE profiles were calculated on the significant ordination axes.

Analysis of similarity (ANOSIM, Clarke & Green 1988) was used to statistically test the significance of differences between the DGGE banding patterns of free-living and particle-associated bacteria. ANOSIM generates a test statistic (R) that is an indication of the degree of separation between groups. A score of 1 indicates complete separation, whereas a score of 0 indicates no separation. Equal to NMDS analyses, ANOSIM is based on a similarity matrix and not the original data matrix. ANOSIM analyses were conducted with software PRIMER 5 version 5.2.9 (PRIMER-E).

Construction of clone libraries. Cloning of the almost complete 16S rRNA gene fragments of free-living and particle-associated bacteria was performed using the pGEM-T-Easy Vector System II (Promega) according to the manufacturer's protocol. Clone libraries were constructed for samples collected in May 2003 (Lake Stechlin only) and November 2003 (all lakes). A total of 19 to 29 clones from each clone library were selected and sequenced as described previously (Allgaier & Grossart 2006).

Phylogenetic analyses. Partial sequences were assembled and corrected manually using the software Chromas version 1.45 (Griffith University). Phylogenetic reconstructions were performed using the ARB software package (http://arb-home.de). 16S rRNA gene sequences of the clone libraries were first checked and classified by the basic local alignment search tool (BLAST; www.ncbi.nlm.nih.gov/BLAST/) and the RDP-classifier of the Ribosomal Database Project (http://rdp.cme.msu.edu/). Sequences were then imported into the ARB database of ca. 52 000 reference sequences, including the closest related sequences determined by BLAST. Sequences were aligned automatically using the integrated alignment module within the ARB package and subsequently corrected manually. For the stability of phylogenetic trees, backbone trees that comprised sequences of only ≥1400 nucleotides were calculated first. Validity of branching patterns of the trees was checked by applying 3 phylogenetic reconstruction methods — neighbor-joining, maximum parsimony, and maximum likelihood — to the appropriate sets of sequences. Sequences ≤1400 nucleotides were added to the trees afterwards according to maximum parsimony criteria. This tool does not correct for evolutionary distances, and does not allow changes in overall tree topology.

Nucleotide sequence accession numbers. Obtained 16S rRNA gene sequences were deposited in GenBank with the following accession numbers: DQ501285 to DQ501378.

RESULTS

Analyses of DGGE banding patterns

DGGE banding patterns of free-living and particle-associated bacteria exhibited distinct differences with regard to bacterial community composition and seasonal dynamics (e.g. Fig. 1). Free-living and particle-associated bacterial communities differed significantly among the 4 lakes ($p \le 0.01$), as indicated by the formation of lake-specific clusters of DGGE profiles in NMDS analyses (Fig. 2A). Within free-living bacteria, DGGE profiles of Lake Breiter Luzin, Lake Stechlin, and the 2 compartments (NE and SW) of Lake Grosse Fuchskuhle exhibited clearly separated clusters, whereas DGGE banding patterns of Lake Tiefwaren overlapped with those of the NE compartment of Lake Grosse Fuchskuhle (Fig. 2A). Although the formation of lake-specific clusters within particle-associated bacteria was statistically significant ($p \ge 0.01$), it was less obvious than for free-living bacterial communities (Fig. 2A).

Comparison of DGGE profiles from each single lake revealed significant differences between free-living and particle-associated bacterial communities (Fig. 2B,
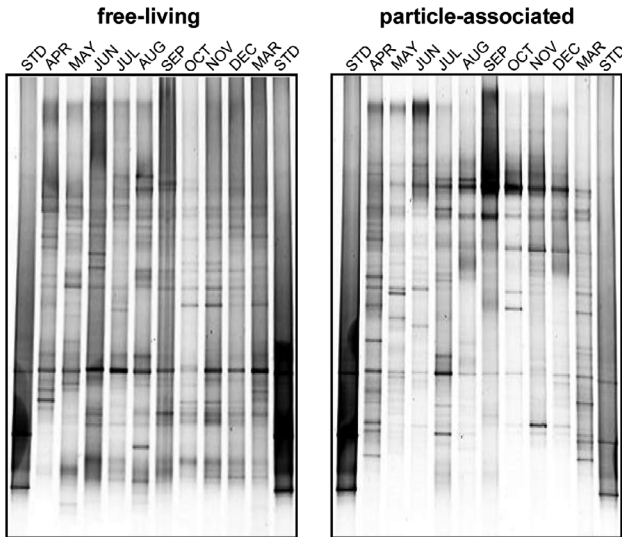
Fig. 1. DGGE profiles of amplified 16S rRNA gene fragments from free-living and particle-associated bacteria from the NE compartment of Lake Grosse Fuchskuhle, collected between April 2003 and March 2004. STD: standard

Table 2). Additionally, NMDS analyses indicated seasonal changes in bacterial community composition as depicted by the formation of season-specific clusters of samples within NMDS plots (Fig. 2B). Season-specific clusters varied among lakes and between bacterial fractions. In general, winter and spring samples of free-living bacteria clustered together and were clearly separated from samples taken in summer and fall. Formation of seasonal clusters within particle-associated bacteria was less pronounced compared with free-living bacterial communities.

Sequence analyses of clone libraries

To obtain more detailed information on the phylogenetic diversity of free-living and particle-associated bacteria of the studied lakes, clone libraries of 16S rRNA gene fragments were constructed and sequenced. Altogether, 12 clone libraries from May (Lake Stechlin only) and November (all lakes) resulted in a total of 277 clones (Table 3). As determined by BLAST and the RDP classifier, 16S rRNA gene sequences of free-living bacteria belonged to *Actinobacteria* (44.4% of total sequences), *Bacteroidetes* (14.6%), *Betaproteobacteria* (10.4%), *Cyanobacteria* (10.4%), *Alphaproteobacteria* (8.3%), *Deltaproteobacteria* (1.4%), Candidate Division OP10 (1.4%), *Gammaproteobacteria* (0.7%), and chloroplasts (3.5%). Contributions of other bacterial phyla such as *Chlorobi*, *Thermomicrobia* or *Acidobacteria* were very low
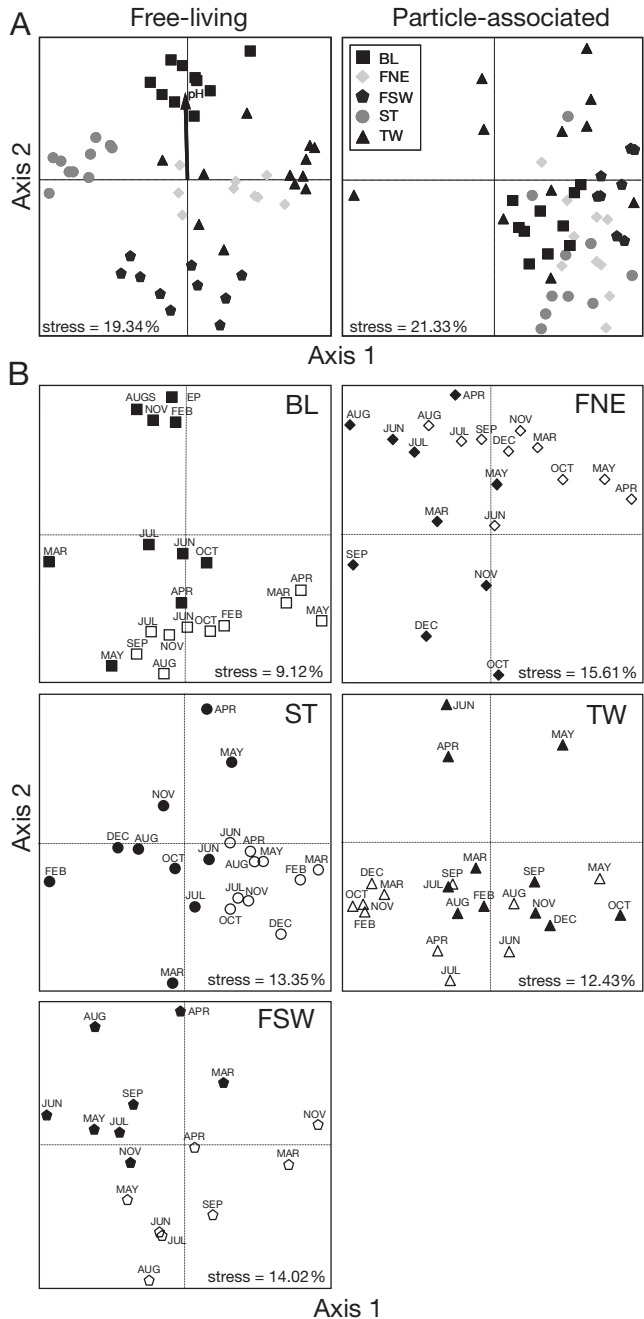
Fig. 2. Non-metric multidimensional scaling (NMDS) ordinations of DGGE banding patterns of free-living and particle-associated bacterial communities from the 4 studied lakes. Results from the first 2 ordination axes are given. (A) NMDS plots of all lakes; free-living bacteria were shown to be strongly correlated with pH. (B) NMDS plots of single lakes. Open symbols: free-living bacteria; solid symbols: particle-associated bacteria. Abbreviations of lakes given in Table 1

(comprising a total of 4.9%). Excluding sequences of chloroplasts from eucaryotic algae, 16S rRNA gene sequences from clone libraries of particle-associated bacteria were affiliated with *Cyanobacteria* (43.5%),

Table 2. ANOSIM statistics from comparison of DGGE profiles of free-living and particle-associated bacteria from each single lake. Global test revealed a sample statistic of 0.499 with a significance of $p \leq 0.001$. FL: free-living; PA: particle-associated. Abbreviations of lakes given in Table 1; values significant at $p \leq 0.05$ in **bold**

	Sample statistic (R)	p
BL (FL vs. PA)	0.462	**0.001**
FNE (FL vs. PA)	0.245	0.08
FSW (FL vs. PA)	0.475	**0.001**
ST (FL vs. PA)	0.459	**0.001**
TW (FL vs. PA)	0.289	**0.001**

Bacteroidetes (24.2%), *Alphaproteobacteria* (8.1%), *Planctomycetes* (4.8%), *Betaproteobacteria* (3.2%), *Gammaproteobacteria* (3.2%), *Verrucomicrobia* (3.2%), Candidate Division OP10 (1.6%), and *Actinobacteria* (1.6%).

In general, clone libraries of free-living bacteria were dominated by *Actinobacteria*, members of *Bacteroidetes*, and *Betaproteobacteria*. *Actinobacteria* sequences occurred in consistently high numbers in all 4 lakes. Members of *Bacteroidetes* were found mainly in Lake Stechlin (May and November), Lake Tiefwaren, and the SW compartment of Lake Grosse Fuchskuhle. No *Bacteroidetes* were found within free-living bacteria of Lake Breiter Luzin. Sequences of *Betaproteobacteria* occurred in relatively high numbers in Lake Stechlin (November only), Lake Breiter Luzin, and the NE compartment of Lake Grosse Fuchskuhle (Table 3). Cyanobacterial 16S rRNA gene sequences were present almost exclusively in the May 2003 clone library of free-living bacteria from Lake Stechlin.

When excluding sequences of chloroplasts from eucaryotic algae, clone libraries of particle-associated bacteria were dominated by sequences of *Cyanobacteria* and members of *Bacteroidetes*. Clones of *Bacteroidetes* were found in Lake Tiefwaren, Lake Stechlin (May), Lake Breiter Luzin, and the NE compartment of Lake Grosse Fuchskuhle (Table 3). Sequences of *Cyanobacteria* were almost only present in Lake Breiter Luzin, Lake Tiefwaren, and Lake Stechlin (May and November). Other phylogenetic lineages such as *Verrucomicrobia* or *Planctomycetes* occurred in low proportions.

Detailed phylogenetic reconstructions of free-living and particle-associated bacterial 16S rRNA gene sequences confirmed the phylogenetic classification retrieved by BLAST and RDP (Fig. 3). Because this study focused on heterotrophic bacteria, no phylogenetic trees were constructed for *Cyanobacteria* or chloroplast sequences. In general, free-living and particle-associated bacteria were equally distributed throughout the constructed phylogenetic trees and no distinct lake-specific or bacterial fraction-specific clusters appeared. A further separation into classes within *Bacteroidetes* is clearly visible in the phylogenetic tree in Fig. 3B. The majority of the 16S rRNA gene sequences of *Bacteroidetes* belonged to Class *Sphingobacteria*. Only 8 sequences were affiliated to Class *Flavobacteria* and 3 sequences to Class *Bacteroidetes*, respectively.

Actinobacterial 16S rRNA gene sequences were phylogenetically affiliated with the freshwater clusters *acI*, *acII*, *acIV*, and *acSTL*, and with the clusters *Soil I-III* and *Microthrix*. Detailed descriptions of the diversity and phylogenetic relationship of actinobacterial sequences of this study are given by Allgaier & Grossart (2006).

Table 3. Phylogenetic classification of 16S rRNA gene sequences derived from 12 clone libraries of free-living and particle-associated freshwater bacterial communities. FL: free-living bacteria; PA: particle-associated bacteria; n: number of sequences retrieved from each fraction. Abbreviations of lakes given in Table 1. [M]Samples from May 2003, [N]samples from November 2003

	ST[M] FL/PA n = 29/20	ST[N] FL/PA n = 23/19	BL[N] FL/PA n = 27/28	FNE[N] FL/PA n = 20/20	FSW[N] FL/PA n = 20/25	TW[N] FL/PA n = 25/21	ΣFL/PA n = 144/133
Alphaproteobacteria	2/–	–/3	2/1	–/1	2/–	6/–	12/5
Betaproteobacteria	1/–	3/1	4/–	5/1	–/–	2/–	15/2
Gammaproteobacteria	–/2	–/–	–/–	–/–	–/–	1/–	1/2
Deltaproteobacteria	–/–	1/–	–/–	1/–	–/–	–/–	2/–
Actinobacteria	7/1	13/–	16/–	11/–	7/–	10/–	64/1
Bacteroidetes	3/3	4/–	–/3	2/2	9/1	3/6	21/15
Candidate Division OP10	1/–	–/1	1/–	–/–	–/–	–/–	2/1
Planctomycetes	–/–	–/1	–/2	–/–	–/–	–/–	–/3
Verrucomicrobia	–/–	–/1	–/–	–/–	–/–	–/1	–/2
other phyla[a]	–/–	1/–	2/2	1/–	–/–	3/2	7/4
Cyanobacteria	12/2	1/3	2/17	–/–	–/–	–/5	15/27
Chloroplasts	3/12	–/9	–/3	–/16	2/24	–/7	5/71

[a]*Chlorobi*, *Fibrobacteres*, *Thermomicrobia*, *Chloroflexi*, Candidate Division TM7, *Acidobacteria* (see also phylogenetic trees)

Fig. 3. (Above and following 2 pages). Maximum likelihood trees of cloned and sequenced 16S rRNA gene fragments from free-living and particle-associated bacteria. Solid lines indicate sequences that were included in primary analyses (sequences ≥1400 nucleotides), whereas dotted lines indicate partial sequences (≤1400 nucleotides) added by maximum parsimony criteria. All sequences from this study shown in **bold, underlined**. Sequences of particle-associated bacteria are additionally shaded grey. GenBank accession numbers and bootstrap values at main branching points are given; scale bar corresponds to 10 base substitutions per 100 nucleotide positions. (A) *Proteobacteria*, (B) *Bacteroidetes*, (C) overall phylogenetic tree of 16S rRNA gene sequences that occur only in low numbers in clone libraries
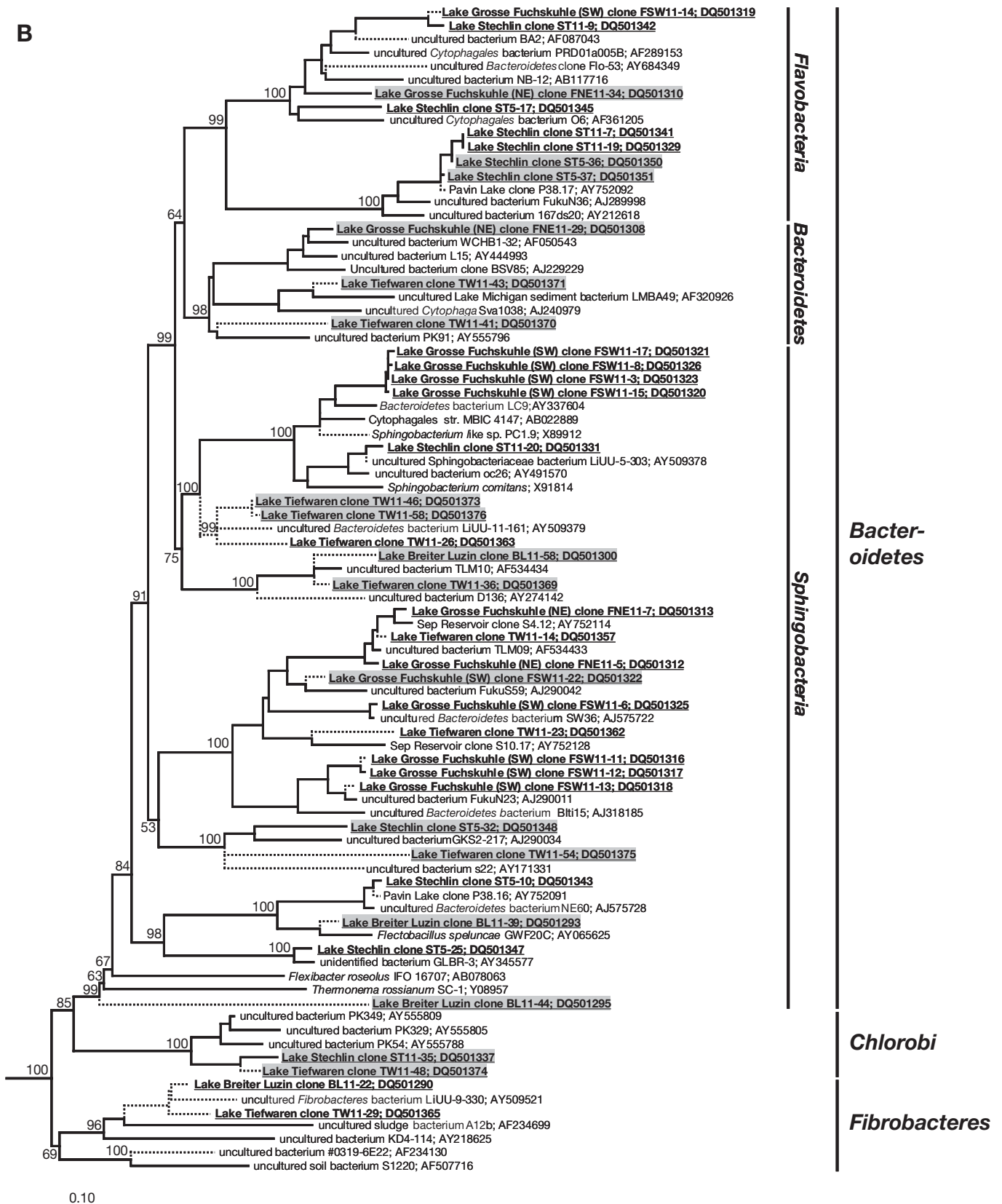
B

Flavobacteria
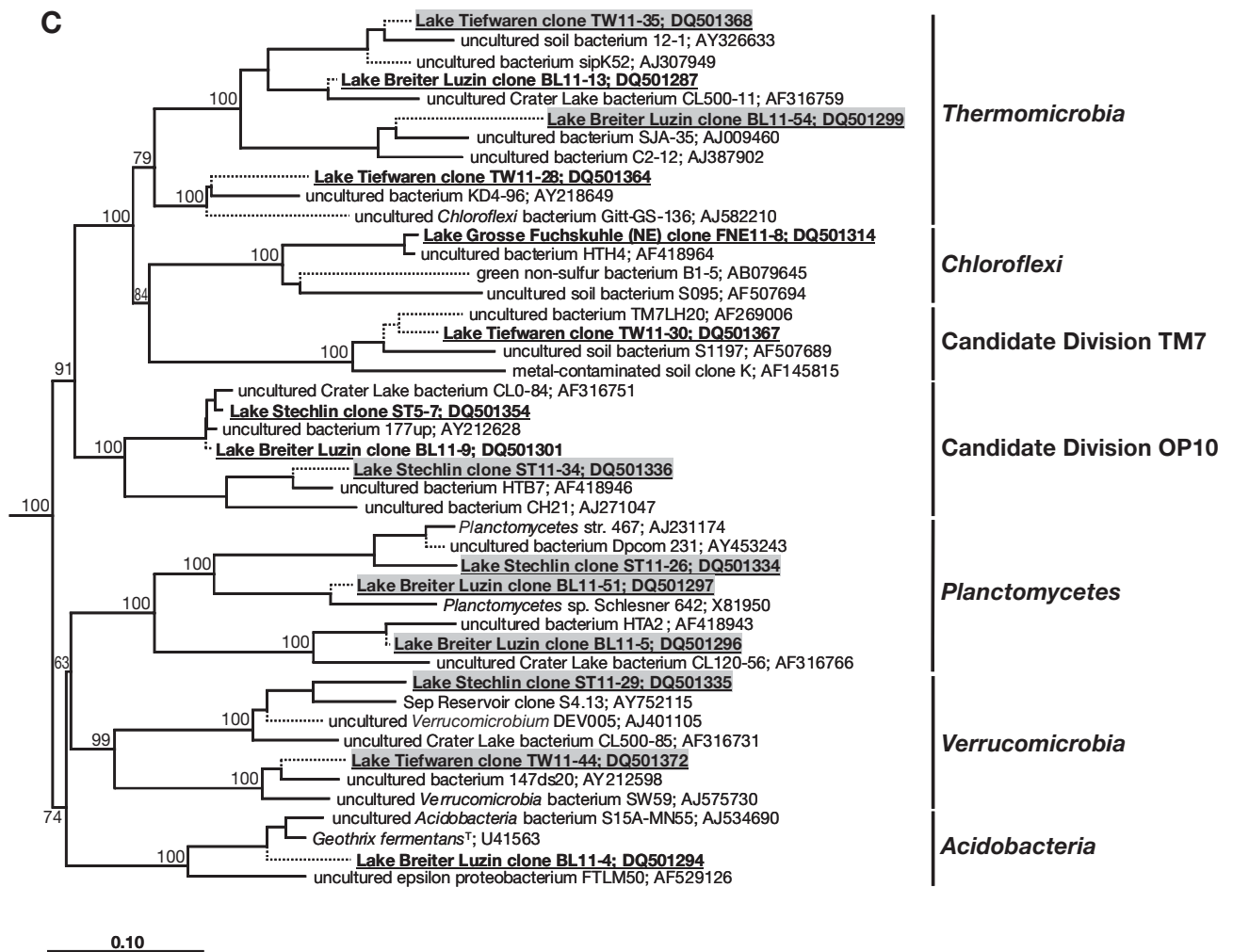
- **Lake Grosse Fuchskuhle (SW) clone FSW11-14; DQ501319**
- **Lake Stechlin clone ST11-9; DQ501342**
- uncultured bacterium BA2; AF087043
- uncultured *Cytophagales* bacterium PRD01a005B; AF289153
- uncultured *Bacteroidetes* clone Flo-53; AY684349
- uncultured bacterium NB-12; AB117716
- **Lake Grosse Fuchskuhle (NE) clone FNE11-34; DQ501310**
- **Lake Stechlin clone ST5-17; DQ501345**
- uncultured *Cytophagales* bacterium O6; AF361205

100, 99

Bacteroidetes

- **Lake Stechlin clone ST11-7; DQ501341**
- **Lake Stechlin clone ST11-19; DQ501329**
- **Lake Stechlin clone ST5-36; DQ501350**
- **Lake Stechlin clone ST5-37; DQ501351**
- Pavin Lake clone P38.17; AY752092
- uncultured bacterium FukuN36; AJ289998
- uncultured bacterium 167ds20; AY212618

100

- **Lake Grosse Fuchskuhle (NE) clone FNE11-29; DQ501308**
- uncultured bacterium WCHB1-32; AF050543
- uncultured bacterium L15; AY444993
- Uncultured bacterium clone BSV85; AJ229229
- **Lake Tiefwaren clone TW11-43; DQ501371**
- uncultured Lake Michigan sediment bacterium LMBA49; AF320926
- uncultured *Cytophaga* Sva1038; AJ240979
- **Lake Tiefwaren clone TW11-41; DQ501370**
- uncultured bacterium PK91; AY555796

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Sphingobacteria

- **Lake Grosse Fuchskuhle (SW) clone FSW11-17; DQ501321**
- **Lake Grosse Fuchskuhle (SW) clone FSW11-8; DQ501326**
- **Lake Grosse Fuchskuhle (SW) clone FSW11-3; DQ501323**
- **Lake Grosse Fuchskuhle (SW) clone FSW11-15; DQ501320**
- *Bacteroidetes* bacterium LC9; AY337604
- Cytophagales str. MBIC 4147; AB022889
- *Sphingobacterium* like sp. PC1.9; X89912
- **Lake Stechlin clone ST11-20; DQ501331**
- uncultured Sphingobacteriaceae bacterium LiUU-5-303; AY509378
- uncultured bacterium oc26; AY491570
- *Sphingobacterium comitans*; X91814

100

- **Lake Tiefwaren clone TW11-46; DQ501373**
- **Lake Tiefwaren clone TW11-58; DQ501376**
- uncultured *Bacteroidetes* bacterium LiUU-11-161; AY509379
- **Lake Tiefwaren clone TW11-26; DQ501363**
- **Lake Breiter Luzin clone BL11-58; DQ501300**
- uncultured bacterium TLM10; AF534434
- **Lake Tiefwaren clone TW11-36; DQ501369**
- uncultured bacterium D136; AY274142

100, 99, 75

- **Lake Grosse Fuchskuhle (NE) clone FNE11-7; DQ501313**
- Sep Reservoir clone S4.12; AY752114
- **Lake Tiefwaren clone TW11-14; DQ501357**
- uncultured bacterium TLM09; AF534433
- **Lake Grosse Fuchskuhle (NE) clone FNE11-5; DQ501312**
- **Lake Grosse Fuchskuhle (SW) clone FSW11-22; DQ501322**
- uncultured bacterium FukuS59; AJ290042
- **Lake Grosse Fuchskuhle (SW) clone FSW11-6; DQ501325**
- uncultured *Bacteroidetes* bacterium SW36; AJ575722
- **Lake Tiefwaren clone TW11-23; DQ501362**
- Sep Reservoir clone S10.17; AY752128
- **Lake Grosse Fuchskuhle (SW) clone FSW11-11; DQ501316**
- **Lake Grosse Fuchskuhle (SW) clone FSW11-12; DQ501317**
- **Lake Grosse Fuchskuhle (SW) clone FSW11-13; DQ501318**
- uncultured bacterium FukuN23; AJ290011
- uncultured *Bacteroidetes* bacterium Blti15; AJ318185

100, 91

- **Lake Stechlin clone ST5-32; DQ501348**
- uncultured bacterium GKS2-217; AJ290034
- **Lake Tiefwaren clone TW11-54; DQ501375**
- uncultured bacterium s22; AY171331
- **Lake Stechlin clone ST5-10; DQ501343**
- Pavin Lake clone P38.16; AY752091
- uncultured *Bacteroidetes* bacterium NE60; AJ575728
- **Lake Breiter Luzin clone BL11-39; DQ501293**
- *Flectobacillus speluncae* GWF20C; AY065625
- **Lake Stechlin clone ST5-25; DQ501347**
- unidentified bacterium GLBR-3; AY345577

100, 53, 84, 100, 98, 100

- *Flexibacter roseolus* IFO 16707; AB078063
- *Thermonema rossianum* SC-1; Y08957

67, 63, 99

- **Lake Breiter Luzin clone BL11-44; DQ501295**

85

Chlorobi

- uncultured bacterium PK349; AY555809
- uncultured bacterium PK329; AY555805
- uncultured bacterium PK54; AY555788
- **Lake Stechlin clone ST11-35; DQ501337**
- **Lake Tiefwaren clone TW11-48; DQ501374**

100

Fibrobacteres

- **Lake Breiter Luzin clone BL11-22; DQ501290**
- uncultured *Fibrobacteres* bacterium LiUU-9-330; AY509521
- **Lake Tiefwaren clone TW11-29; DQ501365**
- uncultured sludge bacterium A12b; AF234699
- uncultured bacterium KD4-114; AY218625
- uncultured bacterium #0319-6E22; AF234130
- uncultured soil bacterium S1220; AF507716

96, 69, 100

100

0.10

Fig. 3. (continued)

C

Lake Tiefwaren clone TW11-35; DQ501368	
uncultured soil bacterium 12-1; AY326633	
uncultured bacterium sipK52; AJ307949	
Lake Breiter Luzin clone BL11-13; DQ501287	
uncultured Crater Lake bacterium CL500-11; AF316759	***Thermomicrobia***
Lake Breiter Luzin clone BL11-54; DQ501299	
uncultured bacterium SJA-35; AJ009460	
uncultured bacterium C2-12; AJ387902	
Lake Tiefwaren clone TW11-28; DQ501364	
uncultured bacterium KD4-96; AY218649	
uncultured *Chloroflexi* bacterium Gitt-GS-136; AJ582210	

Fig. 3. (continued)

Relationships between DGGE profiles and environmental variables

Identification of potential relationships between limnological variables and seasonal changes within free-living and particle-associated bacterial communities were performed by NMDS analyses because of the non-normal distribution of species data. Fifteen limnological variables were used for these analyses: secchi depth, temperature, conductivity, pH, total nitrogen, total phosphorus, oxygen concentration, DOC, total bacterial number, bacterial protein production (total, free-living, and particle-associated), phytoplankton and zooplankton biomass, as well as total primary production.

NMDS analyses were separately performed for free-living and particle-associated bacteria from each single lake, and with a combined data set composed of both bacterial fractions. In addition, 2 comprehensive data sets of free-living and particle-associated bacteria were analysed, comprising DGGE profiles and limnological variables of all studied lakes. Pearson's product moment correlations of the combined data sets of free-living and particle-associated bacteria of the single lakes revealed only weak correlations (Pearson's $r \leq 0.7$, data not shown). In contrast, separate analyses of free-living and particle-associated bacterial communities exhibited several distinct correlations with environmental variables, such as temperature, pH, DOC, bacterial production, primary production, and phytoplankton biomass (Table 4). However, no consistent correlation patterns were observed between all lakes and both bacterial fractions. Comprehensive statistical analyses that included all epilimnetic samples revealed a strong correlation between free-living bacteria and pH ($r = 0.785$, Table 4). As indicated in Fig. 2A, pH was highly correlated with the free-living bacteria of Lake Breiter Luzin.

Table 4. Non-metric multidimensional scaling ordinations and Pearson's product moment correlations between DGGE profiles of free-living and particle-associated bacterial communities and limnological variables. Pearson's r-values ≥0.7 are given for 3 significant ordination axes; limnological variables with r ≤ 0.7 were excluded. First column gives results of comprehensive analyses of epilimnetic samples from all lakes. DOC: dissolved organic carbon; BPP: bacterial protein production; PP: primary production. Abbreviations of lakes given in Table 1

	Epilimnion (all lakes)	BL	FNE	FSW	ST	TW
Free-living						
Secchi depth	–	–	–0.818[a]	–0.846[a]	–	–
Temperature	–	–0.877[a]	–	0.772[a]	0.704[a]	–
O₂ [mg l⁻¹]	–	–	–	–	–0.787[a]	–
pH	0.785[b]	–	–	–	–	–0.747[b]
Conductivity	–	0.895[a]	–	–	0.790[c]	0.713[b]
Total nitrogen	–	–0.705[a]	–	–	–	–
Total phosphorous	–	– 0.738[b]	–	–	–	–0.722[b]
DOC	–	–	–	0.797[a]	–	–
BPP (total)	–	–0.800[a]	–	–	–	–
PP (total)	–	–	–	–	–	0.701[c]
PP (≤3.0, ≥0.2 μm)	–	–	–	–	–0.720[c]	–
Phytoplankton	–	–0.773[b]	–	–	–	–
Particle-associated						
Secchi depth	–	–	–	–	–0.757[c]	–
Temperature	–	–	–	–	–0.725[a]	–
Conductivity	–	–	–	–	0.821[a]	–
Total phosphorous	–	–0.846[c]	–	–	–	–
PP (≤3.0, ≥0.2 μm)	–	–	–	–	–0.745[a]	–
Zooplankton	–	–	–	–	–0.750[a]	–
Phytoplankton	–	–0.831[c]	–	–	–	–

[a,b,c]r-values of ordination axes 1, 2, and 3, respectively

DISCUSSION

Differences in community structure of free-living and particle-associated bacteria

To our knowledge, this is one of the first comprehensive studies of seasonal changes in bacterial community structure and dynamics in freshwater systems that consistently separated free-living from particle-associated bacteria. Our DGGE analyses revealed significant differences between both bacterial fractions in all lakes. This was in accordance with previous studies that used a ≥3.0 μm filtration step to differentiate between free-living and particle-associated bacteria (e.g. Crump et al. 1999, Riemann & Winding 2001, Selje & Simon 2003). Filter pore sizes for separating free-living from particle-associated bacteria are crucial and may greatly bias the analyses of bacterial communities of different size fractions. For example, Hollibaugh et al. (2000) did not observe any differences between particle-associated and free-living bacteria when using 1.0 μm filters for separation, presumably because these filters may have also retained a large portion of free-living bacteria. Also, larger pore sizes may lead to distinct limitations, because large free-living cells (e.g. filamentous bacteria) cannot pass through these filters. As a result, these bacteria are then defined as particle-associated bacteria even though they are in fact free living. Filamentous bacteria are common in freshwater habitats and are selectively predated by bacterivorous protists (Pernthaler et al. 2004, Schauer et al. 2005). However, non-quantitative analyses of the bacterial communities in our study lakes by DAPI and CARD-FISH (catalyzed reporter deposition fluorescence *in situ* hybridization) indicated that filamentous bacteria were in low abundance (data not shown). Nevertheless, we cannot unambiguously state that our separation was always fully successful and yielded the 'real' free-living and particle-associated bacterial fractions. High numbers of cyanobacterial sequences in our clone libraries of particle-associated bacteria indicated that larger cells or colony-forming bacteria remained in the particle-associated fractions, and hence were not included with free-living bacterial communities.

The formation of lake-specific clusters within NMDS analyses of DGGE banding patterns from all lakes was more pronounced for free-living than for particle-associated bacteria (Fig. 2A). Similar effects of environmental conditions on particles among lakes may have led to smaller differences among particle-associated bacterial communities, which may be indicative of adaptation by distinct bacterial communities to these microenvironments (Weiss et al. 1996, Grossart & Simon 1998, Brachvogel et al. 2001). Lake snow aggregates are nutrient rich 'hot-spots' for limnetic bacteria, which leads to higher bacterial abundances, biomasses, and activities in aggregates than in the ambient water (Grossart & Simon 1993, Middelboe et al. 1995, Simon et al. 2002). Although the studied lakes differed in their limnological features, the microenvironment of particles seems to be more similar among the lakes than does that of ambient water.

Dynamics of bacterioplankton communities

NMDS analyses of DGGE banding patterns indicated pronounced seasonal dynamics within free-living and particle-associated bacterial communities of

the 4 studied lakes (Fig. 2B). Seasonal changes in bacterioplankton community structures are common and may be linked to numerous environmental factors, such as phytoplankton succession, protozoan grazing, and viral lysis (Shiah & Ducklow 1994, Hahn & Höfle 1999, Yager et al. 2001, Brussaard et al. 2005). To identify factors controlling changes in bacterial community structure, we compared the DGGE profiles of the respective bacterial communities with 15 limnological variables. Our results indicated strong correlations (Pearson's r ≥ 0.800) between free-living bacteria (Lake Breiter Luzin, NE and SW compartment of Lake Grosse Fuchskuhle) and certain environmental variables such as secchi depth, temperature, and total bacterial production (Table 4). Particle-associated bacteria showed strong correlations with conductivity, total phosphorus, and phytoplankton biomass in Lake Breiter Luzin and Lake Stechlin (Table 4). Statistically significant relationships with these variables have been previously demonstrated for total bacterial communities of different freshwater habitats (see e.g. Stepanauskas et al. 2003, Lindström et al. 2005, Schauer et al. 2005, Yannarell & Triplett 2005). However, it remains unknown how limnological variables control bacterial communities. Inconsistent correlation patterns between the studied lakes suggest adaptation by distinct bacterial communities to lake-specific combinations of variables. Although bacterial communities differed between the studied lakes, trophic state seems to be irrelevant to the determination of bacterial community structure. As shown by our NMDS ordination analyses, differences between free-living and particle-associated bacterial communities indicated no relationship with the trophic gradient of the studied lakes. Dissimilarities among bacterial communities must therefore be caused by other factors.

In addition, changes in bacterial community composition are not only linked to autochthonous processes (e.g. phytoplankton blooms) but also to allochthonous factors. For example, riverine inlets or the input of allochthonous organic matter lead to substantial shifts in bacterial community structure (Crump et al. 2003, Lindström & Bergström 2004). Since all studied lakes are independent from external water inflow, the input of riverine bacteria can be excluded unambiguously. All studied lakes are generally maintained by autochthonous processes, but leaf litter during fall and early winter leads to a significant input of allochthonous carbon into the lakes. For Lake Stechlin it was observed that carbon input by leaves can contribute almost a quarter of the total annual organic carbon input, which significantly increases ^{14}C-glucose uptake by heterotrophic bacteria (H.-D. Babenzien pers. com.). The impact of leaf litter input on the dynamics of the bacterial commu-

nities of the studied lakes is currently unknown and must be investigated in the future. However, formation of seasonal clusters within our NMDS analyses may provide a first indication that the above mentioned processes indeed affect bacterial community structure.

Phylogenetic diversity of freshwater bacterial communities

Clone libraries of 16S rRNA gene fragments indicated distinct phylogenetic differences between free-living and particle-associated bacteria of all lakes. As demonstrated by our phylogenetic analyses, all retrieved 16S rRNA gene sequences belonged to already known bacterial lineages (Hiorns et al. 1997, Glöckner et al. 2000, Zwart et al. 2002). The number of sequenced clones was too low to draw any general conclusion on the quantitative proportions of distinct bacterial lineages. Furthermore, our clone libraries were restricted to 2 selected time points — May (Lake Stechlin) and November 2003 (all lakes) — and hence may be rather limited in their phylogenetic information.

Free-living bacterial communities of the 4 studied lakes were dominated by *Actinobacteria*, *Bacteroidetes*, and *Betaproteobacteria*, whereas particle-associated bacterial communities mainly comprised sequences of *Cyanobacteria* and members of *Bacteroidetes*.

Bacteria of Class *Actinobacteria* were found predominantly in the free-living bacterial fractions of the studied lakes. Only 1 single sequence belonged to the particle-associated bacteria (Table 3). However, *Actinobacteria* do occur regularly on particles, as shown by a study on particle-associated bacterial communities that investigated *Actinobacteria* in detail using a specific DGGE approach (M. Allgaier et al. unpubl. data). Hence, proportions of particle-associated *Actinobacteria* were probably underestimated by our clone libraries. Due to a lack of *Actinobacteria* isolates, almost nothing is known about their physiology and ecological role in freshwater habitats.

Bacteria of the *Bacteroidetes* are common in freshwater habitats but also occur in other aquatic environments (Glöckner et al. 1999, Kirchman 2002, Eiler & Bertilsson 2004). They are known to play an important role in the turnover of organic matter (Cottrell & Kirchman 2000b) and are capable of degrading polymeric substrates such as cellulose and chitin (Kirchman 2002, Jam et al. 2005). Members of *Bacteroidetes* are well known as particle-associated bacteria, but are also common as free-living organisms (Crump et al. 1999, Eiler & Bertilsson 2004, Schauer et al. 2005).

Low numbers of *Betaproteobacteria* in our clone libraries of particle-associated bacteria were in contra-

diction to previous studies, where *Betaproteobacteria* were identified to be one of the dominating bacterial groups of particle-associated bacteria (Weiss et al. 1996, Grossart & Simon 1998, Schweitzer et al. 2001). Members of *Betaproteobacteria* are widely distributed in freshwater habitats, and frequently dominate total bacterial communities. In Lake Grosse Fuchskuhle, *Betaproteobacteria* contributed up to 29% (NE compartment) and 42% (SW compartment) to total bacterial communities (Burkert et al. 2003). Nevertheless, the low numbers of *Betaproteobacteria* sequences in our clone libraries remain unexplained.

Clone libraries of particle-associated bacteria also revealed several sequences of *Verrucomicrobia* and *Planctomycetes*. Even though all sequences of *Verrucomicrobia* were found on particles, it is uncertain whether these bacteria are exclusively adapted to such microenvironments, as was demonstrated for *Planctomycetes* (Neef et al. 1998).

In summary, our results revealed clear seasonal dynamics within free-living and particle associated bacterial communities from all studied lakes. Significant differences were found between both fractions with respect to community structure and phylogenetic diversity. Bacterial communities were strongly correlated with limnological variables such as secchi depth, temperature, conductivity, phosphorus, bacterial production, and phytoplankton biomass. However, no uniform correlation pattern among bacterial communities and measured limnological variables was observed when data from all lakes were compared. This may indicate adaptation by individual bacterial communities to specific environmental conditions in a given lake. Phylogenetically, almost all cloned and sequenced 16S rRNA gene fragments belonged to already known freshwater clusters. Members of the *Bacteroidetes* occurred in both bacterial fractions, whereas *Actinobacteria, Alpha-* and *Betaproteobacteria* mainly occurred within the free-living bacteria. To obtain further information on relationships between bacterioplankton communities and environmental variables, more specific studies on distinct phylogenetic lineages and/or defined seasonal periods are required.

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