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Buesing, Nanna; Filippini, Manuela; Bürgmann, Helmut; Gessner, Mark O.

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# Microbial communities in contrasting freshwater marsh microhabitats

Nanna Buesing<sup>1</sup>, Manuela Filippini<sup>1</sup>, Helmut Bürgmann<sup>2,3</sup> & Mark O. Gessner<sup>1,4</sup>

<sup>1</sup>Department of Aquatic Ecology, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland; <sup>2</sup>Department of Surface Waters, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland; <sup>3</sup>Institute of Terrestrial Ecology, ETH, Zurich, Switzerland; and <sup>4</sup>Institute of Integrative Biology (IBZ), ETH, Zurich, Switzerland

**Correspondence:** Nanna Buesing, Department of Aquatic Ecology, Eawag: Swiss Federal Institute of Aquatic Science and Technology, 6047 Kastanienbaum, Switzerland. Tel.: +41 41 349 21 11; fax: +41 41 349 21 68; e-mail: nanna.buesing@eawag.ch

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## Keywords

bacteria; fungi; DGGE; wetland; freshwater; *Phragmites*.

## Abstract

Heterotrophic microorganisms are widely recognized as crucial components of ecosystems; yet information on their community structure and dynamics in benthic freshwater habitats is notably scarce. Using denaturing gradient gel electrophoresis (DGGE), we determined the composition of bacterial and fungal communities in a freshwater marsh over four seasons. DGGE revealed diverse bacterial communities in four contrasting microhabitats. The greatest compositional differences emerged between water-column and surface-associated bacteria, although communities associated with sediment also differed from those on plant litter and epiphytic biofilms. Sequences of bacterial clones derived from DGGE bands belonged to the *Alphaproteobacteria* (31%), *Actinobacteria* (19%) and *Bacteroidetes* (19%). *Betaproteobacteria* were notably absent. Fungal clones obtained from leaf litter were mainly *Ascomycota*, but two members of the *Basidiomycota* were also identified. Overall, habitat type was the most important factor explaining variation in bacterial communities among samples, whereas temporal patterns in community composition were less pronounced in spite of large seasonal variation in environmental conditions such as temperature. The observed differences among bacterial communities in different microhabitats were not caused by random variation, but rather appeared to be determined by habitat characteristics, as evidenced by largely congruent community profiles of replicate samples taken at 10–100 m distances within the marsh.

## Introduction

The widespread use of molecular biological methods has revealed an astounding diversity of microorganisms in a range of ecosystems (Curtis *et al.*, 2002; Torsvik *et al.*, 2002). This in turn has sparked renewed interest in understanding the distributional patterns of microorganisms and the mechanisms underlying them (Buchan *et al.*, 2003; Lozupone & Knight, 2007; Ramette & Tiedje, 2007). In addition, microbial communities are being increasingly used to address fundamental ecological issues (Prosser *et al.*, 2007), such as the relationship between area and species richness (Reche *et al.*, 2005; Green & Bohannan, 2006), the determinants of community assembly (Lindström *et al.*, 2005; Allgaier & Grossart, 2006) or the importance of biodiversity for ecosystem functioning (Bell *et al.*, 2005; Dang *et al.*,

2005). To be meaningful, all of these studies must be based on an understanding of microbial community structure in natural environments.

One common approach to describe microbial communities in ecosystems is based on the extraction and amplification of rRNA gene sequences and subsequent separation of the PCR fragments by various methods. This includes denaturing gradient gel electrophoresis (DGGE; Muyzer *et al.*, 1993; Loisel *et al.*, 2006), which has been used for a wide range of microbial groups and environments. When combined with sequence analyses of the amplified fragments, this approach can provide not only a profile (band pattern) that allows comparisons of communities over space, time or experimental treatments but also a simple way to reveal phylogenetic affiliations of the dominant organisms present (Burr *et al.*, 2006; Mühling *et al.*, 2008).

However, as with all PCR-based methods, DGGE is subject to the potential limitation that some community members evade detection by PCR and that PCR skews the distribution of alleles relative to the original community (Becker *et al.*, 2000; Lueders & Friedrich, 2003).

Structurally complex ecosystems such as freshwater marshes are useful systems to study the importance of habitat characteristics in determining patterns of microbial communities. Contrasting microhabitats occur in close proximity and are embedded in, and connected by, the aqueous medium. This ensures effective short-range dispersal and identical broad-scale environmental conditions, so that any differences in communities should be due to intrinsic habitat characteristics. Freshwater marshes are also highly productive and provide different types of organic matter to heterotrophic microorganisms (e.g. plant litter, algal detritus, DOC) such that both bacteria and fungi are important components of these ecosystems (Buesing & Gessner, 2006). Currently, information on spatial and temporal patterns of microbial communities in these and other lacustrine ecosystems is scarce, however, as most efforts towards characterizing microbial communities in fresh waters have been devoted to bacterioplankton (e.g. Øvreås *et al.*, 1997; Zwart *et al.*, 2002; Reche *et al.*, 2005). Some information also exists for sediments (Spring *et al.*, 2000), epilithic and epiphytic biofilms (Jackson *et al.*, 2001; Araya *et al.*, 2003; Lyautey *et al.*, 2003; Hempel *et al.*, 2008) and decomposing plant litter (Mille-Lindblom *et al.*, 2006), but communities have not been directly compared among this range of microhabitats.

Earlier analyses that we conducted in a freshwater marsh showed distinct seasonal patterns of microbial productivity in water and epiphytic biofilms, whereas no clear seasonal influence was observed on plant litter and in sediment where bacterial productivity was the highest (Buesing & Gessner, 2006). Fungi also showed substantial productivity in plant litter (Buesing & Gessner, 2006), but their biomass in other aquatic habitats was low (N. Buesing, unpublished data). Temporal changes in these different microhabitats were not always tightly coupled, suggesting that bacterial communities in different habitats undergo at least partly independent dynamics. Pronounced dissimilarities among microhabitats were also apparent in a previous analysis of viral infection rates: only water-column bacteria were infected to a significant extent, whereas infection was barely detectable (< 0.1% of total bacteria) in sediment, plant litter and epiphytic biofilms even though free viruses were abundant (Filippini *et al.*, 2006). A strong variation in bacterial community composition among microhabitats could be one important factor contributing to this unexpected pattern.

The present study thus aimed (1) to assess whether differences exist in bacterial community structure among

microhabitats in a structurally complex aquatic ecosystem, (2) to assess the degree of seasonal changes in bacterial communities in these habitats, (3) to assess the degree of seasonal changes in fungal communities in submerged decomposing plant litter, the major aquatic habitat of fungi in marshes, and (4) to gain insight into the most prominent fungal and bacterial taxa present. We pursued these aims by sampling four aerobic microhabitats in a littoral freshwater marsh once in each season and by characterizing the associated microbial communities by means of DGGE and sequencing of high-intensity bands in the community profiles.

## Materials and methods

### Study site

The study was conducted in a littoral marsh on the eastern shore of Lake Hallwil, a eutrophic, meromictic lake on the Swiss Plateau (47°17'N, 8°14'E) at 449 m a.s.l. Common reed, *Phragmites australis*, extended 850 m along the shore. The stand varied in width between 6 and 20 m and water depth averaged 1 m at the lakeward margin and 35–70 cm in the centre. More information on the study site is provided by Buesing & Gessner (2006).

### Experimental design and sampling

Three randomly selected plots (Ø 1.40 m) were delineated within the marsh and sampled in all four seasons in 2003/2004. Three replicate samples were taken each time in four aerobic microhabitats: the water column, epiphytic biofilms on submerged reed stems, the plant litter layer and the aerobic top sediment layer underneath the plant litter ( $n = 48$ ). Depth-integrated water samples taken using an acid-washed PVC tube were collected in 1-L glass bottles. *Phragmites australis* stems were clipped off just above the sediment surface. The submerged portions with attached epiphytic biofilms were cut into three 10-cm sections and placed in centrifuge tubes containing filtered lake water (pore size 0.2 µm). Sediment samples were collected with a hand-held corer modified from Davis & Steinman (1998). Its diameter of 6.5 cm. The vertical extension of the aerobic sediment was determined using a calibrated oxygen micro-electrode (Model O2NAD-1, Toepffer Lab Systems, Göppingen, Germany) and the corresponding top layer (2–29 mm) was sampled and transferred to sterile centrifuge tubes. Plant litter on the sediment surface was collected using a manually operated bilge pump within an area defined by a custom-made Plexiglas cylinder (30-cm diameter). The collected material was passed over a 1-mm mesh screen, rinsed directly in the field with lake water and placed in a plastic box (Buesing & Gessner, 2006). Samples from all

**Table 1.** Primer pairs used for amplification of bacterial and fungal 16S or 18S rRNA genes

Primer name	References	Target group	Product length	Sequence
GC Clamp			40	5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3'
EUB 341f-GC	Muyzer <i>et al.</i> (1993)	Eubacteria	194	5'-CCT ACG GGA GGC AGC AG-3'
EUB 534r				5'-ATT ACC GCG GCT GCT GG-3'
GC Clamp			40	5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C-3'
Fung f-GC	White <i>et al.</i> (1990); May <i>et al.</i> (2001)	Fungi	350	5'-ATT CCC CGT TAC CCG TTG-3'
NS1				5'-GTA GTC ATA TGC TTG TCT C-3'

compartments were stored in cool boxes and processed immediately upon arrival at the laboratory.

### Sample processing

One hundred millilitres of marsh water was filtered through a 0.2- $\mu\text{m}$  polycarbonate filter. Epiphytic biofilm was carefully scraped off the reed stems with a scalpel and collected in a graduated tube. The volume of the slurry was adjusted to 50 mL with filtered lake water, the tube was vortexed and an aliquot (1 mL) was transferred to an Eppendorf tube. Aerobic sediment was transferred to a centrifuge tube with a 1-mL syringe with the Luer-lock end cut off. Finally, representative subsamples of the collected plant litter were cut into small pieces with a sterile knife and about 80 mg wet mass was stored for later analysis. All samples were stored at  $-80^\circ\text{C}$ .

### DNA extraction and amplification

DNA from all microhabitats was isolated using a soil DNA extraction kit (UltraClean<sup>TM</sup>, Soil DNA Kit, 12800-100, MoBio Laboratories, Solana Beach, CA) following the manufacturer's instructions. The primer pairs used for amplification of bacterial and fungal 16S or 18S rRNA gene are summarized in Table 1. PCR was carried out with a concentration of 0.2  $\mu\text{M}$  of each primer, 0.2 mM of dNTP, 5 mM of  $\text{MgCl}_2$  and 10 ng of DNA. *Taq* polymerase (0.025  $\text{U } \mu\text{L}^{-1}$ ) was added when reaction tubes were at  $95^\circ\text{C}$  (hotstart). Initial denaturation for 5 min at  $94^\circ\text{C}$  was followed by 25 cycles involving denaturation for 1 min at  $95^\circ\text{C}$ , primer annealing for 1 min at  $65^\circ\text{C}$ , decreasing by  $1^\circ\text{C}$  per cycle for 10 cycles to a touchdown annealing temperature of  $55^\circ\text{C}$  and 15 cycles at  $55^\circ\text{C}$ , followed by extension for 1 min at  $72^\circ\text{C}$ . Final extension for 5 min at  $72^\circ\text{C}$  allowed completion of any partial polymerizations. Touchdown PCR was used to reduce the occurrence of misprimers, primer dimers and premature annealing.

### DGGE

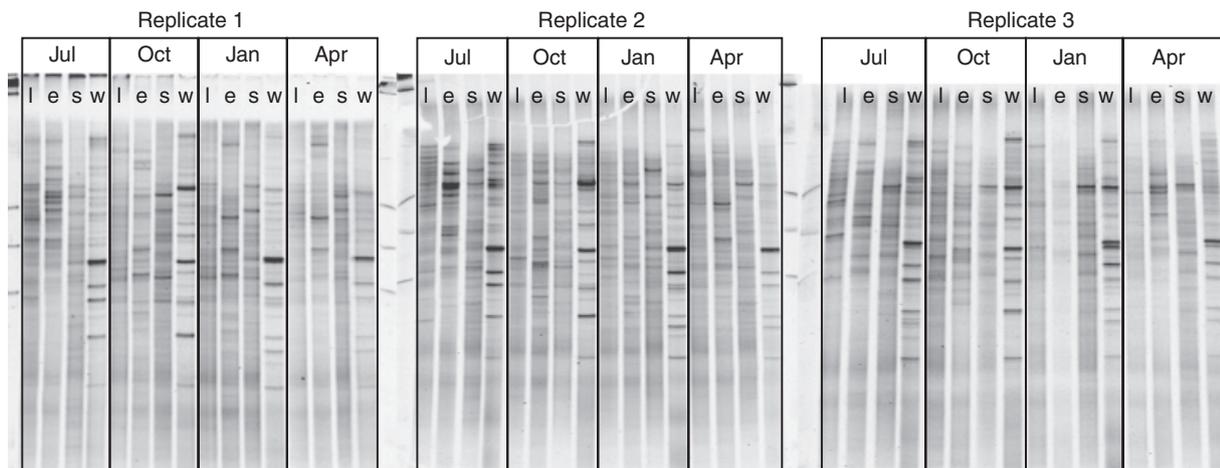
PCR products were analysed by DGGE using a DCode mutation detection system (Bio-Rad, Hercules, CA). Ten to

25  $\mu\text{L}$  of sample (c. 200 ng DNA) was loaded on  $16\text{ cm} \times 16\text{ cm} \times 1\text{ mm}$  gels made of 8% (w/v) polyacrylamide (PAA; acrylamide and *N,N'*-methylene bisacrylamide at a ratio of 37.5:1 (w/w) in  $1 \times$  Tris-acetate-EDTA buffer, pH 8.3) (Sigler *et al.*, 2004). For bacterial samples, a denaturing gradient from 35% to 60% was chosen and for fungal samples a gradient from 20% to 55% was used, with 100% denaturant corresponding to 40% (v/v) formamide and 7 M urea. Gels were run at 200 V and  $60^\circ\text{C}$  for 5 h and were subsequently stained with 1:10 000 diluted GelStar (BioWhittaker Molecular Applications, Rockland, ME). Gel images were taken under UV light using a Bio-Rad Gel Doc EQ system and analyzed using the QUANTITY ONE program, version 4.2.1 (Bio-Rad).

Characteristic high-intensity bands viewed under UV light were cut out from gels with a sterile scalpel and eluted for 24 h at room temperature in 100  $\mu\text{L}$   $1 \times$  TE buffer. To generate template DNA for cloning and sequencing, 2  $\mu\text{L}$  of the eluted rRNA was reamplified with the PCR set-up described above, but this time using primers without a GC clamp. Fragments were cloned before sequencing because preliminary tests had shown that reamplified rRNA from excised band sections loaded directly on polyacrylamide (PAA) gels did not always result in well-defined single bands. Reamplified DGGE fragments were cleaned from primers and deoxyribonucleoside triphosphates using a QIA quick PCR Purification Spin Kit (Qiagen, Hilden, Germany) before cloning.

### Cloning

Purified PCR products obtained with bacterial (3  $\mu\text{L}$ ) and fungal (1.5  $\mu\text{L}$ ) primers were ligated and cloned using the pGEM<sup>®</sup>-T Easy Vectors System (Promega, Madison, WI). Briefly, a plasmid with its DNA insert was transformed using Subcloning Efficiency DH5 $\alpha$  competent cells (Invitrogen, Carlsbad, CA). Successful transformants were selected by blue-white screening according to the instructions of the manufacturer. To isolate plasmid DNA, the Wizard<sup>®</sup> plus SV Minipreps DNA Purification System (Promega) was used following the manufacturer's protocol. DNA was stored at  $-20^\circ\text{C}$  until further analysis. For testing the positions of



**Fig. 1.** Polyacrylamide gels showing rRNA gene amplicon profiles obtained by denaturing gel electrophoresis of bacterial samples taken within a freshwater marsh. Replicates refer to three samples taken at different sites within the marsh. Lane labels indicate the four habitats (l, plant litter; e, epiphytic biofilm; s, sediment; w, water column) and sampling occasions in four seasons (July, October, January and April).

the cloned fragments on the DGGE gels, all clones (dilution 1 : 100) were reamplified using the PCR protocol described above and primers with a GC clamp.

### Sequence analyses

Plasmid inserts were sequenced by Macrogen (Seoul, Korea) and Microsynth (Balgach, Switzerland) using the SP6 universal sequencing primer. Seventeen randomly chosen clones were analysed by both laboratories, which produced identical results.

Sequences were compared with GenBank entries using basic local alignment search tool (BLAST; Altschul *et al.*, 1990), and best matches are reported. Additional reference sequences were retrieved from the *ssu\_jan03.arb* database by aligning all clone sequences in ARB (Strunk & Ludwig, 1996) and adding them to the existing phylogeny using the quick parsimony method. All clone sequences and selected reference sequences were exported to MEGA 3 (Kumar *et al.*, 2004) to perform a phylogenetic analysis with 500 bootstrap samplings using the minimum evolution algorithm, pairwise deletion of missing data and the Kimura 2-parameter distance model (Kimura, 1980). Isolates most closely related to each clone were found using the SEQMATCH tool of the ribosomal database project 2 website (<http://rdp.cme.msu.edu/seqmatch/>) by limiting the search to isolates. The isolate with the highest  $S_{ab}$  score  $> 0.5$  is reported, unless similar  $S_{ab}$  scores were retrieved from isolates belonging to more than one family.

### Data analysis

To minimize the influence of PCR bias, bacterial DGGE bands were scored for presence–absence. This dataset was analysed by correspondence analysis (i.e. indirect gradient

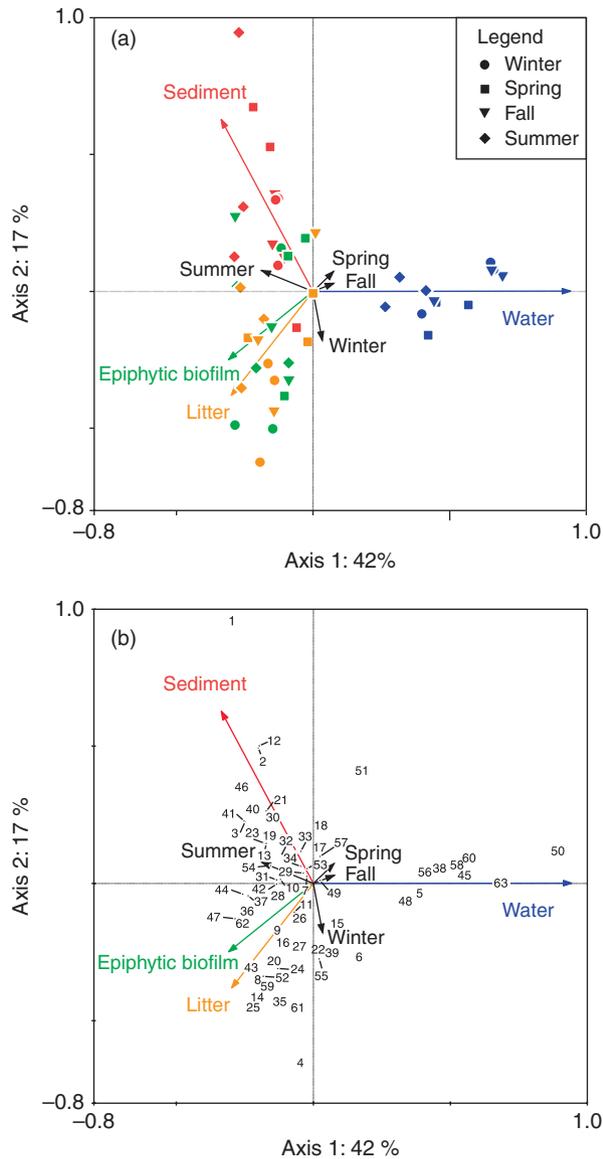
analysis) using CANOCO for Windows 4.5 (Lepš & Šmilauer, 2003), with microhabitat and season superimposed, but not contributing to the factor scores. In addition, Ward's method of hierarchical cluster analysis was used to analyse the bacterial and fungal data, with the distance among communities calculated as Bray–Curtis distances using R version 2.3.1 (R Development Core Team, 2006), and the 'vegan' package (Oksanen *et al.*, 2008). A partial Mantel test (Mantel, 1967) was performed with the matrix of the dice coefficient values (Dice, 1945) and the matrices of the seasons and habitats to test whether distances between communities were significant (FSTAT 2.9.3.2, University of Lausanne, Switzerland; Goudet, 1995). Finally, two-way ANOVA was used to test for differences in DGGE band richness,  $S$ , among communities in different microhabitats and seasons.

## Results

### Microbial community structure in different habitats and seasons

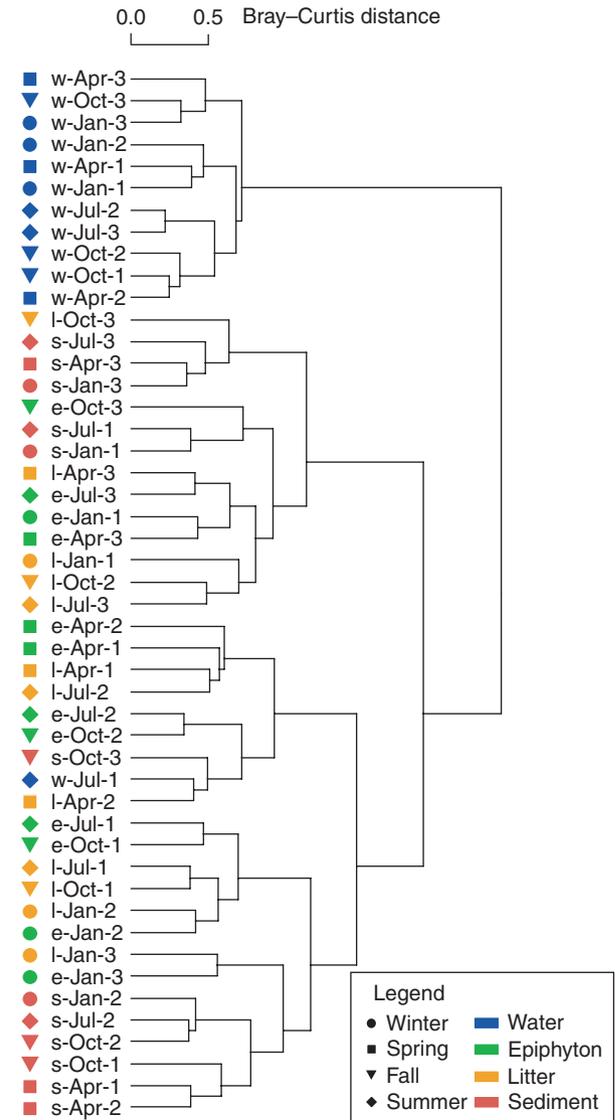
A number of characteristic DGGE bands that could be easily identified across replicates served as internal markers during band alignment (Fig. 1) and allowed us to compare samples across seasons and habitat types. In total, we identified 63 distinct bacterial bands on DGGE gels. The richness of the bands ( $S$ ) was significantly ( $P < 0.05$ ) higher in water samples ( $21.3 \pm 1.1$ ) compared with plant litter ( $16.5 \pm 1.5$ ) and epiphytic biofilms ( $17.6 \pm 1.2$ ), whereas the richness of the DGGE bands from sediment communities ( $18.3 \pm 1.2$ ) was not significantly different from that of any other microhabitat.

The DGGE patterns of bacterial communities in water samples differed markedly from those in the other three



**Fig. 2.** Biplot of a correspondence analysis of DGGE band patterns derived from samples taken on four occasions, one in each season, and in four contrasting microhabitats in a freshwater marsh. The replicate samples were taken on each occasion at three sites in the marsh. Panel (a) shows the bacterial communities inferred from DGGE band patterns and panel (b) shows the associated DGGE bands (i.e. putative species). Numbers in (b) are arbitrary and refer to the bands detected on the gels. See Table 2 for the identity of sequenced bands, which are shown in bold. Seasons and microhabitats were superimposed on the biplot, but did not constrain the factor scores (indirect gradient analysis).

microhabitats, regardless of whether correspondence (Fig. 2a) or cluster analyses (Fig. 3) were performed with presence/absence data and with band-intensity data (not shown). Correspondence analysis clearly distinguished communities in water from those in all other habitats along Axis 1, which accounted for 42% of the total variability (i.e.



**Fig. 3.** Dendrogram of bacterial DGGE band patterns derived from samples taken on four occasions, one in each season, and in four contrasting microhabitats in a freshwater marsh. The replicate samples (1–3) were taken on each occasion (January, April, July and October) at three sites in the marsh. Lower case letters refer to microhabitats (l, plant litter; e, epiphytic biofilm; s, sediment; w, water column). Hierarchical cluster analysis was performed using Ward's method, and Bray-Curtis distances were calculated from band presence-absence data.

inertia) in the data set (Fig. 2a). Nine characteristic bands, five of which have been sequenced, were associated with the water samples (Fig. 2b). The band pattern from sediment communities tended to be distinct from those of plant litter and epiphytic bacterial communities along Axis 2, which accounted for an additional 17% of the total variability. The sediment samples clustered in the upper left quadrant of the biplot (Fig. 2a). Several characteristic bands were associated with these samples (Fig. 2b). In the cluster analysis, all but

one sample from water-column communities were clearly separated from all other samples, reinforcing the result of the correspondence analysis. However, in the cluster analysis, the distinctness of the banding pattern from sediment samples (Fig. 3) was not as clear as in the correspondence analysis (Fig. 2a). Although all but one sediment sample occurred in only three main branches (0.75 distance cutoff), these sediment samples are interspersed with branches of epiphytic biofilm and litter samples. No systematic difference was observed between plant litter and epiphytic biofilm communities with either of the two multivariate analyses (Figs 2a and 3). Overall, bacterial community composition as inferred from DGGE band patterns was strongly influenced by habitat type (Mantel test,  $P < 0.001$ ).

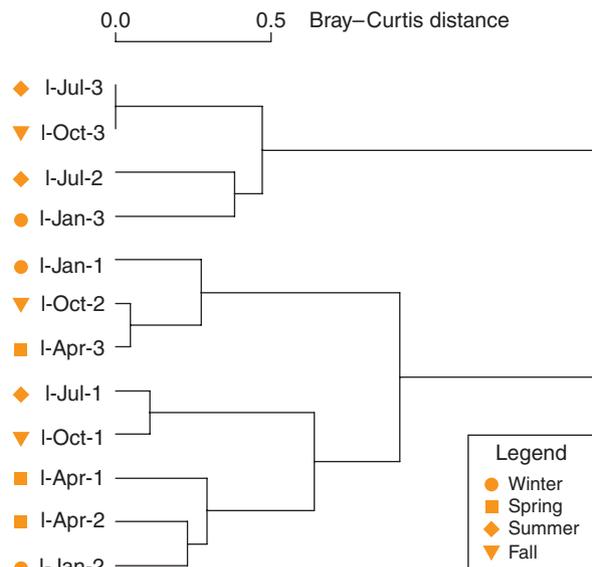
In contrast to these marked differences among some habitat types, no strong seasonal differences were observed for any of the four habitats (Figs 2a and 3, Mantel test,  $P = 0.58$ ). Vectors indicating the four seasons pointed in three different directions on the biplot of Axes 1 and 2 in the correspondence analysis, with spring and fall pointing in the same direction. However, all seasons were located near the centre of the biplot (i.e. vectors were short), indicating that any seasonal differences that might have existed were small or inconsistent.

### Fungal communities in plant litter

DGGE of fragments from plant litter samples generated with primers targeting fungi yielded a total of 18 distinct bands, 13 of which were cloned and sequenced. As observed for bacteria, no seasonal differences were detected in the band pattern generated with these fungal primers (Fig. 4, Mantel test,  $P = 0.25$ ).

### Phylogenetic analysis of sequenced DGGE bands

Despite the relatively short sequences of our 44 cloned DGGE fragments, the topology of the bootstrapped minimum evolution tree (Fig. 5) deviated only slightly from the established phylogeny based on 16S rRNA gene sequences. The main clusters corresponded to known phylogenetic groups and were supported by bootstrap analysis, except for sequences marked Group 1 and 2 in Fig. 5. Sequences of DGGE bands clustered with diverse phylogenetic groups, with all identified groups represented by more than one sequence. Six clones, five of which originated from water samples, were placed in the class *Actinobacteria*. *Actinobacteria* were also dominant among the four sequenced bands that were characteristic for water samples according to Fig. 2b (band nos 5, 38, 48 and 63; Table 2). Three clones clustered with the *Gammaproteobacteria* and eight with the *Alphaproteobacteria*, six of which were most similar to known sequences in the order *Sphingomonadales* (Table 2). All sequences of *Alphaproteobacteria* originated from epiphytic biofilm or plant litter samples. Six clones originating



**Fig. 4.** Dendrogram of fungal DGGE band patterns derived from samples taken on four occasions, one in each season, in a freshwater marsh. Replicate samples (1–3) were taken on each occasion at three sites in the marsh. Hierarchical cluster analysis was performed using Ward's method, and Bray–Curtis distances were calculated from band presence–absence data.

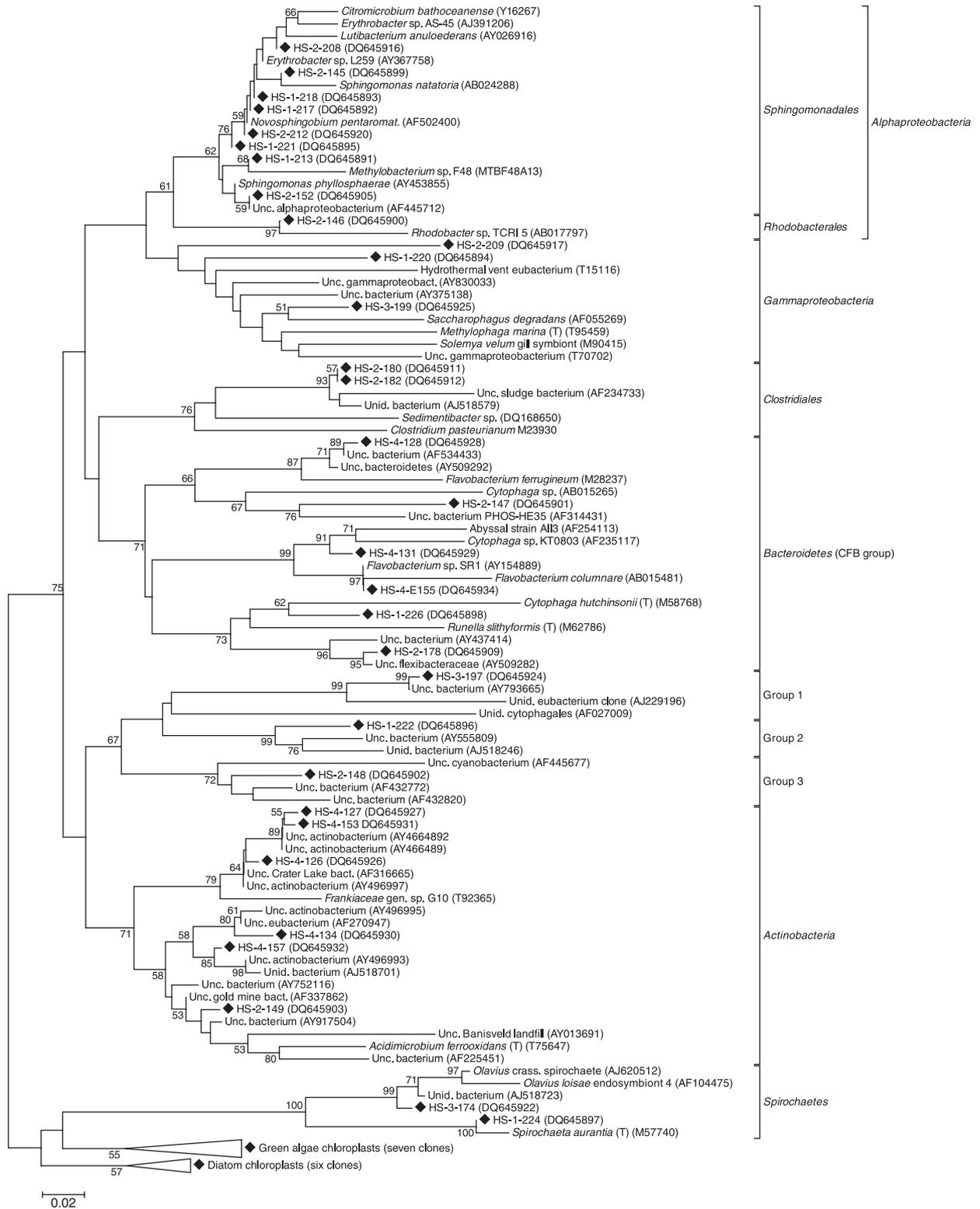
from all habitat types, except sediment, clustered with the *Bacteroidetes* (CFB-group), and only three clones (HS-3-197, HS-2-148, HS-1-222) could not be unambiguously assigned to a phylogenetic group. In the *ARB* analysis, HS-2-148 and HS-1-222 clustered with the *Chlorobi* (CFB-group), but in the minimum evolution phylogenetic analysis (Fig. 5), they were placed close to clone HS-3-197, which, in *ARB*, was assigned to *Cyanobacteria*. Two clones clustered with *Spirochaeta*, and two clustered with the *Clostridiales*. A total of 13 sequences were most closely related to chloroplast sequences from diatoms and green algae. Most of these derived from epiphytic biofilm samples.

Sequence analysis of clones derived from PCR with primers targeting fungi revealed that several bands in the DGGE pattern were not of fungal origin, but belonged to various invertebrate phyla (Fig. 6). Two of the fungal clones (HS-F16 and HS-F14) clustered with known *Basidiomycota*, while the remainder (five clones) were closely related to various *Ascomycota* (Table 3).

## Discussion

### Microbial community patterns across marsh microhabitats

Littoral marshes of lakes comprise various microhabitats that offer a range of conditions for microbial colonization. These microhabitats occur in close spatial proximity and are



**Fig. 5.** Phylogenetic minimum evolution tree of sequenced bacterial clones and selected reference sequences. New entries in the database (GenBank) are marked by a full diamond. The scale bar indicates substitutions per site estimated using the Kimura two-parameter model.

**Table 2.** Sequence analysis of bacterial 16S rRNA gene DGGE bands obtained from different littoral microhabitats of Lake Hallwil, Switzerland

Clone	Band no.	Accession no.	Habitat*	G+C content (%)	Phylogeny†	RDP 2 next isolate‡	Next BLAST matches§	BLAST match origin
HS-4-134	63	DQ645930	w	59.76	<i>Actinobacteria</i>	<i>Acidimicrobium/</i> <i>Microthrix calida</i>	AF270947 (99%/98%) AM690821 (98%/98%)	Soil Freshwater lake
HS-4-157	53	DQ645932	<u>e</u> , l, s, <u>w</u>	57.99	<i>Actinobacteria</i>	<i>Acidimicrobium/</i> <i>Microthrix calida</i>	AY496993 (98%/99%) DQ520164 (98%/99%)	Freshwater lake Freshwater lake
HS-2-149	55	DQ645903	<u>e</u> , s, w	58.24	<i>Actinobacteria</i>	<i>Pseudonocardia/</i> <i>Pseudonocardia</i> <i>thermophila</i>	AM690884 (100%/97%) AY917504 (100%/95%)	Freshwater lake Volcanic deposit
HS-4-126	38	DQ645926	e, l, s, <u>w</u>	58.62	<i>Actinobacteria</i>	<i>Tetrasphaera/</i> <i>Nostocoida limicola</i>	AF316665 (100%/99%) AY496997 (100%/99%)	Freshwater lake Freshwater lake
HS-4-127	48	DQ645927	e, l, s, <u>w</u>	56.90	<i>Actinobacteria</i>	<i>Tetrasphaera/</i> <i>Tetrasphaera</i> <i>australiensis</i>	AY466489 (100%/99%) DQ316380 (100%/99%)	Freshwater sponge Freshwater lake
HS-4-153	5	DQ645931	e, l, <u>w</u>	58.05	<i>Actinobacteria</i>	<i>Tetrasphaera/</i> <i>Tetrasphaera</i> <i>australiensis</i>	AY466489 (100%/99%) DQ316380 (100%/99%)	Freshwater sponge Freshwater lake
HS-2-148	36	DQ645902	<u>e</u> , l, <u>s</u> , w	54.44	(Group 1)	NF <sup>¶</sup>	EF540417 (98%/94%) AF432772 (100%/93%)	Semi-coke Pine rhizosphere soil
HS-3-197	19	DQ645924	e, l, <u>s</u> , w	55.56	(Group 2)	NF	AY793665 (100%/99%) AF314435 (100%/99%)	Lake sediment Batch reactor
HS-1-222	43	DQ645896	<u>e</u> , l, s, w	56.68	(Group 2)	NF	DQ640726 (100%/99%) AM180059 (100%/99%)	Activated sludge Treatment reactor
HS-3-199	25	DQ645925	<u>e</u> , l, s, w	53.61	<i>Gammaproteobacteria</i>	Unclassified <i>Gammaproteobacterial/</i> NEP68	AJ876724 (100%/98%) AM176873 (100%/98%)	River sediment Mangrove sediment
HS-1-220	22	DQ645894	e, l, s, <u>w</u>	54.92	<i>Gammaproteobacteria</i>	<i>Methylomicrobium/</i> <i>Methylomicrobium</i> <i>album</i>	DQ295898 (100%/90%) EF582221 (100%/90%)	Drinking water filter Ocean water
HS-2-209	21	DQ645917	<u>e</u> , l, <u>s</u> , w	53.61	<i>Gammaproteobacteria</i>	Unclassified <i>Gammaproteobacterial/</i> Ellin307	AJ318105 (100%/95%) EF664121 (100%/95%)	Waste gas biofilter Grassland soil
HS-2-146	42	DQ645900	<u>e</u> , l, s, <u>w</u>	56.80	<i>Rhodobacterales</i>	<i>Rhodobacter/</i> <i>Rhodobacter</i> <i>blasticus</i>	DQ065565 (100%/99%) EF392934 (100%/99%)	Freshwater River sediment
HS-2-152	37	DQ645905	<u>e</u> , l, s, w	54.44	<i>Sphingomonadales</i>	<i>Sphingomonas/</i> <i>Sphingomonas</i> <i>phyllosphaerae</i>	AF445712 (100%/100%) EF651276 (100%/99%)	Hot spring Agricultural soil
HS-1-213	7	DQ645891	e, l, s, w	54.44	<i>Sphingomonadales</i>	<i>Sphingomonas/</i> <i>Sphingomonas</i> sp. KIN163	AY136093 (100%/98%) AY792284 (100%/98%)	Freshwater lake Freshwater lake
HS-1-217	26	DQ645892	e, l, s, <u>w</u>	53.25	<i>Sphingomonadales</i>	<i>Blastomonas, others/</i> <i>Blastomonas</i> <i>nataatoria</i> <sup>  </sup>	AB299790 (100%/100%) DQ664250 (100%/100%)	Reed stand in lake Freshwater pond
HS-2-145		DQ645899		53.25				
HS-1-221	24	DQ645895	<u>e</u> , l, s, w	53.25	<i>Sphingomonadales</i>	<i>Blastomonas, others/</i> <i>Erythrobacter</i> <i>longus</i> <sup>  </sup>	DQ378224 (100%/99%) AB299719 (100%/98%)	Polluted soil Reed epiphyton
HS-1-218	28	DQ645893	e, l, s, w	53.25	<i>Sphingomonadales</i>	<i>Blastomonas, others/</i> <i>Sphingomonas</i> <i>nataatoria</i> <sup>  </sup>	EF658786 (100%/100%) AM690965 (100%/100%)	Urban stream Freshwater lake
HS-2-212		DQ645920		53.25				

Table 2. Continued.

Clone	Band no.	Accession no.	Habitat*	G+C content (%)	Phylogeny†	RDP 2 next isolate‡	Next BLAST matches§	BLAST match origin
HS-2-208	17	DQ645916	<b>e</b> , l, s, w	53.25	<i>Sphingomonadales</i>	<i>Blastomonas</i> , others¶ <i>Sphingomonas natatoria</i>	AY149770 (100%/100%) DQ664216 (100%/99%)	Decaying marsh grass Freshwater pond
HS-4-128	56	DQ645928	e, l, s, <b>w</b>	50.26	<i>Bacteroidetes</i>	<i>Terrimonas</i> / <i>Flavobacterium</i> sp. KF030	AF534433 (100%/99%) AY752132 (100%/99%)	Freshwater lake Freshwater lake
HS-2-147	8	DQ645901	<u>e</u> , <u>l</u> , w	51.85	<i>Bacteroidetes</i>	NF	DQ640703 (100%/98%) AB205940 (100%/96%)	Activated sludge Activated sludge
HS-2-178	52	DQ645909	<u>e</u> , <u>l</u> , s, w	56.38	<i>Bacteroidetes</i>	Unclassified <i>Flexibacteraceae</i> / sp. PE03-7G4	AY509282 (100%/98%) AY874043 (100%/98%)	Freshwater lake Freshwater lake
HS-1-226	44	DQ645898	e, l, s	55.85	<i>Bacteroidetes</i>	<i>Reichenbachiella</i> / <i>Bacteroidetes</i> sp. O-014	AY168752 (98%/99%) AF365616 (100%/93%)	Stream biofilm Coral
HS-4-131	6	DQ645929	e, l, <b>w</b>	51.85	<i>Bacteroidetes</i>	<i>Flavobacterium</i> / <i>Flavobacterium</i> sp. 1_4K	EF060996 (100%/100%) AY842553 (100%/100%)	Freshwater lake Freshwater lake
HS-4-155	20	DQ645934	e, l, s, <b>w</b>	53.97	<i>Bacteroidetes</i>	<i>Flavobacterium</i> / <i>Flavobacterium</i> sp. SR1	AY135929 (100%/100%) AF418959 (100%/100%)	Freshwater lake Freshwater lake
HS-3-174	18	DQ645922	e, l, s, w	54.12	<i>Spirochaetes</i>	<i>Spirochaeta</i> / endosymbiont P30-6	AJ518723 (99%/98%) AJ620512 (99%/95%)	Freshwater sediment Sludge worm symbiont
HS-1-224	10	DQ645897	e, l, s, w	52.06	<i>Spirochaetes</i>	<i>Spirochaeta</i> / <i>Spirochaeta</i> sp. MWH-HuW8	AJ565433 (99%/100%) M57740 (99%/99%)	Freshwater pond Freshwater
HS-2-180	41	DQ645911	<b>e</b> , l, <u>s</u> , w	54.71	<i>Clostridiales</i>	<i>Sedimentibacter</i>	DQ979354 (99%/100%)	Soil
HS-2-182	47	DQ645912	<b>e</b> , <u>l</u> , s, w	54.71		<i>Sedimentibacter</i> sp. JN18_A14_H	AJ518579 (99%/98%)	Lake sediment
HS-3-196		DQ645923	<b>s</b>	52.02	Chloroplast/diatoms	(Diatom chloroplasts) <i>Haslea ostrearia</i>	Not searched	
HS-2-150								
HS-3-173		DQ645904	<b>e</b>	52.02				
HS-2-171		DQ645921	<b>s</b>	52.60				
HS-2-210		DQ645907	<b>e</b>	52.60				
HS-2-211		DQ645918	<b>e</b>	52.60				
		DQ645919	<b>e</b>	53.18				
HS-2-170		DQ645906	<b>e</b>	49.11	Chloroplast/green algae	(Green algae chloroplasts)	Not searched	
HS-4-160		DQ645933	<b>w</b>	49.70				
HS-2-179		DQ645910	<b>e</b>	49.11		<i>Pseudendoclonium</i> <i>akinetum</i>		
HS-2-172		DQ645908	<b>e</b>	49.11				
HS-2-207		DQ645915	<b>e</b>	52.02				
HS-2-193		DQ645914	<b>e</b>	52.02				
HS-2-183		DQ645913	<b>e</b>	52.07				

\*Habitats in which this band occurred: e, epiphytic biofilm; l, plant litter; s, sediment; w, water. Dominant habitats are underlined; bold characters indicate the habitat from which the band was cut.

†According to phylogenetic analysis (Fig. 5).

‡Highest scoring isolate match according to RDP 2 search. Genus and the highest scoring species are given. Only S<sub>ab</sub> scores > 0.6 are considered.

§The two closest matches in MEGABLAST searches of database nr/nt are reported. Freshwater clones or isolates are reported when several matches had equal scores. Coverage and similarity of the BLAST hit in parentheses.

¶NF, not found (i.e. no single genus was associated with the query sequence, or S<sub>ab</sub> score was < 0.6).

||Also *Erythrobacter* and *Novosphingobium*.

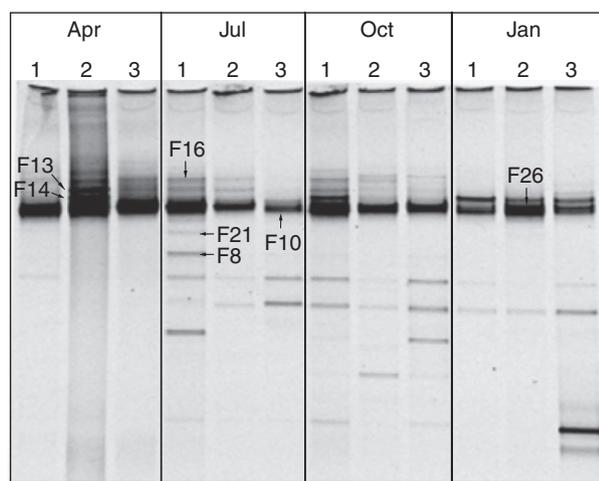
intimately connected by the surrounding water. This situation allows evaluating whether short-range dispersal of microorganisms evens out any differences in microbial community structure across microhabitats, or to what extent microhabitat characteristics override the dispersal-favoured homogenization tendency across communities. In response to this question, our DGGE results indicated that habitat conditions played an important role in shaping bacterial communities in different microhabitats of the marsh, whereas the seasonal variation of environmental

factors, such as the large temperature fluctuations occurring at our study site (Buesing & Gessner, 2006), appeared to have minor or inconsistent effects on either bacterial or fungal communities.

The strongest discrimination occurred between DGGE banding patterns derived from water-column and surface-associated bacteria. However, banding patterns in the surface sediment (layer with a positive redox potential) also differed from those in both the litter layer and epiphytic biofilms on submerged standing-dead plant stems. This result is in agreement with studies in other freshwater systems (i.e. running waters) where the bacterial communities of sediments and epiphytic biofilms were clearly different from the communities in water (Beier *et al.*, 2008) even when low-resolution methods were used to characterize the bacterial communities (Holder-Franklin *et al.*, 1981; Marxsen & Moaledj, 1988). These observations lend support to the hypothesis that distinct bacterial communities partly account for the 'infection paradox' that we previously observed in our marsh, i.e. the conspicuous lack of bacterial infection by viruses in microhabitats other than the water column, despite an abundance of free benthic viruses (Filippini *et al.*, 2006).

The similarity of communities we observed between the other two microhabitats, plant litter and epiphytic biofilms, may be due to the fact that epiphytic biofilms were scraped off submerged standing-dead plant stems. These biofilm communities may have included bacteria involved in litter decomposition, similar to those that colonize plant litter that had fallen onto the sediment.

Our finding of distinct free-living and substrate-associated bacterial communities occurring at a small spatial scale is in line with the results of a global analysis of



**Fig. 6.** Polyacrylamide gel showing rRNA gene amplicon profiles obtained by denaturing gel electrophoresis of litter samples taken within a freshwater marsh and amplified with primers targeting fungi. Lane numbers (1,2,3) refer to three replicate samples taken at different sites within the marsh on four sampling occasions. Numbers preceded by 'F' indicate true fungal clones as given in Table 3.

**Table 3.** Sequence analysis of fungal 18S rRNA gene DGGE bands obtained from submerged plant litter collected in Lake Hallwil, Switzerland

Clone	Accession no.	G+C content (%)	Phylogeny*	Next BLAST matches†	BLAST match origin‡
HS-F8	DQ837572	42.94	Ascomycota	AY879034 (96%/97%) AY879032 (96%/97%)	<i>Lulworthia uniseptata</i> <i>Lulworthia uniseptata</i>
HS-F10	DQ837573	41.21	Ascomycota	DQ678004 (99%/100%) AJ515948 (99%/100%)	<i>Cladosporium cladosporioides</i> Uncultured soil clone
HS-F13	DQ837574	41.50	Ascomycota	DQ384068 (99%/99%) Y11715 (99%/99%)	<i>Lepidosphaeria nicotiae</i> <i>Monodictys castaneae</i>
HS-F21	DQ837577	46.13	Ascomycota	AY544689 (97%/90%) AY484511 (97%/90%)	<i>Carpoligna pleurothecii</i> AFTOL-ID 281 <i>Conioscyphascus varius</i>
HS-F26	DQ837578	41.91	Ascomycota	AY337712 (100%/99%) EF532930 (100%/99%)	<i>Phoma herbarum</i> <i>Phoma</i> sp.
HS-F14	DQ837575	40.23	Basidiomycota	AB085798 (99%/99%) AB000953 (99%/99%)	<i>Cryptococcus carnescens</i> <i>Taphrina maculans</i>
HS-F16	DQ837576	40.74	Basidiomycota	AY336765 (100%/98%) AF518579 (100%/98%)	<i>Fomitopsis pinicola</i> <i>Hymenochaete corrugata</i>

\*According to phylogenetic analysis.

†The two closest matches in MEGABLAST searches of database nr/nt are reported. Coverage and similarity of the blast hit in parentheses.

‡Species designation for the BLAST match or origin of environmental clones.

16S rRNA sequences showing that physical habitat characteristics (i.e. water vs. sediment) are next in importance to salinity in defining bacterial community structure (Lozupone & Knight, 2007). Unexpectedly, other important environmental variables, such as temperature, were far less influential in that large-scale analysis, even though representatives of extreme environments (e.g. hot springs and ice) were amply represented in the data set. This finding by Lozupone & Knight (2007) is also reflected by our data, where no marked seasonal patterns in community structure emerged in the four microhabitats examined. The result is remarkable in view of the large temperature range experienced over the seasonal cycle in the marsh (4–27 °C), the seasonally strong variation in organic matter supply from different sources (e.g. plant litter, phytoplankton) and seasonal changes in dissolved nutrient concentrations (Buesing & Gessner, 2006). The presence of apparently similar microbial communities on four occasions in four seasons at our study site suggests that the previously observed seasonal dynamics in bacterial productivity (Buesing & Gessner, 2006) were due to metabolic responses rather than due to shifts in microbial community composition. This result was particularly unexpected for water samples, because distinct temporal changes of microbial communities have been reported from the pelagic zone of other lakes (e.g. Yannarell *et al.*, 2003).

The differences we found between water, sediment and other substrate-associated communities were not caused by a random variation, but were apparently determined by habitat characteristics. In contrast, seasonal changes, especially in the particle-dominated habitats, were dependent on local or random factors and did not lead to a consistent shift in the communities. Considering that replicate samples were taken in the field tens to hundreds of metres apart, as well as among samples taken in different seasons, whereas communities yielding distinct band patterns between microhabitats were only centimetres to decimetres away from one another suggests a deterministic basis for the observed spatial patterns.

### Composition of bacterial communities

Differences in community composition among microhabitats were indicated by the association of certain DGGE bands with particular microhabitat types (Fig. 2) and by the species identities revealed by our phylogenetic analysis of sequenced bands. For example, high-intensity bands of four of the six identified *Actinobacteria* (clones HS-4-126, 127, 134 and 153) were from water and one occurred in water only (HS-4-134). This dominance of *Actinobacteria* in water samples is in agreement with the regular abundant appearance of some actinobacterial groups in the plankton of lakes and rivers (e.g. Warnecke *et al.*, 2004). Although *Actinobacteria* have commonly been

found in sediments and soils as well (e.g. Warnecke *et al.*, 2004), the relative prominence we observed in water samples may indicate a preference of at least some actinobacterial lineages for a planktonic lifestyle.

Overall, the phylogeny of DGGE bands derived from our water samples suggests typical freshwater communities: *Actinobacteria*, *Bacteroidetes* (CFB-group) and *Alphaproteobacteria* are all among the most frequently found divisions in freshwaters (Glöckner *et al.*, 2000; Zwart *et al.*, 2002; Eiler & Bertilsson, 2004; Tamaki *et al.*, 2005). One unexpected finding, however, is the striking lack of *Betaproteobacteria* among our identified clones, given that members of this division are usually abundant in freshwaters as well (Zwart *et al.*, 2002; Brummer *et al.*, 2003; Briece *et al.*, 2007). The reason for this apparent absence is unknown, but it might be related to the eutrophic conditions in our study lake (c.f. Zwart *et al.*, 2002), particularly in the littoral marsh where our samples were taken.

All other microhabitats (sediment, epiphytic biofilms and plant litter) shared several phylotypes, with the most frequently identified DGGE bands related to *Alphaproteobacteria* (HS-2-152), *Bacteroidetes* (HS-2-178, HS-1-126) and *Chlorobium* (HS-1-122, HS-3-197). Sequence comparisons also showed that nearly all our clones from these habitats were most similar to either environmental or isolated clones from freshwater or other aquatic ecosystems. Even environmental clones of *Bacteroidetes* found as best matches in BLAST searches were from freshwater, even though sequenced isolates in the database typically have a marine origin. These results indicate that the sediment-, epiphytic biofilm- and litter-associated populations we characterized, although distinct from water-column bacteria and, in part, from one another, were nonetheless typical of freshwaters (Table 2) and most probably different from populations in marine and terrestrial environments (Torsvik *et al.*, 2002; Lozupone & Knight, 2007). One exception to this rule was all our sequences assigned to the *Gammaproteobacteria*, which were most closely related to two environmental clones and isolates with a marine origin (Table 2). It should be borne in mind, however, that our sequenced fragments were relatively short (< 200 bp) such that matches with clones in GenBank are not irrevocable.

Both correspondence and cluster analysis failed to discriminate the bacterial communities associated with plant litter and epiphytic biofilms. Accordingly, only a few phylotypes were consistently associated with one or the other of these two habitats. Clone HS-2-182, related to the *Spirochaeta* and mostly found in plant litter, is one of these rare examples. In addition, a number of clones probably related to chloroplasts were uniquely sequenced from epiphytic biofilms, which were dominated by eukaryotic algae, as revealed by occasional inspection. Consequently, caution is needed when assessing the bacterial diversity of eukaryote-dominated habitats based on DGGE banding patterns alone.

## Composition of fungal communities

The same caution may have to be exercised for DGGE analyses of fungal communities. The presence of various clone sequences not related to fungi indicates that our PCR conditions were not stringent enough to preclude amplification of eukaryote sequences other than fungal, particularly those of several invertebrate phyla. It is also possible that our fungal primers were generally not specific enough even though the same primers have proved to be adequate to specifically amplify litter-associated fungi in streams (Nikolcheva *et al.*, 2003) and other freshwater environments (Mille-Lindblom *et al.*, 2006). Furthermore, an assessment of the primer pair with BLAST against nonfungal eukaryotic sequences showed that a number of perfect or near-perfect false-positive matches exist, which may confound the analysis in environments with a high occurrence of animals or animal remains, which is probably the case for the productive marsh studied here. Nevertheless, most sequences of our fungal clones were identified as *Ascomycota*, in line with the fact that this is the most common and diverse phylum of fungi on litter in fresh water, including freshwater marshes (Vijaykrishna *et al.*, 2006; Shearer *et al.*, 2007). Fungal presence in our litter samples in general also concurs with our previous observation that fungi grow actively in the submerged litter layer of the marsh (Buesing & Gessner, 2006), even though fungal biomass and diversity on emergent macrophyte litter has been found to decline upon litter submergence in other marshes (Kuehn *et al.*, 2000; van Ryckegem *et al.*, 2007).

Although the phylogenetic information content in our DGGE fragments allowed reliable identification only at the phylum level, some closest matches obtained in the phylogenetic comparisons suggest interesting affiliations. In particular, we found a sequence with high similarity to a *Lulworthia* species, corroborating a previous record from a tropical lake in China (Cai *et al.*, 2002). This is remarkable because marine and freshwater fungi are thought to be distinctive (Shearer *et al.*, 2007) and *Lulworthia* is considered a marine fungus par excellence (Kohlmeyer *et al.*, 2000). Does its repeated occurrence in fresh water perhaps simply reflect the very incomplete knowledge of fungi in ecosystems (Vijaykrishna *et al.*, 2006)?

## Conclusions

In conclusion, we found diverse DGGE band patterns in the four aerobic microhabitats examined, suggesting a bacterial community in the water column distinct from the communities in the other habitats. The sediment communities also differed from those associated with plant litter and epiphytic biofilm, but these differences were less pronounced. Notwithstanding such dissimilarities, the communities in all

four microhabitats were identified as being typical of freshwaters. The large seasonal variation in environmental conditions over the year appeared not to consistently affect either the bacterial or the fungal communities, except in the water column. The reason for this remarkable lack of a seasonal pattern is unclear, but may be related to the greater heterogeneity of the surface-dominated habitats. Finally, the fungal community colonizing plant litter appeared to be less diverse than the bacterial community on plant litter. While this finding may have also been influenced by methodological bias, its concomitance with a greater bacterial productivity observed in a previous study at the same site is in agreement with the idea that bacteria on submerged plant litter in freshwater marshes assume greater importance than fungi (Buesing & Gessner, 2006).

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** DGGE gel of rRNA gene amplicon profiles of bacterial samples including all band numbers.

**Table S1.** Presence-absence table for all bands shown in Fig. 1.

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