Impact of flooding on soil bacterial communities associated with poplar (Populus sp.) trees

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Abstract

Soil bacterial communities were analyzed in different habitats (bulk soil, rhizosphere, rhizoplane) of poplar tree microcosms (Populus tremula × P. alba) using cultivation-independent methods. The roots of poplar trees regularly experience flooded and anoxic conditions. Therefore, we also determined the effect of flooding on microbial communities in microcosm experiments. Total community DNA was extracted and bacterial 16S rRNA genes were amplified by PCR and analyzed by terminal restriction fragment length polymorphism (T-RFLP) analysis, cloning and sequencing. Clone libraries were created from all three habitats under both unflooded and flooded conditions resulting in a total of 281 sequences. Numbers of different sequences (<97% similarity) in the different habitats represented 16–55% of total bacterial species richness determined from the nonparametric richness estimator Chao1. According to the number of different terminal restriction fragments (T-RFs), all of the different habitats contained approximately 20 different operational taxonomic units (OTUs), except the flooded rhizoplane habitat whose community contained less OTUs. Results of cloning and T-RFLP analysis generally supported each other. Correspondence analysis of T-RFLP patterns showed that the bacterial communities were different in bulk soil, rhizosphere and rhizoplane and changed upon flooding. For example OTUs representing Bacillus sp. were highest in the unflooded bulk soil and rhizosphere. Sequences related to Aquaspirillum, in contrast, were predominant on the poplar roots and in the rhizosphere of flooded microcosms but were rarely found in the other habitats.

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1. Introduction

Soil microbial community structure is affected by plant roots. The rhizosphere is the soil compartment adjacent to and influenced by roots. In the vicinity of roots, growth of microbial populations is enhanced by root exudates such as amino acids, sugars and growth factors [1–3]. In addition, variation in the composition of organic matter in soil affects bacterial diversity [4,5], which is also influenced by various soil physicochemical factors [6,7], soil aggregate size [8] and soil type [9,10]. Consequently, the different soil compartments “bulk soil” (with roots and shoot residue removed), “rhizosphere” (soil influenced by roots), and “rhizoplane” (root surface including endorhizosphere) may be considered as distinct microbial habitats [1,4]. In practice, however, these different compartments are largely defined by the preparation technique. In situ, they merge smoothly from one into the other (e.g. [11]). Furthermore, soil and rhizosphere bacterial community composition apparently varies between different plant species such as annual plants [3,12–14] and trees, i.e. between...
larch and spruce [15], and between pine, spruce and birch [16].

Previous studies have shown that specific bacterial populations are associated with poplar trees. Germaine et al. [17] found a tight association of bacterial poplar tree endophytes (Pseudomonas spp.) with their host plants. Enterobacter and Clostridium species were found in sapwood samples from poplar stems [18]. Recently, a novel aerobic, facultatively methylotrophic bacterium (Methyllobacterium populi) was isolated from poplar trees [19].

Poplar (Populus sp.) is a fast growing soft-wood deciduous tree, which can adapt to anoxic soil conditions caused by flooding. Submerging the root system results in activation of alcoholic fermentation in the roots and leads to increased concentrations of ethanol in the xylem sap, which is transported to the leaves [20,21]. There, ethanol is oxidized under aerobic conditions and partially released as acetaldehyde, but also used for gluconeogenesis [22–24]. The advantage of this adaptation is avoidance of self-poisoning of roots caused by fermentation products such as ethanol [25]. Under flooded conditions the soil resembles an anaerobic freshwater habitat, where the main pathways for mineralisation of organic matter depend on the operation of denitrification, ferric iron reduction, sulfate reduction or methanogenesis [26–29].

To gain insight into the structure of the microbial communities associated with the rhizosphere of poplar trees and the potential community shifts caused by flooding, we used a molecular approach of cloning and fingerprinting of nearly complete 16S rRNA gene fragments. The analysis of terminal restriction fragment length polymorphism (T-RFLP) [30,31] has been shown to be a useful culture-independent tool for studying the complex microbial community composition in soil [32–35] and rhizosphere [4]. We investigated the community structure of Bacteria in three habitats of poplar tree soil microcosms (bulk soil, rhizosphere and rhizoplane) and its response to flooding, using T-RFLP fingerprinting analysis in combination with comparative sequence analysis of cloned environmental 16S rRNA genes.

2. Materials and methods

2.1. Soil samples and poplar tree microcosms

Soil was collected in the year 2000 from a field adjacent to the river Rhine (Kilometer 278.3 to 283.1) at the Polder Altenheim near Offenburg, Germany (Gewässerdirektion Südflicher Oberrhein/Hochrhein, 2000). Soil samples were air-dried and stored at room temperature. The soil was classified as a loam with 40% sand, 45% silt and 15% clay (Analytical Laboratory of the Georg-August University, Faculty of Forest Sciences and Forest Ecology, Göttingen, Germany). The total carbon and total nitrogen contents in the poplar tree microcosms prior to flooding were 5.60% and 0.34%, respectively, as determined with an elemental analyzer (Analytical Chemical Laboratory of the Philipps University, Marburg, Germany). Hybrid poplar trees (Populus tremula × P. alba) were micropropagated and grown in a greenhouse in Freiburg according to Kreuzwieser et al. [23,24]. In order to prevent growth of pathogenic fungi, the shoots of the trees were treated monthly with a fungicide (“Netz-Schweifeli”, Neudorff, Freiburg, Germany). Microcosms were constructed using 13-week-old poplar trees and soil as described by Kreuzwieser et al. [23,24] and incubated in the greenhouse for three weeks. Then, part of the microcosms was flooded with tap water to a level high enough to submerge the root system, and incubation was continued for a further 3 weeks, by which time the unflooded and flooded hybrid poplar trees had reached heights of 50 and 30 cm, respectively.

From two flooded and three unflooded poplar tree microcosms, fractions of bulk soil, rhizosphere and rhizoplane were analyzed. Bulk soil samples were taken from the microcosms at a depth of 2–3 cm containing no visible roots. Rhizoplane samples were prepared by gently washing roots with demineralized water, to remove soil particles defined as rhizosphere samples. Roots were washed further with PBS-buffer (137 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 1.7 mM NaH2PO4 [pH 7.2]) to remove adhering soil particles and were cut into 5–10 mm sized pieces (rhizoplane samples including endorhizosphere). Samples were transported to the laboratory at 4 °C, and were subsequently stored at −20 °C.

2.2. Extraction of DNA and PCR amplification of bacterial 16S rRNA genes

From each replicate microcosm, DNA was extracted in triplicate from bulk soil, rhizosphere and rhizoplane samples as described previously [36]. DNA extracts were further purified using polyvinyl-polypyrrolidone spin columns [37] for bulk soil samples, which contained relatively large amounts of humic material, or using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) for rhizosphere and rhizoplane samples, which were less contaminated.

16S rRNA genes were amplified using the Bacteria-specific primers 27f and 1492/1512r [38]. The reaction mixture (100 μl) contained 1 μl template DNA, 10 μl 10-fold reaction buffer (Invitrogen GmbH, Karlsruhe, Germany), 4 mM MgCl2, 200 μM of each deoxynucleoside triphosphate (AGS, Heidelberg, Germany), 0.3 μM of each primer (MWG Biotech, Ebersberg, Germany) and 1.25 U of Taq DNA-Polymerase (Taq DNA Polymerase recombinant (5 U μl−1), Invitrogen GmbH, Kar-
lsruhe, Germany). For T-RFLP analyses, the reverse primer was labeled at the 5′-terminal with 6-carboxyfluorescein (MWG Biotech, Ebersberg, Germany). For amplification the following protocol was used in the preheated (94 °C) thermal block of a Primus Cycler (MWG Biotech, Ebersberg, Germany): initial denaturation (94 °C for 2 min) followed by 18–22 (cloned PCR product templates) or 30–35 (environmental DNA templates) cycles of denaturation (94 °C for 30 s), annealing (52 °C for 30 s), and elongation (72 °C for 60 s). After terminal elongation (72 °C for 5–7 min), samples were stored at 8 °C until further analysis. PCR products (5 μl) were visualized by standard agarose gel electrophoresis. Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA concentrations were measured by absorption at 260 nm with a photometer (Eppendorf AG, Hamburg, Germany).

2.3. T-RFLP-analysis

16S rRNA gene-based fingerprint patterns of environmental samples were generated by T-RFLP analysis [32,39]. DNA was extracted from different habitats of the poplar tree microcosms and fluorescently labeled PCR products were generated according to the above PCR protocol. Aliquots of purified PCR products (60 ng for environmental DNA amplicons; 30 ng for clonal amplicons), 9 U of restriction enzyme HhaI (Promega, Mannheim, Germany), 1 μl of the appropriate 10× incubation buffer, and 1 μg of bovine serum albumin were combined in a total volume of 10 μl and digested for 3 h at 37 °C. Aliquots (2.5 μl) of the digested amplicons were mixed with 1.7 μl formamide loading dye (Amersham Biosciences, Freiburg, Germany) and 0.8 μl of the internal GeneScan-1000 (ROX) size standard (Applied Biosystems, Weiterstadt, Germany), denatured for 3 min at 95 °C, and immediately placed on ice. T-RFLP analyses were performed on an automated DNA sequencer (373 DNA sequencer, Applied Biosystems, Weiterstadt, Germany) in GeneScan mode as described by Chin et al. [32]. T-RFLP profiles were analyzed with GeneScan 2.1 Software (Applied Biosystems, Weiterstadt, Germany). Terminal restriction fragments (T-RFs) were quantified by peak height using a minimum peak height analysis threshold of 50 relative fluorescence units followed by standardization of DNA quantity [40]. The relative abundance of a detected T-RF within a given T-RFLP pattern was calculated as the respective signal height of this peak divided by the total peak heights of all peaks of the T-RFLP pattern starting from a fragment size of 56 bp to exclude T-RFs caused by primer dimers. T-RFs larger than 928 bp were grouped together. The T-RF representing the chloroplast rRNA gene (i.e. 777 bp) was disregarded. Analysis was performed for each triplicate DNA extract. T-RFs were considered to be identical if their fragment sizes varied in a range of ±1–2 bp among different gels and/or lanes of the same gel and were summarized as operational taxonomic units (OTUs). Ratios were converted to percentages and the results displayed as histograms. The standard error in most cases was less than ±5% of the mean using triplicate DNA extractions. Besides in silico determination of the expected size of the T-RFs, all clones (see below) were tested for their in vitro T-RF pattern in order to detect those showing pseudo-T-RF formation [41].

The overall structures of bacterial communities among the different habitats (bulk soil, rhizosphere, rhizoplane) and treatments (with and without flooding) were compared by analyzing 42 16S rRNA gene-based T-RFLP patterns using correspondence analysis (CA, a multivariate method). The results were visualized in an ordination diagram (CANOCO 4.0 software, Microcomputer Power, Inc., Ithaca, NY, USA). The effect of flooding on numbers of T-RFs was analyzed by one-way ANOVA using the SPSS software (SPSS Inc., Chicago, USA).

2.4. Cloning and sequencing

Bacterial 16S rRNA gene amplicons obtained from the different microcosms and soil compartments were cloned in Escherichia coli JM 109 using the pGEM-T Vector System II cloning kit (Promega, Madison, WI, USA). Clones were selected randomly and checked for correct insert size by vector-targeted PCR and agarose gel electrophoresis. Sequencing was performed with the Big Dye™ Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Weiterstadt, Germany) and primers 343-357f, 536-519b, 1115-1100b, 1512-1492b described by Lane [42] and Weisburg et al. [38] on an automated DNA sequencer (377 DNA Sequencer, Applied Biosystems, Weiterstadt, Germany) as specified by the manufacturer. Bacterial clones were designated BSU (bulk soil unflooded; 55 clones), BSF (bulk soil flooded; 63 clones), RHU (rhizosphere unflooded; 50 clones), RHF (rhizosphere flooded; 78 clones) and RPF (rhizoplane flooded; 35 clones).

2.5. Sequence data and phylogenetic analysis

Sequence data were assembled and checked with the Lasergene software package (DNASTAR, Madison, USA). 16S rRNA gene sequences (~1000 or 1400 bp) were compared with a BLAST search to sequences of the EMBL database (www.ebi.ac.uk). Sequence alignment (Fast Aligner tool version 1.03), calculation of distance matrices and construction of phylogenetic trees were accomplished with the ARB software package (version Linux Beta 030822; http://www.arb-home.de; [43]). Sequences closely related to the cloned 16S rRNA gene
sequences were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank) and integrated into the 16S rRNA gene database (released June 2002, ARB). The terminal sequence positions at the 5' and 3' ends of the 16S rRNA gene sequences (500 bp for partial sequences and 750 bp for full length sequences) were also subjected to a separate treeing analysis (“fractional treeing” [44]) to identify chimeric sequences. Differences in the phylogenetic placement of a fragment pair were considered indicative of chimera formation and such clones were excluded from further analysis. Distance matrices without filter were calculated to estimate clone sequence similarities.

Trees were calculated using the maximum-likelihood based method FastDNAml [43] and Parsimony (PHY-LIP software package (version 3.6a2; J. Felsenstein, Department of Genetics, University of Washington, Seattle, available at http://evolution.genetics.washington.edu/phylip.html). For confirmation of tree topologies, trees were also calculated using the neighbor joining method (PHYLIP, [45]) with the evolutionary model of Felsenstein [46] and using Tree-Puzzle [47] with the Tamura-Nei evolutionary model [48]. Highly variable regions within the 16S rRNA gene were excluded from the analysis by application of 50% base frequency filters. These were based on the sequence dataset of the respective phylogram and resulted in 1309 nucleotide positions. Clone sequences significantly shorter than 1000 bp were added to the tree by using the ARB parsimony tool, which allows the addition of short sequences to phylogenetic trees without changing global tree topology [49].

Bacterial species richness in the clone libraries was estimated using Chao1 [50] as a nonparametric indicator, calculated with EstimateS (version 5.0.1; R. Colwell, University of Connecticut [http://viceroy.eeb.uconn.edu/estimates]) as described by Hughes et al. [51]. For this purpose, a “species” was defined as a group of 16S rRNA gene clones with $\geq 97\%$ sequence similarity [52].

The 16S rRNA gene clone sequences generated in this study were deposited in the EMBL-database under accession numbers AJ863173-AJ863227 (bulk soil unflooded: 55 clones), AJ863228-AJ863290 (bulk soil flooded: 63 clones), AJ863291-AJ863340 (rhizosphere unflooded: 50 clones), AJ863341-AJ863418 (rhizosphere flooded: 78 clones) and AJ863419-AJ863453 (rhizoplane flooded: 35 clones).

3. Results

3.1. T-RFLP-analyses

In order to assess the structure of the bacterial communities in the different habitats (bulk soil, rhizosphere, rhizoplane including endorhizosphere) of flooded and unflooded microcosms of poplar trees, T-RFLP-analysis of bacterial 16S rRNA gene fragments was carried out. In order to compare T-RFLPs, the relative 16S rRNA gene frequencies of individual T-RFs within samples were calculated and displayed as histograms (Fig. 1). In each column the average results of three replicate DNA extractions are displayed. Different columns represent T-RFLP analyses of independent microcosms and different compartments under different conditions, respectively. In total, we detected 43 different T-RFs in 42 bacterial T-RFLP patterns generated by the restriction enzyme HhaI. Approximately 18–25 T-RFs were detected per sample from bulk soil, rhizosphere and rhizoplane (unflooded) (Fig. 1). A T-RF of 777 bp, which was characteristic for chloroplasts, constituted $<0.5\%$ of the relative gene frequency in the different compartments, except in the unflooded and flooded rhizoplane, where it made up about 50% and 15%, respectively. This T-RF was disregarded in further analysis. The number of the different T-RFs did not change significantly ($p > 0.05$) under flooded compared to unflooded conditions in bulk soil and rhizosphere samples. However, the number of T-RFs obtained from the rhizoplane under flooded conditions was significantly ($p < 0.01$) lower (Fig. 1). Bulk soil, rhizosphere and rhizoplane compartments of poplar tree microcosms were clearly distinct based on the relative abundance of T-RFs, although most T-RFs were found to be shared between the different compartments under different conditions. T-RFs with a fragment size of 81 bp (1.3–11.5%), 206 bp (1.1–69.7%), and 340 bp (0.6–6.2%) were detected in all compartments (Fig. 1). In contrast, some fragments were unique to a particular compartment. The T-RFs of 473 bp (1.9–19.4%) and 579 bp (1.6–23.2%) were only found in bulk soil and decreased in abundance after flooding, whereas the T-RF of 294 bp (1.8–3.6%) was exclusively observed in the rhizosphere compartment, and the T-RFs of 640 bp (0.7–11.0%) and 720 bp (0.8–1.1%) only occurred in the rhizoplane habitat (Fig. 1). The strongest change was observed in the flooded rhizoplane compartment, where the T-RF of 206 bp became predominant (Fig. 1). In general, however, differences in T-RFLP-patterns were due to differences in relative abundance rather than the presence or absence of individual T-RFs.

In order to determine the differences between the investigated compartments and conditions, a multivariate statistical analysis (CA: correspondence analysis) of all bacterial OTU frequencies was applied and resulted in two discriminant axes (Fig. 2). CA considers two parameters, the number of T-RFs present and their relative abundance. The closer the scattered data points are to each other, the more similar the bacterial communities are with respect to T-RF composition and abundance. Without flooding, T-RFLP-patterns of bulk soil, rhizosphere and rhizoplane were significantly sepa-
rated from each other (Fig. 2). Flooding showed an effect on all three habitats, which was greatest on the rhizoplane compartment. Flooding decreased the difference in bacterial community structure between the flooded rhizosphere and rhizoplane habitats. T-RFLP-patterns obtained from different plants were similar and clustered tightly together. Twenty eight OTUs showing a relative abundance >14% are displayed in the CA diagram (Fig. 2). Ten OTUs were represented by predominant T-RFs with relative abundances >4% (72, 81, 99, 200, 206, 238, 473, 567, 579 and 640 bp). These OTUs were responsible for separation of the habitats, i.e. bulk soil, rhizosphere and rhizoplane, and indicated the effect of flooding (Fig. 2). The T-RFs of 473 and 579 bp were dominant in the unflooded bulk soil habitat. The unflooded rhizosphere compartment was characterized by the occurrence of T-RFs of 81 and 238 bp, whereas the flooded rhizoplane habitat was clearly influenced by the T-RFs 206 and 567 bp. T-RFs with the fragment length 72, 99, 200 and 640 bp were relevant in the unflooded rhizoplane habitat.

T-RFLP-analysis of the clones allowed assignment of the individual T-RFs to distinct phylogenetic groups (Table 1). The T-RF of 36 bp, representing members of Chloroflexi, Actinobacteria or Nitrospira, was not taken into account, since this T-RF peak could not be discriminated from primer dimers in the T-RFLP-analysis. Seven T-RFs indicated in Fig. 1 could not be assigned to a phylogenetic group, but the remainder (36 out of 43) could be assigned. Several T-RFs shared more than one phylogenetic group (Table 1). In addition to in silico determination of the expected size of the T-RFs, all clones were also tested for their in vitro T-RF formation pattern, in order to detect those showing pseudo-T-RF formation.
Six separate clone libraries of bacterial 16S rRNA genes were amplified from bulk soil (Δ), rhizosphere (□) and rhizoplane (○) from poplar tree microcosms under flooded (closed symbols) and unflooded (open symbols) conditions. Means of triplicate DNA-extractions are displayed. The eigenvalues of the first and second axes in the two-dimensional ordination diagram are as follows: \( \lambda_1 = 0.59 \), \( \lambda_2 = 0.50 \). Different colors represent different plants. Numbers indicate the length [bp] of T-RFs (asterisks) with relative abundance >4% (open asterisks) or >10% (closed asterisks).

Pseudo-T-RFs are detectable as additional, fluorescently labeled fragments in T-RFLP-analysis and must be taken into account when interpreting community T-RFLP-patterns. In fact, 29\% of a total of 281 clones showed formation of pseudo-T-RFs.

### 3.2. 16S rRNA gene clone libraries

Six separate clone libraries of bacterial 16S rRNA gene fragments were constructed, i.e. from bulk soil, rhizosphere and rhizoplane DNA. Samples were taken from poplar tree microcosms under flooded and unflooded conditions. Randomly selected clones were analyzed by T-RFLP and were sequenced. This allowed the assignment of clone sequences to particular T-RFs. Only clones affiliated with plastid-DNA were detected in the unflooded rhizoplane clone library (n = 31), indicating co-extraction of plant-DNA. These clones were excluded from further analysis. In the flooded rhizoplane clone library, on the other hand, only 5\% of the clone sequences were affiliated with plastid-DNA. In a total of 314 bacterial clones analyzed, 33 chimeric sequences were detected and excluded from further analysis. The remaining 281 clone sequences from bulk soil unflooded (BSU: 55 clones), bulk soil flooded (BSF: 63 clones), rhizosphere unflooded (RHU: 50 clones), rhizosphere flooded (RHF: 78 clones) and rhizoplane flooded (RPF: 35 clones) were affiliated with distinct phylogenetic groups within the Bacteria (Table 1). The clone libraries revealed differences in the relative bacterial population structures, which, however, could not be tested statistically, since the clone libraries were not replicated. Most clones of the unflooded bulk soil library were affiliated with Actinobacteria (38\%), followed by clones grouping within the Bacillales (16\%) and Acidobacteria (15\%). Under flooded conditions there was a relative increase in clones affiliated with Alphaproteobacteria and Gammaproteobacteria. Clones related to members of the Bacillales (42\%) and the Alphaproteobacteria (26\%) dominated the rhizosphere clone library. Flooding led to a relative increase in clones clustering with Betaproteobacteria (47\%). All clones from the library of flooded rhizoplane were affiliated with Betaproteobacteria, most of them were related to members of Aquaspirillum species. In total, most clones (50\%) were affiliated with Proteobacteria (Alpha, Beta, Gamma, Delta), followed by clones related to Bacillales, Actinobacteria, and Acidobacteria. Almost all phylogenetic groups in the clone libraries were represented by T-RFs in the T-RFLP analyses of environmental DNA. The clones (a total of 18), of which T-RFs (12 different ones) were not detected in the T-RFLP analyses, are marked in Table 1. Following the phylogenetic analysis presented in Table 1, 114 representative cloned sequences were selected for detailed phylogenetic tree construction, to give an approximate description of the species richness associated with poplar trees (Figs. 3 and 4). The selected clones represented the different phylogenetic groups and exhibited a relative T-RF abundance >4% in the environmental samples.

### 3.3. Phylotype richness

Using the data from the clone libraries, sequences with \( \geq 97\% \) sequence similarities were arbitrarily defined as belonging to the same species. The 16S rRNA gene clones in the bulk soil and rhizosphere libraries under unflooded conditions were grouped into 42 and 32 different species, respectively (Table 2). The total species richness of the bacterial 16S rRNA gene clone libraries was calculated using the nonparametric richness estimator Chao1 [51]. The extrapolated numbers were higher than the observed numbers of species, indicating a large undetected bacterial diversity. Nevertheless, about one-third of the bacterial species present in the unflooded rhizosphere of poplar tree microcosms was apparently detected using the cloning approach. The species richness in the bulk soil was obviously higher than in the rhizosphere since the corresponding 95\% confidence intervals indicated higher species richness than in the rhizosphere (Table 2). Under flooded conditions the libraries from bulk soil, rhizosphere and rhizoplane contained 52, 33 or 6 species, respectively. The extrapolated numbers of species were also higher than those detected experimentally (Table 2). Half of the bacterial community present at the poplar tree rhizoplane under flooded conditions was detected and species richness can be regarded as being much lower than in the other compartments.
Table 1
Distribution of major phylogenetic groups in the bulk soil, rhizosphere and rhizoplane compartments of poplar tree (Populus tremula × P. alba) microcosms under unflooded and flooded conditions, based on the frequencies (n) of 16S rRNA genes in 16S rRNA gene clone libraries and their affiliation to distinct terminal restriction fragments (T-RFs)

<table>
<thead>
<tr>
<th>Phylogenetic group</th>
<th>Contribution of 16S rRNA gene clones in:</th>
<th>Bulk soil</th>
<th>Rhizosphere</th>
<th>Rhizoplane</th>
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<tbody>
<tr>
<td></td>
<td>Bulk soil Unflooded (n = 55) Flooded (n = 63)</td>
<td>Rhizosphere Unflooded (n = 50) Flooded (n = 78)</td>
<td>Rhizoplane Flooded (n = 35)</td>
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<td>T-RF [bp] (n) T-RF [bp] (n) T-RF [bp] (n) T-RF [bp] (n) T-RF [bp] (n)</td>
<td>T-RF [bp] (n) T-RF [bp] (n)</td>
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<tr>
<td>Unknown affiliation</td>
<td>740 (1) 740* (1)</td>
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<tr>
<td>Bacillales</td>
<td>238 (2) 233 (1)</td>
<td>206 (2) 72 (2)</td>
<td>466 (5) 466 (2)</td>
<td>574 (1)</td>
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<td></td>
<td>243 (1) 238 (4)</td>
<td>226 (1) 233 (3)</td>
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<td></td>
<td>547 (1)</td>
<td>233 (3) 238 (7)</td>
<td>473 (1) 574 (1)</td>
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<td>579 (5)</td>
<td>238 (8)</td>
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<td>360 (1)</td>
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<td>Actinobacteria</td>
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<td>143 (2) 143 (1)</td>
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<td>370 (1) 220 (1)</td>
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<td>184* (2) 370 (1)</td>
<td>673 (2) 370 (1)</td>
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<td>238 (1) 441 (1)</td>
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<td>370 (5) 1079 (1)</td>
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<td>192 (1) 99 (6)</td>
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<td>215 (1) 215 (1)</td>
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<td>352 (1)</td>
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<tr>
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<td>36 (1)</td>
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<td>36 (1)</td>
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<td>352 (1)</td>
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<tr>
<td>Acidobacteria</td>
<td>220 (6) 154 (2)</td>
<td>220 (1)</td>
<td>–</td>
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Fragment lengths are shown in base pairs [bp].

n: number of 16S rRNA gene clones analyzed in different compartments.
–: not detected.
* T-RFs marked with an asterisk were only detected in the clone library, but not in the T-RFLP analysis.
Fig. 3. Phylogenetic tree showing the positions of environmental bacterial 16S rRNA gene sequences affiliated with the *Proteobacteria* (PB), recovered from bulk soil (BS), rhizosphere (RH) and rhizoplane (RP) of flooded (F) and unflooded (U) poplar tree microcosms. The tree was constructed with the maximum likelihood method and a filter (50%) for *Proteobacteria*. Scale bar represents 10% sequence difference. Accession numbers of reference sequences are indicated. 16S rRNA gene sequence of *Tropheryma whippeii* was used as outgroup reference. Lengths of T-RFs I; pseudo-T-RFs are marked with asterisks. Branch points marked with solid circles were supported by at least two further phylogenetic calculation methods, while open circles at branch points indicate confirmation by at least one further calculation method.
Fig. 4. Phylogenetic tree showing the positions of environmental bacterial 16S rRNA gene sequences affiliated within the *Bacteria*, recovered from BS, RH and RP of F and U poplar tree microcosms. The tree was constructed with the maximum likelihood method and a filter (50%) for the sequence dataset. Scale bar represents 10% sequence difference. Accession numbers of reference sequences are indicated. 16S rRNA gene sequence of *Pandoraea norimbergensis* was used as outgroup reference. Lengths of T-RFs result from in silico digestion of clonal 16S rRNA gene sequences with *Hha*I; pseudo-T-RFs are marked with asterisks. Branch points marked with solid circles were supported by at least two further phylogenetic calculation methods, while open circles at branch points indicate confirmation by at least one further calculation method.
4. Discussion

Studies on various ecosystems have shown that soil microbial communities are highly diverse [53–56]. The present study was undertaken to characterize microbial communities in unflooded and flooded poplar tree microcosms using the combination of fingerprinting (T-RFLP) and sequencing of 16S rRNA genes. The bacterial communities were structurally different in the bulk soil, rhizosphere and rhizoplane habitats and changed due to flooding, as shown by the composition of the clone libraries (Table 1), visual apparent differences in the T-RFLP patterns (Fig. 1) and CA of the T-RFLP patterns (Fig. 2).

Unflooded bulk soil was dominated by Arthrobacter sp. (T-RF: 473 bp) and Bacillus sp. (T-RF: 579 bp) (Table 1; Figs. 1 and 2). Mahaffee and Kloepper [11] reported that isolates of these genera were dominant in soil samples from cucumber fields. In addition, Bacillus megaterium and Arthrobacter sp. were found as predominant populations in bulk soils from unplanted field plots [14]. Members of these genera are typical of soil environments and exhibit an aerobic-heterotrophic metabolism [57,58].

The unflooded rhizosphere habitat communities differed from the bulk soil communities (Table 1; Figs. 1 and 2). In comparison with the bulk soil, the T-RF of 238 bp increased. This T-RF also represents members of the Bacillales (Bacillus sp.), but different taxa to the T-RF of 579 bp that was dominant in bulk soil (Fig. 4). In addition, the T-RF of 81 bp, representing Alphaproteobacteria, was prominent in the rhizosphere (Fig. 2). This result was in agreement with the clone library data, where clones affiliated within the Bacillales were dominant followed by clones affiliated within the Alphaproteobacteria exhibiting different T-RFs (Table 1). Bacillus species were also dominant in the rhizosphere of barley [59], chrysanthemum [60] and cycad plants [61], whereas Alphaproteobacteria were most frequently found in the rhizosphere of grass [54]. A T-RF of 294 bp length (1.8–3.6%) that was unique for the rhizosphere habitat (Fig. 1) was also found, representing members affiliated with Flexibacter sancti (data not shown).

Flooding of bulk soil led to a relative decrease in T-RFs representing members of the genera mentioned above (Arthrobacter and certain Bacillus species) and a relative increase of members of the Alphaproteobacteria (T-RFs: 81 and 92 bp), Gammaproteobacteria (T-RF: 212 bp) and Deltaproteobacteria (T-RF: 92 bp) (Figs. 1 and 2; Table 1). Members of these taxonomic groups, except the Gammaproteobacteria, have been observed as dominant populations in an agricultural soil [55]. In CA, flooding generally resulted in a shift along axis 1 to the left, so that the bacterial communities in flooded bulk soil became more similar to those in the unflooded rhizosphere, while those in the flooded rhizosphere became more similar to those in the flooded rhizoplane (Fig. 1). For example, a new T-RF of 466 bp (Paenibacillus sp.) appeared in the rhizosphere that was absent in the other habitats (Fig. 1). Daane et al. [62] described the isolation of spore-forming Paenibacillus sp. [63,64] from the rhizosphere of salt marsh plants. Paenibacillus brasiliensis, a bacterium capable of nitrogen fixation, was isolated from the rhizosphere of maize [65]. In the rhizosphere habitat, the T-RF of 206 bp became more dominant after flooding (Fig. 1) and was also characteristic for the flooded rhizoplane habitat (see below).

All analyses (T-RFLP patterns, clone libraries and CA) revealed that the unflooded rhizoplane habitat was distinct from the bulk soil and rhizosphere habitats. CA showed that the unflooded rhizoplane was influenced by the following T-RFs exhibiting relative abundances higher than 10% each: 72, 99, 200 and 640 bp. The clone library of this habitat showed only environmental sequences indicative of plastid-DNA so that an unambiguous affiliation to bacterial clones was not possible. Chelius and Triplett [66] also observed amplification of mitochondria and chloroplast DNA when analyzing roots of Zea mays. If we nevertheless assume that the clone libraries from the rhizosphere and bulk soil compartments also represented the bacterial community of the rhizoplane, the T-RF of 72 bp could be
affiliated to *Bacillus senegalensis* and the T-RF of 99 bp to *Verrucomicrobium* (Table 1). However, the other two T-RFs (200 and 640 bp) were not represented in any clone library and thus could not be affiliated.

The T-RFLP patterns of the rhizoplane habitat revealed a strong decrease in the number of T-RFs upon flooding, in contrast to the other compartments (Fig. 1). By contrast, the relative abundance of chloroplast T-RF, which was disregarded in the analysis shown in Fig. 1, was higher in the unflooded than the flooded rhizosphere. Therefore, it is unlikely that the coextraction of plastid DNA caused a biased evaluation of the flooding effect on the other T-RFs. In addition, CA showed that the bacterial community shifted and became more similar to that of the flooded rhizosphere (Fig. 2). Marilley et al. [67] also observed that phylogenetic diversity decreased in the proximity of plant roots. In this study, members of the *Betaproteobacteria* dominated the flooded rhizoplane habitat community, represented by the T-RFs of 206 and 567 bp. The clone libraries indicated that the T-RF of 206 bp represented *Betaproteobacteria* and revealed that most of the environmental sequences were most closely affiliated with *Aquaspirillum* sp. within the *Comamonadaceae* [68,69] and with an environmental strain obtained from garden soil [70] (Fig. 3), which was capable of denitrification. Members of the genus *Aquaspirillum* are aerobic to microaerophilic, having a respiratory type of metabolism with oxygen as terminal electron acceptor [71]. A few species can grow anaerobically with nitrate, and some species can denitrify. *Aquaspirillum* species have been isolated from a wide variety of fresh water sources, especially those that are stagnant or contain organic matter. Amino acids or the salts of organic acids are used as carbon source. Some species can catabolize ethanol. However, the genus *Aquaspirillum* is very heterogeneous [72]. Lobakova et al. [61] recently described the isolation of bacteria phylogenetically related to *Aquaspirillum* sp. from the root zone of cyclad plants. Clones related to *Aquaspirillum* were also detected in rice root incubations amended with nitrate [73].

Besides *Aquaspirillum*, the 206 bp T-RF possibly represented other betaproteobacterial genera in this habitat, including *Dechloromonas* sp. and *Rhodococcus* sp. (Fig. 3). Previous studies have shown that *Betaproteobacteria* are common on rice roots [74], and in the tomato rhizoplane [75]. Besides *Alphaproteobacteria*, they were the second most abundant bacterial group in the maize rhizoplane [66]. However, *Betaproteobacteria* are physiologically extremely heterogeneous [76]. Members of the genus *Dechloromonas* are facultative anaerobes [77] and exhibit a respiring metabolism, oxidizing acetate with O₂ and other electron acceptors, e.g. nitrate. Scheid et al. [73] observed increased abundance of *Dechloromonas* species when rice root incubations were amended with nitrate. *Rhodococcus* species are able to grow photolithoautotrophically with hydrogen as electron donor [78]. Without light they are able to grow aerobically to microaerobically, exhibiting an O₂-respirative metabolism under these conditions.

It is known that, in addition to PCR artifacts [79,80], the composition of T-RFLP-patterns can be influenced by biased restriction digestion, in particular by pseudo-T-RFs, which result in overestimation of diversity [81,84]. The T-RF of 567 bp in the flooded rhizoplane and rhizosphere habitats was probably due to pseudo-T-RF formation, since this T-RF was found in none of these clone libraries. Most likely, the T-RF of 567 bp was a pseudo-T-RF of *Aquaspirillum* (T-RF: 206 bp) (Table 1 and Fig. 3).

Another possible bias in the assignment of T-RFs to particular bacterial taxa is due to sharing of the same T-RF by different phylogenetic groups. Depending on the habitat, the T-RF of 206 bp may have represented members of the *Betaproteobacteria* (all compartments), the *Gammaproteobacteria* (bulk soil), or the *Baccillales* (unflooded rhizosphere) (Table 1). Hence, the preferential assignment of this T-RF to *Aquaspirillum*, or *Betaproteobacteria* in general, is to some extent equivocal. The T-RF of 66 bp, for example, also was ambiguous, and represented members of both the *Betaproteobacteria* and *Gammaproteobacteria* (Table 1). In contrast, clones with different T-RFs had sequences that were nearly identical, for example clone 17RHF1 (T-RF: 238 bp) and clone 28RHF50 (T-RF: 72 bp) both within the *Baccillales* (Fig. 4). This is also a feature that is frequently encountered in RFLP analysis of environmental samples [82,83].

Although most T-RFs were shared between different compartments and conditions, T-RFLP-patterns were sufficiently different to distinguish the different habitats. In a previous study of bacterial communities associated with field-grown cucumber Mahaffee and Kloepper [11] also observed that communities differed between different habitats. Several reasons may account for the differences in the microbial community structures of bulk soil, rhizosphere, and rhizoplane and the differences between flooded and unflooded conditions. Soil microorganisms differ in their potential to use carbon and electron sources. The diversity of microorganisms in the rhizosphere is influenced by the plant roots and affected by the plant species due to variations in root exudates produced [84,85]. Consequently, different rhizosphere microbial communities are associated with different plants and it has been suggested that plants select for divergent communities on their roots [3,12]. Furthermore, soil type [12,9], soil aggregate size, growth stage, carbon resource heterogeneity [82], and other environmental factors may also influence the microbial community composition in these habitats.

Upon flooding, the T-RFLP-patterns in all compartments changed. Due to the restricted entry of
microbial community. Bossio and Scow [86] reported that flooding caused shifts in the diversity of metabolically active microorganisms using BIOLOG microplates. Flooding also led to a reduction in the availability of suitable electron acceptors resulting in lower rates of metabolically active microorganisms using BIOLOG microplates. Bossio and Scow [86] reported that flooding caused shifts in the diversity of metabolically active microorganisms using BIOLOG microplates. Flooding also led to a reduction in the availability of suitable electron acceptors resulting in lower rates of metabolic potential of theses microbes and their importance to the soil poplar-ecosystem.

As a mechanism to tolerate hypoxic conditions, roots of poplar trees accumulate ethanol, which is transported to the leaves via the xylem sap [20,21] and emitted there under aerobic conditions as acetaldehyde [22–24]. We assume that ethanol was excreted under flooding conditions to a certain extent and may have caused the development of distinct microbial communities. Our results suggest that poplar roots support such a distinct bacterial community. The large number of clone sequences affiliated with Aquaspirillum sp. indicates that this is an important member of the bacterial community of the poplar rhizosphere and rhizosphere under flooded conditions. We conclude that the microbial community present in poplar tree microcosms is complex and dynamic, varying in composition between different compartments as well as varying due to flooding. The reason and the consequence of these changes are not understood. Further study is needed to determine the metabolic potential of theses microbes and their importance to the soil poplar-ecosystem.

Acknowledgments

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References


