

Impact of Plant Functional Group, Plant Species, and Sampling Time on the Composition of *nirK*-Type Denitrifier Communities in Soil^{∇†}

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We studied the influence of eight nonleguminous grassland plant species belonging to two functional groups (grasses and forbs) on the composition of soil denitrifier communities in experimental microcosms over two consecutive years. Denitrifier community composition was analyzed by terminal restriction fragment length polymorphism (T-RFLP) of PCR-amplified *nirK* gene fragments coding for the copper-containing nitrite reductase. The impact of experimental factors (plant functional group, plant species, sampling time, and interactions between them) on the structure of soil denitrifier communities (i.e., T-RFLP patterns) was analyzed by canonical correspondence analysis. While the functional group of a plant did not affect *nirK*-type denitrifier communities, plant species identity did influence their composition. This effect changed with sampling time, indicating community changes due to seasonal conditions and a development of the plants in the microcosms. Differences in total soil nitrogen and carbon, soil pH, and root biomass were observed at the end of the experiment. However, statistical analysis revealed that the plants affected the *nirK*-type denitrifier community composition directly, e.g., through root exudates. Assignment of abundant T-RFs to cloned *nirK* sequences from the soil and subsequent phylogenetic analysis indicated a dominance of yet-unknown *nirK* genotypes and of genes related to *nirK* from denitrifiers of the order *Rhizobiales*. In conclusion, individual species of nonleguminous plants directly influenced the composition of denitrifier communities in soil, but environmental conditions had additional significant effects.

A major focus in ecology is to understand whether and how organisms in ecosystems interact. Since microorganisms are crucial mediators of nutrient cycling in the soil and can thereby affect plant growth, e.g., in symbiosis or by competition for nutrients, the multiple interactions between plants and soil microorganisms are of special interest. Many studies have explored the effect of plants on soil microbial communities in the field or in microcosms, often with special emphasis on the plant rhizosphere (12, 16, 25, 35, 51, 54, 56). Microorganisms in root-associated habitats may respond to the amount, composition, and spectra of root exudates, leading to the development of plant-specific microbial communities (30, 36). However, the root exudation of an individual plant may also depend on its growth conditions and developmental stage, thereby potentially masking species-specific effects (15, 50). Apart from the plants, soil type, soil structure, and specific soil characteristics also affect the microbial community (6, 7, 23, 34), and these soil effects have often been found to be more important than a particular plant species and its root exudates (14). In addition, seasonal changes of environmental conditions may also influence the amount, activity, and composition of soil microorganisms (18, 26, 32, 46, 48, 51).

Most studies of plant-microbe relationships have been focused on the overall microbial community composition based on the analysis of 16S rRNA genes. However, there is also evidence that individual functional groups of soil microorganisms such as methanotrophs (27), ammonia-oxidizing bacteria (29), and denitrifying bacteria (10) are affected by the composition of plant communities. For instance, in an agricultural soil planted with maize, the nitrate-reducing microbial community was distinct from that in unplanted soil (40). Denitrifier communities were also distinct in two soils that differed in their vegetation and soil types (forest and an adjunct meadow) in the Pacific Northwest of the United States (44). Furthermore, the genetic structure of the nitrate-reducing microbial community in soils below grass tufts dominated by *Arrhenatherum elatius*, *Dactylis glomerata*, and *Holcus lanatus* was dependent on the plant species (39). Denitrifiers of the *nirK* type were also found in the rhizospheres of three legume crops, and the diversity and composition of *nirK* transcripts were influenced by plant species identity (49). Apart from effects induced by plants, the community composition of denitrifying bacteria was also shown to be influenced by the seasonal variation of environmental conditions (57).

Although denitrification plays an important role in the N cycle of soil, virtually nothing is known about the association of denitrifying microorganisms with individual grassland plant species. The process of denitrification is driven mainly by facultative anaerobic bacteria, which use oxidized nitrogen compounds as alternative electron acceptors for energy production (60). The key enzyme in dissimilatory denitrification is nitrite reductase, since the ability to reduce nitrite to nitric oxide

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distinguishes nitrate respirers from denitrifiers (60). Two structurally different nitrite reductases are found among denitrifiers. The copper-containing nitrite reductase is encoded by *nirK* and the heme *cd*₁-containing enzyme by *nirS*. Both genes have been effectively appointed as functional marker genes to detect communities of denitrifiers in the environment, e.g., in soils (19, 42), activated sludge (53), and marine environments (5, 9).

This study to analyze denitrifier communities in soil associated with nonleguminous plant species was focused on the analysis of *nirK*-type denitrifiers, because in several former studies (42, 49, 57) *nirK* could be more readily amplified from soils than *nirS*. We hypothesized that plant functional group (grasses and forbs) and plant species identity influence the denitrifier community composition in the soil. The effect of different sampling times on the denitrifier communities was evaluated over two consecutive years. In addition, it was tested whether plant species or plant-mediated differences of soil parameters (total nitrogen and carbon contents, pH, and root biomass) affect the *nirK*-type denitrifier community composition.

MATERIALS AND METHODS

Experimental setup and soil sampling. In autumn 2001, monocultures of eight nonleguminous grassland plants representing two functional plant groups were grown in microcosms (diameter, 20 cm; depth, 100 cm) under ambient environmental conditions in the Ecological Botanical Garden of the University of Bayreuth. Functional plant groups have been defined in the past, in many cases according to morphological traits (31, 41, 55). In the present study two functional plant groups, grasses and forbs, were assigned due to their different root systems. The plant species were the four grasses *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Arrhenatherum elatius*, and *Holcus lanatus* and the four forbs *Geranium pratense*, *Plantago lanceolata*, *Ranunculus acris*, and *Taraxacum officinale*. The plants were cultivated in soil that had been taken from a meadow next to the Ecological Botanical Garden. The original soil type was a stagnic gleysol developed on sandstone. The soil had the following characteristics: pH (CaCl₂), 4.9; total carbon, 0.645%; total nitrogen, 0.07%; NH₄⁺-N (1 M KCl), 19.6 mg kg⁻¹; NO₃⁻-N (1 M KCl), 27.0 mg kg⁻¹. It consisted of 7% sand, 78% silt, and 16% clay (28). As the initial soil characteristics and the weather conditions were identical for all microcosms, these factors were not responsible for differences in denitrifier community composition. The soil was thoroughly mixed and steamed (12 h at 100°C) to kill weed seeds. The plants were grown for 3 years and watered when necessary. Three replicate microcosms were set up for each plant species. The experimental setup is described in detail in the study by Reuter (43). In summer (June) and autumn (September) 2003 and in summer (June) 2004, two soil samples (about 10 g soil) were taken from each microcosm to a depth of 6 cm, pooled, mixed, and immediately stored at -20°C. All 72 soil samples contained plant roots, since the high root density of the grassland plants prevented the separation of rhizosphere and bulk soil. At the end of the experiment, total carbon (C) and nitrogen (N) contents, pH, and root biomass were determined for the upper soil layers (0 to 10 cm) of the microcosms as described previously (43).

DNA extraction. Soil (0.5 g), 0.5 g glass beads (diameter, 0.17 to 0.18 mm; B. Braun Biotech International GmbH, Melsungen, Germany), 800 μl sterile sodium phosphate buffer, and 260 μl sterile sodium dodecyl sulfate buffer were mixed. Cell lysis was done with a bead beater (mini bead beater; BIO 101, Savant, NY) for 45 s with 6.5 ms⁻¹. Samples were centrifuged for 15 min at 20,817 × g at room temperature. Up to 800 μl of the supernatant was transferred to a new tube. After addition of 400 μl sodium phosphate buffer to the soil pellet, cell lysis was repeated. The supernatants were merged and extracted twice with 600 μl phenol-chloroform-isoamyl alcohol (25:24:1) and 600 μl chloroform-isoamyl alcohol (24:1) in phase-lock tubes (Phase Lock Gel Heavy, 2 ml; Eppendorf, Hamburg, Germany) according to the manufacturer's instructions. DNA was precipitated with 0.7 volume of 100% isopropanol at room temperature. After centrifugation (60 min, 20,817 × g, room temperature), the DNA pellet was washed with ice-cold 70% (vol/vol) ethanol and centrifuged again (10 min, 20,817 × g, 4°C). The supernatant was decanted, and the pellet was air dried. Finally, the pellet was resuspended in EB buffer (QIAGEN GmbH, Hilden, Germany). DNA extracts were cleaned up with the Wizard DNA clean-up system (Promega, Mannheim, Germany) according to the manufacturer's recommen-

dations. Subsequently, the purity and the quantity of the DNA were determined by UV spectrophotometry at 260 and 280 nm (Biophotometer; Eppendorf, Hamburg, Germany). The DNA was stored at -20°C.

PCR amplification of *nirK*. The primer pair *nirK1F* and *nirK5R*, used for PCR amplification, was described previously by Braker et al. (4). An amplification reaction mixture contained 25 pmol of each primer (MWG Biotech AG, Ebersberg, Germany), 200 μM of each deoxynucleoside triphosphate (Roche Molecular Diagnostics GmbH, Mannheim, Germany), 400 ng bovine serum albumin (Roche Molecular Diagnostics) μl⁻¹, and 1.25 U of REDAccuTaq LA DNA polymerase (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in 1× reaction buffer provided by the manufacturer. Template DNA (1 μl) and sterile water was added to a final volume of 25 μl reaction solution. For each sample four PCRs were done, and the mixtures were pooled afterwards to minimize PCR artifacts. All PCRs were done in the cyclor Primus 96^{plus} (MWG Biotech). The following touchdown thermal profile was used for amplification: an initial denaturation step of 5 min at 94°C, followed by 10 cycles of 94°C for 30 s, 40 s of primer annealing (in which the temperature started at 57°C and decreased by 0.5°C every cycle), and 40 s at 72°C. Additionally, 27 cycles were performed with an annealing temperature of 55°C. A last step was done at 72°C for 7 min. The quality and the quantity of PCR products were determined by electrophoresis of an aliquot of each PCR on a 1.5% (wt/vol) agarose gel (Biozym Scientific GmbH, Oldendorf, Germany) and by visualization with UV excitation after staining the gel with ethidium bromide (0.5 mg liter⁻¹).

T-RFLP analysis of *nirK*-type denitrifiers. For terminal restriction fragment length polymorphism (T-RFLP) analysis, the reverse PCR primer *nirK5R* was 5' end labeled with 6-carboxyfluorescein. Four PCR products were pooled, and the PCR products of the correct size were excised from the agarose gel and purified with the QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany). The purified PCR products (100 ng) were hydrolyzed with 5 U of the restriction endonuclease HaeIII (New England Biolabs, Frankfurt, Germany) overnight at 37°C. Digests were purified with Autoseq G-50 columns (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. The purified product (2 μl) was mixed with 12 μl deionized HiDi-formamide (Applera, Darmstadt, Germany) and 0.25 μl of an internal DNA length standard (Internal Lane Standard 600; Promega, Mannheim, Germany). RFs were separated on an ABI 310 automated sequencer (Applied Biosystems, Darmstadt, Germany). Afterwards, the lengths of the fluorescently labeled T-RFs were defined by comparison to the internal length standard with GeneScan 3.71 software (Applied Biosystems). Since T-RFs can vary slightly in size, T-RFLP patterns were inspected visually and peak size differences of 1 or 2 base pairs were confirmed by comparing the respective peaks of all patterns. Peaks with a fluorescence of 50 U over the background fluorescence and larger than 60 bp were analyzed by peak height. The relative abundances of T-RFs in a sample, given in percent, were calculated after normalization of peak heights in an iterative standardization procedure as described by Dunbar et al. (13).

Cloning and sequencing of *nirK* genes. Amplified *nirK* genes from soil of the three replicate microcosms of *Holcus lanatus* in summer 2003 and of *Plantago lanceolata* in autumn 2003 were pooled and cloned using the pGEM-T cloning kit (Promega) according to the manufacturer's instructions. *Plantago lanceolata* microcosms were selected for cloning because their T-RFLP patterns included most of the T-RFs also present in soil of the other microcosms. *Holcus lanatus* microcosms were selected for cloning, because they contained a 131-bp fragment of high relative abundance that was specific for some microcosms. White colonies picked at random were screened for inserts of the correct size by PCR amplification of the inserts using vector-specific primers (T7 promoter and M13 reverse primer) as described elsewhere (1). Inserts of the first 36 clones for two pooled *nirK* amplicons were used to generate two random clone libraries. In addition, 200 clones from the two amplicons were screened by T-RFLP to select clones with less-abundant and different terminal restriction sites. Clones corresponding to T-RFs from environmental DNA were chosen for sequencing. PCR products were purified with the QIAquick PCR purification kit (QIAGEN). Inserts were sequenced directly from 70 ng of PCR product with the ABI BigDye Terminator kit (Applied Biosystems) according to the manufacturer's instructions. Afterwards, cycle sequencing reaction products were purified with Autoseq G-50 columns (Amersham Biosciences) and analyzed on an ABI 377 DNA sequencer (Applied Biosystems).

Phylogenetic analysis. Phylogenetic analyses were done with ARB (<http://www.arb-home.de>). Sequences of the *nirK* gene were aligned to sequences from the EMBL database with the ARB Fast Aligner tool. A filter was used for the calculation of phylogenetic trees, including 432 nucleotide positions that excluded insertions and deletions. Trees were constructed with the maximum-likelihood method and with the parsimony and neighbor-joining method to support the tree topology observed with the maximum-likelihood algorithm. A

chimera check was done by calculating two trees based on each half of the sequences. Two possible chimeras were detected and excluded from further analysis. Sequences were analyzed in silico for T-RFs obtained by cleavage with the restriction endonuclease HaeIII, using TRF-CUT (45) for the assignment of theoretical T-RFs to those found by in vitro analysis.

Statistics. Statistics were performed with PC-Ord 4.0 for Windows, CANOCO 4.5, and SPSS 12.0 for Windows.

T-RFs of different length were considered to be indicative of different *nirK* operational taxonomic units (OTUs) present in a sample, and the relative peak heights were used as a measure for the relative abundance of *nirK* OTUs. Effects of the experimental factors plant functional group, plant species, and time and their interactions on T-RFLP profiles were explored by ordination techniques. After an initial detrended correspondence analysis (CA) had indicated that a unimodal response model was more appropriate than a linear model, the data were analyzed by CA and canonical CA (CCA). CA is a method to reduce the dimensionality of a multivariate data set and ordinate samples in an ordination space that corresponds best to the dissimilarities in species composition; i.e., samples with a similar community composition are placed closer together, and samples with a more dissimilar community composition are positioned further apart. In our case, the relative abundance of the T-RFs obtained from each replicate microcosm was used as species abundance. CCA is a method of direct gradient analysis that relates variation in community composition to environmental variation. Because of the nested design of the statistical analyses, covariables and dummy variables were included in the analyses. Covariables are concomitant variables whose effect is eliminated when analyzing the effects of the variables of interest. Dummy variables are nominal variables defined as 1 or 0, which code for the levels of a factor.

First, single CAs were performed with the T-RFLP data sets for the three sampling times to view the structure of the data and the quality of replication. Second, several CCAs were performed to analyze the effects of the factors of interest. These analyses reflected the nested design of the experiment (59) and had different numbers of replicates. The effect of plant group (grasses versus forbs) was tested using a data set consisting of average OTU abundances for the individual plant species. The effect of plant species on *nirK* OTUs was tested using a data set consisting of average OTU abundances for the individual microcosms and with the effect of plant group eliminated by using plant group as a dummy-coded covariable. The effect of time on *nirK* OTU abundance was tested using the data set containing the measurements taken for the individual microcosms at the three sampling times and with the variation due to microcosms eliminated. The effect of interactions on *nirK* OTU abundance was tested using the data set containing the measurements taken for the individual microcosms at the three sampling times and with the variation due to microcosms, time, and plant group (for the interaction of plant group with time) or plant species (for the interaction of plant species with time) eliminated. Monte Carlo permutation tests (based on 5,000 random unrestricted permutations) as available in CANOCO 4.5 (52) were used to test the hypothesis that relative abundances of *nirK* OTUs were related to the factors plant functional group, plant species, and sampling time and the interactions between these factors.

The effect of the experimental factor plant functional group on the soil characteristics was explored by analysis of variance according to the nested experimental design. Thus, the effect of plant functional group was tested against the variation among plant species and that of plant species identity against the variation among the microcosms. Values for total nitrogen content were log transformed prior to the analysis to normalize residuals.

Nucleotide sequence accession numbers. The partial *nirK* gene sequences that were generated in this study have been deposited in the EMBL nucleotide sequence database (accession numbers AM235217 to AM235292).

RESULTS

Analysis of *nirK*-type denitrifier communities. Partial *nirK* genes were successfully amplified from the soil from all plant microcosms, and the *nirK*-type denitrifier community was subsequently resolved by T-RFLP. The majority of the T-RF profiles of soil from triplicate microcosms planted with the same species were similar, indicating that the results were reproducible and representative for the denitrifier community in the soil of these microcosms (see Fig. S1 in the supplemental material for T-RFLP profiles of the replicates at the three sampling times and Fig. S2 in the supplemental material for the corre-

sponding ordination diagrams). However, T-RF profiles of *Holcus lanatus* in summer and autumn 2003 and of *Plantago lanceolata* in summer 2004 showed a relatively higher variability among replicates (see Fig. S1 in the supplemental material for T-RFLP profiles of the replicates at the three sampling times and Fig. S2 in the supplemental material for the corresponding ordination diagrams). Since T-RF profiles were highly reproducible when DNA of one of the replicates was extracted several times (the coefficient of variation of the relative abundance of single T-RFs was less than 1% [data not shown]), it was concluded that differences between patterns from three replicates represent true variation of denitrifier community composition in the microcosms. When the T-RFLP data set of one sampling time was averaged, the histograms showed marked differences among the structures of the denitrifier communities in the soil of microcosms planted with different plants (as an example, the results for summer 2003 are shown in Fig. 1). Some fragments of comparably high relative abundance (e.g., T-RFs of 151 and 185 bp) were detected in the soil of all microcosms but varied in their relative abundances depending on the plant species. In contrast, less-abundant fragments occurred both in relation to several of the plant species (e.g., the 131-bp T-RF was highly abundant with *Holcus lanatus* and less abundant with *Geranium pratense* and *Plantago lanceolata*) and in relation to a single plant species (e.g., the 125-bp T-RF with *Alopecurus pratensis*).

The T-RFLP data set was further analyzed by CCA to analyze the influence of the factors of interest on the composition of the bacterial communities. The functional group of a plant (grasses versus forbs) did not significantly affect the *nirK*-type denitrifier community in the soil ($P = 0.60$ by Monte Carlo permutation test within CCA) (data not shown), but plant species identity significantly influenced the relative abundance of *nirK* T-RFs ($P = 0.0002$ by Monte Carlo permutation test) (Fig. 2). In the ordination diagram, plant species with similar soil denitrifier communities are positioned closely together. For instance, the *nirK*-type denitrifier communities in soil from the plants *Anthoxanthum odoratum* and *Plantago lanceolata* were similar, whereas that from *Holcus lanatus* was very different from those of all other plant species. T-RFs scattering around plant species indicate *nirK* OTUs that typically occurred with a given plant species. Therefore, T-RFs placed in the center of the diagram either represent *nirK* OTUs occurring in the soil from all plant species or represent those that occur only in the soil from *Ranunculus acris*.

There was also a significant general effect of sampling time on the composition of the *nirK*-type soil denitrifier community ($P = 0.0002$ by Monte Carlo permutation test) (Fig. 3). Interestingly, the communities of *nirK*-type denitrifiers in the soil sampled in summers 2003 and 2004 were as different from each other as they were from those in the samples taken in autumn 2003, indicating that effects of microcosm development were at least as important as seasonal effects. Many *nirK* OTUs were placed in intermediate positions, demonstrating their association with two sampling times. For instance, the 248-bp fragment was found in both autumn 2003 and summer 2004, but in autumn 2003 it was detected in the soil from *Ranunculus acris* whereas in summer 2004 it occurred in the soil from *Plantago lanceolata*. Some *nirK* OTUs which were unrelated to sampling time matched with *nirK* OTUs unrelated to plant species (e.g.,

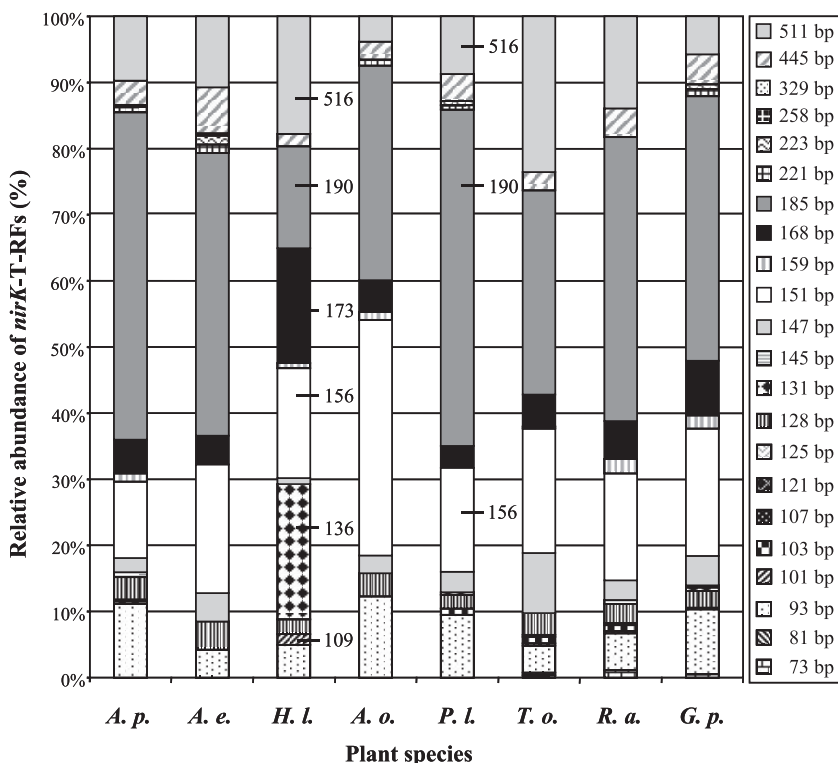


FIG. 1. Average relative abundances of *nirK* T-RFs from soil of replicate plant microcosms in summer 2003 (for data on individual microcosms, see Fig. S1 and S2 in the supplemental material). Peak size is given in base pairs, and the relative abundance of T-RFs is given as percentage of total peak height. Fragment sizes within the graph indicate the sizes (bp) of theoretical T-RFs of clones after in silico analysis. *A. p.*, *Alopecurus pratensis*; *A. e.*, *Arrhenatherum elatius*; *H. l.*, *Holcus lanatus*; *A. o.*, *Anthoxanthum odoratum*; *P. l.*, *Plantago lanceolata*; *T. o.*, *Taraxacum officinale*; *R. a.*, *Ranunculus acris*; *G. p.*, *Geranium pratense*.

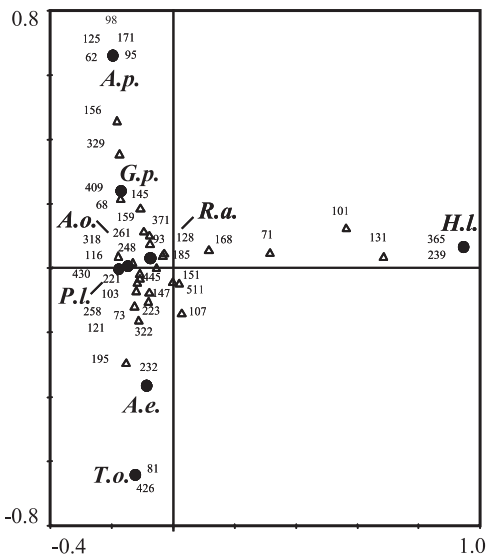


FIG. 2. CCA ordination plot for the effect of plant species identity on the composition of the *nirK*-type denitrifier community in soil, based on the relative abundances of *nirK* T-RFs from the soil of three replicate plant microcosms. Circles indicate plant species and triangles the T-RFs, which are labeled according to fragment size (bp). The eigenvalues of the first and second axes in the ordination diagram are as follows: $\lambda_1 = 0.094$; $\lambda_2 = 0.029$. *A. p.*, *Alopecurus pratensis*; *A. o.*, *Anthoxanthum odoratum*; *A. e.*, *Arrhenatherum elatius*; *H. l.*, *Holcus lanatus*; *G. p.*, *Geranium pratense*; *P. l.*, *Plantago lanceolata*; *R. a.*, *Ranunculus acris*; *T. o.*, *Taraxacum officinale*.

T-RFs of 128, 151, and 185 bp), indicating the general occurrence of the respective denitrifiers in the given soil substrate. When sampling time was defined as a linear contrast to analyze whether there was a continuous development of the community over time, it also affected the composition of the *nirK*-type denitrifier soil community (significance of linear trend component, $P = 0.0002$ by Monte Carlo permutation test within CCA [ordination diagram not shown because of high complexity]), pointing at a trend of microcosm development. However, the interactions of time as a linear term with plant functional group and with plant identity were not significant ($P = 0.96$ and $P = 0.08$ by Monte Carlo permutation test).

In addition to the effect of plant species identity and time, the interaction between the factors plant species and time also influenced soil denitrifier composition significantly ($P = 0.003$ by Monte Carlo permutation test within CCA), indicating that the effect of plant species identity varied with sampling time (see Fig. S3 in the supplemental material for the ordination diagram).

Soil characteristics. At the end of the experiment in summer 2004, total carbon and nitrogen contents, pH, and root biomass per plant were determined for the individual microcosms. Soil total carbon and nitrogen from some of the microcosm replicates deviated unexpectedly from those of the original soil substrate. However, soil planted with *Geranium pratense* had the highest total nitrogen content, while soil planted with *Holcus lanatus* had the highest total carbon content, the highest

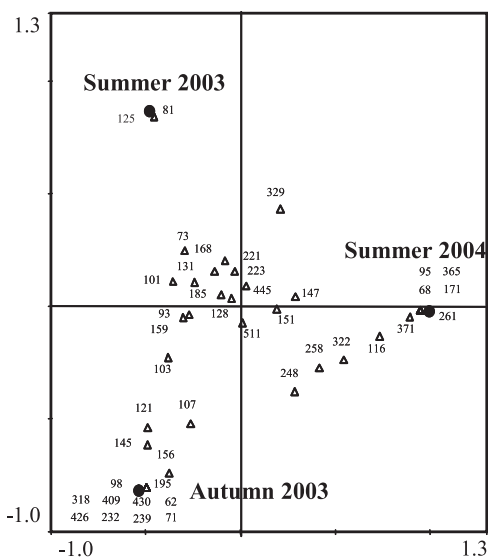


FIG. 3. CCA ordination plot for the effect of sampling time (circles) on the composition of the *nirK*-type denitrifier community in soil. T-RFs (triangles, labeled according to fragment size [bp]) are based on *nirK* T-RFLP data from microcosms. The eigenvalues of the first and second axes in the ordination diagram are as follows: $\lambda_1 = 0.076$; $\lambda_2 = 0.023$.

pH, and the lowest root biomass (Table 1). *Alopecurus pratensis* produced the highest root biomass per plant.

A nested analysis of variance showed that plant functional group affected soil pH ($P < 0.05$). It was significantly higher in soil planted with grasses than in soil planted with forbs. To analyze whether the effects of plant species on denitrifier community composition were mediated by their effects on soil characteristics, the effects of carbon and nitrogen contents (log), pH, and root biomass were eliminated in separate analyses by using them as covariables in CCAs of the effect of plant species identity. This tests whether plant species identity directly affects the denitrifier community or whether the effect of plant species identity on the denitrifier community is mediated by the soil characteristics. Despite the high variability in some of the soil characteristics, the effect of plant species identity on the denitrifier community composition remained, with respect to both the end of the experiment (autumn 2006) and the three sampling times. Thus, this result provides rather conservative

evidence that the plants directly influenced the *nirK*-type denitrifiers, e.g., through root exudates.

Phylogeny of *nirK*. Two representative pooled *nirK* PCR products from the soil from *Holcus lanatus* (summer 2003) and *Plantago lanceolata* (autumn 2003) were cloned, and partial *nirK* gene fragments (516 bp) of clones were sequenced. All cloned and sequenced inserts were *nirK* like. A dendrogram was calculated using our clone sequences as well as additional *nirK* sequences from the EMBL database, including taxonomically characterized isolates and *nirK* sequences of unknown phylogenetic affiliation retrieved from soil and other habitats. The dendrogram for *nirK* from uncultured and cultured denitrifiers showed five major clusters of *nirK* sequences (Fig. 4, clusters I to V) and several subclusters in cluster I and II. The overall topology was supported by neighbor-joining and parsimony analyses, and the clusters and subclusters were defined if sequences were consistently grouping together. To avoid crowding of the tree, we then manually removed the sequences from organisms of unknown phylogenetic affiliation except those obtained in this study. Our *nirK* sequences were distributed within four of the five branches of the *nirK* tree; no sequences grouped in cluster V. The majority of the clones (86 of 109) from soil of both monocultures grouped in cluster I, consisting of hundreds of *nirK* sequences derived from uncultured organisms from soil and activated sludge (data not shown). The *nirK* gene from *Nitrosomonas* sp. strain TA-921 i-NH₄, also grouping in this cluster, is the most closely related sequence from a cultured representative. However, our *nirK* sequences were not closely related to this sequence. The second most abundant group of clones (17) from soil from *Holcus lanatus* clustered within cluster II. Some of these sequences grouped in the vicinity of, but not close to, *nirK* from *Rhizobium sulae* (cluster IIa), one clone was related to *nirK* from *Pseudomonas* sp. (reclassified as a member of the *Rhizobiales* within the *Alphaproteobacteria*) (cluster IIb), and the remainder of the sequences in this cluster formed two distinct subclusters (clusters IIc and e). Two sequences from soil from *Plantago lanceolata* were grouped in cluster III and were related to *nirK* from *Blastobacter denitrificans* and *Bradyrhizobium japonicum*. Four sequences also originating from soil planted with *Plantago lanceolata* were affiliated with *nirK* from *Mesorhizobium* sp.

Assignment of *nirK* sequences to experimentally derived T-RFs. Sequences of *nirK* were cleaved in silico with the restric-

TABLE 1. Characteristics of the soil and root biomass per plant in the microcosms at the end of the experiment

Plant species	Functional group	Mean \pm SEM ^a			
		N (%)	C (%)	pH	Root biomass per plant (g)
<i>Alopecurus pratensis</i>	Grass	0.165 \pm 0.121 a	0.907 \pm 0.030 ab	5.0 \pm 0.04 bc	13.67 \pm 3.67 b
<i>Anthoxanthum odoratum</i>	Grass	0.067 \pm 0.026 a	0.712 \pm 0.058 a	5.0 \pm 0.02 bc	4.35 \pm 1.11 a
<i>Arrhenatherum elatius</i>	Grass	0.057 \pm 0.010 a	1.096 \pm 0.116 ab	5.2 \pm 0.03 c	4.51 \pm 0.79 a
<i>Holcus lanatus</i>	Grass	0.086 \pm 0.008 a	1.406 \pm 0.155 b	5.5 \pm 0.06 d	1.67 \pm 0.36 a
<i>Geranium pratense</i>	Forb	1.003 \pm 0.196 b	0.775 \pm 0.066 a	4.5 \pm 0.02 a	2.91 \pm 0.43 a
<i>Plantago lanceolata</i>	Forb	0.243 \pm 0.044 a	0.957 \pm 0.078 ab	5.0 \pm 0.09 bc	2.86 \pm 1.08 a
<i>Ranunculus acris</i>	Forb	0.044 \pm 0.007 a	0.841 \pm 0.077 a	4.8 \pm 0.14 ab	5.86 \pm 1.52 a
<i>Taraxacum officinale</i>	Forb	0.065 \pm 0.019 a	0.716 \pm 0.098 a	4.8 \pm 0.05 bc	1.98 \pm 0.10 a

^a Results are for three replicates. Different letters indicate significant differences between species ($P < 0.05$ by one-way analysis of variance with Tukey's honestly significant differences test).

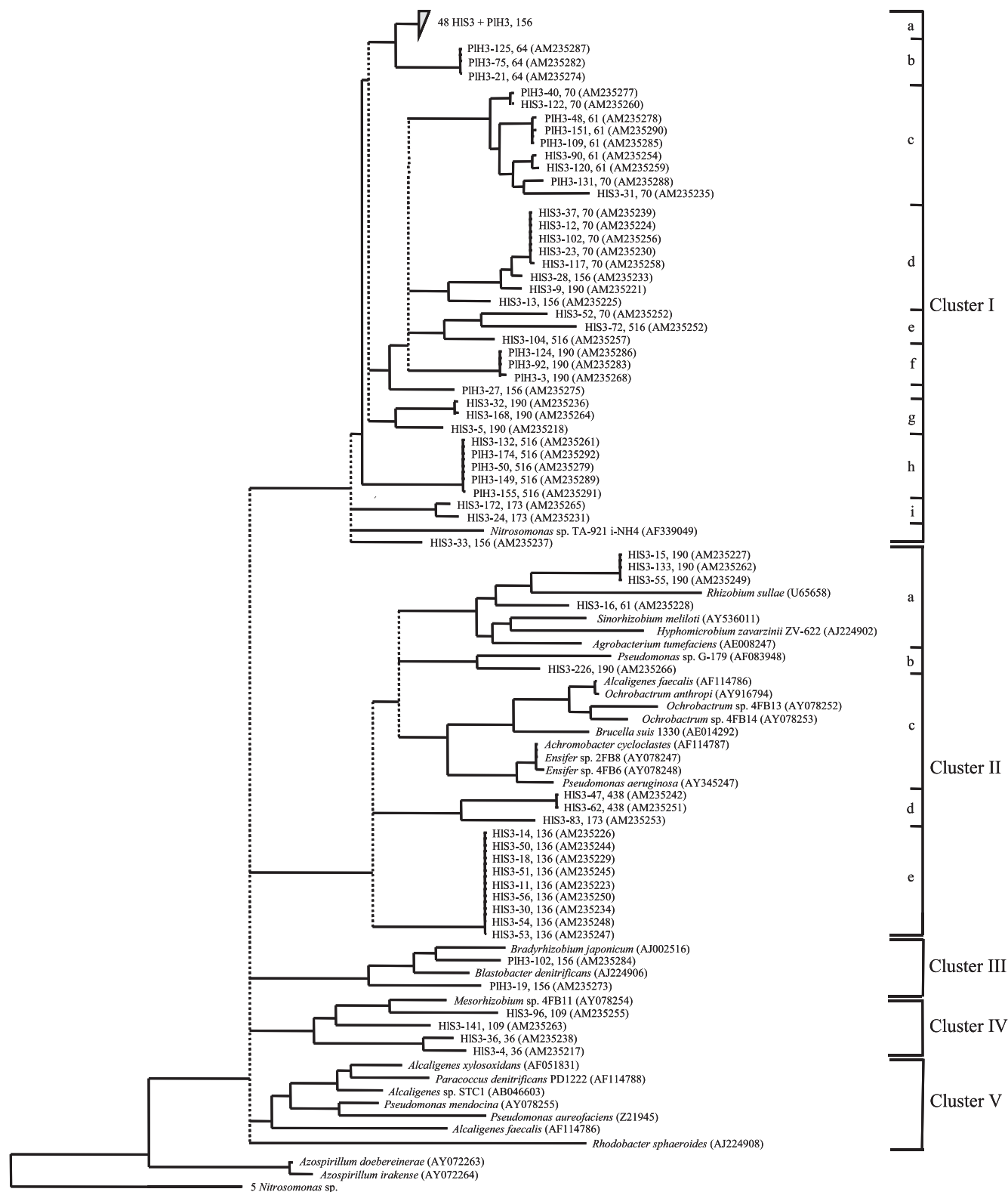


FIG. 4. Maximum-likelihood tree based on partial *nirK* sequences (432 nucleotide positions) from cultured *nirK*-type denitrifiers and sequences from microcosm soil. The tree topology is supported by parsimony and neighbor-joining methods. Dashed lines indicate multifurcations where the tree topology was not consistently resolved. HI, clones obtained from soil of *Holcus lanatus*; PI, clones obtained from soil of *Plantago lanceolata*. S3, summer 2003; H3, autumn 2003. Numbers after the name of the clone indicate the respective sizes of T-RFs after in silico analysis with the restriction enzyme HaeIII. Accession numbers are in parentheses.

tion enzyme HaeIII using the program TRF-CUT and assigned to experimentally derived T-RFs (Fig. 1). T-RFs from in silico analysis differed by 5 to 8 bp from the respective experimental T-RFs. Theoretical T-RFs could be successfully assigned to their respective experimental T-RFs, since the results were confirmed experimentally by T-RFLP-analysis of clones. The theoretical T-RFs of 109, 136, 156, 173, 190, and 516 bp corresponded to the experimental T-RFs of 101, 131, 151, 168, 185, and 511 bp, respectively (Fig. 1). Two sequences with the theoretical T-RF of 438 bp (cluster IId) (Fig. 4) corresponded to an experimental T-RF of 433 bp that was not included in the analysis due to its minor abundance (<1%). In silico analysis also revealed theoretical T-RFs of 36 bp, 61 bp, 64 bp, and 70 bp. However, the corresponding experimental T-RFs were <60 bp in size and could not be determined accurately, since fragments of <60 bp were lacking from the internal standard.

Calculation of theoretical T-RFs demonstrated that 6 of 16 different *nirK* OTUs could be assigned to *nirK* sequences in the clone libraries (Fig. 1 and 4). Sequences with T-RFs of 61, 64, 70, 156 (experimental, 151), 173 (168), 190 (185), and 516 (511) bp clustered in cluster I (Fig. 4). In cluster I, subclusters Ia, Ib, If, Ig, Ih, and Ii consisted of sequences with specific T-RFs. For instance, 48 *nirK* sequences with the 156-bp T-RF formed the distinct subcluster Ia. Sequences with theoretical T-RFs of 64, 70, and 516 bp, the last referring to an abundant T-RF in the soil from all plant microcosms, were grouped either in single subclusters (e.g., Ib or Ih) or in a single cluster (I). However, sequences with different T-RFs clustered in subclusters Ic, Id, and Ie. Sequences with theoretical T-RFs of 156, 173, and 190 bp, which corresponded to the dominant T-RFs in the soil from all plant microcosms (Fig. 1), were found in clusters I, II, and III. One sequence with a T-RF of 61 bp and three sequences with a T-RF of 190 bp were positioned in cluster II, but sequences with these T-RFs were more abundant in cluster I. In contrast to these abundant T-RFs, less-abundant T-RFs referred to sequences in specific subclusters. Subcluster IIe consisted of sequences with the theoretical 136-bp T-RF (experimental 131-bp T-RF) originating from soil from *Holcus lanatus*, where it was most frequently detected. (Fig. 1). This subcluster did not contain *nirK* sequences from cultivated relatives.

Sequences of *nirK* with the unique theoretical T-RF of 109 bp, which were found most frequently in T-RFLP profiles from soil from *Holcus lanatus* but also in soil of *Alopecurus pratensis*, *Taraxacum officinale*, and *Ranunculus acris* (Fig. 1, 2, and 4), were affiliated with *nirK* of *Mesorhizobium* sp. in cluster IV.

DISCUSSION

The aim of this study was to test the hypothesis that non-leguminous grassland plant species, representing two functional groups (grasses versus forbs) with different root systems, exert a species-specific impact on a functional group of soil microorganisms, the *nirK*-type denitrifiers, that is at least as strong as that of the soil. This work was confined to the *nirK* genes as the functional marker for denitrifiers, but focusing on *nirS* in a complementary study would lead to a more complete understanding of the interaction between plants and the functional group of denitrifiers. DNA extraction, PCR amplification, and T-RFLP as a community fingerprinting approach can

suffer from bias (33), but these effects could be minimized, since a standardized DNA extraction procedure for pooled mixed samples from replicate microcosms was used, PCR products were pooled, and T-RF peak heights were standardized. Finally, data were evaluated by CAs which have proven to be effective tools for evaluating data from fingerprinting analyses such as denaturing gradient gel electrophoresis (47, 58) and T-RFLP (2, 9, 26, 37).

The successful amplification of *nirK* genes revealed the presence of *nirK*-type denitrifiers in the soil of all plant microcosms. Given that precondition, we hypothesized at first that the plant functional group (grasses versus forbs) exerts a highly selective effect on *nirK*-type denitrifiers, but the plant functional group did not significantly affect the community composition of *nirK*-type denitrifiers. Due to the small number of replicates (plant species), the statistical power to test this hypothesis was low. There were also no differences in root biomass between soil planted with grasses and soil planted with forbs at the end of the experiment (Table 1). However, the pH was higher in soil planted with grasses than in soil planted with forbs. According to Hinsinger et al. (22), there are various origins of root-mediated pH changes in the rhizosphere, namely, cation-anion exchange balance, organic anion release, root exudation and respiration, and redox-coupled processes. Plant species also may have the ability to react to bulk soil pH values by either reducing or increasing their rhizosphere pH. Grasses have a more intensive root system than forbs, and they are monocots (in contrast to the forbs included in this study) (50), but the differences in taxonomic group, root systems, and pH did not lead to the development of strongly different denitrifier communities in the soil.

In contrast to the functional group of a plant, plant species identity influenced the community composition of *nirK*-type denitrifiers, supporting our hypothesis. This finding agrees with the results of another study, which described significant shifts among microbial communities that occurred in the soil of monocultures planted with three fast-growing tropical hardwood species (8). The authors furthermore reported concomitant differences in soil characteristics (moisture, extractable NO_3^- , percent C, C/N ratio, and pH). Here, differences in denitrifier community composition also coincided with contrasting soil characteristics, e.g., pH, and root biomass, in the microcosms of *Alopecurus pratensis* and *Holcus lanatus*. Other microcosms (*Anthoxanthum odoratum* and *Plantago lanceolata*) with similar soil characteristics harbored similar denitrifier communities, but similar denitrifier communities also thrived in the soil of *Geranium pratense* despite different soil characteristics. Nevertheless, the statistical analysis showed that differences in denitrifier community composition were not induced by differences in soil characteristics and root biomass that were mediated by the plants but that plant species identity affected the *nirK* type directly, e.g., through root exudation. Differences in microbial community composition in association with nonsymbiotic plants may be attributed to differences in the amount and composition of root exudates, because specific exudates may selectively favor some microbial strains over others, thereby altering the microbial community structure (38). Exudation is a dynamic process which varies depending on plant species, physiological status of the plant (50), root zone (24), and nutritional conditions (16). Microorganisms in the vicinity of

plant roots are able to benefit from various carbon sources supplied by the plant and can in turn influence plant nutrition (e.g., via mineralization).

The community composition of *nirK*-type denitrifiers varied among sampling dates. This is in line with the results of other studies that have reported seasonal effects on soil microbial communities (3, 17, 18, 26, 32, 48) and in particular with those of Wolsing and Priemé (57), who reported a significant seasonal shift in the community structure of *nirK*-type denitrifiers in spring, summer, and autumn of a single year. Seasonal changes in weather conditions influence physical soil characteristics such as moisture and temperature and may thus be expected to influence microbial communities. However, community composition not only varied seasonally but also changed directionally with time, suggesting a succession, as indicated by the significant linear trend component of time and the differences in *nirK*-type denitrifier community composition between the summers of 2003 and 2004. This could be due to the development of the plant microcosms with age, due to either aging of the plant itself or a progressing root development in the microcosms. However, data from three sampling times are not sufficient to indicate a consistent trend with time in microcosm development, in particular because the differences among sampling dates varied among individual plant species.

By plotting species and environmental variables, CCAs allow the identification of T-RFs that respond to certain experimental factors (plant functional group, plant species identity, or sampling time). Here, *nirK* OTUs probably represent OTUs specific for the plant rhizosphere, since the high root density of the plants in the microcosms prevented a separate sampling of rhizosphere and bulk soil. The most abundant *nirK* OTUs that were ubiquitous in the soil of all plant species at any sampling time may originate from denitrifiers that are well adapted to the characteristics of the microcosm soil substrate and to the climatic conditions. In contrast, the occurrence of less-abundant *nirK* OTUs such as the 136-bp T-RF (experimental 131-bp T-RF), detected mainly in association with *Holcus lanatus*, may be linked to special environmental factors such as plant identity, sampling time, or the development of individual plant microcosms.

To determine *nirK* sequences corresponding to the T-RFs and to assign theoretical to experimental T-RFs, PCR-amplified *nirK* fragments were cloned, screened by T-RFLP analysis, and sequenced. Clones could be assigned to some but not all T-RFs within the patterns, and most sequences were not closely related to *nirK* from any cultivated organism, indicating denitrifiers with yet-unknown *nirK* genotype. To infer a phylogenetic relationship of denitrifiers without cultivation studies based only on the phylogeny of the respective *nirK* genes is impossible, since a distribution of denitrification genes via horizontal gene transfer is likely to have occurred (11, 20, 21). Several *nirK* sequences were affiliated with *nirK* genes of denitrifiers from the order *Rhizobiales*, namely, *Rhizobium*, *Bradyrhizobium*, *Blastobacter*, and *Mesorhizobium*. This result agrees with other studies that found numerous functional marker genes for denitrification in soils grouping with denitrification genes of these representatives of the *Rhizobiales* (40, 42, 44). *Rhizobiales* usually live in symbiosis with legumes but can also be found free living in soil when leguminous plants are absent.

In conclusion, nonleguminous plant species of temperate grassland exert a species-specific effect on a functional group of soil microorganisms, the *nirK*-type denitrifiers. This effect may be the result of complex interactions between plants, microorganisms, and soil characteristics. Different *nirK* communities at different sampling times and an interaction between the effects of time and plant species identity suggest changes due to seasonal conditions or due to the development of the plant microcosms over time, which differed among plant species. Further research on the effect of root exudates of individual plant species could provide instructive insights into the black box of nonsymbiotic plant-microbe relationships.

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