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ORIGINAL ARTICLE

Seasonality and resource availability control bacterial and archaeal communities in soils of a temperate beech forest

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It was hypothesized that seasonality and resource availability altered through tree girdling were major determinants of the phylogenetic composition of the archaeal and bacterial community in a temperate beech forest soil. During a 2-year field experiment, involving girdling of beech trees to intercept the transfer of easily available carbon (C) from the canopy to roots, members of the dominant phylogenetic microbial phyla residing in top soils under girdled versus untreated control trees were monitored at bimonthly intervals through 16S rRNA gene-based terminal restriction fragment length polymorphism profiling and quantitative PCR analysis. Effects on nitrifying and denitrifying groups were assessed by measuring the abundances of *nirS* and *nosZ* genes as well as bacterial and archaeal *amoA* genes. Seasonal dynamics displayed by key phylogenetic and nitrogen (N) cycling functional groups were found to be tightly coupled with seasonal alterations in labile C and N pools as well as with variation in soil temperature and soil moisture. In particular, archaea and acidobacteria were highly responsive to soil nutritional and soil climatic changes associated with seasonality, indicating their high metabolic versatility and capability to adapt to environmental changes. For these phyla, significant interrelations with soil chemical and microbial process data were found suggesting their potential, but poorly described contribution to nitrification or denitrification in temperate forest soils. In conclusion, our extensive approach allowed us to get novel insights into effects of seasonality and resource availability on the microbial community, in particular on hitherto poorly studied bacterial phyla and functional groups.
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Introduction

Trees release large proportions of their accumulated carbon (C) and nitrogen (N) in the form of tree residues (for example, litter, dead roots) and root exudates to the soil organic matter (Yarwood *et al.*, 2009; Fontaine *et al.*, 2004). The soil organic matter pool provides an important energy source for soil microorganisms, which are the major performing agents in decomposition and soil organic matter transformation, the key processes in terrestrial C and N cycling (Buckley and Schmidt, 2002). Quantity and quality of available C and N control soil microbial population dynamics and microbial processes including nitrification or denitrification (Schimel and Weintraub, 2003; Magill and Aber, 2000). In particular, it was shown that microbial community structures were shaped by N cycle dynamics in forest soils (Högberg *et al.*, 2007; Lejon *et al.*, 2005; Grayston and Prescott, 2005).

Belowground C and N transfer, shaped by trees and through microbial processes, is influenced by external factors such as seasonality. Seasonally alternating climatic conditions take a decisive control on tree physiology, photosynthesis and discharge of C and N into soil (Cannell and Dewar, 1994; Waring and Running, 1998). It can be concluded that cyclic changes in tree physiology have a significant influence on soil microbial communities. In addition, temporal variability in

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the soil microbial community composition was shown in response to seasonal variation in temperature, moisture and plant activity (Koch *et al.*, 2007; Waldrop and Firestone, 2006; Horz *et al.*, 2004; Buckley and Schmidt, 2002).

Seasonal and other temporal alternations in climatic conditions are determinants of soil N cycle dynamics (Cookson *et al.*, 2006; Wolsing and Priemé, 2004; Horz *et al.*, 2004). Soil N cycling includes both reductive and oxidative processes, in which soil microbes have a predominant role (Cabello *et al.*, 2004). Key microbial processes within the soil N cycle are catalyzed by key enzymes, including *amoA* gene encoding a subunit of ammonia monooxygenase in nitrification, as well as *nirS* and *nirK* gene (nitrite reductases) and *nosZ* gene (nitrous oxide (N_2O) reductase) involved in denitrification. The diversity and abundance of microorganisms carrying these genes and the actual link to N_2O emission have been extensively studied in diverse soil ecosystems (for example, Henderson *et al.*, 2010, Leininger *et al.*, 2006; Philippot *et al.*, 2006).

Tree girdling is a procedure to remove the bark and phloem from a tree down to the youngest xylem effectively excluding rhizodeposition into soil and thus restricting resource availability without disturbing the soil-root-microbe ecosystem (Högberg *et al.*, 2001). It was shown that manipulation of C and N availability by tree girdling leads to significant modifications in soil nutrient stoichiometry. Weintraub *et al.* (2007) measured lower dissolved organic C and N as well as an increase over time in nitrate and ammonium in girdled plots of a subalpine forest. Högberg *et al.* (2007) observed a tendency towards higher inorganic N levels in girdled plots of a boreal forest, whereas Ekberg *et al.* (2007) detected a decrease in total organic C in girdled plots of temperate spruce stand. It is thus likely that girdling-related changes in soil chemistry, in particular labile C and N pools have considerable effects on the soil microbial community structure (Dannenmann *et al.*, 2009; Weintraub *et al.*, 2007).

Although these examples show that soil microbial communities are influenced by C and N availability as well as by seasonality, the effects on different bacterial phyla or functional groups in temperate forest soils are still poorly understood. The objective of this study therefore was to assess in depth the microbial community response to C and N availability and to seasonal changes. A 2-year field experiment was carried out in a temperate beech forest (Klausenleopoldsdorf, Lower Austria). The major hypothesis was that seasonality along with altered C and N availability achieved through tree girdling control the community structure of the affected bacterial and archaeal population. Abundance and community structure of the total soil bacterial and archaeal population and specific phyla, that is, acidobacteria, alpha- and beta-proteobacteria and verrucomicrobia, as well as nitrifying and denitrifying microbial communities were investigated. Detected community changes were related to seasonality and tree girdling induced alterations in labile C and N pools as well as to soil moisture and soil temperature variations.

Materials and methods

Experimental site, samplings and geochemical data

The experimental study site was situated in a 65-year old beech forest (forest community *Hordelymo-Fagetum* with main tree species *Fagus sylvatica* L.) in Klausenleopoldsdorf (geographical location: 48°07′N, 16°03′E, 510 m above see level), Lower Austria, approximately 40 km southwest of Vienna. At the study site, representing an extensively managed forest-monitoring site according to the International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests, a mean annual temperature of 7.6 °C and a mean annual precipitation of 768 mm were determined. The soil defined as a dystric cambisol had developed from the Laab formation (Eocene) and major geochemical properties were determined previously (pH value: 4.0; total organic C: 4.36%; total N: 0.33%, C-to-N ratio: 13.1) (Hackl *et al.*, 2004).

The field experiment was started with girdling of trees in May 2006. Tree bark was removed at 10 cm length around the trunk at about 1.5 m above ground. Three girdling plots of 20×20 m were installed, of which only the inner 10×10 m were used for soil samplings. Six replicate control plots without tree girdling measuring 5×5 m were installed. Understory vegetation was removed from all plots and was repeated in the second spring season wherever necessary. During the whole experimental phase, soil temperature and soil moisture were measured continuously (Kaiser *et al.*, 2010). Further details about tree vitality, leaf litter amount and quality as well as fine root biomass in girdled and control plots have been reported by Kaiser *et al.* (2010).

Sampling was performed bimonthly from May 2006 until May 2008. Two replicate samples were taken from each plot (six replicates in total), with four subsamples (soil cores of $10 \times 10 \times 5$–10 cm, depending on the depth of A horizon) collected in each subplot. Generally, sampling was based on a predetermined sampling scheme to avoid sampling of already disturbed soil and to warrant independent soil samples throughout the experimental period. The four sub-samples of each plot were pooled, sieved through 2 mm mesh (5 mm mesh in case of wet soils) and stored at −20 °C. C and N pool data used for statistical purposes in this study were taken from Kaiser *et al.* (2010) and Kitzler *et al.* (unpublished data).

Microbial community structure (terminal restriction fragment length polymorphism (T-RFLP) analysis)

Bulk soil DNA was isolated (FastDNA Spin for Soil Kit, MP Biomedicals, Solon, OH, USA) and extracts were quantified photometrically (Nanodrop

Table 1 Description of primer sets, PCR ingredients and amplification details used for T-RFLP analysis

Target group	*Primer (reference)*	*DNA (ng)*	*Taq (U)*	*$MgCl_2$ (mM)*	*Primer (μM)*	*DNTPs (mM)*	*BSA (mg ml^{-1})*	*Amplification details*
All bacteria	8f (Weisburg *et al.* 1991) 926r (Liu *et al.* 1997)	5	2	1.5	0.15 0.15	0.2	—	95 °C 5 min 30 cycles: 95 °C 30 s, 53 °C 1 min, 72 °C 2 min 72 °C 10 min
All archaea	Ar109f (Lueders and Friedrich, 2000) Ar912r (Lueders and Friedrich, 2000)	10	2	2.0	0.15 0.15	0.2	1.0	95 °C 5 min 35 cycles: 95 °C 60 s, 52 °C 30 s, 72 °C 1 min 72 °C 10 min
Alpha-proteobacteria	8f (Weisburg *et al.*, 1991) Alf685 (Lane, 1991)	10	2	1.5	0.15 0.15	0.2	1.0	95 °C 5 min 95 °C 30 s, 55 °C 1 min, 72 °C 1 min 72 °C 10 min
Beta-proteobacteria	8f (Weisburg *et al.*, 1991) Bet680 (Overmann *et al.*, 1999)	10	2	1.5	0.15 0.15	0.2	1.0	95 °C 5 min 30 cycles: 95 °C 30 s, 55 °C 1 min, 72 °C 1 min 72 °C 10 min
Acidobacteria	Acid31F (Barns *et al.*, 1999) 926r (Liu *et al.*, 1997)	10	2	2.0	0.15 0.15	0.2	2.0	95 °C 5 min 30 cycles: 95 °C 30 s, 45 °C 1 min, 72 °C 1 min 72 °C 10 min
Verrucomicrobia	VMB537f (O'Farrell and Janssen, 1999) VMB1295r (O'Farrell and Janssen, 1999)	10	2	2.5	0.15 0.15	0.2	1.0	95 °C 5 min 35 cycles: 95 °C 1 min, 59 °C 30 s, 72 °C 90 s 72 °C 10 min

Abbreviations: BSA, bovine serum albumin; DNTPs, deoxynucleotide triphosphates.

ND-1000, Nanodrop Technologies, Wilmington, DE, USA). Bacterial and archaeal 16S rRNA genes were PCR-amplified using primers sets targeting total bacteria and archaea as well as four selected bacterial phyla. Based on a soil 16S rRNA gene library of one hundred clones generated before start of the field experiment, acidobacteria (28%), alpha- and beta-proteobacteria (18% and 14%, respectively), as well as verrucomicrobia (16%) have been selected as the four most dominant bacterial community members in the soils of the studied experimental site (Supplementary Table S1; NCBI accession numbers HM364804 to HM364903). For T-RFLP analysis, all forward primers were labeled with 6-carboxyfluorescein at the 5′ ends. From each DNA sample, two replicate PCRs were done. Composition of PCR cocktails and amplification details are provided in Table 1. Amplicons (5 μl) were checked on ethidium bromide-stained 1% (w/v) agarose gels. Replicate amplicons were pooled, purified (Sephadex G-50, GE Healthcare Biosciences, Waukesha, WI, USA), and approximately 200 ng of each amplicon were digested with 5 U *Alu*I (Invitrogen, Carlsbad, CA, USA) at 37 °C for 4 hrs. Before the T-RFLP analysis, digests were purified (Sephadex G-50) and an aliquot of 5 μl was mixed with 15 μl HiDi formamide (Applied Biosystems, Foster City, CA, USA) and 0.3 μl internal size standard (500 ROX Size Standard, Applied Biosystems). Labeled terminal-restriction fragments were denatured at 92 °C for 2 min, chilled on ice and detected on an ABI 3100 automatic DNA sequencer (Applied Biosystems) in the GeneScan mode. Gelquest software package (version 2.2.1, SequentiX, Klein Raden, Germany) was used to compare relative lengths of terminal-restriction fragments with the 500 ROX size standard and to compile electropherograms into a numeric data set, in which fragment length and peak height >50 fluorescence units were used for profile comparison. T-RFLP profiles used for statistical analyses were normalized according to Dunbar *et al.* (2000).

Microbial abundance (quantitative PCR (qPCR) analysis)

For standard preparation, amplicons from each investigated taxonomic group and functional genes (Table 2) were purified (Invisorb Spin PCRapid kit, Invitek, Berlin, Germany), ligated into the StrataClone PCR cloning vector pSC-A (Stratagene, La Jolla, CA, USA), and ligation products were transformed with StrataClone SoloPack competent cells (Stratagene). Specificity of clones used as qPCR

Table 2 Description of primer sets, PCR ingredients and amplification details used for quantitative PCR

Target group	*Primer (reference)*	*Amplification details*
All bacteria	Eub338 (Lane, 1991)	40 cycles
	Eub518 (Muyzer *et al.*, 1993)	95 °C 30 s, 55 °C 35 s, 72 °C 45 s
All archaea	Ar109f (Lueders and Friedrich, 2000)	40 cycles
	Ar912r (Lueders and Friedrich, 2000)	95 °C 30 s, 52 °C 35 s, 72 °C 45 s
Alpha-proteobacteria	Eub338 (Lane, 1991)	40 cycles
	Alf685 (Lane, 1991)	95 °C 30 s, 55 °C 35 s, 72 °C 45 s
Beta-proteobacteria	Eub338 (Lane, 1991)	40 cycles
	Bet680 (Overmann *et al.*, 1999)	95 °C 30 s, 55 °C 35 s, 72 °C 45 s
Acidobacteria	Acid31F (Barns *et al.*, 1999)	40 cycles
	Eub518 (Muyzer *et al.*, 1993)	95 °C 30 s, 55 °C 35 s, 72 °C 45 s
Verrucomicrobia	VMB537f (O'Farrell and Janssen, 1999)	40 cycles
	VMB1295r (O'Farrell and Janssen, 1999)	95 °C 30 s, 59 °C 35 s, 72 °C 45 s
Bacterial *amoA* gene	amoA-1f (Rotthauwe *et al.*, 1997)	45 cycles
	amoA-2r (Rotthauwe *et al.*, 1997)	95 °C 45 s, 57 °C 60 s, 72 °C 60 s
Archaeal *amoA* gene	Arch-amoAF (Francis *et al.*, 2005)	45 cycles
	Arch-amoAR (Francis *et al.*, 2005)	95 °C 45 s, 53 °C 60 s, 72 °C 60 s
Bacterial *nirS* gene	nirSCd3cd (Michotey *et al.*, 2000)	45 cycles
	nirSR3cd (Throbäck *et al.*, 2004)	95 °C 30 s, 58 °C 35 s, 72 °C 45 s, 78 °C 45 s
Bacterial *nosZ* gene	nosZ-f (Henry *et al.*, 2006)	6 touch down cycles
	nosZ1622r (Henry *et al.*, 2006)	94 °C 30 s, 65 °C 45 s (–1 °C), 72 °C 30 s
		40 cycles
		94 °C 30 s, 60 °C 45 s, 72 °C 30 s

standards were checked with Basic Local Alignment Search Tool. Plasmid DNA was isolated (Plasmid Miniprep Kit, Bio-Rad Laboratories, Hercules, CA, USA) and quantified as described above. For qPCR, each 25 µl PCR cocktail contained 12.5 µl iQ Sybr-Green Supermix (Bio-Rad Laboratories), 0.4 µM of each oligonucleotide (Table 2), 1.0 mg ml^{-1} bovine serum albumin and 10 and 50 ng template DNA for taxonomic groups and functional genes, respectively. Apart from the bacterial *amoA* gene assay, all functional gene qPCRs were supplemented with 0.625 µl dimethyl sulfoxide. PCR reactions were run on an iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad Laboratories) and were started with 3 min at 95 °C, followed by amplification cycles specific for each phylum and functional gene (Table 2). Melting curve analysis of amplicons was conducted to confirm that fluorescence signals originated from specific amplicons and not from primer-dimers or other artifacts. Each DNA sample was processed in triplicate reactions, whereas standard curves were generated using duplicate 10-fold dilutions of isolated plasmid DNA. Automated analysis of PCR amplicon quality (for example, PCR baseline subtraction, Ct-threshold setting to the linear amplification phase) and quantity was performed with iCycler Optical System Software Version 3.1 (Bio-Rad Laboratories).

Statistical analyses

Analysis of variance combined with *post hoc* Tukey-B tests (SPSS for Windows, version 11.7, SPSS Inc., Chicago, IL, USA) was performed according to Rasche *et al.* (2006) to determine significant treatment effects (tree girdling, seasonality) on abundance and community structure of the investigated microbial groups and functional genes. Pearson's linear correlation coefficients were calculated for assessing significant relations between microbial abundance and geochemical parameters (SPSS for Windows). Effect of tree girdling on T-RFLP data sets obtained from each target group was further assayed based on Bray–Curtis similarity coefficients (Legendre and Legendre, 1998). Therefore, a similarity matrix was generated for all possible pairs of samples of each target group. This similarity matrix was used for analysis of similarity (ANOSIM) statistics (Clarke and Green, 1988) to test the hypothesis that bacterial, archaeal and taxonomic communities were altered by tree girdling over the investigation period. ANOSIM generates a test statistics, *R*. The magnitude of *R* indicates the degree of separation between two communities, with a score of 1 indicating complete separation and 0 indicating no separation. Calculation of similarity coefficients and ANOSIM were carried out using Primer six for Windows (version 6.1.5, Primer-E Ltd., Plymouth, UK). To test the influence of environmental variables on the microbial community structure, canonical correspondence analyses were carried out in Canoco (version 4.5 for Windows, PRI Wageningen, the Netherlands) (Lepš and Šmilauer, 2003). Presence or absence as well as relative height of terminal-restriction fragments were used as 'species' data whereas geochemical data were included in the analysis as 'environmental' variables. Resulting ordination biplots approximated the weighted differences between the individual communities (T-RFLP patterns) with respect to each of the geochemical factors, which were represented as arrows. The length of the corresponding arrows indicated the relative importance of the geochemical factor in explaining

variation in the six microbial T-RFLP profiles, whereas the angle between arrows indicated the degree to which they were correlated. A Monte Carlo permutation test based on 1000 random permutations was used to calculate the impact of geochemical variables on community patterns.

Results

Microbial community structure (T-RFLP analysis)
Compared with tree girdling, seasonality had the greatest, significant influence on the total bacterial and archaeal community structure as well as on the four selected phyla ($P<0.001$) (Table 3). The community structure of the total archaea was changed by tree girdling ($P<0.001$), whereas total bacterial was not ($P>0.05$). Alpha-proteobacteria and acidobacteria showed a detectable community change due to tree girdling ($P<0.01$), whereas beta-proteobacteria and verrucomicrobia appeared not altered ($P>0.05$). A clear interaction between the two factors 'seasonality' and 'tree girdling' was determined indicating an interrelated influence of both factors on the community dynamics of the bacterial and archaeal community ($P<0.05$) (Table 3).

ANOSIM detected a distinct tree girdling-induced community change among archaea, which was confirmed by several significant R values ranging between 0.124 and 0.913 indicating distinct structural differences between two individual archaeal communities (control versus tree girdling) ($P<0.05$) (Table 4). Based on R values, greatest community differentiations became measurable during early fall and winter months. A comparable trend was determined for total bacteria, although the differences were less pronounced as compared with the total archaea explained by a smaller number of significant R values. For alpha-proteobacteria and acidobacteria, ANOSIM calculated several significant R values, whereas for beta-proteobacteria and verrucomicrobia only at three sampling dates significant tree girdling effects ($P<0.05$) were found.

Canonical correspondence analysis was used to test the significant dependence of the community differentiations on tree girdling and seasonality-related changes in geochemical parameters (Table 5, Figure 1). Multivariate testing, based on 1000 Monte Carlo permutations, confirmed the significance of the two canonical axes ($P<0.05$). The total percentage variance of the microbiota-environment relation ranged between 47.6% (verrucomicrobia) and 70.8% (total bacteria) (Table 5). The first canonical axis attributed the greatest influence explaining the microbiota-environment relation. The strong relation between the T-RFLP data sets and geochemical data was substantiated by high correlation coefficients of at least 0.390 (Table 5). Figure 1 illustrates the relationship between the community changes and geochemical data and shows further the clear seasonality and tree girdling related community differentiations overall the 2-year experimental period. Generally, higher soil moisture was determined in the second experimental year, whereas higher soil temperatures were measured in the first year of the field experiment (Kaiser *et al.*, 2010). These soil climatic differentiations were clearly reflected in all six T-RFLP community patterns, in which distinct community shifts were observed between the first and the second year (Figure 1). The first year samples tended to cluster if higher soil temperatures and lower soil moisture were observed, whereas the opposite effect was determined for the second year samples. When explaining the effect of determined chemical parameters on assayed microbial communities, nitrate contents were in average highest in the first year of investigation and peaked during summer months (Kaiser *et al.*, 2010). Dissolved organic nitrogen (DON) showed a clear decrease in the second year as compared to the first year, whereas no clear trend was observable for dissolved organic carbon (DOC) and ammonia (Kaiser *et al.*, 2010). These changes in chemical parameters were clearly reflected in alterations within the soil microbial communities (Figure 1). In particular, the first year samples tended to cluster with high nitrate values, whereas the second year samples indicated a clear correlation with high DON values. In general, seasonality effect was overwhelming the effect of tree girdling.

Microbial abundance (qPCR analysis)
Analysis of variance determined significant effects of seasonality on bacterial and archaeal abundance

Table 3 Analysis of variance to determine significant effects of seasonality and tree girdling on the community structure (TRFLP analysis) and abundance (qPCR analysis) of the soil prokaryotic population assessed in a bimonthly time grid from May 2006 to May 2008

Parameter	*Seasonality*	*Tree girdling*	*Interaction*
Community structure (T-RFLP analysis)			
Total bacteria	***[a]	n.s.	**
Total archaea	***	***	***
Alpha-proteobacteria	***	*	*
Beta-proteobacteria	***	n.s.	**
Acidobacteria	***	**	**
Verrucomicrobia	***	n.s.	**
Abundance (qPCR analysis)			
Total bacteria	***	n.s.	**
Total archaea	***	**	*
Alpha-proteobacteria	***	**	**
Beta-proteobacteria	***	n.s.	n.s.
Acidobacteria	***	**	**
Verrucomicrobia	***	n.s.	**
Bacterial *amoA* gene	***	***	***
Archaeal *amoA* gene	***	**	*
Bacterial *nirS* gene	***	n.s.	n.s.
Bacterial *nosZ* gene	***	n.s.	n.s.

[a]Significance levels: n.s.: $P>0.05$; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Table 4 Output of analysis of similarity (ANOSIM) revealing the tree girdling effect on soil bacterial and archaeal community structure (tree girdling versus control) over the whole field experiment period starting in May 2006 until May 2008

Sampling	*Total bacteria*	*Total archaea*	*Alpha-proteobacteria*	*Beta-proteobacteria*	*Acidobacteria*	*Verrucomicrobia*
May 2006	0.033[a]	0.115	0.035	0.078	0.057	0.022
June 2006	0.330**[b]	0.093	0.120	0.320**	0.189*	0.091
August 2006	0.111	0.106	0.017	0.044	0.037	0.085
October 2006	0.017	0.304*	0.011	0.078	0.089	0.078
December 2006	0.033	0.815**	0.143*	0.065	0.194*	0.057
February 2007	0.204*	0.724**	0.144*	0.080	0.107	0.313**
April 2007	0.250*	0.211*	0.287*	0.033	0.124	0.015
June 2007	0.193*	0.124*	0.270**	0.094	0.337**	0.457**
July 2007	0.078	0.448**	0.056	0.063	0.146*	0.169
September 2007	0.257*	0.913**	0.617**	0.620**	0.348*	0.007
November 2007	0.165*	0.472**	0.150*	0.089	0.196*	0.163
January 2008	0.067	0.500**	0.026	0.030	0.017	0.057
March 2008	0.015	0.328**	0.152*	0.093	0.174*	0.069
May 2008	0.457**	0.441**	0.222*	0.113*	0.270*	0.237*

[a]*R* indicates the degree of separation between two populations, with a score of 1 indicating complete separation and 0 indicating no separation.
[b]Levels of significance between two tested populations: $*P<0.05$; $**P<0.01$.

Table 5 Relationship between the soil prokaryotic community shifts and soil environment dynamics as revealed by canonical correspondence analyses and Monte Carlo permutation tests performed separately for each of the six 16S rRNA gene-based T-RFLP data sets and geochemical data which comprised of dissolved soil organic carbon, dissolved soil organic nitrogen, soil nitrate as well as soil ammonia (original geochemical data have been discussed by Kaiser *et al.*, unpublished data)

Prokaryotic group	*Monte Carlo permutation test of canonical axes*	*Percentage variance of microbiota-environment relation*		*Microbiota-environment correlation*	
		Canonical axis 1	*Canonical axis 2*	*Canonical axis 1*	*Canonical axis 2*
Total bacteria	***	47.3	23.5	0.555	0.685
Total archaea	***	35.7	21.3	0.710	0.523
Alpha-proteobacteria	**	29.1	28.1	0.409	0.518
Beta-proteobacteria	*	49.4	18.4	0.552	0.469
Acidobacteria	***	39.3	25.9	0.547	0.531
Verrucomicrobia	*	25.6	22.0	0.455	0.390

Significance levels: $*P< 0.05$; $**P< 0.01$; $***P< 0.001$.

as measured by qPCR of 16S rRNA genes and functional genes ($P<0.001$) (Table 3, Figure 2). Seasonality effect was most pronounced for beta-proteobacteria, which showed abundance shifts of 236 and 242% for the control (average 1.14×10^{10} 16S rRNA gene copies) and girdled (average 1.18×10^{10}) plots, respectively, over the whole experimental period. The smallest seasonality effect was determined for total archaea (average 6.13×10^{7}) with 63% variation in the control plots over the whole experiment. However, abundance of total archaea measured in the girdled plots (average 1.09×10^{8}) revealed a seasonality related fluctuation of 112%. The other microbial groups assayed in the control and girdled plots took intermediate positions with seasonal changes ranging between 65% (verrucomicrobia in control plots) and 97% (alpha-proteobacteria in girdled plots). In contrast to seasonality, tree girdling had a minor effect and was only significant for total archaea, alpha-proteobacteria and acidobacteria ($P<0.05$). For these three groups, significantly higher 16S RNA gene copies were determined in the girdled plots as compared with the control plots (44%, total archaea; 18%, alpha-proteobacteria and acidobacteria). No significant differences were found for beta-proteobacteria (3% higher in girdled plots) and verrucomicrobia (2% higher in girdled plots). Contrastingly, 16S rRNA gene copies tended to be 7% greater in control plots in comparison with girdled plots when analyzing total bacterial abundance ($P>0.05$). Except for beta-proteobacteria, a clear interaction between both factors could be calculated indicating an interrelated influence of 'seasonality' and 'tree girdling' on the abundance of bacterial and archaeal communities ($P<0.05$) (Table 3).

A highly significant effect of seasonality was detected for archaeal and bacterial *amoA* gene, *nirS* gene and *nosZ* gene abundance ($P<0.001$) (Table 3, Figure 3). All assayed functional genes revealed higher gene copies in the second experimental year (June 2007 to May 2008) as compared with the first investigation period (May 2006 to May 2007). Abundance of bacterial and archaeal *amoA* genes

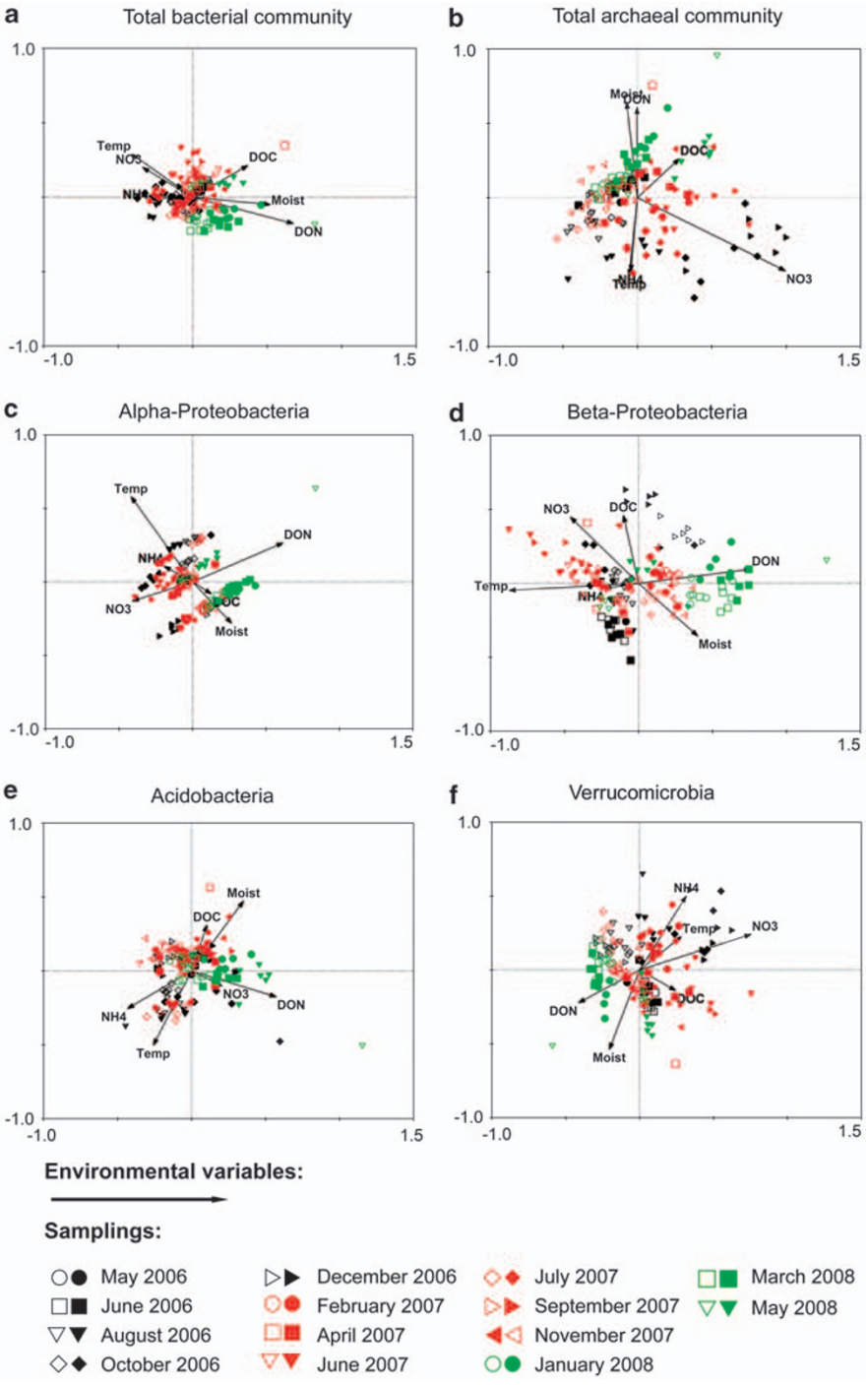

Figure 1 Biplots of canonical correspondence analysis of the six individual 16S rRNA gene-based T-RFLP data sets obtained from (**a**) total bacteria, (**b**) total archaea, (**c**) alpha-proteobacteria, (**d**) beta-proteobacteria, (**e**) acidobacteria and (**f**) verrucomicrobia. Sampling years (Seasonality) are indicated with colors (2006, green; 2007, red; 2008, black), whereas control and tree girdling samples are shown with open and bold symbols, respectively. Geochemical data (soil moisture (Moist), soil temperature (Temp), soil nitrate (NO3), soil ammonia (NH4), dissolved soil organic carbon (DOC) and dissolved soil organic nitrogen (DON) are presented with black arrows.

showed greater seasonal fluctuations as compared with *nirS* and *nosZ* genes. Over the whole project period, greatest seasonal changes on archaeal *amoA* gene abundance were found in control plots (average 1.41×10^7, 187% variation), whereas the effect was smallest for archaeal *amoA* gene copies in girdled plots (average 3.14×10^7, 145%). Bacterial *amoA* gene copies took an intermediate position and their seasonal responses were similar to those of archaeal *amoA* genes. Although seasonality related shifts of the quantities of *nirS* and *nosZ* genes were highly significant ($P<0.001$), their overall seasonal

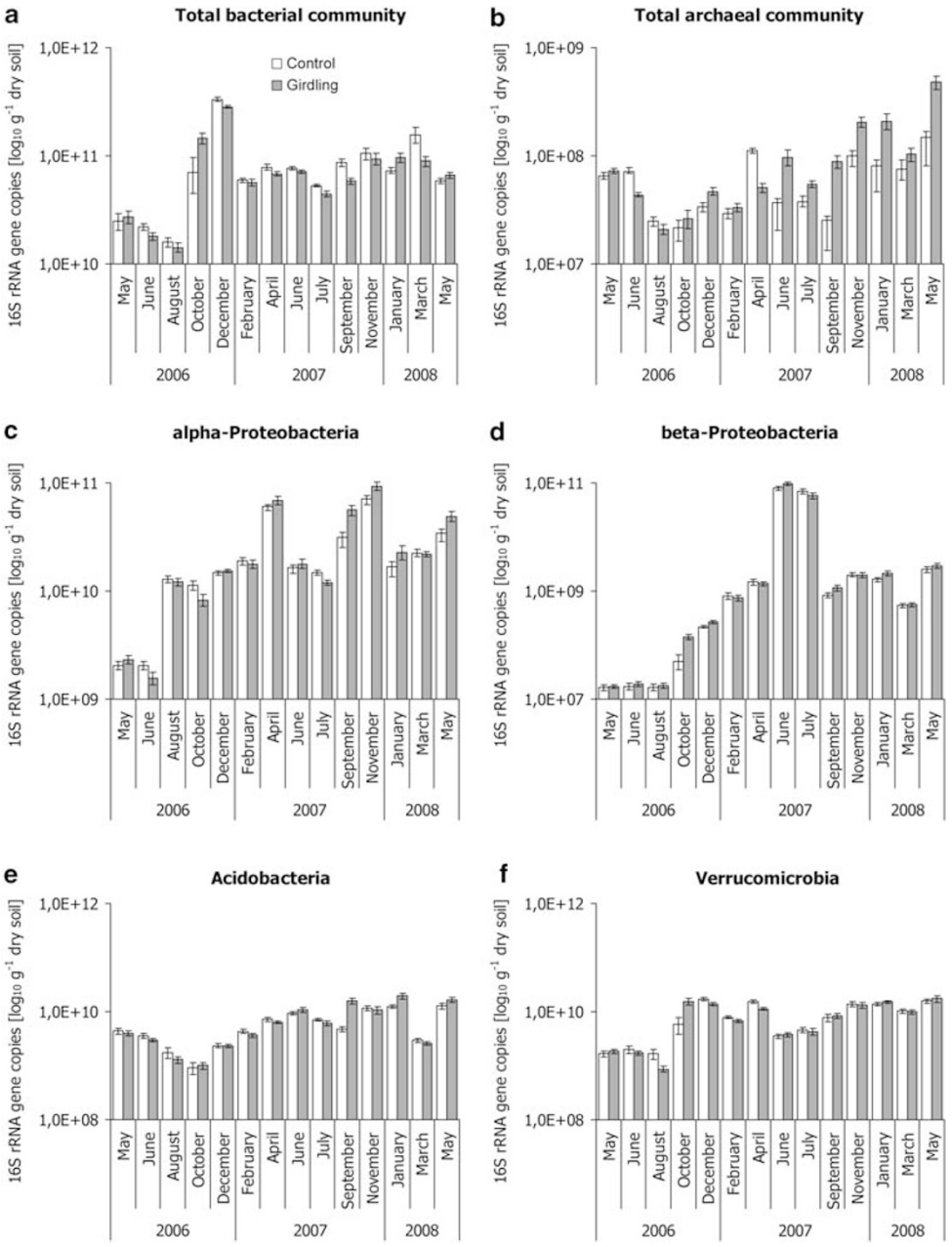

Figure 2 Quantitative PCR data of the six assayed microbial groups (**a**) total bacteria, (**b**) total archaea, (**c**) alpha-proteobacteria, (**d**) beta-proteobacteria, (**e**) acidobacteria and (**f**) verrucomicrobia as determined over a 2-year period starting in May 2006. Presented data (16S rRNA gene copies per gram dry soil) are average values calculated from six individual soil samples per treatment along with standard errors. White columns show the control plots, whereas grey bars represent the tree girdling plots.

alternation was less intense as compared with the assayed *amoA* genes. Greatest seasonal differentiations were determined for *nosZ* gene in the control plots (107%), whereas smallest differences were determined for *nirS* gene copies in the control plots (91%). Copy numbers of the *nirS* gene behaved similarly to those of the *nosZ* gene, but with slightly greater seasonal changes. Distinct differences between copy numbers of assayed functional genes were determined between control and tree girdling plots. Archaeal *amoA* gene revealed 55% higher numbers in girdled plots (6.83×10^7) as compared with control plots (1.41×10^7) ($P<0.01$), whereas 71% higher copies of bacterial *amoA* gene were measured in girdled plots (average 6.83×10^7) as compared with the corresponding controls (average 1.99×10^7) ($P<0.001$). Effect of tree girdling was not significant for *nirS* and *nosZ* genes. Copy numbers of *nosZ* gene showed 14% lower values in girdled plots as compared with controls (2.04×10^8 versus 2.33×10^8), whereas *nirS* gene abundance was not significantly changed by tree girdling (2.43×10^8

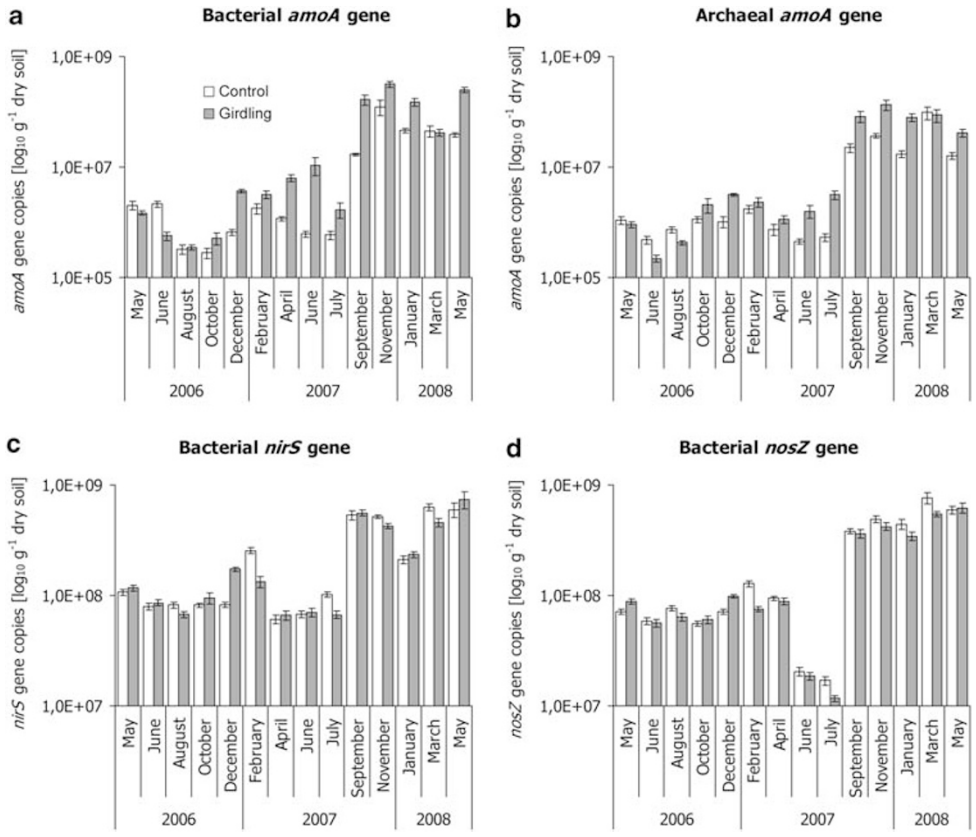

Figure 3 Abundance of the six assayed functional genes for (**a**) bacterial ammonia monooxygenase (*amoA* gene), (**b**) archaeal ammonia monooxygenase (*amoA* gene), (**c**) bacterial nitrite reductase (*nirS* gene) and (**d**) bacterial nitrous oxide reductase (*nosZ* gene), as determined by quantitative PCR data over a 2-year period starting in May 2006. The numbers of gene copies are presented per gram dry soil and are average values calculated from six individual soil samples per treatment along with standard errors. White columns show the control plots, whereas grey bars represent the tree girdling plots.

(controls) versus 2.35×10^8 (tree girdling)) ($P>0.05$). Significant interactions between seasonality and tree girdling were determined for both *amoA* genes ($P<0.05$). Generally, it needs to be pointed out that very small abundance differences may have occurred due to variations in the used qPCR assays. In the latter discussion only those abundance differences were considered for which statistical significances were obtained.

Linear correlation coefficients between microbial abundance and geochemical data

Positive correlations, except for total archaea, verrucomicrobia and functional genes, were calculated for DOC (range from $r=0.164$ ($P<0.05$, acidobacteria) to $r=0.239$ ($P<0.01$, alpha-proteobacteria)), showing increased gene abundance when high DOC contents were determined. DON revealed a positive correlation with total archaea ($r=0.299$, $P<0.01$), acidobacteria ($r=0.166$, $P<0.05$) both *amoA* genes (bacterial *amoA* gene, $r=0.228$; archaeal *amoA* gene, $r=0.226$) and the *nosZ* gene ($r=0.352$) ($P<0.01$) (Table 6). For soil nitrate, positive correlations were determined between total bacteria ($r=0.254$, $P<0.01$), and soil ammonia was positively correlated with beta-proteobacteria ($r=0.187$, $P<0.05$) and acidobacteria ($r=0.301$, $P<0.01$). Negative correlations were calculated between soil ammonia and total archaea ($r=-0.278$, $P<0.01$) as well as verrucomicrobia ($r=-0.269$, $P<0.01$) reflecting decreased abundance with increasing soil ammonia levels. Although soil nitrate was only correlated with *nosZ* gene ($r=-0.256$), soil ammonia was negatively correlated with all functional genes (at least $r=-0.235$) ($P<0.01$). Total N_2O emission was positively correlated with total archaeal abundance ($r=0.476$), acidobacteria ($r=0.527$), verrucomicrobia ($r=0.393$), archaeal *amoA* ($r=0.283$), *nirS* ($r=0.445$) and *nosZ* ($r=0.424$) ($P<0.01$) genes. Soil temperature was negatively correlated with all assayed groups (range from $r=-0.163$ ($P<0.05$, acidobacteria) to $r=-0.421$ ($P<0.01$, verrucomicrobia)), except with beta-proteobacteria ($r=0.475$, $P<0.01$), indicating an abundance decrease with higher soil temperatures. Similar trends were observed for soil moisture (range from $r=-0.233$ ($P<0.01$, total bacteria) to $r=-0.398$ ($P<0.01$, beta-proteobacteria)); however, for total archaea and acidobacteria an increase in abundance was determined when higher soil moisture occurred ($r=0.327$ ($P<0.01$, total archaea) and

Table 6 Pearson's linear correlation coefficients between prokaryotic abundance as measured by 16S rRNA gene-based quantitative PCR and geochemical data (geochemical data taken from Kaiser *et al.* (in revision) and B Kitzler *et al.* (unpublished data)

Prokaryotic group	*Dissolved soil organic carbon*	*Dissolved soil organic nitrogen*	*Soil nitrate*	*Soil ammonia*	*Total nitrous oxide emission*	*Soil temperature*	*Soil moisture*
Total bacteria	0.174*	n.s.	0.254**	n.s.	n.s.	−0.320**	−0.233**
Total archaea	n.s.	0.299**	n.s.	−0.278**	0.476**	−0.192*	0.327**
Alpha-proteobacteria	0.239**	n.s.	n.s.	n.s.	n.s.	−0.243**	−0.265**
Beta-proteobacteria	0.194*	n.s.	n.s.	0.187*	n.s.	0.475**	−0.398**
Acidobacteria	0.164*	0.166*	n.s.	0.301**	0.527**	−0.164*	0.289**
Verrucomicrobia	n.s.	n.s.	n.s.	−0.269**	0.393**	−0.421**	n.s.
Bacterial *amoA* gene	n.s.	0.228**	n.s.	−0.235**	n.s.	−0.259**	0.268**
Archaeal *amoA* gene	n.s.	0.226**	n.s.	−0.294**	0.283**	−0.330**	0.247**
Bacterial *nirS* gene	n.s.	n.s.	n.s.	−0.330**	0.445**	−0.308**	0.320**
Bacterial *nosZ* gene	n.s.	0.352**	−0.256**	−0.310**	0.424**	−0.456**	0.418**

Significance levels: n.s.: $P > 0.05$; *$P < 0.05$; **$P < 0.01$.

$r = 0.289$ ($P < 0.01$, acidobacteria)). Functional genes were negatively correlated with soil temperature (range from $r = -0.259$ (bacterial *amoA* gene) to $r = -0.456$ (*nosZ* gene)), whereas positive correlations were determined between functional genes and soil moisture (range from $r = 0.247$ (archaeal *amoA* gene) to $r = 0.418$ (*nosZ* gene)) ($P < 0.01$).

Discussion

Previous studies on the effects of seasonality and resource availability on dynamics of soil microbial communities under field conditions have been restricted in resolution by the use of wide sampling intervals and short investigation periods, and have focused on broad microbial domains rather than specific phyla. To overcome these limitations, we used a bi-monthly sampling scheme during a 2-year tree girdling field experiment period to study in detail the effect of seasonality and resource availability on the soil microbial community. Structure (T-RFLP analysis) and abundance (qPCR) of microbial communities were analyzed at different taxonomic scales including bacterial and archaeal domains and specific bacterial phyla, which occur prominently in the assayed soil, that is, acidobacteria, alpha- and beta-proteobacteria and verrucomicrobia, as well as nitrifying and denitrifying bacteria and archaea. Our results showed that resource availability due to seasonal variation, but also due to tree girdling resulted in specific short- and medium-term changes in community structure and abundance of archaea and bacteria as well as representatives of selected phyla. Generally, microbial communities were altered by seasonal effects to a larger extent than by altered root exudation. These community alterations were partly ascribed to the influence of seasonality and tree girdling on physicochemical parameters such as DOC) and DON, nitrate, ammonia, as well as soil temperature and soil moisture.

Seasonality in temperate forest soils is reflected by alterations in soil moisture and soil temperature, being acknowledged control factors of soil microbial communities (Stres *et al.*, 2008; Tabuchi *et al.*, 2008; Cleveland *et al.*, 2007). Both parameters were responsible for compositional shifts in soil bacterial communities determined in this study. Similarly, changes in soil temperature and moisture appeared to be determinants of the archaeal community in the present field study. Also Shen *et al.* (2008) and Tourna *et al.* (2008) have found temporal shifts in archaeal abundance, and Stres *et al.* (2008) and Tourna *et al.* (2008) evidenced responsiveness of soil archaea to variations in soil temperature and soil moisture. Seasonal changes in soil climate were further closely linked to short- and medium-term variations in resource availability, which further correlated with the quantity and quality of organic matter entering the soil, as it was also previously suggested (Bell *et al.*, 2009; Cookson *et al.*, 2006; Krave *et al.*, 2002). Consequently, total bacterial communities and individual phyla studied were clearly shaped by supply of DOC, DON and mineral N (that is, ammonia, nitrate), which is in agreement with previously published data (Drenovsky *et al.*, 2004; Zak *et al.*, 2003; Alden *et al.*, 2001).

Abundance of acidobacteria was positively correlated with soil DOC and mineral N contents, which is in agreement with previous reports (Tabuchi *et al.*, 2008; Hayatsu *et al.*, 2008; Ruppel *et al.*, 2007). Several members of this phylum have been evidenced to be facultative or obligatory anaerobic organotrophs (Jones *et al.*, 2009; Fierer *et al.*, 2007). Hence, their abundance may be particularly favored by high soil moisture together with effects on community structure, as we found in the present field study and was proven by other reports (for example, Janssen, 2006). Throughout the field experiment, acidobacterial communities were more abundant and underwent significant structural changes in girdled, C-limited plots. This may signify their high metabolic versatility to be well-adapted to resource limitation and their ability to decompose complex C substrates deriving from the rather recalcitrant soil organic matter pool (Ward *et al.*, 2009; Hansel *et al.*, 2008; Eichorst *et al.*, 2007; Fierer *et al.*, 2007).

Girdling prevents the uptake of available nutrients such as ammonia and nitrate by trees (Högberg *et al.*, 2001), and therefore resulted in relatively higher mineral N concentrations in soils of girdled plots. We found significantly higher bacterial *amoA* gene copies in girdled plots than in controls substantiating our assumption that N availability is a crucial controlling factor for ammonia oxidizing bacteria (Fierer *et al.*, 2009). No correlation was seen between ammonia oxidizing bacteria and soil nitrate content. However, net changes in the soil nitrate pool do not reflect nitrifying activity, as apart from microbes plants also utilize nitrate as N source (Adair and Schwartz, 2008). Copies of bacterial *amoA* genes were further positively correlated with DON. DON is the precursor of ammonia (mineralization) and thus is essential for the constant replenishment of the ammonia pool as substrate for nitrification, thus indicating that DON is essential for maintaining ammonia oxidizing bacteria metabolism (You *et al.*, 2009; Brierley *et al.*, 2001). Moist soil conditions pronouncing the diffusion of substrates (for example, nitrate and ammonia) to microbes offered obviously a favourable environment for sustaining and increasing the abundance of ammonia oxidizing bacteria, which is in agreement with previously published information (Fierer *et al.*, 2009; Adair and Schwartz, 2008).

We found that seasonality and varying resource availability changed the abundance of ammonia oxidizing archaea (AOA). Decreasing soil temperature was correlated with increasing AOA abundance, which is in contrast to the results of a soil microcosm study by Tourna *et al.* (2008). However, Urakawa *et al.* (2008) and Caffrey *et al.* (2007) investigated marine ecosystems in which decreased phylogenetic diversity and abundance of AOA were found with increasing temperature, respectively. Based on these contradictory results, we suggest further experiments under field conditions to substantiate that decreasing soil temperature promotes AOA abundance. Our results suggested a potential dependence of AOA on ammonia availability, which was supported by recent studies (He *et al.*, 2007; Santoro *et al.*, 2008). Because of the negative correlation between AOA and ammonia concentrations, we conclude that the ammonia decrease may have been the consequence of pronounced ammonia oxidation activity in the assayed temperate soils, whereas Valentine, (2007) proposed that AOA seem to be better adapted to low ammonia concentrations in soil. However, it remains poorly investigated to which extent soil AOA react to different concentrations of ammonium in soils (Jia and Conrad, 2009; Chen *et al.*, 2008).

N_2O emissions were positively correlated with the abundance of total archaea and AOA indicating that archaea may be directly involved in denitrification processes. Although denitrification is often considered a bacterial process, the measured high abundance of archaea suggested that denitrification was probably also widespread among the archaea studied in this field experiment. But further research is required to substantiate this assumption as only limited information is available for archaeal denitrification in soil ecosystems so far (for example, Bartossek *et al.*, 2010; Hayatsu *et al.*, 2008). However, it has been confirmed that several archaeal members perform both assimilatory and dissimilatory reduction processes to produce for example, N_2O (Hayatsu *et al.*, 2008; Cabello *et al.*, 2004; Zehr and Ward, 2002), and their actual contribution to denitrification was proven by the presence of denitrification genes (for example, *nir* and *nos* genes) in the genomes of several archaeal species (Bartossek *et al.*, 2010; Cabello *et al.*, 2004).

In conclusion, our field study in a temperate ecosystem including tree girdling to induce soil C limitation is the first field survey that has been performed for two consecutive years with a bimonthly sampling scheme. This approach allowed us to get a detailed insight into short- and medium-term effects of seasonality and resource availability on the soil microbial community, which has been explored at domain level as well as at a smaller taxonomic scale using selected bacterial phyla and functional groups. We showed that community structure and abundance of archaea and acidobacteria appeared to be particularly altered by these two factors, reflecting their potentially high metabolic versatility in the assayed soils. Further, our extensive field survey revealed that belowground C allocation along with seasonal climatic influences changed the abundance of nitrifying and denitrifying bacteria and archaea and showed a sound correlation with treatment-related dynamics of physicochemical parameters in the investigated soils. Based on our proposed assumptions, it will be essential to promote future research to further explore and understand the role of various phylogenetic and functional groups in terrestrial environments as well as their individual response to various environmental parameters and particularly their resilience to climate change (Cruz-Martínez *et al.*, 2009; Youssef and Elshahed, 2009).

Conflict of interest

The authors declare no conflict of interest.

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