Microbial Ecology

Influence of Ozone on Litter Quality and Its Subsequent Effects on the Initial Structure of Colonizing Microbial Communities

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Abstract

Ozone is considered as the main factor in air pollution related to a decline of forest in North America and Europe. In the present study, the effect of changed litter quality, due to ozone stress to trees, on the microbial communities colonizing the subsequent litter was investigated. Litter bag technique using beech and spruce litter from ozone-stressed and control trees, was combined with 16S and 18S rRNA-based fingerprinting methods and cloning to characterize phylogenetic diversity. Litter bags were incubated for 2 and 8 weeks in a beech–spruce mixed forest. Differences between the structure of microbial communities colonizing control and ozone-exposed litter were evident by fingerprints of 16S and 18S rRNA RT-PCR products. RT-PCR products, from litter degraded for 8 weeks, were cloned to identify the bacterial and fungal groups. Clones similar to members of Actinobacteria dominated the bacterial libraries, whereas effects of changed litter quality were mainly observed for the Proteobacteria. Fungal libraries were dominated by clones similar to Ascomycota members. Reduced proportion of clones similar to Basidiomycota and Zygomycota in library from ozone-stressed spruce trees and Chytridiomycota from ozone-stressed beech trees was observed when compared to their control counterparts. As hypothesized, changed litter quality due to elevated O_3 did influence the structure of litter-colonizing microbial communities. However, these differences were not as pronounced as those between the two plant species.

Introduction

Ozone is a greenhouse gas that is continuously accumulating in the lower atmosphere. Its concentration has doubled from preindustrial concentrations to approximately 50 ppb [17]. Elevated O_3 is known to cause biochemical and physiological changes in most plants [3, 11, 27], like decreased photosynthesis, reduced plant growth, and changes in C allocation in many woody plants [31]. It also alters tissue nutrient concentrations [6, 28, 29], which in turn may affect decomposition rates. However, impacts of ozone on nutrient dynamics vary with study and species. For example, studies with coniferous species reported no effects of ozone on N concentrations [13, 26] or increase in N with increasing ozone [6, 32].

Due to changes in plant physiology, microbial communities in soil and rhizosphere might be indirectly influenced by higher ozone concentrations in the atmosphere. Changed litter quality might influence initial microbial colonization of the litter and its subsequent degradation. Litter fall and its degradation constitute an important aspect of nutrient cycling and energy transfer in most ecosystems [20]. Growth and productivity of forest ecosystems depend mainly on the amount, quality, and rate of decomposition of the corresponding plant litter [22].

Despite the importance of litter degradation, little is known about the microbial communities colonizing leaf litter in situ and how degradation is affected by changed litter quality due to environmental stresses like enhanced ozone levels. Beech covers much of the afforested land in Germany today; however, for economic reasons, beech has been replaced by conifers, especially spruce. Therefore, we focused on the litter from both plant species and its microbial degradation. Aneja et al. [5] have shown that

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difference in decomposition rates between beech leaves and spruce needles is related to differences in the composition of the microbial decomposer communities colonizing the two litter types. In this study, both soil site and litter quality were reported as important parameters influencing the initial colonization and subsequent succession of microbial communities. In the present study, we hypothesize that changes in the biochemical constituents of plant litter, produced under elevated O_3, would elicit changes in active microbial colonizers and influence their subsequent succession during decomposition.

Therefore, the main aim of this investigation was to determine the effects of changed litter quality of beech and spruce, due to enhanced ozone, on the structure of initial microbial colonizers. For this, litter bag technique, which involves incubating bags of a defined mesh size with litter under investigation (beech and spruce) in soil followed by the analysis of degradation kinetics and microbial colonization at selected time points (early time points in the present study), was used. rRNA analysis was chosen as a marker molecule for microbial community studies; as for bacteria, there is a good evidence that active microbes have in general higher RNA contents in their cells compared to dormant cells or bacteria with low activity profiles. However, this correlation has not been proven for fungal communities. Therefore, for fungal communities, rRNA is more an indicator for the presence of a phylotype than for its activity. To analyze microbial community structure, a two-step approach was chosen including fingerprinting of PCR products for screening all samples obtained and cloning and sequencing of PCR products for a detailed analysis of selected samples.

Materials and Methods

Beech and Spruce Litter. Beech and spruce litter was collected from Kranzberg forest, a beech and spruce mixed forest located in southeastern Germany (latitude 48° 25′ N; longitude 11°40′E; altitude 490 m) in autumn 2002. Leaf litter was collected from litter traps placed in control and ozone-exposed (150 ppb; ozone exposure since 1998) sites. Thus, the collected litter had never reached the ground. Details about the setup of the ozone fumigation have been published elsewhere [36]. Freshly collected litter was then dried at 70°C for 96 h and stored at room temperature in sealed bags in dark. rRNA extraction, cDNA synthesis, and PCR amplification with bacterial and fungal primers (see below) from the dried litter material gave no amplification signal. Also, amplification of DNA gave no indication for bacteria and fungi that were still in a viable state on the litter material.

Litter Bags and Decomposition Reaction. Nylon litter bags (10×10 cm; mesh size 40 μm) were each filled with 4 g of dried litter according to ISO 17155. For each litter type, 10 bags were filled and were buried in August 2003 into the upper 5 cm (A_h-horizon) of the respective control and ozone-exposed sites. Both sites were lacking of a significant O horizon. After 2 weeks, five bags each of both beech and spruce litter were removed. The remaining bags were removed after 8 weeks. All bags were shock-frozen at −100°C and stored as individual bags until further analysis.

Biochemical Characterization of Litter. For sugar and starch measurements, dried, undegraded litter and frozen, degraded litter were freeze-dried and milled. Each bag was processed separately. Sugars were extracted with hot water (80°C) from 20 mg of sample in three steps (one time with 1 ml water and two times with 0.5 ml water). The protocol involved incubation with water for 10 min at 80°C followed by centrifugation at 10,000×*g* for 5 min. The supernatant was collected from the three steps and pooled. From the remaining pellet, starch was extracted enzymatically using heat stable amylase (1250 U ml^{-1}; Sigma) for 30 min at 60°C and amyloglucosidase (3 U ml^{-1}; Sigma) overnight at 37°C and measured as glucose. Sugars and starch were analyzed using HPLC with a BIORAD Aminex HPX-87C column (at 85°C; 0.6 ml flow) with water as mobile phase. A refractive index detector (RI 1755, Bio–Rad) was used for detection. All samples were analyzed twice.

Cellulose determination was adopted from Updegraff [33]. The test was miniaturized using 2 ml Eppendorf screw-cap vials and a starting amount of 0.1 g plant material. The residue of acetic/nitric acid treatment was quantified gravimetrically after lyophilization.

Lignin was determined according to Bruce and West [7] with 60 mg of finely powdered 1:1 mixtures of plant tissue and diatomaceous earth. The thioglycolic acid conjugate was dissolved in oxygen-free 0.5 N NaOH and quantified photometrically. Organosolv lignin (Sigma-Aldrich, Taufkirchen, Germany) was used as a reference.

RNA Extraction and RT-PCR Amplification for Bacteria and Fungi. RNA extraction and cDNA synthesis from litter were performed as described by Aneja et al. [4]. Each bag was processed separately for molecular analysis. Primer pair 968f-GC and 1401r [15], with an annealing temperature of 54°C, was used in specific RT-PCR reaction targeting bacteria. For fungi, primer pair NS1 and NS2-GC at an annealing temperature of 52°C [37] was used in the reaction. One microliter of synthesized cDNA was used in 48 μl PCR reaction, which consisted of 5 μl of 10× reaction buffer, 5 μl of 3% BSA, 2.5 μl of dimethylsulfoxide, 3 μl of 25 mM $MgCl_2$ for bacteria (for fungi, 5 μl was added), 5 μl of 2 mM deoxyribonucleoside triphosphate mixture (dNTP), and 1 μl each of 10 μM primer pair. The reaction involved hot start at 95°C for 10 min followed by the addition of 2.5 U of *Pfu* DNA Polymerase (Stratagene, Amsterdam, The Netherlands) for bacteria and Taq DNA Polymerase (Invitrogen, Karlsruhe, Germany) for fungi. The cycling parameters were 94°C for 1 min, annealing for 1 min, and 72°C for 1 min for 30 cycles followed by a final extension at 72°C for 10 min. RT-PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). DNase-treated nucleic acids, without being reverse-transcribed, were used as controls in RT-PCR to check for residual DNA in RNA preparations (data not shown).

Denaturing Gradient Gel Electrophoresis. Denaturing gradient gel electrophoresis (DGGE) for bacterial RT-PCR products was performed using 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 37:1) with a 48–58% denaturant. For the analysis of fungal communities, gradient was reduced to 35–45%. A 100% denaturant is defined as 7 M urea plus 40% formamide [1]. Appropriate volumes containing about 2 μg of the purified RT-PCR products, measured by absorbance at 260 nm, were loaded. The gels were electrophoresed at 60°C at 50 V for 17 h using D-Gene system (Bio–Rad Laboratories, Munich, Germany) and silver-stained using the protocol described by Heukeshoven and Dernick [16].

Image Analysis. Dried DGGE gels were scanned using HP Scanjet 7400c. Profiles obtained were analyzed by clustering via the unweighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) using GelCompar II Software (Applied Maths, Kortrijk, Belgium). Only within-gel comparisons, taking into account only the presence and absence of bands, were performed for the profiles as variability between two gels was too big for a proper analysis. The position tolerance was set at 1%, and background subtraction was applied. Cophenetic correlation, which is a parameter to express the consistence of a cluster, was calculated using the same software.

Cloning of RT-PCR Products and Sequencing. Purified RT-PCR products for 16S and 18S rRNA from beech and spruce litter incubated for 8 weeks in their respective control or ozone-exposed sites were cloned. Approximately 100 ng of purified bacterial RT-PCR products was cloned into pCR®-Blunt II-TOPO® vector of Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Karlsruhe, Germany) as described by the manufacturer. This ligation mix was incubated for 30 min at room temperature. Same amount of purified fungal RT-PCR products was cloned into pCR® 2.1 vector of TA Cloning Kit (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. Ligations were incubated at 14°C overnight.

Two microliters of ligation mix was transformed into chemically competent One Shot® DH5α™-T1^R cells provided in the kit following the manufacturer's instructions. Colonies were inoculated in LB medium (supplemented with 50 μg ml^{-1} kanamycin). Plasmids were isolated using Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany) and screened for inserts of correct size by *Eco*RI digestion (MBI Fermentas, Heidelberg, Germany) at 37°C for 1 h.

Screening of the Libraries by RFLP. Hundred clones with the desired insert were screened from each of the four libraries (a total of 800 clones; BO: beech litter from ozone-exposed site, SO: spruce litter from ozone-exposed site, incubated in the ozone-exposed site for 8 weeks; BC: beech litter from control site, and SC: spruce litter from control site, incubated in the control site for 8 weeks) by digestion with *Msp*I (MBI Fermentas, Heidelberg, Germany) at 37°C for 4 h. Digests were resolved on 4% high resolution agarose (Qbiogene, Heidelberg, Germany), and representative samples of the different RFLP patterns observed were then sequenced on ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the manufacturer's instructions. Other restriction enzymes were tested using amplified ribosomal genes from a pure culture collection (bacteria and fungi) of the site under study. However, the best resolution was obtained with *Msp*I. Rarefaction curves were generated using the software Analytic Rarefaction provided on the web (http://www.uga.edu/~strata/software/). Before analysis, sequences were checked for plausibility, and chimeric sequences were eliminated from further analysis.

Nucleotide Sequence Accession Numbers. Nucleotide sequences determined in this study have been submitted to the GenBank database under accession numbers DQ069861–DQ069890.

Results

Biochemical Characteristics of Undegraded and Degraded Litter. Overall rates of degradation of beech and spruce litter were comparable after 8 weeks (Table 1). The mineralization rates were in accordance with the asymptotic model of litter decay wherein high rates were observed initially (2 weeks) followed by reduced mineralization. After 2 weeks, a tendency for high mineralization rates for litter from ozone-stressed trees was observed. However, at the 8-week time point, the rates were comparable for both control and ozone-exposed litter of both species.

Similar levels of sugars were observed for beech and spruce litter from both control and ozone-exposed sites (Table 2). However, litter from ozone-exposed sites had

Table 1. **Degradation rates of beech and spruce litter during 2 and 8 weeks of incubation in soil sites**

Litter type	*2 weeks (mg d^{-1})*	*8 weeks (mg d^{-1})*
BC	7+/−0,3	4+/−0,2
BO	11+/−0,5	5+/−0,2
SC	4+/−0,2	3+/−0,1
SO	7+/−0,3	4+/−0,2

BC: beech litter from control site, BO: beech litter from ozone-exposed site, SC: spruce litter from control site, and SO: spruce litter from ozone-exposed site

higher starch amounts compared to control litter of the same species. Effect of ozone on the amount of starch was more prominent in the case of beech litter. The C/N ratio for spruce litter was not affected by the ozone treatment and was about 30. The C/N ratio for beech was a little bit lower (26) and was also not influenced by the ozone treatment (data not shown). During the course of degradation, there was a steady decrease in starch for beech litter, whereas for spruce, an increase in the same was observed. Sugars decreased during the course of decomposition, with the exception of beech litter from ozone-exposed site (BO). Among the polymeric substances, cellulose and lignin amounts were comparable for the litter of the two species and also among the litter from control and ozone-exposed sites (data not shown). Lignin content, per gram of dry weight, increased by more than 30% (compared to the initial undegraded litter) for both litter types during the course of decomposition, whereas there was only a marginal decrease in cellulose concentration (<5%).

Analysis of Colonizing Bacterial Communities. Dried undegraded litter got colonized during the incubation period in soil by bacteria and fungi, which had moved into the litter bags. 16S rRNA was used as molecular marker for the analysis of active bacterial communities of the soil site and colonizing bacterial communities from degraded litter. All samples yielded the desired RT-PCR product, and no residual DNA was detected in PCR reactions with DNase-treated nucleic acids without being reverse-transcribed. Moreover, no 16S rRNA or the corresponding gene could be detected in dried undegraded litter (data not shown). DGGE profiles from the control and ozone-exposed soil sites were equally complex with respect to the number of bands observed (Fig. 1). However, bacterial community structure of the incubated litter types was significantly different even after 2 weeks of incubation. Each litter type (BO and SO: ozone-exposed beech and spruce litter, respectively; BC and SC: control beech and spruce litter, respectively) showed its unique bacterial community fingerprint. Analysis of the DGGE profiles using GelCompar II revealed two main clusters. The major cluster represented differences in the bacterial community structure due to the plant species from which the litter originated. Comparable number of bands was observed in the profiles from ozone-stressed and control litter. The succession of bacterial community from 2 to 8 weeks, although being comparable for both ozone-stressed and control spruce litter (about 87% similarity between 2 and 8 weeks profiles), showed greater difference for ozone-stressed beech litter. These profiles were more than 95% similar with replicate samples (data not shown). Although the bacterial fingerprints changed from 2 to 8 weeks, indicating a succession of the colonizing bacterial communities, the effects were less pronounced compared to the differences due to plant species.

To identify the bacterial colonizers, 16S rRNA RT-PCR products from beech and spruce litter incubated for 8 weeks were cloned, and 100 clones, with the insert of correct size, were then screened by restriction fragment length polymorphism (RFLP) with *Msp*I. Ten different RFLP patterns were observed on resolving the digests on high-resolution agarose. Representative samples for each RFLP type were sequenced, and the sequences were used to assign the clones to bacterial groups using the NCBI BLAST program. Clones similar to sequences reported in other studies, but mentioned as "unclassified" in the NCBI database, have been grouped together as "unclassified" in the present study also. Figure 2 shows the broad phylogenetic distribution of clones within each library. A majority of the clones were highly similar (>96% homology) to the members of Actinobacteria with higher proportions observed in beech libraries. Sequences similar to the members of different subgroups of Proteobacteria were also present although in less proportions (<15%). Effects of ozone on litter quality and its subsequent bacterial colonization were evident by the marked absence of clones similar to the members of the γ-subdivision of Proteobacteria from SO library and

Table 2. **Values of sugars and starch in undegraded and degraded beech and spruce litter**

Litter type & degradation period	*Sugars[a] (mg g^{-1})*	*Starch (mg g^{-1})*
BC	5+/−0,3	0.3+/−0,1
BC 2 weeks	3+/−0,1	ND
BC 8 weeks	4+/−0,1	ND
BO	4+/−0,2	8+/−0,3
BO 2 weeks	4+/−0,1	2+/−0,1
BO 8 weeks	4+/−0,2	2+/−0,1
SC	5+/−0,2	ND
SC 2 weeks	4+/−0,1	1+/−0,1
SC 8 weeks	1+/−0,2	2+/−0,1
SO	4+/−0,1	6+/−0,3
SO 2 weeks	3+/−0.1	3+/−0,1
SO 8 weeks	2+/−0,1	0,7+/−0,1

[a] sugar levels mentioned are the sum of glucose, xylose, fructose and sucrose. ND: not detected. BC: beech litter from control site, BO: beech litter from ozone-exposed site, SC: spruce litter from control site, and SO: spruce litter from ozone-exposed site, respectively, degraded for 2 and 8 weeks

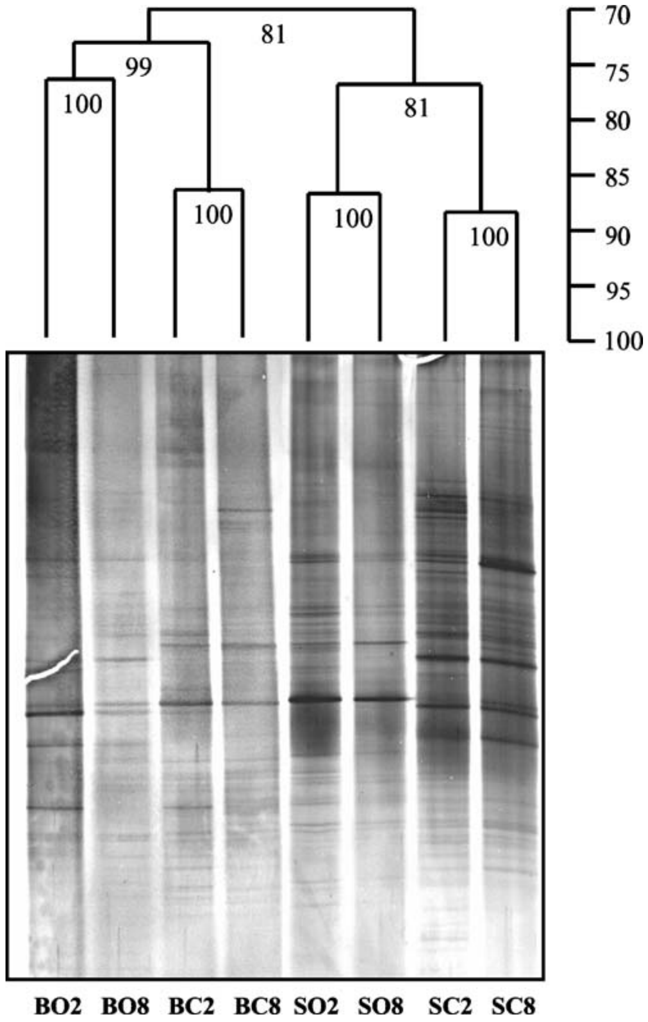

Figure 1. DGGE profiles and UPGMA tree showing the similarities between the patterns of 16S RT-PCR products from litter collected from ozone-exposed and control sites and degraded for 2 and 8 weeks in their respective sites. BO: beech litter from ozone-exposed site, BC: beech litter from control site, SO: spruce litter from ozone-exposed site, and SC: spruce litter from control site. Numbers 2 and 8 stand for 2 and 8 weeks, respectively, of degradation of litter. Scale represents percent similarity. Numbers at branch points are the cophenetic correlation values, which express the consistency of the cluster.

the β-subdivision of Proteobacteria from SC library. An opposite trend was observed in the case of beech litter where clones similar to the members of the γ-subdivision of Proteobacteria were present in increased proportion in BO library. Clones similar to the members of β-subdivision of Proteobacteria were absent from beech libraries.

On performing rarefaction analysis by plotting the number of RFLP types observed as a function of the number of clones screened, a plateau, as expected for full coverage of the bacterial diversity in the library, was obtained after screening 60 clones for each library type (Fig. 3). No new RFLP type was observed even when the number of clones analyzed were increased to 100. Therefore, a total of 400 clones analyzed from the four libraries (100 clones each from BC, BO, SC, and SO) are true representation (within the limits of experimental protocols) of the bacterial diversity present in the system. It is interesting to note that clear differences in the maximum number of RFLP types were visible, comparing beech and spruce litter. As for spruce litter, only 10 different RFLP types could be obtained, whereas for beech litter, up to 20 RFLP types were visible. An additional influence of the ozone treatment on the number of RFLP types was only evident for beech litter, with a reduced number of RFLP types obtained. Overall, these data support the results obtained by the DGGE fingerprints that the litter type (beech or spruce) exerted the strongest influence on the structure of the colonizing bacterial communities.

Analysis of Colonizing Fungal Communities. The 18S rRNA was targeted to characterize the fungal communities from the two soil sites, as well as colonizing communities from beech and spruce litter. RT-PCR products were obtained for all the samples using NS1 and NS2 primers. Similar to 16S, no 18S rRNA or its corresponding gene could be detected in dried undegraded litter (data not shown). On resolving the purified 18S rRNA RT-PCR products by DGGE, reduced number of bands was observed when compared to bacterial profiles after the same time points (Fig. 4). No major differences in the number of bands were observed between the DGGE profiles from control and ozone-exposed soil sites, and cluster analysis of the profiles from the litter revealed two clusters, the major one being for the plant species. For both beech and spruce, the succession of fungal communities from 2 to 8 weeks was comparable for ozone-stressed and control litter types. High reproducibility as observed earlier for bacterial profiles was also observed for 18S profiles from replicate samples (data not shown).

For the identification of fungal groups colonizing both litter types and also to look for ozone specific effects, 18S RT-PCR products were cloned to generate libraries followed by RFLP analysis as done for bacterial clone libraries. On resolving the RFLP digests on high-resolution agarose, 20 different RFLP patterns were observed. Representative samples for each RFLP type were sequenced, and sequences were used to assign the clones to fungal groups using the NCBI BLAST program. Figure 5 shows the broad phylogenetic distribution of the clones within each library. Majority of the clones (approximately 70–75% in beech libraries and 85–90% in spruce libraries) had sequences similar (≥98%) to the members of Ascomycota. Sequences similar to the members of other groups such as Basidiomycota,

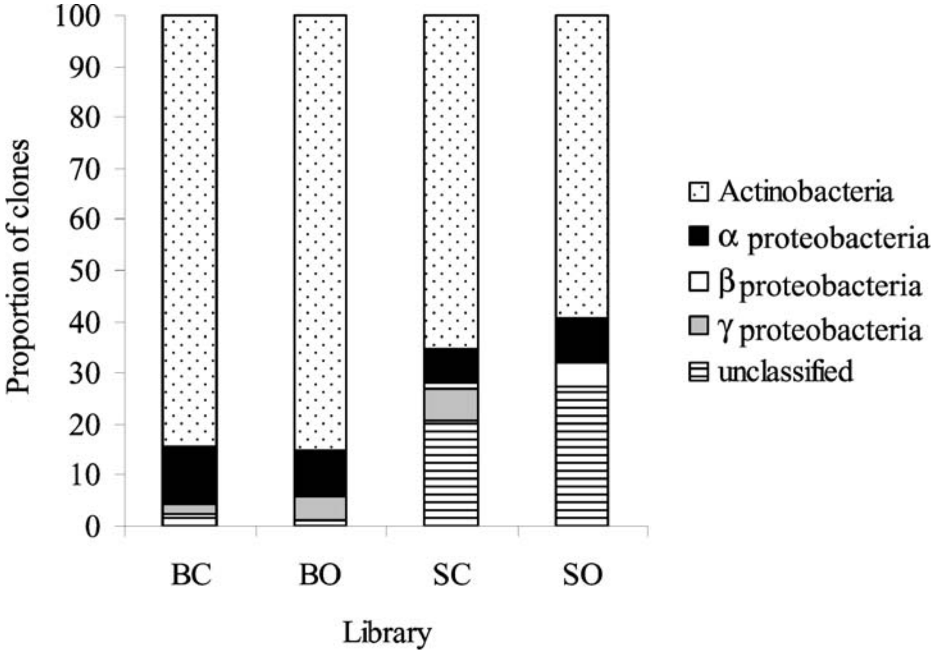

Figure 2. Relative distribution of clones to different bacterial groups. For abbreviations, refer to Fig. 1.

Zygomycota, and Chytridiomycota were present in lower proportions. Effects of ozone on litter quality and subsequently on fungal colonization were evident by the reduced proportion of clones similar to the members of Chytridiomycota in BO library. Clones similar to the members of Basidiomycota were present in lower proportions, whereas clones similar to Chytridiomycota were absent from spruce libraries.

Furthermore, effects of changed litter quality, due to ozone, on fungal colonizers were evident when the distribution of clones similar to the different members of Ascomycota was analyzed. Within Ascomycota, high proportions of clones were more than 98% similar to the 18S rRNA sequence of *Penicillium* and *Trichoderma* (Fig. 6). Clones similar to *Penicillium* were present in increased proportions in BO library. Similarly, clones similar to 18S rRNA sequence of *Trichoderma* were present in increased proportion in SO library, whereas clones similar to *Leptodontidium* were decreased in proportion in SO library.

As done for bacterial libraries, rarefaction curves were constructed for the four libraries. A plateau was obtained after screening 60 clones for each library type. No new RFLP type was observed even when the number of clones was increased to 100 (Fig. 3). Therefore, a total of 400 clones analyzed from the four libraries (100 clones each from BO, BC, SO, and SC) indicate the full coverage of fungal diversity colonizing the litter. The maximum number of RFLP types is depending similarly to the bacterial data on the plant material. It is interesting to note that the effect is opposite to what has been found for bacterial RFLP types. Beech litter which shows a huge diversity in bacterial RFLP types has a very low diversity of fungal RFLP types (up to five). In contrast, spruce litter which has a lower number of bacterial RFLP types has a significant higher amount of different fungal types. An additional effect of the ozone treatment was not visible.

Discussion

Microbial communities and their function can be influenced directly or indirectly by various biotic and abiotic factors. Although it is well known that microbes are highly sensitive to ozone [18] and ozone is used for disinfection of drinking water in several countries [10], it is unlikely that ozone has a direct effect on microbial communities in soil, as the highly reactive ozone is degraded in the first few millimeters of soil. Only if very high concentrations are used, direct effects of ozone on the soil biota can be observed. Loranger et al. [19], for example, has shown direct effects of enhanced ozone concentrations on selected groups of soil fauna (Collembola and Acari) in a temperate forest. The effect on soil microbes was not characterized in this study. However, most other studies have shown that ozone has no direct effect on living biota in soil [21]. In contrast, there are several indications that an indirect effect of ozone via changed organic matter composition of the soil on microbial community structure and function is possible [25]. This is despite of the possibility that these indirect effects might be much less pronounced than a direct action of the ozone on bacteria and fungi. Also, our results do not support the hypothesis that an increased ozone concentration in the air has a direct effect on the microflora of the bulk soil.

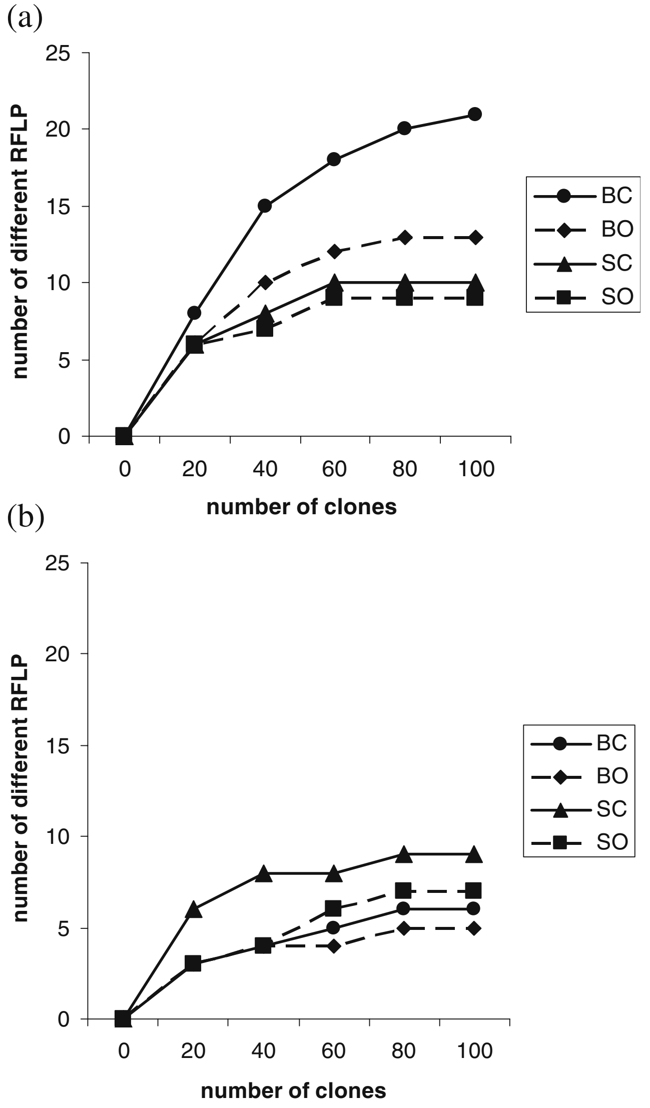

Figure 3. Rarefaction curves showing the extent of microbial diversity covered by clone libraries. (a) bacterial clone libraries, (b) fungal clone libraries.

As evident in both DGGE profiles and clone libraries, differences between the microbial colonizers from control and ozone-exposed sites were less prominent than the differences between beech and spruce colonizers.

Differences in the colonization of investigated litter types by microbes may be linked to litter quality although no significant differences were observed between the lignin and cellulose amounts between the control and ozone-exposed litter of the two species. Moreover, the amounts of these components did not differ significantly even between beech and spruce. Similar results have earlier been reported by Vesterdal [34] in a 4-year degradation experiment using natural litter from the forest. In the present study, however, higher levels of starch were observed in the litter from ozone-exposed site. Ozone has been shown to reduce phloem loading in plants [3], and this could result in higher starch levels in the litter from ozone-exposed site.

It has been hypothesized that changes in macromolecular C (e.g., lignin and tannin) degrading functional groups are more critical to litter degradation processes than changes in simple C (e.g., sugars, starch, etc.) degrading functional groups because of lower species richness of microbes with the enzymatic capacity for macromolecular C degradation [30]. In contrast, low redundancy of microbial community involved in litter degradation was clearly visible in our study, as only slight changes in the litter quality resulted in significant changes in the bacteria and fungi colonizing the litter material. Furthermore, the number of bands in the DGGE fingerprints (about 30 for bacterial fingerprints and 15 for fungal fingerprints) and the high number of clones to be analyzed before saturation was obtained confirm that

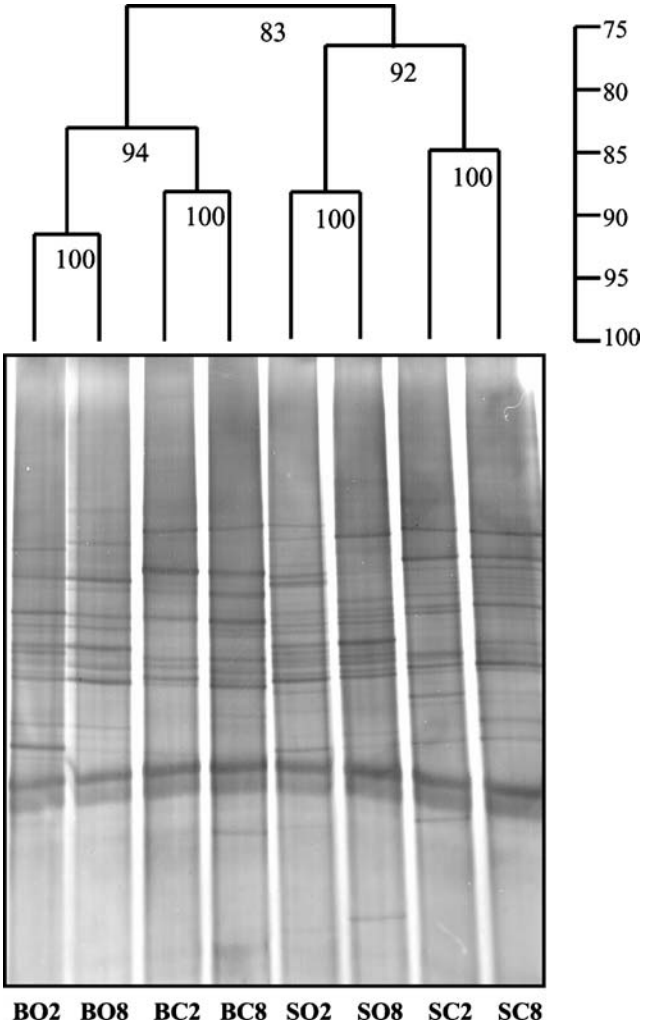

Figure 4. DGGE profiles and UPGMA tree showing the similarities between the patterns of 18S RT-PCR products from litter collected from ozone-exposed and control sites and degraded for 2 and 8 weeks in their respective sites. For abbreviations, refer to Fig. 1.

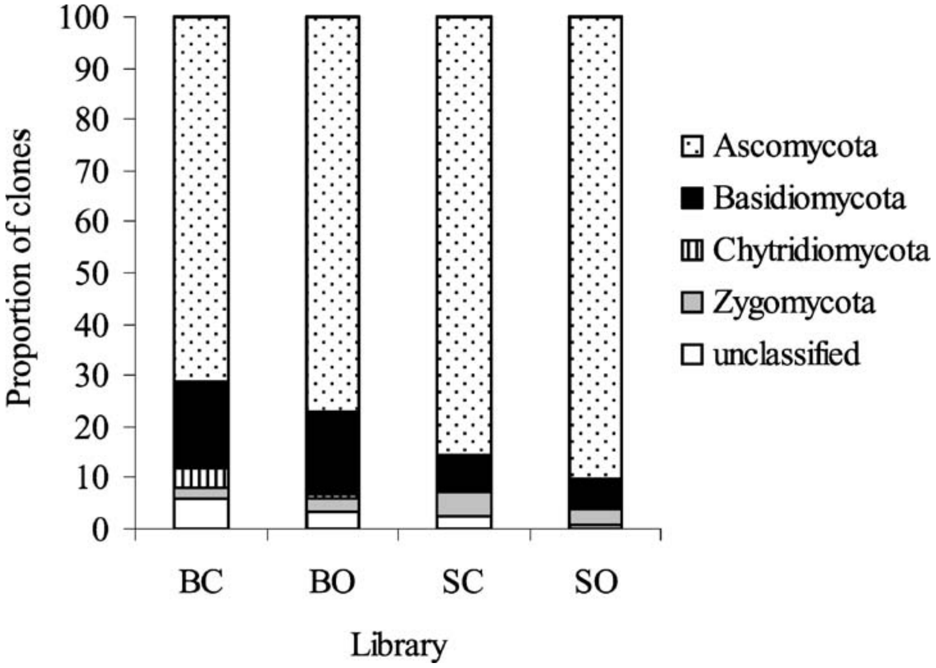

Figure 5. Relative distribution of clones similar to different fungal groups. For abbreviations, refer to Fig. 1.

many bacteria and fungi are able to colonize the litter material. This substantiates the finding of Waldrop and Firestone [35], who examined the degradation of four ^{13}C-labeled compounds (starch, xylose, vanillin, and pine litter), quantified rates of associated enzyme activities, and identified microbial groups utilizing the ^{13}C-labeled substrates in soils under oaks and in adjacent open grasslands. Although the microbial groups responsible for starch, xylose, and vanillin degradation differed in the two soils, the turnover rates were very similar.

Bacterial and fungal colonizers identified by the clone libraries from the litter material are mostly known to be involved in litter degradation. For example, Akasaka et al. [2] identified Actinobacteria to be the most dominant bacterial group in rice plant residue degradation. Also, members of α-Proteobacteria have earlier been shown to be the key degraders of salt marsh grass, *Spartina alterniflora* [8]. Previous studies of fungal community of southeastern US salt marshes have identified several species of ascomycetous fungi as major

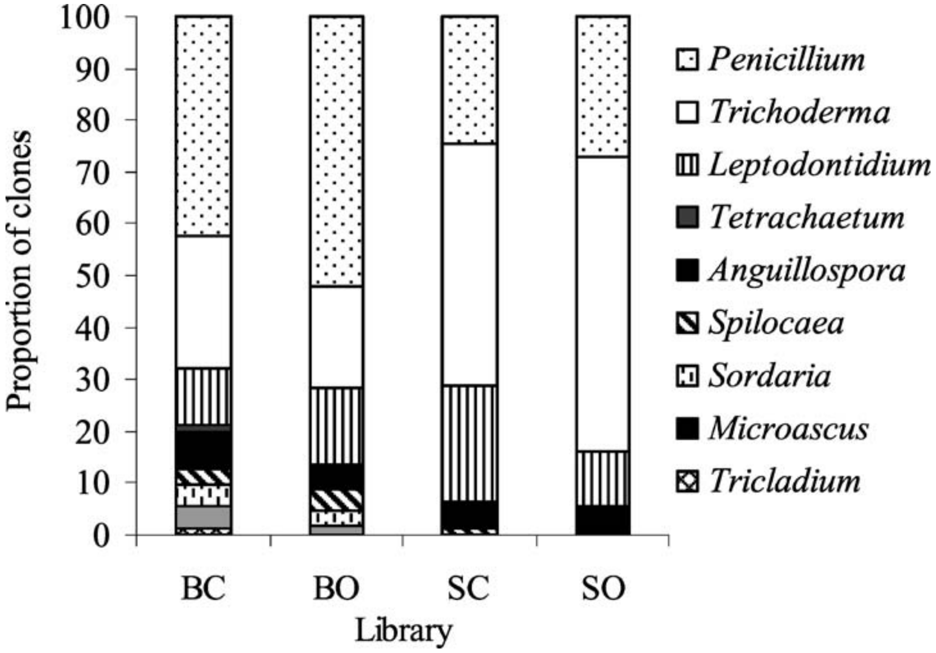

Figure 6. Relative distribution of clones similar to different genera of Ascomycota. For abbreviations, refer to Fig. 1.

decomposers of *S. alterniflora* blades based on both traditional culture- and microscopy-based methods [23, 24] as well as using molecular approaches [9, 12]. Cox et al. [12] and Frankland [14] have earlier shown the effect of litter quality on the sequence of fungal succession during the course of degradation using cultivation-dependent techniques. They followed fungal succession on fallen leaves of *Quercus* (oak), *Betula* (birch), *Corylus* (hazel) and *Fraxinus* (ash) in broad-leaved, U.K. woodland on mull humus. It was reported that the succession patterns were different for the various litter types.

In this study, the effect of ozone on litter quality, with respect to its subsequent microbial colonization, has been shown. Our experiments were designed to investigate the initial colonization of litter. As seen mainly for the beech litter material, this time is too short for an overall assessment of degradation. Therefore, additional long-term degradation experiments are required for a better understanding of the dynamics of involved microbial community during the whole process of litter degradation.

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