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MOLECULAR COMMUNITY RESPONSE OF FOREST SOIL BACTERIA TO FERTILIZATION AND TREE GIRDLING

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Abstract

Previous studies have found that soil microbial communities are influenced by a wide variety of environmental factors and that the availability of various resources is crucial for determining microbial community structures. A field experiment in a beech-forest located in lower Austria was carried out in which rhizodeposition was manipulated by tree girdling, the soil was treated with artificial nitrogen fertilizer or left untreated as control. Forest soil microbial community compositions were assayed by 16S rRNA gene-based T-RFLP analysis. Further, quantitative real-time PCR analysis was used for analyzing the treatment effects on the abundance of prokaryotic forest soil communities. In detail, total bacterial and archaeal communities as well as specific bacterial taxa (e.g. Acidobacteria, Verrucomicrobia, Proteobacteria and high-GC gram positive bacteria) were assessed. In addition, qPCR analysis was used to quantify functional genes involved in soil nitrogen cycling (bacterial *amoA*, archaeal *amoA*, bacterial *nirS* and bacterial *nosZ*). Community profiling revealed obvious soil microbial diversity differentiations due to season and soil treatments. In general, the impact of nitrogen fertilization was only minor in comparison to the effect of the season and tree girdling. Changes in the abundance of soil microbes and functional nitrogen cycling genes were rather minor or transient in comparison to community differentiations. In general, a winter peak of increased abundance was determined, and a stronger response of archaea to changing environmental conditions and resource availabilities was measured in contrast to bacteria. In conclusion, varying environmental conditions at different time points of the year as well as changing resource availability had a strong influence on the diversity of forest soil microbial communities, whereas the abundance of soil microbial communities was only minor affected.

1 Introduction

1.1 What is soil?

Soil represents one of the most complex and diverse ecosystems on the Earth and displays the Earth's most important resource (Dubey et al., 2006; Young and Crawford, 2004). It is composed of inorganic mineral components of various particle sizes, shapes and chemical characteristics as well as of a soil organic matter (SOM) component in multiple stages of decomposition. Further, plant roots, soil gases, soil water and the living soil population (the "edaphon") contribute to the composition of soil (Standing and Killham, 2007).

The living component of the soil is composed of bacteria, archaea, fungi and algae, along with soil fauna (micro-, meso- and macrofauna) (Paul and Clark, 1989). The high diversity and abundance of microbial, plant, and animal life and their interaction with soil influence ecosystem function. In particular, the activities of the soil microbiota are the key ecosystem processes that sustain life on the Earth. Especially, the recycling or mineralization of elements such as carbon, nitrogen and phosphorus can be mainly attribute to the functioning of soil microbiota (Fitter et al., 2005).

The influence of soil microorganisms on their inhabited ecosystem is derived from their enormous abundances, their high phylogenetic diversities and the huge number of diverse biochemical reactions they catalyze in soil (Arias et al., 2005; Copley, 2000; Dubey et al., 2006; Torsvik and Ovreas, 2007). Prokaryotes inhabiting soil are involved in almost all biogeochemical cycles, and they play a major role in soil nutrient cycling processes since they possess enormous metabolic diversity and versatility. Further, they influence soil aggregation, soil structure, soil fertility and pathogenicity (Dubey et al., 2006; Kirk et al., 2004; Torsvik and Ovreas, 2002).

1.2 Soil microorganisms in forest ecosystems

Soil microbes play a central role in the productivity, efficiency and health of forest soil ecosystems and in maintaining soil ecosystem functioning and sustainability. The phenotypic and genotypic diversity of microbial communities in forest soils is extraordinary. Soil microbes are an essential component of the biotic community in forests as they are mainly responsible for the breakdown (decomposition) of organic and some inorganic compounds deriving from animal feces, dead plant and animal material, plant litter, as well as from

synthetic sources, such as atmospheric decomposition of nutrients (e.g. nitrogen). The organic resource decomposed by microbes in forest soils is further supplied through metabolites produced via photosynthetic processes released by plant roots (rhizodeposition) (Parkin, 1993; Van Elsas et al., 2007).

The products of microbial decomposition, along with microbial biomass itself, contribute to soil organic matter and, amongst others, determine the productivity and ecological functioning of soils. Microbially mineralized nutrients are taken up by plants (in forests mainly by forest trees), while others are immobilized in the microbial biomass, released to atmospheric gases, or scavenged by other microorganisms (Atlas and Bartha, 1981; Yanagita, 1990).

Microbial communities in soil act as both a source and a sink of atmospheric gases. They have a measurable effect on atmospheric chemistry and global climate, including the greenhouse effect, by influencing the budget of gases such as CO_2, CH_4, H_2, N_2O, and NO (Avrahami et al., 2002; Conrad, 1996; Philippot et al., 2006; Robertson et al., 2000).

Soil bacterial communities are influenced by a wide range of climatic, environmental and edaphic factors. The composition of microbial communities is usually subjected to seasonal fluctuations and may vary between different locations. Soil characteristics such as soil type and surface topography (Ovreas and Torsvik, 1998; Parkin, 1993), as well as soil pH (Högberg et al., 2007), soil moisture (Drenovsky et al., 2004) and soil temperature (Avrahami and Conrad, 2003) have been proven to influence the diversity and functioning of soil microbial communities. Further, resource and nutrient availability (Drenovsky et al., 2004), above-ground plant growth (Loreau, 2001), and soil pollution (Powell et al., 2003) determine the diversity and functioning of soil microbial communities.

However, a complete understanding of how microbial community dynamics in soils is influenced by biotic and abiotic factors is still lacking. Therefore, further investigations are needed to enhance the knowledge on the highly complex interactions between microbial communities and their complex environments in which they are embedded.

1.3 Nutrient cycling in forests

The major part of nutrient cycling in forest soils is carried out by soil microorganisms (Hackl et al., 2004). A broad range of phylogenetically different bacterial groups are involved in the decomposition of soil organic matter (SOM) and thereby play an important role in forest nutrient cycling. The amount of available carbon and nitrogen can influence microbial processes in soil (Magill and Aber, 2000; Schimel and Weintraub, 2003; Vance and Chapin,

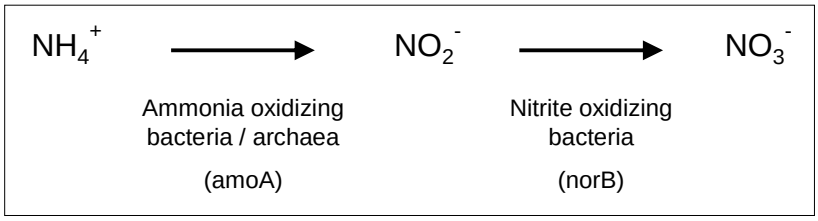
2001), and hence, resource availability and microbial decomposition processes in forest soils are closely interrelated.

1.3.1 Forest nitrogen cycling

One of the most important and most complex nutrient cycles in soil ecosystems is the nitrogen cycle. Various microbial groups are involved in the transformation of both organic and inorganic nitrogenous compounds and thus influence nitrogen cycling in soils through the main ecologically relevant microbial processes of nitrification and denitrification. Moreover, bacteria may be specialized on the fixation of atmospheric nitrogen (Atlas and Bartha, 1981).

1.3.1.1 Nitrification

Nitrification is a chemoautotrophic oxidation process, which typically occurs in two-stages. It involves the microbial oxidation of ammonia (NH_4^+) to nitrite (NO_2^-) and then to nitrate (NO_3^-). In the first step, ammonia is oxidized to nitrite by autotrophic ammonia-oxidizing bacteria (AOB) that can synthesize the enzyme ammonia monooxygenase (He et al., 2007). Nitrite is then oxidized to nitrate by autotrophic nitrite oxidizers (Prosser, 2007).

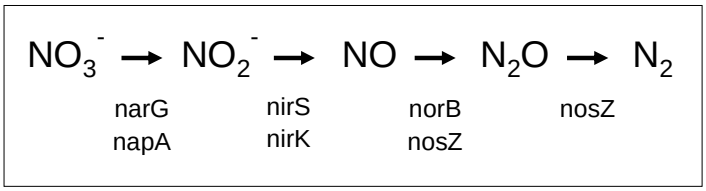


Most studies on nitrification commonly concentrate on ammonia-oxidizing bacteria (AOB) carrying out the first step of nitrification. The key enzyme of aerobic ammonia oxidizers responsible for the transformation of ammonia to nitrite is ammonia monooxygenase. The responsible gene encoding for the alpha-subunit of this enzyme (*amoA*) is frequently used as a phylogenetic marker gene for studying ammonia-oxidizing bacteria (Avrahami and Conrad, 2003; He et al., 2007) (Avrahami et al., 2002; Purkhold et al., 2000).

Autotrophic aerobic ammonia oxidizing organisms were thought to belong to the domain of bacteria. However, recently, ammonia-oxidizing archaea (AOA) have been proven to be the most important group of ammonia oxidizers. Additionally, (Leininger et al., 2006) found a higher abundance of archaeal *amoA* gene copies as compared to bacterial *amoA* genes in soils of diverse origin.

1.3.1.2 Denitrification

The anaerobic reduction of nitrate (NO_3^-), nitrite (NO_2^-) and nitric oxide (NO) to nitrous oxide (N_2O) or nitrogen gas (N_2) is defined as denitrification (Philippot and Hallin, 2005).



This stepwise reduction process is the major biological mechanism by which fixed nitrogen in the soil returns to the atmosphere (Philippot and Hallin, 2005). The reduction of soluble nitrite to gaseous nitric oxide is an important part of the denitrification process as it is the first step that leads to gas formation. As mentioned above, microbial denitrification yield in greenhouse effect gases that essentially influence global climate and play a significant role in global warming and the destruction of the stratospheric ozone layer.

Nitrite reductase is one of the key enzymes in the denitrification process catalyzing nitrite to nitric oxide. Therefore, nitrite reductase is frequently used as a functional marker for the analysis of denitrifying bacteria. Two nitrite reductase enzymes exist that are functionally equivalent although they have a different genetic structure (Avrahami et al., 2002; Coyne et al., 1989). The copper-containing nitrite reductase is encoded by *nirK*, which is frequently detected in soils (Prieme et al., 2002). The *nirS* gene, encoding the cytochrome cd_1-containing nitrite reductase, has been frequently found in marine sediments (Braker et al., 2000) and in soils (Sharma et al., 2006; Throbäck et al., 2004).

The *nosZ* gene codes for the catalytic subunit of the nitrous oxide reductase, which is a homodimeric multicopper enzyme. Some denitrifying bacteria lack the gene encoding nitrous oxide reductase and therefore emit nitrous oxide as an end product of their denitrification process (Philippot et al., 2006). In order to better understand nitrous oxide emissions from soils, it is important to analyze also those bacteria that are able to reduce nitrous oxide.

Denitrifying bacteria show a high taxonomic diversity and are widespread among phylogenetically unrelated groups (Avrahami et al., 2002). The ability to denitrify is not only limited to bacteria but was also found in some archaea and in fungi (Philippot and Hallin, 2005).

1.3.2 Forest carbon cycling

The global carbon cycle is composed of the fixation of carbon dioxide (CO_2) into organic material by autotrophic organisms performing photosynthesis, and the decomposition of this fixed organic carbon to carbon dioxide by heterotrophic decomposing organisms (Högberg et al., 2001). The carbon balance in forest soils is regulated by CO_2 fixation and soil organic carbon decomposition, and hence soil carbon dynamics clearly affect forest carbon balance (Schlesinger and Andrews, 2000). The soil process by which plant-fixed CO_2 returns to the atmosphere is referred as to soil respiration (Schlesinger and Andrews, 2000). Furthermore, there are complex feedbacks between C inputs from trees to soil and nutrient cycling processes regulating soil productivity (Millard et al., 2007).

The majority of carbon that is stored in the microbial biomass is finally metabolized to CO_2 released to the atmosphere, or is incorporated into complex humic complexes which may act as intermediate energy source in labile organic fractions or remain stable in highly, recalcitrant complexes (Prosser, 2007; Yanagita, 1990).

High-molecular weight compounds cannot be accumulated by soil microbes, and hence have to be degraded by microbially produced enzymes that are released into the soil matrix (“extracellular enzymes”). In detail, plant litter e.g. is mainly composed of polymers which are mainly degraded by these microbial, extracellular enzymes. Additionally, many interacting microbial taxa are required to decompose plant litter consisting of a range of different compounds (Weintraub et al., 2007) (Kandeler et al., 2005).

1.3.3 The rhizosphere

The major part of labile carbon sources that are easily degradable by soil microbes are root exudates (Bertin et al., 2003; Hutsch et al., 2002). A high amount of photosynthetically fixed carbon is released into the soil during the vegetation period (Hutsch et al., 2002), and hence rhizodeposition is considered to be a strong effector of a wide range of soil processes mediated by the soil microbiota (Paterson et al., 2007). Roots exude a variety of low-molecular weight organic compounds, including sugars, amino acids and organic acids, and a large proportion of these root-derived carbon substances are rapidly metabolized by microorganisms (Bertin et al., 2003; Hutsch et al., 2002). In comparison to plant litter, most plant root exudates are directly degraded by bacteria without extracellular enzymatic decomposition (Weintraub et al., 2007). Furthermore, the release of root exudates and their subsequent decomposition by soil microbes is a key process linking atmospheric and terrestrial carbon fluxes (Paterson et al., 2007).

The root-soil interface, defined as rhizosphere, is the soil environment with the greatest microbial activity within soil. Through the release of a wide variety of organic and inorganic compounds, roots affect soil microbial communities in their immediate vicinity. Beside the impact on soil bacteria, roots also influence soil structure, aeration and biological activity (Bertin et al., 2003).

In order to better understand the influence of the rhizosphere on related soil processes, a new method named tree girdling was developed. Girdling terminates the transport of photosynthates from the leaves to the roots, while the water transport in trees is not disrupted. Girdling does not result in the immediate death of roots and it does not immediately change the principal physical soil parameters (Högberg et al., 2001). Hence, tree girdling is an effective method for isolating the effects of rhizodeposition on soil functioning without disturbing the surrounding soil environment (Subke et al., 2004; Weintraub et al., 2007).

1.4 Methodological approaches to study microbial diversity

Microbial diversity is intensively studied because it is still not well-understood how the distribution and abundance of soil bacterial communities are controlled. Various approaches have been used to study population structures and shifts.

1.4.1 Cultivation-dependent approaches

The traditional way to study soil microorganisms is to analyze their metabolism, morphology and physiology after cultivation in pure culture (Kent and Triplett, 2002). However, the portion of microorganisms accessible through conventional culture techniques amounts only 1% (Torsvik and Ovreas, 2002). Hence, cultivation-dependent methods only expose information about a very minor portion of the bacterial species existing in soil. Further, enrichment cultures are selective for fast growing microorganisms adapted to grow in culture media (Kassen and Rainey, 2004). Consequently, bacteria cultured from soil samples do not necessarily have significant functions in their natural soil environment. Frequently, the most abundant bacteria inhabiting soils cannot be grown in culture (Dubey et al., 2006).

1.4.2 Cultivation-independent approaches

Because of the obvious insufficiency of cultivation-dependent approaches for studying the diversity, abundance and function of soil bacteria, various culture-independent methods, including fatty acid analysis and DNA- and RNA-based techniques, have been developed.

Generally, cultivation-independent methods can be separated into biochemical-based and molecular-based techniques (Kirk et al., 2004).

1.4.2.1 Phospholipid fatty acid analysis

A frequently used biochemical method is the analysis of phospholipid fatty acids (PLFA) as a microbial biomarker existing in soil (Dubey et al., 2006). PLFAs are part of the membranes of all living cells and the strong correlation between the PLFA compositions with the phylogeny of microbes is appropriate for the qualitative and quantitative investigation of microbial community structure and diversity. Therefore, this technique has been extensively used for determining similarities or differences between soil microbial communities (Zelles, 1999). A clear restriction of this method is the inability to detect community components at a fine taxonomic level. It is generally difficult to distinguish between organisms and to identify the individual species accounting for similarities or differences among soil bacterial communities (Dubey et al., 2006; Zelles, 1999).

1.4.2.2 Molecular methods

In 1990, Torsvik et al. presented the first study on the analysis of soil prokaryotes by means of their DNA. Since then, various different DNA-based techniques have been generated to assess microbial community structure and diversity. Through the availability of the polymerase chain reaction (PCR) it became possible to amplify target genes (mostly 16S ribosomal RNA genes) by PCR (using universal or specific primers) after DNA isolation from soil samples. The resulting products can finally be separated in different ways (depending on the technique) for studying prokaryotic diversity, identifying individual prokaryotes and predicting phylogenetic relationships (Dubey et al., 2006; Kirk et al., 2004; Pace, 1997).

The16S ribosomal RNA (16S rRNA) gene is very well suited and commonly used for phylogenetic analysis of bacteria as it is a molecular chronometer present in all organisms. 16S rRNA genes are evolutionary conserved among all cellular life forms and have a low rate of evolutionary change. They possess a defined structure and are functionally redundant. Certain 16S rDNA areas are conserved, whilst others are highly variable (Woese, 1987). The variable domains within the rRNA genes contain sequence regions specific for groups of related organisms or even individual species, which makes rRNA genes highly useful for studying microbial communities in the soil environment and for examining the phylogenetic relationships among soil prokaryotes (Kent and Triplett, 2002; Woese, 1987).

Initially, sequencing of rRNA genes cloned from isolated soil DNA was commonly used for phylogenetic analysis of soil bacteria (Rappe and Giovannoni, 2003; Woese, 1987). Although sequencing has become routine, sequencing thousands of clones is laborious and very expensive, and hence, at present sequencing is usually not the method of choice for comprehensively analysing microbial communities. However, new high-throughput sequencing methods have been developed, which have been also increasingly applied to study microbial community structures.

Apart from rRNA gene sequencing, a number of PCR-based fingerprinting approaches have been developed for studying the structure of bacterial communities. PCR-based molecular fingerprinting methods include, amongst others, amplified rDNA restriction analysis (ARDRA), single strand conformational polymorphism (SSCP) analysis, ribosomal intergenic spacer analysis (RISA), denaturing/temperature gradient gel electrophoresis (DGGE/TGGE) analysis and more recently terminal restriction fragment length polymorphism (T-RFLP) analysis (Dubey et al., 2006; Kent and Triplett, 2002; Kirk et al., 2004). These methods do not directly offer phylogenetic information, but they yield high resolution and provide information about changes in the whole microbial community structure in soil (Kent and Triplett, 2002). Most community fingerprinting methods assess differences in the community composition between different samples, treatments, or locations, or they provide information on changes in microbial communities over time (Kent and Triplett, 2002).

1.4.2.3 Terminal restriction fragment length polymorphism

T-RFLP analysis is currently one of the most powerful and frequently used methods for rapidly detecting and comparing the diversities of bacterial communities after PCR-based DNA amplification from soil samples (Marsh, 1999). Like ARDRA, the investigation of T-RFLPs is based on variation in the position of restriction sites among DNA fragments. T-RFLP analysis utilizes the same principle as ARDRA, except that one PCR primer, commonly the forward primer, is labelled with a fluorescent dye and only the labelled terminal restriction fragments (T-RFs) are detected after the restriction digest, which greatly simplifies the genetic fingerprint (Liu et al., 1997).

The sizes of the T-RFs are determined by comparison to those of an internal standard consisting of DNA fragments of known length (Abdo et al., 2006; Tiedje et al., 1999). Commonly, an automated DNA sequencer is used for separating the T-RFs by capillary electrophoresis and visualizing the excitation of the applied dye (Blackwood et al., 2003; Liu

et al., 1997). T-RFLP analysis provides qualitative information, and also quantitative information about each T-RF detected, including size in base pairs and intensity of fluorescence. The high resolution of automated electrophoresis instruments and the high sensitivity of fluorescence detection increase the number of individual species detectable compared to methods that use standard gel electrophoresis (Kent and Triplett, 2002).

1.4.2.4 Quantitative real time PCR

A methodological approach that has gained increasing interest for detecting specific genes in soil samples and for quantifying the abundance of soil microorganisms is real-time-PCR (RT-PCR), also referred to as quantitative PCR (qPCR) (Dubey et al., 2006; Fierer et al., 2005). This technique allows the simultaneous amplification and detection of specific DNA sequences present in soil. Increases in PCR product can be measured throughout the reaction by the incorporation of a fluorescent dye into the DNA. Fluorescence intensity increases as PCR product accumulates during each amplification cycle, and the fluorescence intensity is proportional to the amount of PCR product formed (Raeymaekers, 2000). As each amplification cycle theoretically doubles the number of molecules, it is possible to determine the concentration of target DNA that was initially present in the total extract of soil DNA (Kubista et al., 2006; Peirson and Butler, 2007).

2 Objectives and hypotheses

As forests represent the natural vegetation cover in the majority of landscapes in Central Europe and as almost 50% of the total area in Austria presently consists of forested land (Hackl et al., 2004), studying the composition and diversity of bacterial communities and their interrelationship with the vegetation and the soil environment in forests is of great interest.

The overall aim of this study was to analyze the relationship between resource availability and the composition and structure of microbial communities as previous studies showed that soil microorganisms and microbially-driven processes might be limited by available resources (Schimel and Weintraub, 2003; Schmidt et al., 2007; Vance and Chapin, 2001). The influence of changes in resource availability through enhanced nitrogen deposition and reduced plant carbon input on the diversity and abundance of various bacterial groups was determined in this study. Amongst others, Lipson et al. (2002), Lipson and Schmidt (2004) and Schmidt et al. (2007) demonstrated that soil bacteria respond to varying environmental conditions at different times of the year. Therefore, the seasonal pattern of the soil bacterial community was also examined in this work. Furthermore, we chose the bacterial and archaeal *amoA* genes, as well as the bacterial *nirS* and *nosZ* gene for analyzing nitrifying and denitrifying communities in soil.

T-RFLP analysis was selected as the method of choice for determining microbial communities and their structures in this study. Additionally, as qPCR is a method that allows comprehensive and detailed description of soil microbial community structures (Fierer et al., 2005; Kubista et al., 2006), it was selected for the analysis of the abundance of different bacterial groups present in forest soil. Soil bacteria were analyzed to test the following three hypotheses:

- In temperate forest ecosystems, the course of the season influences structure and function of forest soil bacterial communities.
- Resource availability has an effect on the structure and function of bacteria inhabiting forest soils, and varying nutrient availabilities select for different microbial communities, as different microbial groups exhibit different nutrient requirements.
- Changes in resource availability are strongly interrelated with the course of the season, and hence, the disposability of important nutrients, e.g. carbon and nitrogen, for soil bacteria varies within the course of the season.

3 Materials and Methods

3.1 Study site and field experiments

A temperate, mature 62-year old beech-forest (*Hordelymo-Fagetum*) located in Klausenleopoldsdorf, Lower Austria, approximately 40 km west of Vienna was selected as a model system for analyzing variations in microbial community structure and function due to the course of the season, and after nitrogen fertilization and tree girdling.

3.1.1 Field experiment 1 – Seasonality

For identifying seasonal changes in microbial community structure and function, six replicate 10 x 10 m plots were installed at the Klausenleopoldsdorf forest. Soils from each replicate plot were sampled monthly from August 2007 until March 2008. The soil samples were transported in cooling boxes to the laboratory, stored at 4°C, and homogenized by passage through a 2 mm sieve (a 5 mm sieve was used when soils were to wet for sieving through 2 mm) before further laboratory analysis.

3.1.2 Field experiment 2 – Fertilization

The Klausenleopoldsdorf study site is located in an area which receives low loads of N deposition (10 kg N ha^{-1} yr^{-1}) (Hahn et al., 2000). Six replicate 10 x 10 m fertilization plots were established and fertilized every two weeks with ammonium-nitrate (NH_4NO_3) to simulate elevated N deposition of approximately 30 kg N ha^{-1} yr^{-1}. Every two months, soil samples were collected from each replicate plot. Soil samples from the six replicate plots of field experiment 1 were used as a control for the fertilization experiment.

3.1.3 Field experiment 3 – Tree girdling

In three 25 x 25 m girdling plots the trees were girdled at the beginning of the growing season by taking off the bark around the stem to prevent rhizodeposition and to reduce soil carbon input. Soil samples only within the inner 10 x 10 m plots were taken every second month. Accordingly, samples from the control plots were obtained.

3.2 DNA isolation

Microbial soil DNA was isolated using the FastDNA® SPIN Kit for Soil ((MP Biomedicals, Solon, Ohio, USA) according to the manufacturer's instructions. The DNA extracts were quantified photometrically (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA).

Table 1: List of primers used for T-RFLP and qPCR analysis

Target	Primer	Nucleotide sequence	Reference
Eubacteria	Eub338	ACCTACGGGAGGCAGCAG	(Lane, 1991)
Eubacteria	Eub518	ATTACCGCGGCTGCTGG	(Muyzer et al., 1993)
16S all bacteria	8f	AGAGTTTGATCCTGGCTCAG	(Weisburg et al., 1991)
16S all bacteria	926r	CCGTCAATTCCTTTRAGTTT	(Liu et al., 1997)
Acidobacteria	Acid31	GATCCTGGCTCAGAATC	(Barns et al., 1999)
Verrucomicrobia	VMB537f	GCCAGCAGCCGCGGTAATACA	(Petroni et al., 2000)
Verrucomicrobia	VMB1296r	GCAGMCT[BtndT]CAATCTGAA CTGRGC	(Petroni et al., 2000)
Alpha-Proteobacteria	Alf685	TCTACGRATTTCACCYCTAC	(Lane, 1991)
Beta-Proteobacteria	Bet680	TCACTGCTAAACCYG	(Overmann et al., 1999)
Archaea	Ar109f	ACKGCTCAGTAACACGT	(Lueders and Friedrich, 2000)
Archaea	Ar912rt	CTCCCCCGCCAATTCCTTTA	(Lueders and Friedrich, 2000)
Actinobacteria	Actino235	CGCGGCCTATCAGCTTGTTG	(Stach et al., 2003)
Bacterial *amoA*	amoA-1f	GGGGTTTCTACTGGTGGT	(Rotthauwe et al., 1997)
Bacterial *amoA*	amoA-2r	CCCCTCKGSAAAGCCTTCTTC	(Rotthauwe et al., 1997)
Archaeal *amoA*	Arch-amoAF	STAATGGTCTGGCTTAGACG	(Francis et al., 2005)
Archaeal *amoA*	Arch-amoAR	GCGGCCATCCATCTGTATGT	(Francis et al., 2005)
Bacterial *nirS*	nirSCd3aF	AACGYSAAGGARACSGG	(Michotey et al., 2000)
Bacterial *nirS*	nirSR3cd	GASTTCGGRTGSGTCTTSAYG	(Throbäck et al., 2004)
Bacterial *nosZ*	nosZ-f	CGYTGTTCNTCGACAGCCAG	(Henry et al., 2006)
Bacterial *nosZ*	nosZ1622r	CGCRASGGCAASAAGGTSCG	(Henry et al., 2006)

3.3 Terminal restriction-fragment length polymorphism (T-RFLP) analysis

Bacterial and archaeal 16S rRNA genes were PCR amplified using primer sets targeting the total bacterial and archaeal community as well as bacterial phylogenetic taxa (Acidobacteria, Verrucomicrobia, Alpha-Proteobacteria and Beta-Proteobacteria). The used primers / primer combinations are listed in Table 1 / 2. For T-RFLP analysis, the forward primers were labelled with 6-carboxyfluorescein (6-Fam) at the 5' end. Two replicate PCR

reactions were performed for each primer set. The reactions were carried out with a Biometra T1 thermocycler, applying an initial denaturation step of 5 min at 95°C and a final elongation step of 5 min at 72°C. The amplification steps were specific for each phylogenetic group and are listed in Table 2. The PCR mixtures contained 1 x PCR reaction buffer, 0.15 µM of each primer (Table 2), 200 µM (each) dATP, dCTP, dGTP, and dTTP, and 2 U of Firepol (Solis BioDyne OU, Tartu, Estonia) (for amplification of the total bacterial community) or Taq DNA polymerase (Invitrogen) (for the rest of the amplified phylogenetic groups). Further ingredients (DNA template, $MgCl_2$ and bovine serum albumin (BSA)) were specific for each phylogenetic group and are listed in Table 2. PCR products (5 µl) were verified by electrophoresis in 1% (w/v) agarose gels.

Table 2: Description of primer sets, PCR ingredients and amplification details used for T-RFLP analysis

Target	Primer-Combination	DNA [ng]	$MgCl_2$ [mM]	BSA [mg ml^{-1}]	Amplification details		
					Temp. (°C)	Time	Cycles
16S all bacteria	8f-FAM / 926r	5	1.5	--	95° 95°/53°/72° 72°	5’ 30’’/1’/2’ 10’	1 30 1
All Archaea	109f-FAM / 912rt	10	2.0	1.0	95° 95°/52°/72° 72°	5’ 1’/30’’/1’ 5’	1 35 1
Acidobacteria	Acid31-FAM / 926r	10	2.0	2.0	95° 95°/45°/72° 72°	5’ 30’’/1’/1’ 10’	1 30 1
Verruco-microbia	VMB537f-FAM / VMB1295r	10	2.5	1.0	95° 95°/59°/72° 72°	5’ 1’/30’’/90’’ 5’	1 35 1
Alpha-Proteobacteria	8f-FAM / Alf685r	10	1.5	1.0	95° 95°/55°/72° 72°	5’ 30’’/1’/1’ 5’	1 30 1
Beta-Proteobacteria	8f-FAM / Bet680r	10	1.5	1.0	95° 95°/55°/72° 72°	5’ 30’’/1’/1’ 5’	1 30 1

Replicate amplicons were pooled, purified with Sephadex™ G-50 (GE Healthcare Biosciences, Waukesha, WI, USA) and 10 µl purified PCR products (approximately 200 ng of DNA) were digested with 5 U of restriction enzyme *Alu*I (Promega Corporation, Madison, WI, USA) in a 20 µl reaction at 37°C for 3 h. Prior to T-RFLP analysis, digests were again purified with Sephadex™ G-50 (GE-Healthcare).

Five µl of *Alu*I digested PCR products were mixed with 15 µl of deionized formamide (Applied Biosystems, Warrington, UK) and 0.3 µl of internal size standard (GeneScan™ -500 ROX™ Size Standard, Applied Biosystems, Warrongton, UK). The reactions were denaturated at 92°C for 2 min and immediately chilled on ice prior to T-RFLP analysis. Fluorescently labelled terminal restriction fragments (T-RFs) were detected by capillary electrophoresis using an ABI 3100 automated DNA sequencer (Applied Biosystems 3100 Genetic Analyzer) in the GeneScan mode. The relative lengths of the labelled terminal restriction fragments were determined by comparing them with the 500 ROX™ internal size standard.

GelQuest DNA fingerprint analysis software (version 2.1.2, SequentiX, Klein Raden, Germany) was used to compile the electropherograms of each sample into numeric data, where both fragment length and peak height were used as parameters for profile comparison. All T-RFs with peaks between 50 and 400 bp and peak heights of > 50 fluorescence units were included in the further analysis. T-RFs that differed by less than 1 bp were clustered. Normalization of T-RFLP profiles was performed as suggested by Dunbar et al. (Dunbar et al., 2000) and normalized data were then used for statistical analysis.

3.4 Quantitative real-time PCR (qPCR) analysis

Bacterial and archaeal 16S rRNA genes were quantitatively PCR amplified using primer sets targeting the total bacterial and archaeal community, bacterial phylogenetic taxa (Acidobacteria, Verrucomicrobia, high-GC Gram-positive bacteria, Alpha-Proteobacteria and Beta-Proteobacteria), as well as bacterial and archaeal functional genes involved in nitrogen cycling (bacterial *amoA*, archaeal *amoA*, bacterial *nirS* and bacterial *nosZ*). The used primers / primer combinations are listed in Table 1 / 3. Three replicate PCR reactions were performed for each primer set. The 25 µl PCR mixtures included 1 x iQ™ SYBR® Green Supermix (Bio-Rad) (including 100 mM KCl, 40 mM Tris-HCl, 0.4 mM (each) dATP dCTP, dGTP and dTTP, 50 U/ml iTaq DNA polymerase, 6 mM MgCl2, SYBR Green I, 20nM fluoresein, and stabilizers), 10 µM of each primer (Table 3) and 10 mg/ml BSA. Further ingredients (DNA template and dimethylsulfoxid (DMSO)) were specific for each phylogenetic group and are listed in Table 3. Real-time PCR was performed on an iCycler iQ thermocycler (Bio-Rad) with the protocol for each target microbial and functional group as shown in Table 3. After an initial procedure at 95°C for 3 min the assays for the target groups were run using 40 cycles, followed by 1 min at 95°C and 1 min at 58°C.

Table 3: Description of primer sets, PCR ingredients and amplification details used for qPCR analysis

Target	Primer-Combination	DNA [ng]	DMSO [mg ml^{-1}]	Amplification details		
				Temp. (°C)	Time	Cycles
16S all bacteria	Eub338f / Eub518r	10	--	95° 95°/54°/72° 95° 58° 58°	3’ 30’’/35’’/45’’ 1’ 1’ 10’’	1 40 1 1 80*
All Archaea	109f / 912rt	10	--	95° 95°/52°/72° 95° 58° 58°	3’ 30’’/35’’/45’’ 1’ 1’ 10’’	1 40 1 1 80*
Acidobacteria	Acid31 / Eub518r	10	--	95° 95°/54°/72° 95° 58° 58°	3’ 30’’/35’’/45’’ 1’ 1’ 10’’	1 40 1 1 80*
Verruco-microbia	VMB537f / VMB1295r	10	--	95° 95°/59°/72° 95° 58° 58°	3’ 30’’/35’’/1’ 1’ 1’ 10’’	1 40 1 1 80*
Alpha-Proteobacteria	338f / Alf685r	10	--	95° 95°/54°/72° 95° 58° 58°	3’ 30’’/35’’/45’’ 1’ 1’ 10’’	1 40 1 1 80*
Beta-Proteobacteria	338f / Bet680r	10	--	95° 95°/54°/72° 95° 58° 58°	3’ 30’’/35’’/45’’ 1’ 1’ 10’’	1 40 1 1 80*
High-GC gram positive bacteria	Actino235 / Eub518r	10	--	95° 95°/54°/72° 95° 58° 58°	3’ 30’’/35’’/45’’ 1’ 1’ 10’’	1 40 1 1 80*

* +0.5°C/cycle

Table 3. Extended.

Target	Primer-Combination	DNA [ng]	DMSO [µl]	Amplification details		
				Temp. (°C)	Time	Cycles
Bacterial amoA	amoA-1f / amoA-2r	50	--	95° 95°/57°/72°/78° 95° 58° 58°	3’ 1’/1’/1’/1’ 1’ 1’ 10’’	1 45 1 1 80*
Archaeal amoA	Arch-amoAF/ Arch-amoAR	50	0.625	95° 95°/53°/72° 95° 58° 58°	5’ 45’’/1’/1’ 1’ 1’ 10’’	1 45 1 1 80*
Bacterial nirS	nirSCd3aF / nirSR3cd	50	0.625	95° 95°/58°/72°/78° 95° 58° 58°	3’ 30’’/35’’/45’’/45’’ 1’ 1’ 10’’	1 45 1 1 80*
Bacterial nosZ	nosZ-f / nosZ1622r	50	0.625	95° 94°/65°[-1°/cycle]/72° 94°/60°/72° 95° 58° 58°	3’ 30’’/45’’/30’’ 30’’/45’’/30’’ 1’ 1’ 10’’	1 6 40 1 1 80*

* +0.5°C/cycle

Fluorescence intensity was measured after each amplification cycle, hence fluorescence increases as PCR product accumulates during each amplification step due to intercalation of SybrGreen into the amplified DNA. The amplified products (5 µl) were run on a 1% agarose gel to confirm the specificity of the amplification. To confirm that the fluorescence signal originated from specific PCR products and not from primer-dimers or other artefacts, melting curve analysis of the PCR products was conducted following the four temperature steps by measuring fluorescence continuously as the temperature increased from 58°C to 95°C.

For generating standard curves, plasmid standards containing the target regions were generated for each primer set. Plasmid DNA concentrations were determined on a Nanodrop ND-1000 and the copy numbers were calculated directly from the concentration of the

plasmid DNA, assuming that dsDNA of 1000 bp in length corresponds to 1.52 pmol. Standard curves were generated by subjecting triplicate ten-fold serial dilutions of a known copy number of the plasmid DNA to real-time PCR assays.

The triplicate SQ values obtained by qPCR were pooled and after calculating the copy numbers per gram dry soil, the data were subjected to statistical analysis.

3.5 Statistical analysis

Analysis of variance combined with post hoc Tukey-B tests (SPSS for Windows, version 15.0) was used to determine significant season and treatment effects on the T-RFLP and qPCR data sets. The values of peak height of terminal restriction fragments, as well as microbial abundances obtained by qPCR were examined for significant differences in relation to the season, tree girdling and nitrogen fertilization. Additionally, the T-RFLP data set was further subjected to resemblance analysis (Primer 6 for Windows, version 6.1.5) for investigating similarities between different seasons and treatments and for constructing dendrograms, based on hierarchical cluster analysis (complete linkage).

4 Results

4.1 T-RFLP

To explore the influence of the course of the season and of the girdling and fertilization treatments on the structural composition of the prokaryotic communities in the Klausenleopoldsdorf forest soil, soil samples were taken monthly from August 2007 until March 2008 and analyzed by normalized terminal restriction fragment length polymorphism (T-RFLP) fingerprints.

4.1.1 Season-effect

The course of the season revealed significant differences within each of the tested bacterial and archaeal communities ($P < 0.05$) (Table 4). In particular, the effect of seasonal changes on the total bacterial community structure, and within the total bacterial community on Alpha-Proteobacteria was highly significant ($P < 0.001$), whereas the effect on Acidobacteria ($P < 0.05$), Verrucomicrobia ($P < 0.05$), Beta-Proteobacteria ($P < 0.01$) and the total archaeal community ($P < 0.01$) was less significant (Table 4).

Table 4: Analysis of variance of the effect of seasonal variations on tested bacterial and archaeal taxa

	Significance level[a]	
Taxon	Season[b]	
Total bacterial community	0.000	***
Total archaeal community	0.004	**
Acidobacteria	0.042	*
Verrucomicrobia	0.014	*
Alpha-Proteobacteria	0.000	***
Beta-Proteobacteria	0.003	**

[a]Significance levels: *P < 0.05; **P < 0.01; ***P < 0.001
[b]tested month: August 2007 – March 2008

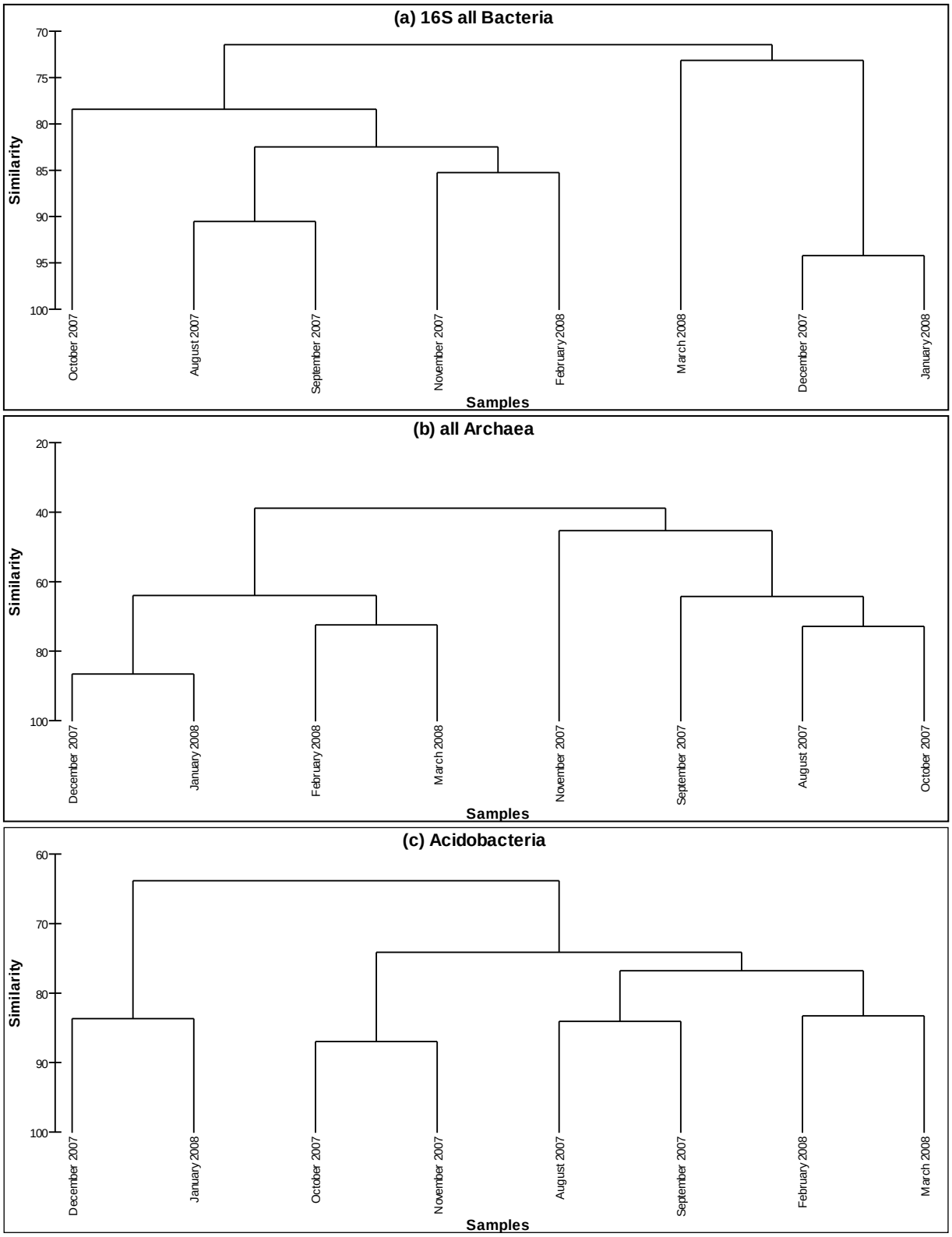


Fig. 1. Dendrograms based on hierarchical cluster analysis (complete linkage) of the normalized T-RFLP data showing community structure similarities in the control soil samples taken from August 2007 until March 2008.
(a) Bacteria, (b) Archaea, (c) Acidobacteria, (d) Verrucomicrobia, (e) alpha-Proteobacteria and (f) beta-Proteobacteria

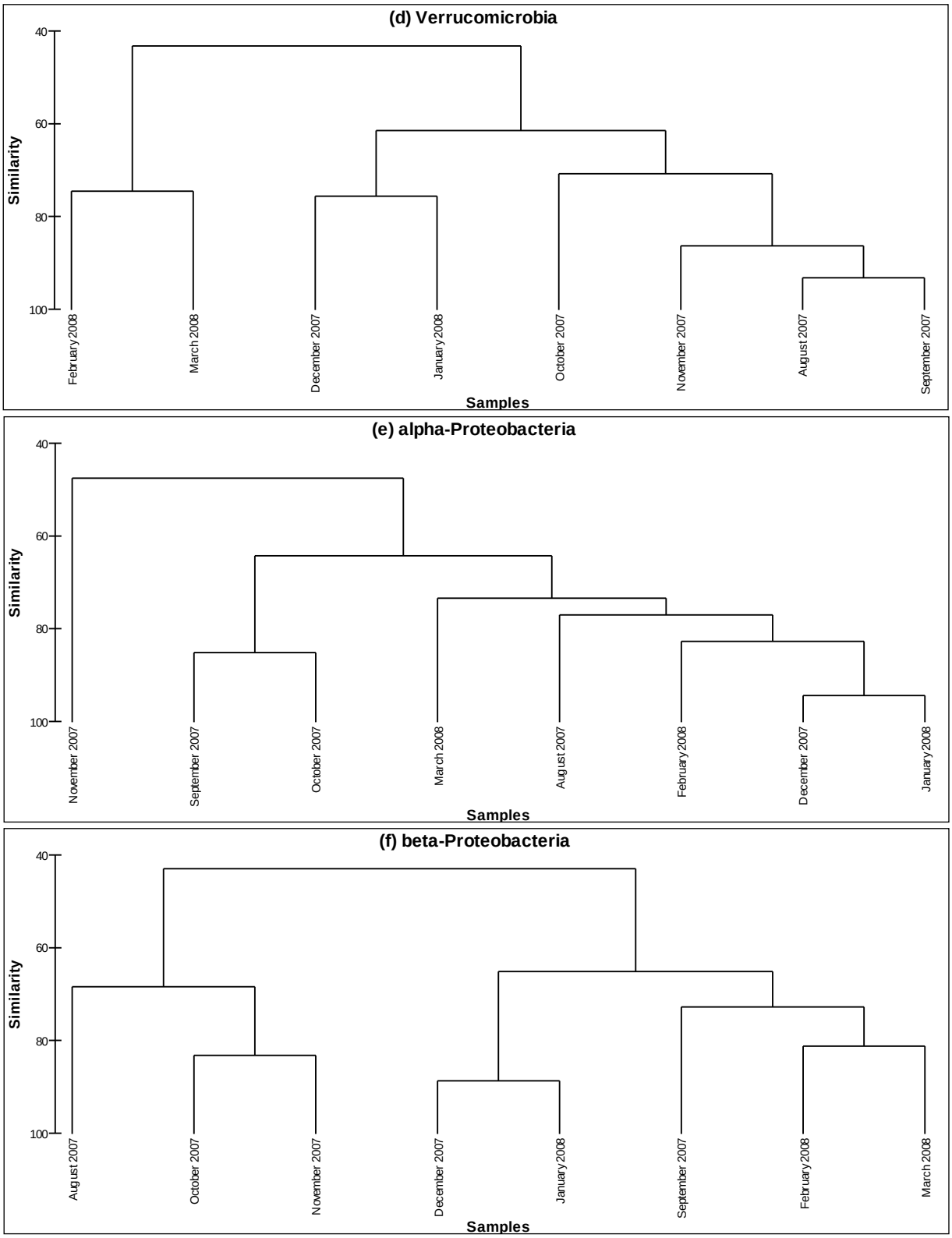


Fig. 1. Extended.

The total bacterial community and the community of Acidobacteria revealed similar trends. The community structure of both all bacteria and Acidobacteria in the coldest months (December 2007 and January 2008) could be separated from those in the other analyzed

months (August 2007 – November 2007; February 2008 and March 2008), however, with the exception of March 2008 for the total bacterial community (Fig 1a and c).

Like for the total bacterial community and for Acidobacteria, verrucomicrobial communities could be grouped together in the winter months (December 2007 and January 2008). However, unlike for the total bacterial community and for Acidobacteria, the community composition of Acidobacteria in February and March 2008 appeared to be even more different compared to those in the rest of the analyzed months.

Exceptional December 2007, a clear separation of the years 2007 and 2008 was detectable for the total archaeal community (Fig. 1b). Archaea as well as Beta-Proteobacteria showed a strong community shift in December 2007, which persisted until March 2008 (Fig. 1b and f).

Other than the rest of the analyzed bacterial taxa, Alpha-Proteobacteria displayed no clear tendency. Nevertheless, as the community composition showed significant differences, strong changes seemed to appear over the whole tested time period.

Generally, in summer months as well as in winter months the composition of the bacterial and archaeal communities was quite similar. Moreover, it could be demonstrated that community structures in months coming one after another were often similar (Fig. 1). For example, the community composition of the total bacterial community as well as of all tested bacterial taxa was very similar in December 2007 and January 2008, and, with the exception of the total bacterial community and alpha-Proteobacteria, also in February and March 2008 (Fig. 1a and c-f) ($P > 0.05$). For Archaea no significant difference was found between August and September 2007, December 2007 and January 2008 and February and March 2008 (Fig. 1b) ($P > 0.05$). Contrastingly, months that are further away from each other, like August and March 2008, often showed a low level of similarity (Fig. 1).

4.1.2 Soil treatment-effect

Apart from the season influence, the bacterial and archaeal community structures were also affected by the two soil treatments, girdling and fertilization (Table 5). The total bacterial population and investigated bacterial taxa, with one exception (Verrucomicrobia in March 2008) showed only a significant treatment effect in September 2007 and November 2007 ($P < 0.05 – P < 0.01$). From the two soil treatments, tree girdling in particular affected bacterial population structures. Related effects on the archaeal community were detectable all over the investigation period ($P < 0.05 – P < 0.01$).

Table 5: Bray Curtis analysis of similarity between the community structure of bacteria, archaea and specific bacterial subgroups after each individual treatment

Taxa	Treatment comparison	R value[a]			
		Sept. 07	Nov. 07	Jan. 08	Mar. 08
16S all Bacteria	C vs. G	**0,252***	**0,174***	0,041	0,009
	C vs. F	**0,241***	**0,276***	-0,07	-0,069
	G vs. F	**0,357****	**0,339****	0,122	0,009
all Archaea	C vs. G	**0,959****	**0,413****	**0,378****	**0,161***
	C vs. F	-0,078	**0,654****	-0,059	-0,143
	G vs. F	**0,72****	**0,244***	**0,191****	**0,161***
Acidobacteria	C vs. G	**0,248***	**0,37***	0,006	0,126
	C vs. F	-0,02	0,013	-0,1	-0,057
	G vs. F	**0,231***	0,235	0,024	0,248
Verrucomicrobia	C vs. G	0,137	**0,265***	0,011	-0,013
	C vs. F	-0,063	-0,1	-0,048	0,217
	G vs. F	0,163	**0,28****	-0,087	**0,433****
Alpha-Proteobacteria	C vs. G	**0,569****	**0,985****	0,143	-0,002
	C vs. F	0,117	**0,987****	-0,069	-0,011
	G vs. F	**0,385****	0,211	0,106	-0,031
Beta-Proteobacteria	C vs. G	**0,319****	0,152	-0,011	0,078
	C vs. F	-0,03	**0,252***	-0,054	-0,03
	G vs. F	**0,324****	**0,293***	0,037	0,078

Key to abbreviations: C, control; G, girdling; F, fertilization
[a]R statistics: 1 = complete separation; 0 = no separation
*P < 0.05; **P < 0.01

By analyzing similarity relationships it could be demonstrated that seasonal variations affected the total bacterial community as well as proteobacterial communities much stronger than the individual treatments, since control, girdling and fertilization of the individual months mostly build a common branch (Fig. 2a and e-f). Although resource availability significantly effected the composition of the total bacterial and proteobacterial communities in September and November 2007 ($P < 0.05 – P < 0.01$) (Table 5), its influence was definitely weaker than that of the season.

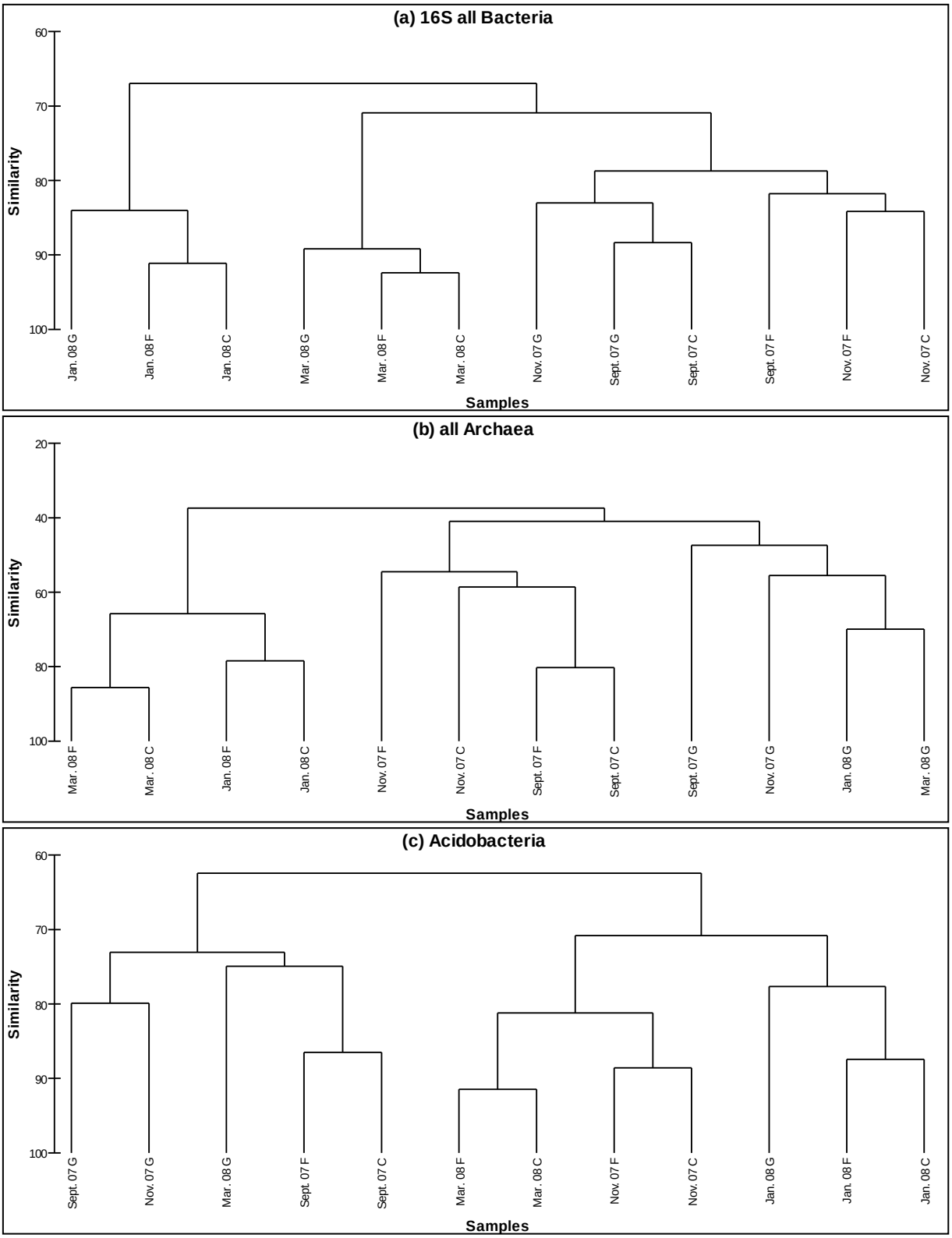


Fig. 2: Dendrograms based on hierarchical cluster analysis (complete linkage) of the normalized T-RFLP data derived from soil samples taken every two months showing the similarity of community structures after each individual treatment from September 2007 until March 2008.
(a) Bacteria, (b) Archaea, (c) Acidobacteria, (d) Verrucomicrobia, (e) Alpha-Proteobacteria and (f) Beta-Proteobacteria

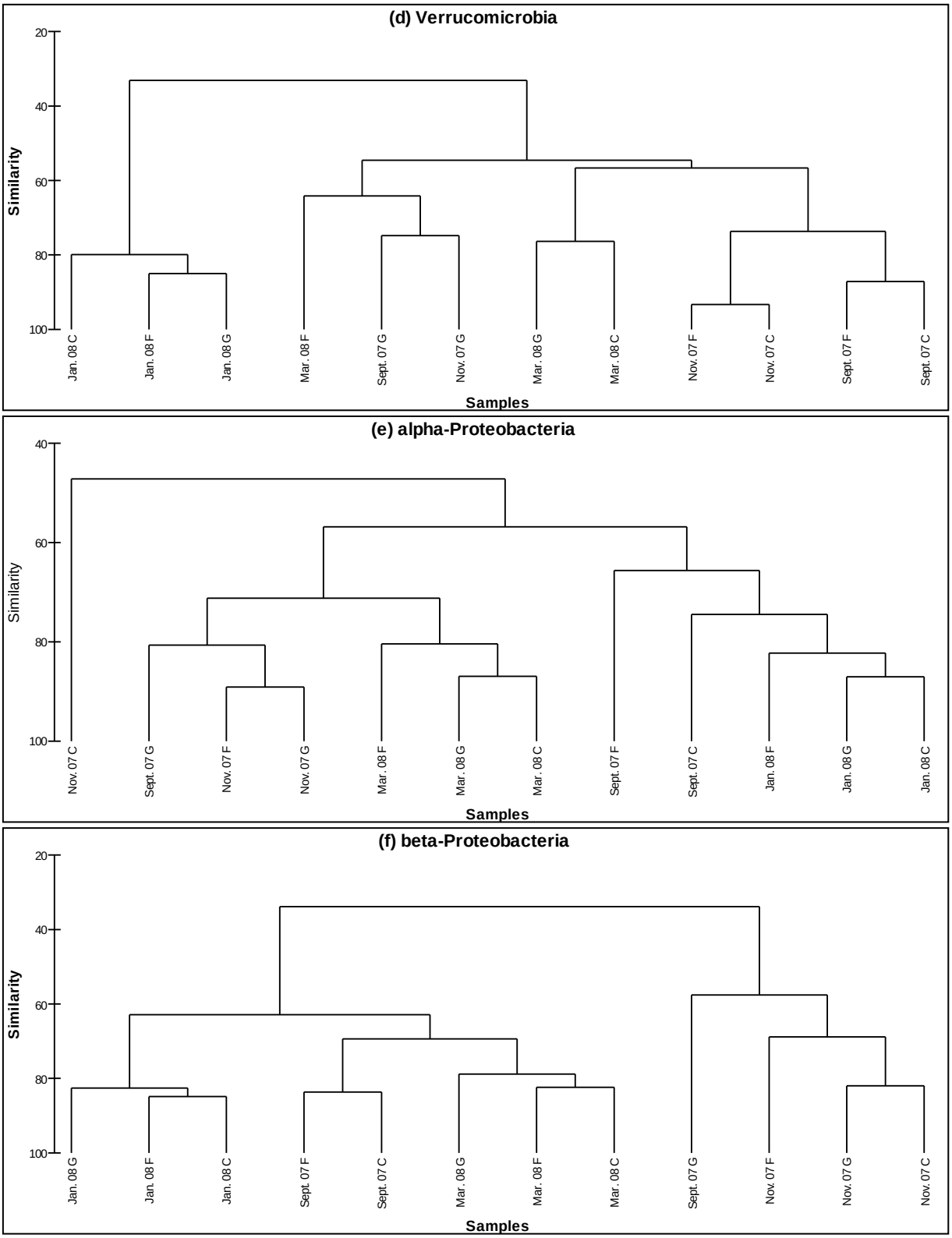


Fig. 2. Extended.

Verrucomicrobia and Acidobacteria were clearly affected by seasonal variations, however, other than for the total bacterial community and Proteobacteria, the impact of the resource availability was at least equally strong. In particular the availability of carbon strongly affected these two bacterial taxa, since they showed a separation of the girdling

treatment from the other samples in September and November 2007 and for Acidobacteria additionally in March 2008 (Fig. 2c-d).

Generally, all analyzed bacterial taxa were influenced by the course of the season, since the January 2008 samples were grouped together on one sub branch in all cases (Fig. 2c-f). Simultaneously, the community composition of all examined bacterial taxa was affected by tree girdling, as the September 2007 girdling treatment was separated from the other September 2007 samples in all cases. However, the extent of impact of the season and resource availability differed for the individual bacterial taxa. In general, the impact of fertilization was very low (Fig. 2c-f).

Archaeal structures were significantly different over the complete experiment period when comparing control with the girdling treatment (September 2007, November 2008 and January 2008: $P < 0.01$; March 2008: $P < 0.05$) (Table 5). The difference was greatest in September 2007 ($R = 0.959$), but decreased continuously from September 2007 until March 2008 ($R = 0.161$). In Fig. 2b, all tested girdling samples were grouped in one branch confirming the results of Bray Curtis similarity analysis showing that girdling strongly affects archaeal community composition. Unlike for the total bacterial community and Proteobacteria, the girdling effect on Archaea was definitely stronger than the impact of the season. Further, a significant difference in the archaeal community composition after comparison of control and fertilization treatment could be detected in November 2007 ($P < 0.01$), whereas in September 2007, January 2008 and March 2008 the communities were similar (Table 5). Fig. 2b shows that control and fertilization plots were highly similar in the individual months. The impact of tree girdling on Archaea was strongest, followed by the season, and fertilization coming last.

4.2 Quantitative real-time PCR

The abundance of bacteria and archaea in the forest soil in Klausenleopoldsdorf was determined at different time points of the year and after tree girdling and nitrogen fertilization by quantitative real-time PCR.

4.2.1 Season-effect

Comparison of the tested months from August 2007 until March 2008 revealed only small changes in abundance for the total bacterial community, the total archaeal community and for the six analyzed bacterial taxa Acidobacteria, Verrucomicrobia, alpha-Proteobacteria, beta-Proteobacteria and high-GC gram positive bacteria. Although the changes in abundance

were small, overall they were highly significant in all cases ($P < 0.001$). The season-related changes in bacterial and archaeal abundance are shown in Fig. 3.

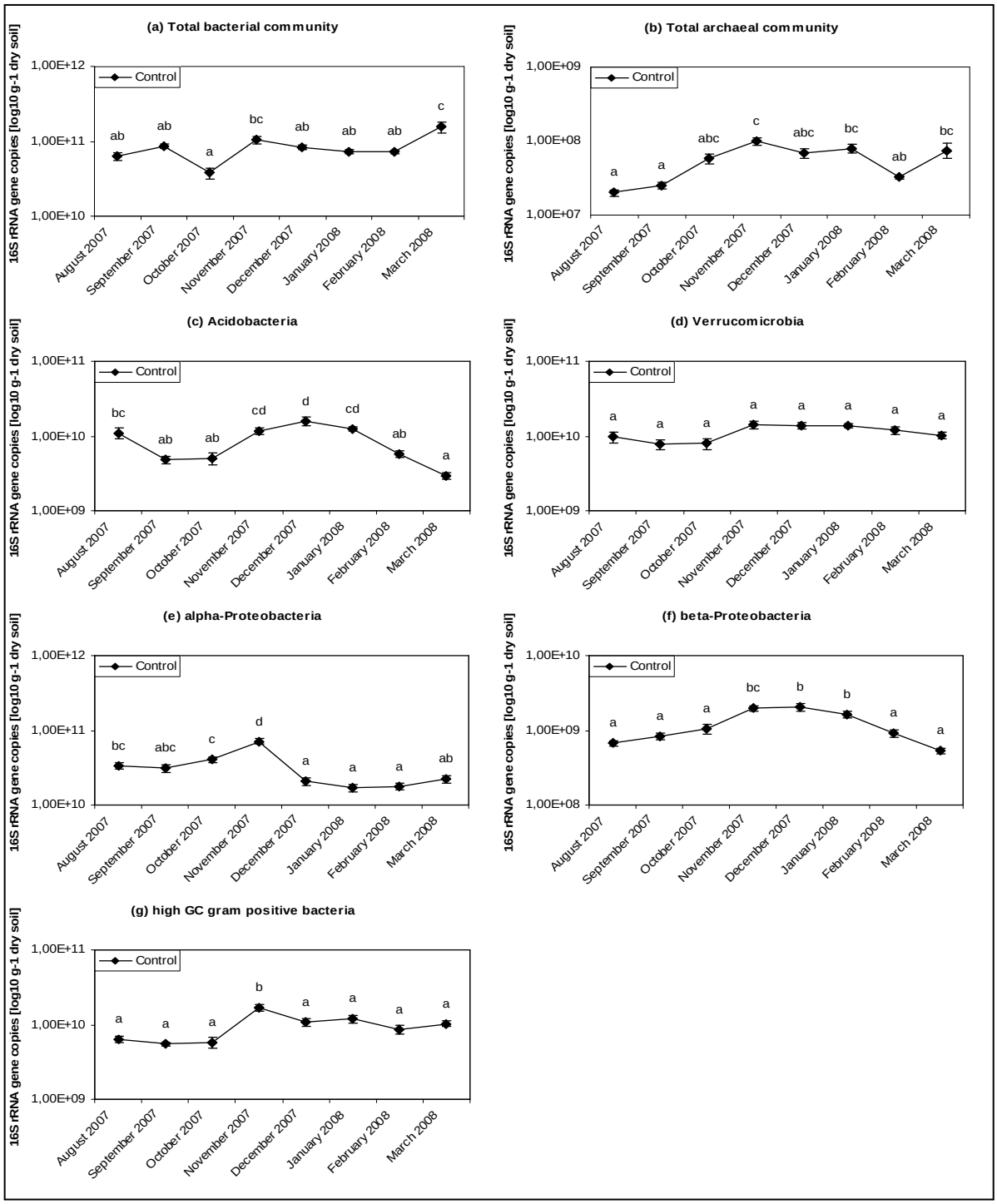


Fig. 3. *Abundance (16S rRNA gene copies) of (a) the total bacterial community, (b) the total archaeal community (c) Acidobacteria, (d) Verrucomicrobia, (e) alpha-Proteobacteria, (f) beta-Proteobacteria and (g) high GC gram-positive bacteria from August 2007 until March 2008. Different letters indicate significant differences between the abundances.*

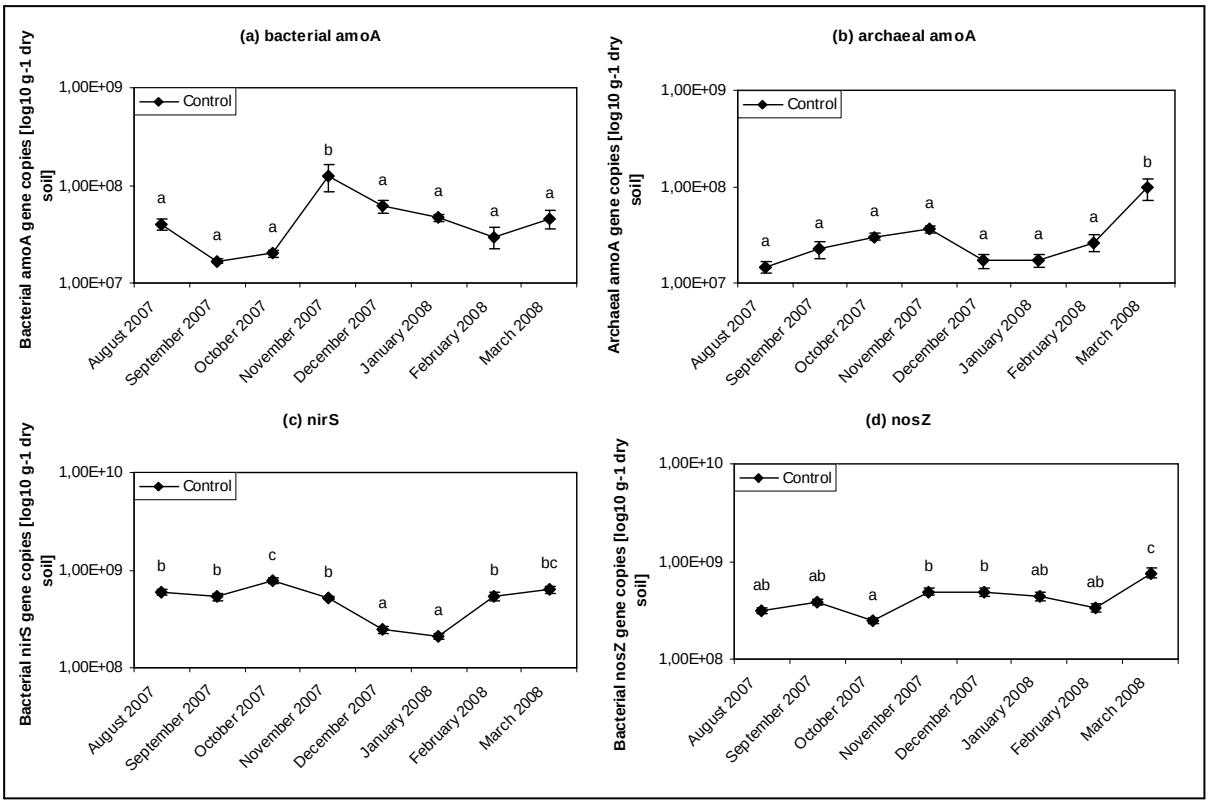


Fig. 4. *Abundance (gene copies) of bacterial nitrification and denitrification genes from August 2007 until March 2008. Different letters indicate significant differences between the abundances. (a) bacterial amoA, (b) archaeal amoA, (c) bacterial nirS and (d) bacterial nosZ*

Moreover, the season influenced the abundance of both bacterial and archaeal *amoA* genes participating in nitrification ($P < 0.001$ and $P < 0.05$), and bacterial *nirS* and *nosZ* genes involved in denitrification ($P < 0.001$ in both cases) (Fig. 4). However, according to bacteria and archaea, the differences were only small. Generally, the greatest season-related changes in abundance occurred between October and November 2007 (Fig. 3 and 4).

Comparison of archaeal as well as bacterial abundances in the different months showed similar trends. A slight increase beginning from August 2007 was detected, which ended with a peak in November 2007, however, with the exception of October 2007 for the total bacterial community (Fig. 3a-b). This increase in abundance was overall followed by a decrease from November 2007 until February 2008. In March 2008 the abundance of the total bacterial and archaeal community clearly increased and reached about the same level as the winter peak in November 2007. In general, changes in the abundance of Archaea were somewhat greater than that of Bacteria.

Like for the total bacterial and archaeal communities, acidobacterial, proteobacterial and high-GC gram-positive bacterial abundances were clearly higher in winter 2007. This initial increase was followed by a decrease until March 2008, and other than for the total

bacterial and archaeal communities, no final increase in abundance was detected (Fig. 3c and e-g). Whereas the decrease after the winter peak was continuous for Acidobacteria, Beta-Proteobacteria and high-GC gram-positive bacteria, Alpha-Proteobacteria showed a strong significant decrease immediately after the winter peak (Fig. 3c and e-g).

Verrucomicrobia showed the smallest changes in abundance between the different tested months. The abundance stayed constant over the complete analyzed time, with a slight trend towards a minimal increase in November 2007 (Fig. 3d). Comparable, bacterial *nosZ* gene copies showed only minimal changes, with a seasonal pattern similar to that of the total bacterial community (Fig. 4d).

The abundance of both, bacterial and archaeal *amoA* genes in November 2007 was higher compared to October 2007 (Fig. 4a-b), indicating a change due to autumn environmental conditions. The winter peak appeared to be especially strong for the bacterial *amoA* gene (Fig. 4a). The greatest difference in archaeal *amoA* abundance could be detected from February to March 2008 (Fig. 4b).

For bacterial *nirS* gene copies a small increase could be detected between September and October 2007, whereas in December 2007 and January 2008 the abundance was slightly lower (Fig. 4c).

4.2.2 Treatment-effect

Comparison of both total bacterial and total archaeal abundances as well as the abundances of the analyzed bacterial taxa in the collected soil samples overall revealed highly significant differences in relation to the course of the season ($P < 0.001$), while the impact of the treatments was only highly significant for archaea ($P < 0.001$) but not for bacteria ($P > 0.05$) (Table 6). Acidobacterial and alpha-proteobacterial communities significantly changed in response to the treatments ($P < 0.01$ and $P < 0.05$), while Verrucomicrobia, beta-Proteobacteria and high GC gram positive bacteria were overall not significantly affected by the treatments ($P > 0.05$), (Table 6). Comparison of abundances affected by the course of the season and the treatments in combination showed highly significant differences for archaea and all examined bacterial subgroups ($P < 0.001$), and significant differences for bacteria ($P < 0.01$) (Table 6).

The abundance of the four analyzed functional genes (bacterial and archaeal *amoA*, bacterial *nirS* and bacterial *nosZ*) overall was significantly influenced by both the season and the treatments ($P < 0.05$) and also by a combination of the two factors season and treatment (P

< 0.001) (Table 7). The changes in abundance of bacterial *amoA*, *nirS* and *nosZ* genes due to the season were highly significant (P < 0.001).

Table 6. Analysis of variance of the effect of each individual factor on soil bacterial and archaeal communities

Taxon	Significance level[a]					
	Season[b]		Treatment[b]		Interaction[b]	
Total bacterial community	0.000	***	0.370	-	0.003	**
Total archaeal community	0.000	***	0.000	***	0.000	***
Acidobacteria	0.000	***	0.002	**	0.000	***
Verrucomicrobia	0.000	***	0.230	-	0.000	***
Alpha-Proteobacteria	0.000	***	0.045	*	0.000	***
Beta-Proteobacteria	0.000	***	0.061	-	0.000	***
High-GC gram + bacteria	0.000	***	0.155	-	0.000	***

[a]Significance levels: *P < 0.05; **P < 0.01; ***P < 0.001
[b]Individual factors: tested months: August 2007 until March 2008; treatments: control, fertilization, tree girdling; interaction: season in combination with treatment.

Table 7. Analysis of variance of the effect of each individual factor on functional bacterial and archaeal genes

Functional genes	Significance level[a]					
	Season[b]		Treatment[b]		Interaction[b]	
Bacterial amoA	0,000	***	0,000	***	0,000	***
Archaeal amoA	0,014	*	0,000	***	0,000	***
nirS	0,000	***	0,019	*	0,000	***
nosZ	0,000	***	0,003	**	0,000	***

[a]Significance levels: *P < 0.05; **P < 0.01; ***P < 0.001
[b]Individual factors: tested months: August 2007 until March 2008; treatments: control, fertilization, tree girdling; interaction: season in combination with treatment.

Consistent with the results shown in Table 6, neither nitrogen fertilization, nor tree girdling showed great differences in bacterial abundance compared to the control (Fig. 5a) (P > 0.05). Further, for all analyzed bacterial taxa and the bacterial *nirS* and *nosZ* genes, the season had a greater influence on their abundances than the individual treatments (Fig. 5c-f and Fig. 6c-d)).

For Archaea and both archaeal and bacterial *amoA* genes, the girdled plots clearly differed in abundance from the rest of the treatments with an increased abundance in each of the evaluated months, whereas control and fertilization showed similar abundances in the individual months (Fig 5b and Fig. 6a-b). This suggests that the overall effect of the treatments was mainly due to tree girdling, with fertilization playing only a minor role.

Compared to the effect of the season, the influence of tree girdling on the abundance of Archaea and *amoA* genes was clearly stronger.

In total, tree girdling clearly influenced archaea and both *amoA* genes to a greater extent than seasonal variations, whereas bacteria, all examined bacterial taxa and bacterial *nirS* and *nosZ* genes were only minimally influenced by the treatments (Table 6 and 7; Fig. 5 and 6).

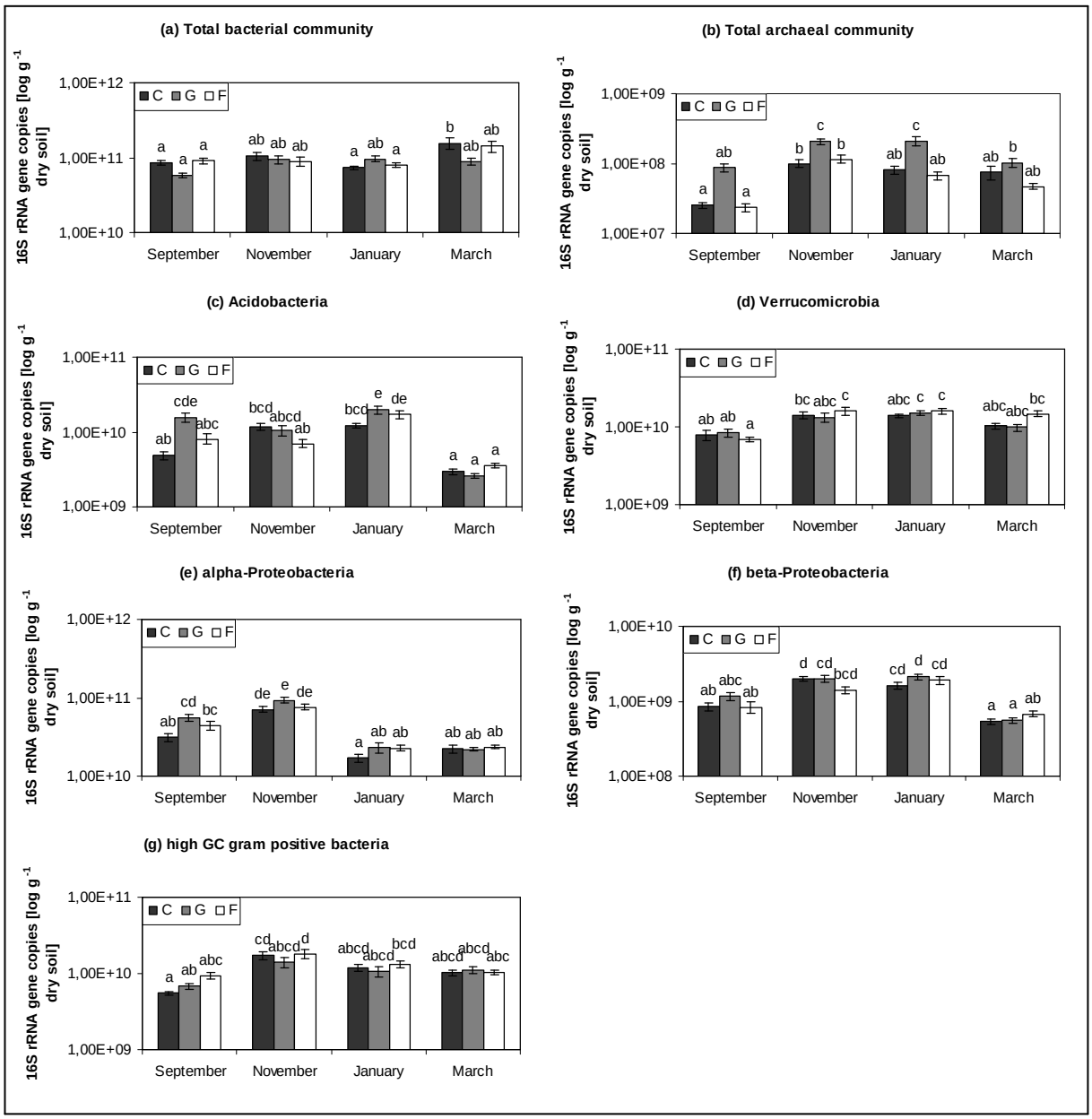


Fig. 5. *Abundance (16S rRNA gene copies) of (a) the total bacterial community, (b) the total archaeal community (c) Acidobacteria, (d) Verrucomicrobia, (e) alpha-Proteobacteria, (f) beta-Proteobacteria and (g) high GC gram-positive bacteria in the control soil and after tree girdling and fertilization from September 2007 until March 2008. Different letters indicate significant differences between the abundances.*

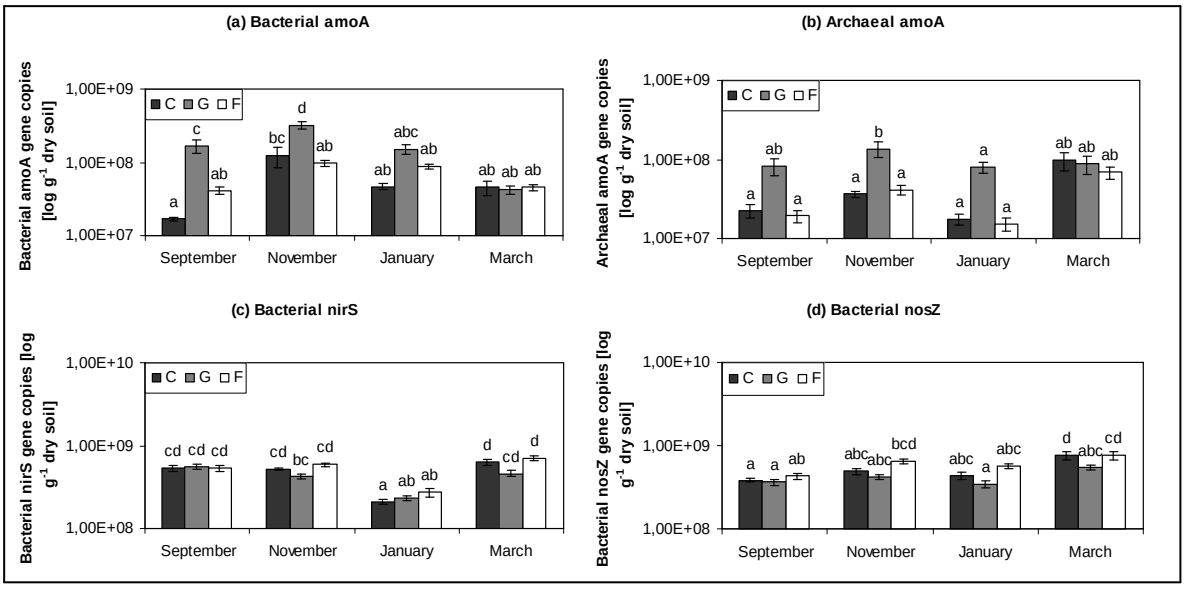


Fig. 6. *Abundance (gene copies) of bacterial and archaeal nitrification and of bacterial denitrification genes in the control soil and after tree girdling and fertilization from September 2007 until March 2008. Different letters indicate significant differences between the abundances.*
(a) bacterial amoA, (b) archaeal amoA, (c) bacterial nirS and (d) bacterial nosZ

5 Discussion

The soil is a dynamic environment in which many parameters (e.g. temperature, soil pH and soil moisture) may influence the community structure and abundance of the soil microbial community. The objective of this study was to investigate the effect of the course of the season as well as of nitrogen fertilization and tree girdling on structural and functional characteristics of soil microbial communities. For this purpose, structural community fingerprints based on 16S rRNA gene differences were generated and analyzed for corresponding treatment effects. Further, microbial abundances and the abundance of functional genes involved in the nitrogen cycle were assessed by quantitative real-time PCR. The results demonstrated that microbial communities were affected by the season, tree girdling and to a lesser extent by nitrogen fertilization.

5.1 T-RFLP

5.1.1 Season-effect

The microbial community structure differentiations assayed in the Klausenleopoldsdorf soil in this study were tightly coupled with the seasonal alterations. It could be therefore concluded that varying environmental conditions at different time points of the year play an important role in influencing the composition of microbial populations. This assumption is based on previous studies by Sinsabaugh et al. (2002) and Thormann et al. (2003), who studied the metabolic and genetic potential under changing environmental conditions. In detail they confirmed that seasonal variations lead to differences in enzyme activity and fungal community structures, respectively, during the decomposition of soil organic matter. In addition, Nemergut et al. (2007) confirmed the dramatic change of microbial communities in recently deglaciated soils on very short time scales.

In a similar study, Lipson and Schmidt (2004) reported that the phylogeny of soil bacterial communities changed dramatically from season to season in alpine tundra soils, whereas Monson et al. (2006) confirmed that seasonal patterns of soil respiration could largely be explained by seasonal succession of microbial communities in coniferous forests of the Rocky Mountains. The community differences between summer months and winter months obtained in this study were confirmed by studies performed by Monson et al. (2006), Lipson and Schmidt (2004) and Schmidt et al. (2007).

Even at a finer taxonomic scale the phylogenetic makeup of bacterial communities changed from season to season. The total bacterial community, as well as Acidobacteria,

revealed distinct community compositions in winter months compared to the rest of the examined months. It is likely that these similarities in community changes are due to the fact that Acidobacteria represent the main part of the total bacterial community in the analyzed soil. Beta-Proteobacteria, which are less abundant than Acidobacteria, showed a similar community shift in winter, however, unlike for the total bacterial community and Acidobacteria this shift persisted until spring. For Verrucomicrobia the shift in community composition occurred in spring rather than in winter. Likewise, Lipson and Schmidt (2004) found seasonal changes in community composition at fine taxonomic scales by constructing and analysing clone libraries of 16S rDNA from alpine soil samples of the Colorado Rocky Mountains collected in winter, spring and summer. A shift in beta-proteobacterial communities was found in winter, confirming the results of the present study. However, the results obtained by Lipson and Schmidt (2004) do not exactly come along with the results observed in the present study for the other examined bacterial taxa. Whereas in the Klausenleopoldsdorf forest soil an obvious change in the acidobacterial community was observed in winter, the strongest change examined by Lipson and Schmidt (2004) occurred in spring. Moreover, while in the present study a shift in verrucomicrobial communities was determined, Lipson and Schmidt (2004) detected similar verrucomicrobial communities in all seasons.

Archaea were generally thought to be restricted to exceptional environments with extreme conditions such as high temperatures, high salt concentrations or high and low pH values. However, recent studies demonstrated that archaea, especially those involved in ammonia oxidation, do also exist in „ordinary“ natural environments such as soil and water (Fierer et al., 2007; Francis et al., 2005; Leininger et al., 2006). Accordingly, assessed archaea in the assayed soil revealed great variations in their community composition at different seasons. The similarity of the total bacterial community structure at different times of the year tended to be higher than the resemblance of archaeal communities (Fig. 1a and b) and the differences between the tested months tended to be greater for archaea than for bacteria. These results suggest that archaea might be more sensitive than bacteria to altering conditions, however, additional analyses are still required to confirm this hypothesis.

In conclusion, obtained results strengthen the suggestion that different seasons representing varying environmental conditions influence the community structure and abundance of bacteria and archaea.

5.1.2 Soil treatment effect

In the present study, the two soil treatments, tree girdling and nitrogen fertilization, affected the total bacterial and archaeal communities in the analyzed soil, suggesting that the microbial population structure is dependent on the availability of nitrogen and carbon. This suggestion was confirmed by Zechmeister-Boltenstern et al. (2004) who demonstrated that microbial communities in European forests with high nitrogen inputs differed significantly from forests of unpolluted areas. Correspondingly, in our study nitrogen fertilization affected the composition of soil microbes. However, the effect of tree girdling was stronger than fertilization, indicating a strong dependence of microbial communities on the presence of carbon provided by rhizodeposition, whereas the sensitivity of soil microbial populations to the disposability of nitrogen was only moderate. Previous studies indicated that microbial utilization of root exudate compounds was proven to be a strong determinant of soil microbial community (Paterson et al., 2007). In addition, Högberg et al. (2007) showed that terminating tree belowground carbon allocation by girdling lead to significant changes in fungal soil communities in Fennoscandian boreal forests. Furthermore, it has been demonstrated that the amount of available nutrients, especially carbon and nitrogen, are major determinants of the soil microbial community (Schimel and Weintraub, 2003; Vance and Chapin, 2001). In addition, it was suggested that different microbial communities exhibit different nutrient requirements and that varying nutrient availabilities select for different microbial groups (Fenchel et al., 1998; Makino et al., 2003). Nevertheless, for many microbial groups the season effect was more pronounced than the effect resource availability. In contrast to bacterial populations, archaea were substantially more affected by girdling than by seasonal changes.

In conclusion, the availability of carbon and to a lower extent also nitrogen combined with the course of the season play a major role in controlling bacterial community composition. However, it can be suggested that additional factors (e.g. vegetation cover, soil chemical properties, pH, soil water content) may have a lasting effect on the community structure of soil microbial populations (Drenovsky et al., 2004; Hackl et al., 2004).

5.2 Quantitative real-time PCR

5.2.1 Season-effect

The abundances of soil microbes changed only slightly in response to seasonal variations in the present study. Similarly, the abundances of the analyzed functional genes bacterial *amoA*, archaeal *amoA*, bacterial *nirS* and bacterial *nosZ* were also influenced by the

course of the season, however, the effect of season was rather small. These results suggested that varying environmental conditions at different time points of the year changed bacterial and archaeal abundances as well as the abundance of genes conducting nitrogen cycling only to a small extent.

Generally, the greatest season-related changes in the abundance occurred between October and November 2007. A winter peak was determined for the prokaryotic abundance and abundance of analyzed functional genes involved in nitrogen cycling. Correspondingly, it has been shown that microbial biomass peaked under late-winter snow packs and that a high percentage of plant litter decomposition takes place in the winter in many seasonally snow-covered ecosystems (Hobbie and Chapin, 1996; Lipson and Schmidt, 2004; Taylor and Jones, 1990). Furthermore, it was shown that the under-snow peak microbial biomass declined during snowmelt (Lipson and Schmidt, 2004), which supports our findings.

5.2.2 Soil-treatment effect

In contrast to the general strong influence of soil treatments on forest soil microbial composition, a general low change in the abundance of soil bacteria was determined. Nevertheless, according to the T-RFLP results, tree girdling seemed to have a stronger effect than nitrogen fertilization, suggesting that the availability of carbon is more important than the availability of nitrogen for both microbial community structure and abundance. In contrast to the rather small changes for soil bacterial abundances, a clear effect on the abundance of archaea was determined due to tree girdling that confirmed the suggestion that archaea can more rapidly respond to environmental changes and to changes in resource availability compared to bacteria. According to the effects on bacteria, nitrogen fertilization had only a minor effect on the abundance of archaea.

Both, bacteria and archaea seemed to be influenced by an interaction between season and treatment. Exceptional for the total bacterial community for which the combination of season and soil treatment was not significant, all other examined taxa were significantly affected by this interaction suggesting that both interacting factors.

Recently, ammonia oxidizing and denitrifying soil microbial communities have been extensively studied by DGGE and T-RFLP analysis (Avrahami et al., 2002; Deiglmayr et al., 2006; Patra et al., 2006), but only little data are available focusing on the abundance of functional nitrogen cycling genes by quantitative real-time PCR. Recently, He et al. (2007) analyzed the abundance of ammonia-oxidizing bacteria and archaea after fertilizing soil with nitrogen alone as well as with nitrogen in combination with other fertilizers (phosphorus,

potassium) and detected strong changes in population size due to fertilizer applications. Inconsistent with these results, in our study, nitrogen fertilization had only a minor effect on the abundance of *amoA* genes. These functional nitrogen cycling genes clearly responded to reduced carbon availability after tree girdling, whereas changes in bacterial and archaeal *amoA* gene abundances due to seasonal variations and after nitrogen fertilization were only minor. Although bacterial *nirS* and *nosZ* genes showed an overall treatment effect concerning their abundances, this effect was not as strong as for the *amoA* genes, suggesting that the presence of *amoA* genes responded more quickly to environmental changes compared to *nirS* and *nosZ* genes.

In general, our findings suggest that the availability of low carbon compounds, which was reduced by tree girdling, was influencing the abundance and community structure of the soil microbial population. In addition, nitrogen fertilization revealed changes in the abundance and community structure, however, the fertilization effect was comparably small. In conclusion, the course of the season together with the availability of nitrogen and carbon resources determined microbial populations in the examined forest soil.

6 Final conclusions and outlook

The presented work provides a better understanding of microbial community structure and function in forest soils influenced by various environmental factors. In summary, our experimental set-up, the field experiment combined with T-RFLP analysis and qPCR measurements, was appropriate to demonstrate that the course of the season, nitrogen fertilization as well as the availability of carbon (demonstrated by tree girdling) were important factors determining the structure, function and abundance of soil microbial communities. Further, achieved results showed that the abundance of the investigated microbial communities and functional genes were strongly influenced by interactions between analyzed factors. Analysis of microbial communities under different environmental conditions and resource availabilities has provided information about microbial population diversity, composition and abundance in forest ecosystems. Additionally, new insights into the abundance of functional genes involved in forest soil nitrogen cycling were delivered. However, further analysis of functional genes would greatly increase the understanding of the role of bacteria in forest soil processes. In addition, it has to be considered that complex ecosystematic networks are involved in determining forest soil microbial communities and the abundance of nitrogen cycling genes. Furthermore, the consequence of the individual parameters tested in this study may be different under different environmental conditions. Consequently, additional molecular work is required to understand the phylogeny and function of microbial populations in forest soil ecosystems in more detail. Finally, a better understanding of the complex interactions between soil microorganisms and their environmental habitats is still required, which can then be used as baseline to further evaluate the factors influencing microbial communities.

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Zusammenfassung

Bisherige Studien haben gezeigt, dass mikrobielle Gemeinschaften im Boden durch viele verschiedene Umweltfaktoren beeinflusst werden und dass die Verfügbarkeit verschiedener Ressourcen die Struktur von mikrobiellen Gemeinschaften mitbestimmt. In einem Feldexperiment in einem Buchenwald in Niederösterreich wurde die Rhizodeposition durch „Baumgirdling“ manipuliert, der Boden wurde mit künstlichem Stickstoffdünger behandelt oder blieb als Kontrolle unbehandelt. Die Zusammensetzung der mikrobiellen Gemeinschaften im Waldboden wurde durch 16S rRNA basierende T-RFLP Analyse untersucht. Weiters wurde mithilfe von quantitativer real-time PCR die Auswirkung der verschiedenen Behandlungen auf die Abundanz der prokaryotischen Gemeinschaften im Waldboden analysiert. Es wurden sowohl die gesamte Population der Bakterien und Archaeen, als auch spezifische bakterielle Taxa (z.B. Acidobakterien, Verrucomikrobien, Proteobakterien und gram-positive Bakterien mit hohem GC Gehalt) untersucht. Weiters wurden funktionelle Gene die im Stickstoffkreislauf eine wichtige Rolle spielen (*amoA* von Bakterien und Archaeen, sowie *nirS* und *nosZ* von Bakterien) mithilfe von qPCR quantifiziert. Die Profile der mikrobiellen Gemeinschaften zeigten klare Unterschiede in der mikrobiellen Diversität aufgrund des Saisonverlaufs und der Bodenbehandlungen. Grundsätzlich war der Einfluss der Stickstoffdüngung im Vergleich zu den Auswirkungen des Saisonverlaufs und des „Baumgirdlings“ eher gering. Veränderungen in der Abundanz der Bodenmikroorganismen und der funktionellen Stickstoffkreislauf-Gene waren im Vergleich zu den Gemeinschaftsunterschieden ebenfalls eher gering bzw. nur vorübergehend von Bedeutung. Im Allgemeinen wurde ein Peak mit erhöhter Abundanz im Winter festgestellt, und Archaeen reagierten stärker auf veränderte Umweltbedingungen und Ressourcenverfügbarkeiten als Bakterien. Insgesamt hatten variierende Umweltbedingungen zu verschiedenen Jahreszeiten sowie unterschiedliche Ressourcenverfügbarkeiten einen starken Einfluss auf die Diversität von mikrobiellen Gemeinschaften im Waldboden, während die Abundanz der mikrobiellen Gemeinschaften im Boden nur wenig beeinflusst wurde.

Curriculum vitae

The author of the present thesis, Daniela Knapp, was born in Gmünd, Austria, on the 15th of January 1984. In 2002, she began the study of Molecular Biology at the University of Vienna. She specialized on genetics, microbiology/immunology and developmental biology. From 2007 until 2008, she worked on her diploma thesis in microbiology under the supervision of Ao. Univ.-Prof. Dr. Andreas Richter. The scientific work was carried out under the supervision of Priv.-Doz. DI Dr. Angela Sessitsch at the Austrian Research Centers GmbH, Department of Bioresources. The results of her research work are presented in this Masters thesis.

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