Institut für Bodenökologie
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# Degradation of beech and spruce litter - Influence of soil site and litter quality on microbial communities

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*Dedicated to my parents*

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# INDEX

## ABBREVIATIONS

| | |
|---|---|
| α | alpha (-subgroup of proteobacteria) |
| °C | degree centigrade |
| APS | ammonium persulphate |
| β | beta (-subgroup of proteobacteria) |
| bp | base pair |
| BSA | bovine serum albumin |
| cDNA | complementary DNA |
| cm | centimetre |
| δ | delta (-subgroup of proteobacteria) |
| DEPC | diethylene pyrocarbonate |
| DGGE | denaturing gradient gel electrophoresis |
| $dH_2O$ | distilled water |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribose nucleic acid |
| DNase | deoxyribonuclease |
| EDTA | ethylene diamine tetra acetic acid |
| e.g. | for example |
| et al. | et alteri |
| Fe | iron |
| γ | gamma (-subgroup of proteobacteria) |
| g | gram |
| h | hours |
| K | potassium |
| kg | kilogram |
| klx | kilolux |
| l | litre |
| LB | Luria Bertani (-medium) |
| μ | micron ($10^{-6}$) |
| M | molar |
| m | milli ($10^{-3}$) |
| Mg | magnesium |
| min | minute |

| | |
|---|---|
| mRNA | messenger RNA |
| N | nitrogen |
| n | nano ($10^{-9}$) |
| P | phosphorus |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| pmol | pico moles ($10^{-12}$) |
| ppb | parts per billion |
| ppm | parts per million |
| RAP-PCR | RNA arbitrarily primed PCR |
| rDNA | ribosomal DNA |
| RFLP | restriction fragment length polymorphism |
| RNA | ribose nucleic acid |
| RNase | ribonuclease |
| rRNA | ribosomal RNA |
| RT | reverse transcription |
| TAE | tris acetic acid EDTA buffer |
| TBE | tris boric acid EDTA buffer |
| TEMED | tetramethylethylenediamine |
| V | volt |
| viz. | videlicet (namely) |
| vol. | volume |
| vol/vol | volume / volume |
| w/v | weight / volume |
| Zn | zinc |

# 1. INTRODUCTION

## 1.1 Role of plant litter in ecosystem functioning and stability

In forest ecosystems, litter fall is the largest source for the return of organic material and nutrients to the soil. The ecological significance of litter degradation and humus formation can be studied at different levels. At the biosphere level, an understanding of decomposition is important as soils represent a major sink of carbon. The amount of carbon that is stored in soil as humus and related stable organic compounds is not being circulated through the atmosphere. Thus, an understanding of the factors influencing the amount of humus formed and the stability of that humus are important in predicting global carbon budgets (Shaver et al., 1992; Schlesinger and Andrews, 2000).

At the ecosystem level, decomposition is important for somewhat different reasons. Nutrient cycling is closely tied to decomposition and the availability of nutrients in a given soil is due in large part to the decay dynamics of organic matter in that soil. It is also responsible for the formation of humic substances that contribute to soil fertility as well as the long-term storage of carbon. In addition, the accumulation of organic matter in soil can greatly enhance the nutrient holding capacity of the soil and affect the soil pH.

The ecological significance of decomposition and humus formation can also be viewed from the perspective of microbial ecology. Decomposition generally follows a sequential pattern with different classes of organic compounds dominating the decay process as it proceeds. In general, degradation of water soluble and low molecular weight compounds, e.g. sugars and phenolics, dominate the first stage of litter decay. Next, hemicelluloses, especially those that are arabinan-based, begin to disappear. Somewhat later, cellulose degradation is the dominant activity and finally lignin degradation becomes dominant. Although individual processes may dominate a particular stage of decomposition, any or all the processes may occur throughout the decay continuum. This general pattern has been observed in many studies (Mindermann, 1968; Berg et al., 1982; Aber et al., 1984). These changes in the substrate quality lead to succession of different bacterial and fungal groups involved in degradation, with different groups and species dominating different stages of decomposition (Cox et al., 1997; Dilly and Munch, 2001; Buchan et al., 2003). This process supports diversity in the microbial population by supplying a rich set of intermediate degradation products and serving as energy and nutrient sources for different microbial subpopulations. To follow the succession of

microbial colonizers during decomposition, bacterial and fungal communities were analyzed in the present study after two and eight weeks.

## 1.2 Plant litter and decomposition

### 1.2.1 Plant litter composition

The bulk of plant litter consists of varying amounts of several major classes of organic compounds. There are four principal groups of soluble organic material in litter: sugars, phenolics, hydrocarbons and glycerides. The soluble sugars are predominantly mono- and oligosaccharides that had been involved in metabolic processes of the plant. The soluble phenolics are low molecular weight compounds that serve either as protectants against herbivory, are lignin precursors, or are waste products. Phenolics are highly variable in their solubility and many have a tendency to condense into less soluble forms or to react with larger molecules. The relative proportions of these compounds vary with the plant part (e.g. leaves, stems, roots, bark) and among species.

Lignin often makes up between 15 - 40% of the litter mass. In some extreme cases, litter can have lignin contents as low as 4% or as high as 50%. Lignin, in contrast to cellulose, is a highly variable molecule. The initial composition of lignin varies with the plant species, and the variation is enough to make the lignin of each species unique. The types of lignin formed in deciduous and coniferous trees are different. Deciduous species contain varying ratios of syringyl and guaiacyl types of lignin whereas conifers have mainly guaiacyl lignin (Fengel and Wegener, 1983).

In addition to lignin, the quantitatively most common components in plant litter are the different polymer carbohydrates such as cellulose and main hemicelluloses. Of these, cellulose, the most common component, is made up of glucose units connected with β-1-4 bonds forming long chains of molecules organised into fibres. Cellulose may constitute between 10 - 50% of the litter mass. Hemicelluloses are polymers of sugars other than glucose that also form long chains of molecules that are built into the fibre. The proportions of hemicelluloses vary among litter species. Differences in the major hemicelluloses are primarily reflected in the concentrations of xylose and mannose. Deciduous leaves (e.g. beech) are lower in mannans whereas Norway spruce needles have higher levels. The ratio of hemicelluloses to cellulose ranges from 0.7 - 1.2 with higher ratios often seen in deciduous

litter (e.g. beech) compared to coniferous litter (e.g. spruce). Hemicelluloses may together make up as much as 30 - 40% fibre.

Similarly, all plant litter contains essential nutrients such as N, P, S, K, Ca, Mg, Mn and Fe, but the concentrations vary with the litter species. For example, leaf litter of nitrogen fixing genus alder (*Alnus*) has very high levels of N (often above 3%); in contrast, pine needle litter is more N-poor (often below 0.4%). Plant species is thus one dominant factor in determining the litter quality (Berg and McClaugherty, 2003).

### 1.2.2 Factors affecting decomposition

Decomposition of litter is a complex suite of processes that strongly affect the mineralization and immobilization of mineral nutrients and their cycling and is a principal pathway for the return of nutrients to the soil (Miller, 1984). As decomposition of litter involves a complex series of sequential processes that are both plant species and soil site dependent, plant leaf litter undergoes changes in its composition from its initial state during decomposition, with some litter components disappearing rapidly, some slowly and some begin to disappear only after a time delay (Berg and McClaugherty, 2003). Chemical composition of litter, environmental conditions and especially characteristics of the soil environment are the key factors that influence decomposition rates of litter.

#### *1.2.2.1 Chemical composition of litter as site dependent parameter*

Numerous studies suggest that litter decay and nutrient release are controlled by a wide variety of chemical properties, the most studied being the N, P and lignin content of the litter. Its chemical composition determines both the composition of the microbial community and the course and pattern of decomposition process. In many studies, the N concentration of the litter and the C:N ratio was strongly correlated with litter decay rates (Witkamp, 1966; Coulson and Butterfield, 1978; Berg and Staaf, 1980; Taylor et al., 1989; Tian et al., 1992a). In other studies, P concentrations and C:P ratios appeared to be good predictors of decay rates (Coulson and Butterfield, 1978; Schlesinger and Hasey, 1981; Staaf and Berg, 1982; Berg et al., 1987; Vitousek, 1994). In other cases, lignin concentration and lignin:N ratio in plant litter was a good predictor of litter decomposition (Meentemeyer, 1978; Berg and Staaf, 1980; Melillo et al., 1982; Berg, 1984; Tian et al., 1992b; Van Vuuren et al., 1993). These parameters and their effects on litter decomposition are plant species and soil site dependent. In general, plant species from nutrient-poor environments produce litter that is more difficult to decompose than litter of species from nutrient-rich environments. Low-nutrient species

generally have higher C:N ratios and higher concentrations of decay-resistant plant compounds than high-nutrient species (Schlesinger and Hasey, 1981; Aber and Melillo, 1982; Pastor et al., 1984; French, 1988; Horner et al., 1988; Nicolai, 1988; Berendse et al., 1989; Chapin, 1991; Van Vuuren et al., 1993). Due to these interspecific differences in decomposability of the litter, it has been postulated that species from nutrient-poor environments slow down the rate of nutrient cycling in their habitat, whereas species from nutrient-rich habitats have an accelerating effect on the rate of nutrient cycling (Chapin, 1991; Field et al., 1992; Vitousek, 1994). In the past few years, these postulated effects of plants on the rate of nutrient cycling have been demonstrated in a number of studies (Berendse, 1990; Wedin and Tilman, 1990; Wedin and Pastor, 1993). Besides interspecific variation in litter decomposition rates, it is expected that components of global change, such as increased levels of nitrogen deposition from the atmosphere, may alter litter quality and thereby increase litter decomposition and nutrient release rates from nutrient poor litter (Chapin, 1991; Field et al., 1992; Aerts and De Caluwe, 1994, Vitousek, 1994). Indeed, increased N supply leads to increased litter N concentrations (Miller et al., 1976; Coulson and Butterfield, 1978; Oren et al., 1988) and litters with higher N concentrations decompose faster (Coulson and Butterfield, 1978; Hunt et al., 1988). As a result, there might be a positive feedback between nitrogen supply rate and litter decomposition and nutrient release at the intraspecific level.

#### *1.2.2.2 Global environmental influences*

Climate is a strong regulatory factor in litter composition as shown on a larger scale using actual evapotranspiration (Meentemeyer, 1978) and directly influences litter decomposition rates, especially of newly shed litter. Among the many environmental variables, temperature and moisture appear to be the critical factors. Both of these factors act by directly influencing the microbial communities involved in litter degradation. The effect of climate decreases as decomposition proceeds and lignin degradation is the rate-limiting factor. The predictive changes in the atmosphere, e.g. elevated $CO_2$ and ozone, have shown to have effects on the quality of litter and thereby on the functioning of forest ecosystems (Boerner and Rebbeck, 1995; Ball, 1997; Andersen, 2003).

Ozone is a normal constituent of the stratosphere but is also accumulating in the lower atmosphere. Its concentration has doubled from pre-industrial concentrations to approximately 50 ppb (Olszyk et al., 2001). Over the last few decades, ozone concentrations have increased 1 to 2% per year (Fishman, 1991). Increases in ambient ozone concentrations have been shown to cause changes in physiological processes of trees (Mikkelsen and Ro-

Poulsen, 1994) and thus the corresponding changes to tree growth and productivity (Pearson and Mansfield, 1994). Comprehensive reviews on the biochemical and physiological changes in plants caused by ozone are available (Chappelka and Chevone, 1992; Runeckles and Chevone, 1992; Andersen, 2003). The magnitude and direction of these changes are plant species specific and depend upon the experimental conditions. Generally, in coniferous species, ambient, or slightly elevated ozone levels increased photosynthesis rates compared to filtered air (Eamus et al., 1990), but with a further increase of ozone levels gas exchange rates declined. Broad-leaved trees appeared to be more sensitive, with ozone exposures causing decreased stomatal conductance and assimilation rates compared to filtered air (Le Thiec et al., 1994). Impacts of ozone on nutrient dynamics vary with study and species. Studies with coniferous species reported no effects of ozone on N concentrations (Reich et al., 1988; Edwards et al., 1992) or increases in N with increasing ozone (Baker et al., 1994; Temple and Riechers, 1995). Elevated $O_3$ has the potential to alter soil microbial communities and their function through its influence on plant litter production. It also alters the nutrient concentrations of plant tissue (Schier, 1990; Baker et al., 1994; Samuelson et al., 1996), which in turn may affect decomposition rates. In the present study, ozone stress was given to beech and spruce plants in the greenhouse and to mature trees in Kranzberger forest. The aim of the study was to identify the effects of changed litter quality, due to ozone stress, on the structure and function of the colonizing microbial community.

#### *1.2.2.3 Soil Site*

##### *1.2.2.3a Abiotic factors*

Although litter chemical composition and climate appear to dominate as regulating factors in decomposition over a regional scale, numerous soil site-specific factors are important in regulating decomposition at the local or even microscale. Soil factors include both physical and chemical properties. Texture is perhaps the most important physical property of soil because it influences nutrient and water dynamics, porosity and permeability, and surface area. Jenkinson (1977), in one of the first long-term studies to examine the effects of soil properties on decomposition, reported that clayed soils are able to hold on to more biologically degradable soil organic matter than sandier soils.

Chemical properties include pH, cation exchange capacity, organic matter content and nutrients. Jenkinson (1977) reported slow initial degradation rates in acidic soils. Among mineral nutrients, soil N status is considered the most common limiting factor and has

received the most attention. Effects of adding N to decomposing substrates depend upon the stage of decomposition. Studies with agricultural residues have found little effect of added N, except occasionally during the initial stages of decay (Lueken et al., 1962; Knapp et al., 1983, Bremer et al., 1991). However, when considering a longer period of decay, the effects of added N on decay rate appear to be negligible, and may even become negative (Fog, 1988). Soil chemical composition, including the availability of nutrients, has an influence on the chemical composition of leaves and thus also on chemical composition of foliar litter.

*1.2.2.3b Biotic factors*

The abundance and composition of soil fauna and microbial communities have been shown to affect the rate of litter breakdown (Schaefer and Schauermann, 1990). Wachendorf et al. (1997) estimated that faunal activity accounted for approximately 50% and 6% of the decomposition rate of the same litter in mull and moder soil types respectively, whereas for these same two sites, microorganisms accounted for decomposition rates of 18% and 70%. Some studies have also demonstrated the importance of soil fauna in decomposition by affecting the composition and activities of microbial communities (Verhoef and Brussaard, 1990; Anderson, 1988). Though soil-ingesting animals have been shown to play an important role in both aggregate turnover and in litter breakdown (Martin and Marinissen, 1993), microorganisms mainly carry out litter decomposition in forest soils (Teuben, 1991; Scholle et al., 1992). Plant litter in temperate and boreal coniferous forests is decomposed through microbial activity and the quantitative contribution of microorganisms to decomposition is considered to be above 95% with soil animals being responsible for the remaining maximum 5% (Berg et al., 2003). However, biochemical decomposition processes are microbial and the involved microorganisms with their metabolic activities, which are soil site-specific, result in transformation of mineral nutrients and organic products often involved in humification.

The ingrowth of microorganisms, mainly fungi, into the litter, may begin prior to litter fall, but the ingrowth of decomposers takes place when the litter has reached the ground. The faster growing microorganisms start invading the litter, with part of the litter C becoming microbial biomass and some part mineralized to $CO_2$. The composition of the microbial community that invades litter depends on the properties of the litter, soil site characteristics and changes in those properties over time. Role of several species of bacteria and fungi in litter degradation has been identified in previous studies (Frankland, 1992; Rosenbrock et al., 1995; Cox et al., 1997; Cox et al., 2001). In small-scale experiments of short duration, using defined cohorts of plant litters and more precise analytical methods, the effects of litter

colonization by key fungal species during microbial succession become apparent (Frankland, 1992; Cox et al., 2001). Under laboratory conditions, where much of the environmental variability encountered in the field can be factored out, forest soil material from different sites and associated microbial communities emerge as important variables in rates of litter decomposition (Prescott, 1996; Chadwick et al., 1998). The structure of soil microbial community involved in the decomposition of organic matter in forest ecosystems is in turn also influenced by the amount and quality of litter-input dependent on plant species composition (Bååth et al., 1995; Chadwick et al., 1998).

### 1.3 Methods to study microbial colonization and tools to investigate microbial community structure and function

The diversity of soil microbial communities has been investigated for many years using methods based on isolation and cultivation of the microbes. Numerous studies have used litter bag technique in combination with cultivation dependent techniques to identify the microbial degraders and to follow succession of microbial communities during the course of decomposition (Frankland, 1992; Rosenbrock et al., 1995; Cox et al., 1997; Cox et al., 2001; Dilly and Munch, 2001; Buchan et al., 2003). Such techniques are known for their selectivity and are not considered representative of the extent and diversity of the community. The proportion of cells, which can be cultured, is only a small fraction of the total population (Amann et al., 1995) and few data are available concerning how closely they reflect the actual composition of these communities.

The limitations of culture-dependent techniques have been a considerable handicap to microbial ecology. Ecological inferences based on the metabolic properties of cultivated bacteria are, by necessity, unrepresentative of the natural populations from which they were obtained (Brock, 1987). Although the biases of cultivation-based approaches were recognized by Winogradsky (1949), it is only recently that means have been developed to study the uncultivated majority. First Zuckerkandl and Pauling (1965), then Woese's advances in microbial phylogeny (Woese et al., 1985) coupled with developments in molecular biology provided the necessary methods to allow the identification of uncultivated bacteria.

To get a better insight into the bacterial and fungal community structure, methods independent of cultivation should be used. The cultivation independent methods are based on either membrane lipids or nucleic acids as markers. The analysis of microbial membrane lipids, specifically phospholipids fatty acids (PLFA) is an effective tool for determining the

microbial community structure in environmental samples (Lechevalier, 1977) and has also been applied to investigate microbial communities decomposing conifer litters (Wilkinson et al., 2002).

Using nucleic acid as molecular marker, methods that rely on direct amplification of rRNA genes (rDNA) have been rapidly replacing cultivation as an approach to compare the bacterial communities of various habitats (Ferris and Ward, 1997; Torsvik et al., 1998; Duineveld et al., 2001; Gremion et al., 2003). Fungal diversity in environmental samples, e.g. decaying wood and rhizosphere soil, has also been analysed using denaturing gradient gel electrophoresis (DGGE) of amplified 18S ribosomal DNA (rDNA) (Kowalchuk et al., 1997; Smit et al., 1999; Vainio and Hantula, 2000). The development of techniques for the analysis of 16S and 18S rRNA sequences in natural samples has greatly enhanced our ability to detect and identify bacteria and fungi from natural environments (Olsen et al., 1986; Pace et al., 1986). This has confirmed earlier estimates that only a small percentage of microbial species have been isolated in culture (Ward et al., 1990; Barns et al., 1994; Choi et al., 1994).

Molecular biological techniques are not free from limitations and biases, especially when applied to environmental samples. As with other techniques used by soil microbiologists, biases appear at the stage of sampling and storage before extraction of nucleic acids. Problems are encountered at the stage of DNA / RNA extraction, which includes the reliable and reproducible lysis of all bacterial cells and fungal mycelia, extraction of intact nucleic acids, and the removal of substances, such as humic acids, bacterial exopolysaccharides and proteins, which may inhibit polymerase activity during PCR and DNA digestion with restriction enzymes (Trevors and van Elsas, 1995; Clegg et al., 1997; van Elsas et al., 1997; Cullen and Hirsch, 1998; Frostegård et al., 1999; Gelsomino et al., 1999). Generally, soil samples are recommended to be refrigerated or frozen if they are not analysed further. Miller et al. (1999) found that DNA yields decreased rapidly for refrigerated samples and decreased slowly over several weeks for frozen samples. Therefore, it is recommended to use freeze-dried samples (Miller et al., 1999). Impurities in the extracted total DNA significantly reduce the rate of DNA re-association, decreasing precision of the re-association estimates of the soil microbial diversity. In addition, the source of the DNA, which is originating not only from active bacterial cells but also from dead or dormant cells, and persistent DNA adsorbed onto soil particles, can affect the interpretation of the re-association kinetics, leading to an

overestimation of detected genomes / species (Torsvik et al., 1990; Stahl, 1997; Clegg et al., 1998).

Inspite of the above mentioned limitations, molecular techniques generate valuable information on microbial diversity and community structure in environmental samples, taking into accounts both the culturable and unculturable fractions of microorganisms (van Elsas et al., 1998). Combining of different methods that work at a broad scale with those that can be used to identify particular groups or species of bacteria or fungi seems to be a way of avoiding several limitations associated with analysis of complex communities (Øvreås and Torsvik, 1997; Muyzer, 1998; Nüslein and Tiedje, 1998; Øvreås et al., 1998; Macnaughton et al., 1999; Sandaa et al., 1999). These methods include genetic fingerprinting, which provides a global picture of the genetic structure of the microbial community and PCR fragment cloning followed by restriction and / or sequence analysis, which enable assessment of the diversity of the community in terms of the number of different species, and to a lesser extent, the relative abundance of these species. Nucleic acid based techniques can also be combined with PLFA analysis, which has been used to describe the relative abundances of the different microbial groups in various environmental samples.

### 1.3.1 Analysis of structural diversity

The gene coding for ribosomal RNA associated with the small ribosomal subunit (16S in prokaryotes and 18S in eukaryotes) has been extensively used to characterize bacterial and fungal communities. This gene is particularly suited for such studies as all bacteria and fungi harbour this gene, which is essential for ribosome functioning in protein synthesis. Therefore, their evolutionary relationships can be deduced (molecular clock) and cells can be identified by *in situ* hybridisation targeting abundant ribosomes in cells. Additionally, a large number of 16S and 18S sequences of different organisms are stored in databases. These sequences in several highly conserved regions can be and have been used to design PCR primers.

Numerous studies have applied rRNA as a molecular target to characterize microbial communities (Ferris and Ward, 1997; Heuer and Smalla, 1997; Felske et al., 1998; Duineveld et al., 2001; Gremion et al., 2003). In nearly all of these studies, novel microbial linkages have been discovered, confirming our lack of understanding of the microbial species that inhabit soils and their potentially important roles in ecosystem functions.

In recent years, a number of analyses have focussed on the characterization of soil microbial communities based on rRNA as opposed to rDNA (Felske et al., 1996; Sessitsch et al., 2002; Purdy et al., 2003). Like rDNA, rRNA has both conserved and highly variable regions that permit the discrimination of taxa at multiple taxonomic levels. In addition, use of rRNA offers certain principle advantages over rDNA techniques. As rRNA sequences are typically present in cells in higher copy number than rDNA sequences, they should be easier to detect (Moran et al., 1993). When ribosomes are extracted directly from soil samples, free nucleic acids and many dormant microorganisms are excluded and only rRNA from active cells is detected (Felske et al., 1997). Because ribosomes are the sites of protein synthesis, cellular ribosome content (and thus rRNA content) has been shown to be directly correlated with metabolic activity and growth rate under steady state conditions (Wagner, 1994). Therefore, a high proportion of rRNA sequences detected in soil samples should correspond to metabolically active and growing microorganisms. Exceptions to this general observation have also been reported (Kerkhof and Kemp, 1999; Oerther et al., 1999). Results with rRNA can be readily compared with those for simultaneously extracted DNA to estimate both the active and total community (Felske et al., 1996).

Fingerprinting of amplified 16S and 18S genes can be performed using denaturing gradient gel electrophoresis (DGGE). The technique enables the separation of fragments of the same length depending on differences in sequences. This offers a culture-independent tool for tracking dominant bacterial and fungal populations in space and time and to assess community changes (Muyzer et al., 1993). Compared to RFLP / ARDRA that is appropriate for fine-structure analysis of specific components of microbial community structure, the patterns of DGGE or TGGE are more useful for a direct comparison of structural diversity between different microbial communities from different natural sites or environments perturbed by any way. In addition, the possibility of hybridisation with group- or species-specific probes or excision of bands and its sequencing provides additional information of the microbial community composition that is impossible to obtain with RFLP / ARDRA (Kowalchuk et al., 1998; Muyzer and Smalla, 1998; Macnaughton et al., 1999; Yang and Crowley, 2000).

### 1.3.2 Analysis of functional diversity

Changes in the active microbial communities may lead to changes in the functions performed by the community. A number of methods are available that enable one to make investigations at the functional level. Garland and Mills (1991) proposed that substrate utilisation, using

commercially available Biolog plates, could be used to characterise the functional differences among microbial communities. The application of the substrate utilisation assay has been studied extensively in various environments such as soils, water (Garland and Mills, 1991) and wastewater systems (Victorio et al., 1996).

Another method to investigate functional diversity is stable isotope probing, which typically involves the application of a stable isotope-labelled substrate to the environment of interest and then assessing the assimilation of the isotope into microbial biomarkers. Typical biomarkers that have been used for stable isotope studies include nucleic acids (Manefield et al., 2002a, 2002b; Radajewski et al., 2000), phospholipids fatty acids (Treonis et al., 2004) and ergosterol (Malosso et al., 2004).

Functional studies have also been done at the mRNA level. Direct extraction of mRNA from soil (Sayler et al., 1989) and quantification of mRNA by an RNase protection assay (Fleming et al., 1993) have been used for naphthalene dioxygenase in soil and for soluble methane monooxygenase in aquifer sediments (Stapleton et al., 1998). Reverse transcriptase PCR (RT-PCR) amplification of mRNA for soluble methane monooxygenase in aquifer sediments (Selvaratnam et al., 1995) and for lignin peroxidase in soils (Bogan et al., 1996) has also been performed. One of the major prerequisites for such methods is knowledge of the sequence flanking the target region for the design of amplification primers for PCR. This limits the choice of primers and thus methods that bypass the requirement of prior sequence information are needed.

For generating RNA profiles, Liang and Pardee (1992) introduced mRNA differential display RT-PCR (DDRT-PCR) in which an oligo (dT) primer is used in the reverse transcription reaction and an additional primer is used in the PCR step. The use of oligo (dT) renders this technique useful for eukaryotic gene expression where mRNA is polyadenylated. Fleming et al. (1998) optimised and applied the differential display technique to identify differentially expressed genes using pure cultures and soil microcosms to identify genes induced by toluene. The high percentage of false positives generated by DDRT-PCR strongly limits the usability of the method (Zegzouti et al., 1997). A derivative of DDRT-PCR, RNA arbitrarily primed-PCR (RAP-PCR) described by Welsh et al. (1992) has the potential to bypass these limitations. RAP-PCR uses an arbitrary primer or random hexanucleotide mixtures (Abu Kwaik and Pederson, 1996) at a low annealing temperature for cDNA synthesis reactions.

Due to low stringency temperatures the arbitrary primer is able to bind at random sites within the template that show limited, but not complete complementarities. Menke and Mueller-Roeber (2001) have described the optimisation of the protocol for fingerprinting plant cells.

In the present study, RAP-PCR was optimized for generating metabolic fingerprints of microbial communities colonizing the litter. RAP-PCR products were also cloned to identify the transcripts. In addition, 16S and 18S rRNA DGGE fingerprints were used to compare the bacterial and fungal communities colonizing the litter. Furthermore, clone libraries were generated with these RT-PCR products to identify the key colonizers.

### 1.4 Objectives

The present study hypothesized that the structure and function of the microbial communities colonizing litter are influenced by both the site of degradation (soil site-specific microbial communities) and litter quality (biochemical components of litter). Ozone has been identified in many previous studies to alter tree physiology, growth and litter production. Furthermore, the study hypothesized that changed litter quality, due to ozone stress to plants, influences nutrient turnover rates and the colonizing microbial communities.

The main objectives of the present study were:

- **(i)** to identify the effects of soil site on bacterial and fungal colonization of beech and spruce litter
- **(ii)** to identify the effects of litter quality on bacterial and fungal colonization of beech and spruce litter
- **(iii)** to identify the key bacterial and fungal groups that colonize beech and spruce litter and follow succession of microbial communities
- **(iv)** to generate and compare the metabolic profiles of beech and spruce litter degraded in different soil sites and for different time points
- **(v)** to study the effects of ozone stress on litter quality and its subsequent colonization by bacteria and fungi.

To achieve these objectives, litter bag technique (Swift and Anderson, 1989) with litter degraded in different soil sites was combined with RNA based (16S and 18S rRNA) molecular analysis of the microbial colonizers. Clone libraries were made to identify the bacterial (16S libraries) and fungal (18S libraries) groups colonizing the different litter types and also to follow their succession during the course of decomposition. In addition, RAP-PCR method was optimized and metabolic fingerprints generated from degraded litter material.

# 2. MATERIALS AND METHODS

## 2.1 Litter materials

### 2.1.1 Litter production in green house with and without ozone stress

Three years old beech and spruce plants were grown in greenhouse from 14.02.02 until 21.03.02 under controlled conditions in a sandy loam soil taken from a beech-spruce mixed forest ($A_h$-horizon; 20% clay, 32% silt, 48% sand). Twenty rectangular polyethylene boxes (60 x 45 x 40 cm) were filled with about 70 kg of soil per box. Forty plants each of beech and spruce were planted in containers with four plants per container. During the growth period temperature was maintained at 25°C with a photoperiod of 14 h $day^{-1}$ (approximately 14 klx). The plants were watered equally every alternate day with 2.1 l of water and fertilized once a week with the same volume of nutrient solution (Table 1).

**Table 1.** Concentration of various macro- and micronutrients in the fertilizer solution.

| **Macronutrients** | **Molarity** |
|---|---|
| $KNO_3$ | 14.5 mM |
| $MgSO_4.7H_2O$ | 1 mM |
| $KH_2PO_4$ | 1.3 mM |
| $Na_2HPO_4$ | 0.4 mM |
| $CaCl_2.2H_2O$ | 600 µM |
| $FeSO_4$-EDTA | 100 µM |
| **Micronutrients** | **Molarity** |
| $Al_2(SO_4)_3.18H_2O$ | 2 nM |
| KJ | 2 nM |
| KBr | 2 nM |
| $SnCl_2.2H_2O$ | 2 nM |
| LiCl | 2 nM |
| $MnCl_2.4H_2O$ | 2 µM |
| $H_3BO_3$ | 10 µM |
| $ZnSO_4.7H_2O$ | 1 µM |
| $CuSO_4.5H_2O$ | 1 µM |
| $NiSO_4.6H_2O$ | 2 nM |
| $Co(NO_3)_2.6H_2O$ | 2 nM |
| $(NH_4)_6Mo_7O_{24}$ | 10 nM |

The growth of plants was monitored during this period by their requirement for $CO_2$, which increased from 4.6 l (09.03.02) to 20 l (20.03.02). $CO_2$ concentration was maintained at 400 ppm throughout the growth period. Litter was removed from the plants by hand at the end of the experiment (Figure 1).


**Figure 1.** Beech and spruce plants grown in green house for production of litter (14.02.02 - 21.03.02).
**(a)**: Plants at end of growth period; **(b)**: after removal of leaves and needles.

For experiments with ozone as a stress factor, three years old beech and spruce plants from the nursery of Bavarian Forest Service (Staatliche Baumschule und Samen- klenge, Laufen, Germany) were grown in greenhouse from 26.03.03 until 23.06.03 using the same soil and conditions described above. Forty plants each of spruce and beech were planted in containers with four plants per container. Half of the beech and spruce plants were put under ozone stress (300 ppb) from 21.05.03 to 23.06.03. The other halves were used as controls (Figure 2a and 2b). Ozone stress was given during the photoperiod and ozone stressed plants showed typical symptoms of ozone exposure like red lesions on the leaf surface (Figure 2c) and crumpling of leaves (Figure 2d). Leaves and needles were removed from the control and ozone stressed plants by hand at the end of the experiment.

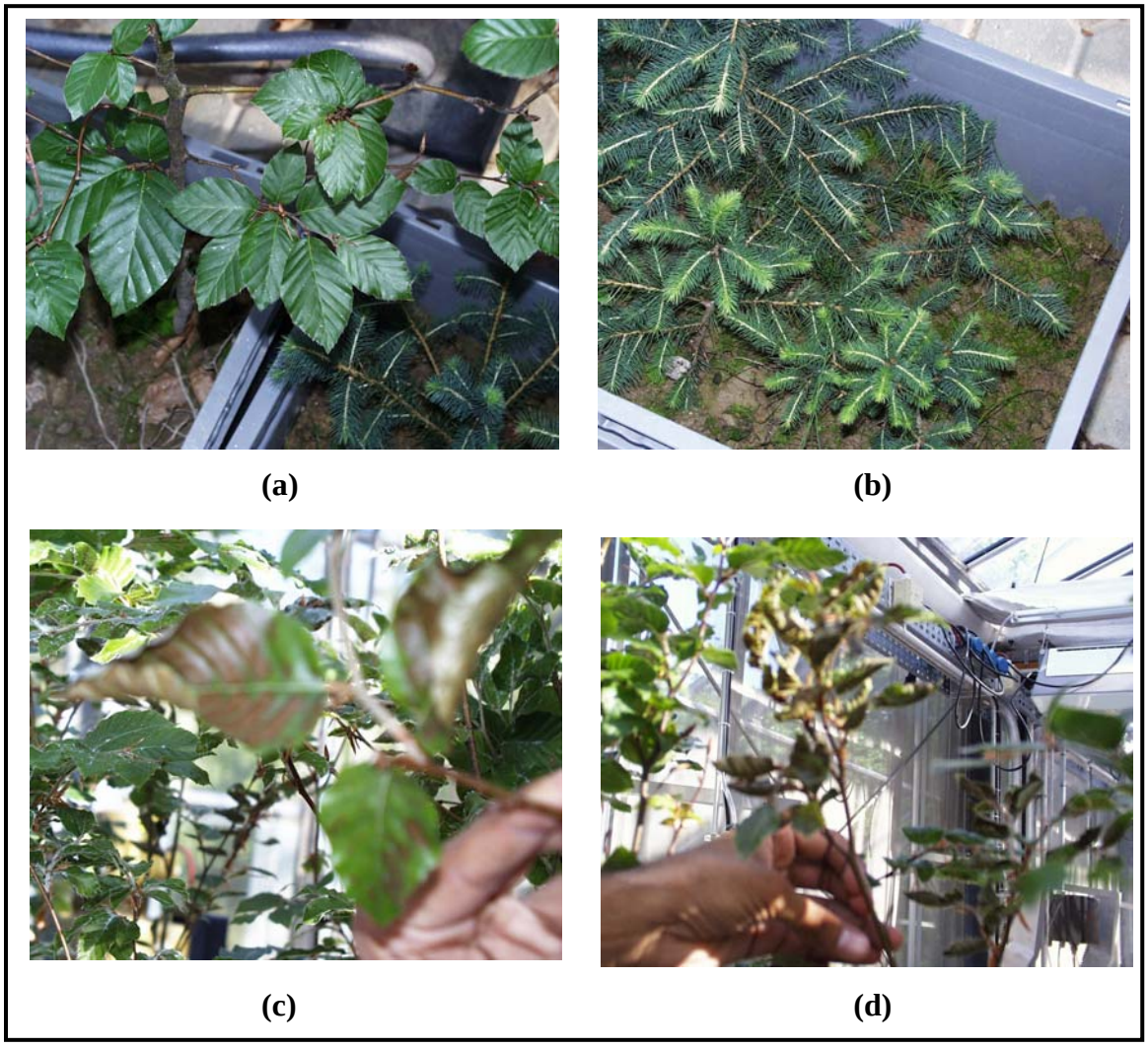


**Figure 2.** Beech and spruce plants grown in green house for production of litter (26.03.03 - 23.06.03).
**(a)** & **(b)**: control beech and spruce plants at end of growth period, respectively; **(c)** & **(d)**: ozone exposed beech plants with appearance of red lesions and curling of leaves.

### 2.1.2 Collection of natural litter from Kranzberger forest (2003)

Beech and spruce litter was collected from Kranzberger forest, a beech and spruce mixed forest, near the city of Freising, Bavaria, Germany. Leaf and needle litter was collected from both control and ozone exposed (150 ppb) sites from the nets in autumn. This litter had never reached the ground before being used in the experiment (Figure 3).

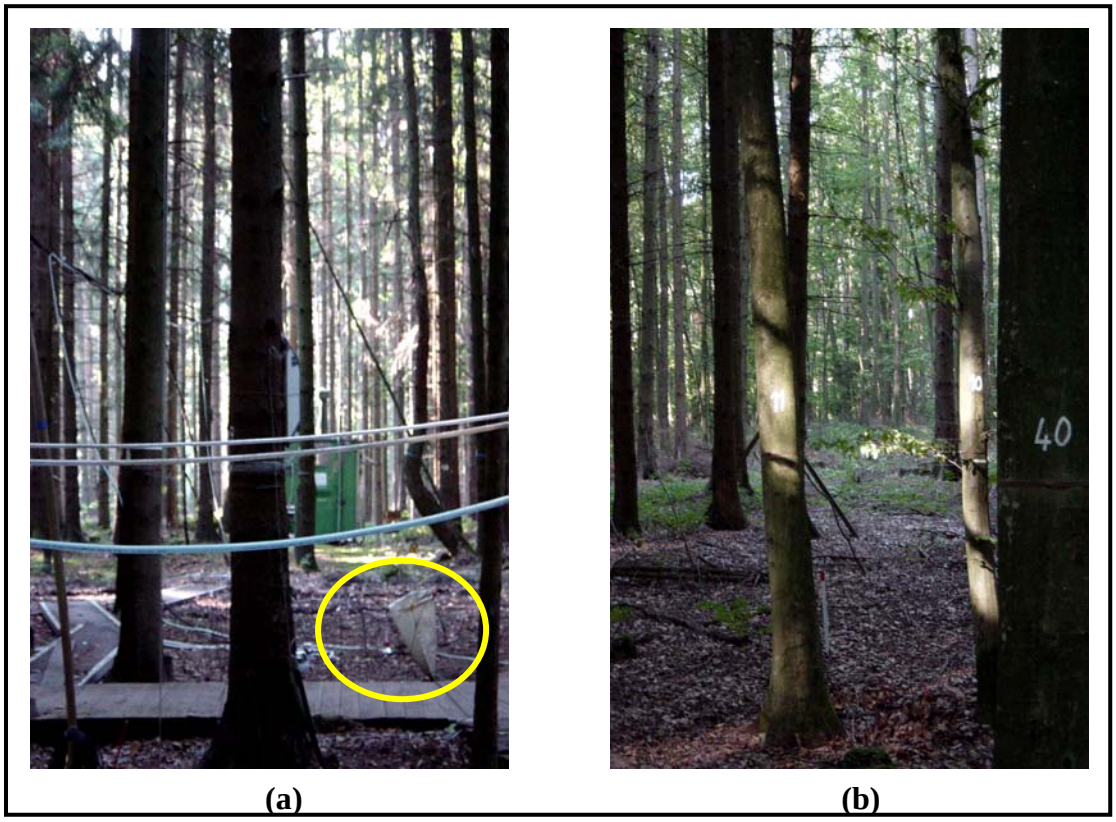


**Figure 3.** Experimental sites in Kranzberger forest from where natural beech and spruce litter were collected and degradation experiments conducted.
**(a)**: Ozone exposed site with litter traps (encircled); **(b)**: Control site.

### 2.1.3 Handling and litter bag filling

Litter material was dried at 70°C for 96 h and stored at room temperature in sealed bags. Nylon litter bags (10 cm x 10 cm; mesh size 40 µm) were each filled with 4 g of dried litter. Beech leaves were crushed by hand before filling in the litter bags. The litter bags were then closed by stitching. Figure 4 shows a closed litter bag filled with litter.

**Figure 4.** Litter bag of mesh size 40 µm (10 x 10 cm) filled with dried litter and closed by stitching before incubating in soil sites for degradation experiments.

## 2.2 Degradation experiments in soil sites

### 2.2.1 Experimental sites

Four different soil sites, grass field (G), maize field (M), beech forest (B) and beech-spruce mixed forest (BS) were selected for conducting the decomposition experiments with greenhouse produced litter. The soil from all four sites was classified as sandy loam and was comparable to the soil used in the greenhouse experiment for plant growth. Ozone stressed and control soil sites of Kranzberger forest were selected for performing the decomposition experiments with natural litter collected from Kranzberger forest.

### 2.2.2 Field experiments with greenhouse produced litter (2002 and 2003)

Ten bags of each litter type per soil site were buried on 28.08.02 in the $A_h$-horizon of the following four sites: G, M, B and BS. Five bags each of both beech and spruce litter were removed from the four sites on 11.09.02. The remaining bags were removed on 24.10.02.

The experiments in 2003 were made using the litter from control and ozone stressed plants grown in the same year (section 2.1.1). Twenty bags of each litter type (10 from control litter and 10 from ozone stressed litter) were buried in each of the four different sites on 04.09.03. Five bags of both control and ozone stressed litter (for both beech and spruce) were removed on 18.09.03. The remaining bags were removed on 30.10.03. Three bags were used for molecular studies and were stored at -80°C.

### 2.2.3 Field experiments with natural litter in Kranzberger forest (2003)

Ten nylon bags were filled for each litter type (section 2.1.2) viz. beech control litter, beech ozone stressed litter, spruce control litter and spruce ozone stressed litter. Incubation of the ozone stressed litter and control litter was carried out in the ozone exposed and control sites, respectively. The bags were put in the respective sites on 25.07.03. Half of the litter bags were removed on 08.08.03 and the other half were removed on 19.09.03. Three bags were used for molecular studies and were stored at -80°C.

## 2.3 Dry weight measurement

Out of the recovered five bags, two were dried at 70°C for 96 h to measure the amount of remaining litter. Remaining dry weight was used to calculate the degradation rates per day.

## 2.4 Molecular analysis of microbial communities

### 2.4.1 Buffers and media

#### 2.4.1.1 CTAB extraction buffer (Griffiths et al. 2000)

| | |
|---|---|
| Solution A (10% CTAB – 0.7 M NaCl) | |
| CTAB | 10.00 g |
| NaCl | 4.09 g |
| DEPC treated distilled water | 100.00 ml |
| Solution B (240 mM potassium phosphate buffer) | |
| potassium phosphate buffer (pH 8.0) | 3.26 g |
| DEPC treated distilled water | 100.00 ml |

Mixed equal volumes of Solutions A and B.

#### 2.4.1.2 LB medium (Sambrook and Russell, 2001)

| | |
|---|---|
| Peptone | 10.00 g |
| NaCl | 10.00 g |
| Yeast extract | 5.00 g |

Volume was adjusted to 1 l using distilled water. pH was adjusted to 7.5.

#### 2.4.1.3 30% Polyethelene glycol – 1.6 M NaCl (Griffiths et al. 2000)

| | |
|---|---|
| Polyethelene glycol | 30.00 g |
| NaCl | 9.36 g |
| DEPC treated water | 100.00 ml |

#### 2.4.1.4 50x TAE buffer (Sambrook and Russell, 2001)

| | |
|---|---|
| Tris base | 242.00 g |
| EDTA | 18.60 g |
| Glacial Acetic Acid | 57.10 ml |

Volume was adjusted to 1 l using distilled water. pH was adjusted to 8.0.

#### 2.4.1.5 5x TBE buffer (Sambrook and Russell, 2001)

| | |
|---|---|
| Tris-base | 54.00 g |
| Boric Acid | 27.50 g |
| EDTA | 2.92 g |

Volume was adjusted to 1 l using distilled water. pH was adjusted to 8.0.

### 2.4.2 Nucleic acid extraction

Nucleic acid extraction from the soil and frozen litter material was performed using the method of co-extraction of DNA and RNA described by Griffiths et al. (2000). This involved bead beating and solvent extraction of the nucleic acids (Figure 5). Precautions were taken to prevent degradation of RNA by RNases. All glassware were baked overnight at 200°C and rinsed with diethylene pyrocarbonate (DEPC) treated water. All solutions were prepared with DEPC treated water (2 h with 0.1% DEPC at 37°C, followed by autoclaving at 121°C for 20 min).

To obtain pure DNA, RNA was removed by incubating with RNase A (Sigma, Munich, Germany) at a final concentration of 100 μg $ml^{-1}$ at 37°C for 10 min. Prior to reverse-transcription, DNA was removed from RNA by treatment with DNase (1 U $\mu l^{-1}$; RNase free; Promega, Mannheim, Germany) according to the protocol described below.

- DNase digestion reaction:

| | |
|---|---|
| Nucleic acid | 24 μl |
| RQ1 RNase-Free DNase 10x Reaction Buffer | 3 μl |
| RQ1 RNase-Free DNase | 3 μl |

- Incubated at 37°C for 30 min.
- 3 μl of RQ1 DNase Stop Solution was added to terminate the reaction.
- Incubated at 65°C for 10 min to inactivate the DNase.

### 2.4.3 cDNA synthesis

Reverse transcription of RNA was performed in a final reaction mixture of 20 μl as described in Table 2 using Omniscript RT Kit (Qiagen, Hilden, Germany).

**Table 2.** Set up for cDNA synthesis.

| Component | Volume / Reaction | Final concentration |
|---|---|---|
| 10x Buffer RT | 2.0 μl | 1x |
| dNTP Mix (5 mM each dNTP) | 2.0 μl | 0.5 mM each dNTP |
| Primer* (10 μM) | 2.0 μl | 1 μM |
| RNase inhibitor (10 U $\mu l^{-1}$) | 1.0 μl | 10 U 20 $\mu l^{-1}$ reaction |
| Omniscript Reverse Transcriptase | 1.0 μl | 4 U 20 $\mu l^{-1}$reaction |
| RNase-free water | Variable | To make final vol. 20 μl |
| Template RNA (see section 2.4.2) | Variable | Up to 2 μg $reaction^{-1}$ |

*Random hexamer (NNNNNN) was used in the reaction.

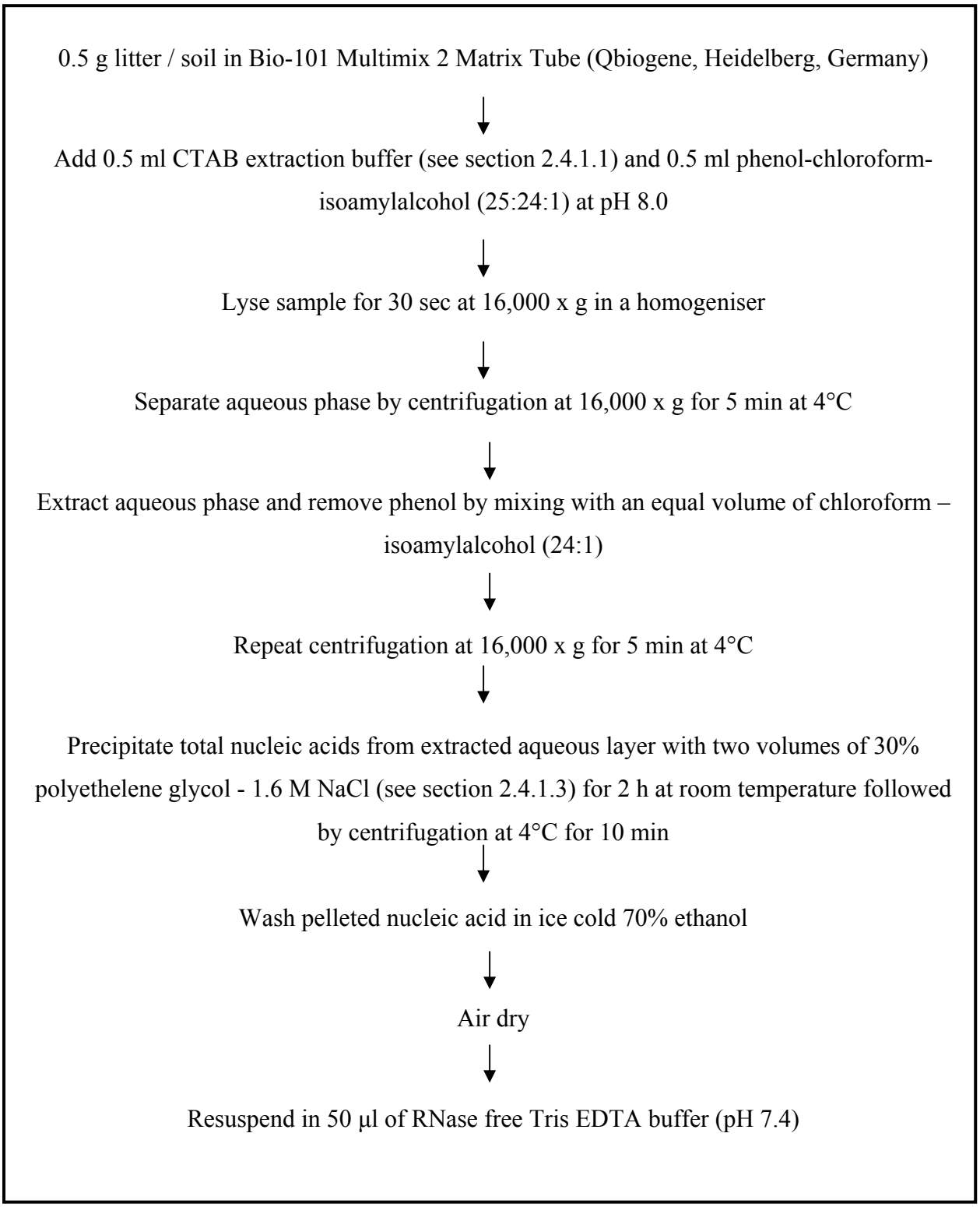


**Figure 5.** Flow diagram for extraction of nucleic acids from soil according to Griffiths et al.'s (2000) method of co-extraction of DNA and RNA.

The reaction mixture was incubated at 37°C for 90 min. The reaction was stopped by heating to 93°C for 5 min followed by rapid cooling on ice.

### 2.4.4 PCR and RT-PCR

Amplifications were performed for different targets as described in the Table 3. Table 4 shows the volumes (in µl) of different components used in PCR and RT-PCR mix in 50 µl reactions. The primer stocks used were 10 pmol $\mu l^{-1}$.

**Table 3.** Primers and cycling conditions used for various targets investigated in the present study.

| Target | Primers used (Reference) | Cycling Programme (denaturation / annealing / elongation) | No. of cycles |
|---|---|---|---|
| 16S rRNA | F984-GC & R1378 (Nübel et al., 1996) | 94°C-1 min / 54°C-1 min / 72°C-1 min | 35 |
| 18S rRNA | NS1 & NS2-GC (White et al., 1990) | 94°C-1 min / 52°C-1 min / 72°C-1 min | 35 |
| Archaea | w036 & w039-GC (Leclerc et al., 2001) | 94°C-1 min / 56°C-30 sec / 72°C-1 min | 30 |

**Table 4.** Amounts of various components in the amplification reactions.

| Component \ Target | 16S | 18S | Archaea |
|---|---|---|---|
| Buffer (10x) | 5 | 5 | 5 |
| $MgCl_2$ (25 mM) | - | 5 | 3 |
| dNTP (2 mM) | 5 | 5 | 5 |
| Primer (Forward) | 1 | 2 | 1 |
| Primer (Reverse) | 1 | 2 | 1 |
| Bovine serum albumin (3%) | 5 | 5 | 5 |
| Dimethyl sulfoxide (DMSO) | 2.5 | 2.5 | 2.5 |
| Template (DNA / cDNA)[c] | 1 | 1 | 1 |
| Nuclease free water | 28.5 | 16.5 | 25.5 |
| Enzyme | 1[a] | 1[b] | 1[b] |

[a] Cloned *Pfu* DNA Polymerase (2.5 U $\mu l^{-1}$, Stratagene, Amsterdam, The Netherlands)
[b] Taq DNA Polymerase (5 U $\mu l^{-1}$, Invitrogen, Karlsruhe, Germany).
[c] See section 2.4.2 and 2.4.3

Prior to the cycling conditions, a hot start of 94°C for 5 min was performed for all samples. Enzyme was added to the reaction mix during the pause. Cycles were followed by a final extension at 72°C for 10 min. All PCR and RT-PCR products were stored at 4°C for further analysis. PCR and RT-PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany).

### 2.4.5 RNA arbitrarily primed (RAP) PCR

Fingerprinting of total RNA was performed using RAP-PCR protocol. Table 5 shows the volumes of different components used in RAP-PCR mix in a 25 µl reaction. Thermal cycler was programmed as: 94°C for 5 min to denature, 37°C for 5 min for low stringency annealing of the primer and 72°C for 5 min for extension for 2 cycles. This was followed by 45 cycles of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C. Final extension was programmed at 72°C for 10 min followed by cooling at 4°C. The 10 mer primer (5'TCACGATGCA3') described by Williams et al. (1990), was used in the present study.

**Table 5.** Set up for RAP-PCR.

| Component | Vol. in µl |
|---|---|
| Buffer (10x) | 2.5 |
| $MgCl_2$ (50 mM) | 1.0 |
| dNTP (2 mM) | 1.25 |
| Primer (100 pmol) | 0.5 |
| BSA (3%) | 2.5 |
| DMSO | 1.25 |
| cDNA* | 1.0 |
| Nuclease free water | 14.75 |
| Taq DNA Polymerase | 0.25 |

* see section 2.4.3 for cDNA synthesis.

### 2.4.6 Gel Electrophoresis

#### *2.4.6.1 Agarose gel electrophoresis*

PCR and RT-PCR reactions (see section 2.4.4) were checked for products and their approximate concentrations on 1.5% agarose (Biozym, Oldendorf, Germany) gels prepared in 1x TAE buffer (see section 2.4.1.4) and run at 100 V for 1.5 h in 1x TAE buffer followed by 15 min staining with ethidium bromide (0.5 mg $l^{-1}$).

### *2.4.6.2 Polyacrylamide gel electrophoresis (PAGE)*

6% non-denaturing polyacrylamide gels (ratio of acrylamide to bisacrylamide, 29:1) were prepared with the following constituents as described by Sambrook and Russell (2001).

| | |
|---|---|
| 30% Acrylamide solution (Bio-Rad Laboratories, Munich, Germany) | 7.98 ml |
| $dH_2O$ | 23.74 ml |
| 5x TBE (see section 2.4.1.5) | 8.00 ml |
| 10% ammonium persulphate (APS) | 250 µl |
| TEMED | 17 µl |

Appropriate volumes containing about 2 µg of RAP-PCR products (see section 2.4.5), measured by absorbance at 260 nm, were loaded. The gels were electrophoresed at 50 V for 17 h in 1x TBE (see section 2.4.1.5) using D-Gene system (Bio-Rad Laboratories, Munich, Germany).

### *2.4.6.3 Denaturing gradient gel electrophoresis (DGGE)*

DGGE was performed using 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 37:1) with a gradient of 48 - 58% denaturant for bacteria and 35 – 40% denaturant for fungi as in Table 6. 100% denaturant is defined as 7 M urea plus 40% formamide (Abrams and Stanton, 1992). Appropriate volumes containing about 2 µg of the purified PCR and RT-PCR products (see section 2.4.4), 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).

**Table 6.** Amounts of various components used in DGGE.

| Percentage of denaturant | Component | Volume of component |
|---|---|---|
| 35% | 0% | 7.8 ml |
| | 100% | 4.2 ml |
| | APS | 50 µl |
| | TEMED | 10 µl |
| 40% | 0% | 7.2 ml |
| | 100% | 4.8 ml |
| | APS | 50 µl |
| | TEMED | 10 µl |
| 48% | 0% | 6.2 ml |
| | 100% | 5.8 ml |
| | APS | 50 µl |
| | TEMED | 10 µl |
| 58% | 0% | 5 ml |
| | 100% | 7 ml |
| | APS | 50 µl |
| | TEMED | 10 µl |
| Stacking Solution | 0% | 8 ml |
| | APS | 35 µl |
| | TEMED | 8 µl |

### 2.4.7 Silver staining

PAGE (see section 2.4.6.2) and DGGE (see section 2.4.6.3) gels were silver stained using a modified version of the protocol described by Heukeshoven and Dernick, 1986 (see Table 7).

### 2.4.8 Image analysis

Dried PAGE (see section 2.4.6.2) and DGGE (see section 2.4.6.3) gels were scanned using HP Scanjet 7400c. The obtained RAP-PCR and DGGE profiles were analysed by clustering via the unweighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) using GelCompar II Software (Applied Maths, Kortrijk, Belgium). The position

tolerance was set at 1% and background subtraction was applied. Both strong and weak bands were included in the analysis, thus taking into account only the presence and absence of bands at specific positions. Cophenetic correlation, which is the measure of the consistency of the cluster, was calculated using the same software.

**Table 7.** Protocol for silver staining.

| Solution | Components | Duration |
|---|---|---|
| **Fixing** | | >30 min |
| Glacial acetic acid | 25 ml | |
| Milli Q water | 225 ml | |
| **Washing** | | 3 x 2 min |
| Milli Q water | 250 ml | |
| **Silver reaction** | | 25 min |
| $AgNO_3$ | 0.37 g | |
| Formaldehyde (37% w/v) | 0.25 ml | |
| Milli Q water | 250 ml | |
| **Washing** | | 2 x 1 min |
| Milli Q water | 250 ml | |
| **Developing** | | 5 min or more |
| $Na_2CO_3$, anhydrous | 6.25 g | |
| Formaldehyde (37% w/v) | 0.25 ml | |
| Sodium thiosulphate (2% w/v)* | 0.25 ml | |
| Milli Q water | adjust vol. to 250 ml | |
| **Stopping** | | 10 min |
| EDTA-$Na_2$ x 2H2O | 3.65 g | |
| Milli Q water | adjust vol. to 250 ml | |
| **Preserving** | | >30 min |
| Glycerol (100%) | 30 ml | |
| Ethanol | 75 ml | |
| Milli Q water | 195 ml | |

*Sodium thiosulphate stock solution (2% w/v):
$Na_2S_2O_3$ x $5H_2O$ 3.14 g
Milli Q water adjust vol. to 100 ml and filter sterilize

### 2.4.9 Cloning

RAP-PCR products (see section 2.4.5) and purified 18S RT-PCR products (see section 2.4.4) were cloned into pCR®2.1 vector of TA Cloning Kit (Invitrogen, Karlsruhe, Germany) as described below.

| | |
|---|---|
| Purified RAP-PCR / RT-PCR product | 3 µl (approx. 150 ng) |
| 10X ligation buffer | 1 µl |
| Sterile water | 3 µl |
| pCR®2.1 vector (25ng/µl) | 2 µl |
| T4 DNA ligase | 1 µl |

Ligations were incubated at 14°C overnight. Purified 16S RT-PCR products (see section 2.4.4) were cloned into pCR®-Blunt II-TOPO® vector of Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Karlsruhe, Germany) as described below.

| | |
|---|---|
| Purified RT-PCR product | 3 µl (approx. 150 ng) |
| Salt Solution | 1 µl |
| Sterile water | 1 µl |
| pCR®-Blunt II-TOPO® vector | 1 µl |

This ligation mix was incubated for 30 min at room temperature.

2 µl of ligation mixture was transformed into chemically competent One Shot® DH5α™-T1$^R$ cells provided in the kit following manufacturer's instructions. Colonies were inoculated in LB medium (see section 2.4.1.2; supplemented with 50 µg ml$^{-1}$ kanamycin). 100 µl of Xgal solution (Qbiogene, Germany) was spread on the plates to be used for the plating of transformants of RAP-PCR and 18S RT-PCR products. Plasmids were isolated using Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany). Purified plasmids were tested for inserts by *Eco*RI digestion (MBI Fermentas, Heidelberg, Germany). Digestion was set using the following protocol.

| | |
|---|---|
| 10x Buffer *Eco*RI | 2 µl |
| Plasmid | 5 µl |
| Restriction enzyme (10 U $\mu l^{-1}$) | 0.1 µl |
| Nuclease-free water | 12.9 µl |

Digestion was incubated at 37°C for 1 h. This was followed by inactivation of the restriction enzyme at 65°C for 10 min. The digested products were checked on 1.5% agarose (see section 2.4.6.1) at 100 V for 1 h.

### 2.4.10 Restriction fragment length polymorphism (RFLP)

Plasmids were digested in a final volume of 20 µl by *Msp*I (MBI Fermentas, Heidelberg, Germany) using the following protocol.

| | |
|---|---|
| 10x Buffer $Y^+$/Tango | 2 µl |
| Plasmid (see section 2.4.9) | 5 µl |
| Restriction enzyme (10 U $\mu l^{-1}$) | 0.1 µl |
| Nuclease-free water | 12.9 µl |

Digestions were incubated at 37°C for 4 h. This was followed by inactivation of the restriction enzyme at 65°C for 10 min. The digested products were checked on 4% high resolution agarose (Qbiogene, Heidelberg, Germany) at 100 V for 2 h. Preparation of high resolution agarose gel was the same as described for normal agarose gel in section 2.4.6.1.

### 2.4.11 Sequencing and sequence analysis

Inserts were sequenced on ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, USA) using CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, CA). The following protocol was followed.

| | |
|---|---|
| DTCS Quick Start Master Mix | 8 µl |
| -47 Sequencing Primer (1.6 pmol $\mu l^{-1}$; included in kit) | 2 µl |
| Template | 135-150 ng |
| $dH_2O$ | adjust vol. to 20 µl |

Thermal cycling programme followed was: 96°C for 20 sec, 50°C for 20 sec, 60°C for 4 min for 30 cycles. Ethanol precipitation was performed as described below.

- 4 µl of stop solution (1.5 M NaOAc + 50 mM EDTA prepared fresh by mixing equal volumes of 3 M NaOAc and 100 mM EDTA) and 1 µl of 20 mg $ml^{-1}$ glycogen was added to each reaction.
- 60 µl cold 95% (vol/vol) ethanol / $dH_2O$ was added and centrifuged at 14,500 x g at 4°C for 15 min. The supernatant was discarded and the pellet rinsed twice with 200 µl of 70% (vol/vol) ethanol / $dH_2O$ and centrifuged at 14,500 x g at 4°C for 2 min.
- Pellets were vacuum dried for 40 min and resuspended in 40 µl of sample loading solution (provided in the kit) before loading on to the sequencer.

Sequences were compared with NCBI BLAST and assigned to different bacterial and fungal groups following NCBI classification.

### 2.4.12 Rarefaction analysis

Rarefaction is used to estimate diversity (and place confidence limits on diversity) if sample size had been lower than it actually was. Rarefaction curves were generated using the software "Analytic Rarefaction" provided on the web (http://www.uga.edu/~strata/software/Software.html). The program uses the rarefaction equations for the expected number of species (E) given by Hurlbert (1971) and for the variance of the expected number of species (Var) given by Heck et al. (1975).

### 2.4.13 Nucleotide sequence accession numbers

The clone sequences determined in this study have been submitted to GenBank under accession numbers AY556485–AY556534, AY681354–AY681471, AY682123–AY682194 and AY683239–AY683321.

# 3. RESULTS

## 3.1 Chemical characteristics of litter produced in greenhouse (green litter)

Total C, total N, lignin, sugars, phenolics and mineral nutrients were measured by different collaborators of the project. The calculated C:N and lignin:N ratios are presented together with lignin, phenolics and sugar amounts in table 8. Differences between the deciduous (beech) and coniferous (spruce) litter were evident by higher lignin amounts and lignin:N ratios in beech litter. Higher values of C:N, sugars and phenolics were observed for spruce litter compared to beech. Ozone stress to the plants also affected the litter quality of both species. Higher values were observed for lignin amounts and lignin:N ratio of litter from ozone stressed plants. Additionally, reduced sugar levels were observed for litter from ozone stressed plants compared to the control litter.

**Table 8.** Chemical characteristics of dried, undegraded, green, beech and spruce litter.

| Litter type | C:N | Lignin (mg/g) | Lignin:N | Sugars* (mg/g) | Phenolics (µmol/g) |
|---|---|---|---|---|---|
| BOG | 22 | 317 | 15 | 51 | 39 |
| BCG | 21 | 298 | 13 | 68 | 37 |
| SOG | 30 | 170 | 11 | 76 | 117 |
| SCG | 27 | 142 | 8 | 92 | 117 |

*sugar levels mentioned are the sum of glucose, xylose, fructose and sucrose.

BOG: **b**eech litter, **o**zone stressed, from **g**reenhouse; BCG: **b**eech litter, **c**ontrol, from **g**reenhouse; SOG: **s**pruce litter, **o**zone stressed, from **g**reenhouse; and SCG: **s**pruce litter, **c**ontrol, from **g**reenhouse, respectively.

[Sugars were measured by F. Fleischmann, Technical University Munich, Life Science Centre, Weihenstephan; Lignin amounts were measured by W. Heller, Institute for Biochemical Plant Pathology and total C and N were measured by F. Buegger, Institute of Soil Ecology, GSF-National Research Center for Environment and Health, Neuherberg; Measurements were made in duplicates and variability between replicates was <5%.]

Levels of mineral nutrients Mg, K, Fe and Zn are shown in figure 6. Beech and spruce litter differed with respect to the Mg and Fe amounts while the amounts of K and Zn were comparable for both species. Effects of ozone on litter quality were evident by the reduced levels of Mg and K in the litter from ozone stressed plants compared to the control litter (Figure 6a). An opposite trend was observed for the levels of Fe, where higher levels were found in litter collected from ozone stressed plants (Figure 6b). These trends were observed for both beech and spruce litter.

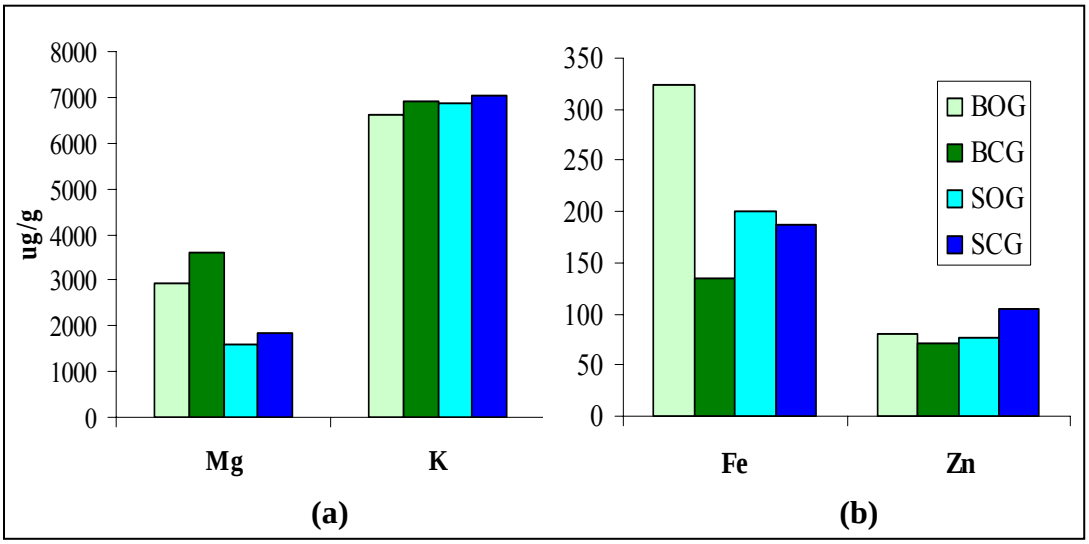


**Figure 6.** Levels of nutrients in control and ozone stressed dried, undegraded, green, beech and spruce litter. **(a)** macronutrients (Mg, K); **(b)** micronutrients (Fe, Zn).
BOG: **b**eech litter, **o**zone stressed, from **g**reenhouse; BCG: **b**eech litter, **c**ontrol, from **g**reenhouse; SOG: **s**pruce litter, **o**zone stressed, from **g**reenhouse; and SCG: **s**pruce litter, **c**ontrol, from **g**reenhouse, respectively.
[Mineral nutrients were determined by A. Goettlein].

### 3.2 Chemical characteristics of litter from Kranzberger forest (natural litter)

As done for green litter, total C, total N, lignin, sugars, phenolics and mineral nutrients were measured for natural litter also. They are tabulated and presented in table 9. In contrast to the results with green litter, higher lignin and lignin:N ratios were observed for spruce litter compared to beech. Natural litter also had more than 10 fold reduced sugar levels and here, the levels were comparable for both the species. Phenolics were also present in reduced amounts when compared to the green litter and were in the same order for both beech and spruce litter. Similar to the results obtained with green litter, higher values were observed for lignin amounts and lignin:N ratio of litter from ozone stressed trees. Sugar levels also showed a trend similar to that observed with green litter. Thus, important parameters that determine the rate of decay (lignin and lignin:N) showed similar trends in response to ozone stress in both green litter removed from the plants grown in the greenhouse and natural litter collected from the forest.

**Table 9.** Chemical characteristics of dried, undegraded, natural, beech and spruce litter.

| Litter type | C:N | Lignin (mg/g) | Lignin:N | Sugars* (mg/g) | Phenolics (µmol/g) |
|---|---|---|---|---|---|
| BOF | 31 | 787 | 52 | 4 | 15 |
| BCF | 29 | 660 | 45 | 5 | 13 |
| SOF | 34 | 840 | 60 | 4 | 11 |
| SCF | 32 | 803 | 58 | 5 | 12 |

*sugar levels mentioned are the sum of glucose, xylose, fructose and sucrose.

BOF: **b**eech litter, **o**zone stressed, from Kranzberger **f**orest; BCF: **b**eech litter, **c**ontrol, from Kranzberger **f**orest; SOF: **s**pruce litter, **o**zone stressed, from Kranzberger **f**orest; and SCF: **s**pruce litter, **c**ontrol, from Kranzberger **f**orest, respectively.
[Sugars were measured by F. Fleischmann, Technical University Munich, Life Science Centre, Weihenstephan; Lignin amounts were measured by W. Heller, Institute for Biochemical Plant Pathology and total C and N were measured by F. Buegger, Institute of Soil Ecology, GSF-National Research Center for Environment and Health, Neuherberg; Measurements were made in duplicates and variability between replicates was <5%.]

Beech and spruce litter also differed with respect to the concentrations of the four mineral nutrients (Figure 7). Ozone stress also influenced the levels of mineral nutrients in both beech and spruce. High levels of Mg and K were observed for ozone stressed litter of both species (Figure 7a). This is in contrast to what was observed for green litter where ozone stress resulted in reduced concentrations of Mg and K.

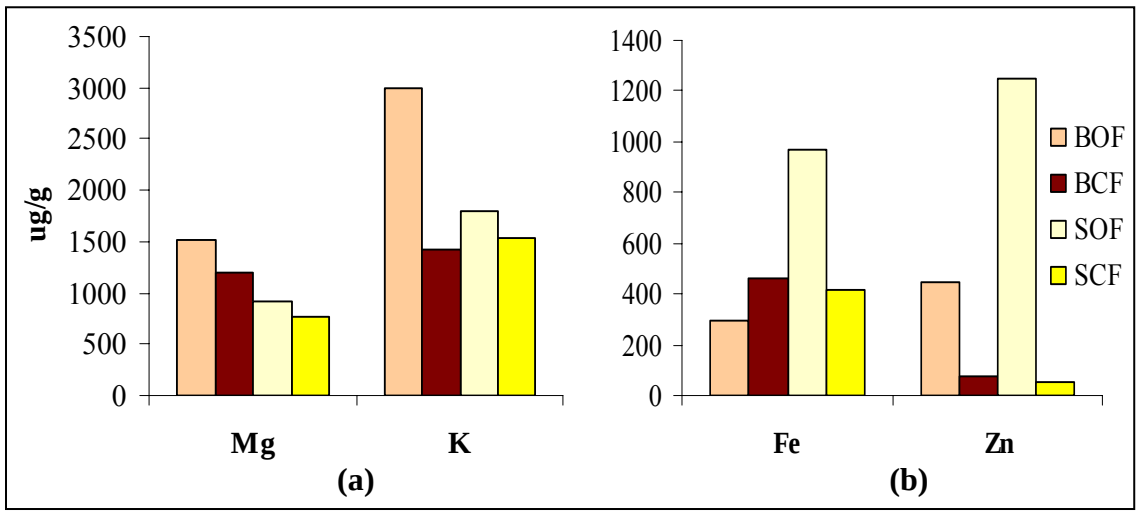


**Figure 7.** Levels of nutrients in control and ozone stressed dried, undegraded, natural, beech and spruce litter. **(a)** macronutrients (Mg, K); **(b)** micronutrients (Fe, Zn).
BOF: **b**eech litter, **o**zone stressed, from Kranzberger **f**orest; BCF: **b**eech litter, **c**ontrol, from Kranzberger **f**orest; SOF: **s**pruce litter, **o**zone stressed, from Kranzberger **f**orest; and SCF: **s**pruce litter, **c**ontrol, from Kranzberger **f**orest, respectively.
[Mineral nutrients were determined by A. Goettlein].

Levels of Fe showed different patterns in beech and spruce natural litter. Reduced Fe levels were observed for beech litter from ozone stressed trees compared to the control counterpart (Figure 7b) whereas for spruce litter, the trend was opposite. High levels of Zn were found in both beech and spruce litter from ozone stressed trees compared to control litter. Such effects of ozone on Zn concentrations were not observed in green litter.

### 3.3 Detection of RNA in dried undegraded litter by RAP-PCR

Presence of RNA in dried, undegraded litter was checked by RAP-PCR technique as described in section 2.4.5. No RAP-PCR products could be observed for any of the replicates of beech or spruce litter (both green and natural litter), thus negating the presence of RNA in dried undegraded litter material (Figure 8).

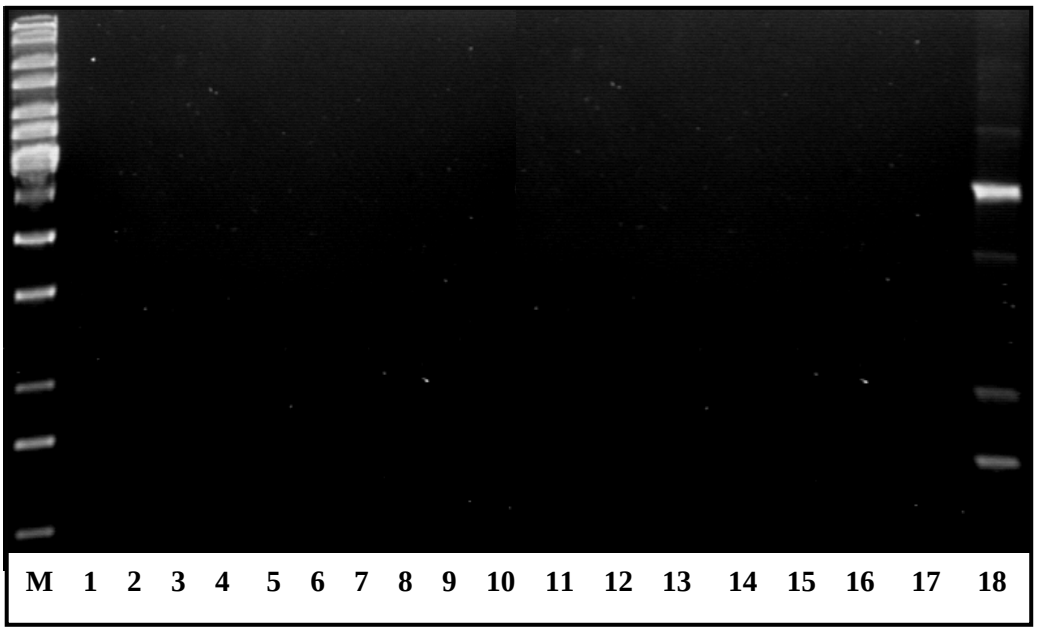


**Figure 8.** RAP-PCR profiles with dried, undegraded, beech and spruce litter.
Lane M: 1 kb DNA ladder; lanes 1 & 2 – replicate samples of control, green beech litter; lanes 3 & 4 – replicate samples of ozone stressed, green beech litter; lanes 5 & 6 - replicate samples of control, green spruce litter; lanes 7 & 8 - replicate samples of ozone stressed, green spruce litter; lanes 9 & 10 - replicate samples of control, natural beech litter; lanes 11 & 12 - replicate samples of ozone stressed, natural beech litter; lanes 13 & 14 - replicate samples of control, natural spruce litter; lane 15 & 16 - replicate samples of ozone stressed, natural spruce litter; lane 17 – negative control; lane 18 – cDNA of *E.coli* used as positive control.

### 3.4 Degradation of control green litter in non-forest and forest soil sites

#### 3.4.1 Microbial communities in the four different soil sites

Soil samples from the four sites selected for the experiment (see section 2.2.1) yielded discrete bands with the predicted size of 473 bp (433 bp insert + 40 bp GC-clamp) for 16S rDNA and rRNA (Figure 9). BSA and DMSO had to be added to the amplification reaction during optimisation to prevent inhibitory effects of organic matter and humic substances present in soil and for efficient denaturation.

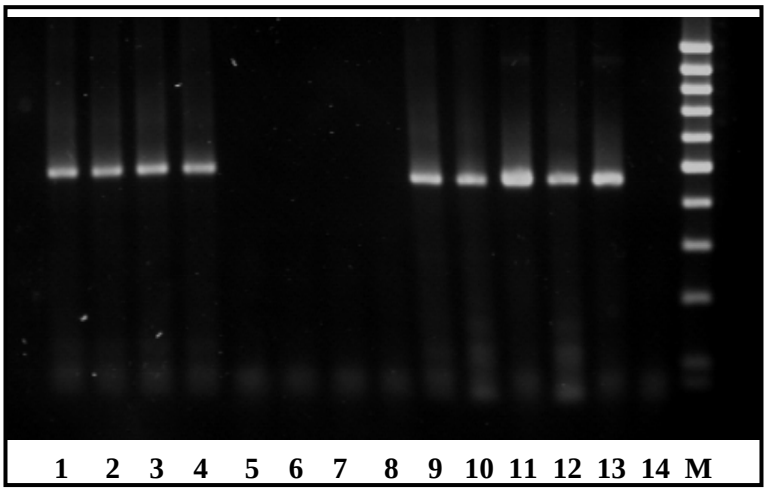


**Figure 9.** Agarose gel of PCR and RT-PCR fragments for 16S rDNA and rRNA from soil samples.
Lanes 1, 2, 3, 4 - PCR products for grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**) sites, respectively; lanes 5, 6, 7, 8 - DNase$^{+}$RT$^{-}$ PCR products for **G**, **M**, **B** and **BS** sites respectively; lanes 9, 10, 11, 12 - RT-PCR products for **G**, **M**, **B** and **BS** sites respectively; lane 13 - PCR with *E.coli* as positive control; lane 14 - negative control; lane M: 100 bp marker.

Positive results, with the desired band size of 601 bp (561 bp insert + 40 bp GC-clamp), were also obtained for the four soil sites with 18S specific primer after standardizing the amount of BSA and DMSO required for the protocol (Figure 10).

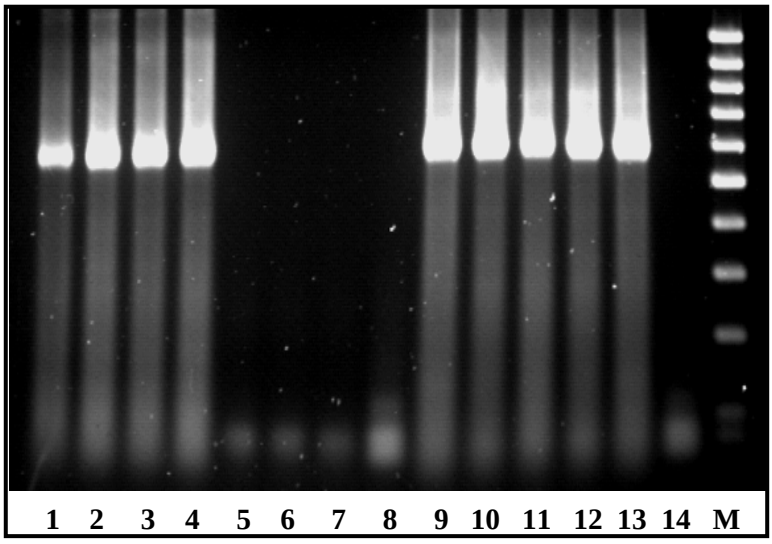


**Figure 10.** Agarose gel of PCR and RT-PCR fragments for 18S rDNA and rRNA from soil samples.
Lanes 1, 2, 3, 4 - PCR products for grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**) sites, respectively; lanes 5, 6, 7, 8 - $DNase^{+}RT^{-}$ PCR products for **G**, **M**, **B** and **BS** sites respectively; lanes 9, 10, 11, 12 - RT-PCR products for **G**, **M**, **B** and **BS** sites respectively; lane 13 - PCR with DNA of *Trichoderma* sp. as positive control; lane 14 - negative control; lane M: 100 bp marker.

PCR and RT-PCR products were purified using QIAquick PCR purification kit (Qiagen, Hilden) to get rid of single stranded nucleic acids and primer artefacts. Purified 16S PCR and RT-PCR products from each of the four soil sites were resolved by denaturing gradient gel electrophoresis (DGGE) on a denaturant gradient of 48 – 58% to compare the total and active bacterial communities of the different sites. Comparable number of bands (15 - 20) was obtained in the DNA derived profiles for all the four sites (Figure 11). Distinct differences were observed between the DNA and RNA derived profile for a given soil site implying differences between the total and active fraction of bacteria. On comparing the profiles with the GelCompar II software, two separate clusters were obtained, one formed by the profiles derived from forest sites and the other by the non-forest sites. These two major clusters were approximately 60% similar to each other. Within each cluster, the DNA and RNA derived profiles of the same soil site clustered together. Higher similarity values were observed between the DNA and RNA derived profiles for the non-forest sites compared to the forest sites. Similar clustering of the profiles was observed with replicate soil samples and also multiple nucleic acid extractions from the same sample.

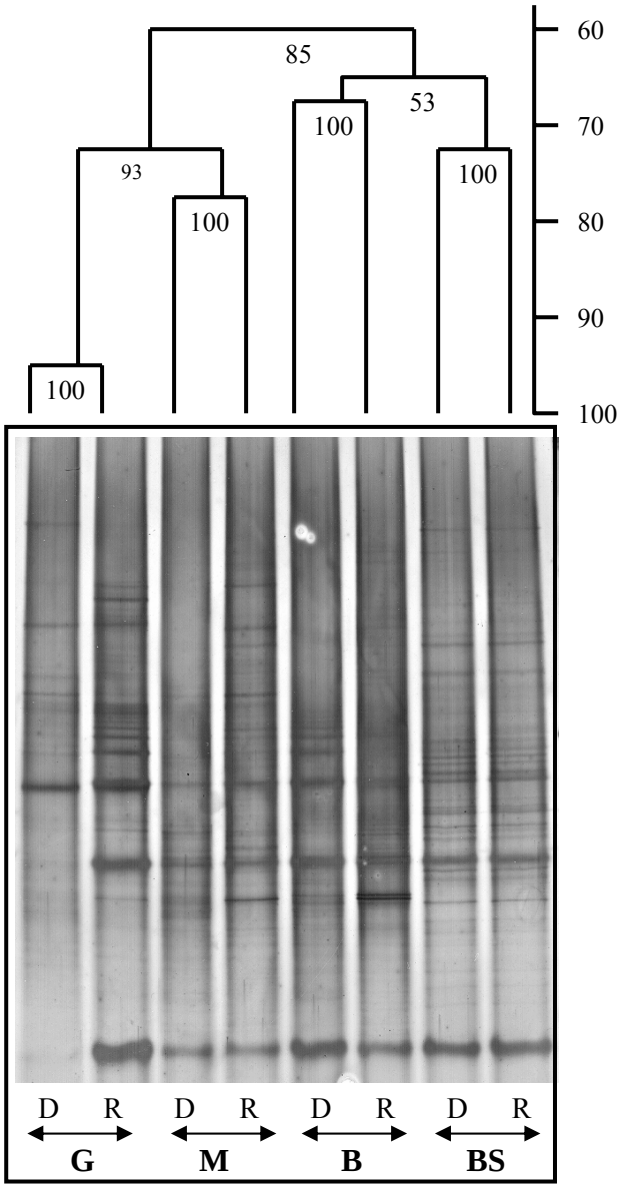


**Figure 11.** Denaturing gradient gel electrophoresis (DGGE) profiles and UPGMA tree showing the similarities between the patterns of 16S PCR and RT-PCR products from the four soil samples.
D and R: DNA and RNA derived profiles, respectively. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**). Scale represents % similarity. Numbers at branch points are the cophenetic correlation values, which express the consistency of the cluster.

As done with 16S products, purified 18S PCR and RT-PCR products were resolved by DGGE on a gradient of 35 – 40% to compare the total and active fungal communities of the four soil sites. The fingerprints from the forest sites had a higher number of bands (20 - 25) indicating them to be more diverse compared to the non-forest sites where the number was reduced to about 15 (Figure 12). As earlier observed for bacterial profiles, cluster analysis of these profiles revealed two major clusters**,** one formed by the forest sites and the other by the non-

forest sites. Within each cluster, distinct differences were observed between the DNA and RNA derived profiles of the same site. Similar clustering pattern was observed with replicate samples of the soil sites and also after multiple nucleic acid extraction of the same sample though the proportion of similarity and differences varied.

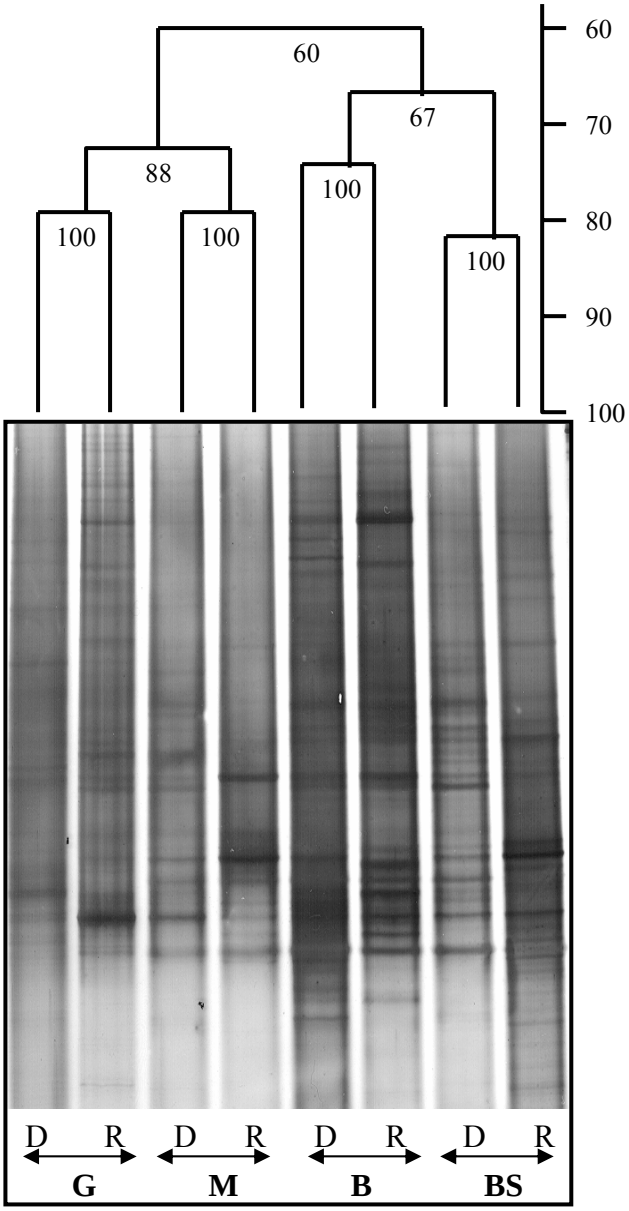


**Figure 12.** DGGE profiles and UPGMA tree showing the similarities between the patterns of 18S PCR and RT-PCR products from the four soil samples.
D and R: DNA and RNA derived profiles, respectively. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**). Scale represents % similarity. Numbers at branch points are the cophenetic correlation values, which express the consistency of the cluster.

Due to these observed differences between the DNA and RNA derived profiles of both bacterial and fungal communities, RNA was targeted for analysis of bacterial and fungal communities colonizing beech and spruce litter at different time points as the main aim of the present study was to identify the active bacterial and fungal colonizers.

### 3.4.2 Degradation rates of litter

Dry weight was measured for beech and spruce litter degraded in a particular soil site for two and eight weeks and used to calculate the degradation rate. Decomposition of both litter types showed a typical two step kinetics, with high degradation rates in the first two weeks followed by reduced rates at eight weeks time point (Table 10). Spruce litter showed a tendency for rapid mineralization compared to beech litter. This turnover pattern was observed for all sites. No clear impact of the soil sites where the bags had been placed, on the loss of litter biomass, could be detected.

**Table 10.** Degradation rates of litter after two and eight weeks of degradation in the four soil sites.

| Litter type | Deg. rate after 2 weeks (mg/day) | | | | Deg. rate after 8 weeks (mg/day) | | | |
|---|---|---|---|---|---|---|---|---|
| | **G** | **M** | **B** | **BS** | **G** | **M** | **B** | **BS** |
| BCG | 85 | 78 | 71 | 78 | 25 | 27 | 25 | 23 |
| SCG | 121 | 114 | 121 | 114 | 37 | 30 | 37 | 41 |

BCG: **b**eech litter, **c**ontrol, from **g**reenhouse; and SCG: **s**pruce litter, **c**ontrol, from **g**reenhouse, respectively. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**). Variation between two replicate bags / sample was <5%.

### 3.4.3 Metabolic profiling of microbial communities

#### *3.4.3.1 Total RNA fingerprinting of microbial communities using RAP-PCR*

Total RNA profiles were generated from two and eight weeks degraded litter using a 10 mer primer. The obtained RAP-PCR products were resolved by PAGE. Higher number of bands (20 - 30) with a size from 300 – 1200 bp was observed for litter degraded in forest sites compared to the non-forest sites (Figure 13). Significant differences in the fingerprints were evident when patterns for the same litter type degraded in different sites and different litter types in the same site were compared. A clear succession of the microbial community during the degradation process between two and eight weeks was also evident. The reproducibility of

the obtained fingerprints from multiple nucleic acid extractions from the same litter bag was very high (similarity values greater than 98% between replicates). In addition, similarity values between true replicates (different litter bags with same litter material, incubated in the same soil for the same period) were significant (>95%).

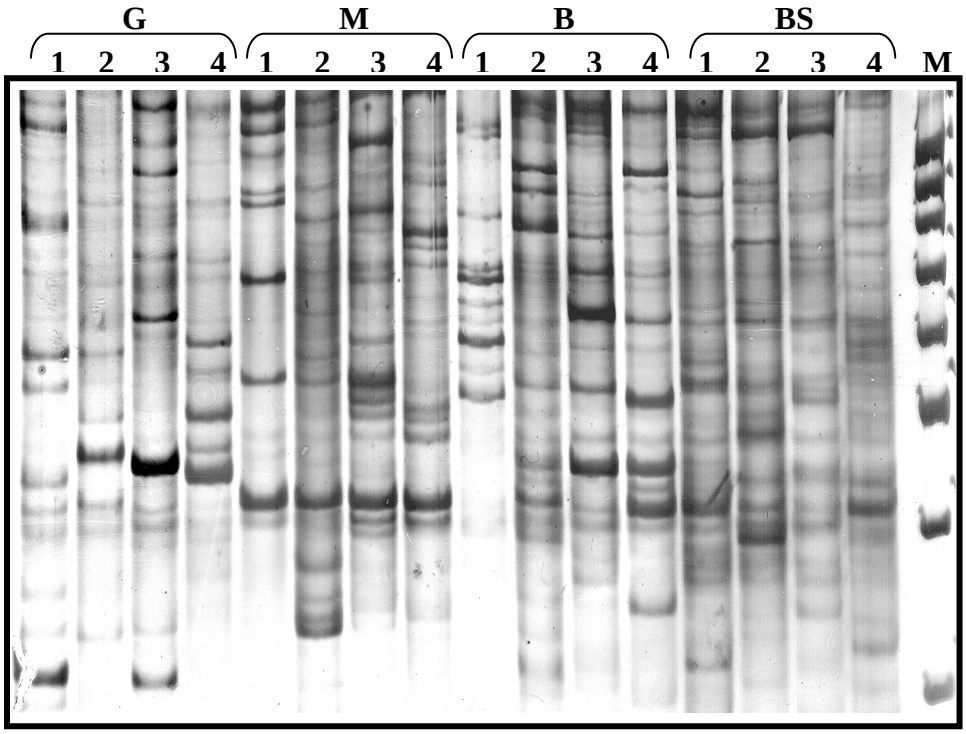


**Figure 13.** RAP-PCR profiles with cDNA from litter degraded for two and eight weeks in the four sites.
lane 1: BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter degraded for two weeks in the respective site; lane 2: BCG litter degraded for eight weeks in the respective site; lane 3: SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) degraded for two weeks in the respective site; lane 4: SCG litter degraded for eight weeks in the respective site; lane M - 100 bp marker. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**).

Cluster analysis of the total RNA fingerprints revealed two major clusters, one formed by the profiles from beech and spruce litter degraded in non-forest soil sites and the other consisting of the profiles from beech and spruce litter degraded in the forest sites (Figure 14). The only exception was beech litter that was incubated for two weeks in the beech forest soil. Such clustering of the non-forest and forest sites was also observed when the bacterial and fungal communities of the four soil sites were compared. These results provided the initial evidence that the microbial community structure and function were different for same litter in different soil sites (soil site effects), for different litter types in the same soil (litter type effects) and were changing over time (succession).

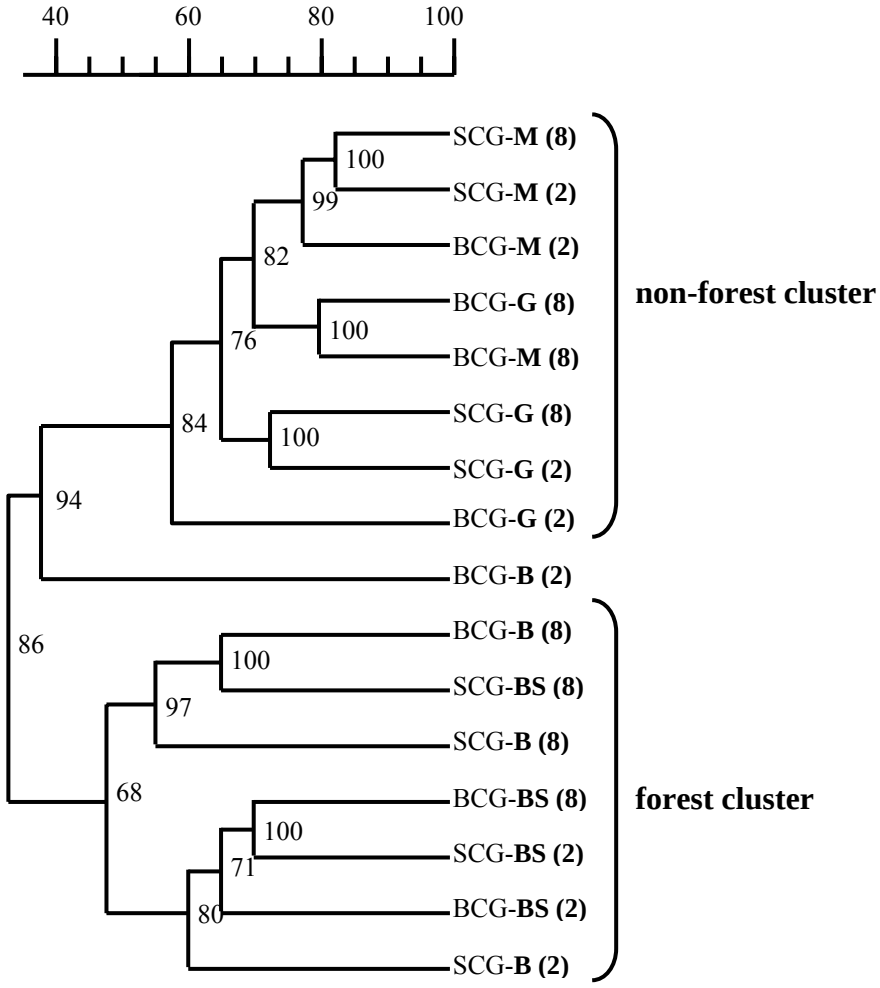


**Figure 14.** UPGMA tree of the RAP-PCR profiles from litter degraded for two and eight weeks in the four different soil sites.
BCG: **b**eech litter, **c**ontrol, from **g**reenhouse; and SCG: **s**pruce litter, **c**ontrol, from **g**reenhouse, respectively. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**). Numbers in parentheses indicate the weeks of degradation. Scale represents percent similarity. Numbers at the branch points are the cophenetic correlation values, which are the measure of consistency of a cluster.

### *3.4.3.2 Clone libraries of RAP-PCR products*

Beech-spruce mixed forest (BS) soil was selected for detailed analysis of the RAP profiles as it was the normal site of degradation for beech and spruce litter. RAP-PCR products for the four samples (beech and spruce litter degraded in BS site for 2 and 8 weeks) were cloned to generate RAP libraries. 100 clones from each library were screened for inserts by *Eco*RI digestion and clones with insert size within 400 – 700 bp were selected for further analysis. These selected clones were screened by RFLP using *Msp*I. In total, twenty different RFLP patterns were observed (Figure 15).

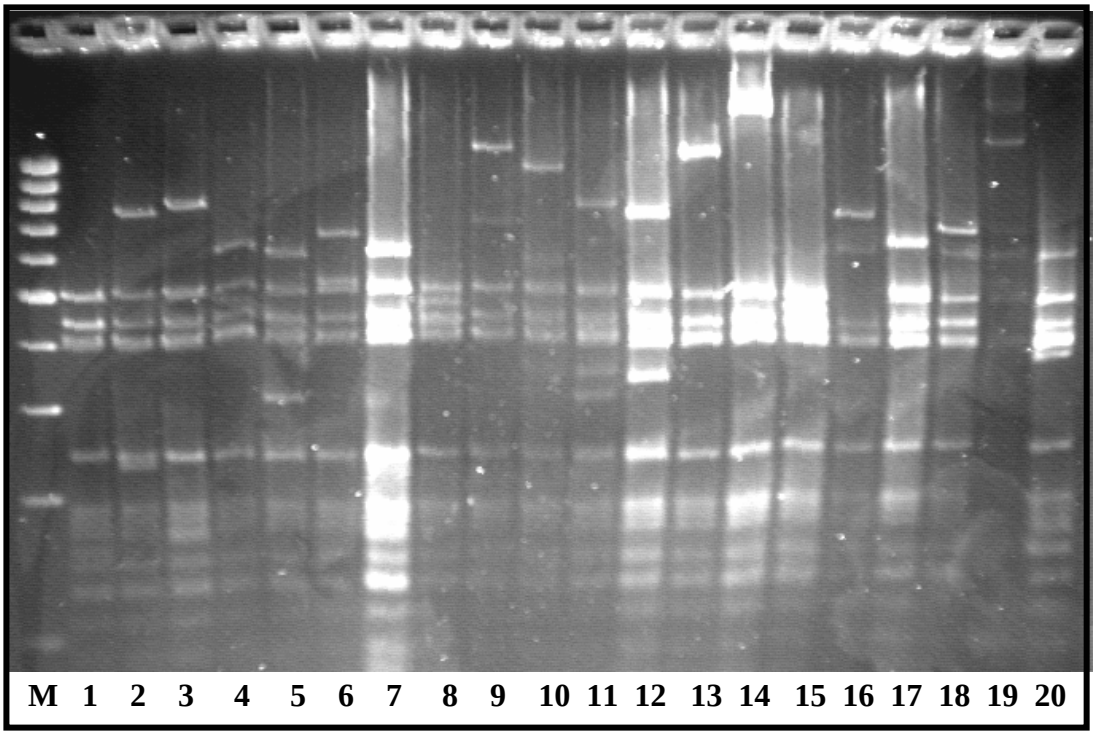


**Figure 15.** 3% high resolution gel showing the 20 different RFLP patterns observed in RAP clone libraries from litter degraded in BS site.
lane M - 100 bp marker; lanes 1 – 20 – patterns 1 to 20.

On sequencing, it was revealed that eleven RFLP patterns showed highest similarity to ribosomal sequences and three had no similarity to any published sequence in the NCBI database (Table 11). The ribosomal transcripts included sequences similar to 16S rRNA sequences of members of β- and γ- subgroups of proteobacteria. Also included were sequences similar to 18S rRNA sequences of members of Ascomycota and Zygomycota. In addition, some sequences similar to 23S rRNA were also obtained. The non-ribosomal fraction of the libraries included sequences similar to that of bacterial esterase, bacterial cytochrome P450 hydroxylase, Excinuclease ABC Subunit A (bacterial DNA repair) and AUR 1 (phospholipid metabolism in *S. cerevisiae*).

**Table 11.** Sequence similarities of different RFLP types, obtained from RAP-libraries of litter degraded in BS site, to known sequences in the NCBI database.

| Pattern | Sequence similarity | Group | % similarity | Accession number |
|---|---|---|---|---|
| **I** | *Trichoderma viride* (18S rRNA) | Ascomycota | 97 | AF548104 |
| **II** | *Tritirachium sp.* (18S rRNA) | Ascomycota | 97 | AY231440 |
| **III** | *Legionella pneumophila* (16S rRNA) | Gamma proteobacteria | 91 | AF134574 |
| **IV** | Uncultured rhizosphere ascomycete (18S rRNA) | Ascomycota | 97 | AJ506006 |
| **V** | *Tritirachium sp.* (18S rRNA) | Ascomycota | 97 | AY231440 |
| **VI** | *Nitrosomonas europaea* (23S rRNA) | Beta proteobacteria | 95 | NC_004757 |
| **VII** | *Melanospora zamiae* (18S rRNA) | Ascomycota | 96 | AY046578 |
| **VIII** | Mycobacterial esterase | - | 44* | AE007022 |
| **IX** | PTPRF**, interacting protein (liprin), alpha 1(PPFIA1) of *H. sapiens* | - | 89* | NM_003626 |
| **X** | *Bradyrhizobium* sp. (16S rRNA) | Alpha proteobacteria | 100 | AB069654 |
| **XI** | no match | - | - | - |
| **XII** | Hypothetical protein from *Gibberella zeae* | - | 33* | XM_382559 |
| **XIII** | *Blastocladiella emersonii* (28S rRNA) | Chytridiomycota | 97 | X90411 |
| **XIV** | *Legionella pneumophila* (16S rRNA) | Gamma proteobacteria | 91 | AF134574 |
| **XV** | *Backusella ctenidia* (18S rRNA) | Zygomycota | 98 | AF157122 |
| **XVI** | Aur1 of *Saccharomyces cerevisiae* | - | 50* | U49090 |
| **XVII** | no match | - | - | - |
| **XVIII** | Probable Excinuclease ABC Subunit A of *Ralstonia solanacearum* | - | 60* | NC_003295 |
| **XIX** | cytochrome P450 hydroxylase of *Streptomyces lavendulae* | - | 40* | AF127374 |
| **XX** | no match | - | - | - |

* Similarity at amino acid level ** Protein tyrosine phosphatase receptor type f

### 3.4.4 Microbial communities colonizing litter after two and eight weeks

#### *3.4.4.1 RT-PCR for 16S and 18S rRNA from degraded litter samples*

Colonizing bacterial and fungal communities were analysed by DGGE of 16S and 18S rRNA RT-PCR products respectively, using litter material from the incubated litter bags. The desired band of 473 bp was obtained for both the litter types in all the four sites. Similar results, with the expected band of 601 bp, were also obtained for all litter samples with 18S specific primer pair (Figure 16).

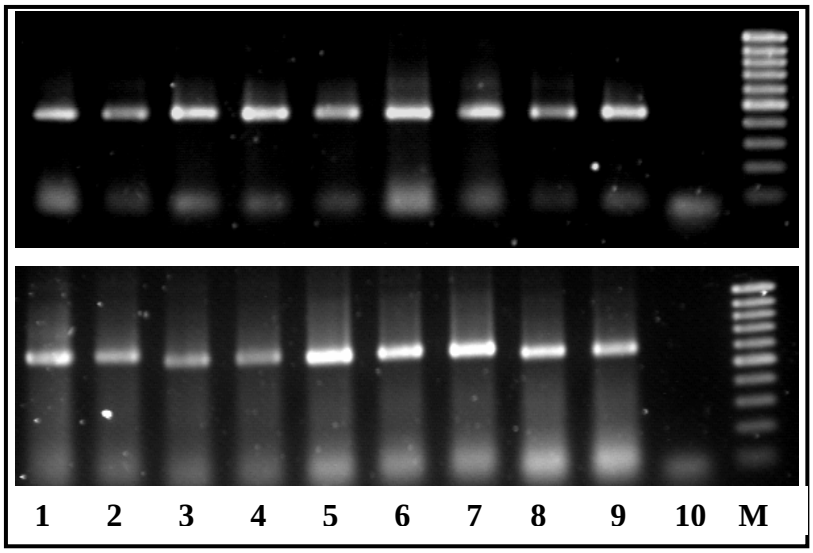


**Figure 16.** Agarose gels of RT-PCR fragments for 16S (upper) and 18S (lower) rRNA from litter degraded in the four sites.
lanes 1, 2, 3, 4 – RT-PCR products for BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter degraded in grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**) sites, respectively; lanes 5, 6, 7, 8 - RT-PCR products for SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter degraded in **G**, **M**, **B** and **BS** sites, respectively; lane 9 – positive control (DNA of *E.coli* for 16S and *Trichoderma* sp. for 18S); lane 10 – negative control, lane M – 100 bp marker.

Archaeabacterial community colonizing both litter types was targeted using specific primers, w036 and w039-GC (see section 2.4.4). The desired band of 582 bp could not be amplified from the degraded litter after the two time points. However, using the same primer pair, desired amplicons could be detected using the cDNA of the four soil sites indicating the group to be active in soil but not colonizing the litter (Figure 17). Hence, no further analysis of the archaeal community was performed.

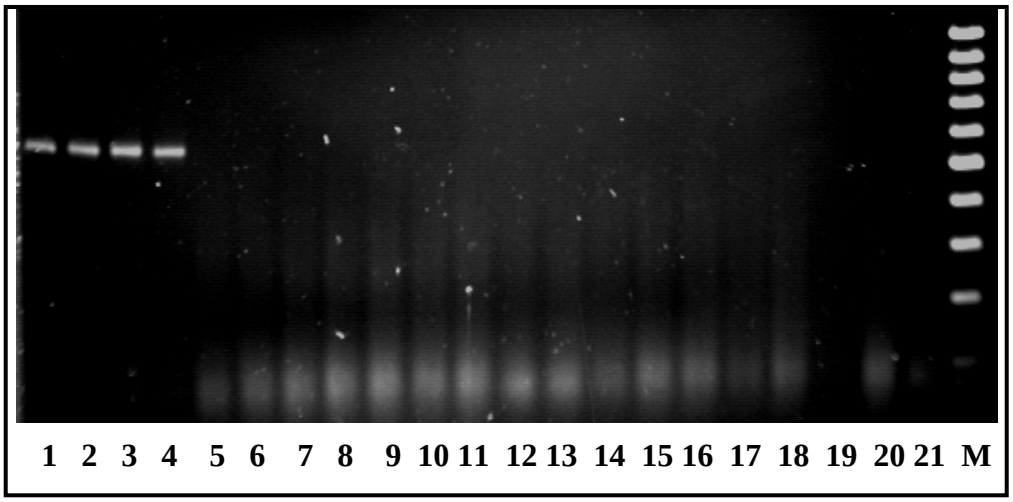


**Figure 17.** Agarose gel of RT-PCR fragments for 16S rRNA specific for archaea from soil samples and litter degraded in the four sites.
lanes 1, 2, 3, 4 – RT-PCR products for grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**) sites, respectively; lanes 5, 6, 7, 8 - RT-PCR products for BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter in **G**, **M**, **B** and **BS** sites respectively for two weeks; lanes 9, 10, 11, 12 - RT-PCR products for SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter degraded in **G**, **M**, **B** and **BS** sites respectively for two weeks; lanes 13, 14, 15, 16 - RT-PCR products for BCG litter degraded in **G**, **M**, **B** and **BS** sites respectively for eight weeks; lanes 17, 18, 19, 20 - RT-PCR products for SCG litter degraded in **G**, **M**, **B** and **BS** sites respectively for eight weeks; lane 21 – negative control, lane M – 100 bp marker.

#### *3.4.4.2. Bacterial communities*

Distinct profiles were obtained for each sample on resolving the purified 16S RT-PCR products by DGGE (Figure 18 a-d). Fingerprints generated from the replicates taken from the same site and at the same time point were highly similar (>95%). Independent from the litter type and the sites of incubation, after two weeks 20 – 25 distinct bands were obtained in all profiles from the extracted rRNA with highest number of bands in the soil profiles. The positions of most of the major bands in the fingerprints from litter samples were different, providing evidence that the structure of the bacterial community is highly dependent on the litter type and the sites of incubation. In contrast, when comparing bacterial DGGE profiles from litter samples, which had been incubated for eight weeks, differences were not only visible with respect to position of bands in the fingerprints but also in the overall number of bands, indicating differences in total diversity and structure of the bacterial communities. The increase in diversity from two to eight weeks was dependent on the soil site. The increase in the number of bands for both beech and spruce litter was comparable in the two non-forest sites while for the forest sites, it was the BS site that supported greater diversity compared to the B site.

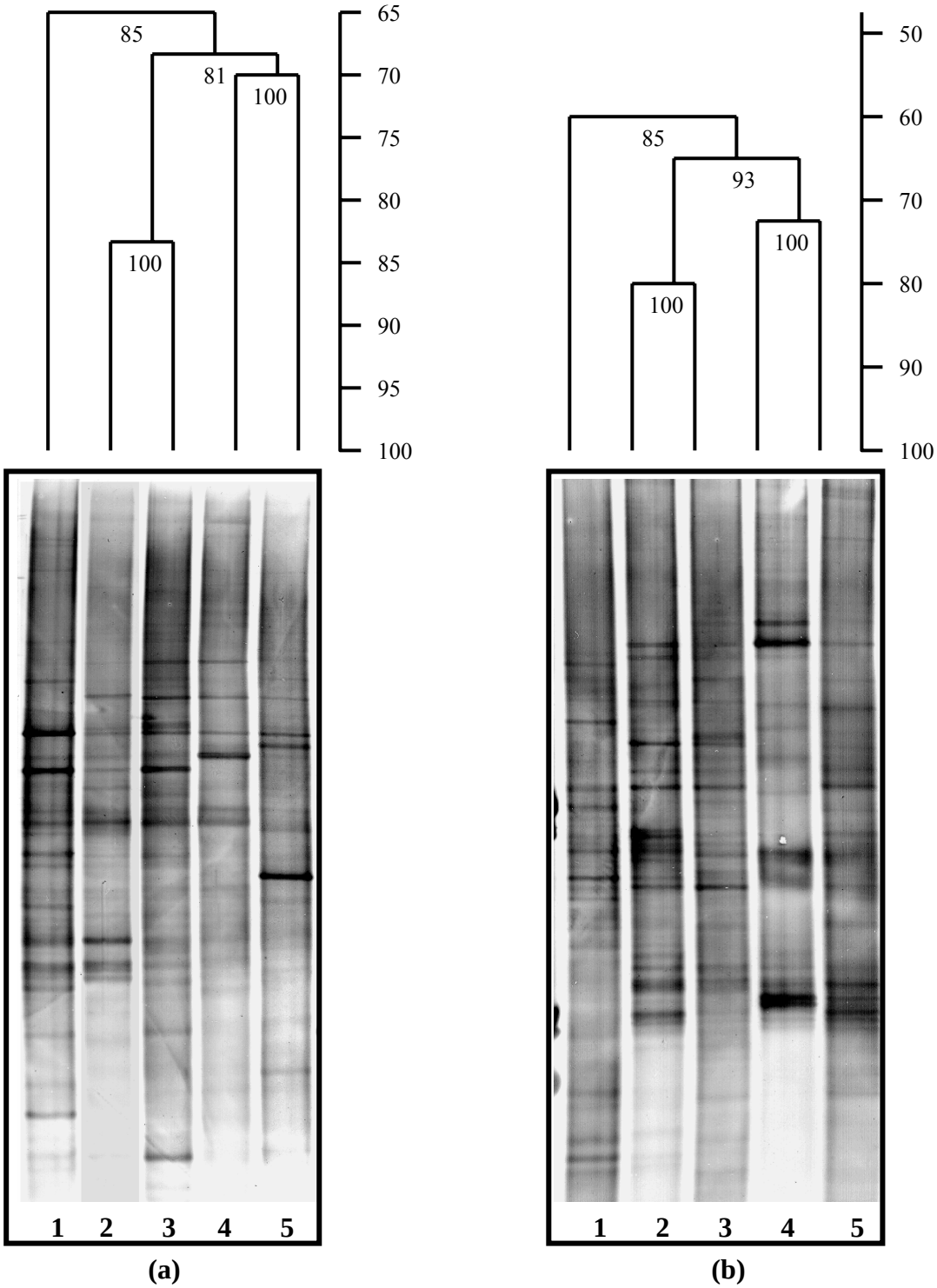


**Figure 18 (a) & (b).** DGGE profiles and UPGMA tree showing the similarities between the patterns of 16S RT-PCR products from litter degraded for two and eight weeks in grass field (**G**) **(a)** and maize field (**M**) **(b)**.
lane 1: soil site; lane 2: BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter after two weeks degradation; lane 3: BCG litter in respective sites after eight weeks degradation; lane 4: SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter after two weeks degradation; lane 5: SCG litter in respective sites after eight weeks degradation. Scale represents % similarity. Numbers at branch points are the cophenetic correlation values, which express the consistency of the cluster.

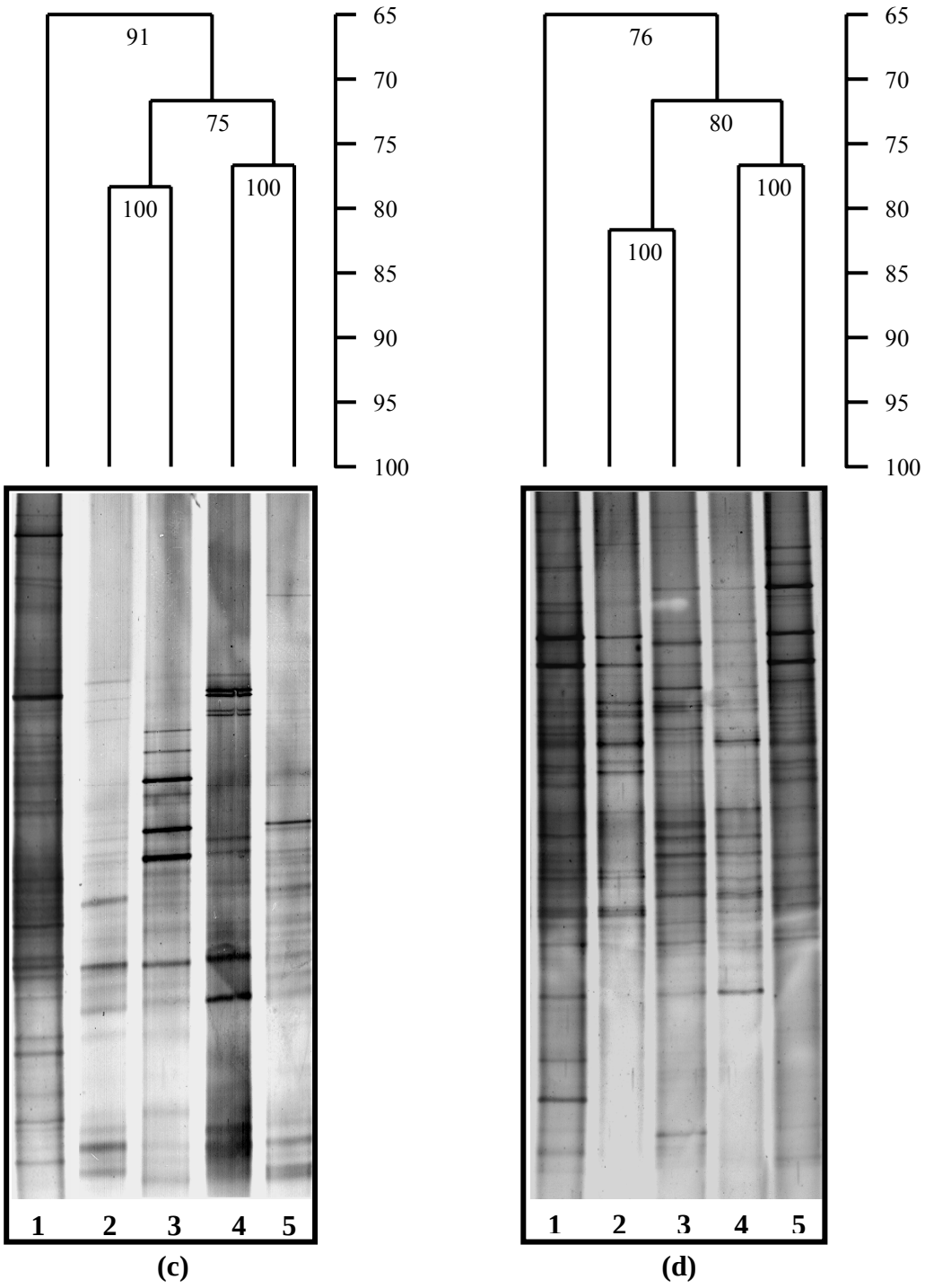


**Figure 18 (c) & (d).** DGGE profiles and UPGMA tree showing the similarities between the patterns of 16S RT-PCR products from litter degraded for two and eight weeks in beech forest site (**B**) **(c)** and beech spruce mixed forest site (**BS**) **(d)**.
lane 1: soil site; lane 2: BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter after two weeks degradation; lane 3: BCG litter in respective sites after eight weeks degradation; lane 4: SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter after two weeks degradation; lane 5: SCG litter in respective sites after eight weeks degradation. Scale represents % similarity. Numbers at branch points are the cophenetic correlation values, which express the consistency of the cluster.

Analysis of the DGGE profiles revealed two clusters, one for each litter type, which were 60 - 65% similar to the profile of the respective soil site, reflecting the selective effect of litter type on the bacterial community. This pattern of clustering was observed for all the four sites.

#### *3.4.4.3 Bacterial clone libraries*

Bacterial RT-PCR products from beech and spruce litter degraded in beech-spruce mixed forest (BS) site were cloned to identify the colonizing bacterial groups and to follow their succession over time. Beech-spruce mixed forest (BS) site was selected for detailed analysis as it was the normal site of degradation for beech and spruce litter. The amplified 394 bp (excluding the primer sequence and GC-clamp) sequences were used to assign the clones to bacterial groups using NCBI BLAST programme. A total of 200 clones were analysed from the four libraries (50 clones each from B2, B8, S2 and S8). Figure 19 shows the broad phylogenetic distribution of clones within each library. Sequences showing a match of 96% or higher to any member of a known bacterial group were assigned to that particular group. Majority of the clones were highly similar (>96% homology) to members of different subgroups of proteobacteria. Sequences similar to members of other groups such as Acidobacteria, Actinobacteria and Gemmantimonadetes were also present, though in less proportions (<25%). Clones similar to sequences reported in earlier studies, but mentioned as "unclassified" in the NCBI database, have been grouped together as "unclassified" in the present study. After two weeks of incubation, beech and spruce libraries had much more bacterial groups in common as compared to eight weeks libraries indicating litter type to become the selecting parameter by the second time point (eight weeks). In beech libraries, clones similar to γ-proteobacteria members were the most dominant and their proportion increased after eight weeks. Clones similar to members of α- and β-subdivision of proteobacteria, Actinobacteria and Acidobacteria were present in two weeks library but were absent from libraries after eight weeks. In spruce libraries, most of the clones had sequences similar to members of α-, β-, γ- and δ-proteobacteria. There was a much higher increase in the proportion of clones similar to α-proteobacteria members as compared to the increase in the proportion of γ-proteobacteria in eight weeks library. In addition, a decrease in the proportion of clones similar to β- and δ-proteobacteria members was observed for eight weeks degraded spruce litter.

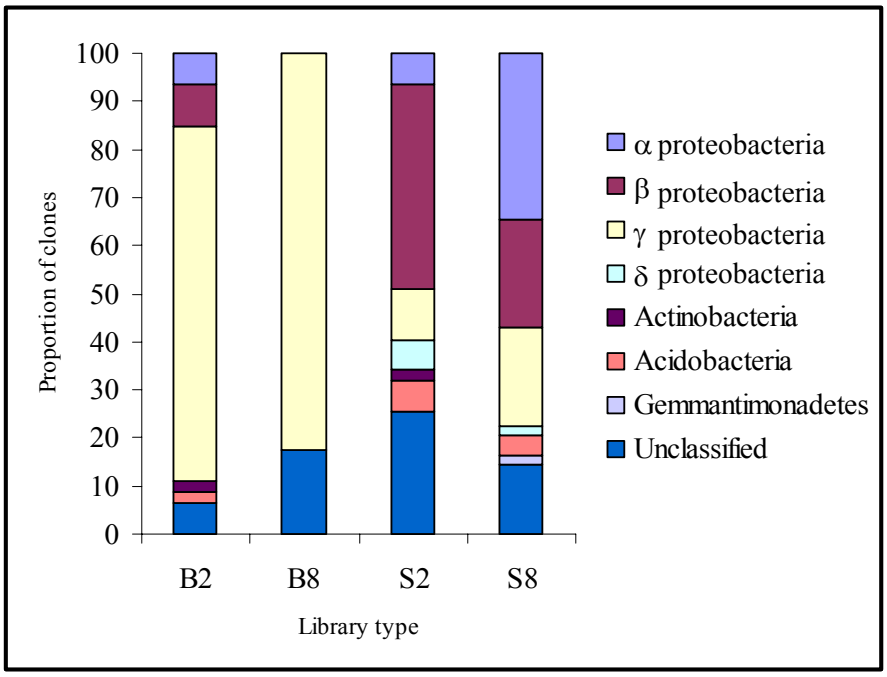


**Figure 19.** Relative distributions of clones to different bacterial groups from litter degraded in BS soil site.
B2 and B8: libraries of BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter degraded in beech-spruce mixed forest (**BS**) site for two and eight weeks, respectively; S2 and S8: libraries of SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter degraded in **BS** site for two and eight weeks, respectively.

### *3.4.4.4 Rarefaction curves of bacterial clone libraries*

To assess the extent of bacterial diversity targeted in the clone libraries, number of bacterial groups observed in a library was plotted as a function of the number of clones screened by analytical rarefaction software (Figure 20). A plateau, as expected for full coverage of library, was obtained after screening 44 clones for beech litter after 2 weeks (B2), 12 clones for beech litter after 8 weeks (B8), 42 clones for spruce litter after 2 weeks (S2) and 46 clones for spruce litter after 8 weeks (S8). Therefore, 50 clones screened per library are true representation of the bacterial diversity present in the litter bags.

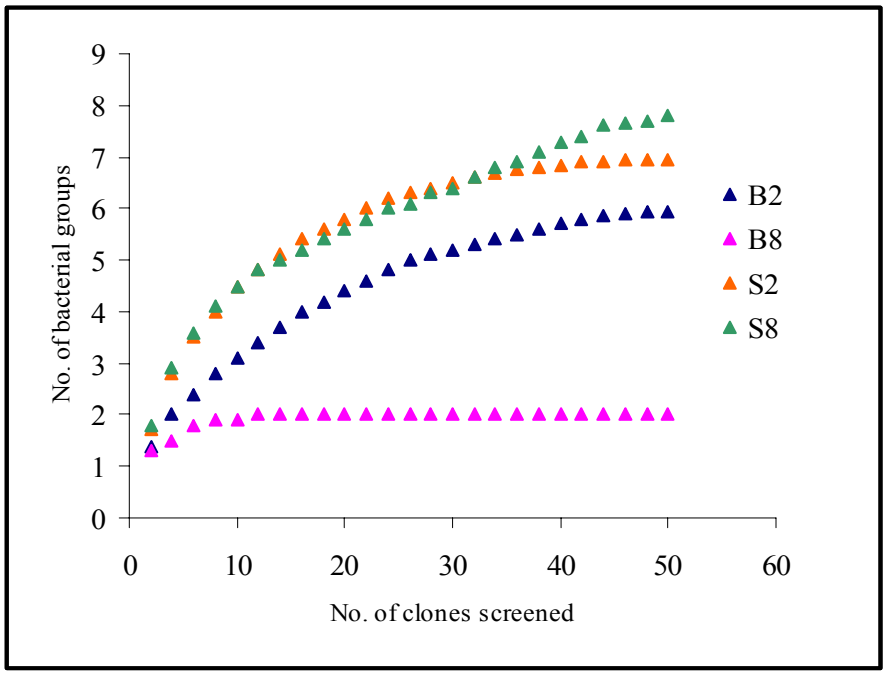


**Figure 20.** Rarefaction curves showing the extent of bacterial diversity covered by the four libraries.
B2 and B8: libraries of BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter degraded in beech-spruce mixed forest (**BS**) site for two and eight weeks, respectively; S2 and S8: libraries of SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter degraded in **BS** site for two and eight weeks, respectively.

### *3.4.4.5 Fungal communities*

All litter samples yielded positive 18S RT-PCR products of 601 bp, with the primer pair used (Figure 16). On resolving these RT-PCR products by DGGE, distinct profiles were obtained for each sample (Figure 21 a-d). Fingerprints generated from the replicates taken from the same site and at the same time point were more than 95% similar. As earlier observed for bacterial DGGE profiles, high number of bands were observed in the soil profiles (about 20) compared to litter samples (10 - 15). However, the increase in diversity from two to eight weeks, as was observed for bacterial profiles from both beech and spruce litter in all the four sites, was not revealed in the fungal profiles. On comparing the obtained DGGE profiles, two major clusters, one for each litter type, were obtained for each of the four sites. Similar clustering was observed when the bacterial 16S rRNA profiles were compared though the proportion of similarity varied.

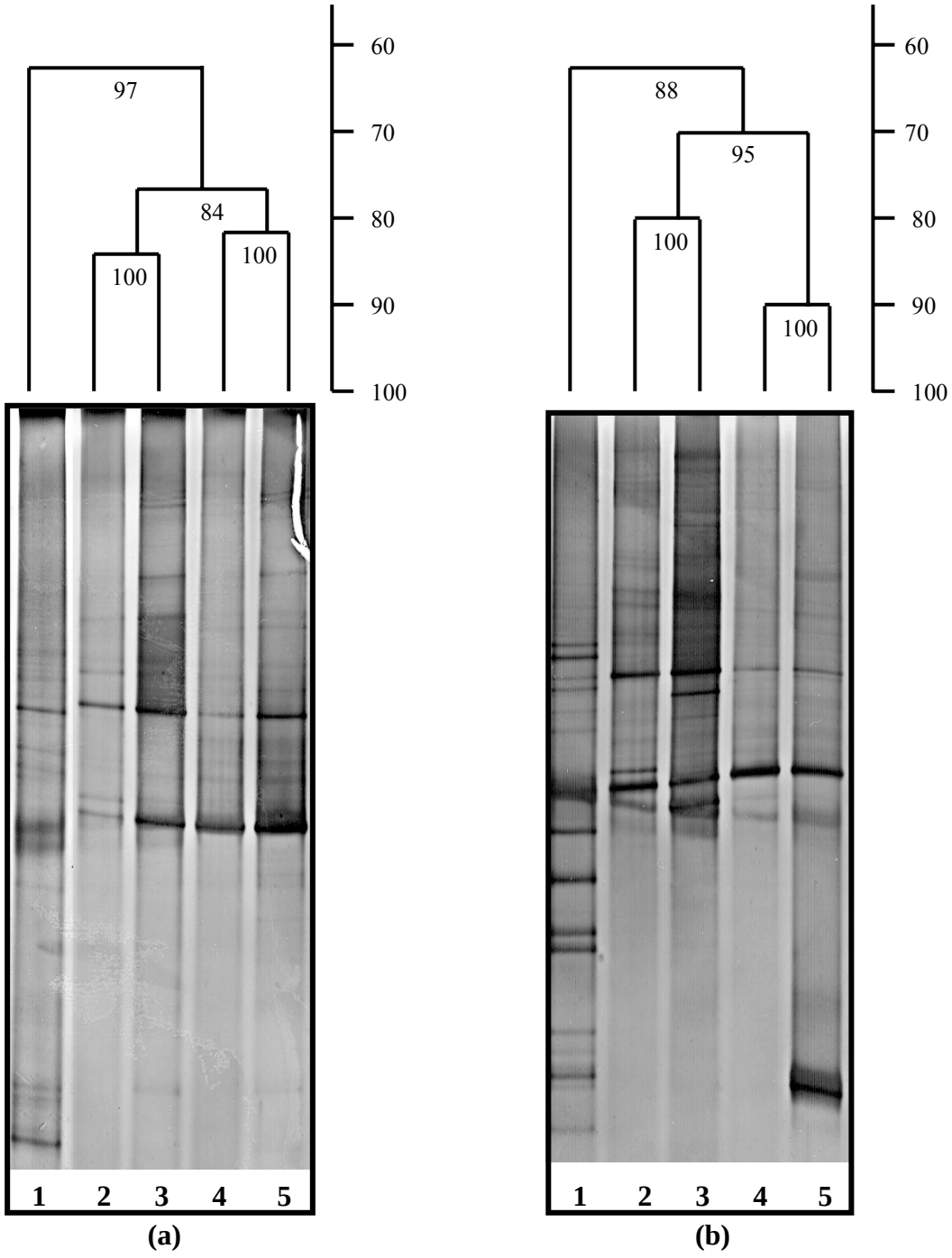


**Figure 21 (a) & (b).** DGGE profiles and UPGMA tree showing the similarities between the patterns of 18S RT-PCR products from litter degraded for two and eight weeks in grass field (**G**) **(a)** and maize field (**M**) **(b)**.
lane 1: soil site; lane 2: BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter after two weeks degradation; lane 3: BCG litter in respective sites after eight weeks degradation; lane 4: SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter after two weeks degradation; lane 5: SCG litter in respective sites after eight weeks degradation. Scale represents % similarity. Numbers at branch points are the cophenetic correlation values, which express the consistency of the cluster.

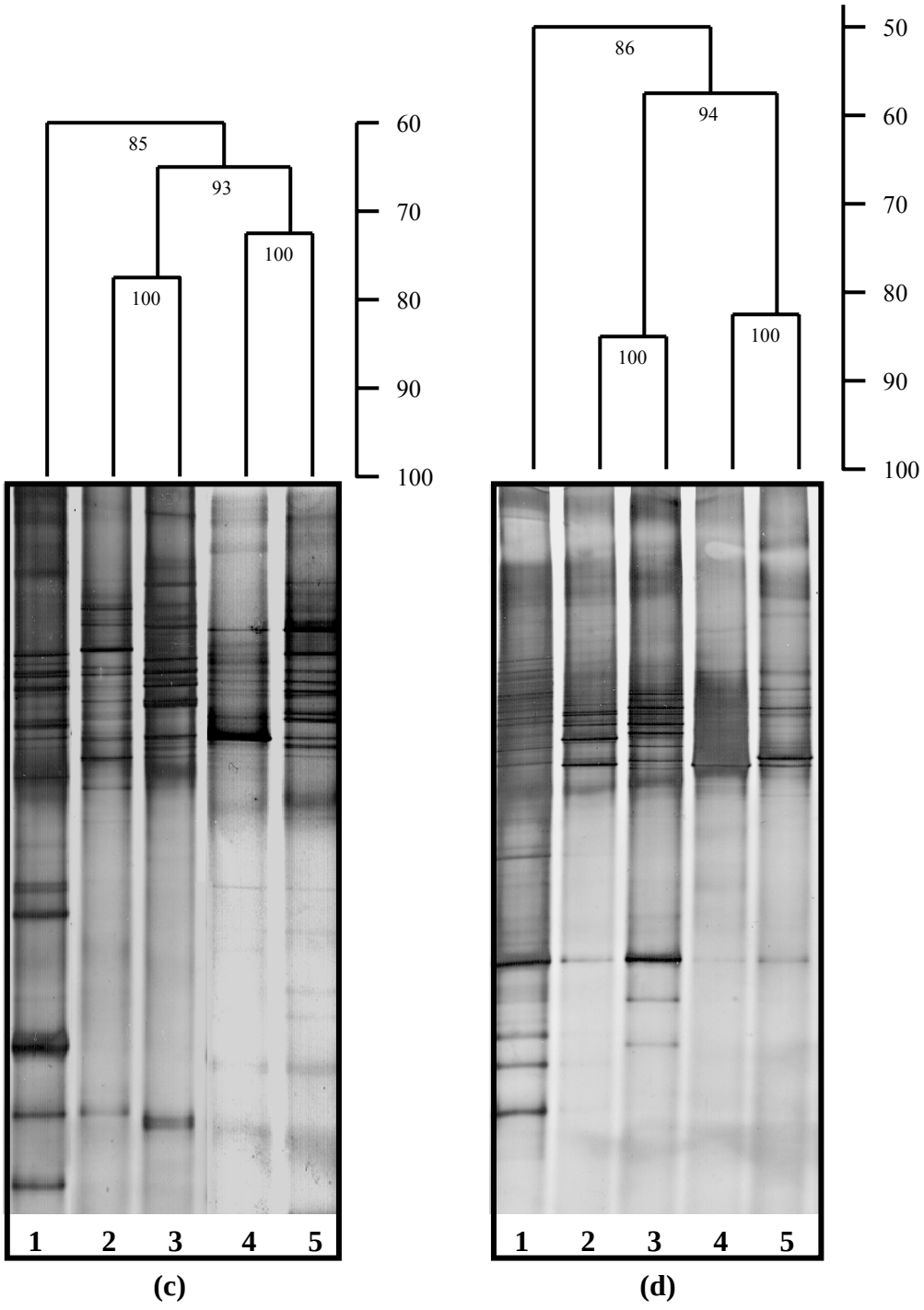


**Figure 21 (c) & (d).** DGGE profiles and UPGMA tree showing the similarities between the patterns of 18S RT-PCR products from litter degraded for two and eight weeks in beech forest (**B**) **(c)** and beech spruce mixed forest (**BS**) site **(d)**.
lane 1: soil site; lane 2: BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter after two weeks degradation; lane 3: BCG litter in respective sites after eight weeks degradation; lane 4: SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter after two weeks degradation; lane 5: SCG litter in respective sites after eight weeks degradation. Scale represents % similarity. Numbers at branch points are the cophenetic correlation values, which express the consistency of the cluster.

Greater differences were observed between the two and eight weeks profiles of fungal community colonizing beech and spruce litter, which had been incubated in the B forest site. For the other three sites, the profiles were about 80% or more similar to each other. Within the non-forest sites, the cluster formed by the fungal fingerprints of the two litter types was more than 60% similar to the 18S profile of the soil site. This similarity value was reduced for the BS forest site.

#### *3.4.4.6 Fungal clone libraries*

For the identification of fungal groups colonizing both litter types and their succession during the course of decay, 18S RT-PCR products from beech and spruce litter degraded in the BS site for two and eight weeks were cloned to generate libraries. The amplified 521 bp sequences (excluding primer sequences and GC-clamp) were used to assign the clones to fungal groups using NCBI BLAST programme. Sequences showing a match of 94% or higher to any member of a known fungal group were assigned to that particular group. As done for bacterial libraries, a total of 200 clones were analysed from the four libraries (50 clones each from B2, B8, S2 and S8). Majority of the clones (97% in B2, 85% in B8, 98% in S2 and 70% in S8 libraries) had sequences similar (≥98%) to members of Ascomycota. Sequences similar to members of other groups such as Zygomycota (present only in beech litter libraries with increased proportion in eight weeks), Basidiomycota (present only in spruce litter library after eight weeks) and Glomeromycota (present only in spruce litter library after eight weeks) were present. Clones similar to sequences reported in earlier studies, but mentioned as "unclassified" in the NCBI database, have been grouped together as "unclassified" in the present study and were present in B8 and S2 libraries at less than 5% proportions (Figure 22).

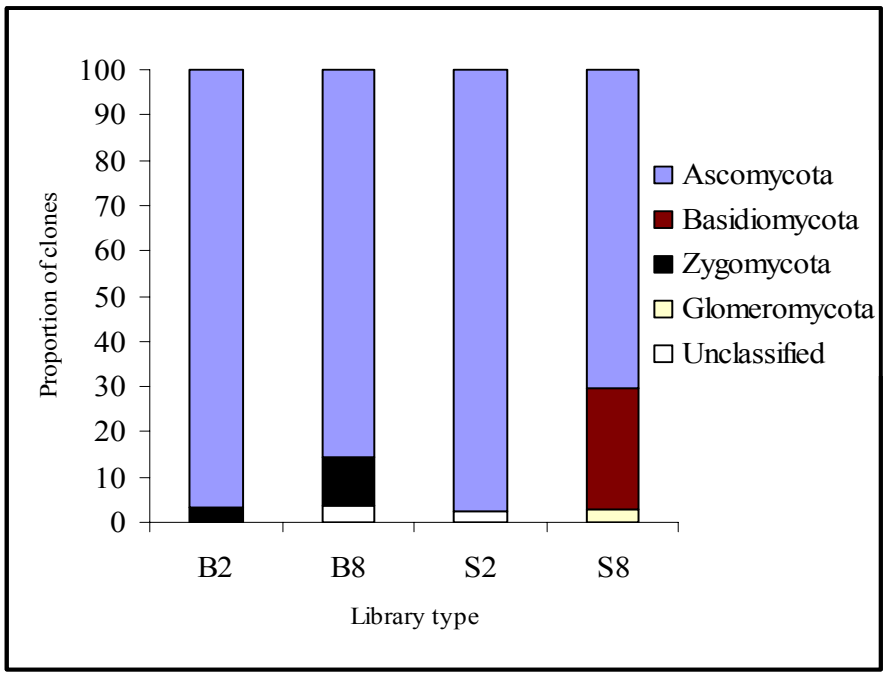


**Figure 22.** Relative distributions of clones to different fungal groups from litter degraded in BS soil site.
B2 and B8: libraries of BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter degraded in beech-spruce mixed forest (**BS**) site for two and eight weeks, respectively; S2 and S8: libraries of SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter degraded in **BS** site for two and eight weeks, respectively.

Within Ascomycota, high proportions of clones were more than 98% similar to the 18S rRNA sequence of *Penicillium* (Figure 23). There was a reduction in the proportion of such clones from 40% in B2 library to 15% in B8 library. However, clones similar to 18S rRNA sequence of *Trichoderma* increased in proportion from 26% (2 weeks) to 35% (8 weeks) in beech libraries. Also, clones similar in sequence to *Myrothecium*, which were absent from beech two weeks library, were present in significant proportions after eight weeks. In contrast to beech libraries, clones similar to the 18S rRNA sequence of *Penicillium* were observed in increased proportions after eight weeks in spruce libraries. Moreover, sequences similar to *Trichoderma* constituted about 13% of the clones in spruce two weeks library but were not detected in eight weeks library.

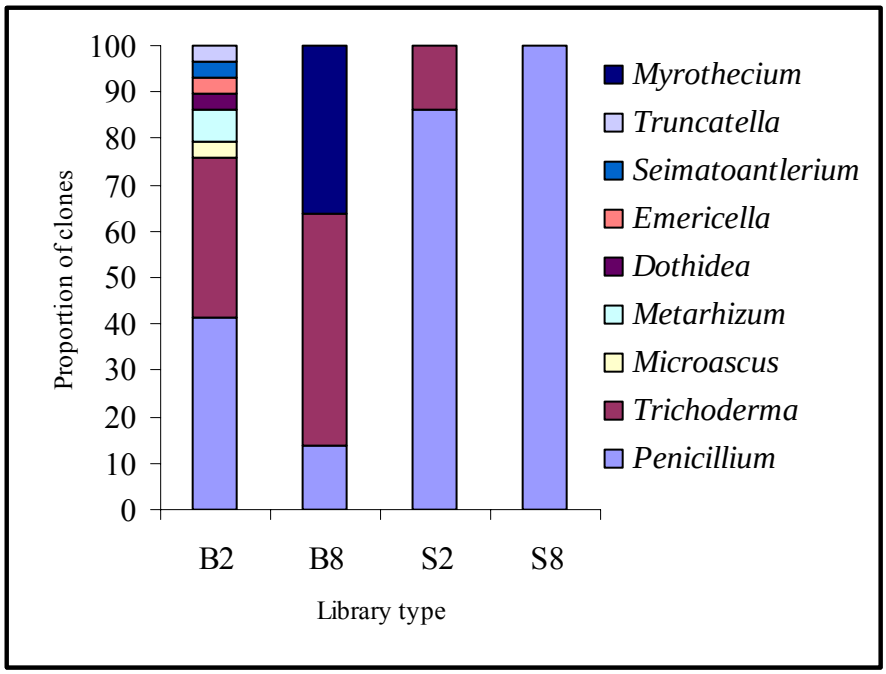


**Figure 23.** Relative proportions of clones similar to different genera of Ascomycota in 18S libraries from degraded beech and spruce litter.
B2 and B8: libraries of BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter degraded in beech-spruce mixed forest (**BS**) site for two and eight weeks, respectively; S2 and S8: libraries of SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter degraded in **BS** site for two and eight weeks, respectively.

### *3.4.4.7 Rarefaction curves of fungal clone libraries*

As done for bacterial libraries, to assess the extent of fungal diversity covered in the clone libraries, number of fungal groups observed in a library was plotted as a function of the number of clones screened by analytical rarefaction software (Figure 24). A plateau, as expected for full coverage of library, was obtained after screening 40 clones for beech litter after 2 weeks (B2), 30 clones for beech litter after 8 weeks (B8), 40 clones for spruce litter after 2 weeks (S2) and 44 clones for spruce litter after 8 weeks (S8). Therefore, 50 clones screened per library are true representation of the fungal diversity present in the litter bags.

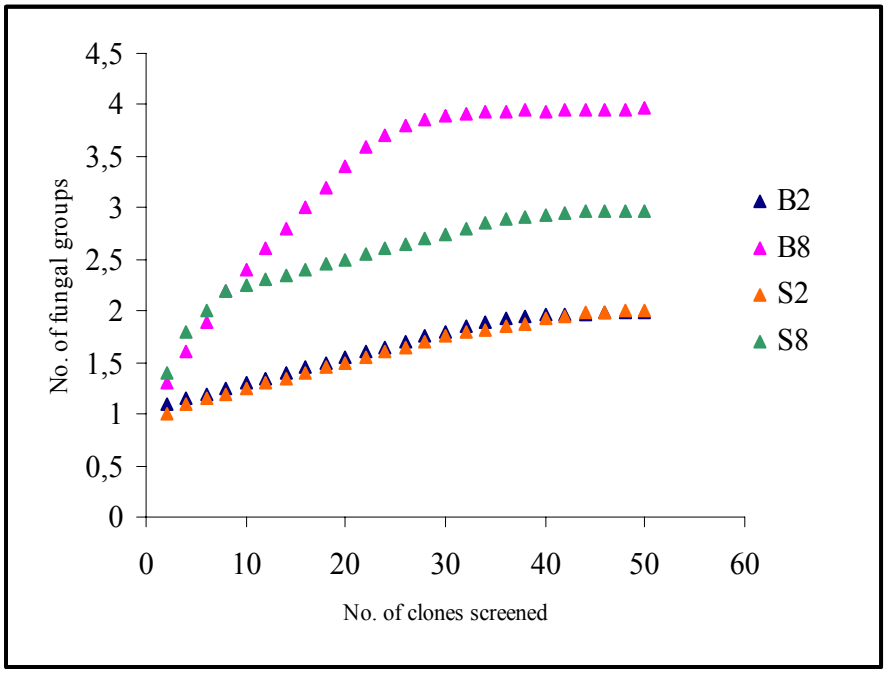


**Figure 24.** Rarefaction curves showing the extent of fungal diversity covered by the four libraries.
B2 and B8: libraries of BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter degraded in beech-spruce mixed forest (**BS**) site for two and eight weeks, respectively; S2 and S8: libraries of SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter degraded in **BS** site for two and eight weeks, respectively.

## 3.5 Degradation of control and ozone stressed green beech and spruce litter in non-forest and forest soil sites

### 3.5.1 Degradation rates of litter

Dry weight was measured for litter from control and ozone stressed beech and spruce plants degraded in the particular soil site after two and eight weeks. Degradation rates of control and ozone stressed litter of both the species were in accordance with the earlier results (see section 3.4.2) where higher mineralization rates were observed during the first two weeks followed by reduced rates (Table 12). Within the forest sites, a tendency for spruce litter to degrade faster than beech litter was observed. Such a trend reflecting high mineralization rates for spruce compared to beech in the same soil sites, was also observed earlier (see section 3.4.2). Effects of ozone on litter mass loss rates were observed for both beech and spruce. A trend, which reflected higher mineralization rates for litter from ozone stressed plants, was observed.

**Table 12.** Degradation rates of litter after two and eight weeks of degradation in four different soil sites.

| Litter type | Deg. rate after 2 weeks (mg/day) | | | | Deg. rate after 8 weeks (mg/day) | | | |
|---|---|---|---|---|---|---|---|---|
| | **G** | **M** | **B** | **BS** | **G** | **M** | **B** | **BS** |
| BOG | 25 | 11 | 18 | 21 | 14 | 11 | 13 | 12 |
| BCG | 14 | 7 | 16 | 13 | 9 | 5 | 9 | 11 |
| SOG | 21 | 11 | 39 | 43 | 12 | 5 | 14 | 18 |
| SCG | 7 | 7 | 29 | 27 | 6 | 5 | 12 | 16 |

BOG: **b**eech litter, **o**zone stressed, from **g**reenhouse; BCG: **b**eech litter, **c**ontrol, from **g**reenhouse; SOG: **s**pruce litter, **o**zone stressed, from **g**reenhouse; and SCG: **s**pruce litter, **c**ontrol, from **g**reenhouse, respectively. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**). Variation between two replicate bags / sample was <5%.

### 3.5.2 *Total RNA fingerprinting of microbial communities using RAP-PCR*

Analysis of the RAP-PCR profiles in section 3.4.3.1 revealed that the metabolic fingerprints of the microbial community colonizing beech and spruce litter were dependent on the soil type, i.e. forest sites and non-forest sites. To study the effect of changed litter quality due to ozone stress to plants, on microbial colonization and activity in the different soil sites, total RNA profiles were generated from litter degraded for two and eight weeks in the four different soil sites. The profiles from litter degraded in the non-forest sites were less complex compared to those from the forest sites with respect to the number of bands observed (Figure 25 & 27). Reduced complexity of the profiles from litter degraded in the non-forest sites was also observed earlier (see section 3.4.3.1). As earlier observed, significant differences in the fingerprints were evident when patterns for the same litter type degraded in different sites (soil site effects) and different litter types in the same site (litter type effects) were compared. A clear succession of the microbial community during the degradation process between two and eight weeks was also evident.

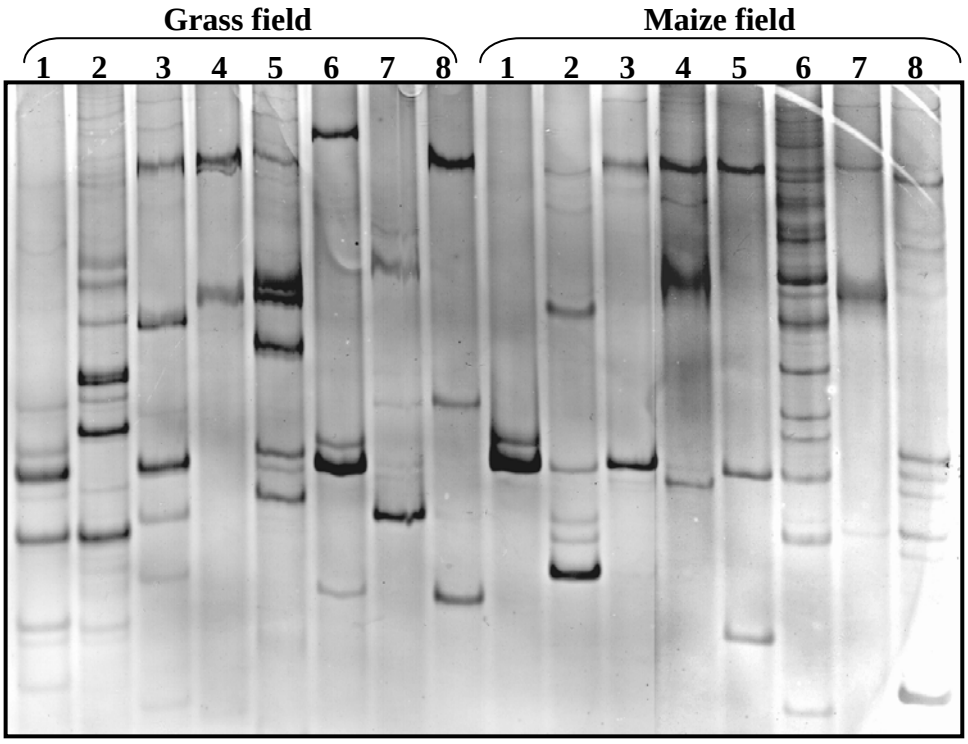


**Figure 25.** RAP-PCR profiles with cDNA from litter degraded for two and eight weeks in the non-forest sites.
lane 1: BOG (**b**eech litter, **o**zone stressed, from **g**reenhouse) litter after 2 weeks; lane 2: BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) after 2 weeks; lane 3: BOG litter after 8 weeks; lane 4: BCG litter after 8 weeks; lane 5: SOG (**s**pruce litter, **o**zone stressed, from **g**reenhouse) after 2 weeks; lane 6: SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) after 2 weeks; lane 7: SOG litter after 8 weeks; lane 8: SCG litter after 8 weeks, respectively.

The reproducibility of the obtained fingerprints from multiple nucleic acid extractions from the same litter bag and also from replicate bags was as high as 95%. Cluster analysis of the total RNA fingerprints from non-forest sites revealed four clusters, one for each plant species in the two different sites (Figure 26). The profiles of ozone stressed and control litter types were more than 70% similar to each other after eight weeks. This was observed for both plant species and in both the non-forest sites.

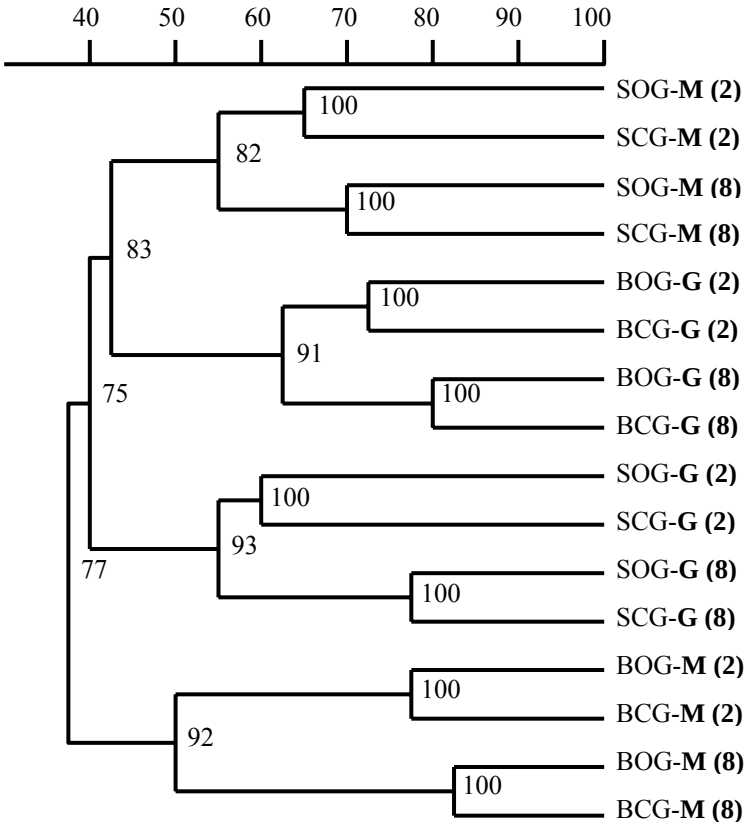


**Figure 26.** UPGMA tree of the RAP-PCR profiles from litter degraded for two and eight weeks in the non-forest sites.
BOG: **b**eech litter, **o**zone stressed, from **g**reenhouse; BCG: **b**eech litter, **c**ontrol, from **g**reenhouse; SOG: **s**pruce litter, **o**zone stressed, from **g**reenhouse and SCG: **s**pruce litter, **c**ontrol, from **g**reenhouse, respectively. The soil sites have been abbreviated as: grass field (**G**) and maize field (**M**). Numbers in parentheses indicate the weeks of degradation. Scale represents percent similarity. Numbers at the branch points are the cophenetic correlation values, which are the measure of consistency of a cluster.

As done for the litter degraded in the non-forest sites, RAP-PCR profiles were also generated from the ozone stressed and control litter types of both species, degraded in the two different forest sites. Each sample had a distinct profile with comparable number of bands (20 - 30) in all the profiles with the exception of control spruce litter degraded in BS site for eight weeks (Figure 27). Effects of soil site, litter type and the succession of microbial communities during the course of degradation were also evident. In contrast to what was observed with non-forest sites, the profiles from litter degraded in the forest sites formed two major clusters upon analysis (Figure 28). Each cluster was specific for each soil site. As also observed earlier in

the non-forest sites, the profiles from ozone stressed and control litter of a particular species degraded in the same site for same time period clustered together, though the proportion of similarity varied. The lone exception to this clustering pattern was the control spruce litter degraded in the BS site for eight weeks.

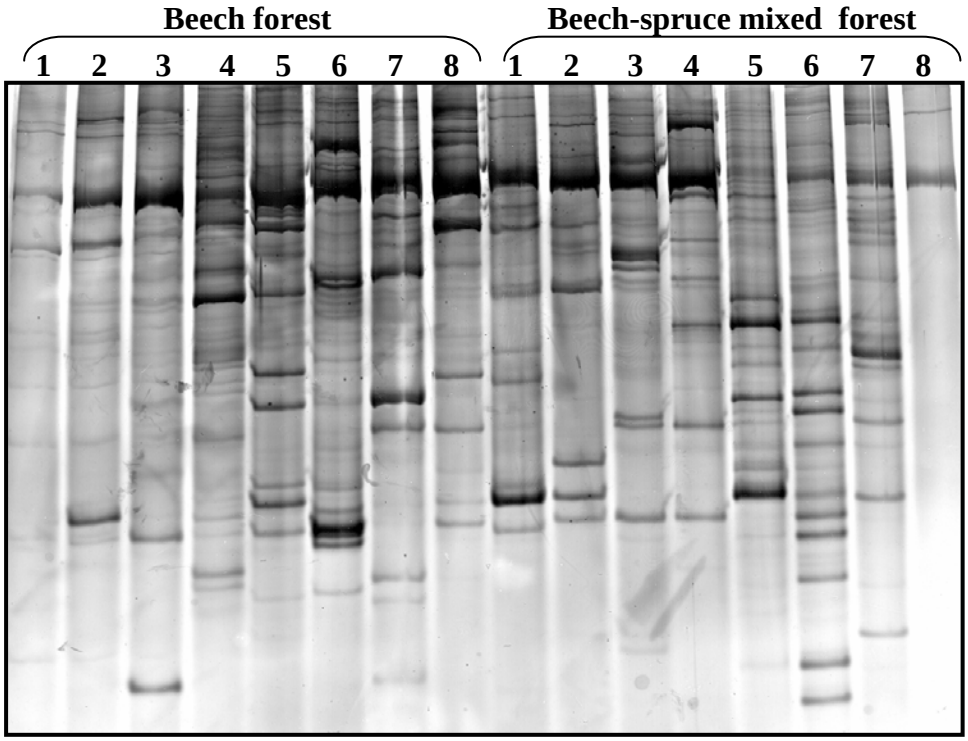


**Figure 27.** RAP-PCR profiles with cDNA from litter degraded for two and eight weeks in the forest sites.
lane 1: BOG (**b**eech litter, **o**zone stressed, from **g**reenhouse) litter after 2 weeks; lane 2: BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter after 2 weeks; lane 3: BOG litter after 8 weeks; lane 4: BCG litter after 8 weeks; lane 5: SOG (**s**pruce litter, **o**zone stressed, from **g**reenhouse) litter after 2 weeks; lane 6: SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter after 2 weeks; lane 7: SOG litter after 8 weeks; lane 8: SCG litter after 8 weeks, respectively.

These results in the non-forest and forest sites with control and ozone stressed litter of two different species confirmed the hypothesis that both soil site and litter type influenced microbial colonization of litter. They also provided the initial evidence that ozone stress to the plants affected their litter quality that subsequently influenced the structure and function of the colonizing microbial communities. Based on these results, bacterial and fungal communities were targeted separately and analysed.

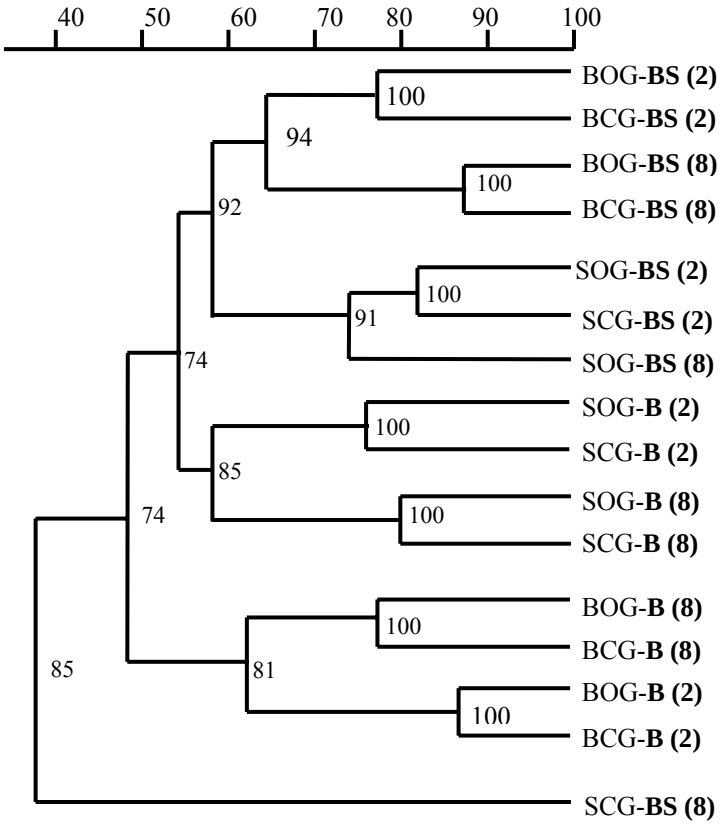


**Figure 28.** UPGMA tree of the RAP-PCR profiles from litter degraded in the forest sites for two and eight weeks.
BOG: **b**eech litter, **o**zone stressed, from **g**reenhouse; BCG: **b**eech litter, **c**ontrol, from **g**reenhouse; SOG: **s**pruce litter, **o**zone stressed, from **g**reenhouse and SCG: **s**pruce litter, **c**ontrol, from **g**reenhouse, respectively. The soil sites have been abbreviated as: beech forest (**B**) and beech-spruce mixed forest (**BS**). Numbers in parentheses indicate the weeks of degradation. Scale represents percent similarity. Numbers at the branch points are the cophenetic correlation values, which are the measure of consistency of a cluster.

### 3.5.3 Bacterial communities

Besides the effects of soil sites and plant species, effects of ozone on litter quality of both species and subsequently on its bacterial colonization were evident by the DGGE profiles of 16S rRNA RT-PCR products from control and ozone stressed litter degraded in different soil sites. After two weeks, irrespective of the soil site and litter type, 20 - 25 bands were observed in all the profiles (Figure 29 a & b). Pattern analysis generated two major clusters, one by the forest sites and the other by the non-forest sites (Figure 30). Such a clustering was also observed earlier when the bacterial communities of the four soil sites were compared (see section 3.4.1) and also in RAP-PCR profiles (see section 3.4.3.1), revealing that at the initial

time point of two weeks, it was the soil site which was the deciding parameter for the development of bacterial community. This clustering pattern was unaffected by multiple nucleic acid extractions from replicate samples. Differences were observed between the profiles of control and ozone stressed litter incubated in the same soil site indicating their colonizing bacterial communities to be different. Such differences were observed for both beech and spruce litter in all the four sites of incubation.

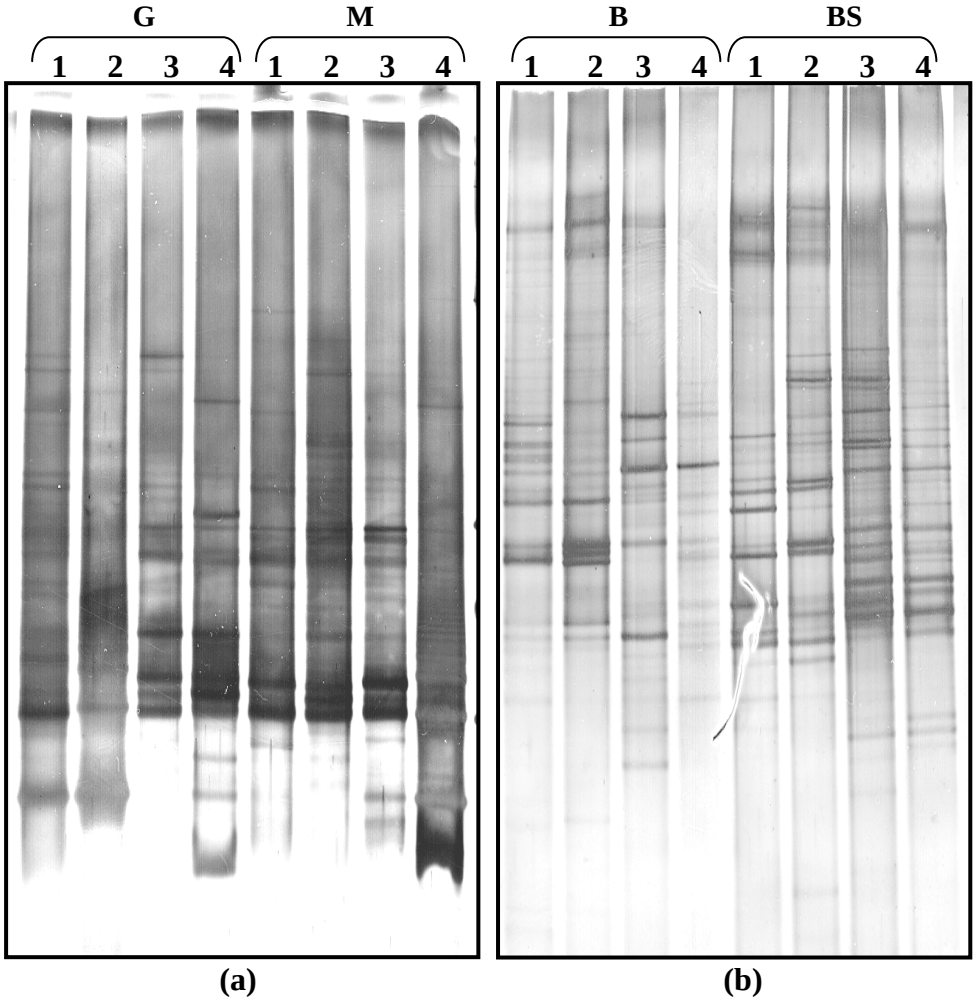


**Figure 29 (a) & (b).** DGGE profiles of 16S RT-PCR products from litter degraded for two weeks in non-forest **(a)** and forest sites **(b)**.
lane 1: BOG (**b**eech litter, **o**zone stressed, from **g**reenhouse) litter in respective site; lane 2: BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter in respective site; lane 3: SOG (**s**pruce litter, **o**zone stressed, from **g**reenhouse) litter in respective site; lane 4: SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter in respective site. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**).

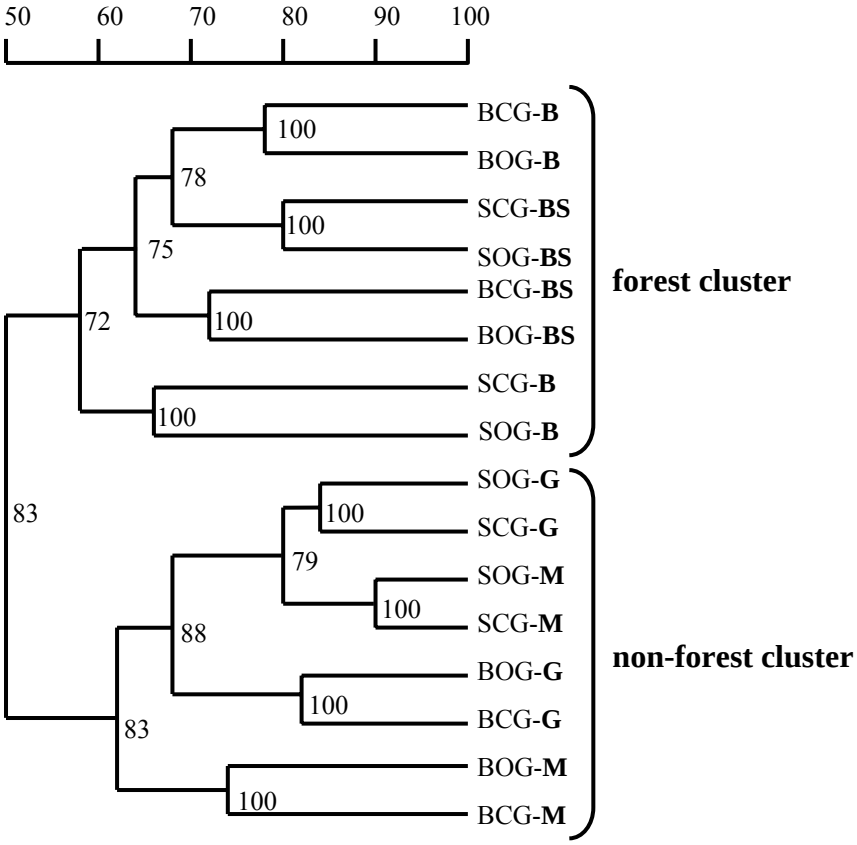


**Figure 30.** UPGMA tree showing the similarities between the DGGE patterns of 16S RT-PCR products from litter degraded for two weeks in the four soil sites.
BOG: **b**eech litter, **o**zone stressed, from **g**reenhouse; BCG: **b**eech litter, **c**ontrol, from **g**reenhouse; SOG: **s**pruce litter, **o**zone stressed, from **g**reenhouse and SCG: **s**pruce litter, **c**ontrol, from **g**reenhouse, respectively. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**). Scale represents percent similarity. Numbers at the branch points are the cophenetic correlation values, which are the measure of consistency of a cluster.

DGGE profiles generated from litter samples that had been incubated for eight weeks were highly complex with an increase in the number of bands when compared to the profiles after two weeks (Figure 31 a & b). Increase in diversity of bacterial communities from two to eight weeks was also observed earlier (see section 3.4.4.2). Pattern analysis of the DGGE profiles generated two major clusters, one for each litter type (Figure 32). Within each cluster, differences were observed between the bacterial profiles of control and ozone stressed litter. This was observed in all the four soil sites. The clustering together of bacterial profiles from same litter type degraded in the four different soil sites, highlights the fact that by eight

weeks, litter species plays a dominant role and is a selecting parameter for the bacterial communities unlike two weeks when soil site plays a pivotal role.

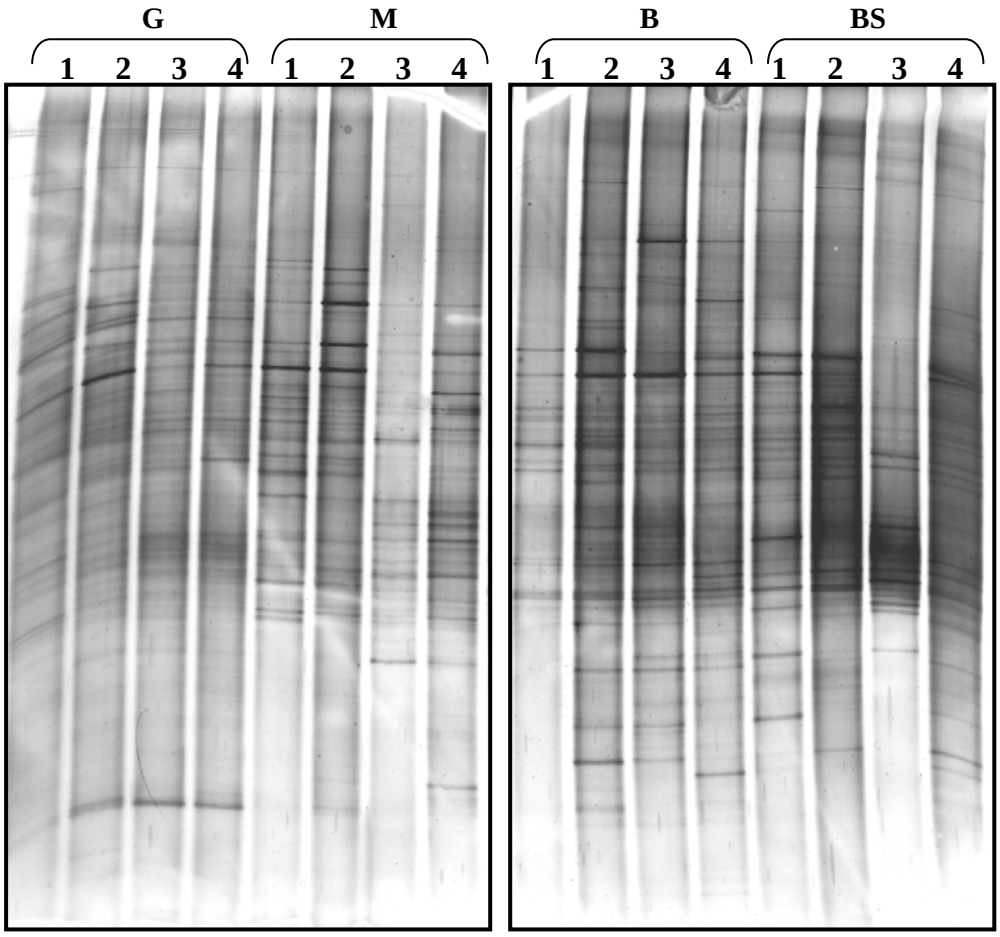


**Figure 31 (a) & (b).** DGGE profiles of 16S RT-PCR products from litter degraded for eight weeks in non-forest **(a)** and forest sites **(b)**.
lane 1: BOG (**b**eech litter, **o**zone stressed, from **g**reenhouse) litter in respective site; lane 2: BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter in respective site; lane 3: SOG (**s**pruce litter, **o**zone stressed, from **g**reenhouse) litter in respective site; lane 4: SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter in respective site. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**).

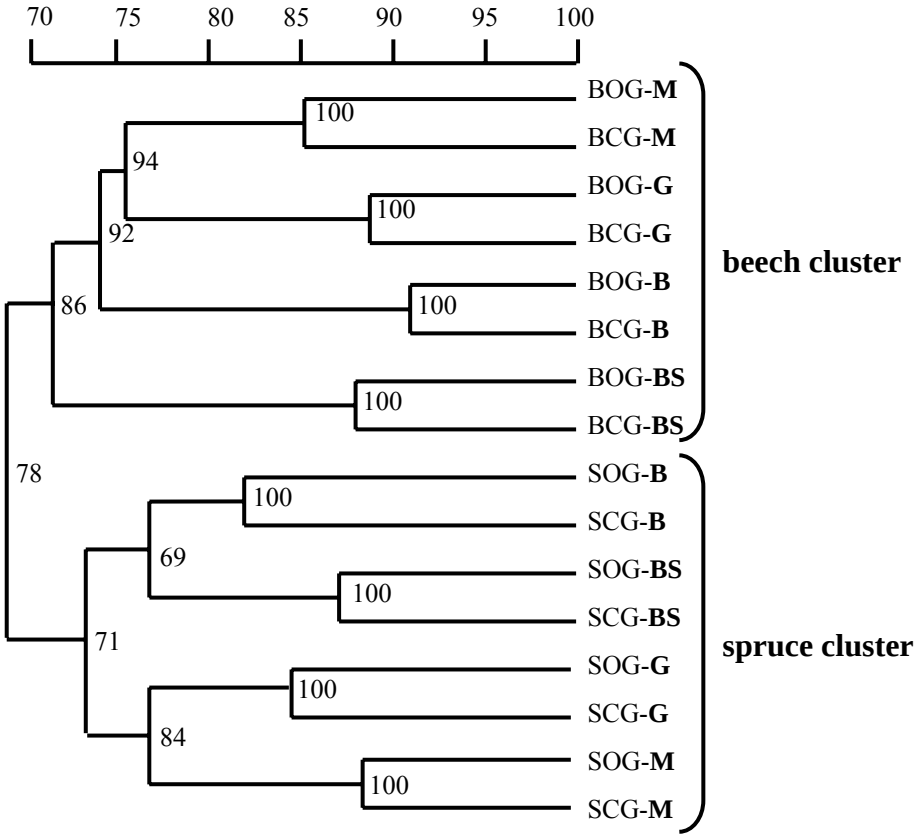


**Figure 32.** UPGMA tree showing the similarities between the DGGE patterns of 16S RT-PCR products from litter degraded for eight weeks in the four soil sites.
BOG: **b**eech litter, **o**zone stressed, from **g**reenhouse; BCG: **b**eech litter, **c**ontrol, from **g**reenhouse; SOG: **s**pruce litter, **o**zone stressed, from **g**reenhouse and SCG: **s**pruce litter, **c**ontrol, from **g**reenhouse, respectively. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**). Scale represents percent similarity. Numbers at the branch points are the cophenetic correlation values, which are the measure of consistency of a cluster.

### 3.5.4 Fungal communities

Fungal communities colonizing both litter types in different soil sites were analysed by DGGE fingerprinting of 18S rRNA RT-PCR products using litter material from the incubated litter bags. DGGE fingerprints were also used to screen for differences between the fungal communities colonizing litter from control and ozone stressed plants of beech and spruce litter (Figure 33 a & b). In comparison to the 16S DGGE profiles, reduced number of bands were observed in the 18S profiles (12 - 15). Inspite of the less number of bands, differences were observed between the 18S profiles of beech and spruce litter incubated in the same soil site indicating their colonizing fungal communities to be different. Effects of changed litter

quality due to ozone stress, on the colonizing fungal communities were also evident by the differences in their DGGE profiles. This was observed for both beech and spruce litter in all the four sites of incubation.

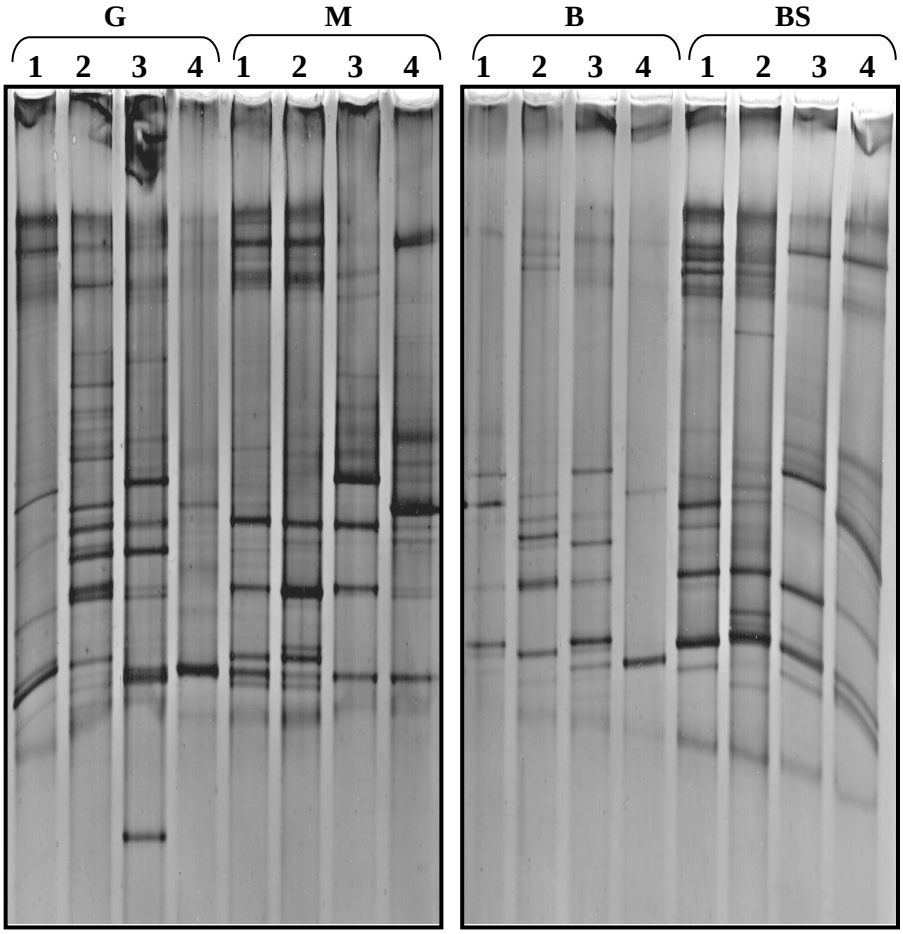


**Figure 33 (a) & (b).** DGGE profiles of 18S RT-PCR products from litter degraded for two weeks in the non-forest sites **(a)** and forest sites **(b)**.
lane 1: BOG (**b**eech litter, **o**zone stressed, from **g**reenhouse) litter in respective site; lane 2: BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter in respective site; lane 3: SOG (**s**pruce litter, **o**zone stressed, from **g**reenhouse) litter in respective site; lane 4: SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter in respective site. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**).

Cluster analysis of the DGGE patterns revealed two major clusters, one formed by the profiles from litter degraded in the forest sites and the other by the same in non-forest sites (Figure 34). Similar clustering was observed with replicate samples and also with multiple nucleic acid extractions from the same samples, though the proportion of similarity varied. At two weeks time point, soil site appeared to be the dominant factor influencing the build up of the

fungal community in the different litter types. Similar clustering was also observed with 18S DGGE profiles of the four soil sites (see section 3.4.1) and also with the 16S DGGE patterns after two weeks (see section 3.5.3).

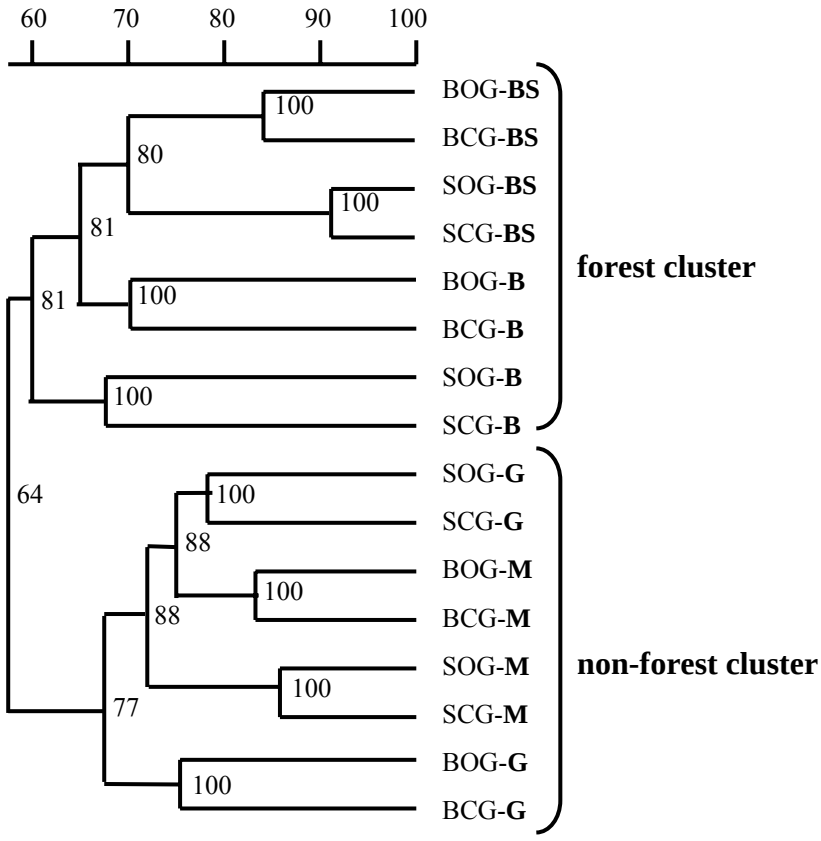


**Figure 34.** UPGMA tree showing the similarities between the DGGE patterns of 18S RT-PCR products from litter degraded for two weeks in the four soil sites.
BOG: **b**eech litter, **o**zone stressed, from **g**reenhouse; BCG: **b**eech litter, **c**ontrol, from **g**reenhouse; SOG: **s**pruce litter, **o**zone stressed, from **g**reenhouse and SCG: **s**pruce litter, **c**ontrol, from **g**reenhouse, respectively. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**). Scale represents percent similarity. Numbers at the branch points are the cophenetic correlation values, which are the measure of consistency of a cluster.

18S DGGE profiles were also generated from litter samples that had been incubated for eight weeks (Figure 35 a & b). The increase in diversity and complexity, as observed for bacterial DGGE profiles, were not observed in fungal profiles. However, effects of changed litter quality due to ozone stress to plants, on its fungal colonization were also observed for both beech and spruce, and were evident in all the four sites. Comparing the profiles and generating UPGMA tree, once again reveals two major clusters, one with the forest sites and the other with the non-forest sites (Figure 36). Within each cluster, effects of litter type on the

fungal community are evident by the clustering together of the same litter type degraded in the two different forest and non-forest sites. This was unlike the 16S DGGE profiles where after eight weeks, separate clusters were observed for the two species (see section 3.5.3).

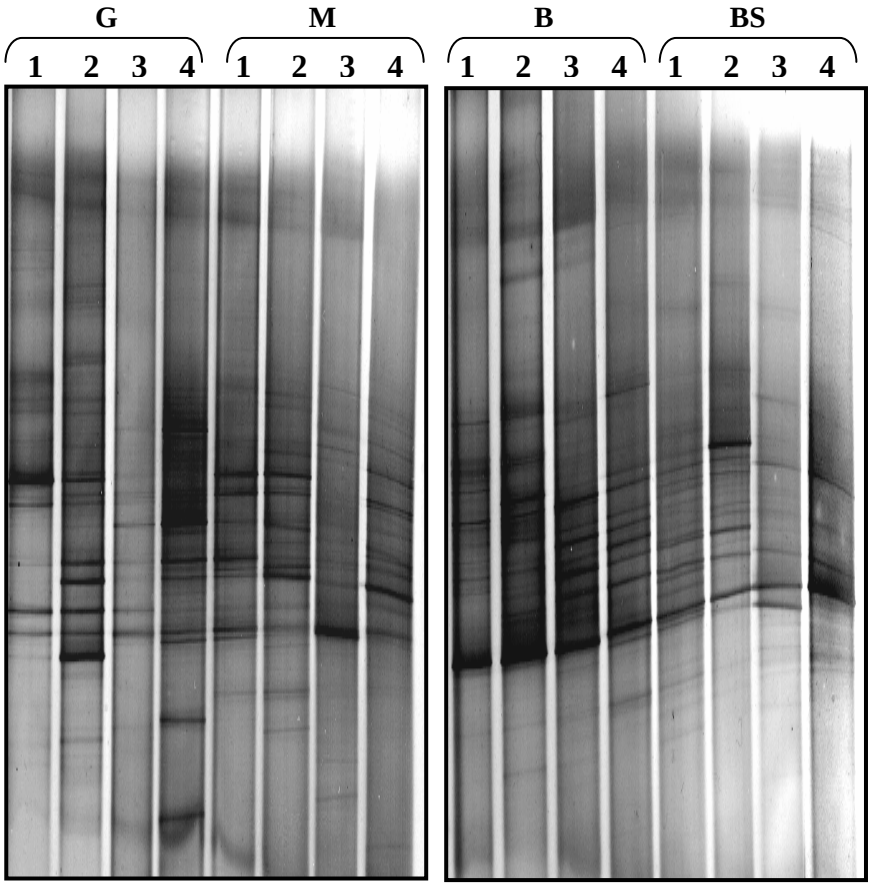


**Figure 35 (a) & (b).** DGGE profiles of 18S RT-PCR products from litter degraded for eight weeks in non-forest sites **(a)** and forest sites **(b)**.
lane 1: BOG (**b**eech litter, **o**zone stressed, from **g**reenhouse) litter in respective site; lane 2: BCG (**b**eech litter, **c**ontrol, from **g**reenhouse) litter in respective site; lane 3: SOG (**s**pruce litter, **o**zone stressed, from **g**reenhouse) litter in respective site; lane 4: SCG (**s**pruce litter, **c**ontrol, from **g**reenhouse) litter in respective site. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**).

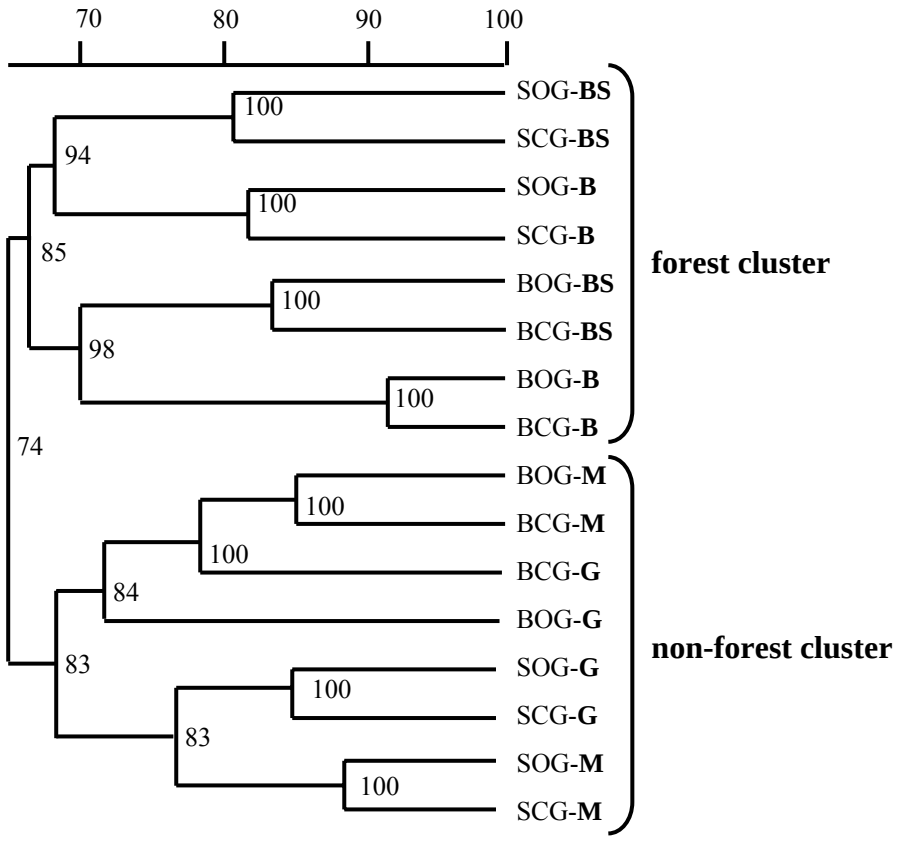


**Figure 36.** UPGMA tree showing the similarities between the DGGE patterns of 18S RT-PCR products from litter degraded for eight weeks in the four soil sites.
BOG: **b**eech litter, **o**zone stressed, from **g**reenhouse; BCG: **b**eech litter, **c**ontrol, from **g**reenhouse; SOG: **s**pruce litter, **o**zone stressed, from **g**reenhouse and SCG: **s**pruce litter, **c**ontrol, from **g**reenhouse, respectively. The four soil sites have been abbreviated as: grass field (**G**), maize field (**M**), beech forest (**B**) and beech-spruce mixed forest (**BS**). Scale represents percent similarity. Numbers at the branch points are the cophenetic correlation values, which are the measure of consistency of a cluster.

In the above experiments in Schäftlarn forest, effects of soil site and litter type on the microbial community structure and function were studied. Ozone stress to the plants affected litter quality, which resulted in differences in the bacterial and fungal colonization of the litter. Such differences were visualized by DGGE fingerprints of the bacterial and fungal communities colonizing both litter types in the four soil sites and two time points. It was observed that the structure of the initial colonizing microbial community was dependent upon the soil site. As decomposition proceeds, the selective effect of litter quality on the microbial community becomes prominent. Succession of the bacterial and fungal communities during the course of decomposition was also observed. These results provided the basis for the following experiments in Kranzberger forest using natural litter from both control and ozone exposed sites.

### 3.6 Degradation of natural litter in respective forest soil sites

#### 3.6.1 Microbial communities of "ozone stressed" and "control" soil sites

Total nucleic acids were extracted from the ozone stressed and control soil sites and primers F984-GC and R1378 were used to amplify the hypervariable V3-V6 region of 16S rDNA and rRNA. Purified 16S RT-PCR products from each of two soil sites were resolved by DGGE to compare their active bacterial communities (Figure 37).

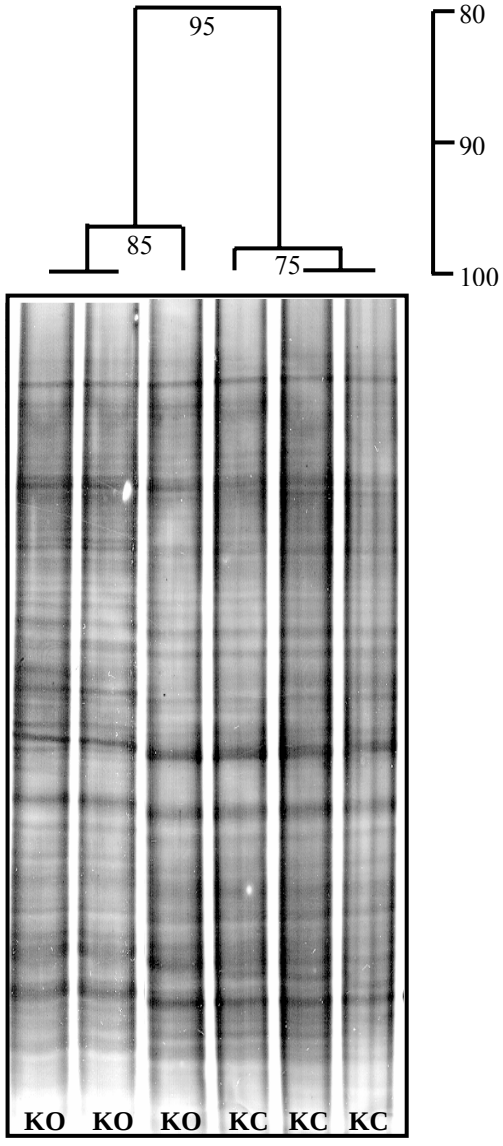


**Figure 37.** DGGE profile and UPGMA tree showing the similarities between the patterns of 16S RT-PCR products of three replicates from soil sites.
KO: **K**ranzberger, **o**zone stressed soil site; KC: **K**ranzberger **c**ontrol soil site. Scale represents % similarity. Numbers at branch points are the cophenetic correlation values, which express the consistency of the cluster.

Approximately 40 bands were observed in each sample and the profiles derived from ozone stressed and control sites were largely but not completely similar to each other. Replicates for each of the sites were more than 97% similar to each other while the active bacterial community of the two sites was 80% similar.

Primer pair NS1 and NS2-GC was used to amplify a region of 18S rRNA. As done for 16S products, purified 18S RT-PCR products were also resolved by DGGE to compare the active fungal communities of the two soil sites (Figure 38).

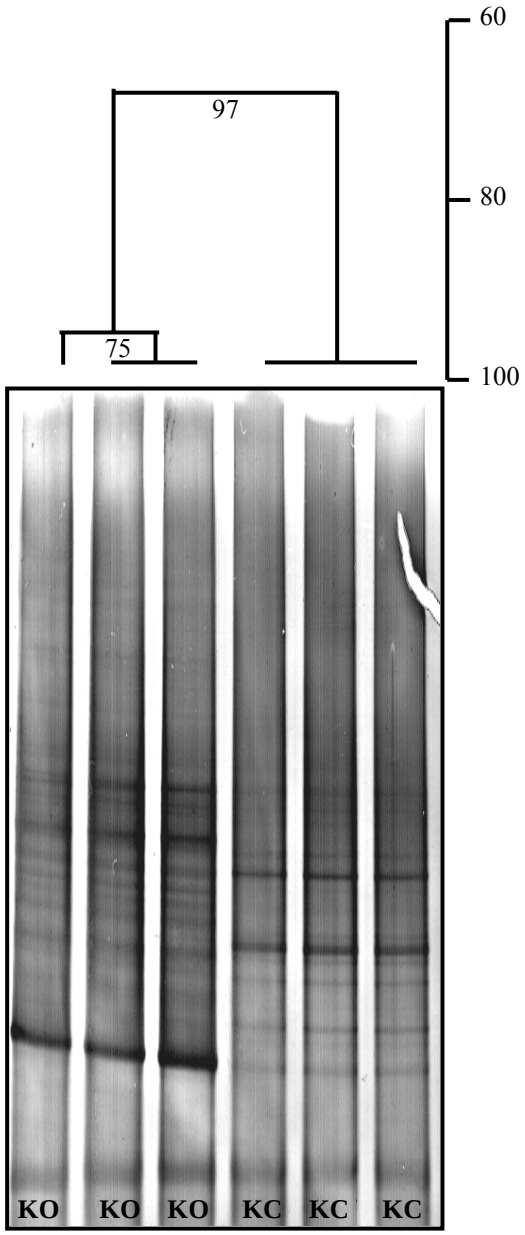


**Figure 38.** DGGE profile and UPGMA tree showing the similarities between the patterns of 18S RT-PCR products of three replicates from soil sites.
KO: **K**ranzberger, **o**zone stressed soil site; KC: **K**ranzberger **c**ontrol soil site. Scale represents % similarity. Numbers at branch points are the cophenetic correlation values, which express the consistency of the cluster.

Comparable number of bands (about 20) were obtained in the profiles from ozone stressed and control sites. As earlier observed for bacterial DGGE profiles, the replicates were more than 97% similar while distinct differences were observed between active fungal communities of the control and ozone stressed sites. The fungal communities of the two sites were approximately 70% similar. These differences between the microbial communities of the two sites could be due to the direct effects of ozone on the soil microbial communities or indirectly either due to the effects of ozone on rhizodeposition by trees or litter quality.

### 3.6.2 Degradation rates of litter

Dry weight was measured for both control litter and litter from ozone stressed trees degraded in their respective sites for two and eight weeks. Degradation rates (Table 13) were lower than what were observed for green litter (see section 3.4.2 and 3.5.1). Moreover, the tendency for high mineralization of spruce compared to beech, observed with green litter, was not evident with natural litter. The degradation rates were, however, in accordance with the asymptotic model of litter decay wherein high rates were observed initially followed by reduced mineralization. Same trend was also observed with green litter (see section 3.4.2 and 3.5.1). After two weeks, tendency for high mineralization rates for litter from ozone stressed trees was observed. However, unlike with green litter, the rates were comparable for both control and ozone stressed litter of both the species at eight weeks time point.

**Table 13.** Dry weight of beech and spruce litter after two and eight weeks of degradation in the respective sites.

| Litter type | Deg. rate after 2 weeks (mg/day) | Deg. rate after 8 weeks (mg/day) |
|---|---|---|
| **BOF** | 11 | 5 |
| **BCF** | 7 | 4 |
| **SOF** | 7 | 4 |
| **SCF** | 4 | 3 |

BOF: **b**eech litter, **o**zone stressed, from Kranzberger **f**orest; BCF: **b**eech litter, **c**ontrol, from Kranzberger **f**orest; SOF: **s**pruce litter, **o**zone stressed, from Kranzberger **f**orest and SCF: **s**pruce litter, **c**ontrol, from Kranzberger **f**orest, respectively. Variation between two replicate bags / sample was <5%.

### 3.6.3 Total RNA fingerprinting of microbial communities using RAP-PCR

Metabolic fingerprints of the colonizing microbial community were generated by RAP-PCR using the 10 mer primer. These profiles were then resolved by PAGE. Comparable number of bands (18 – 20) was obtained in all the samples (Figure 39). The profiles from replicate samples were highly reproducible (>95% similar).

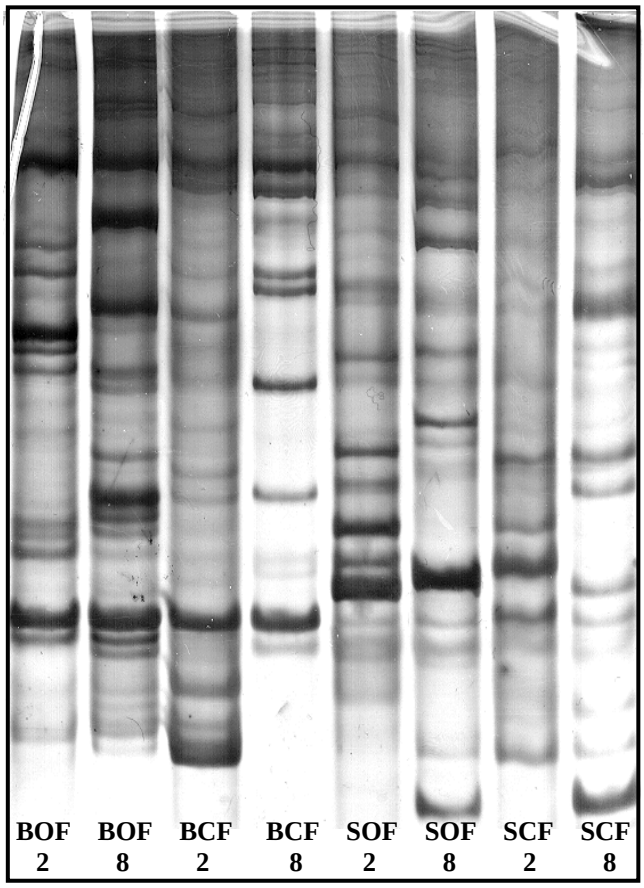


**Figure 39.** RAP-PCR profiles with cDNA from litter degraded for two and eight weeks. BOF: **b**eech litter, **o**zone stressed, from Kranzberger **f**orest; BCF: **b**eech litter, **c**ontrol, from Kranzberger **f**orest; SOF: **s**pruce litter, **o**zone stressed, from Kranzberger **f**orest and SCF: **s**pruce litter, **c**ontrol, from Kranzberger **f**orest, respectively; numbers 2 and 8: two and eight weeks of degradation of litter, respectively.

Cluster analysis of the profiles revealed the effects of changed litter quality due to ozone stress, on the subsequent microbial community structure and function. The profiles from the ozone stressed litter of both species formed one major cluster (Figure 40). Similar clustering was also observed with profiles from replicate samples. Greater differences were observed between the profiles of two and eight weeks degraded control litter compared to the ozone

counterpart. These results with the 10 mer primer provided the initial leads to investigate into the bacterial and fungal communities in more detail.

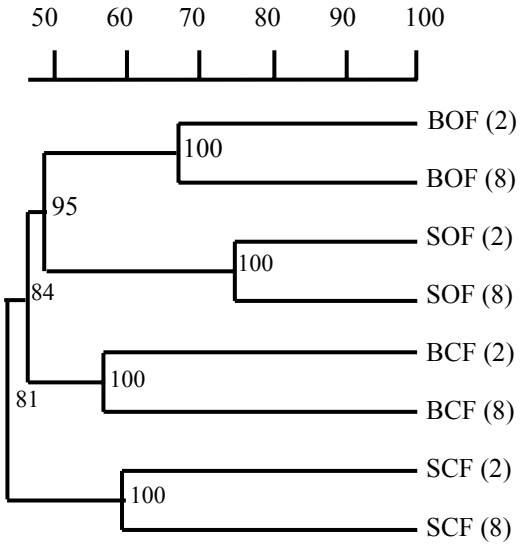


**Figure 40.** UPGMA tree showing the similarity of RAP-PCR profiles from litter degraded for two and eight weeks.
BOF: **b**eech litter, **o**zone stressed, from Kranzberger **f**orest; BCF: **b**eech litter, **c**ontrol, from Kranzberger **f**orest; SOF: **s**pruce litter, **o**zone stressed, from Kranzberger **f**orest and SCF: **s**pruce litter, **c**ontrol, from Kranzberger **f**orest, respectively. Numbers in parenthesis indicate the weeks of degradation. Scale represents % similarity. Numbers at branch points are the cophenetic correlation values, which express the consistency of the cluster.

### 3.6.4 Microbial communities after two and eight weeks

#### *3.6.4.1 Bacterial communities*

Denaturing gradient gel electrophoresis of RT-PCR products of 16S rRNA, extracted from the degraded litter inside the incubated litter bags, was used to screen for characterizing and comparing the bacterial communities degrading different litter types, viz. beech litter from ozone stressed trees, beech litter from control trees, spruce litter from ozone stressed trees and spruce litter from control trees. All litter samples yielded positive results with the primer pair used and distinct DGGE profiles were obtained for each sample (Figure 41). The number of bands in the profiles from the ozone stressed and control litter was comparable (approximately 30) and the increase in diversity from two to eight weeks as observed with green litter (see section 3.4.4.2 and 3.5.3), was not observed with natural litter. The succession of bacterial community from two to eight weeks was comparable for ozone stressed and control spruce litter (about 87% similarity between two and eight weeks

profiles), while for beech, greater difference was observed between the two and eight weeks profiles of ozone stressed litter. These profiles were more than 95% similar with replicate samples.

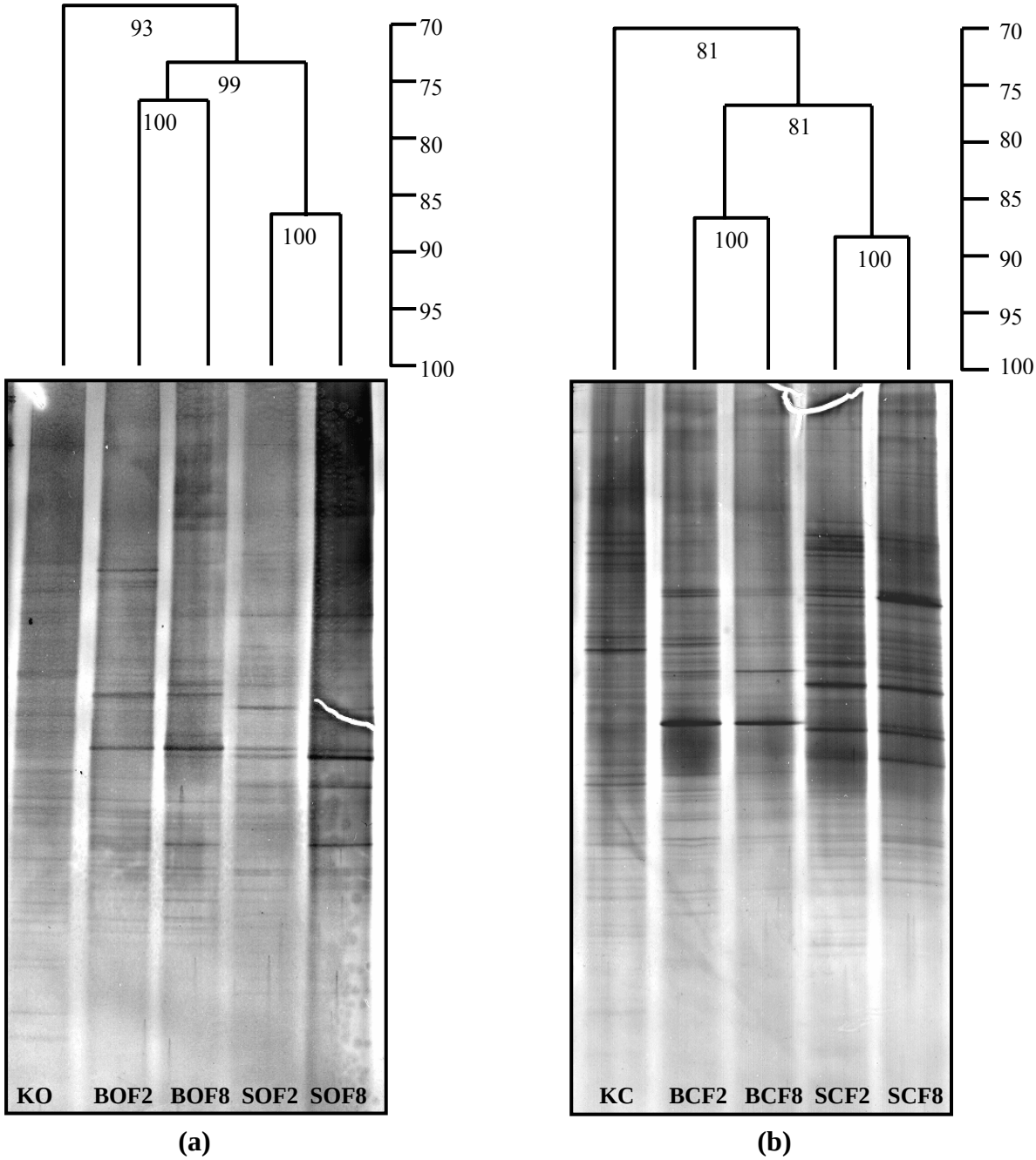


**Figure 41 (a) & (b).** DGGE profiles and UPGMA tree showing the similarities of the patterns of 16S RT-PCR from ozone stressed litter **(a)** and control litter **(b)** degraded for two and eight weeks in their respective sites.
KO: **K**ranzberger site, **o**zone stressed; KC: **K**ranzberger **c**ontrol site; BOF: **b**eech litter, **o**zone stressed, from Kranzberger **f**orest; BCF: **b**eech litter, **c**ontrol, from Kranzberger **f**orest; SOF: **s**pruce litter, **o**zone stressed, from Kranzberger **f**orest and SCF: **s**pruce litter, **c**ontrol, from Kranzberger **f**orest, respectively; numbers 2 and 8: two and eight weeks of degradation of litter, respectively. Scale represents % similarity. Numbers at branch points are the cophenetic correlation values, which express the consistency of the cluster.

### *3.6.4.2 Bacterial clone libraries*

16S rRNA clone libraries were made to identify the key bacterial groups colonizing both species of litter and also to identify the effects of changed litter quality, due to ozone, on the colonizing bacterial groups. Bacterial RT-PCR products from beech and spruce litter degraded for eight weeks were cloned and 100 clones from each library with the insert of correct size (checked by *Eco*RI digestion; see section 2.4.9), were then screened by RFLP with *Msp*I (section 2.4.10). Ten different RFLP patterns were observed on resolving the RFLP digests on high resolution agarose (Figure 42).

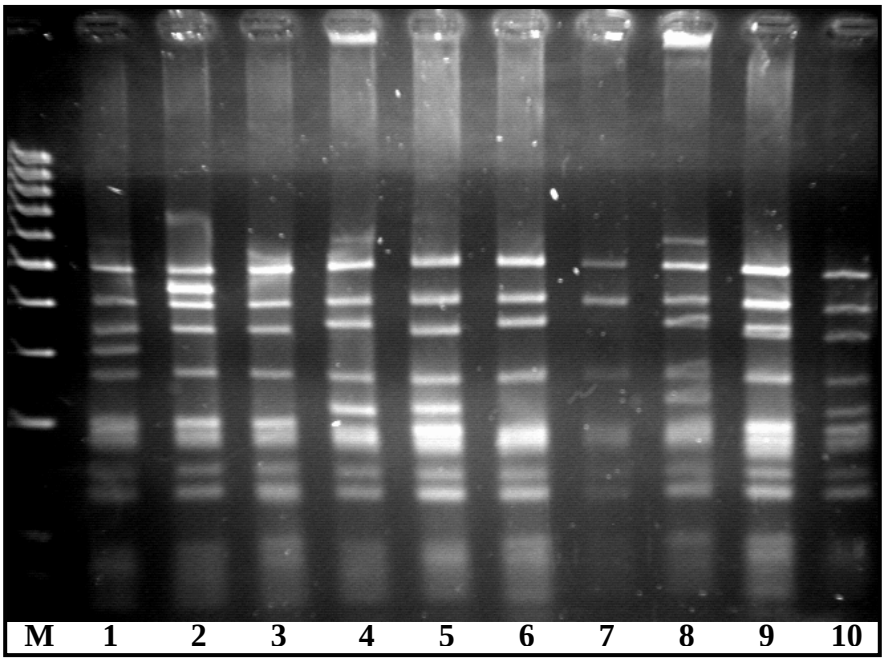


**Figure 42.** 3% high resolution gel showing the 10 different RFLP patterns observed in 16S clone libraries from eight weeks degraded litter.
lane M - 100 bp marker; lanes 1 – 10 – patterns 1 to 10.

Representative samples for each RFLP type were sequenced and the 394 bp sequences were used to assign the clones to bacterial groups using NCBI BLAST programme. Majority of the clones were highly similar (>96% homology) to members of actinobacteria (Figure 43). Sequences similar to members of different subgroups of proteobacteria were also present, though in less proportions (<15%). This was in contrast to the results with 16S libraries from green litter where clones similar to proteobacteria constituted the major fraction (see section 3.4.4.3). Clones similar to sequences reported in earlier studies, but mentioned as "unclassified" in the NCBI database, have been grouped together as "unclassified" in the

present study. Proportion of clones similar to Actinobacteria members was reduced in spruce libraries compared to beech litter libraries. Moreover, significant proportions of clones in the spruce litter libraries constituted the unclassified group while the proportion of such clones was less than 5% in beech libraries. Effects of changed litter quality, due to ozone stress to the trees, on subsequent bacterial colonization, were evident by the marked absence of clones similar to members of γ-proteobacteria from SOF library. An opposite trend was observed in beech litter where clones similar to members of γ-proteobacteria were present in increased proportion in BOF library compared to BCF library. Clones similar to members of β-proteobacteria were present only in spruce litter libraries with higher proportion in SOF library.

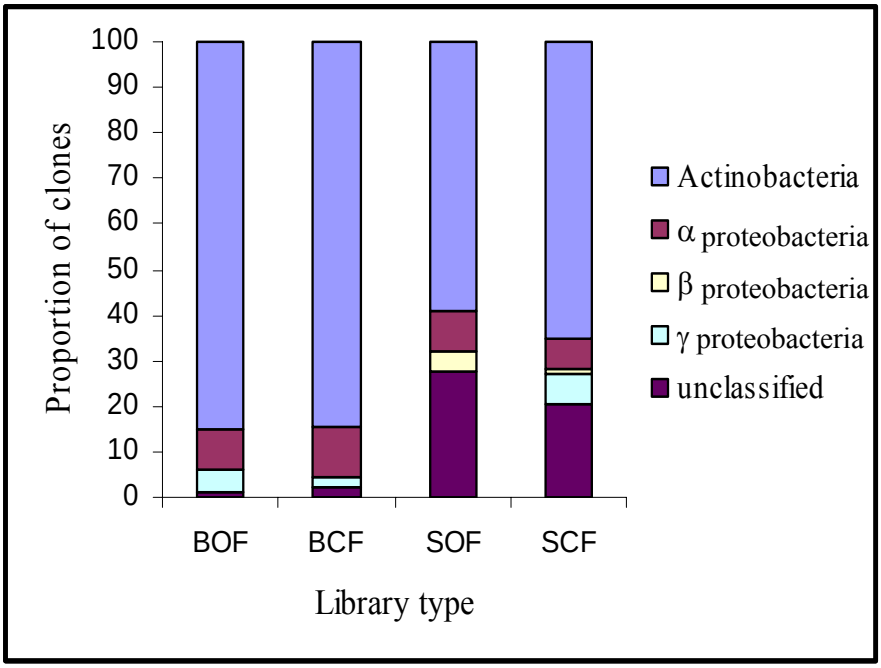


**Figure 43.** Relative distributions of clones to different bacterial groups from eight weeks degraded litter.
BOF: **b**eech litter, **o**zone stressed, from Kranzberger **f**orest; BCF: **b**eech litter, **c**ontrol, from Kranzberger **f**orest; SOF: **s**pruce litter, **o**zone stressed, from Kranzberger **f**orest and SCF: **s**pruce litter, **c**ontrol, from Kranzberger **f**orest, respectively.

### *3.6.4.3 Rarefaction curves of bacterial clone libraries*

Assessment of the proportion of bacterial diversity targeted in the libraries was made by the analytical rarefaction analysis wherein the number of bacterial groups observed in a library were plotted as a function of the number of clones screened (Figure 44). A plateau, as expected for full coverage of the bacterial diversity in the library, was obtained after screening

60 clones for each library type. No new RFLP type was observed even when the number of clones analysed were increased to 100.

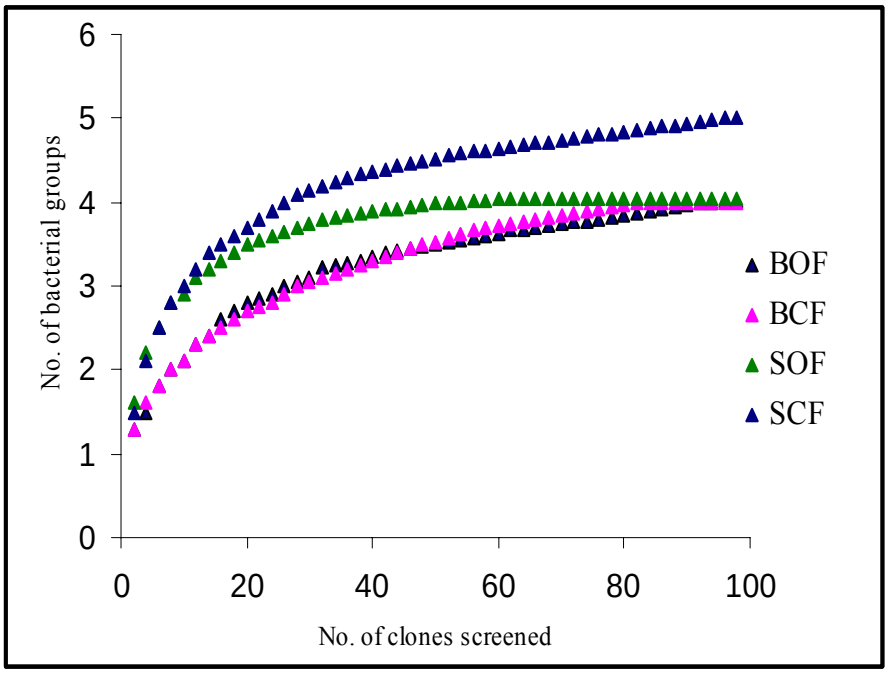


**Figure 44.** Rarefaction curves showing the extent of bacterial diversity covered by the four libraries.
BOF: **b**eech litter, **o**zone stressed, from Kranzberger **f**orest; BCF: **b**eech litter, **c**ontrol, from Kranzberger **f**orest; SOF: **s**pruce litter, **o**zone stressed, from Kranzberger **f**orest and SCF: **s**pruce litter, **c**ontrol, from Kranzberger **f**orest, respectively.

### *3.6.4.4 Fungal communities*

18S rRNA was targeted to characterize the fungal communities colonizing beech and spruce litter using RNA extracted from the corresponding litter bags. RT-PCR products were obtained for all the samples using NS1 and NS2 primers. On resolving the purified 18S rRNA RT-PCR products by DGGE, comparable number of bands (approximately 20) was observed for both ozone stressed and control litter type of both the species (Figure 45). For both beech and spruce, the succession of fungal communities from two to eight weeks was comparable for ozone stressed and control litter types. High reproducibility as earlier observed for bacterial DGGE profiles was also observed with the 18S profiles from replicate samples.

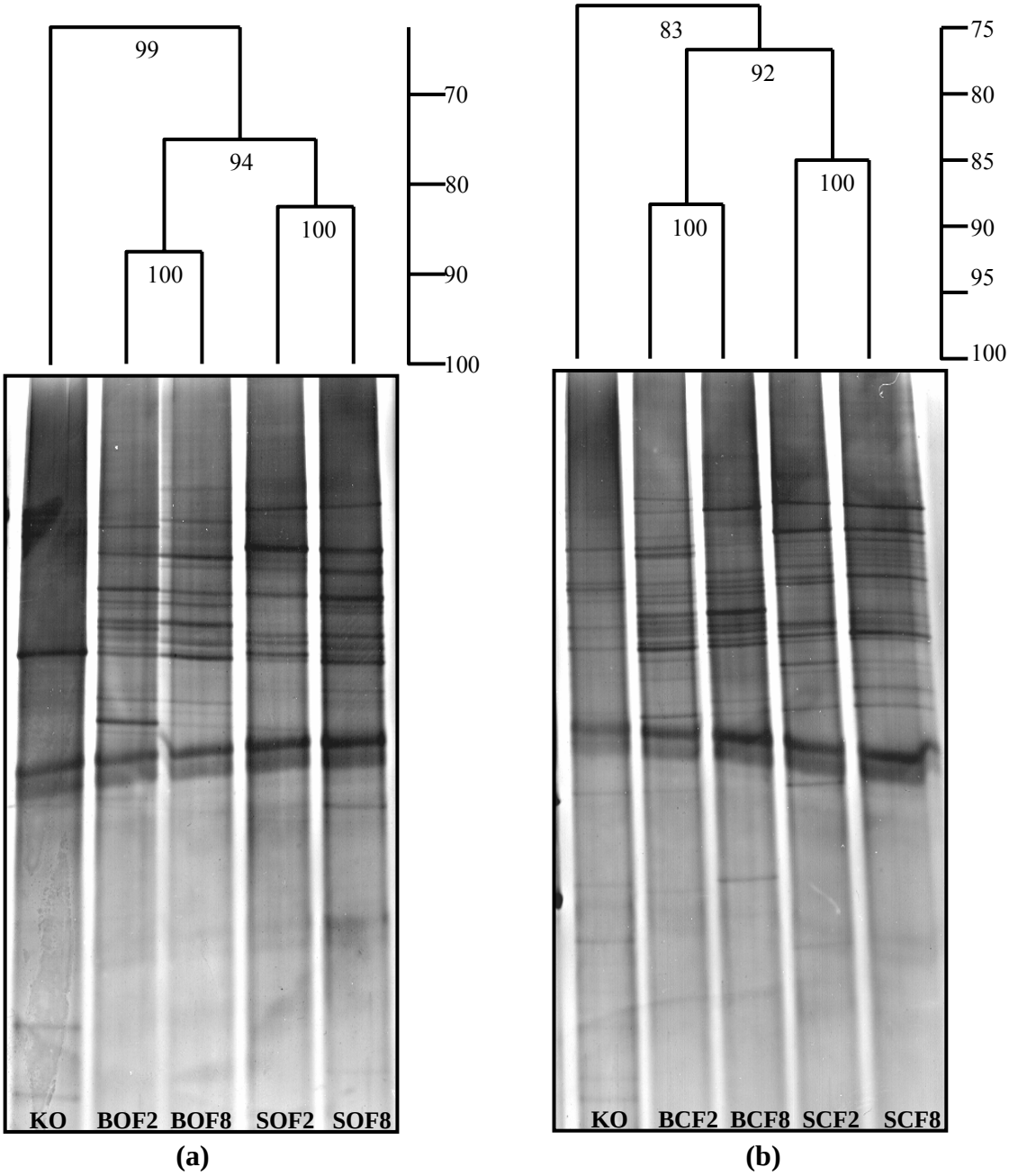


**Figure 45 (a) & (b).** DGGE profiles and UPGMA tree showing the similarities of the patterns of 18S RT-PCR products from ozone stressed litter **(a)** and control litter **(b)** degraded for two and eight weeks in their respective sites.
KO: **K**ranzberger site, **o**zone stressed; KC: **K**ranzberger **c**ontrol site; BOF: **b**eech litter, **o**zone stressed, from Kranzberger **f**orest; BCF: **b**eech litter, **c**ontrol, from Kranzberger **f**orest; SOF: **s**pruce litter, **o**zone stressed, from Kranzberger **f**orest and SCF: **s**pruce litter, **c**ontrol, from Kranzberger **f**orest, respectively; numbers 2 and 8: two and eight weeks of degradation of litter, respectively. Scale represents % similarity. Numbers at branch points are the cophenetic correlation values, which express the consistency of the cluster.

### *3.6.4.5 Fungal clone libraries*

For the identification of fungal groups colonizing both litter types and also to look for ozone specific effects, fungal RT-PCR products were cloned to generate libraries and 100 clones (per library) with the insert of correct size (checked by *Eco*RI digestion; see section 2.4.9) were then screened by RFLP with *Msp*I (section 2.4.10). On resolving the RFLP digests on high resolution agarose, twenty different RFLP patterns were observed (Figure 46). Representative samples for each RFLP type were sequenced and sequences (521 bp) used to assign the clones to fungal groups using NCBI BLAST programme.

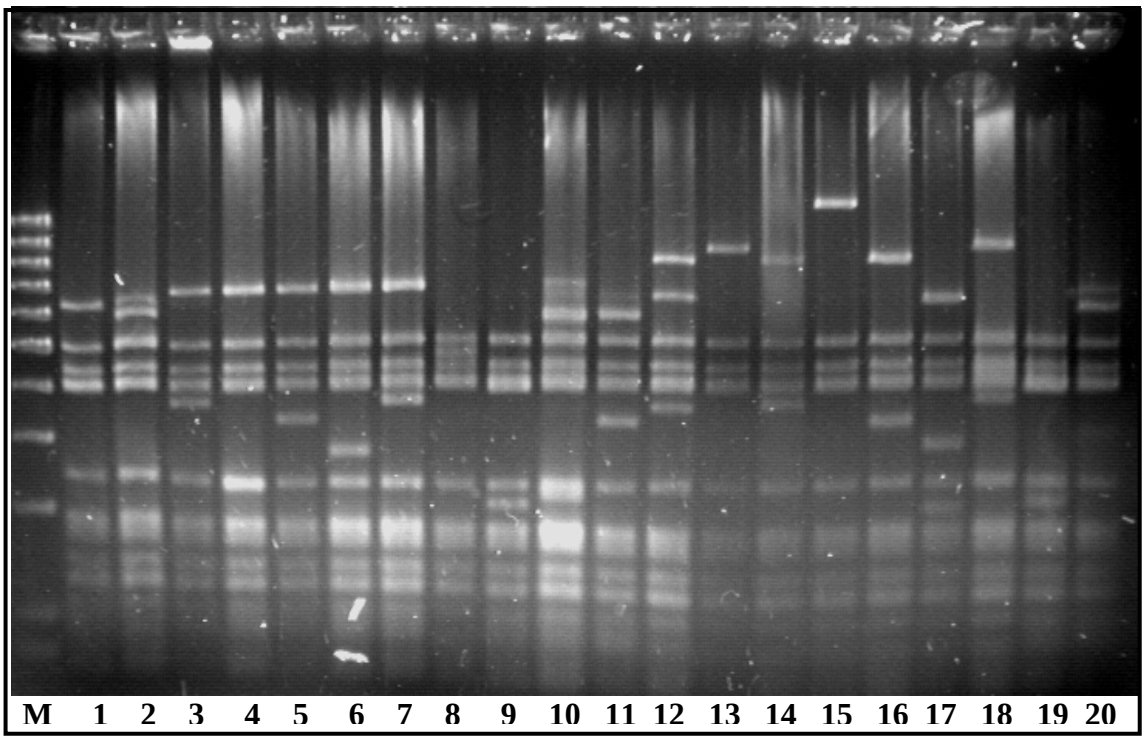


**Figure 46.** 3% high resolution gel showing the 20 different RFLP patterns observed in 18S clone libraries from eight weeks degraded litter.
lane M - 100 bp marker; lanes 1 – 20 – patterns 1 to 20.

Majority of the clones (>70% in beech libraries and 85% in spruce libraries) had sequences similar (≥98%) to members of Ascomycota (Figure 47). Ascomycota was also observed as the major fungal group in 18S libraries from green litter (see section 3.4.4.6). Sequences similar to members of other groups such as Basidiomycota, Zygomycota and Chytridiomycota were present in lower proportions. Clones similar to sequences reported in earlier studies, but mentioned as "unclassified" in the NCBI database, have been grouped together as "unclassified" in the present study.

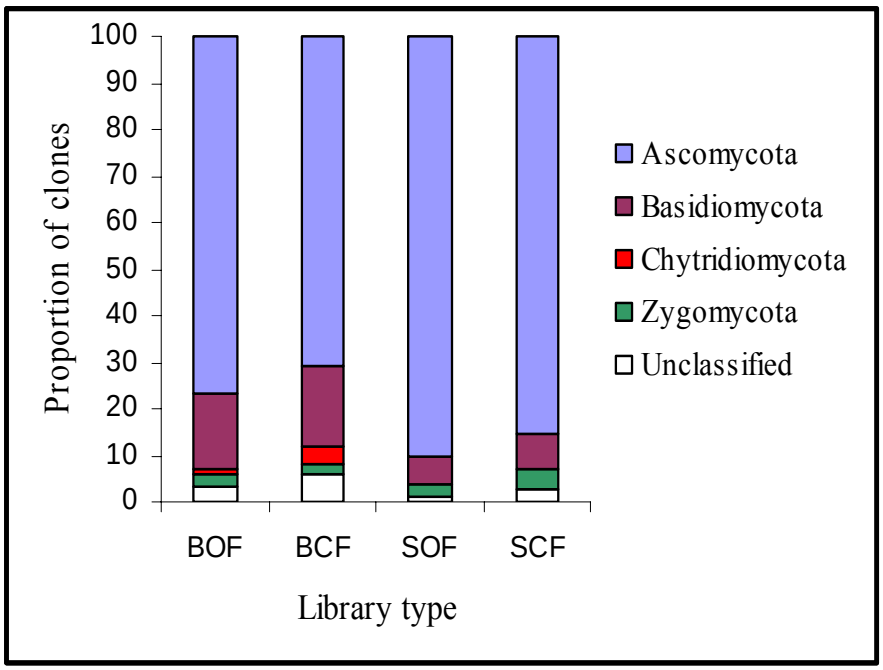


**Figure 47.** Relative distributions of clones to different fungal groups from eight weeks degraded litter.
BOF: **b**eech litter, **o**zone stressed, from Kranzberger **f**orest; BCF: **b**eech litter, **c**ontrol, from Kranzberger **f**orest; SOF: **s**pruce litter, **o**zone stressed, from Kranzberger **f**orest and SCF: **s**pruce litter, **c**ontrol, from Kranzberger **f**orest, respectively.

Effects of changed litter quality, due to ozone, on fungal community were evident by the reduced proportion of clones similar to members of Basidiomycota and Zygomycota in SOF library and Chytridiomycota in BOF library compared to their control counterparts. Clones similar to members of Chytridiomycota were absent from spruce libraries. Effects of ozone on litter quality were also evident when the distribution of clones similar to different members of Ascomycota was analysed.

Within Ascomycota, high proportion of clones were more than 98% similar to the 18S rRNA sequence of *Penicillium* and *Trichoderma* (Figure 48). The proportion of clones similar in sequence to 18S rRNA sequence of *Penicillium* increased from 40% in BCF library to 50% in BOF library while clones similar in sequence to 18S rRNA sequence of *Trichoderma* decreased. On the other hand, in spruce libraries, the proportion of clones similar to 18S rRNA sequence of *Trichoderma* increased from 46% in SCF library to 54% in SOF library. Clones similar to these two genera constituted the major proportion of Ascomycota in 18S libraries from green litter also (see section 3.4.4.6).

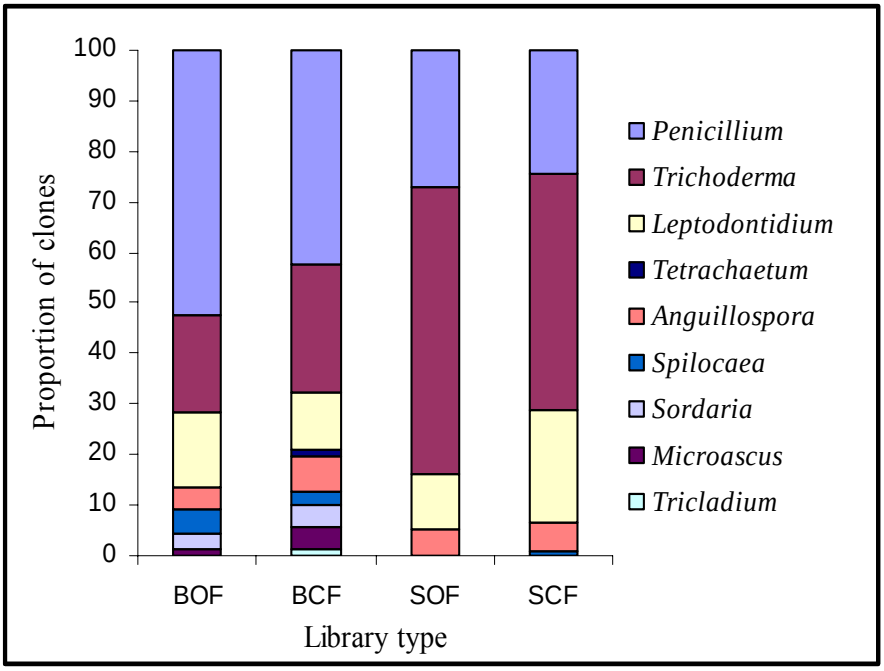


**Figure 48.** Relative proportions of clones similar to different genera of Ascomycota in 18S libraries from beech and spruce litter degraded for eight weeks.
BOF: **b**eech litter, **o**zone stressed, from Kranzberger **f**orest; BCF: **b**eech litter, **c**ontrol, from Kranzberger **f**orest; SOF: **s**pruce litter, **o**zone stressed, from Kranzberger **f**orest and SCF: **s**pruce litter, **c**ontrol, from Kranzberger **f**orest, respectively.

### *3.6.4.6 Rarefaction curves of fungal clone libraries*

As done for bacterial libraries, rarefaction analysis curves were generated for the four 18S libraries also (Figure 49). A plateau was obtained after screening 70 clones for each library type. No new fungal group was observed even when the number of clones was increased to 100. With 100 clones screened per library, the curves indicate the full coverage of fungal diversity colonizing the litter.

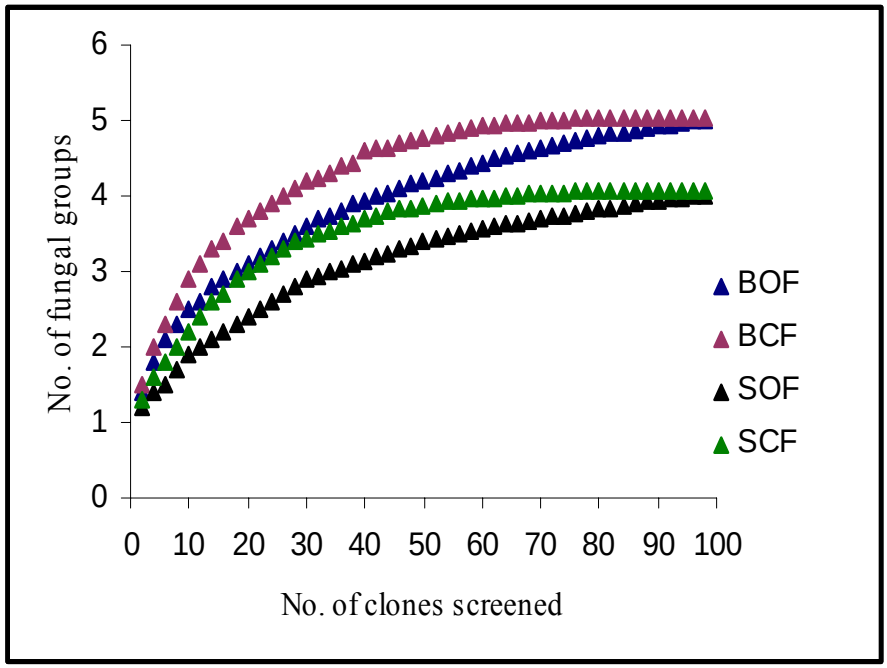


**Figure 49.** Rarefaction curves showing the extent of fungal diversity covered by the four libraries.
BOF: **b**eech litter, **o**zone stressed, from Kranzberger **f**orest; BCF: **b**eech litter, **c**ontrol, from Kranzberger **f**orest; SOF: **s**pruce litter, **o**zone stressed, from Kranzberger **f**orest and SCF: **s**pruce litter, **c**ontrol, from Kranzberger **f**orest, respectively.

# 4. DISCUSSION

Leaf litter decomposition is a key process governing nutrient availability and hence primary production in terrestrial ecosystems. Climatic and chemical factors regulate litter decomposition process mainly by affecting soil microorganisms, viz. both the activity and composition of the microbial community and by litter quality (Swift and Anderson, 1989). Relatively little is known how the initial litter quality and subsequent changes in the chemical composition of litter during decomposition, affect the colonizing microbial communities. Different substrate quality of conifer litter, (e.g. spruce) compared to deciduous litter, (e.g. beech) might affect the diversity of the microbial communities involved in the turnover of forest soil organic carbon and nitrogen. Moreover, a different substrate quality due to ozone stress, might affect the diversity of the forest soil microbial communities and warrants attention. Hence, effects of litter quality on the colonizing microbial communities need to be investigated. Therefore, the main objective of the present study was to investigate the effect of litter type and quality on the colonizing microbial communities. Using cultivation independent, RNA based molecular techniques clear effects of soil site, litter type (beech and spruce litter) and litter quality (control and ozone stressed litter) on the bacterial and fungal communities were evident. This leads to a better understanding and identification of the microbes colonizing different litter types.

## 4.1 Effects of plant species and ozone on litter quality

Substrate composition is one of the most important factors influencing the decomposition of plant residues in soils. Despite the fact that the build-up of thick organic horizons in spruce monocultures is well known, the mechanisms which are responsible for the accumulation of organic matter remains little understood. Various biochemical parameters were measured for beech and spruce litter both from plants in greenhouse (green litter) and from Kranzberger forest (natural litter).

Green spruce litter had higher sugar levels, soluble phenolics and low lignin and lignin:N ratio compared to green beech litter. Effects of ozone on litter quality were evident by higher lignin amounts and lignin:N ratio in litter from ozone stressed plants. The litter from these plants also had reduced sugar amounts. Lignin concentration and lignin to nitrogen ratio of plant litter has been shown to be inversely related to mass loss rates (Meentemeyer, 1978; Berg and Staaf, 1980; Melillo et al., 1982; Berg, 1984; Tian et al., 1992b; Van Vuuren et al., 1993). Ozone stress also affected the level of mineral nutrients in both beech and spruce litter.

Beech and spruce have been shown to differ in their sensitivity to ozone (Landolt et al., 2000). Ozone has been shown to alter leaf nutrient contents that affect litter quality. Scherzer et al. (1998) showed that ozone reduced foliar N concentrations in yellow-poplar leaves, which resulted in slower decomposition following senescence. Other studies have also shown a change in foliar N concentration in response to ozone treatment, affecting C:N ratio and litter quality (Berg and Staff 1980; Andersen et al., 2001).

Natural litter from Kranzberger had low sugars, soluble phenolics and high lignin and lignin:N ratios compared to green litter. With normal litter, much of the nitrogenous compounds and sugars are readsorbed by the plant prior to leaf fall (Prescott et al., 1989). As green litter was removed from the plants, there was no retranslocation of nutrients. This could be one reason for high sugar levels and low lignin:N ratios observed in green litter compared to natural litter. Other factors like differences in plant age, soil site and environmental conditions (temperature, moisture, photoperiod etc.), however, cannot be ruled out.

In contrast to the results with green litter, natural spruce litter collected from Kranzberger forest had higher levels of lignin and lignin:N ratios compared to beech litter from the same site. Sugar levels were comparable for beech and spruce litter. These biochemical differences between beech and spruce natural litter are in contrast to the report by Vesterdal (1999) where similar concentrations of mineral nutrients and condensed C compounds such as lignin, were reported for beech leaves and spruce needles of different origin. Effects of ozone on litter quality were in accordance to what was observed with green litter. Higher values were observed for lignin amounts and lignin:N ratio of litter from ozone stressed trees. Similar results have also been reported by Kim et al. (1998) where blackberry (*Rubus cuneifolus*) litter exposed to elevated ozone had greater permanganate lignin than control treatments. Thus, important parameters that determine the rate of decay (lignin and lignin:N) showed similar trends in response to ozone stress in both green litter and natural litter.

This changed litter quality due to ozone stress, may affect the normal process of decomposition by microorganisms, thereby also influencing the functioning of forest ecosystems. Decomposition of leaf litter is the key process linking the nitrogen and carbon cycles and the release of nutrients to the soil, all of which are then made available for plant uptake. Long-term shifts in decomposition could change the structure, function and persistence of an ecosystem by altering nutrient cycling rates. Majority of the studies about

the effect of ozone on litter quality and its degradation have investigated total N, total C, C:N ratios, lignin amounts and mass loss rates. Effects of the changed litter quality due to ozone, on the colonizing microbial community have not yet been described. Studying these effects was one of the objectives of the present study.

### 4.2 Microbial community analysis of soil sites selected for degradation

Small subunit ribosomal RNA and its genes have been extensively used to study microbial diversity in environmental samples. 16S rDNA and rRNA has been commonly used to study the bacterial diversity in various ecosystems (Rheims et al., 1996; Felske et al., 1998, Duineveld et al., 2001; Sharma et al., 2004a). Fungal diversity in environmental samples, e.g. decaying wood and rhizosphere soil, has been analysed using 18S rDNA as a molecular marker (Kowalchuk et al., 1997; Smit et al., 1999; Vainio and Hantula, 2000).

DNA-based detection assays do not discriminate between dormant and active populations. DNA obtained from environmental samples could originate from dormant cells, dead cells, or even from free DNA. Adsorption of DNA at mineral surfaces, especially in soil, could harbour more or less intact nucleic acids a long time after lysis of the source organism (Lorenz and Wackernagel, 1987). DGGE allows the analysis of a large number of samples and is a good tool for initial screening of similarities or differences between different communities (Heuer and Smalla, 1997). In the present study, the method was employed to investigate the microbial communities of the soil sites, microbial communities colonizing the different litter materials incubated in different soils and also for screening experimental variations between the replicates.

Detection of RNA, which typically has a short half-life, provides evidence of activity that can be correlated with the physiochemical conditions. As the ribosome per cell ratio is roughly proportional to growth rate of bacteria, with few exceptions (Wagner, 1994), 16S rRNA has been regarded as an indicator of total bacterial activity. To characterize the metabolically active bacteria and fungi in the soil samples, 16S and 18S rRNA were targeted in RT-PCR using defined primer systems. The resulting DGGE profiles were compared with those obtained by DNA target analysis. Each soil site had a distinct bacterial and fungal DGGE profile, indicating the microbial communities to be specific for each site. Comparison of the DGGE profiles revealed two major clusters, one formed by the forest sites and the other by the non-forest sites, thus providing the evidence for the effect of soil site on its microbial community structure.

Unlike previous report by Duineveld et al. (2001), wherein a reduced number of bands in RNA generated profiles was reported as compared to DNA-based profiles, this study shows an equally complex RNA profile when compared to the respective DGGE profile generated by DNA. However, it needs to be mentioned that in the study by Duineveld et al. (2001), rhizosphere soil was characterized. In the rhizosphere, plant exudates exert a selective effect and result in the development of microbial community that is plant rhizosphere specific (Sharma et al., 2004a and 2004b). Comparison of DNA and RNA profiles of the four different soil sites revealed marked differences between the total and metabolically active microbial communities. As the present study aimed to characterize the active microbial community colonizing beech and spruce litter, RNA extracted from the degraded litter, was targeted in all subsequent molecular analysis.

### 4.3 Degradation rates of litter

Results of dry weight measurement from experiments in non-forest and forest soil sites using green litter (see section 3.4.2 and 3.5.1) and natural litter (see section 3.6.2) were in accordance with the asymptotic model of litter decay (Johansson, 1994; Johansson et al., 1995). Rate of degradation was observed to be high during the first two weeks but was comparatively reduced thereafter. This was observed for both litter species (beech and spruce) in all the soil sites included in the study. During the initial weeks, the least recalcitrant components present, e.g., sugars, starch and low molecular weight extractives, are decomposed rapidly followed by a slow decay of lignin (Hammel, 1997). As litter decomposition proceeds, concentration of lignin and lignin-like substances increase because they are resistant to decay and enzymes catalyse the polymerisation of lignin-like substances and other forms of stabilized organic matter (Rutigiano et al., 1996). Another possibility could be the limitation of mineral nutrients in the system. Elevated concentrations of N and P have been shown to accelerate leaf decomposition via enhanced microbial activity (Sridhar and Bärlocher, 2000).

Unlike expected, green spruce litter was observed to degrade faster compared to green beech litter. This trend correlated well with low lignin and high sugar levels observed in the former (see section 3.1). Rapid mineralization of spruce compared to beech has also been reported by Albers et al. (2004). However, with natural litter from Kranzberger, no significant differences were observed between the degradation rates of natural beech and spruce litter after eight weeks. Similar results have earlier been reported by Vesterdal (1999) in a four-year

degradation experiment using beech and spruce litter materials of different origin in a range of study sites.

Dry weight measurements from the experiments with ozone stressed and control green litter (see section 3.5.1) and from experiments with natural litter (see section 3.6.2), revealed the effects of ozone on litter quality with respect to its subsequent mineralization. Compositional changes in leaf structural characteristics, such as lignin content, would be expected to alter rates of litter decomposition. Though ozone stress resulted in higher lignin levels and high lignin:N ratios, both of which indicate low mineralization rates, it was the ozone stressed litter that mineralized rapidly compared to its control counterpart. The effect of ozone on degradation rates was more pronounced at the first time point (two weeks) and was observed for both beech and spruce litter. It has been shown that N remobilization from foliage into the plant is not complete at the time of foliage abscission in ozone exposed plants (Matyssek et al., 1993, Patterson and Rundel, 1995). Greater N content of senesced litter from ozone stressed trees, could increase rates of decomposition in the initial weeks when the least recalcitrant components, e.g. sugars, starch etc., are degraded. In previous studies, high N concentration in litter have been shown to support high degradation rates in the initial phase of decomposition (Berg and McClaugherty, 2003).

High lignin and ligin:N ratios and low sugar levels observed in natural litter, correlate with its low degradation rates compared to green litter. With normal litter, much of the nitrogenous compounds are reabsorbed by the plant prior to leaf fall (Prescott et al., 1989). Green litter was removed from the plant and so, there was no retranslocation of nutrients. As a result, this green litter was rich in nutrients that are otherwise limiting in normal litter and supported greater microbial activity than in the normal case. Besides, the differences between the degradation rates of natural and green litter could also be attributed to differences in soil site, plant age and environmental conditions.

### 4.4 Total RNA fingerprinting as a useful tool for microbial community analysis

The present study demonstrates that a short arbitrary primer may be used to reproducibly generate RNA fingerprints from complex microbial communities. Identification of microorganisms from environmental samples has been revolutionized by the application of techniques like PCR and FISH. While both the techniques are very powerful methods, conventional primers for PCR and oligos for FISH require sequence information from the

relevant genes. RAP-PCR employs short arbitrary primers and thus, no prior sequence information about the organisms is needed. Application of RAP-PCR to describe microbial gene transcription from environmental samples has been restricted so far mainly to pure cultures. Fleming et al. (1998), for example, could show the induction of genes by toluene using *P. putida* and by salicylate using *B. cepacia* as model organisms. In the present study RAP-PCR, without radioactivity, was successfully applied to characterize the microbial community colonizing beech and spruce litter. In a recent study by Frias-Lopez et al. (2004), RAP-PCR was used to identify differentially expressed genes in bacteria associated with coral black band disease.

As the initial litter material was free of RNA and the mesh size of the litter bags was 40 µm, all RNA extracted from the litter material after incubation in the different soils can be assigned to microbial activity. Succession of the microbial communities during the course of degradation was observed when the RAP-profiles of litter degraded for two weeks were compared with that of eight weeks degraded litter. Each litter sample had its own unique metabolic profile and both soil site effects and litter type effects were evident. UPGMA tree generated for the RAP-profiles showed two major clusters, one each by the profiles of litter degraded in forest and non-forest sites, with one exception (beech litter incubated for two weeks in the beech forest soil). These results provided the evidence that the microbial communities colonizing the two litter types in non-forest sites were different from those in the forest sites.

RAP-PCR products for both beech and spruce litter incubated in the BS soil site were also cloned to generate libraries. The sequences under consideration were in the range of 400 – 700 bp, which are not enough to link a particular sequence to a particular species. Therefore, no attempts to assign sequences to any species were made. Not surprisingly, clone libraries were dominated by ribosomal RNA, as it is known to have higher stability than mRNA and is also present in higher proportion in an organism. Majority of the ribosomal sequences were similar to 16S rRNA sequences of known members of proteobacteria and 18S rRNA sequences of members of Ascomycota. However, the presence of bacterial esterase, bacterial cytochrome P450 hydroxylase, Excinuclease ABC Subunit A (bacterial DNA repair) and AUR 1 (phospholipid metabolism in *S. cerevisiae*) in the generated libraries shows that at least 20% of the cDNA is of messenger RNA origin. Furthermore, sequences of three RFLP patterns had no direct match in the database even when amino acid sequences were used for

screening, thus highlighting the fact that the present databases are limiting with respect to the environmental sequences.

In subsequent experiments with ozone stressed and control green litter (see section 3.5.2), effects of soil sites and litter quality on microbial community structure and function were evident in RAP profiles. RAP-PCR profiles from natural litter degradation experiment (see section 3.6.3) provided the initial evidence that changed litter quality due to ozone stress also affected the structure and function of the colonizing microbial communities. In summary, RAP-PCR was successfully applied to study microbial activity in degrading leaf litter. The method provides a rapid tool to assess similarities and differences between samples and also changes induced in the community over time. It can also be applied to study the active microbial community of an environmental sample without the prior knowledge of sequences in the system. The technique has also been successfully applied to study rhizosphere soil microbial communities (Sharma et al., 2004b).

### 4.5 Effect of soil site and litter type on the colonizing microbial communities

The results from RAP-profiles provided the initial evidence that both soil site and litter type influence the colonizing microbial communities and their succession during the course of degradation. To gain a detailed insight into the bacterial and fungal communities, specific primers targeting 16S rRNA and 18S rRNA were used in RT-PCR reactions. The present study aimed to investigate if the natural microbial community specific for a particular site had a greater effect on litter colonization or if the litter type was the determining factor in the development of colonizing microbial community. By comparing the DGGE profiles of the same litter degraded in different soil sites, it was observed that the diversity of the microbial community colonizing the litter, was determined by the soil site. The diversity of bacterial and fungal communities colonizing beech and spruce litter was comparable between the two non-forest sites. Within the forest sites, the bacterial communities colonizing beech and spruce litter incubated in the beech-spruce mixed forest site were more diverse compared to those from the beech forest site. An opposite trend was observed with fungal DGGE profiles. Fungal communities colonizing beech and spruce litter in beech forest site were more diverse than their counterparts from the beech-spruce mixed forest. This was evident at both the time points. Within each site, the effects of litter type were evident by the close clustering of two and eight week profiles of each litter type. Litter quality lead to the selection of specific bacterial and fungal members from the total microbial diversity present in the soil system. Differences between the profiles of beech and spruce litter degraded in the same site can be

attributed to the differences in various biochemical characteristics of the two litters described in section 3.1 and discussed in section 4.1. Succession of microbial community during the course of decomposition was evident by DGGE and also confirmed by cloning. Succession of microbial communities during organic matter turnover in litter bags has also been shown earlier using cultivation dependent techniques (Frankland, 1992; Cox et al., 1997; Dilly and Munch, 2001; Buchan et al., 2003).

### 4.5.1 Bacterial communities

16S rRNA clone libraries were made to identify the key bacterial groups colonizing both litter types incubated in BS site. Different subdivisions of proteobacteria constituted the major proportion of clones in the four libraries. Dominance of proteobacteria was earlier reflected in RAP-libraries where different subdivisions of proteobacteria constituted the bacterial fraction. Clones similar in sequence to members of γ-subdivision of proteobacteria dominated the bacterial libraries derived from beech litter. The proportion of such clones increased from two to eight weeks. Sequences similar to *Pseudomonas* dominated the γ-proteobacterial fraction of the libraries from degraded beech litter. Furthermore, sequences similar to members of Actinobacteria and Acidobacteria were also present. In a related study, Wahbeh and Mahasneh (1985), following the *in situ* degradation of seagrass *Halophila stipulacea*, showed that *Pseudomonas, Actinomyces* and *Arthrobacter* were the most prominent genera involved in decomposition. All of the above mentioned genera are capable of degrading structural carbohydrates.

For spruce litter, major proportion of the clones belonged to different subdivisions of proteobacteria. There was an increase in the proportion of clones similar to α-proteobacteria and γ-proteobacteria in eight weeks library. Members of α-proteobacteria have also earlier been shown to be key degraders of salt marsh grass, *Spartina alterniflora* (Buchan et al., 2003). Unlike in spruce litter library after eight weeks, where sequences similar to α-, β- and δ- proteobacteria members constituted more than 50% of the colonizing bacterial community, their absence from beech library after eight weeks highlight the selective nature of litter type on the colonizing bacterial community.

### 4.5.2 Fungal communities

In all the four libraries, clones similar in sequence to members of Ascomycota constituted the major proportion of clones. Ascomycota was also observed as the major fungal group in RAP-libraries. Previous studies of fungal community of south-eastern U. S. salt marshes have

identified several species of ascomycetous fungi as major decomposers of *S. alterniflora* blades, based on both traditional culture- and microscopy-base methods (Newell and Porter, 2000, Newell, 2001a, 2001b) as well as using molecular approaches (Buchan et al., 2002). In 18S rRNA libraries from spruce litter, clones similar to Basidiomycota members were present in significant proportions in library after eight weeks but were absent from two weeks library. A succession of functional groups of fungi linked to plant litter quality occurs during the process of decomposition in which Basidiomycota species may dominate over Ascomycota during the later stages of decomposition, because they can synthesise enzymes required to degrade complex polymers (Deacon, 1997). Cox et al. (1997) and Frankland (1992) have earlier shown the effect of litter quality on the sequence of fungal succession during the course of degradation using cultivation dependent techniques. Absence of Zygomycota members from spruce libraries and Basidiomycota members from beech libraries reflects the selective nature of plant litter.

This selective effect of litter type could be the reason for reduced diversity observed in the clone libraries after eight weeks. In contrast, DGGE profiles show an increase in diversity (bacteria) or comparable diversity (fungi) from two to eight weeks. However, it also needs to be mentioned that fingerprints such as DGGE resolve individual sequences whereas clone libraries are based on bacterial or fungal groups. Moreover, one organism can produce more than one band in a DGGE profile due to sequence heterogeneity in rRNA operons (Nübel et al., 1996).

### 4.6 Effect of ozone on litter quality and on the colonizing microbial communities

Ozone has been shown to affect leaf nutrient levels and also litter quality (Scherzer et al., 1998; Andersen et al., 2001). Effects of ozone on various biochemical parameters of litter were observed in the present study too and have been discussed in section 4.1. From the dry weight measurements, a trend for rapid mineralization of litter from ozone stressed plants and trees, compared to control litter type was observed (see section 4.3). DGGE profiles of 16S and 18S RT-PCR products were used to compare the underlying microbial communities. In experiments with the green litter in four different soil sites, distinct differences were observed in the microbial communities colonizing control and ozone stressed litter of the same type (beech or spruce) degraded in the same site. There was an increase in the diversity of bacterial communities from two to eight weeks while fungal diversity was comparable for the two time points. In a related study investigating the decomposition of wheat and rye grass litter filled in

litter bags and incubated in different agricultural soil sites, Dilly et al. (2004) reported an increase in diversity of microbial community during the course of litter decomposition using the number of bands observed in DGGE profiles as an indicator of diversity. Cluster analysis of the DGGE profiles revealed that at the initial time point of two weeks, it was the soil site that was the deciding factor for the build up of the microbial communities. By eight weeks, litter type plays a dominant role and is a selecting parameter for microbial communities.

To validate these results in natural system, litter degradation experiments were done in Kranzberger forest using natural litter from that site. Clear differences were observed between the DGGE profiles of microbial communities colonizing beech and spruce litter. These differences can be attributed to the differences in their biochemical composition (see section 3.2 and 4.1). An increase in diversity in bacterial communities from two to eight weeks, as observed in green litter, was however not visible with natural litter. Effects of changed litter quality due to ozone stress (see section 3.2 and 4.1), on the colonizing microbial communities were evident by DGGE.

These observations, both with green litter and natural litter, support the hypothesis that changes in litter quality caused by ozone, do influence the subsequent microbial colonization of the litter. Bacterial and fungal groups colonizing natural beech and spruce litter (both ozone stressed and control) after eight weeks were identified by making clone libraries. Ozone may affect early stages of decomposition by altering populations of leaf surface organisms, before or after senescence. Magan et al. (1996), found a shift in phyllosphere fungi on Scots pine (*Pinus sylvestris*), Sitka spruce (*Picea sitchensis*) and Norway spruce (*Picea abies*) exposed to ozone, but the potential effect of these changes on subsequent litter decomposition remains uncertain.

### 4.6.1 Bacterial communities

16S rRNA clone libraries were made to identify the key bacterial groups colonizing the different litter types. Actinobacteria dominated the bacterial clone libraries derived from all litter types. There was not much difference in the proportion of such clones between the control litter and the litter from ozone stressed trees. However, significant difference was observed between beech and spruce libraries. In a related study by Akasaka et al. (2003), actinobacteria was observed to be the most dominant bacterial group in rice plant residue degradation. Wahbeh and Mahasneh (1985), following the *in situ* degradation of seagrass *Halophila stipulacea*, showed that most prominent genera involved in decomposition were

*Actinomyces*, *Arthrobacter*, *Bacillus* and *Pseudomonas*, all of which are capable of degrading structural carbohydrates. Dominance of Actinobacteria was in contrast to the libraries from green litter, where instead proteobacteria dominated the four libraries (see section 4.5.1). Green litter was rich in sugars and had low lignin amounts (see section 4.1). Thus, it supported the typically r-strategic proteobacteria. Unlike proteobacteria, Actinobacteria are K-strategic and typically soil bacteria. They could therefore colonize the nutrient poor (low sugars, high lignin) natural litter. Moreover, as the experiments with green litter and natural litter were carried out in different soil sites and time, the influence of site-specific factors and other environmental conditions, in addition to litter quality, cannot be negated. Clones similar to members of α- proteobacteria were also present in varying proportions in all libraries. Members of α-proteobacteria have earlier been shown to be key degraders of salt marsh grass, *Spartina alterniflora* (Buchan et al., 2003). Absence of sequences similar to β-proteobacteria members from beech libraries, highlight the selective nature of litter type on the degrading bacterial community. Effects of changed litter quality due to ozone, on the bacterial community are evident by the absence of clones similar to members of γ-proteobacteria and an increase in the proportion of clones similar to β-proteobacteria members in SOF library. An opposite trend was observed in the case of beech litter where clones similar to members of γ-proteobacteria were present in increased proportion in BOF library. A significant proportion of clones in spruce libraries were similar to sequences reported in earlier studies, but mentioned as "unclassified" in the NCBI database. There was a slight increase in the proportion of such clones in SOF library.

### 4.6.2 Fungal communities

Sequences similar to Ascomycota members dominated the four fungal libraries, followed by Basidiomycota and Zygomycota. Dominance of Ascomycota was also observed in libraries from green litter. The proportion of major fungal groups was more similar between control litter and litter from ozone stressed trees of the same species than between beech and spruce. Previous studies of fungal community of south-eastern U. S. salt marshes have identified several species of ascomycetous fungi as major decomposers of *S. alterniflora* blades, based on both traditional culture- and microscopy-base methods (Newell and Porter, 2000; Newell, 2001a, 2001b) as well as using molecular approaches (Buchan et al., 2002). Effects of ozone on litter quality and subsequently on the degrading fungal communities were evident by the reduced proportion of clones similar in sequence to members of Chytridiomycota in BOF library and clones similar in sequence to Basidiomycota and Zygomycota in SOF library.

Chytridiomycota members were absent from spruce litter library reflecting the selective nature of plant litter. Cox et al. (1997, 2001) and Frankland et al. (1992) have earlier shown the effect of litter type 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 different litter type.

### 4.7 Outlook

In this study, the effects of soil site and litter quality on the colonizing microbial community was presented. There is considerable body of literature on the effects of ozone on plant growth and physiological and biochemical changes caused in the plant (Andersen, 2003) but relatively few reports are available on the effect of ozone on litter quality. Moreover, most of the studies dealing with the effect of ozone on litter quality, address the total C, total N, lignin amounts and mass loss rates of the litter. How this changed litter quality affects microbial community, which is pivotal to litter degradation, humus formation and nutrient turnover processes has not been addressed yet. In the present study, cultivation independent techniques have been applied to show the effect of changed litter quality due to ozone, on the colonizing microbial communities. 16S and 18S rRNA used as molecular probes provided a detailed information about bacterial and fungal groups involved in leaf litter colonization.

However, it must be considered that leaves in litter bags may face a slightly different microclimate and microhabitat compared to natural environment, so the results in the field may differ slightly from that in the litter bag (Tam et al., 1990). The mesh size of the bag allows bacteria and fungi, but other large invertebrates that play significant roles in leaf litter decay are excluded from the study. The litter bag method may underestimate actual decomposition but reflects trends and allows comparison among species and sites (Wieder and Lang, 1982). Influence of soil site on the mass loss and nutrient release from decomposing beech and spruce litter has previously been reported by Vesterdal (1999). However, microbial communities, which play a key role in such soil processes, were not described. Succession of microbial communities during organic matter turnover in litter bags has been shown earlier using cultivation dependent techniques (Cox et al., 1997; Dilly and Munch, 2001; Buchan et al., 2003). It is generally accepted that cultivation methods recover less than 1% of the total microorganisms present in environmental samples (Amann et al., 1995); therefore, microbial investigations based only on cultivation strategies can not reflect

the microbial diversity and activity present. In the present study, using 16S and 18S rRNA as molecular probes, detailed information about bacterial and fungal groups colonizing leaf litter was obtained. New groups in addition to those reported in earlier studies (Cox et al., 1997; Dilly and Munch, 2001; Buchan et al., 2003) were also observed as active colonizers, thus reflecting an upper hand of the methods employed.

Additional long-term degradation experiments with natural litter are required for the better understanding of the dynamics of the involved microbial community during litter degradation. Studies that combine the techniques of fluorescence in-situ hybridization (FISH) and *in-situ* PCR are needed to provide greater insights into the bacterial and fungal groups involved in degradation and their enzymatic activities *in situ*. These would lead to a better understanding of the structure and function of the microbial communities. Besides, quantitative estimations of carbon and nitrogen allocation to different pools (e.g. leaching, microbial biomass, mineralization to $CO_2$ and $N_2$) as well as analysis of physical soil structure and stability would significantly enhance the present knowledge of carbon and nitrogen fluxes during decomposition and its long term influence on soil.

# 5. SUMMARY

Litter degradation constitutes an essential aspect of nutrient cycling and energy transfer in most ecosystems. Plant litter in temperate and boreal coniferous forests is decomposed through microbial activity and the quantitative contribution of microorganisms to decomposition is considered to be above 95% with soil animals being responsible for the remaining maximum 5% (Berg et al., 2003). The present study was based on the hypothesis that both soil site and litter quality influence the build up and colonization of microbial communities in litter. It was also hypothesized that ozone stress to the plants and trees affects the litter quality that then subsequently affects its microbial colonization. To address these hypotheses, the classic litter bag technique using beech and spruce litter from plants with and without ozone stress was combined with RNA-based fingerprinting method to get information about litter degradation on one hand and the corresponding active microbial communities on the other.

Litter bags were filled with green beech and spruce litter and were incubated for two and eight weeks in the upper soil of non-forest (grass field and maize field) and forest (beech forest and beech-spruce mixed forest) sites, with comparable soil properties. Dry weight measurements revealed a two-step process (asymptotic model) with high degradation rates during the first two weeks, followed by slow decomposition. High mineralization rates for spruce compared to beech were observed. On generating metabolic profiles using 10 mer primer in RAP-PCR, it was observed that the profiles derived from litter degraded in forest sites were comparatively more complex than those from non-forest soil sites. 16S and 18S rRNA were used as molecular markers for analysis of bacterial and fungal colonizers. DGGE fingerprinting of the RT-PCR products revealed the effect of soil site on the diversity of the colonizing microbial communities. Litter type resulted in the selection of specific microbes from the total diversity present in the soil sites. RT-PCR products for beech and spruce litter incubated in the beech-spruce mixed forest were also cloned to identify the colonizing bacterial and fungal groups. Clones similar to members of different subdivisions of proteobacteria dominated the bacterial libraries. Clones similar to members of Ascomycota dominated the four fungal libraries. *Penicillium*, *Trichoderma* and *Myrothecium* constituted the major proportion of clones within Ascomycota. Effect of litter type on fungal community was evident by the absence of Basidiomycota members from beech litter libraries and Zygomycota members from spruce litter libraries.

Ozone is considered as the main factor in air-pollution related decline of forest in North America and Europe. Previous studies have reported the effects of ozone on litter quality. In the present study, ozone stress was given to plants and mature trees, respectively, to study the effects of ozone on litter quality and degradation rates as well as on microbial colonization. With both green litter from plants in greenhouse and with natural litter from Kranzberger forest, a tendency for rapid mineralization of litter from ozone stressed plants and trees compared to control litter was observed by dry weight measurements. Differences between the microbial communities (both their structure and function) colonizing the control and ozone stressed litter of the same species were investigated by RAP-PCR. DGGE fingerprinting of the 16S and 18S RT-PCR products revealed that changed litter quality, due to ozone stress, resulted in differences in the structure of the colonizing bacterial and fungal communities. Such differences were observed for both beech and spruce litter. Clone libraries (both 16S and 18S) were constructed to identify the bacterial and fungal groups colonizing natural litter at eight weeks. Clones similar to members of Actinobacteria dominated the bacterial libraries from natural litter unlike the earlier observed dominance of proteobacteria in the clone libraries derived from green litter. Effects of ozone on litter quality and subsequently on the colonizing bacterial community were evident by the absence of γ-proteobacteria from ozone stressed spruce litter library. Clones similar to member of β-proteobacteria were present in reduced proportions in control spruce litter library. In libraries generated from beech litter, reduced proportions of α–proteobacteria and increased proportions of γ-proteobacteria were observed in the libraries from ozone stressed litter compared to its control counterpart. In the fungal libraries, as earlier observed for 18S libraries generated from green litter, clones similar to members of Ascomycota dominated. Effects of changed litter quality due to ozone, on fungal communities were evident by the reduced proportion of clones similar to members of Basidiomycota and Zygomycota in SOF library and Chytridiomycota in BOF library compared to their control counterparts. Clones similar to members of Chytridiomycota were absent from spruce libraries. Effects of ozone on litter quality were also evident when the distribution of clones similar to different members of Ascomycota was analysed.

In this study, the decay parameters for litter were shown to be soil site-specific and plant species dependent. Using two different plant species, four different soil sites and two time points, it could be shown that the diversity of the microbial colonizers were dependent on the site (soil type, vegetation, pH etc.). Litter type results in the selection of specific microbial

groups from the total diversity present in soil site. Ozone stress resulted in differences in both litter degradation rates and the colonizing microbial communities as hypothesized.

# 6. REFERENCES

**Aber, J. D. and Melillo, J. M. (1982)** Nitrogen immobilization in decaying hardwood leaf litter as a function of initial nitrogen and lignin content. Can. J. Botany 60:2263–2269.

**Aber, J. D., McClaugherty, C. A. and Melillo, J. M. (1984)** Litter decomposition in Wisconsin forests - Mass loss, organic-chemical constituents and nitrogen. University of Wisconsin Research Bulletin R3284, University of Wisconsin, Madison, WI.

**Abrams, E. S. and Stanton, V. P. (1992)** Use of denaturing gradient gel electrophoresis to study conformational transitions in nucleic acids. Method Enzymol. 212:71-104.

**Abu Kwaik, Y. and Pederson, L. L. (1996)** The use of differential display-PCR to isolate and characterize a *Legionella pneumophila* locus induced during the intracellular infection of macrophages. Mol. Microbiol. 21:543-556.

**Aerts, R. and De Caluwe, H. (1994)** Nitrogen use efficiency of *Carex* species in relation to nitrogen supply. Ecology 75:2362–2372.

**Akasaka, H., Izawa, T., Ueki, K. and Ueki, A. (2003)** Phylogeny of numerically abundant culturable anaerobic bacteria associated with degradation of rice plant residue in Japanese paddy field soil. FEMS Microbiol. Ecol. 43:149-161.

**Albers, D., Migge, S., Schaefer, M. and Scheu, S. (2004)** Decomposition of beech leaves (*Fagus sylvatica*) and spruce needles (*Picea abies*) in pure and mixed stands of beech and spruce. Soil Biol. Biochem. 36:155-164.

**Amann, R. I., Ludwig, W. and Schleifer, K. H. (1995)** Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143-169.

**Andersen, C. P. (2003)** Source-sink balance and carbon allocation below ground in plants exposed to ozone. New Phytol. 157:213-228.

**Andersen, C. P. Hogsett, W. E., Plocher, M., Rodecap, K. and Lee, E. H. (2001)** Blue-wild-rye grass competition increases the effect of ozone on ponderosa pine seedlings. Tree Physiol. 21:319-327.

**Anderson, J. M. (1988)** Spatio-temporal effects of invertebrates on soil processes. Biol. Fert. Soils 6:216–227.

**Bååth, E., Frostegård, A., Pennanen, T. and Fritze, H. (1995)** Microbial community structure and pH response in relation to soil organic matter quality in wood-ash fertilized, clear-cut or burned coniferous forest soils. Soil Biol. Biochem. 27:229-240.

**Baker, T. R., Allen, H. L., Schoenberger, M. M. and Kress, L. W. (1994)** Nutritional response of loblolly pine exposed to ozone and simulated acid rain. Can. J. Forest Res. 24:453-461.

**Ball, A. S. (1997)** Microbial decomposition at elevated $CO_2$ levels: effect of litter quality. Glob. Change Biol. 3:379-386.

**Barns, S. M., Fundyga, R. E., Jeffries, M. W. and Pace, N. R. (1994)** Remarkable Archaeal diversity detected in a Yellowstone National Park hot spring environment. P. Natl. Acad. Sci. USA 91:1609-1613.

**Berendse, F. (1990)** Organic matter accumulation and nitrogen mineralization during secondary succession in heathland ecosystems. J. Ecol. 78:413–427.

**Berendse, F., Bobbink, R. and Rouwenhorst, G. (1989)** A comparative study on nutrient cycling in wet heathland ecosystems. II. Litter decomposition and nutrient mineralization. Oecologia 78:338–348.

**Berg, B. (1984)** Decomposition of root litter and some factors regulating the process: long-term root litter decomposition in a Scots pine forest. Soil Biol. Biochem. 16:609–618.

**Berg, B. and McClaugherty, C. (2003)** Initial litter chemical composition. In: Plant litter: Decomposition, humus formation and carbon sequestration (Berg, B. and McClaugherty, C., Eds.), pp 49-78, Springer Verlag, Germany.

**Berg, B., Virzo De Santo, A., Rutigliano, F. A., Fierro, A. and Ekbohm, G. (2003)** Limit values for plant litter decomposing in two contrasting soils—influence of litter elemental composition Acta Oecol. 24:295-302.

**Berg, B. and Staff, H. (1980)** Decomposition rate and chemical changes of Scots pine needle litter. II. Influence of chemical composition. In: Structure and Function of Northern Coniferous Forests - An ecosystem study (Persson, T., Ed.), Ecological Bulletins (Stockholm) 32:373-390.

**Berg, B., Hannus, K., Popoff, T. and Theander, O. (1982)** Changes in organic-chemical components during decomposition. Long-term decomposition in a Scots pine forest I. Can. J. Botany 60:1310-1319.

**Berg, B., Muller, M. and Wessen, B. (1987)** Decomposition of red clover (*Trifolium pratense*) roots. Soil Biol. Biochem. 19:589–594.

**Boerner, E. J. and Rebbeck, J. (1995)** Decomposition and nitrogen release from leaves of three hardwood species grown under elevated $O_3$ and /or $CO_2$. Plant Soil 170:149-157.

**Bogan, B. W., Schoenike, B., Lamar, R. T. and Cullen, D. (1996)** Manganese peroxidase mRNA and enzyme activity levels during bioremediation of polycyclic aromatic

hydrocarbon-contaminated soil with *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 62:2381-2386.

**Bremer, E., van Houtum, W. and van Kessel, C. (1991)** Carbon dioxide evolution from wheat and lentil residues affected by grinding, added nitrogen, and absence of soil. Biol. Fert. Soils 11:221-227.

**Brock, T. D. (1987)** The study of microorganisms *in situ*: progress and problems. Symposium of the Society for General Microbiology 41:1–17.

**Buchan, A., Newell, S. Y., Moreta, J. I. and Moran, M. A. (2002)** Molecular characterization of bacterial and fungal decomposer communities in a southeastern U.S. saltmarsh. Microbial Ecol. 43:329-340.

**Buchan, A., Newell, S. Y., Butler, M., Biers, E. J., Hollibaugh, J. T. and Moran, M. A. (2003)** Dynamics of bacterial and fungal communities on decaying salt marsh grass. Appl. Environ. Microbiol. 69:6676-6687.

**Chadwick, D. R., Ineson, P., Woods, C. and Piearce, T. G. (1998)** Decomposition of *Pinus sylvestris* litter in litter bags: influence of underlying native litter layer. Soil Biol. Biochem. 30:47-55.

**Chapin, F. S. (1991)** Effects of multiple environmental stresses on nutrient availability and use. In: Response of plants to multiple stresses (Mooney, H. A., Winner, W. E. and Pell, E. J., Eds.), pp 68–88, Academic Press, San Diego, California, USA.

**Chappelka, A. H. and Chevone, B. I. (1992)** Tree responses to ozone. In: Surface level ozone exposures and their effects on vegetation (Lefohn, A. S. Ed.) pp 271-324, Lewis Publishers, Chelsea, MI.

**Choi, B. K., Paster, B. J., Dewhirst, F. E. and Gobel, U. B. (1994)** Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. Infect. Immun. 62:1889-1895.

**Clegg, C. D., Ritz, K. and Griffiths, B. S. (1997)** Direct extraction of microbial community DNA from humified upland soils. Lett. Appl. Microbiol. 25:30-33.

**Clegg, C. D., Ritz, K. and Griffiths, B. S. (1998)** Broad-scale analysis of soil microbial community DNA from upland grasslands. Antonie Leeuwenhoek 73:9-14.

**Coulson, J. C. and Butterfield, J. (1978)** An investigation of the biotic factors determining the rates of plant decomposition on blanket bog. J. Ecol. 66:631–650.

**Cox, P., Fischer, P. J. and Anderson, J. M. (1997)** Experiments in fungal survival of two common pine litter colonisers. Mycologist 11**:**55-58.

**Cox, P., Wilkinson, S. P. and Anderson, J. M. (2001)** Effects of fungal inocula on the decomposition of lignin and structural polysaccharides in *Pinus sylvestris* litter. Biol. Fert. Soils 33:246-251.

**Cullen, D. W. and Hirsch, P. R. (1998)** Simple and rapid method for direct extraction of microbial DNA from soil for PCR. Soil Biol. Biochem. 30:983–993.

**Deacon, J. W. (1997)** Modern mycology. Blackwell Scientific Publications, Boston.

**Dilly, O. and Munch, J. C. (2001)** Shifts in physiological capabilities of the microbiota during the decomposition of leaf litter in black alder (*Alnus glutinosa* (Gaertn.) L.) forest. Soil Biol. Biochem. 33:921-930.

**Dilly, O., Bloem, J., Vos, A. and Munch, J. C. (2004)** Bacterial diversity in agricultural soils during litter decomposition. Appl. Environ. Microbiol. 70:468-474.

**Duineveld, B. M., Kowalchuk, G. A., Keijzer, A., van Elsas, J. D. and van Veen, J. A. (2001)** Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. Appl. Environ. Microbiol. 67:172-178.

**Eamus, D., Barnes, J. D., Mortensen, L., Ro-Poulsen, H. and Davison, A. W. (1990)** Persistent stimulation of $CO_2$ assimilation and stomatal conductance by summer ozone fumigation in Norway spruce. Environ. Pollut. 63:365–379.

**Edwards, G. S., Kelly, J. M. and Mays, P. A. (1992)** Ozone, acidic precipitation, and soil Mg impacts on soil and loblolly pine seedling nutrient status after three growing seasons. Water Air Soil Poll. 63:281-294.

**Felske, A., Backhaus, H. and Akkermmans, A. D. L. (1997)** Direct ribosome isolation from soil. In: Molecular Microbial Ecology Manual, supplement 3 (Akkermmans, A. D. L., van Elsas, J. D. and de Bruijn, F. J. Eds.) pp 1.2.4/1-10, Kluwer Academic Publishers, Dordrecht, The Netherlands.

**Felske, A., Engelen, B., Nubel, U. and Backhaus, H. (1996)** Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. Appl. Environ. Microbiol. 62:4162-4167.

**Felske, A., Wolterink, A., van Lis, R. and Akkermans, A. D. L. (1998)** Phylogeny of the main bacterial 16S rRNA sequences in Drenste A grassland soils (The Netherlands). Appl. Environ. Microbiol. 64:871-879.

**Fengel, D. and Wegener, G. (1983)** Wood: chemistry, ultrastructure, reactions, pp 613, Walter De Gruyter, New York.

**Ferris, M. J. and Ward, D. M. (1997)** Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 63:1375-1381.

**Field, C. B., Chapin, F. S., Matson, P. A. and Mooney, H. A. (1992)** Responses of terrestrial ecosystems to the changing atmosphere: a resource–based approach. Ann. Rev. Ecol. Syst. 23:201–235.

**Fishman, J. (1991)** The global consequences of increasing tropospheric ozone concentrations. Chemosphere 7:685-695.

**Fleming, J. T., Sanseverino, J. and Sayler, G. S. (1993)** Quantitative relationship between naphthalene catabolic gene frequency and expression in predicting PAH degradation in soils at town gas manufacturing sites. Environ. Sci. Technol. 27:1068-1074.

**Fleming, J. T., Sanseverino, J. and Sayler, G. S. (1998)** Optimization of differential display of prokaryotic mRNA: Application to pure culture and soil microcosms. Appl. Environ. Microbiol. 64:3698-3706.

**Fog, K. (1988)** The effect of added nitrogen on the rate of decomposition of organic matter. Biol. Rev. 63:433-462.

**Frankland, J. C. (1992)** Mechanisms in fungal succession. In: The Fungal Community: its Organisation and Role in the Ecosystem (Carroll, G. C. and Wicklow, D. T., Eds.), pp 383-402, Marcel Dekker, New York.

**French, D. D. (1988)** Some effects of changing soil chemistry on decomposition of plant litters and cellulose on a Scottish moor. Oecologia 75:608–618.

**Frias-Lopez, J., Bonheyo, G. T. and Fouke, B. W. (2004)** Identification of differential gene expression in bacteria associated with coral black band disease by using RNA-arbitrarily primed PCR. Appl. Environ. Microbiol. 70:3687-3694.

**Frostegård, A., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., Jeannin, P., Nesme, X. and Simonet, P. (1999)** Quantification of bias related to the extraction of DNA directly from soils. Appl. Environ. Microbiol. 65:5409-5420.

**Garland, J. L. and Mills, A. L. (1991)** Classification and characterisation of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilisation. Appl. Environ. Microbiol. 57**:**2351-2359.

**Gelsomino, A., Keyzer-Wolters, A. C., Cacco, G. and van Elsas, J. D. (1999)** Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis. J. Microbiol. Meth. 38:1-15.

**Gremion, F., Chatzinotas, A. and Harms, H. (2003)** Comparative 16S rDNA and rRNA sequence analysis indicates that Actinobacteria might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. Environ. Microbiol. 5:896-907.

**Griffiths R. I., Whiteley, A. S., O'Donnel, A. G. and Bailey, M. J. (2000)** Rapid method for the coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA- based microbial community composition. Appl. Environ. Microbiol. 66:5488-5491.

**Hammel, K. E. (1997)** Fungal degradation of lignin. In: Driven by Nature: Plant Litter quality and Decomposition (Cadish, G. and Giller, K. E., Eds.), pp 33-45, CAB International, Wallingford.

**Heck, K. L., Belle, G. V. and Simberloff, D. (1975)** Explicit calculation of the rarefaction diversity measurement and the determination of sufficient sample size. Ecology 56:1459-1461.

**Heuer, H. and Smalla, K. (1997)** Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. In: Modern Soil Microbiology (van Elsas, J. D., Trevors, J. T. and Wellington, E. M. H., Eds.), pp 353-373, Marcel Dekker, Inc, New York.

**Heukeshoven, J. and Dernick, R. (1986)** Neue Ergebnisse zum Mechanismus der Silberfaerbung. In: München - Electrophorese Forum (Radola, B. J., Ed.), pp 22-27, VCH, Weinheim, Germany.

**Horner, J. D., Gosz, J. R. and Cates, R. G. (1988)** The role of carbon-based plant secondary metabolites in decomposition in terrestrial ecosystems. Am. Nat. 132:869–883.

**Hunt, H. W., Ingham, E. R., Coleman, D. C., Elliot, E. T. and Reid, C. P. P. (1988)** Nitrogen limitation of production and decomposition in prairie mountain meadow, and pine forest. Ecology 69:1009–1016.

**Hurlbert, S. H. (1971)** The nonconcept of species diversity: a critique and alternative parameters. Ecology 52:577-586.

**Jenkinson, D. S. (1977)** Studies on the decomposition of plant material in soil V. The effects of plant cover and soil type on the loss of carbon from $^{14}C$ labelled ryegrass decomposition under field conditions. J. Soil Sci. 28:424-434.

**Johansson, M. B. (1994)** Decomposition rates of Scots pine needle litter related to site properties, litter quality and climate. Can. J. Forest Res. 24:1771-1781.

**Johansson, M-B., Berg, B. and Meentemeyer, V. (1995)** Litter mass loss rates in late stages of decomposition in a climate transect of pine forests. Long-term decomposition in a Scots pine forest. Can. J. Botany 73:1509-1521.

**Kerkhof, L. and Kemp, P. (1999)** Small ribosomal RNA content in marine Proteobacteria during non-steady-state growth. FEMS Microbiol. Ecol. 30:253-260.

**Kim, J. S., Chappelka, A. H. and Miller-Goodman, M. S. (1998)** Decomposition of blackberry and broomsedge bluestem as influenced by ozone. J. Environ. Qual. 27:953-960.

**Knapp, E. B., Elliot, L. F. and Campbell, G. S. (1983)** Microbial respiration and growth during the decay of wheat straw. Soil Biol. Biochem. 15:319-323.

**Kowalchuk, G. A., Bodelier, P. L. E., Heiling, G. H. J., Stephen, J. R. and Laanbroek, H. J. (1998)** Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR-DGGE and oligonucleotide probe hybridisation. FEMS Microbiol. Ecol. 27:339-350.

**Kowalchuk, G. A., Gerards, S. and Woldendorp, J. W. (1997)** Detection and characterization of fungal infections of *Ammophila arenaria* (Marram grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18S rDNA. Appl. Environ. Microbiol. 63:3858-3865.

**Landolt, W., Bühlmann, U., Bleuler, P. and Bucher, J. B. (2000)** Ozone-exposure-response relationships for biomass and root/shoot ratio of beech (*Fagus sylvatica*), ash (*Fraxinus excelsior*), Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*). Environ. Pollut. 109:473-478.

**Le Thiec, D., Dixon, M. and Garrec, J. P. (1994)** The effects of slightly elevated ozone concentrations and mild drought stress on the physiology and growth of Norway spruce, *Picea abies* (L.) Karst. and beech, *Fagus sylvatica* (L.), in open-top chambers. New Phytol. 128:671–678.

**Lechevalier, M. P. (1977)** Lipids in bacterial taxonomy-a taxonomist's view. Crit. Rev. Microbiol. 5:109-210.

**Leclerc, M., Delbes, C., Godon, J. J. and Moletta, R. (2001)** Single strand conformation polymorphism monitoring of 16S rDNA archaea during start-up of an anaerobic digester. FEMS Microbiol. Ecol. 34:213-220.

**Liang, P. and Pardee, A. B. (1992)** Differential display of eukaryotic messenger RNA by means of polymerase chain reaction. Science 257:967-971.

**Lorenz, M. and Wackernagel, W. (1987)** Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. Appl. Environ. Microbiol. 53:2948-2952.

**Lueken, H., Hutcheon, W. L. and Paul, E. A. (1962)** The influence of nitrogen on the decomposition of crop residues in the soil. Can. J. Soil Sci. 42:276-288.

**Macnaughton, S. J., Stephen, J. T., Venosa, A. D., Davis, G. A., Chang, Y. J. and White, D. C. (1999)** Microbial population changes during bioremediation of an experimental oil spill. Appl. Environ. Microbiol. 65:3566-3574.

**Magan, N., Smith M. K. and Kirkwood I. A. (1996)** Effects of atmospheric pollutants on phyllosphere and endophytic fungi. In: Fungi and environmental change (Frankland, G. C., Magan, N. and Gadd, G. M., Eds.), pp 90-101, Cambridge University Press, UK.

**Malosso, E., English, L., Hopkins, D. W. and O'Donnell, A. G. (2004)** Use of 13C-labelled plant materials and ergosterol, PLFA and NLFA analyses to investigate organic matter decomposition in Antarctic soil. Soil Biol. Biochem. 36:165– 175.

**Manefield, M., Whiteley, A. S., Griffiths, R. I. and Bailey, M. J. (2002a)** RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. Appl. Environ. Microbiol. 68:5367– 5373.

**Manefield, M., Whiteley, A. S., Ostle, N., Ineson, P. and Bailey, M. J. (2002b)** Technical considerations for RNA-based stable isotope probing: an approach to associating microbial diversity with microbial community function. Rapid Commun. Mass Sp. 16:2179– 2183.

**Martin, A. and Marinissen, J. C. Y. (1993)** Biological and physico-chemical processes in excrements of soil animals. Geoderma 56:331-347.

**Matyssek, R., Günthardt-Goerg, M. S., Landolt, W. and Keller, T. (1993)** Whole-plant growth and leaf formation in ozonated hybrid poplar (*Populus* x *euramericana*). Environ. Pollut. 81:207-221.

**Meentemeyer, V. (1978)** Macroclimate and lignin control of litter decomposition rates. Ecology 59:465–472.

**Melillo, J. M., Aber, J. D. and Muratore, J. F. (1982)** Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. Ecology 63:621–626.

**Menke, U. and Mueller-Roeber, B. (2001)** RNA fingerprinting of specific plant cell types: Adaptation to plants and optimisation of RNA Arbitrarily Primed PCR (RAP-PCR). Plant Mol. Biol. Rep. 19:33-48.

**Mikkelsen, T. N. and Ro-Poulsen, H. (1994)** Exposure of Norway spruce to ozone increases the sensitivity of current year needles to photoinhibition and desiccation. New Phytol. 128: 153–163.

**Miller, D. N., Bryant, J. E., Madsen, E. L. and Ghiorse, W. C. (1999)** Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl. Environ. Microbiol. 65:4715-4724.

**Miller, H. G. (1984)** Dynamics of nutrient cycling in plantation ecosystems. In: Nutrition of Plantation Forest (Boven, G. D. and Nambiar, E. K. S., Eds.), pp 53-78, Academic Press, London.

**Miller, H. G., Cooper, J. M. and Miller, J. D. (1976)** Effects of nitrogen supply on nutrients in litterfall and crown leaching in a stand of Corsican pines. J. Appl. Ecol. 13:233–248.

**Mindermann, G. (1968)** Addition, decomposition, and accumulation of organic matter in forests. J. Ecol. 56:355-362.

**Moran, M. A., Torsvik, V. L., Torsvik, T. and Hodson, R. E. (1993)** Direct extraction and purification of rRNA for ecological studies. Appl. Environ. Microbiol. 59:915-918.

**Muyzer, G. (1998)** Structure, function and dynamics of microbial communities: the molecular biological approach. In: Advances in Molecular Ecology (Carvalho, G. R., Ed.), pp 87-117, IOS Press, Amsterdam, The Netherlands.

**Muyzer, G. and Smalla, K. (1998)** Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Leeuwenhoek 73:127-141.

**Muyzer, G., De Waal, E. C. and Uitterlinden, A. G. (1993)** Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes for 16S rRNA. Appl. Environ. Microbiol. 59:695-700.

**Newell, S. Y. (2001a)** Multiyear patterns of fungal biomass dynamics and productivity within naturally decaying smooth cordgrass shoots. Limnol. Oceanogr. 46:573-583.

**Newell, S. Y. (2001b)** Spore-expulsion rates and extents of blade occupation by ascomycetes of the smooth-cordgrass standing-decay system. Bot. Mar. 44:277-285.

**Newell, S. Y. and Porter, D. (2000)** Microbial secondary production from saltmarsh grass shoots and its known potential fates. In: Concepts and controversies in tidal marsh ecology (Wienstein, M. P. and Kreeger, D. A., Eds.), pp 159-185. Kluwer Academic, Dordrecht, The Netherlands.

**Nicolai, V. (1988)** Phenolic and mineral content of leaves influences decomposition in European forest ecosystems. Oecologia 75:575–579.

**Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., Ludwig, W. and Backhaus, H. (1996)** Sequence heterogeneities of genes encoding 16S rRNAs in

*Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. 178:5636-5643.

**Nüslein, K. and Tiedje, J. M. (1998)** Characterization of the dominant and rare members of a young Hawaiian soil bacterial community with small-subunit ribosomal DNA amplified from DNA fractionated on the basis of its guanine and cytosine composition. Appl. Environ. Microbiol. 64:1283-1289.

**Oerther, D. B., de los Reyes F. L. 3rd, Hernandez, M. and Raskin L. (1999)** Simultaneous oligonucleotide probe hybridization and immunostaining for in situ detection of Gordona species in activated sludge. FEMS Microbiol. Ecol. 29:129-136.

**Olsen, G. J., Lane, D. J., Giovannoni, S. J. and Pace, N. R. (1986)** Microbial ecology and evolution: a ribosomal RNA approach. Annu. Rev. Microbiol. 40:337-365.

**Olszyk, D. M., Johnson, M. G., Phillips, D. L., Seidler, R. J., Tingey, D. T. and Watrud, L. S. (2001)** Interactive affects of $CO_2$ and $O_3$ on a ponderosa pine plant/litter/soil mesocosm. Environ. Pollut. 115:447-462.

**Oren, R., Schulze, E.-D., Werk, K. S. and Meyer, J. (1988)** Performance of two *Picea abies* (L.) Karst stands at different stages of decline. VII. Nutrient relations and growth. Oecologia 77:163–173.

**Øvreås, L. and Torsvik, V. (1997)** Microbial diversity and community structure in two different agricultural soil communities. Microbial Ecol. 36:303-315.

**Øvreås, L., Jensen, S., Daae, F. L. and Torsvik, V. (1998)** Microbial community changes in a perturbed agricultural soil investigated by molecular and physiological approaches. Appl. Environ. Microbiol. 64:2739-2742.

**Pace, N. R., Stahl, D. A., Lane, D. J. and Olsen, G. J. (1986)** The analysis of natural microbial populations by ribosomal RNA sequences. Adv. Microb. Ecol. 9:1-55.

**Pastor, J., Aber, J. D., McClaugherty, C. A. and Melillo, J. M. (1984)** Aboveground production and N and P cycling along a nitrogen mineralization gradient on Blackhawk Island, Wisconsin. Ecology 65:256–268.

**Patterson, M. T. and Rundel, P. W. (1995)** Stand characteristics of ozone-stressed populations of *Pinus jeffreyi* (Pinaceae): extent, development and physiological consequences of visible injury. Am. J. Bot. 82:150-158.

**Pearson, M. and Mansfield, T. A. (1994)** Effects of exposure to ozone and water stress on the following season's growth of beech (*Fagus sylvatica* L.). New Phytol. 126:511–515.

**Prescott, C. E. (1996)** Influence of forest floor type on rates of litter decomposition in microcosms. Soil Biol. Biochem. 28:1319-1325.

**Prescott, C. E., Corbin, J. P. and Parkinson, D. (1989)** Biomass, productivity and nutrient-use efficiency of aboveground vegetation in four Rocky Mountain coniferous forests. Can. J. Forest Res. 19:309-317.

**Purdy, K. J., Nedwell, D. B. and Embley, T. M. (2003)** Analysis of the sulfate-reducing bacterial and methanogenic archaeal populations in contrasting Antarctic sediments. Appl. Environ. Microbiol. 69:3181-3191.

**Radajewski, S., Ineson, P., Parekh, N. R. and Murrell, J. C. (2000)** Stable- isotope probing as a tool in microbial ecology. Nature 403:646– 649.

**Reich, P. B., Schoettle, A. W., Stroo, H. F. and Amundson, R. G. (1988)** Effects of ozone and acid rain on white pine (*Pinus strobus*) seedlings grown in five soils III. Nutrient relations. Can. J. Botany 66:1517-1531.

**Rheims, H., Sproer, C., Rainey, F. A. and Stackebrandt, E. (1996)** Molecular biological evidence for the occurrence of uncultured members of the actinomycete line of descent in different environments and geographical locations. Microbiology 142:2863-2870.

**Rosenbrock, P., Buscot, F. and Munch, J. C. (1995)** Fungal succession and changes in the fungal degradation potential during the initial stage of litter decomposition in a black alder forest (*Alnus glutinosa* (L.) Gaertn.). Eur. J. Soil Biol. 31:1-11.

**Runeckles, V. C. and Chevone, B. I. (1992)** Crop responses to ozone. In: Surface level ozone exposures and their effects on vegetation (Lefohn, A. S., Ed.), pp 189-260, Lewis Publishers, Chelsea, MI.

**Rutigiano, F. A., De Santo, A. V., Berg, B., Alfani, A. and Fioretto, A. (1996)** Lignin decomposition in decaying leaves of *Fagus sylvatica* L. and needles of *Abies alba* Mill. Soil Biol. Biochem. 28:101-106.

**Sambrook**, **J. and Russell, D. W. (2001)** Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

**Samuelson, L. J., Kelly, J. M., Mays, P. A. and Edwards, G. S. (1996)** Growth and nutrition of *Quercus rubra* L. seedlings and mature trees after three seasons of ozone exposure. Environ. Pollut. 91:317-323.

**Sandaa, R.-A., Torsvik, V., Enger, O., Dane, F. L., Castberg, T. and Hahn, D. (1999)** Analysis of bacterial communities in heavy metal-contaminated soils at different levels of resolution. FEMS Microbiol. Ecol. 30:237-251.

**Sayler, G. S., Fleming, J., Applegate, B., Werner, C. and Nikbakht, K. (1989)** Microbial community analysis using environmental nucleic acid extracts. In: Recent advances in

microbial ecology (Hattori, T. Y., Ishida, Y. M., Morita, R. and Uccida, A., Eds.), pp 658-660, Japan Scientific Societies Press, Tokyo, Japan.

**Schaefer, M. A. and Schauermann, J. (1990)** The soil fauna of beech forests: comparison between a mull and a moder soil. Pedobiologia 34:299-314.

**Scherzer, A. J., Rebbeck, J. and Boerner, R. E. J. (1998)** Foliar nitrogen dynamics and decomposition of yellow-poplar and eastern white pine during four seasons of exposure to elevated ozone and carbon dioxide. Forest Ecol. Manag. 109:355-366.

**Schier, G. A. (1990)** Response of yellow-poplar (*Liriodendron tulipifera* L.) seedlings to simulated acid rain and ozone 2. Effect on throughfall chemistry and nutrients in the leaves. Environ. Exp. Bot. 30:325-331.

**Schlesinger, W. and Andrews, J. (2000)** Soil respiration and the global carbon cycle. Biogeochemistry 48:7-20.

**Schlesinger, W. H. and Hasey, M. M. (1981)** Decomposition of chaparral shrub foliage: losses of organic and inorganic constituents from deciduous and evergreen leaves. Ecology 62:762–774.

**Scholle, G., Wolters, V. and Joergensen, R. G. (1992)** Effects of mesofauna exclusion on the microbial biomass in two modern profiles. Biol. Fert. Soils 12:253–260.

**Sessitsch, A., Gyamfi, S., Stralis-Pavese, N., Weilharter, A. and Pfeifer, U. (2002)** RNA isolation from soil for bacterial community and functional analysis: evaluation of different extraction and soil conservation protocols. J. Microbiol. Meth. 51:171-179.

**Selvaratnam, S., Schoedel, B. A., McFarland, B. L. and Kulpa, C. F. (1995)** Application of reverse transcriptase PCR for monitoring expression of the catabolic *dmpN* gene in a phenol-degrading sequencing batch reactor. Appl. Environ. Microbiol. 61:3981-3985.

**Sharma, S., Aneja, M. K., Mayer, J., Schloter, M. and Munch, J. C. (2004a)** Characterization of bacterial community structure in rhizosphere soil of grain legumes. Microbial Ecol. (Accepted).

**Sharma, S., Aneja, M. K., Mayer, J., Schloter, M. and Munch, J. C. (2004b)** RNA fingerprinting of microbial community in the rhizosphere soil of grain legumes. FEMS Microbiol. Lett. 240:181-186.

**Shaver, G. R., Billings, W. D., Chapin, F. S., Giblin, A. E., Oechel, W. C., Nadelhoffer, K. J. and Rastetter, E. B. (1992)** Global change and the carbon balance of arctic ecosystems. Bioscience 42:433–441.

**Smit, E., Leeflang, P., Glandorf, B., van Elsas, J. D. and Wernars, K. (1999)** Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes

encoding 18S rRNA and temperature gradient gel electrophoresis. Appl. Environ. Microbiol. 65:2614-2621.

**Sridhar, K. R. and Bärlocher, F. (2000)** Initial colonization, nutrient supply, and fungal activity on leaves decaying in streams. Appl. Environ. Microbiol. 66:1114-1119.

**Staaf, H. and Berg, B. (1982)** Accumulation and release of plant nutrients in decomposing Scots pine needle litter. II. Long-term decomposition in a Scots pine forest. Can. J. Botany 60:1561–1568.

**Stahl, D. A. (1997)** Molecular approaches for the measurement of density, diversity, and phylogeny. In: Manual of Environmental Microbiology (Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenbach, L. D. and Walter, M. V., Eds), pp 102-114, ASM Press, Washington, D.C.

**Stapleton, R. D., Ripp, S., Jimenez, L., Cheol-Koh, S., Fleming, J. T. and Sayler, G. S. (1998)** Nucleic acid analytical approaches in bioremediation: site assessment and characterization. J. Microbiol. Meth. 32:165-178.

**Swift, M. J. and Anderson, J. M. (1989)** Decomposition. In: Tropical rain forest ecosystems-Biogeographical and ecological studies (Lieth, H. and Werger, M. J. A., Eds.), pp 547-569, Elsevier, Amsterdam, The Netherlands.

**Tam, N. F. Y., Vrijmoed, L. L. P. and Wong, Y. S. (1990)** Nutrient dynamics associated with leaf decomposition in a small subtropical mangrove community in Hong Kong. B. Mar. Sci. 47:68-78.

**Taylor, B. R., Parkinson, D. and Parsons, W. F. J. (1989)** Nitrogen and lignin content as predictors of litter decay rates: a microcosm test. Ecology 70:97–104.

**Temple, P. J. and Riechers, G. H. (1995)** Nitrogen allocation in ponderosa pine seedlings exposed to interacting ozone and drought stresses. New Phytol. 130:97-104.

**Teuben, A. (1991)** Nutrient availability and interactions between soil arthropods during decomposition of coniferous litter: a mesocosm study. Biol. Fert. Soils 10:256–266.

**Tian, G., Kang, B. T. and Brussaard, L. (1992a)** Biological effects of plant residues with contrasting chemical composition under humid tropical conditions—decomposition and nutrient release. Soil Biol. Biochem. 24:1051–1060.

**Tian, G., Kang, B. T. and Brussaard, L. (1992b)** Effects of chemical composition on N, Ca and Mg release during incubation of leaves from selected agroforestry and fallow plant species. Biogeochemistry 16:103–119.

**Torsvik, V., Daae, F. L., Sandaa, R. A. and Øvreås, L. (1998)** Novel techniques for analysing microbial diversity in natural and perturbed environments. J. Biotechnol. 64:53-62.

**Torsvik, V., Goksoyr, J. and Dane, F. L. (1990)** High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. 56:782-787.

**Treonis, A. M., Ostle, N. J., Stott, A. W., Primrose, R., Grayston, S. J. and Ineson, P. (2004)** Identification of groups of metabolically-active rhizosphere microorganisms by stable isotope probing of PLFAs. Soil Biol. Biochem. 36:533– 537.

**Trevors, J. T. and van Elsas, J. D. (1995)** Nucleic Acids in the Environment: Methods and Applications, (Trevors, J. T. and van Elsas, J. D., Eds.), pp 256, Springer-Verlag, Heidelberg.

**Vainio, E. J. and Hantula, J. (2000)** Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol. Res. 104:927-936.

**van Elsas, J. D., Duarte, G. E., Rosado, A. S. and Smalla, K. (1998)** Microbiological and molecular methods for monitoring microbial inoculants and their effects in the environment. J. Microbiol. Meth. 32:133-154.

**van Elsas, J. D., Mantynen, V. and Wolters, A. (1997)** Soil DNA extraction and assessment of the fate of Mycobacterium chlorophenolicum strain PCP-1 in different soils by 16S ribosomal RNA gene sequence based most-probable-number PCR and immunofluorescence. Biol. Fert. Soils 24:188-195.

**Van Vuuren, M. M. I., Berendse, F. and De Visser, W. (1993)** Species and site differences in the decomposition of litters and roots from wet heathlands. Can. J. Botany 71:167–173.

**Verhoef, H. A. and Brussaard, L. (1990)** Decomposition and nitrogen mineralization in natural and agroecosystems: the contribution of soil animals. Biogeochemistry 11:175–211.

**Vesterdal, L. (1999)** Influence of soil type on mass loss and nutrient release from decomposing foliage litter of beech and Norway spruce. Can. J. Forest Res. 29:95-105.

**Victorio, L., Gilbride, K. A., Allen, D. G. and Liss, S. N. (1996)** Phenotypic fingerprinting of microbial communities in wastewater treatment systems. Water Res. 30:1077-1086.

**Vitousek, P. M. (1994)** Beyond global warming: ecology and global change. Ecology 75:1861–1876.

**Wachendorf, C., Irmler, U. and Blume, H. P. (1997)** Relationship between litter fauna and chemical changes during litter decomposition under different moisture conditions. In: Driven by nature: Plant litter quality and decomposition (Cadisch, G. and Giller, K. E., Eds.), pp135-144, CAB International, Wallingford.

**Wagner, R. (1994)** The regulation of ribosomal RNA synthesis and bacterial cell growth. Arch. Microbiol. 161:100-106.

**Wahbeh, M. I. and Mahasneh, A. M. (1985)** Some aspects of decomposition of leaf litter of the seagrass *Halophila stipulacea* from the gulf of Aqaba (Jordan). Aquat. Bot. 21:237-244.

**Ward, D. M., Weller, R. and Bateson, M. M. (1990)** 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature 345:63-65.

**Wedin, D. A. and Pastor, J. (1993)** Nitrogen mineralization dynamics in grass monocultures. Oecologia 96:186–192.

**Wedin, D. A. and Tilman, D. (1990)** Species effects on nitrogen cycling: a test with perennial grasses. Oecologia 84:433–441.

**Welsh, J., Chada, K., Dalal, S. S., Cheng, R., Ralph, D. and McClelland, M. (1992)** Arbitrarily primed PCR fingerprinting of RNA. Nucleic Acid Res. 20:4965-4970.

**White, T. J., Bruns, T., Lee, S. and Taylor, J. W. (1990)** Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications (Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. Eds.), pp 315-322, Academic Press Inc., New York.

**Wieder, R. K. and Lang, G. E. (1982)** A critique of the analytical methods used in examining decomposition data obtained from litter bags. Ecology 63:1636-1642.

**Wilkinson, S. C., Anderson, J. M., Scardelis, S. P., Tisiafouli, M., Taylor, A. and Wolters, V. (2002)** PLFA profiles of microbial communities in decomposing conifer litters subject to moisture stress. Soil Biol. Biochem. 34:189-200.

**Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. (1990)** DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.

**Winogradsky, S. (1949)** Microbiologie du sol, problèmes et méthodes. Barneoud Frères, France.

**Witkamp, M. (1966)** Decomposition of leaf litter in relation to environment, microflora and microbial respiration. Ecology 47:194–201.

**Woese, C. R., Stackebrandt, E., Macke, T. J. and Fox, G. E. (1985)** A phylogenetic definition of the major eubacterial taxa. Syst. Appl. Microbiol. 6:143-151.

**Yang, C.-H. and Crowley, D. E. (2000)** Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. Appl. Environ. Microbiol. 66:345-351.

**Zegzouti, H., Marty, C., Jones, B., Bouquin, T., Latché, A., Pech, J. C. and Bouzayen, M. (1997)** Improved screening of cDNAs generated by differential display enables the selection

of true positives and the isolation of weakly expressed messages. Plant Mol. Biol. Rep. 15:236-245.

**Zuckerkandl, E. and Pauling, L. (1965)** Molecules as documents of evolutionary history. J. Theor. Biol. 8:357-366.

# 7. APPENDIX

## 7.1 Figure legends

## 7.2 Table legends

## 7.3 Curriculum Vitae

**Name:** Manish Kumar Aneja

**Date of birth:** 11.10.1977

**Place of birth:** New Delhi, India

**Academic qualifications / research experience:**

2002-2004 Ph.D under the guidance of Prof. J. C. Munch and Dr. Michael Schloter at Institute for Soil Ecology, GSF-National Research Centre for Environment and Health, Neuherberg, Germany.
Title: Degradation of beech and spruce litter - Influence of soil site and litter quality on microbial communities.

2000-2001 Research project in the lab of Dr. Gerhard Behre at GSF-Hematologikum, Munich, Germany.
Title: Identification and functional characterization of target proteins of AML1-ETO fusion protein.

1998-2000 Master of Biotechnology, All India Institute of Medical Sciences, New Delhi, India.
Title: Sequence analysis of the peptide repeats within the *cg*2 gene of *Plasmodium falciparum* and their correlation with Chloroquine resistance.

1999 Training at National Institute of Immunology, New Delhi, India.
Title: Production of D-*myo*-inositol 1,2-cyclic monophosphate using recombinant *Bacillus subtilis* and it's purification using ion-exchange chromatography.

1995-1998 Bachelor of Science (Microbiology), University of Delhi, New Delhi, India.

1982-1995 Faith Academy Senior Secondary School, New Delhi, India (Class I -XII)

**Publications**

1. Sharma I, **M. K. Aneja,** S. Biswas, V. Dev, M. A. Ansari, S. T. Pasha and Y. D. Sharma (2001) Allelic variation in the cg2 gene does not correlate with chloroquine resistance among Indian *Plasmodium falciparum* isolates. Int. J. Parasitol. 31(14):1669-1672.
2. Sharma S., **M. Aneja**, M. Schloter and J. C. Munch (2003) Structural and functional characterization of microbial diversity in the rhizospheres of three grain legumes. FEMS Microbiol. Lett. 222:Supplement 1, pp 46.
3. **Aneja M. K.**, S. Sharma, J. C. Munch and M. Schloter (2004) RNA fingerprinting - a new method to screen for differences in plant litter degrading microbial communities. J Microbiol. Meth. 59(2):223-231.

4. Sharma S., **M. K. Aneja,** M. Schloter and J. C. Munch (2004) RNA fingerprinting of microbial community in the rhizosphere soil of grain legumes. FEMS Microbiol. Lett. 240 (2):181-186.
5. Sharma, S., **M. K. Aneja,** J. Mayer, J. C. Munch and M. Schloter (2005) Diversity of transcripts of nitrite reductase genes (*nirK* and *nirS*) in rhizosphere of grain legumes. Appl. Environ. Microbiol. 71:2001-2007.
6. Sharma S, **M. K. Aneja**, J. Mayer, J. C. Munch and M. Schloter (2005) Characterization of bacterial community structure in rhizosphere soil of grain legumes. Microbial Ecology (In Press).
7. **Aneja, M. K.,** S. Sharma, J. C. Munch and M. Schloter (2004) Effect of soil site and litter quality on the microbial colonization of beech and spruce litter. FEMS Microbiol Ecol. (Submitted).
8. **Aneja, M. K.,** S. Sharma, J. C. Munch and M. Schloter. Influence of ozone on litter quality and its subsequent effect on the composition of microbial populations involved in the turnover process (In preparation).