Changes in the bacterioplankton community of oligotrophic Lake Stechlin (northeastern Germany) after humic matter addition

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ABSTRACT: In an effort to better understand the dynamics of members of the bacterioplankton community in relation to humic matter (HM) addition, and to provide insight into the ecology of common and persistent as well as transient freshwater bacteria, we designed a study with a batch and a dilution approach. We used single vs. repeated HM additions in incubations with bacterial communities from the epilimnion (0–10 m) and hypolimnion (40 m) of oligotrophic Lake Stechlin (northeastern Germany). Molecular methods were applied for detailed phylogenetic characterization of bacterial community composition (BCC) every 2 wk over 8 wk of incubation at *in situ* temperature. Whereas no significant differences in the development of BCC in batch vs. dilution cultures were observed, the BCC of epilimnetic and hypolimnetic samples greatly differed. This indicates that HM addition led to the establishment of a highly specific but different BCC depending on the source community in combination with the respective *in situ* temperature. Further, DGGE banding patterns revealed a high variability in the BCC of epilimnetic and hypolimnetic samples. *Betaproteobacteria* were consistently present and specific *Alphaproteobacteria*, such as members of the *Roseisalinus* group, *Bacteroidetes*, and *Deltaproteobacteria* were enriched only after HM addition. Other phylogenetic groups, including *Actinobacteria* and *Gammaproteobacteria*, were only sporadically present. Our approach resulted in the cultivation of a variety of bacteria such as *Lysobacter*, *Methylobacterium*, *Pseudomonas*, *Rhodopila*, and *Variovorax* species. The addition of HM selected for specific HM-degrading bacterial phylotypes, which are found at different depths even in the clear waters of Lake Stechlin.

KEY WORDS: Humic matter · Bacterioplankton community · Lake Stechlin · DGGE · CARD-FISH

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INTRODUCTION

In freshwater systems, highly diverse bacterial populations allow for rapid and efficient adaptation to changes in substrate availability (Crump et al. 2003). Such associated changes in substrate availability indicate the ecological role of major phylotypes in the bacterioplankton community (Lindström 2000), as well as their resistance to changes in environmental conditions (Pearce et al. 2005). Our current understanding of the relationship between growth of individual bacterial populations and availability of particular sub-

strates or nutrients is still rudimentary (Pernthaler & Amann 2005). To aid in the analysis of effects of nutrient loading on the most abundant and metabolically active bacterial groups, combinations of molecular techniques and improved isolation approaches have recently been used (Hahn 2003, Hahn et al. 2003). For example, DNA fingerprint methods such as DGGE, coupled with sequencing of individual 16S rRNA gene fragments (bands), have often been used with catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) to rapidly determine the dominant bacterial members including those linked to

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substrate availability in aquatic environments (Crump et al. 2003, Castle & Kirchman 2004, Gich et al. 2005, Pearce et al. 2005, Grossart et al. 2008). Furthermore, to link the structure and function of freshwater bacterioplankton, molecular techniques are combined with improved isolation techniques to isolate dominant and significant microorganisms (Hahn et al. 2004).

In most freshwater systems, the concentration and quality of the organic matter source, i.e. humic matter (HM), vary greatly in time and space (Sachse et al. 2001), and recent studies have shown that *Actinobacteria* and *Betaproteobacteria* are generally the major groups of common freshwater bacterial phylotypes (Zwart et al. 2002) that respond differently to a sudden pulse of humic matter (Burkert et al. 2003). In addition, drastic shifts in bacterioplankton community composition (BCC) may occur after such sudden pulses (Crump et al. 2003).

Interestingly, certain phylotypes have unique persistence patterns (Haukka et al. 2005), and dominant bacteria that have a better ability to utilize allochthonous substances are being found, such as *Betaproteobacteria* (Burkert et al. 2003). Although changes in BCC can be dramatic even between years (Newton et al. 2006), the extent of this variation and the dynamics of different members of the community are still unclear.

Thus, we designed a study using a batch and a dilution approach to provide insight into the ecology of common and persistent as well as transient freshwater bacteria of oligotrophic Lake Stechlin (northeastern Germany) after single vs. repeated HM additions. Further, we used different bacterial source communities (epilimnion: 0–10 m; hypolimnion: 40 m), and incubated bacteria at their respective *in situ* temperatures. Individual members of the community were phylogenetically characterized to reveal detailed changes in BCC. Following HM addition, there were marked changes in BCC depending on the bacterial source community in combination with the respective *in situ* temperature.

MATERIALS AND METHODS

Study site. Lake Stechlin is one of the clearest lakes in northern Germany and is classified as a dimictic oligotrophic lake (Casper 1985) with low anthropogenic impact. Since this lake has been continuously studied for almost 50 yr, it has considerable ecological significance and serves as a reference lake e.g. for the European Water Framework Directive (http://ec.europa.eu/environment/water/water-framework/index_en.html).

The lake is situated in a forest mainly composed of beech and pine trees and lies at the northern border of

Brandenburg, close to the Mecklenburg Lake District (53°10' N, 13°02' E). It was formed by the melting of dead ice after the glaciations ca. 12 000 yr ago. The lake has a maximum depth of 69.5 m and an area of 4.3 km², with hypolimnetic oxygen saturation levels of up to 60%.

Experimental design. The present study is based on 2 experimental approaches (batch and dilution cultures), each using 2 bacterial community sources (epilimnion: 0–10 m; hypolimnion: 40 m) incubated at *in situ* temperatures. HM extracted from nearby humic Lake Grosse Fuchskuhle (FUKU) was added to duplicate water samples (1 l) from oligotrophic Lake Stechlin (Fig. 1). For batch cultures, only a single addition of HM into the water at the beginning of the experiment was performed. For dilution cultures, the bacterioplankton was introduced into fresh HM via repeated dilution every 2 wk over 8 wk of incubation. DGGE analyses of BCC were performed at 2 wk intervals. Bacterial isolates were obtained and sequences acquired from isolates and DGGE bands were phylogenetically characterized.

Sample collection. Water samples from Lake Stechlin were taken in early spring (March 2005) from 0–10 (epilimnion, pooled) and 40 m (hypolimnion) depths and filtered through 0.8 µm Nucleopore filters (Sartorius) to remove large organisms such as algae and bacterivores.

Preliminary testing of the effects of incubation and HM addition. A preliminary experiment was conducted to test whether the applied incubation conditions (batch culture) would lead to a pronounced shift in BCC compared to the initial lake water sample after 18 d. Additionally, we tested whether HM addition alone had the potential to substantially change BCC after 18 d of incubation. The exact incubation conditions are given below. As indicated in Fig. A1 in the appendix, our incubation conditions did not substantially affect BCC. On the other hand, a pronounced shift in BCC occurred 18 d after HM addition as compared to the initial sample (T_0 without and with HM) and the 18 d sample without HM. This indicates that addition of HM did not change the initial BCC and that our experimental approach is suitable for studying the effects on BCC following HM addition.

Establishment and maintenance of batch and dilution cultures. We added sterile-filtered natural HM (RO-isolate from FUKU; 50 mg l⁻¹ final conc.), sterile trace mineral salts, and nutrients to 1 l lake water samples in 2 l sterile bottles. The RO-isolate of HM was derived from the epilimnetic water of FUKU via reverse osmosis as prepared by Sachse et al. (2001). The trace mineral salts and nutrients were: 75.0 mg MgSO₄ · 7H₂O, 43.0 mg Ca(NO₃)₂ · 4H₂O, 16.0 mg NaHCO₃, 5.0 mg KCl, 3.7 mg K₂HPO₄ · 3H₂O, 4.4 mg Na₂EDTA,

Fig. 1. Flow diagram of batch and dilution cultures at 2 wk sampling intervals. This approach was used for water samples from 0–10 m and 40 m

3.2 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1.0 mg H_3BO_3 , 0.2 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.02 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 mg $\text{CuSO}_4 \cdot 6\text{H}_2\text{O}$, 0.01 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.006 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 5.0 mg nutrient broth (Merck), 5.0 mg tryptic soy broth (Difco), 5.0 mg peptone (Difco), and 5.0 mg yeast extract (Difco). Our protocol is a slight revision of the inorganic basal medium (IBM) used by Hahn et al. (2003) for growth and isolation of ultramicrobacteria of *Actinobacteria*.

Batch cultures were not manipulated during incubation (Fig. 1). For dilution cultures, a 5 ml aliquot was taken at 2 wk intervals and diluted into 1 l of fresh lake water prepared as mentioned above. All cultures were incubated on a rotary shaker at 100 rpm in the dark at *in situ* temperatures (15°C in the epilimnion; 4°C in the hypolimnion) for 8 wk. Samples were taken from all cultures every 2 wk (Fig. 1).

Isolation of bacteria. To acquire bacterial isolates, we plated a 1 ml water sample from the batch and the dilution cultures onto agar plates at the end of the experiment. The plates were prepared from epilimnic or hypolimnic water supplemented with sterile-filtered natural HM, sterile trace mineral salts and nutrients (see above), and agar (1.5%, Difco). Pure cultures were derived after subsequent isolation and recultivation. The phylogenetic affiliation of all bacteria was determined using PCR, DGGE and sequencing analyses (see below).

Total cell counts and CARD-FISH. Total bacterial abundance was determined every 2 wk using epifluo-

rescence microscopy after staining with DAPI (0.2 mg 100 ml^{-1}). Numbers of *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Eubacteria* were determined using an improved protocol for CARD-FISH (Sekar et al. 2003). Briefly, 10 ml from each culture were collected on Nucleopore filters (0.2 μm , Sartorius) and fixed with 50% (v/v) ethanol in 1% (v/v) saline phosphate buffer. All filters were stored at -20°C until further processing. For quantification of *Actinobacteria*, *Alphaproteobacteria*, and *Betaproteobacteria*, probes HGC69a (Roller et al. 1994), ALF968 (Glöckner et al. 1999), and BET42a (Manz et al. 1992) were used. Eubacterial abundance was determined with the probe mix EUB 338 I-III, which detects most *Eubacteria*, even including *Planctomycetes* and *Verrucomicrobia* (Daims et al. 1999). Stained bacteria were counted with a Leica DR-MB epifluorescence microscope at 1000 $\times$ magnification, and at least 10 randomly selected fields of view (each with 10 000 μm^2) were examined from each culture. Mean and standard deviations were calculated.

DNA extraction and PCR amplification of 16S rRNA gene fragments. To characterize particle-associated (PA) and free-living (FL) bacteria of the natural water samples before the beginning of the experiment (T_0), 100 ml of natural lake water samples were filtered through 5.0 μm and then through 0.2 μm Nucleopore membranes (Sartorius). To characterize temporal changes in the BCC of each culture, 100 ml of sample were taken and filtered through 0.2 μm Nucleopore filter (Sartorius) at 2 wk intervals.

Extraction of genomic DNA was performed using a protocol with lysozyme, proteinase K and zirconium beads. Filters were cut into sections and placed in 2 ml Eppendorf reaction tubes. Zirconium beads (500 mg), sodium dodecyl sulfate (25%), and Na phosphate buffer (120 mM; pH 8) were added to the filter sections, which were then vortexed for 10 min at maximum speed to mechanically disrupt the bacteria. The bacteria were further treated with lysozyme (10 mg ml^{-1}) and proteinase K (10 mg ml^{-1}) overnight. After addition of NH_4 phosphate (7.5 M) and isopropanol (0.7 $\times$ vol), the DNA was precipitated and subsequently washed 2 $\times$ with ethanol and finally resuspended in 50 to 100 μl tris-ethylene-diaminetetraacetic acid (TE) buffer, and stored at -20°C until use.

DGGE analyses. Primer pairs specific for *Eubacteria* (341f and 907r; Muyzer et al. 1993) were used to amplify the 566 bp long 16S rRNA gene fragments. At the 5'-end of each forward primer, an additional 40 bp GC-rich nucleotide sequence (GC-clamp) was added to stabilize migration of the DNA fragments in the DGGE gel (Muyzer et al. 1993). The reaction mixtures for the PCR amplification contained 2 to

5 µl template DNA, 200 nM of each of the appropriate primers, 250 µM of each deoxyribonucleoside triphosphate, 2 mM of MgCl₂, 5 µl of 10× PCR buffer, and 0.5 U of BIOTAQ Red DNA polymerase (Bioline) in a total reaction volume of 50 µl. PCR reactions were performed in a Gradient Cyclor PT-200 (MJ Research) using an initial denaturation step 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 (10 min) and subsequent cooling at 4°C completed the reaction. The amplified DNA was purified using the Nucleo Spin Extract II PCR purification kit (Macherey-Nagel) and stored at –20°C. DGGE was performed in a 7% (v/v) polyacrylamide gel with a denaturing gradient that was modified from 40 to 70% (*Eubacteria*) of urea and formamide (Allgaier et al. 2007). Prior to loading of PCR products onto DGGE gels, DNA was quantified on agarose gels using a quantitative DNA ladder (low DNA mass ladder, Invitrogen). After electrophoresis at 100 V for 18 h, DNA bands were stained with 1× SYBR gold (Molecular Probes) and documented using an Alpha-Imager 2200 transilluminator (Biozym).

Representatives of all bright and well-defined DGGE bands were excised and eluted in sterile water at 4°C overnight. The eluted DNA was re-amplified with primer 341f without the GC-clamp. The PCR products were purified using the NucleoSpin extract kit (Macherey-Nagel), quantified on agarose gels using a quantitative DNA ladder (high DNA mass ladder, Invitrogen), and sequenced using primer 341f together with BigDye Xterminator purification kit (Applied Biosystems). Some bands with identical running behavior were sequenced to determine sequence identity. Sequences obtained with ABI Prism 3100-Avant genetic analyzer (Applied Biosystems) were checked and corrected manually with the program Chromas (version 1.45, Technelysium).

Analyses of DGGE profiles. DGGE banding patterns were analyzed using the software package GelCompar II, version 3.5 (Applied Maths). Within GelCompar II, a band-based binary presence/absence table was calculated according to a similarity index using Dice correlation. The distance among clusters was calculated using UPGMA. A Mantel test using Monte Carlo randomization with 999 randomized runs (PCord, version 5.01) was conducted to compare the similarity matrices acquired.

Phylogenetic analysis. Phylogenetic analyses of the partial 16S rRNA gene sequences were done using the ARB software package (<http://arb-home.de>). The retrieved sequences were imported into an ARB database of 52 000 reference sequences, including the closest related sequences determined by BLAST

(www.ncbi.nlm.nih.gov/BLAST/). Sequences were first aligned automatically by the integrated alignment module within the ARB package, and subsequently corrected manually.

For stability of the phylogenetic tree, a backbone tree was calculated with sequences ≥1400 nucleotides. Consistency of branching patterns was checked by applying 2 phylogenetic reconstruction methods to the appropriate set of sequences, namely neighbor-joining and maximum parsimony. Subsequently, sequences ≤1400 nucleotides were added to the tree according to maximum parsimony criteria. To exclude highly variable positions within the 16S rRNA gene sequences, a 50% base frequency filter for *Eubacteria* was calculated. This filter uses only those sequence positions of the alignment where 50% of the analyzed sequences have identical entries. Thus, the phylogenetic calculations became more robust and potential alignment errors were excluded.

The partial sequences of the 16S rRNA gene fragments obtained in this study were deposited in GenBank with the accession numbers EU432278 to EU432330.

RESULTS

Abundance and dynamics of bacteria

At the beginning of the incubations, total bacterial numbers were 3.4×10^5 cells ml^{–1} for the 0–10 m sample, and 1.03×10^6 cells ml^{–1} for the 40 m sample. Subsequently, bacterial numbers peaked in epilimnic batch cultures (0–10 m) at 4 and 8 wk (6.4×10^5 and 6.5×10^5 cells ml^{–1}, respectively), but dropped to a low at 6 wk (0.37×10^5 cells ml^{–1}) in the 40 m samples (Fig. 2). For dilution cultures, bacterial numbers peaked at 6 wk for both 0–10 and 40 m samples (1.40×10^6 and 1.57×10^6 cells ml^{–1}, respectively; Fig. 2). An increase at 8 wk was also found for *Eubacteria* determined by CARD–FISH analyses, which accounted for up to 80.14 and 93.17% of total bacteria in the 0–10 and 40 m dilution cultures, respectively.

There were no consistent differences in the abundance of *Actinobacteria*, *Alphaproteobacteria*, and *Betaproteobacteria* between bacterial source communities during the 8 wk incubation (data not shown). Respective percentage contributions (±SD) of the 3 major bacterial groups to total bacteria in the 0–10 and 40 m samples (lowest to highest over the entire incubation) were: *Actinobacteria* 0 to $5.2 \pm 1\%$ and 0 to $7.9 \pm 1\%$; *Alphaproteobacteria* 0 to $15.4 \pm 0.8\%$ and 0 to $30.4 \pm 3\%$; *Betaproteobacteria* 5.1 ± 1 to $27.5 \pm 4\%$ and 13.5 ± 2 to $65.8 \pm 8\%$, respectively.

Fig. 2. Changes in total bacterial numbers (+SD) in batch and dilution cultures of water from 2 different bacterial source communities following addition of humic matter (DAPI counts)

Comparison of BCC at 0–10 vs. 40 m

To compare BCC after HM addition, we distinguished the BCC of PA and FL fractions of initial lake water samples (T_0) and compared them with those of samples from batch and dilution cultures using DGGE and cluster analysis (Dice index of DGGE banding patterns). In batch cultures from both the 0–10 and 40 m samples at T_0 , the BCC of FL fractions formed a distinct cluster (Fig. 3a), in contrast to those of the PA fractions. After HM addition, the BCC from both 0–10 and 40 m samples formed distinct clusters including all 4 time points, i.e. weeks 2 to 8. In dilution cultures from both the 0–10 and 40 m samples at time T_0 , the BCC of the FL and PA fractions formed separate clusters (Fig. 3b). After addition of HM, the BCC from the 0–10 m sample showed 2 subclusters depending on incubation time, while the BCC from the 40 m sample showed a single subcluster that varied with time and the BCC tended to be more similar to each other towards the end of the incubation.

Comparison of BCC in batch vs. dilution cultures

A cursory look at the DGGE banding patterns of BCC obtained after 8 wk of incubation from either 0–10 or 40 m samples showed different banding patterns in the batch and dilution cultures (Fig. 4). How-

Fig. 3. Cluster analysis of eubacterial DGGE banding patterns of (A) batch culture and (B) dilution culture. T_0 : start of the experiment; FL: free-living bacteria; PA: particle-associated bacteria

ever, a Mantel test comparing the similarity matrices from batch and dilution cultures (Dice correlation; Fig. 3) showed a positive correlation ($r = 0.556$, $p = 0.001$). Thus, both batch and dilution cultures lead to similar changes in the BCC following HM addition. The single marked difference between batch and dilution approaches was in the relative position of PA in the 0–10 m sample compared to that in the 40 m sample at T_0 (Fig. 3). This was attributable to a difference in total band intensity in the 40 m PA sample in the batch culture (Fig. 4).

NOTE: Errors were found in Fig. 4B after publication. A corrected version is shown here

Fig. 4. DGGE gels of bacterial communities (using primers targeting 16S rRNA genes of Eubacteria) upon addition of humic matter: (A) batch culture, (B) dilution culture. FL: free-living bacteria, PA: particle-associated bacteria in initial samples at time zero (T_0), α : Alphaproteobacteria, β : Betaproteobacteria, γ : Gammaproteobacteria, Bd: Bacteroidetes, Ab: Actinobacteria, C: Cyanobacteria, F: Firmicutes, δ : Deltaproteobacteria. The standard is displayed to the right of each set. Sequenced bands are marked by arrows and the respective band numbers are given in Table A1

Phylogeny of DGGE bands

The DGGE fingerprint patterns of batch and dilution cultures are shown in Fig. 4 and the phylogenetic affiliation of the sequences are given in Table A1. The dominant bands in the initial lake water sample at T_0 represented bacteria belonging to typical freshwater groups (Zwart et al. 2002, Allgaier & Grossart 2006b). FL *Betaproteobacteria* were closely related to uncultured clones L2Sp-25 (*Methylophilus* group) and MWH-IPGL7W22 (*Polynucleobacter* sp.). FL *Actinobacteria* were closely related to uncultured clones

L50W-39 and L50Sp-28, DGGE bands DRC-PA-DG1 and 30g1, and clones L50Sp-17 and L-2-64. PA bacteria were mainly related to a photoautotrophic cyanobacterium, which has a 99% similarity to a diatom chloroplast (Table A1)/chloroplast.

A high variability in the DGGE banding patterns of *Eubacteria* was observed. In addition, the following trends were seen after 8 wk of incubation: (1) a few bacterial phylotypes, which formed major bands in the T_0 sample, continued to be present after HM addition; (2) bacteria related to *Actinobacteria*, which formed major bands in the T_0 sample, declined after HM addition.

tion; and (3) bacterial phylotypes, that were not detected in the T_0 sample exhibited DGGE bands after HM addition. Each of these trends is presented in detail.

(1) *Betaproteobacteria* were common in the T_0 samples and persisted after HM additions (Fig. 4). The maximum percentages indicate the preferential growth of specific *Betaproteobacteria* populations in the presence of HM. Their most prominent representative belongs to the *Polynucleobacter necessarius* cluster (Burkert et al. 2003, Grossart et al. 2008) and is almost identical to isolate MHW-IPGL7W22 (Fig. 4a & Table A1; Wu & Hahn 2006). Its consistent presence in the 0–10 m sample during the entire incubation indicates that this bacterium is favored by HM additions.

Another *Betaproteobacterium*, closely related to clone CbS1s.06 (*Propionivibrio* group), was detected in the HM 0–10 m batch sample and was likely to be present in the T_0 sample (as inferred from the similar banding pattern). This bacterium became increasingly prominent during the 8 wk incubation, indicating that it is able to tolerate and actively grow in the presence of HM.

(2) Several FL *Actinobacteria* were present in the T_0 samples from the lake, but were no longer detected upon HM addition. These bacteria were affiliated to uncultured clones L50W-39 and L50Sp-28 in the 0–10 m samples; and to DGGE bands DRC-PA-DG1 and 30g1, and clones L50Sp-17 and L-2-64 in the 40 m samples. The *Betaproteobacterium* closely related to clone L2Sp-25 (*Methylophilus* group) also disappeared after HM addition.

(3) Some bacteria were not detected in the DGGE banding pattern of the T_0 sample but were detected after HM addition. In the 0–10 m batch sample, a single phylotype, which is an *Alphaproteobacterium* closely related to an uncultured bacterium of the *Roseisalinus* group (DGGE band ESBAC-6), was detected only after HM addition. In contrast, in the 0–10 m dilution sample, multiple bacteria were detected only after HM addition; e.g. several *Betaproteobacteria* related to bacterium S09 (*Herbaspirillum* group), clone CbS1s.22 (*Acidovorax* group), clone YE-28 (*Acidovorax* group), clone A50Su-14 (*Comamonas* group), and clone 268ds10 (*Aquabacterium* group); a *Deltaproteobacterium* related to bacterium K18 (*Anaeromyxobacter* group); and a *Bacteroidetes* related to clone IRD10C04 (*Brumimicrobium* group).

Similarly in the 40 m sample, HM addition in batch cultures revealed *Betaproteobacteria* related to clone TBa-14 (*Janthinobacterium* group), clone Toolik_Jun 2005_Tussock_170 (*Duganella* group), clone YE-28 (*Acidovorax* group), and clone L1A.9A11 (*Rhodoferrax* group). HM addition in dilution cultures showed *Betaproteobacteria* related to clone 5RHF33 (*Duganella*

group), clone A3m (*Acidovorax* group), clone C2.2rn (*Leptothrix* group), clone CbR3s.06 (*Methylophilus* group), and bacterium LH10 (*Acidovorax* group).

Some bacteria occurred only sporadically after HM additions but did not show a consistent pattern, such as *Gammaproteobacteria* related to clone Kas174B (*Gemmatimonas* group) and to clone FTL201 (*Pseudomonas* group), which were detected at 2 and 4 wk after HM addition in the 0–10 and 40 m batch cultures, respectively.

The phylogenetic analysis of all 41 sequences representing the major DGGE bands acquired from both batch and dilution cultures revealed that 18 sequences (44 %) belonged to *Betaproteobacteria*, 12 (29 %) to *Actinobacteria*, 3 (7 %) to *Cyanobacteria*, 2 (5 %) to *Bacteroidetes*, 2 (5 %) to *Alphaproteobacteria*, 2 (5 %) to *Gammaproteobacteria*, 1 (2 %) to *Deltaproteobacteria*, and 1 (2 %) to *Firmicutes*. Although most of the sequences fell into well-established groups, 10 (24 %) had no nearest isolate relative and 8 (20 %) had a similarity of 97 % or less to their nearest isolate relative (Table A1).

Cultivation of bacterial isolates

A total of 11 bacterial isolates was obtained (Table A2), all of which were subsequently screened by PCR with eubacterial primers and used for further analysis. The cultures were identified as members of 3 different phylogenetic groups (Table A2): 4 *Alphaproteobacteria* (36 %), 4 *Betaproteobacteria* (36 %), and 3 *Gammaproteobacteria* (27 %). Several isolates could be easily grown on plates after HM addition using our cultivation approach.

Although the isolates were grown on solid media with the same constituents as used in both batch and dilution cultures, the isolates were different from the major bands in our DGGE gels, but were related to them: *Alphaproteobacteria* were related to bacterium N7 (*Methylobacterium*, ISO 115), clone 28RHF13 (*Devosia*, ISO 117), bacterium MB12 (*Rhodopila*, ISO 117b), and clone P385 (*Gluconacetobacter*, ISO 118); *Betaproteobacteria* were related to CHNCT9 (*Variovorax*, ISO 103b+109), bacterium oxSCC-30 (*Rhodoferrax*, ISO 121), and bacterium S-99 (*Herbaspirillum*, ISO 123); and *Gammaproteobacteria* were related to clone S011D (*Lysobacter*, ISO 110), strain SPS-9, and clone VAS26 (*Pseudomonas*, ISO 113 and ISO 116b).

Inferences on freshwater bacterial phylogeny

The phylogenetic relationships of all sequenced DGGE bands and isolates are given in Fig. 5. Most of the DGGE bands and isolates belonged to various

Fig. 5. Phylogenetic tree of 16S rRNA gene sequences of all DGGE bands and isolates from initial natural water samples of Lake Stechlin and from the batch and dilution cultures with added humic matter. Solid lines indicate sequences that were analyzed using maximum likelihood criteria (sequences of >1400 nucleotides), and broken lines indicate sequences that were added to the tree using maximum parsimony criteria (sequences of <1400 nucleotides). Sequences isolated in the present study are highlighted in grey, and GenBank accession numbers are given in parentheses. B and D: batch and dilution cultures, respectively. Bootstrap values at the main branching points are given. Phylogenetic groupings are highlighted with gray boxes

clusters of *Alphaproteobacteria*, *Bacteroidetes*, *Betaproteobacteria*, *Deltaproteobacteria*, or *Gammaproteobacteria*, which do not belong to specific freshwater clusters. *Actinobacteria*, however, were mainly members of the freshwater cluster *acI-B*. Inferences were based on groupings made by Glöckner et al. (2000), Urbach et al. (2001), Zwart et al. (2002), Warnecke et al. (2004), Gich et al. (2005), Allgaier & Grossart (2006a), and Newton et al. (2006).

DISCUSSION

We observed pronounced differences in the development of BCC following HM addition to epilimnic vs. hypolimnic samples kept at their respective *in situ* temperatures. This suggests that the bacterial source community, in combination with temperature, is important for the establishment of specific bacterial communities following HM input. These results are partially supported by the findings of Langenheder et al. (2005), who demonstrated that medium and inoculum both separately influence BCC in batch cultures. These changes in BCC reflect the presence of 2 types of bacteria: (1) specific bacteria that grow only under selected conditions, and (2) metabolically versatile taxa that quickly adapt to new substrates.

Moreover, our study shows that BCC changes in a similar manner whether kept in batch or dilution cultures. This indicates that single and repeated HM pulses select for a similar bacterial community structure. Thus, BCC is not regulated by selection due to single or repeated HM addition. Instead, the history and taxa of the source community seem to be stronger regulating factors in shaping the BCC (see also Kirchner et al. 2004, Langenheder et al. 2006).

Presence of specific bacteria following HM addition

HM addition to the native BCC of oligotrophic Lake Stechlin selected for a highly specific bacterial commu-

nity. Addition of HM favored the growth of *Betaproteobacteria* such as a member of the *Polynucleobacter* sub-clade identical to isolate MHW-IPGL7W22. Related *Betaproteobacteria* are well adapted to their natural organic matter field in humic Lake Grosse Fuchskuhle (Grossart et al. 2008). For example, several isolates of the *Polynucleobacter* sub-clade were found to grow on low molecular weight substances such as acetate and propionate, which are known to be by-products of photolysis and anaerobic humic matter degradation (Hahn et al. in press). Since we cannot rule out a possible change in the chemical features of our HM concentrate during the isolation procedure, there is a slight chance that HM or its byproducts, such as acetate, may explain the exceptional persistence of this bacterium. Several other *Betaproteobacteria*, including *Acidovorax*, *Herbaspirillum*, and *Methylophilus* species, were also favored by HM addition and are present in a variety of humic lakes (Burkert et al. 2003, Pearce et al. 2005, Newton et al. 2006, Grossart et al. 2008). They represent a major fraction of limnetic bacterioplankton (Métthe et al. 1998, Crump et al. 1999, Zwart et al. 2002) and their abundance is significantly increased by supplementation with HM-rich soil DOM, which correlated well with the total metabolic activity of the bacterioplankton (Perez & Sommaruga 2006).

In contrast, freshwater *Actinobacteria* of the *ac* clade, which is one of the most abundant groups of freshwater bacteria in temperate lakes, decreased in abundance after HM addition, suggesting that most of them are not able to utilize HM or its byproducts. These *Actinobacteria* are an autochthonous component of many lakes, including Lake Stechlin (Warnecke et al. 2005, Allgaier & Grossart 2006a, Newton et al. 2006). We were unable to obtain *Actinobacteria* of the *ac* clade in pure culture; hence, their metabolic capacities still need to be investigated. Their low abundance in our HM cultures, however, may suggest that growth of most *Actinobacteria* of the *ac* clade in lakes is supported by environmental factors other than inorganic nutrients and HM (Allgaier & Grossart 2006a).

Several other bacteria that were detected at different incubation times, but which did not show consistent patterns of growth after HM addition, were *Actinobacteria* (*Frigoribacterium*), *Alphaproteobacteria* (*Methylocystis* and *Roseisalinus*), *Bacteroidetes* (*Brumimicrobium* and *Crocinitomix*), *Betaproteobacteria* (*Aquabacterium*, *Comamonas*, *Duganella*, *Janthinobacterium*, *Leptothrix*, *Propionivibrio*, and *Rhodospirillum*), and *Gammaproteobacteria* (*Gemmatimonas*). Most of these bacteria have been previously found in plankton, lake sediment, groundwater and drinking water (Höfle et al. 1999, Meijer et al. 1999, Jung et al. 2004, Bowman et al. 2005, Labrenz et al. 2005, Loy et

al. 2005, Xiao et al. 2005, Lindner et al. 2007). Their growth may have benefited from changes in pH and the presence of other bacteria and/or their metabolic products in the cultures. It is also possible that their growth is favoured in mixed culture, and thus might be independent of HM itself. These bacteria are presumably able to rapidly adapt to conditions following HM addition or are generalists capable of utilizing a wide range of organic matter including HM and its degradation products.

Cultivation of bacteria after HM addition

HM addition either in batch or dilution cultures can be successfully used for isolation of rare bacteria, such as those related to *Devosia*, *Lysobacter*, *Pseudomonas*, *Rhodopila*, and *Variovorax* species. Our simple isolation approach does not account for important environmental conditions, such as hydrodynamics, protozoan predation, and viral lysis. However, it has the advantage of isolating some potentially important HM-degrading bacteria. Isolates on HM-rich media, such as our *Pseudomonas* isolates, are known to degrade a variety of polymeric and aromatic compounds (Brenner et al. 2005). *Variovorax* species have been previously found after nutrient enrichment of samples from an Antarctic freshwater lake (Pearce et al. 2005). It has been suggested that *Methylobacterium* species can actively utilize HM (Moosvis et al. 2005). Thus, addition of HM via allochthonous supply into Lake Stechlin may select for specific phylotypes that are not necessarily major bacterial phylotypes in the lake, but which are important HM-degrading bacteria. This shows that potentially important HM-degrading bacteria are present in microbial populations even in oligotrophic Lake Stechlin. Their isolation, in addition to controlled community manipulation experiments, could provide more subtle phylogenetic differences and determine ecologically relevant features of these bacteria in the future.

Acknowledgements. We thank E. Mach for technical assistance during sampling and for measurement of dissolved organic carbon. This work was supported by a PhD scholarship to K.M.L.H.S. from the Deutscher Akademischer Austauschdienst (DAAD), by the Leibniz Foundation, and by a course travel grant to K.M.L.H.S. from Boehringer Ingelheim Fonds.

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Table A1. Phylogenetic affiliation of sequenced DGGE (*Eubacteria*) bands in bath and dilution cultures

Band no.	Accession no.	Phylogenetic group	Nearest published relative	Similarity (%)	Accession no.	Nearest isolate relative	Similarity (%)	Accession no.
Batch culture								
5	EU432278	<i>Actinobacteria</i>	Clone L50W-39	99	AJ966208			
6	EU432279	<i>Actinobacteria</i>	Clone L50Sp-28	99	AJ966233	Z34; Gram-positive bacterium	99	AF488669
7	EU432280	<i>Actinobacteria</i>	DGGE gel band DRC-PA-DG1	99	EF088491	Z8; Gram-positive bacterium	99	AF488643
9	EU432281	<i>Cyanobacteria</i>	Clone ES3-64	99	DQ463283	<i>Stephanodiscus minutulus</i> plastid	99	AY221720
13	EU432282	<i>Alphaproteobacteria</i>	DGGE band ESBAC-6	100	AM261466			
14	EU432283	<i>Betaproteobacteria</i>	Clone CbS1s.06	98	EF014671	Mz12; Betaproteobacterium	98	AF531017
15	EU432284	<i>Betaproteobacteria</i>	Clone TaH4	100	AM072347	MWH-IPGL7W22; Betaproteobacterium	100	AJ876403
16	EU432285	<i>Gammaproteobacteria</i>	Clone Kas174B	98	EF203205	<i>Gemmatimonas aurantiaca</i>	97	AB072735
21	EU432286	<i>Bacteroidetes</i>	Clone PRD18E03	99	AY948034			
23	EU432287	<i>Betaproteobacteria</i>	Clone TBa-14	96	DQ791335	ZS20-3; <i>Janthinobacterium</i> sp.	96	EF471898
24	EU432288	<i>Betaproteobacteria</i>	Clone Toolik_Jun2005_Tussock_170	98	DQ510499	RCP_C1; <i>Zoogloea ramigera</i>	97	DQ922742
25	EU432289	<i>Betaproteobacteria</i>	Clone YE-28	100	EF419228	RBE2CD-120; Betaproteobacterium	100	EF111258
27	EU432290	<i>Betaproteobacteria</i>	Clone L1A.9A11	100	AY989166	R-7724; Antarctic bacterium	100	AJ440986
28	EU432291	<i>Gammaproteobacteria</i>	Clone FTL201	99	AF529093	cse024; Rape rhizosphere bacterium	99	AJ295357
29	EU432292	<i>Alphaproteobacteria</i>	Clone AKYH1402	99	AY922106	KS12; <i>Methylocystis</i> sp.	98	AJ458494
30	EU432293	<i>Cyanobacteria</i>	P140; <i>Aulacoseira ambigua</i> chloroplast	99	AJ536463	<i>Haslea wawrikae</i> chloroplast	97	AF514855
32	EU432295	<i>Actinobacteria</i>	P38.3; Uncultured lake bacterium	99	AY752085	Bacterium rJ7	93	AB021325
Dilution culture								
1	EU432296	<i>Betaproteobacteria</i>	Clone L2Sp-25	100	AJ966145	wo51; Betaproteobacterium	100	AF530995
5	EU432297	<i>Actinobacteria</i>	DGGE band 30g1	99	EF016571			
6	EU432298	<i>Actinobacteria</i>	Clone L50Sp-28	100	AJ966233	Z34; Gram-positive bacterium	99	AF488669
7	EU432299	<i>Actinobacteria</i>	Clone L50Sp-17	99	AJ966223	Z8; Gram-positive bacterium	99	AF488643
8	EU432300	<i>Actinobacteria</i>	Clone L-2-64	100	DQ675460			
9	EU432301	<i>Betaproteobacteria</i>	S06; <i>Herbaspirillum</i> sp.	99	DQ387439	S06; <i>Herbaspirillum</i> sp.	99	DQ387439
12	EU432302	<i>Betaproteobacteria</i>	Clone CbS1s.22	100	EF014687			
13	EU432303	<i>Betaproteobacteria</i>	Clone TaH4	99	AM072347	MWH-IPGL7W22; Betaproteobacterium	99	AJ876403
15	EU432304	<i>Betaproteobacteria</i>	Clone YE-28	99	EF419228	RBE2CD-120; Betaproteobacterium	99	EF111258
16	EU432305	<i>Betaproteobacteria</i>	Clone A50Su-14	98	AJ965856	CHX; <i>Brachymonas petroleovorans</i>	97	AY275432
17	EU432306	<i>Betaproteobacteria</i>	Clone 268ds10	99	AY212718	HTCC303; Betaproteobacterium	99	AY429685
18	EU432307	<i>Deltaproteobacteria</i>	K18; Deltaproteobacterium	96	AY146669	K18; Deltaproteobacterium	96	AY146669
19	EU432308	<i>Bacteroidetes</i>	Clone IRD18C04	100	AY947919			
23	EU432309	<i>Actinobacteria</i>	DGGE band 29g1	100	EF016569			
24	EU432310	<i>Actinobacteria</i>	Clone L2Sp-24	99	AJ966144			
25	EU432311	<i>Actinobacteria</i>	Clone L50Sp-28	100	AJ966233	Z34; Gram-positive bacterium	99	AF488669
26	EU432312	<i>Actinobacteria</i>	Clone L50Sp-17	100	AJ966223	Z8; Gram-positive bacterium	100	AF488643
27	EU432313	<i>Firmicutes</i>	Clone L-2-64	100	DQ675460			
28	EU432314	<i>Cyanobacteria</i>	P140; <i>Aulacoseira ambigua</i> chloroplast	99	AJ536463	<i>Haslea wawrikae</i> chloroplast	98	AF514855
29	EU432315	<i>Betaproteobacteria</i>	Clone 5RHF33	99	AJ863414	Wuba73; Betaproteobacterium	99	AF336362
30	EU432316	<i>Betaproteobacteria</i>	Clone A3m	100	AM421649	Strain LI2-55; Betaproteobacterium	99	AJ964892
32	EU432317	<i>Betaproteobacteria</i>	Clone C2.2m	99	AM421622	MG68; <i>Aquabacterium</i> sp.	98	AJ746125
33	EU432318	<i>Betaproteobacteria</i>	Clone CbR3s.06	96	EF014645	R1-B20; Aquatic bacterium	96	AB195752
35	EU432319	<i>Betaproteobacteria</i>	LH10; Betaproteobacterium	100	DQ535024	LH10; Betaproteobacterium	100	DQ535024

Table A2. Phylogenetic affiliation of sequenced isolates

Isolate no.	Accession no.	Source	Phylogenetic group (Proteobacteria)	Nearest published relative	Similarity (%)	Accession no.	Nearest isolate relative	Similarity (%)	Accession no.
103b	EU432320	0–10 m	Beta	CHNCT9; <i>Variovorax</i> sp.	99	EF471221	CHNCT9; <i>Variovorax</i> sp.	99	EF471221
109	EU432321	0–10 m	Beta	CHNCT9; <i>Variovorax</i> sp.	100	EF471221	CHNCT9; <i>Variovorax</i> sp.	100	EF471221
110	EU432322	40 m	Gamma	Clone S011D	100	AM158337	<i>Lysobacter ginsengisoli</i>	99	AB245363
113	EU432323	0–10 m	Gamma	SPS-9; <i>Pseudomonas</i> sp.	99	AM689989	SPS-9; <i>Pseudomonas</i> sp.	99	AM689989
115	EU432324	0–10 m	Alpha	N7; <i>Methylobacterium</i> sp.	99	EF423366	N7; <i>Methylobacterium</i> sp.	99	EF423366
116b	EU432325	0–10 m	Gamma	Clone RX39	100	AM398417	PSA A4(4); <i>Pseudomonas</i> sp.	100	DQ628969
117	EU432326	0–10 m	Alpha	Clone 28RHF13	100	AJ863374	DS-56; <i>Pseudodoevosia insulae</i>	100	EF012357
117b	EU432327	0–10 m	Alpha	MB12; Drinking water bacterium	100	AY328843	MB12; Drinking water bacterium	100	AY328843
118	EU432328	0–10 m	Alpha	Clone P385	98	DQ263521	Bacterium Ellin5134	98	AY234551
121	EU432329	40 m	Beta	oxSCC-30; Unidentified bacterium	99	AJ387873	FXS33; Glacier bacterium	99	AY315176
123	EU432330	40 m	Beta	S-99; <i>Herminiimonas fonticola</i>	100	DQ011678	S-99; <i>Herminiimonas fonticola</i>	100	DQ011678

Fig. A1. Dendrogram of DGGE banding patterns of the preliminary test: effects of humic matter (HM) addition and incubation on bacterial community composition (BCC). T₀: start of the experiment