

**Impact of plant identity, diversity and
composition on diversity, composition and
function of *nirK*-type denitrifying
microorganisms in temperate grassland soil**

Dissertation

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PLEDGE

I certify that the present thesis entitled

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was accomplished without any unlawful device. I did not use any other than the described literature sources or technical devices.

This work has never been submitted before in this or a similar form to any other university and has not been used before any examination.

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The following publication is in preparation for re-submission by the date of the present thesis:

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TABLE OF CONTENTS

Abbreviations	IV
Summary/Zusammenfassung	V
1. Introduction	1
1.1 Influence of plants on soil microorganisms	1
1.2 Denitrification in soil	2
1.3 Role of the greenhouse gas nitrous oxide (N ₂ O)	3
1.4 Diversity and function of ecosystems	4
2. Objectives	5
3. Material and methods	6
3.1 Chemicals, gases, and solutions	6
3.2 Soil sampling and site characteristics	7
3.3 Determination of soil properties and nitrate concentrations	10
3.4 Measuring nitrous oxide (N ₂ O)	11
3.5 Incubation experiments with soil slurries	13
3.5.1 Soil slurries with and without the addition of acetylene	13
3.5.2 Denitrifier enzyme activity method (DEA)	14
3.6 Most probable number (MPN) of nitrate reducing microorganisms	14
3.7 Qualitative molecular analyses of the denitrifier community	15
3.7.1 DNA extraction from soil and amplification by polymerase chain reaction (PCR)	15
3.7.2 Agarose gelelectrophoresis	17
3.7.3 Terminal restriction fragment length polymorphism (T-RFLP)	17
3.7.4 Diversity indices	18
3.7.5 Phylogenetic analyses	18
3.8 Quantitative molecular analysis of the denitrifier community: MPN-PCR	20
3.9 Statistics	20

4. Results	23
4.1 Plant microcosms	23
4.1.1 Influence of plant functional group, plant identity, and sampling time on the <i>nirK</i> -type denitrifier communities	23
4.1.2 Richness, Shannon diversity, and Shannon evenness of the <i>nirK</i> -type denitrifier communities	29
4.2 Planted mesocosms (lysimeters)	30
4.2.1 Soil nitrate concentrations	30
4.2.2 Net- and gross N ₂ O production rates and denitrifier enzyme activity (DEA)	31
4.2.3 MPN of nitrate reducing microorganisms	36
4.2.4 Influence of plant diversity, plant combination, and sampling time on the <i>nirK</i> -type denitrifier communities	36
4.2.5 Richness, Shannon diversity, and Shannon evenness of the <i>nirK</i> -type denitrifier communities	43
4.2.6 Interactions between denitrifier diversity and functioning	44
4.2.7 Phylogenetic analyses of amplified <i>nirK</i> genes from microcosm and mesocosm soil	49
4.2.8 Quantification of <i>nirK</i> -type denitrifying microorganisms by MPN-PCR	53
5. Discussion	54
5.1 Plant microcosms	54
5.1.1 Plant-specific impacts on the <i>nirK</i> -type denitrifier community composition	54
5.1.2 Seasonal variations of the <i>nirK</i> -type denitrifier community composition	56
5.1.3 Description of the <i>nirK</i> -type denitrifier community structure by diversity indices	57
5.2 Planted mesocosms (lysimeters)	58
5.2.1 Impacts of plant diversity, plant combination, and time on the <i>nirK</i> -type denitrifier community	58
5.2.2 Interdependencies between soil nitrate concentrations, N ₂ O production rates, denitrifier enzyme activity, MPN, plants, and denitrifier	

communities.....	61
5.2.3 Phylogenetic analyses	66
5.2.4 MPN-PCR as a quantification method	67
5.3 Aspects of diversity-function relationships	68
6. Conclusions and Outlook	69
Appendix	71
References	123
Acknowledgements	136
Curriculum vitae	137
Contributions to symposia and conferences	138

I. Abbreviations

ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
bp	Base pairs
BSA	Bovine serum albumin
CA	Correspondence analysis
CCA	Canonical correspondence analysis
Comb.	(Plant) combination
DEA	Denitrifier enzyme activity
Div.	Diversity
DNA	Desoxyribonucleic acid
dNTP	Desoxynucleosidtriphosphate
GC-ECD	Gas chromatograph with electron capture detector
MPN	Most probable number
NH ₄ ⁺	Ammonium
nir	Nitrite reductase
N ₂ O	Nitrous oxide, dinitrogenoxide
NO	Nitric oxide, nitrogenoxide
NO ₃ ⁻	Nitrate
OTU	Operational taxonomic unit
P	(Statistical) probability
PCR	Polymerase chain reaction
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
v/v	Volume per volume

II. Summary/Zusammenfassung

Ein Forschungsschwerpunkt der mikrobiellen Ökologie ist die Aufdeckung und das Verständnis von Wechselwirkungen zwischen Pflanzen und im Boden lebenden Mikroorganismen, zu denen auch die denitrifizierenden heterotrophen Mikroorganismen gehören. Sie sind fakultativ anaerob und können bei Sauerstoffmangel Nitrat dissimilatorisch zu Nitrit, Stickstoffmonoxid, Distickstoffoxid (N_2O) und Stickstoff reduzieren. Der zweite Schritt der Denitrifikation, d. h. die Reduktion von Nitrit zu Stickoxid, wird durch das Enzym Nitritreduktase katalysiert. Dieses Enzym wird durch zwei funktionell gleichwertige Gene kodiert: *NirK* und *nirS*.

Die Ziele meiner Arbeit waren erstens zu prüfen, ob die Identität von Pflanzenarten, ihre Diversität, d. h. die Anzahl der Pflanzenarten, und die Kombination von Pflanzenarten die Diversität und die Zusammensetzung von denitrifizierenden Mikroorganismengemeinschaften beeinflussen. Zweitens habe ich postuliert, dass die Diversität und die Zusammensetzung der Denitrifizierer einen Einfluß auf ihre Funktion, d. h. die Produktion des Gases N_2O hat.

Um diese Hypothesen zu prüfen, habe ich zwei Experimente durchgeführt. Im ersten Experiment wurde der Einfluss acht einzelner, nicht zu den Leguminosen zählender Pflanzenarten auf die Zusammensetzung der denitrifizierenden Mikroorganismen untersucht. Von den acht Pflanzenarten waren vier Gräser (*Arrhenatherum elatius*, *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Holcus lanatus*) und vier Kräuter (*Geranium pratense*, *Taraxacum officinale*, *Plantago lanceolata*, *Ranunculus acris*), so dass auch der Einfluß der beiden funktionellen Pflanzengruppen auf die Denitrifizierer untersucht werden konnte. Zusätzlich habe ich getestet, ob sich die Zusammensetzung der Denitrifizierergemeinschaft über die Zeit ändert und ob es saisonale Unterschiede gibt. Die acht genannten Pflanzenarten wurden hierfür in Monokulturen/Mikrokosmen in demselben Bodensubstrat kultiviert. Im Rahmen meines zweiten Experimentes habe ich den Einfluss der Pflanzendiversität (Anzahl der Pflanzenarten), der Artenzusammensetzung der Pflanzen und der Zeit auf die Denitrifizierer untersucht. Von den oben genannten acht Pflanzenarten wurden Kombinationen von 0, 2, 4 und 8 Pflanzen in Lysimetern/Mesokosmen in demselben Bodensubstrat kultiviert. In diesem Experiment wurden die Netto- und die Bruttoproduktion von N_2O und die Enzymaktivität der Denitrifizierer (DEA) als funktionale Komponente bestimmt.

In beiden Experimenten wurde Gesamt-DNA aus dem Boden extrahiert, die *nirK*-Gene mittels PCR amplifiziert und mit der Methode des terminalen Restriktionsfragmentlängenpolymorphismus (T-RFLP) analysiert. Der Einfluss der verschiedenen Faktoren auf die Diversität und Zusammensetzung der Denitrifizierer wurde mittels verschachtelter Varianzanalysen (ANOVA) und Korrespondenzanalysen (CA) sowie kanonischer Korrespondenzanalysen (CCA) untersucht. Amplifikate von *nirK* aus den Bodenproben wurden kloniert und sequenziert, um wichtige *nirK*-Genotypen in den Böden identifizieren zu können.

In allen untersuchten Bodenproben der Mikro- und Mesokosmen fanden sich Denitrifizierer mit einem *nirK* Gen. Die funktionelle Pflanzengruppe (Gräser vs. Kräuter) hatte keinen Effekt auf die *nirK*-Denitrifizierergemeinschaft im Boden, aber die individuellen Pflanzenarten beeinflussten die relativen Häufigkeiten der *nirK*-T-RFs. Auch wirkte sich der Probenahmezeitpunkt und die Wechselwirkung des Probenahmezeitpunktes mit der einzelnen Pflanzenart auf die Zusammensetzung der Denitrifizierergemeinschaft aus.

Im zweiten Experiment zeigte sich, dass die Pflanzendiversität und die Pflanzenkombination einen signifikanten Einfluss auf die Zusammensetzung der *nirK*-Denitrifizierer haben. Ferner wurde ein genereller Effekt des Probenahmezeitpunktes, ein gerichteter Effekt der Zeit und Effekte der Wechselwirkungen zwischen Zeit und Pflanzendiversität und Zeit und Pflanzenkombination festgestellt.

Die Netto- und Bruttoproduktionsraten von N₂O wurden hauptsächlich von der Pflanzendiversität, der Pflanzenkombination, dem Probenahmezeitpunkt und der Interaktion von Probenahmezeitpunkt und Pflanzendiversität beeinflusst. Die Enzymaktivität der Denitrifizierer war abhängig von der Pflanzendiversität, der Pflanzenkombination, dem Probenahmezeitpunkt, der Interaktion von Probenahmezeitpunkt mit der Pflanzenkombination und der Zusammensetzung der *nirK*-Denitrifizierer.

Die phylogenetische Analyse der *nirK*-Sequenzen aus den Böden der Pflanzenmikro- und mesokosmen ergab, dass die Mehrzahl der *nirK* Sequenzen mit *nirK* Sequenzen verwandt waren, die von unkultivierten Organismen aus Böden und Klärschlämmen stammen und am ehesten mit *nirK* aus *Nitrosomonas* sp. TA-921 als dem nächsten kultivierten Organismus verwandt sind. Einige der Sequenzen zeigten eine Verwandtschaft zu *nirK* Genen von Denitrifizierern der Ordnung *Rhizobiales*, namentlich *Bradyrhizobium*, *Rhizobium* und *Mesorhizobium*.

Die Ergebnisse zeigen, dass einzelne, nicht zu den Leguminosen gehörende Pflanzenarten sowie die Pflanzendiversität und die Pflanzenkombination spezifische Effekte auf die Zusammensetzung der *nirK*-Denitrifizierer ausüben und teilweise indirekt ihre Funktion, d. h. die Produktion von N_2O und die Enzymaktivität beeinflussen.

1. INTRODUCTION

1.1 Influence of plants on soil microorganisms

A major focus in ecology is to understand the interactions between plants and soil microorganisms. Since microorganisms are crucial mediators of nutrient-cycling in soil and can thereby affect plant growth, e.g. in symbiosis or by competition for nutrients, 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 (Grayston et al. 1998, Innes et al. 2004, Miethling et al. 2000, Vetterlein and Jahn 2004, Wieland et al. 2001, Yang and Crowley 2000). 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 (Kowalchuk et al. 2002, Marschner et al. 2001). However, the root exudation of an individual plant may also depend on its growth conditions and developmental stage thereby potentially masking species-specific effects (Duineveld et al. 1998, Smalla et al. 2001).

Although evidence is increasing that individual plant species can influence microorganisms in their rhizosphere or in soil, the effects of plant species diversity and composition on soil microorganisms remain largely unexplored. Malý et al. (2000) found that plant diversity and composition did not significantly affect microbial biomass C and N. Wardle and Nicholson (1996) found that the effect of increasing plant species richness influenced soil microbial biomass positively or negatively. Another study showed that the changes in microbial biomass were small or not detectable (Spehn et al. 2000). In contrast to measuring effects on microbial biomass, analyses based on the 16S rRNA genes have been carried out. Kowalchuk et al. (2002) found that plant species diversity and composition had little effect on microbial communities inhabiting bulk soil, but differences in the diversity of microbial communities were observed in the rhizosphere of the plant species. Recently, Nunan et al. (2005) reported an influence of plant community composition, but not of individual plant species, on bacterial communities in grassland soils.

Apart from the plants, soil type, soil structure and its general characteristics also affect the microbial community (Brodie et al. 2002, Buckley and Schmidt 2003, Kowalchuk et al. 2002), and these soil effects have often been found to be more important than the identity of the plant species that excrete root exudates (Grivan et al. 2003). In addition, seasonal changes of environmental conditions may also influence

the amount, activity and composition of soil microorganisms (Griffiths et al. 2003, Kennedy et al. 2005, Rogers and Tate 2001, Smalla et al. 2001).

As already mentioned, most studies of plant-microbe relationships have focused on the overall composition of the microbial community based on the analysis of the 16S rRNA genes. However, there is also evidence that individual functional groups of soil microorganisms like methanotrophs (Knief et al. 2003), ammonia-oxidizing bacteria (Kowalchuk et al. 2000) and denitrifying bacteria (Cavigelli and Robertson 2000) 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 of unplanted soil (Philippot et al. 2002). 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 USA (Rich et al. 2003). 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 (Patra et al. 2006). Denitrifiers of the *nirK*-type were also found in the rhizosphere of three legume crops, and the diversity and composition of *nirK* transcripts was influenced by plant species identity (Sharma et al. 2005). Apart from effects induced by plants, the community composition of denitrifying bacteria has also been shown to be influenced by the seasonal variation of environmental conditions (Wolsing and Priemé 2004).

1.2 Denitrification in soil

An important part of the natural N-cycle is soil denitrification, since it is the pathway that returns nitrogen compounds back to the atmosphere. Denitrification is mainly driven by facultative anaerobic bacteria, which use oxidized nitrogen compounds as alternative electron acceptors for energy production when the availability of oxygen is limited (Tiedje 1994). Denitrifying bacteria can contribute to N-losses from soil via the production of the gases NO, N₂O and N₂.

The dissimilatory nitrate reduction consists of four single reactions which are catalyzed by the enzymes nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase. These enzymes are encoded by the genes *nar*, *nir*, *nor* and *nos*.



The gases nitric oxide (NO), nitrous oxide (N₂O) and dinitrogen (N₂) are released to the atmosphere where NO and N₂O contribute to stratospheric ozone decay and the greenhouse effect (Houghton et al. 1996).

The key enzyme in dissimilatory denitrification is nitrite reductase, since the ability to reduce nitrite to nitric oxide separates denitrifiers from nitrate respirers. Two forms of nitrite reductase have been found in nature. One is the product of the *nirK* gene encoding the copper-containing nitrite reductase, the other is the product of the *nirS* gene encoding the cytochrome *cd*₁ containing nitrite reductase. According to Zumft (1997) both genes have never been found together in the same cell but are physiologically equivalent. During the last years *nirK* has been effectively appointed as a functional marker gene for phylogenetically diverse denitrifying microorganisms in soils (Henry et al. 2004, Priemé et al. 2002,), activated sludge (Throbäck et al. 2004), and marine environments (Braker et al. 2000, Oakley et al. 2007). In a number of studies *nirK* could be more readily amplified from soils than *nirS* (Priemé et al. 2002, Sharma et al. 2005, Wolsing and Priemé 2004).

1.3 Role of the greenhouse gas nitrous oxide (N₂O)

Over the past decades the atmospheric N₂O concentration increased dramatically due to anthropogenic contributions (Sowers 2001). N₂O has an estimated lifetime of 120 years, and its primary sink in the atmosphere is the stratosphere, where it contributes to ozone depletion and the greenhouse effect (Prather et al. 1995). Therefore, the N₂O balance is critical to the ecosystem of our planet. The proposed sources of N₂O are terrestrial soils, aquatic systems, chemical industries, and combustion of fossil fuels and biomass. Soils are thought to account for over half of the total N₂O inputs to the atmosphere (Prather et al. 1995). In terrestrial ecosystems N₂O production is mainly driven by microbial nitrification and denitrification (Granlin and Bockmann 1994), but chemodenitrification (van Cleemput 1998) and fungal transformation processes (Laughlin and Stevens 2002) have been observed, too. It is generally assumed that N₂O production in oxic soil originates from nitrification and N₂O production in anoxic soil from denitrification (Bouwman 1996), but anaerobic niches in oxic soil can also be a site of denitrification (Conrad 1996), especially in deeper soil layers (Müller et al. 2004). Furthermore, some aerobic denitrifying bacteria can

produce low levels of nitrous oxide (Takaya et al. 2003) and nitrifier denitrification of ammonia oxidizers is a possible source, too (Wrage et al. 2001).

Although there are many sources of N_2O production, the major sink of N_2O is denitrification carried out by microorganisms (Conrad 1996). The rate of microbial denitrification depends on the soil water content, temperature, pH, carbon supply, nitrate and nitrite availability, the induction of denitrifying enzymes and the community composition of denitrifying microorganisms (Cavigelli and Robertson 2000, Cavigelli and Robertson 2001). Denitrifier community composition may be influenced by competition for carbon with other heterotrophs, since the majority of denitrifiers are aerobic heterotrophs and may seldom use their denitrification capacity (Tiedje 1988). Hence, the input of organic carbon by plants into soils may affect the diversity and composition of denitrifier communities thus influencing N_2O emissions.

1.4 Diversity and function of ecosystems

A major issue in the past and current discussion about biodiversity is the question, whether the loss of diversity results in decreased functioning of ecosystems. The idea that a diverse ecosystem is a more stable ecosystem is outlined in the insurance hypothesis (Yachi and Loreau 1999). It proposes that the capacity of an ecosystem to withstand disturbances, species losses or species invasions depends on the redundancy of species that can stabilize or compensate functions and that it depends on the capability of species to respond differently to these disturbances. A higher diversity increases the probability that such species are present in an ecosystem. However, a low level of diversity in combination with a huge variety of reactions or functions of these species can also suffice for the insurance of an ecosystem (McCann 2000, Yachi and Loreau 1999).

Hooper and Vitousek (1997) have proposed the “niche complementarity effect”. Based on experiments with plants, niche complementarity can take place in space (rooting depths), time (resource demand during growth), and in nutrient preference (e.g. nitrate vs. ammonium vs. dissolved organic N). They suggest that a higher diversity of plants increases the amount of resources that can be used and recycled by species. Therefore the resources or the nutrients will be lost to a lesser degree from the ecosystem (e.g. via leaching).

2. OBJECTIVES

The objectives of my work can be summarized in two hypotheses:

1. The diversity and composition of plant communities affects the diversity and composition of denitrifier communities.
2. The diversity and composition of denitrifier communities affects their functioning, i.e. the reduction of N₂O emission from soil. A higher denitrifier diversity should result in decreased N₂O emission, since N₂O and other nitrogen oxides can be more efficiently used by diverse populations than by less diverse populations (“complementarity effect”).

The present study consists of two separate experiments. In the first experiment, I analysed the *nirK*-containing denitrifier community associated with different non-leguminous plant species, representing two functional groups of plants (grasses and forbs), from unimproved temperate grasslands. Grasses have a more intensive root system compared to forbs (Strasburger et al. 1999). Hence I assumed that the functional group of a plant and the plant species identity influence denitrifier diversity and composition and that denitrifier community composition differs over time or at least over seasons. In this experiment, eight typical grassland plant species from Western Europe were cultivated in individual microcosms containing the same soil.

In the second experiment, different plant combinations with varying levels of plant diversity were cultivated in mesocosms (lysimeters) filled with the same soil substrate. Plant combinations consisted of 0, 2, 4, and 8 plant species. In this experimental setup N₂O net and gross production and denitrifier enzyme activity were measured as functional characteristics. In both experiments, DNA was extracted from soil, *nirK*-genes were amplified by PCR and analysed by terminal restriction fragment length polymorphism (T-RFLP). Denitrifier diversity and community composition were analysed by nested analysis of variance (ANOVA) and by correspondence analysis (CA) or canonical correspondence analysis (CCA). Amplicons of *nirK* from the soil samples were cloned and sequenced to identify important genotypes of the denitrifier communities.

Tris-HCl, pH 8	0.5 M
NaCl	0.1 M

Phenol-Chloroform-Isoamylalkohol (ready to use)
25:24:1 (v/v/v), pH 8

Chloroform-Isoamylalkohol (ready to use)
24:1 (v/v)

Tris-EDTA (TE-buffer) (Sambrook et al. 1989)

Tris-HCl	10 mM
EDTA	1 mM
adjusted to pH 8 with HCl	

Solutions for agarose gelelectrophoresis:

Tris-acetate-EDTA (TAE-buffer) (10× ready to use) pH 8.3

Loading buffer 6×

Saccharose	50% [w/v]
Bromophenolblue	0.1% [w/v]
Urea	4 M
EDTA	50 mM

DNA-Ladder (50×, 12.5 µl each)

1 kb-Ladder, 250 µg [µg/µl]	25 µl
Loading buffer 6×	125 µl
H ₂ O	475 µl

Solutions for MPN-counts

Minimal medium was prepared according to Jeter and Ingraham (1981), that mainly consisted of nutrient broth (NB), potassiumnitrate and a potassiumphosphate buffer.

3.2 Soil sampling and site characteristics

Soil samples were taken from plant microcosms and planted mesocosms (lysimeters) located in the Ecological Botanical Garden of the University of Bayreuth. The plants are typical species of unfertilized or unimproved fresh meadows.

Plants of microcosms and mesocosms were cultivated in the same soil, that had been taken from a meadow next to the Ecological Botanical Garden. The original soil type was a stagnic gleysol (international soil classification; Schachtschabel et al. 1998), developed on sandstone interspersed with red and violet clay layers (www.

bayern.de/ wwa-bt/trinkwasser/seiten/geologie.htm). The soil had the following characteristics: $\text{pH}_{(\text{CaCl}_2)}$ 4.9, $\text{NH}_4^+\text{-N}_{(1\text{ M KCl})}$ 19.6 mg kg⁻¹, $\text{NO}_3^-\text{-N}_{(1\text{ M KCl})}$ 27.0 mg kg⁻¹, and it consisted of 7% sand, 78% silt and 16% clay (Kossmann 2005). The soil was thoroughly mixed and steamed (12 hours at 100°C) to kill weed seeds. Plant microcosms (Ø 20 cm, depth 100 cm) and mesocosms (1.3 × 1.3 m, depth 100 cm) were established in autumn 2001 and kept under ambient environmental conditions.

The city of Bayreuth is part of the “Oberpfälzer Senke”, located at the eastern border of the “Fränkische Alb” and the western border of the “Fichtelgebirge”. The mean temperature of January in Bayreuth is -0.1°C, of July 18.2°C. The mean annual rainfall is 630 mm.

Soil samples were taken in summer 2003 (18.-19.6), autumn 2003 (23.-24.9), winter 2003 (11.-12.12), summer 2004 (7.-8.6), and autumn 2004 (9.-10.9). Heavy ground frost in winter 2003 prevented soil sampling of the fragile plant microcosms (monocultures). Only the mesocosms (lysimeters) were sampled.

The soil was sampled to 7 cm depth and afterwards stored at 4°C for measuring functional characteristics or frozen at -20°C for DNA extraction. The detailed procedure of soil sampling is described in the following sub-sections.

Plant microcosms/monocultures

In autumn 2001 monocultures of eight non-leguminous grassland plants representing two plant functional groups were established in microcosms (Ø 20 cm, depth 100 cm) in the Ecological Botanical Garden of the University of Bayreuth (a picture of the microcosms is shown in the appendix (Fig. 19)). The experimental set-up is described in detail in the study of Reuter (2005). Plant functional groups had been defined in the past, in many cases according to morphological traits (Ledeganck et al. 2003, Pokorny et al. 2005, Viketoft et al. 2005). In the present study I assigned two plant functional groups, grasses and forbs, due to their different root systems. The plant species were the four grasses *Alopecurus pratensis*, *Arrhenatherum elatius*, *Anthoxanthum odoratum*, and *Holcus lanatus*, and the four forbs *Plantago lanceolata*, *Taraxacum officinale*, *Ranunculus acris*, and *Geranium pratense*. The plants were grown for three years and watered if necessary. In addition, they were fertilized twice a year (with an amount of the NPK-fertilizer “Blaukorn” corresponding to 50 kg per ha). Foreign plant species were removed manually. Three replicate microcosms were set up for each species (Table 1). In summer and autumn 2003 and in summer 2004

two soil samples were taken near the plant from each microcosm with a (cork) corer to a depth of 7 cm (about 20 g soil), pooled, thoroughly mixed and homogenized with a spoon, transferred to a sterile 50 ml Falcon tube 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.

Table 1. Plant species, plant types and plant functional groups

Plant species	German/english names	Plant type	Plant functional group
<i>Alopecurus pratensis</i>	Wiesenfuchsschwanz/ Meadow foxtail	Tall grass	Grass
<i>Arrhenaterum elatius</i>	Wiesenglatthafer/ Tall oat grass	Tall grass	Grass
<i>Anthoxanthum odoratum</i>	Gemeines Ruchgras/ Sweet vernal grass	Small grass	Grass
<i>Holcus lanatus</i>	Wolliges Honiggras/ Yorkshire fog	Small grass	Grass
<i>Plantago lanceolata</i>	Spitzwegerich/ Ripwort plantain	Rosette forb	Forb
<i>Taraxacum officinale</i>	Gemeiner Löwenzahn/ Common dandelion	Rosette forb	Forb
<i>Ranunculus acris</i>	Scharfer Hahnenfuß/ Meadow buttercup	Forb	Forb
<i>Geranium pratense</i>	Wiesenstorchschnabel/ Meadow cranesbill	Forb	Forb

Planted mesocosms (lysimeters)

In autumn 2001 eight plant species (given in Table 1) were established in different combinations and with different levels of plant diversity (Table 2) in 28 mesocosms (lysimeters, $1.3 \times 1.3 \times 1.3$ m) in the Ecological Botanical Garden of the University of Bayreuth (two pictures of the mesocosms are shown in the appendix (Fig. 20 and 21)). The experimental set-up is described in detail in the study of Kossmann (2005). The plants were grown for four years and watered if necessary. They were mown and

fertilized twice a year (with an amount of the fertilizer “Blaukorn” corresponding to 50 kg per ha). Foreign plant species were removed manually. Five replicate mesocosms were set up for each combination and diversity level. Three replicate mesocosms were set up for the unplanted variant. In summer, autumn, and winter 2003, and in summer and autumn 2004 four soil samples were randomly taken from each mesocosm with a (cork) corer to a depth of 7 cm (about 50 g soil), pooled, thoroughly mixed and homogenized with a spoon. About 20 g of the soil sample was transferred to a sterile 50 ml Falcon tube and immediately stored at -20°C . The remainder of the soil sample was transferred to a small plastic bag and stored at 4°C (for measuring functional and soil characteristics). All soil samples contained plant roots, since the high root density of the grassland plants prevented the separation of rhizosphere and bulk soil. The soil samples which were stored at 4°C were sieved (< 2 mm) to homogenize the samples and remove plant roots before soil characteristics and functional characteristics were measured.

Table 2. Plant diversity and combination in the mesocosms (lysimeters)

Diversity	Plant species combination	Plant functional group
0	None	None
2a	<i>Holcus lanatus</i> + <i>Arrhenaterum elatius</i>	Two grasses
2b	<i>Holcus lanatus</i> + <i>Geranium pratense</i>	Grass and forb
2c	<i>Holcus lanatus</i> + <i>Plantago lanceolata</i>	Grass and forb
4	<i>Holcus lanatus</i> + <i>Arrhenaterum elatius</i> + <i>Geranium pratense</i> + <i>Plantago lanceolata</i>	Two grasses and two forbs
8	as 4, + <i>Alopecurus pratensis</i> + <i>Anthoxanthum odoratum</i> + <i>Taraxacum officinale</i> + <i>Ranunculus acris</i>	Four grasses and four forbs

3.3 Determination of soil properties and nitrate concentrations

Soil water contents were determined gravimetrically. Soil (5-10 g) was weighted, dried at 105°C overnight and weighted again.

Calculation: Water content [weight %] = $(B_f - B_t) * B_t^{-1} * 100$

B_f = fresh soil [g]

B_t = dry soil [g]

To determine pH values, an amount of soil equalling 10 g dry weight was suspended in 10 ml tap water. The soil slurries were incubated for two hours at room temperature before the pH values were determined with a glass electrode (Mettler TOLEDO, In Lab[®] 427) and a pH-meter (Microprocessor pH-Meter 539, WTW).

Nitrate concentrations in soil were assessed with an ion chromatograph (IC) (Sykam, Fürstfeldbruck) (Table 3) after extraction from soil with distilled water (seradest). An amount of soil equalling 2 g dry weight was suspended in 10 ml distilled water and shaken at 150 rpm for 1 hour at 4°C. Afterwards the soil suspension was filtered (round filter 2095, Schleicher & Schuell GmbH, Dassel, Germany) and nitrate was measured with the IC. 1 mM NaNO₃ was used as a standard. Data were evaluated with the software Peak simple (version 2.66, SRI Instruments, Torrence, USA).

Table 3. Parameters of IC

Column	6 cm stainless steel column, Ø 4.6 mm; packing: styrol/ divinobenzol, grain size 10 µm temperature: 70°C
Oven	S4110
HPLC-pump	S1121
Suppressor-system	S4260A + S420B with separation-column, LCA A14; 2.5 cm, Ø 3 mm
Eluent	5 mM Na ₂ CO ₃ + modifier
Flow-rate	1.5 ml min ⁻¹
Detector	Conductivity-detector S3111 UV/vis detector (UVIS 204, Linear, USA)
Tableau	S7121
Autosampler	S5200 (Schambeck SFD GmbH, Bad Honnef, Germany)

3.4 Measuring nitrous oxide (N₂O)

N₂O was measured with a gas chromatograph (GC) (Carlo Erba Instruments, GC 8000) connected to an electron capture detector (ECD) (Table 4). Soil slurries had been manually shaken to equilibrate the gas and the liquid phase of the slurries before the gas samples were taken out with 100 µl or 1 ml Pressure-Lok-Syringes[®] (VICI,

Baton Rouge, Louisiana, USA), which had been flushed with N₂. The minimum detection limit of the system was 40 ppb_v. Linearity of the calibration was given between 40 ppb_v und 35 ppm_v. Before and after each measurement calibration gas was injected to check the results.

Measurements with acetylene in the gas phase required an occasional heating of the oven up to 70°C for 30 minutes, because the column had to be cleaned of remaining acetylene.

Data were evaluated with the software Peak Simple (version 2.66, SRI Instruments, Torrence, USA).

Table 4. Parameters of GC-ECD

Column	4 m stainless steel column, Ø 1/8"; packing material: Hay Sep [®] N, 75cc, mesh 80/100; pre-column: natron on solid medium for CO ₂ -adsorption
Carrier gas	ECD-nitrogen; make-up-gas: 5% CH ₄ in argon
Detector	ECD; ⁶³ Ni
Temperature	Injector: 60°C Oven: 50°C Detector basis: 300°C Detector: 350°C
Flow	1.8 nA
Attenuation	2 ⁰

Calculation of N₂O-release:

The measured N₂O concentration [ppm_v] was converted to the amount of substance [μmol] and related to soil dry weight.

$$F_x = \frac{M_v * p * (V_g + V_w * \alpha)}{R * T * TS}$$

F_x: released N₂O amount [μmol N₂O g⁻¹ TS]

M_v: mixing ratio of N₂O [bar] (1 ppm_v = 1×10⁻⁶ bar)

p: pressure [bar]

V_g: volume of the gas phase [l]

V_w: volume of the liquid phase [l]

α : Bunsenkoefficient (for N₂O at 25°C: 0.561 (Encyclopedie des Gaz – L’Air Liquide))

R: common gas constant (0.083144 [l bar K⁻¹ mol⁻¹])

T: temperature [K]

TS: dry weight of soil[g]

Calculation of N₂O production rates:

The calculated N₂O release was converted to µg N₂O-N. Afterwards the slope in the linear range of the N₂O release was used to determine the N₂O production rates.

$$R_x = \frac{\Delta F_v * MG * a}{\Delta t}$$

R_x: N₂O production rate [µg N₂O-N g⁻¹ TS h⁻¹]

F_x: released N₂O amount [µmol N₂O g⁻¹ TS]

MG: molecular weight (N₂O: 44 g mol⁻¹)

a : transformation factor of N₂O to N₂O-N (= 0.6363)

t: time [h]

3.5 Incubation experiments with soil slurries

3.5.1 Soil slurries with and without the addition of acetylene

All soil slurry experiments were done with sieved (< 2 mm) and cool stored (4°C) soil samples from the mesocosms. An amount of soil equalling 10 g dry weight was put in 120 ml serum bottles, 10 ml sterile filtered tap water was added, and the serum bottles were capped with butyl stoppers. The soil slurries were manually shaken and flushed with nitrogen for 30 minutes to remove any residual oxygen. Afterwards the pressure in the bottles was adjusted to normal pressure. The evaluation of the measured data is described in chapter 3.4. The final results were studied by nested analysis of variance (ANOVA), described in chapter 3.9.

Assay without the addition of acetylene:

Anaerobic soil slurries were incubated at 25°C and were shaken on a horizontal shaker at 200 rpm to determine the net or actual denitrification rate. After an initial measurement (0 h) gas samples were taken hourly for 8-10 h. After 8-10 h the N₂O release was not linear any more.

Assay with the addition of acetylene:

For the determination of gross or potential denitrification rates the acetylene inhibition technique was applied (Ryden and Focht 1979, Yoshinari et al. 1977). This method is based on the inhibited reduction of N_2O to N_2 : N_2O accumulates in the gas phase and can be more easily measured than N_2 (Yoshinari et al. 1977). Therefore the anaerobic gas slurries were provided with a volume of acetylene that equals 10% acetylene in the gas phase (Ryden and Focht 1979). The corresponding amount of N_2 was removed from the gas phase. After an initial measurement (0 h) gas samples were taken hourly for 8-10 h. After 8-10 h the linearity of N_2O release was no longer given. The plateau of N_2O accumulation was reached after 11 h.

3.5.2 Denitrifier enzyme activity method (DEA)

The DEA assay (Smith and Tiedje 1979) enables the assessment of the potential activity of pre-existing denitrifying enzymes in a soil sample. It is based on the principle that the rate of the N_2O production is proportional to enzyme concentration when no other factors are limiting. Thus, nitrate and glucose are added to anaerobic soil slurries. Additionally, 10% acetylene in the gas phase blocks the N_2O reduction. Chloramphenicol was added to prevent the bacteria from growing and producing new enzymes.

According to Murray and Knowles (2004) an amount of soil equalling 10 g dry weight was put in serum bottles. The slurries were amended with 25 ml filtered tap water that contained glucose, potassium nitrate (both 10 mM) and chloramphenicol (0.1 g/l; Murray and Knowles 1999). The serum bottles were capped with butyl stoppers, oxygen was removed by flushing the bottles with N_2 for 30 minutes, and the pressure was adjusted to normal pressure. Soil slurries were incubated at 25°C and shaken on a horizontal shaker at 200 rpm. Gas samples were taken hourly from 0-5 h. As recommended in the work of Murray and Knowles (2004) the syringe was flushed with N_2 before gas samples were taken out of the bottles to prevent an addition of oxygen to the gas samples. The results were evaluated by nested analysis of variance (ANOVA), described in chapter 3.9.

3.6 Most probable number (MPN) of nitrate reducing microorganisms

MPN was only done with mesocosm soil samples of summer 2004. An amount of soil equalling 1g dry weight was suspended in 9 ml autoclaved tap water and shaken

at 150 rpm for 1 h at 4°C. Aliquots (100 µl) of this suspension were used for further dilutions. From each sample three dilution series (1:10) were prepared until 10⁻⁹. The incubation was done in Deep Well™ plates with 96 wells (Nunc GmbH, Wiesbaden, Germany) at 15°C for four weeks. A negative control contained no soil inoculum. Griess-reagent and zinc powder (both from Merck, Darmstadt, Germany) were used for the detection of nitrite in the solutions. If the solutions remained transparent the nitrate or nitrite had been reduced. The calculation of MPN values was done according to Schwarz (2002). The results were evaluated by nested analysis of variance (ANOVA), described in chapter 3.9.

3.7 Qualitative molecular analyses of the denitrifier community

3.7.1 DNA extraction from soil and amplification by polymerase chain reaction (PCR)

Soil (0.5 g), 0.5 g glass-beads (Ø 0.17-0.18 mm, B. Braun Biotech Int. GmbH, Melsungen, Germany), 800 µl sterile sodium phosphate buffer and 260 µl sterile SDS buffer were mixed. Cell lysis was done with a bead-beater (BIO 101 Mini Bead Beater, Savant, New York, USA) for 45 s with 6.5 m⁻¹. Samples were centrifuged for 15 minutes at 14,000 rpm and 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-isoamylalcohol (25:24:1) and 600 µl chloroform-isoamylalcohol (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 volumes of 100% isopropanol at room temperature. After centrifugation (60 minutes, 14,000 rpm at room temperature) the DNA pellet was washed with ice-cold (-20°C) 70% (v/v) ethanol and centrifuged again (10 min, 14,000 rpm at 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 Wizard® DNA Clean-up-System (Promega, Mannheim, Germany) according to the manufacturer's recommendations. Subsequently, the purity and 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.

The primer pair nirK1F and nirK5R (Braker et al. 1998) was used for the detection of *nirK*-type denitrifier bacteria. *Pseudomonas sp.* G-179 served as a positive refer-

ence in the PCR. For T-RFLP-analysis the nirK5R Primer was replaced by a nirK5R primer labelled with carboxyfluorescein (= FAM) at the 5`-end.

PCR reactions were prepared for a final volume of 25 µl. For each sample four PCR reactions were done and pooled afterwards to minimize PCR artefacts. In every reaction a positive and a negative control (without DNA) were included. All PCR reactions were done in the cycler Primus 96^{plus} (MWG Biotech, Ebersberg, Germany). Table 5 shows the primers, table 6 the temperature profile of the PCR reactions, and table 7 shows PCR solutions and components.

Table 5. Oligonucleotidprimers for amplification of *nirK* from soil DNA

Name	Sequence (5' - 3')	Reference
nirK1F	GG(A/C)ATGGT(G/T)CC(C/G)TGGCA	Braker et al. (1998)
nirK5R	GCCTCGATCAG(A/G)TT(A/G)TGG	Braker et al. (1998)

Table 6. Temperature profile of *nirK*-amplification

Temperature [°C]	Time [min]	Cycles	Process
94	5.00	1	Denaturing
94	0.30		Denaturing
57 – 52.5	0.40	10	Primer-annealing
72	0.40		Elongation
94	0.30		Denaturing
55	0.40	27	Primer-annealing
72	0.40		Elongation
72	7.00	1	Elongation
4	∞		Cooling

Table 7. Components and concentrations for 25 µl PCR reaction

Solution	Final concentration
H ₂ O	ad 25 µl
10× buffer	1× buffer (50 mM Tris-HCl, 15 mM (NH ₄) ₂ SO ₄ (pH 9.3), 2.5 mM MgCl ₂ , 1% Tween 20)
dNTP's	200 µM each
Forward-Primer	25 pmol

Reverse-Primer	25 pmol
BSA	400 ng μl^{-1}
Taq-polymerase	1.25 U μl
DNA	20-40 ng

3.7.2 Agarose gelelectrophoresis

Agarose gelelectrophoresis was used to check the quality and quantity of the PCR amplicons. The separation of the products was done with 1.5% [w/v] agarosegels (120 V, 45-60 min run time), buffered by $1\times$ TAE-buffer. A 1-kb-ladder (Invitrogen GmbH, Karlsruhe, Germany) was used as a size standard. Gels were stained in 0.001% [v/v] ethidium bromide solution and photographed (INTAS, Gel Jet Imager, Göttingen, Germany).

PCR products of the correct size were excised from the agarose gel and purified with the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany), because in some cases PCR byproducts of unexpected sizes were amplified, too. Afterwards DNA was quantified photometrically (Biophotometer, Eppendorf, Hamburg, Germany).

3.7.3 Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is a fingerprinting technique which allows the comparison of complex microbial communities in environmental samples. The method is based on differences in the position of restriction sites within sequences. In contrast to restriction fragment length polymorphism (RFLP) only DNA fragments of different length with a terminal label can be detected, fragments without a label are not detected.

The purified PCR products (100 ng) were hydrolysed with 5 U of *Hae*III (New England Biolabs, Frankfurt, Germany) overnight at 37°C (Avrahami et al. 2002). Digestions were cleaned up with Autoseq G-50 columns (Amersham Biosciences, Piscataway, NJ, USA) 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). The restriction fragments were separated on an ABI 310 automated sequencer (Applied Biosystems, Darmstadt, Germany). Since T-RFs can vary slightly in size, T-RFLP patterns were inspected visually and peak size

differences of one or two bp were confirmed by comparing the respective peaks of all patterns. Afterwards, the lengths of the fluorescently labeled terminal restriction fragments (T-RFs) were defined by comparison to the internal length standard with GeneScan 3.71 software (Applied Biosystems). Peaks with a fluorescence of 50 U over the background fluorescence and larger than 60 bp were analysed by peak height. Peak heights were normalized in an iterative standardization procedure according to Dunbar et al. (2000), and the relative abundances of T-RFs in a sample, given in percentages, were calculated.

3.7.4 Diversity indices

Diversity indices were calculated with PC-Ord 4.0 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 of *nirK*-OTUs relative abundance. With this dataset the diversity indices species richness (i.e. the number of *nirK*-OTUs), Shannon-Wiener index and Shannon evenness index were determined according to Hill et al. (2003) (calculation with ln). The effects of the experimental factors plant group (grasses vs. forbs), plant species, plant diversity, plant combination and sampling time on the diversity indices were evaluated by nested analysis of variance (ANOVA), described in chapter 3.9.

3.7.5 Phylogenetic analyses

Plant microcosms

Amplified *nirK* genes were cloned using the pGEM-T cloning kit (Promega) according to the manufacturer's instructions. Prior to cloning, four single PCR reactions were pooled for each sample to minimize amplification artefacts. 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 (Avrahami et al. 2002). Inserts of the first 36 clones for two *nirK* amplicons (one from the three replicates from soil of *Holcus lanatus* in summer 2003, one from the three replicates from soil of *Plantago lanceolata* in autumn 2003) were sequenced to generate two random clone libraries. In addition, 200 clones from the two amplicons and 20 clones from another amplicon (one from the three replicates from soil of *Alopecurus pratensis* in summer 2003) were screened by T-RFLP to select clones with less abundant and different terminal restriction sites. Clones corre-

sponding 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 reactions were purified with Autoseq G-50 columns (Amersham Biosciences) and analysed on an ABI 377 DNA sequencer (Applied Biosystems).

Phylogenetic analyses were done with ARB (<http://www.arb-home.de>). Sequences of *nirK* 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. The tree was constructed with the maximum likelihood method and with the parsimony and neighbour 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 analysed *in silico* for terminal restriction fragments obtained by cleavage with the restriction endonuclease *HaeIII* using T-RF-CUT (Ricke et al. 2005) for the assignment of theoretical T-RFs to those found by *in vitro* analysis.

Planted mesocosms (lysimeters)

Amplified *nirK* genes from the soil of the plant mesocosms were phylogenetically analysed as described for the genes from the soil of the plant monocultures. 200 clones from three *nirK* amplicons (one from the three replicates from the unplanted soil in autumn 2003, one from the three replicates from the unplanted soil in summer 2004, and one from the five replicates from the soil with the highest plant diversity in summer 2004) 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.

Nucleotide sequence accession numbers

The partial *nirK* gene sequences that were generated in the plant microcosm experiment have been deposited in the EMBL nucleotide sequence database (accession numbers AM235217 to AM235292). The partial *nirK* gene sequences that were generated in the plant mesocosm experiment (lysimeters) have not been deposited in a nucleotide sequence database yet.

3.8 Quantitative molecular analysis of the denitrifier community: MPN-PCR

MPN-PCR was only done with the mesocosm soil samples of summer 2004. Before MPN-PCR was performed, the DNA concentration of the extracts was determined quantitatively with Pico Green® for double-stranded DNA according to the manufacturer's instructions (MoBiTec GmbH, Göttingen, Germany). Afterwards all DNA extracts of summer 2004 were diluted to 2 ng/μl. PCR amplification was done as described in chapter 3.7.1. The MPN-PCR was done following the instructions of Kowalchuk et al. (2004). First, 10⁻¹ serial dilutions of purified soil DNA are prepared to determine the minimum concentration "c", which gives a positive amplification by PCR with the respective primers. From the second minimum concentration (10× c), triplicate 1:3 serial dilutions of the DNA solution are prepared and amplified with PCR. Afterwards the less dilute solution giving 2, 1 or 0 positive amplifications per triplicates is determined and can be used for further calculations.

The results were evaluated by nested analysis of variance (ANOVA), described in chapter 3.9.

3.9 Statistics

Statistics were performed with CANOCO 4.5 and SPSS 12.0 for Windows.

Plant microcosms

The effects of the experimental factors plant functional group (grasses vs. forbs), plant species, sampling time and the interactions of interest on the measured variables (e.g. diversity indices) were studied by analysis of variance (ANOVA) according to the nested experimental design (Zar 1999). Thus, the effects of plant functional group were tested against the variation among plant species, and those of plant species identity against the variation among the microcosms, and the effect of time against the residual. The effect of the interaction between plant functional group and time was tested against the interaction between plant identity and time.

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, time and the interactions on T-RFLP-profiles were explored by ordination techniques. After an initial detrended correspondence analysis had indicated that an unimodal response model was more

appropriate than a linear model, the data were analysed by correspondence analysis (CA) and canonical correspondence analysis (CCA). A CA is a method to describe the structure of a dataset. In addition, a CCA allows to relate community variation to environmental variation. It uses the individual T-RFs obtained from each replicate microcosm and their relative abundance as input variables and calculates the position of all the T-RFs in a two-dimensional ordination. Samples with a similar community composition are placed closer together, samples with a dissimilar community composition are positioned further apart. Because of the nested design of the experiment covariables and dummy variables were included in the analyses. Covariables are concomitant variables whose effect is partialled out when analysing the effects of the variables of interest. Dummy variables are nominal variables defined as 1 or 0, that code for the levels of a factor.

First, single CAs were performed with the T-RFLP-datasets for the three sampling times to view the structure of the data and the quality of replication. Second, several CCAs were performed to reflect the nested design of the experiment and the different number of replicates for the effect of the factors of interest. The effect of plant functional group (grasses vs. forbs) was tested using a dataset consisting of average OTU abundances for the individual plant species. The effect of plant species on *nirK*-OTUs was tested using a dataset consisting of average OTU abundances for the individual microcosms and with the effect of plant functional group partialled out by using the two plant groups as a dummy-coded covariable. The effect of time on the pattern of *nirK*-OTU abundance was tested using the dataset containing the measurements taken for the individual microcosms at the three sampling times and with the variation due to individual microcosms and time partialled out. The effect of interactions on the pattern of *nirK*-OTU abundance was tested using the dataset containing the measurements taken for the individual microcosms at the sampling times and with the variation due to individual microcosms and single effects of the factors partialled out. Monte Carlo permutation tests (based on 5000 random unrestricted permutations with blocks defined by the covariable microcosm) as available in CANOCO 4.5 (ter Braak and Šmilauer 2002) were used to test the hypothesis that relative abundances of *nirK*-OTUs were related to plant functional group, plant species, sampling time and the interactions between these factors.

Planted mesocosms (lysimeters)

The effects of the experimental factors plant diversity (e.g. the number of plant species), plant combination, sampling time and the interactions of interest on the measured variables (nitrate concentration, N₂O rate measurements, MPN data, MPN-PCR data, diversity indices) were studied by analysis of variance (ANOVA) according to the nested experimental design (Zar 1999). The effects of plant diversity were tested against the variation among plant combinations, that of plant combinations against the variation among the individual mesocosm, and the effect of time against the residual. The effect of the interaction between plant diversity and time was tested against the interaction between plant combination and time.

In addition, effects of the experimental factors on T-RFLP-profiles were explored by ordination techniques. After an initial detrended correspondence analysis had indicated that an unimodal response model was more appropriate than a linear model, the data were analysed by correspondence analysis (CA) and canonical correspondence analysis (CCA).

First, single CAs were performed with the T-RFLP-datasets for the five sampling times to view the structure of the data and the quality of replication. Second, several CCAs were performed to reflect the nested design of the experiment and the different number of replicates for the effect of the factors of interest. The effect of plant diversity was tested using a dataset consisting of average OTU abundances for the different diversity levels. The effect of plant combination on *nirK*-OTUs was tested using a dataset consisting of average OTU abundances for the individual mesocosms and with the effect of plant diversity partialled out by using the plant diversity as a dummy-coded covariable. The general effect of time on the pattern of *nirK*-OTU abundance was tested using the dataset containing the measurements taken for the individual mesocosms at the sampling times and with the variation due to individual mesocosms and time partialled out. The specific effect of time (summer and autumn of one year against summer and autumn of the other year) on OTU abundance was tested using a dataset containing the measurements for the respective sampling times and with the variation due to individual mesocosms partialled out. The effect of interactions on OTU abundance was tested using the dataset containing the measurements taken for the individual mesocosms at the five sampling times and with the variation due to individual mesocosms and the main effects partialled out. Monte Carlo permutation tests (based on 5000 random unrestricted permutations with blocks defined by the

covariable mesocosm) as available in CANOCO 4.5 (ter Braak and Šmilauer 2002) were used to test the hypothesis that relative abundances of *nirK*-OTUs were related to plant diversity, plant combination, sampling time and the interactions between these factors.

To detect interdependencies between measured variables, the measured variables of interest were included as a factor in the analysis of variance or in the analysis of covariance (ANCOVA). Different positions of a factor in the hierarchical design together with the part of variance explained by a factor can reveal interdependencies of two measured variables. Covariables were used to partial effects out.

Relationships between plant diversity, plant species composition and function were studied by including sample scores along the first and the second ordination axis (calculated within CCA) as a factor in the nested analysis of variance.

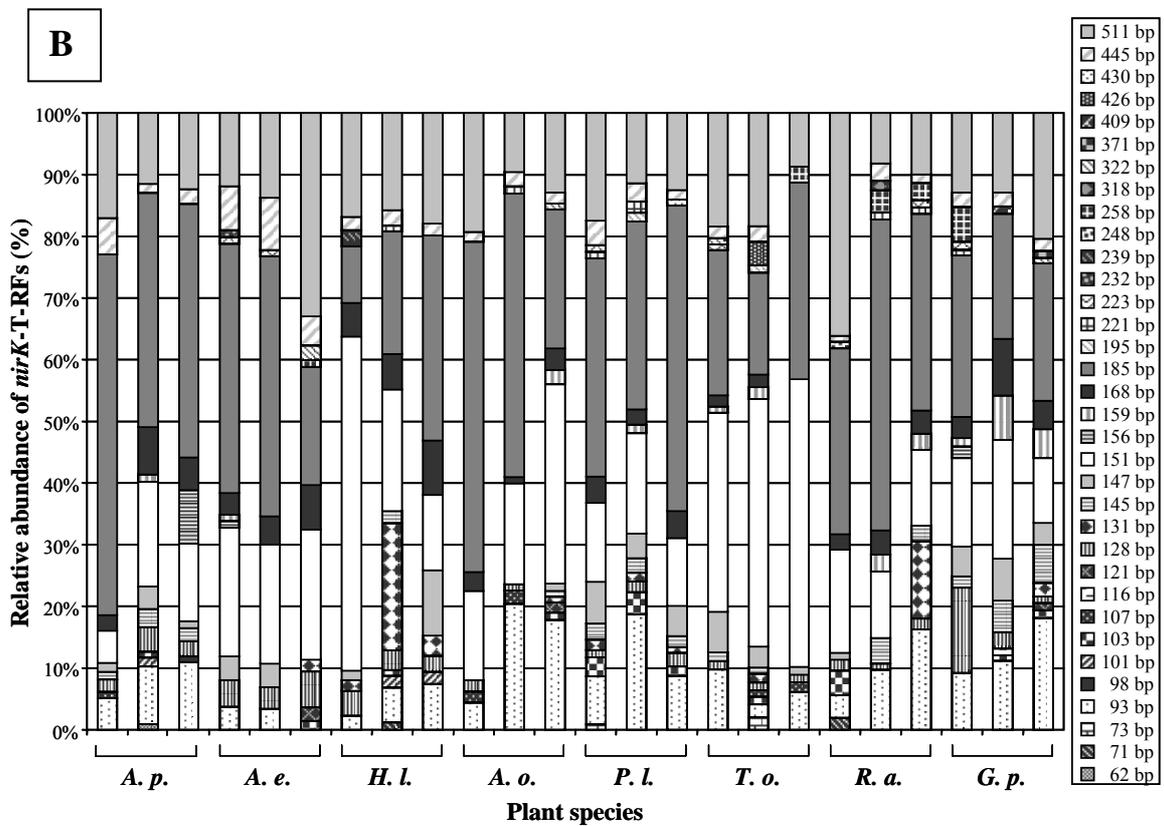
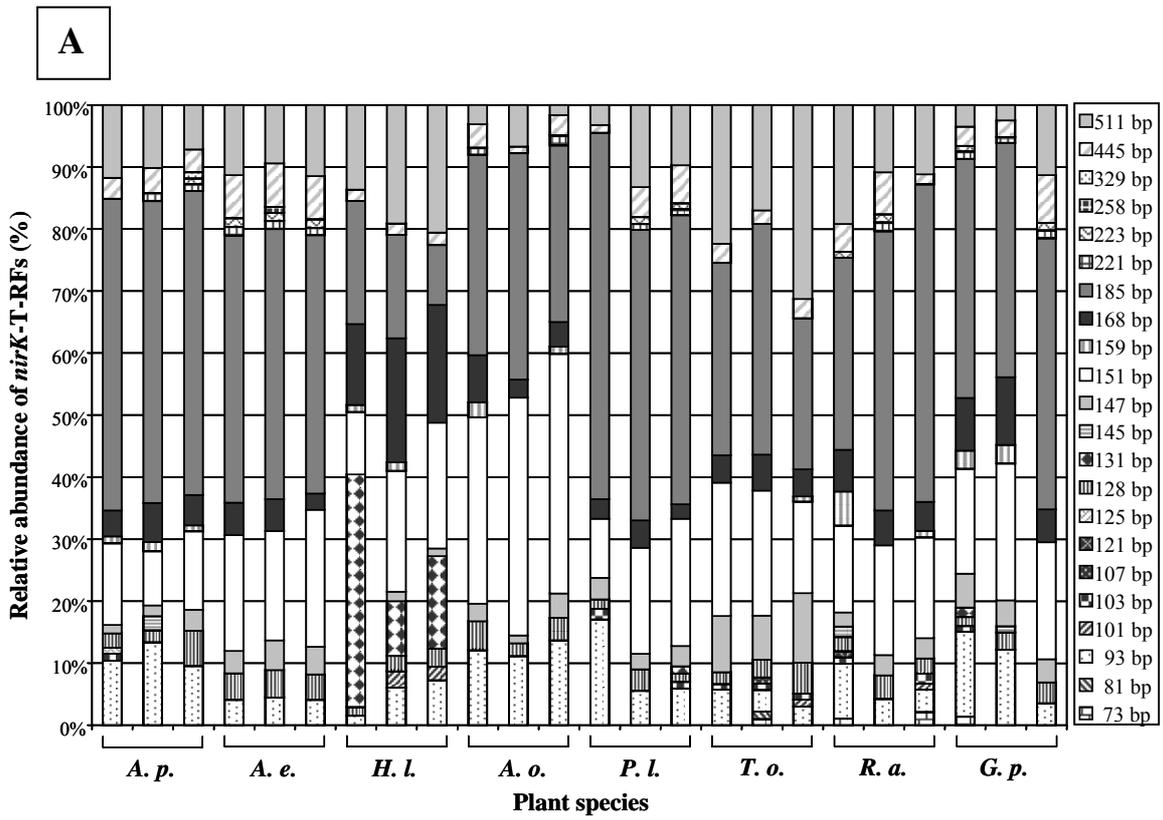
4. RESULTS

4.1 Plant microcosms

The denitrifier community in the soil of plant microcosms was only investigated with qualitative molecular techniques. Since the same soil was used for the establishment of plant microcosms and mesocosms, the results referring to the soil are described in chapter 4.2.

4.1.1 Influence of plant functional group, plant identity, and sampling time on the *nirK*-type denitrifier communities

NirK genes were successfully amplified from the soil of all plant microcosms, and the *nirK*-type denitrifier community was subsequently resolved by T-RFLP. 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. T-RF-profiles were also highly reproducible when DNA of one of the replicates was extracted several times (the standard deviation of the relative abundance of single T-RFs was less than 0.1%). T-RFLP analysis showed marked differences in the composition of the denitrifier community in the soil of microcosms planted with different plants (Fig. 1).



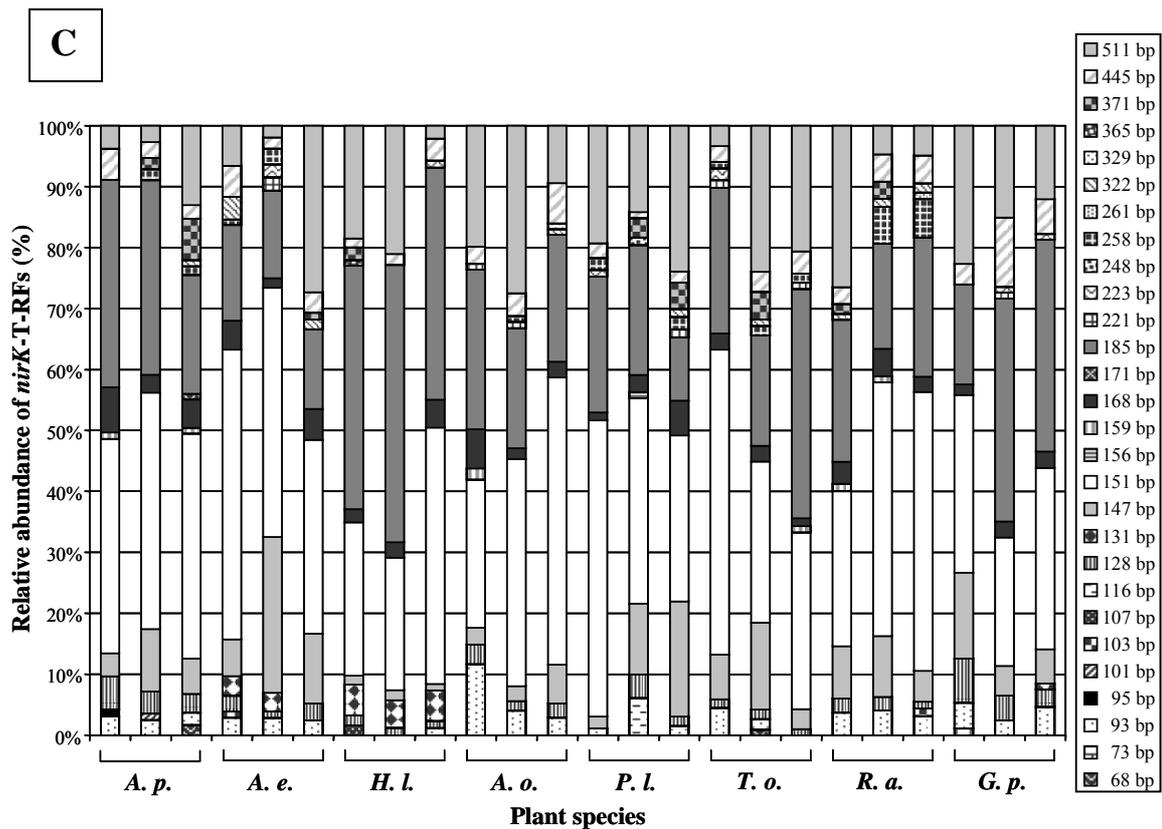
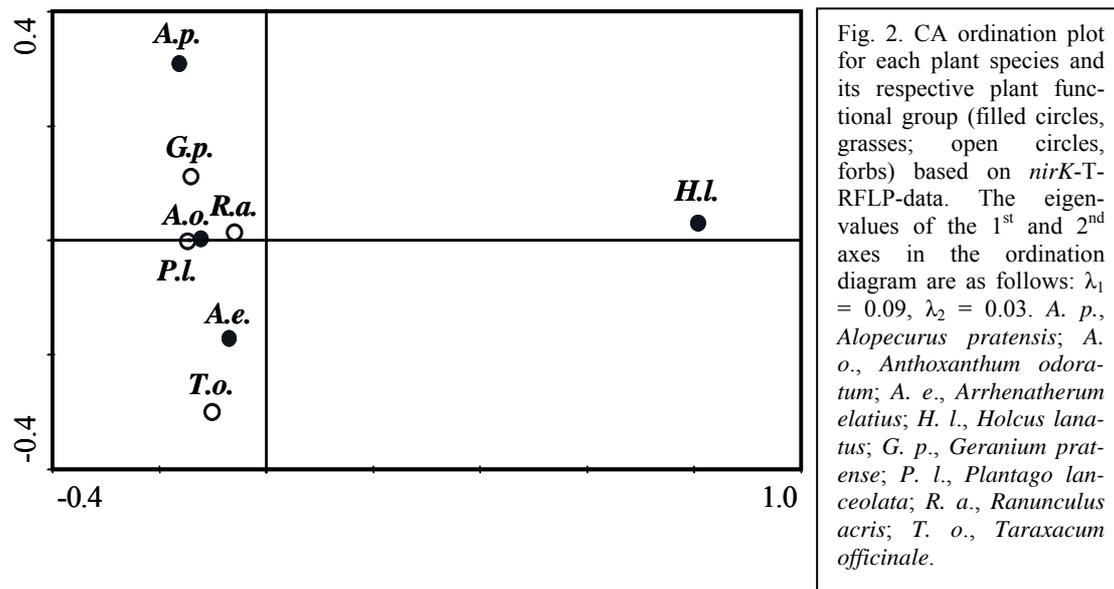


Fig. 1. Relative abundance of *nirK* terminal restriction fragments (T-RFs) from soil of plant microcosms in summer 2003 (A), autumn 2003 (B) and summer 2004 (C). Peak size is given in base pairs, the relative abundance of T-RFs in percentage of total peak height. *A. p.*, *Alopecurus pratensis*; *A. 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*.

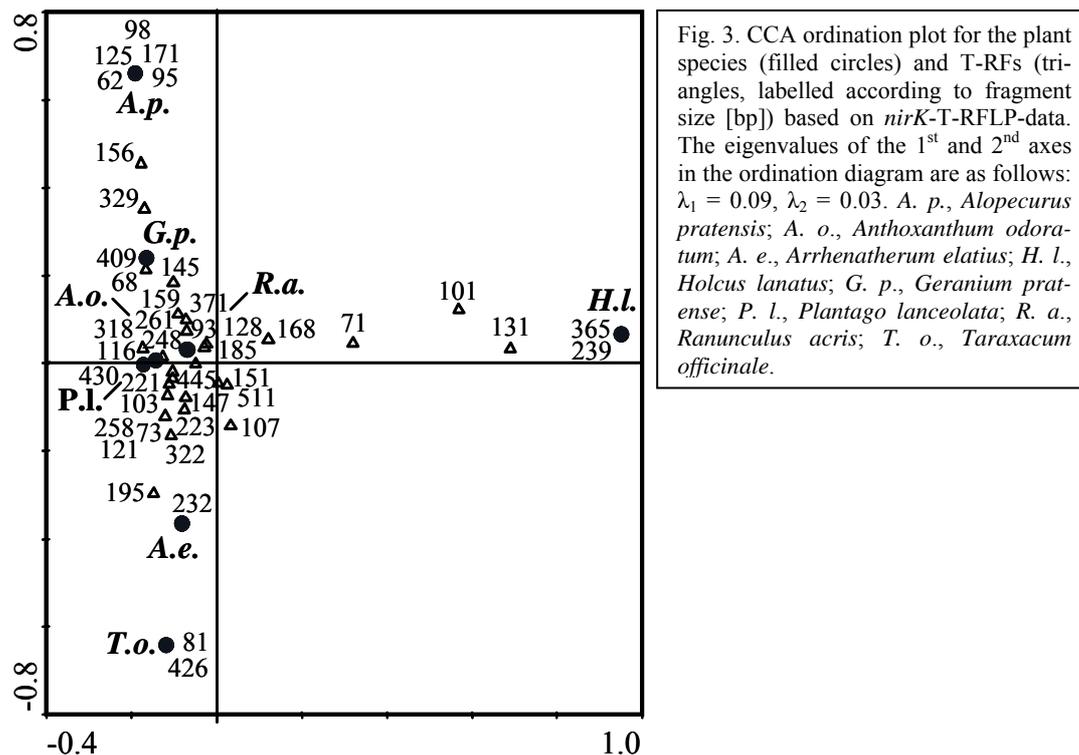
Some fragments of comparably high relative abundance (e.g. T-RFs of 128, 151, 168, 185 and 511 bp) were detected in soil of all microcosms at all sampling times (Fig. 1), but varied in their relative abundances depending on the plant species and the sampling time. In contrast, less abundant fragments occurred both in relation to some of the plant species at all sampling times (e.g. the 131 bp T-RF with *Holcus lanatus*) and in relation to plant species at two of the three sampling times (e.g. the 81 bp T-RF with *Taraxacum officinale* in summer 2003 and with *Plantago lanceolata* in summer 2004). In addition, less abundant fragments also occurred in relation to a single plant species at one sampling time (e.g. the 125 bp T-RF with *Alopecurus pratensis* in summer 2003).

The T-RFLP dataset was analysed by ordination techniques (i.e. CA and CCA) that reflected the nested design of the experiment. The results of CA or CCA can be visualized in an ordination diagram (the CA ordination diagrams that correspond to the T-RFLP-histograms (Fig. 1) are shown in the appendix (Fig. 1, 2 and 3)). The functional group of a plant (grasses vs. forbs) did not affect the *nirK*-type denitrifier community in the soil ($P = 0.60$; Monte Carlo permutation test within CCA) (Fig. 2). The *nirK*-type denitrifier communities in soil planted with grasses were not clearly separated from denitrifier communities in soil planted with forbs.



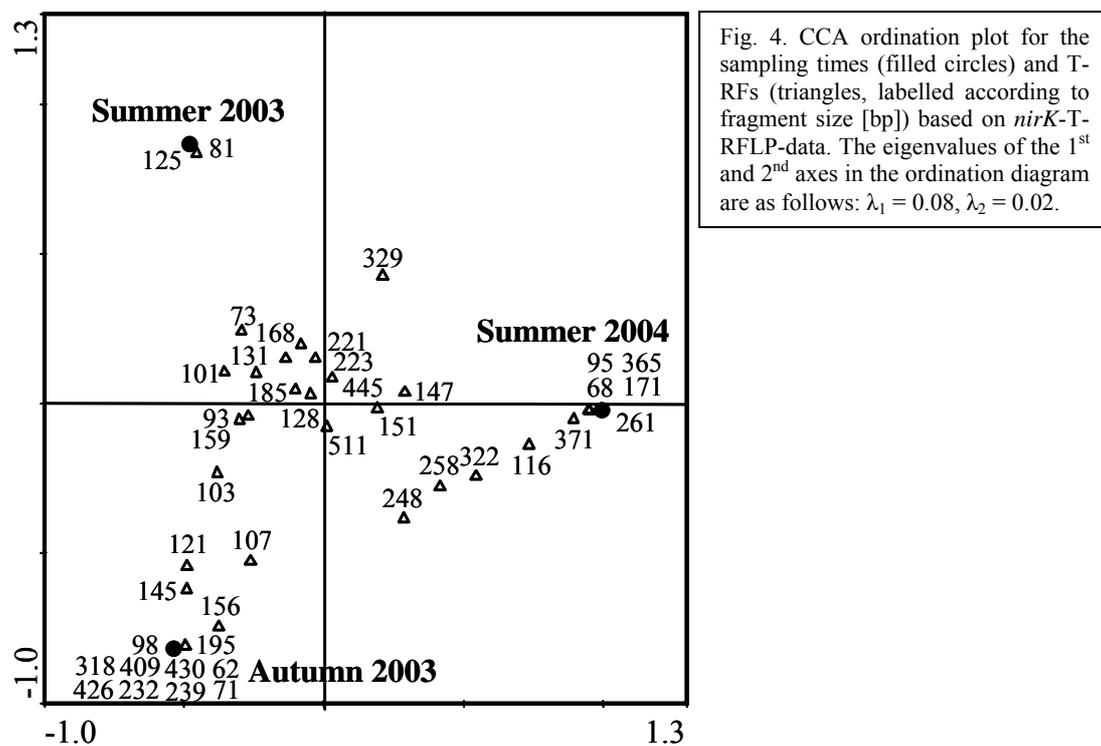
The community in the soil of *Holcus lanatus* was very dissimilar from the other ones and therefore positioned distantly. The communities in the soil of *Alopecurus pratensis* and *Taraxacum officinale* were also positioned distantly from those of the other plants. The communities in the soils of *Anthoxanthum odoratum* and *Plantago lanceolata* were the most similar ones. Although the communities in soil of three of the forbs (*Plantago lanceolata*, *Ranunculus acris* and *Geranium pratense*) were positioned closely together, the community in soil of the third forb (*Taraxacum officinale*) was placed distantly from those.

In contrast to the plant functional group, individual plant species influenced the relative abundance of *nirK*-T-RFs ($P = 0.0002$; Monte Carlo permutation test) (Fig. 3). In the ordination diagram T-RFs scattering around plant species indicate *nirK*-OTUs that typically occurred with a given plant species. Plant species with a similar soil denitrifier community are positioned closely together.



For instance, soil from the plants *Plantago lanceolata*, *Anthoxanthum odoratum* and *Ranunculus acris* had a similar denitrifier community, whereas that from *Holcus lanatus* was very different from that of all other plant species. T-RFs placed in the center of the diagram either represent *nirK* operational taxonomic units (OTUs) occurring in the soil of all plant species or those that occur in the soil of *Ranunculus acris*.

There was also a general effect of sampling time on the composition of the *nirK*-type denitrifier soil community ($P = 0.0002$; Monte Carlo permutation test within CCA) (Fig. 4).



Interestingly, the communities of *nirK*-type denitrifiers in the soil sampled in the two summers 2003 and 2004 were as different from each other as they were from 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 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*. I also observed that *nirK*-OTUs which were unrelated to sampling time matched with *nirK*-OTUs unrelated to plant species (e.g. T-RFs of 128, 151, and 185 bp) indicating the general occurrence of the respective denitrifiers in the given soil.

When sampling time was defined as a linear factor to analyse whether there was a continuous development of the community over time, it also affected the composition of the *nirK*-type denitrifier soil community (the linear trend component was $P = 0.0002$; Monte Carlo permutation test within CCA) (ordination diagram not shown, because of high complexity), but the interactions of time as a linear factor with plant functional group and with plant identity were not significant ($P = 0.96$ and $P = 0.08$; Monte Carlo permutation test).

In addition, the interaction of plant functional group and sampling time on soil denitrifier community composition was not significant ($P = 0.42$; Monte Carlo per-

mutation test within CCA). However, the interaction of plant species identity and time influenced soil denitrifier composition ($P = 0.003$; Monte Carlo permutation test within CCA) indicating that the effects of individual plant species varied with sampling time (see appendix Fig. 4 for the ordination diagram).

4.1.2 Richness, Shannon diversity, and Shannon evenness of the *nirK*-type denitrifier communities

The diversity and evenness of a community can be described by diversity indices. Here, richness (i.e. the number of *nirK*-OTUs), Shannon diversity and Shannon evenness indices were used as additional characters to analyse differences between the *nirK*-containing denitrifier communities. The impact of the experimental factors (i.e. plant functional group, plant species, sampling time, and interactions between these factors) on denitrifier diversity indices was tested by nested ANOVAs according to the hierarchical experimental design (Table 8).

Table 8. Effects of the experimental factors (plant functional group etc.) on diversity indices according to the nested experimental design. The effects of plant functional group were tested against the variation among plant species identities, that of plant identity against the variation among the individual microcosms, and the effect of time against the residual. The effect of the interaction between plant group and time was tested against the interaction between plant identity and time. ANOVA, (+) $P < 0.1$; (*) $P < 0.05$; n.s. not significant.

	F-values		
	Richness (i.e. number of <i>nirK</i> -OTUs)	Shannon diversity index	Shannon evenness index
Plant func. group	6.90 (*)	2.06 n.s.	0.08 n.s.
Plant identity	1.02 n.s.	1.70 n.s.	2.88 (*)
Microcosm	1.07 n.s.	1.08 n.s.	0.95 n.s.
Sampling time	3.71 (*)	2.31 n.s.	0.90 n.s.
Plant group x time	3.27 (+)	0.86 n.s.	0.74 n.s.
Plant identity x time	0.73 n.s.	1.68 n.s.	2.15 (*)

Richness, i.e. the number of *nirK*-OTUs, was affected by plant functional group, sampling time and the interaction between these two factors. The mean richness was 10.55 in soil of grasses and 11.58 in soil of forbs, and it was highest in autumn 2003 (11.97). Shannon diversity was unrelated to the factors tested.

Shannon evenness was influenced by plant identity and the interaction of plant identity with time. The mean evenness of *nirK*-OTUs was highest in soil of *Geranium pratense* (0.792) and lowest in soil of *Alopecurus pratensis* (0.715). The evenness for all plant species was highest in summer 2003 (mean 0.747).

The results of the phylogenetic analysis of amplified *nirK* genes from the plant microcosm soil and the assignment of theoretically to experimentally derived T-RFs are shown and described together with the results of the planted mesocosms in figure 14, table 20 and chapter 4.2.8.

4.2 Planted mesocosms (lysimeters)

Soil samples of the mesocosms in winter 2003, summer 2004 and autumn 2004 contained 14-16, 6-11, and 5-9% water [% weight], respectively. To equilibrate the differences in water content before the measurements, the corresponding amount of soil (i.e. dry weight) was taken.

4.2.1 Soil nitrate concentrations

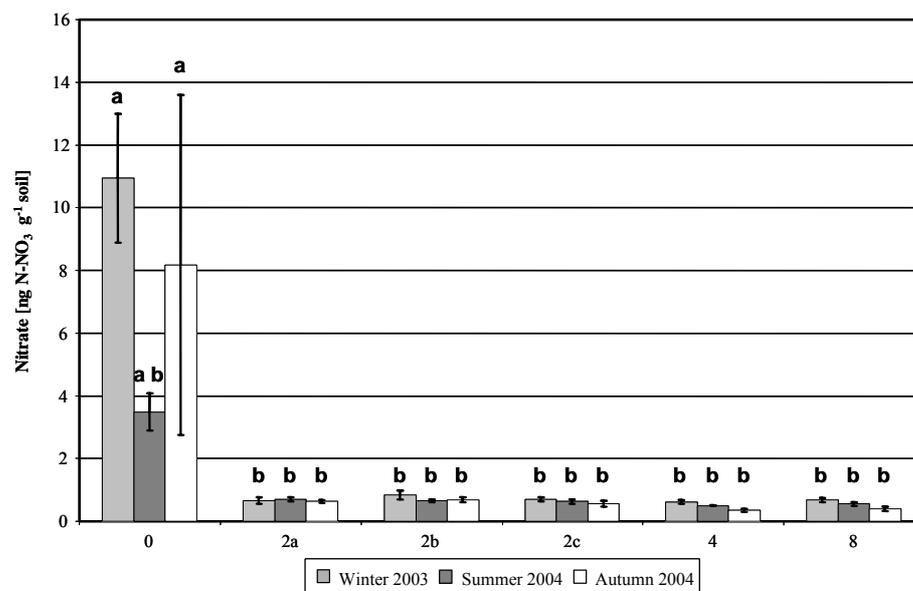


Fig. 5. Nitrate concentrations of mesocosm soil samples from the upper soil layer (0-7 cm). Data are means (n=5, plot 0 n=3), bars indicate standard errors of the mean. Different letters indicate significant differences (Tukey's test, $P < 0.05$). (0, unplanted; 2a, *Holcus lanatus* and *Arrhenatherum elatius*; 2b, *Holcus lanatus* and *Geranium pratense*; 2c, *Holcus lanatus* and *Plantago lanceolata*; 4, *Holcus lanatus*, *Arrhenatherum elatius*, *Geranium pratense*, *Plantago lanceolata*; 8, like 4, and *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Taraxacum officinale*, *Ranunculus acris*).

In winter 2003 and autumn 2004, but not in summer 2004, nitrate concentrations in the upper soil layer of unplanted mesocosms were significantly higher than in the upper soil layer of planted mesocosms (Fig. 5). Among the planted mesocosms no significant differences in nitrate concentrations were found in the soils in winter 2003, summer 2004, and autumn 2004. Plant diversity, plant combination, and the interaction of plant diversity with time significantly affected nitrate concentrations (Table 9). Increasing plant diversity was related with less nitrate concentrations in mesocosm soils, but this effect varied with sampling time and was mainly a result of differences between unplanted and planted mesocosms at the three sampling times (Fig. 5).

Table 9. Effects of the experimental factors (plant diversity etc.) on nitrate concentrations according to the nested experimental design. The effects of plant diversity were tested against the variation among plant combinations, that of plant combinations against the variation among the individual mesocosms, and the effect of time against the residual. The effect of the interaction between plant diversity and time was tested against the interaction between plant combination and time. Plant diversity was used as a covariable. ANCOVA, (+) $P < 0.1$; $P (*) < 0.05$; $P (***) < 0.001$; n.s. not significant.

	F-value
(log) Plant diversity	5.77 (+)
Plant combination	29.82 (***)
Mesocosm	0.25 n.s.
Sampling time	1.34 n.s.
(log) Div. x time	5.81 (*)
Plant comb. x time	0.71 n.s.

4.2.2 Net- and gross N₂O production rates and denitrifier enzyme activity (DEA)

As outlined in chapter 3.5, soil slurries were prepared to measure net- and gross-production rates of N₂O and to determine the denitrifier enzyme activity (DEA). The measured N₂O production rates were not in situ rates, but measured under anoxic conditions with standardized temperature and water content. The pH-values in all soil slurries were 6.1-6.4. The amounts of substrate were in situ.

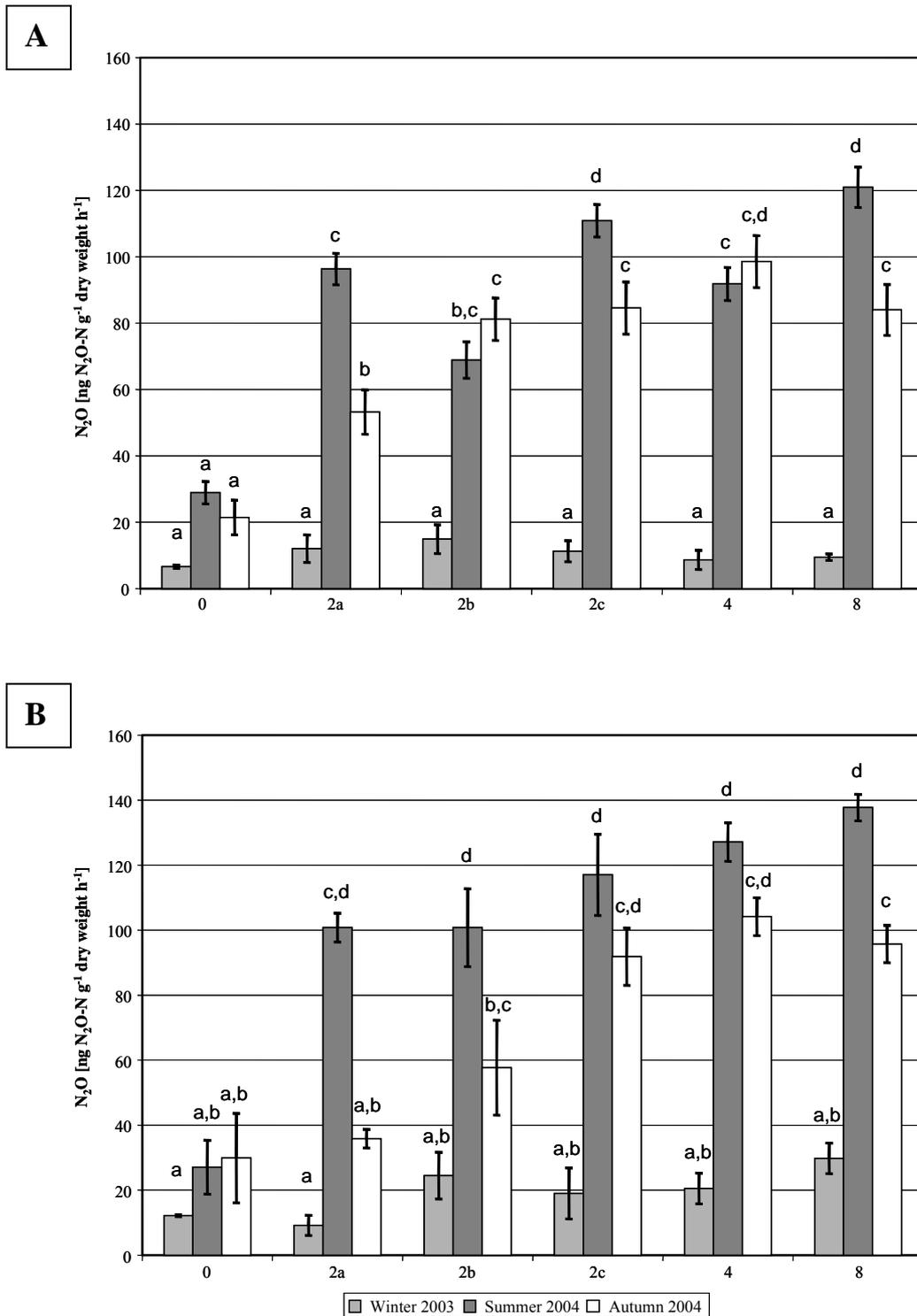


Fig. 6. N_2O production rates of mesocosm soil. A, net production; B, gross production. Data are means ($n=5$, plot 0 $n=3$), bars indicate standard errors of the mean. Different letters indicate significant differences (Tukey's test, $P < 0.05$). (0, unplanted; 2a, *Holcus lanatus* and *Arrhenatherum elatius*; 2b, *Holcus lanatus* and *Geranium pratense*; 2c, *Holcus lanatus* and *Plantago lanceolata*; 4, *Holcus lanatus*, *Arrhenatherum elatius*, *Geranium pratense*, *Plantago lanceolata*; 8, like 4, and *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Taraxacum officinale*, *Ranunculus acris*).

In unplanted soil, net- and gross N_2O production rates did not show any significant differences at the three sampling times (Fig. 6). In addition, both rates of unplanted

soil were similar to the rates of planted soils in winter 2003. In summer and autumn of the following year both rates were significantly different between unplanted and planted soil (with the exception of soil in mesocosm 2a in autumn). Among the planted soils net N₂O production differed among plant combinations and between sampling times. Gross N₂O production rates of planted soil were similar to each other in summer and were similar to each other in autumn (with the exception of 2a). Generally, both rates were lowest in winter, intermediate in autumn and highest in summer.

In two cases, gross production rates were surprisingly smaller than net production rates (2a and 2b in autumn). The other mesocosm soils showed relatively similar or higher gross production rates than net production rates.

Plant diversity, plant combination, sampling time and the interaction of plant diversity with time significantly influenced the net and gross N₂O production rates (Table 10). The interaction of plant combination with time only influenced the net N₂O production. Unfortunately, the individual mesocosms also influenced the net N₂O production indicating a high variability between replicate mesocosms.

Table 10. Effects of experimental factors (plant diversity etc.) on N₂O production rates according to the nested experimental design. Plant diversity was used as a covariable. ANCOVA, (+) P < 0.1; P (*) < 0.05; P (***) < 0.001; n.s. not significant.

	F-values	
	Net N ₂ O production	Gross N ₂ O production
(log) Plant diversity	12.9 (*)	14.3 (*)
Plant combination	2.7 (+)	3.7 (*)
Mesocosm	4.2 (***)	1.6 n.s.
Sampling time	220.4 (***)	81.1 (***)
(log) Div. x time	3.4 (+)	4.5 (*)
Plant comb. x time	6.7 (***)	1.8 n.s.

Net and gross N₂O production rates were found to be linearly related (Fig. 7) in mesocosm soil. Gross N₂O production rates were fifteen times higher than net N₂O production rates.

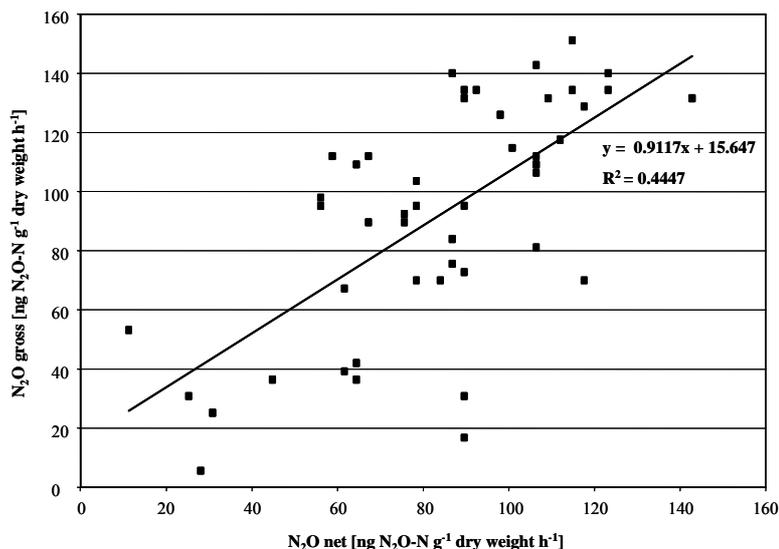


Fig. 7. Scatter plot and linear regression of net and gross N₂O production rates of mesocosm soil.

The DEA method was used to estimate the potential activity of pre-existing denitrifying enzymes in soil. DEA was determined by measuring the rate of N₂O production in the presence of acetylene from soil samples placed under anaerobic conditions and supplied with excess carbon source (glucose) and nitrate. Chloramphenicol was added to inhibit the growth of bacteria and the synthesis of new denitrifying enzymes.

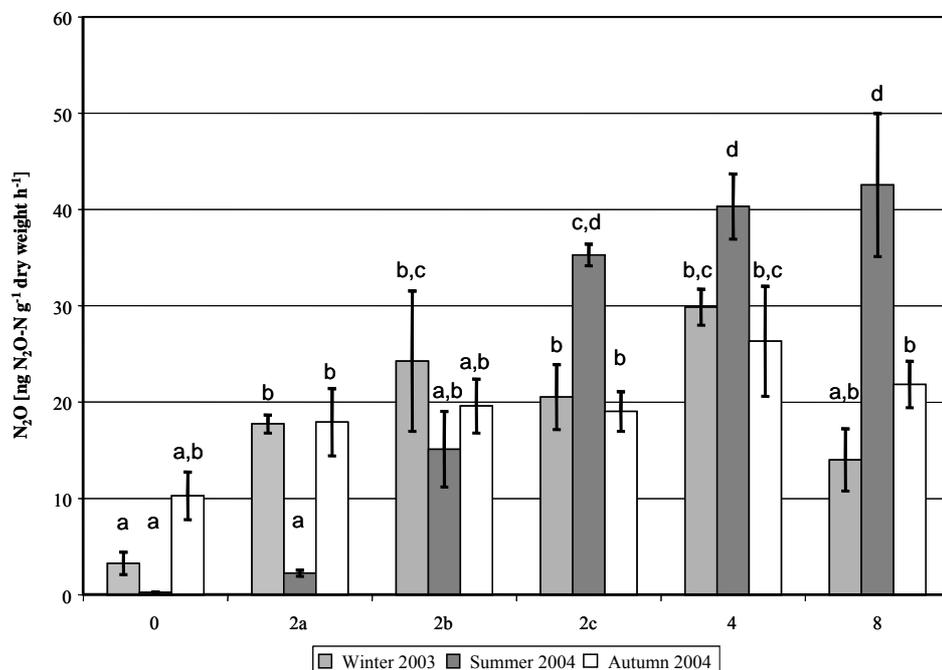


Fig. 8. Denitrifying enzyme activity of mesocosm soil. Data are means (n=5, plot 0 n=3), bars indicate standard errors of the mean. Different letters indicate significant differences (Tukey's test, P < 0.05). (0, unplanted; 2a, *Holcus lanatus* and *Arrhenatherum elatius*; 2b, *Holcus lanatus* and *Geranium pratense*; 2c, *Holcus lanatus* and *Plantago lanceolata*; 4, *Holcus lanatus*, *Arrhenatherum elatius*, *Geranium pratense*, *Plantago lanceolata*; 8, like 4, and *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Taraxacum officinale*, *Ranunculus acris*).

DEA was about half as high as net and gross N₂O production rates (Fig. 8), either because it was measured from 0 to 5 h (the net and gross rates were measured from 0 to 8-10 h) or because the growth of the denitrifiers and the synthesis of new denitrifying enzymes were efficiently inhibited by chloramphenicol.

In unplanted soil the denitrifying enzyme activity was generally lower than in planted soil, and it did not differ between winter, summer and autumn (Fig. 8). The enzyme activities in winter and autumn were similar within planted soils, whereas in summer a clear trend of higher activity in soils with increasing plant diversity was observed.

Plant diversity, plant combination, sampling time and the interaction of plant combination with sampling time significantly affected denitrifier enzyme activity (Table 11).

Table 11. Effects of experimental factors (plant diversity etc.) on denitrifier enzyme activity according to the nested experimental design. Plant diversity was used as a covariable. ANCOVA, (+) $P < 0.1$; $P (*) < 0.05$; $P (***) < 0.001$; n.s. not significant.

	F-value
(log) Plant diversity	7.4 (+)
Plant combination	10.4 (***)
Mesocosm	0.9 n.s.
Sampling time	3.9 (*)
(log) Div. x time	2.8 n.s.
Plant comb. x time	5.3 (***)

4.2.3 Most probable number (MPN) of nitrate reducing microorganisms

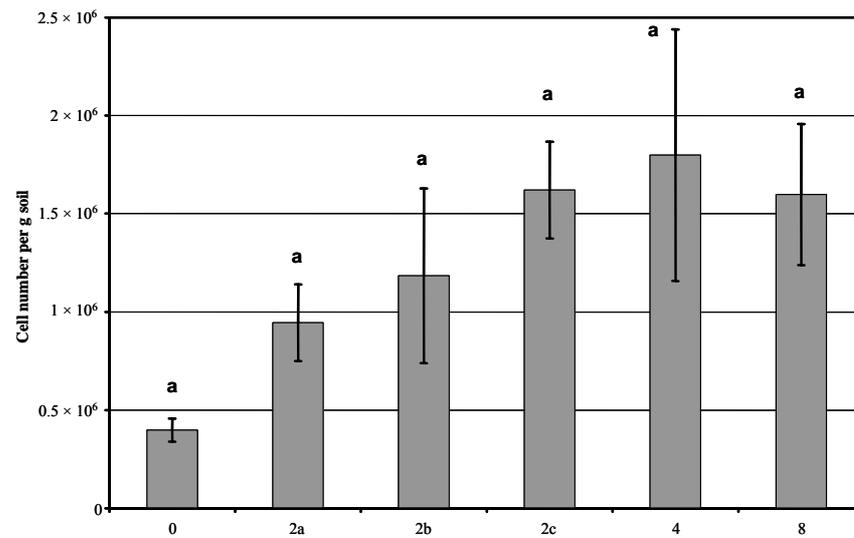


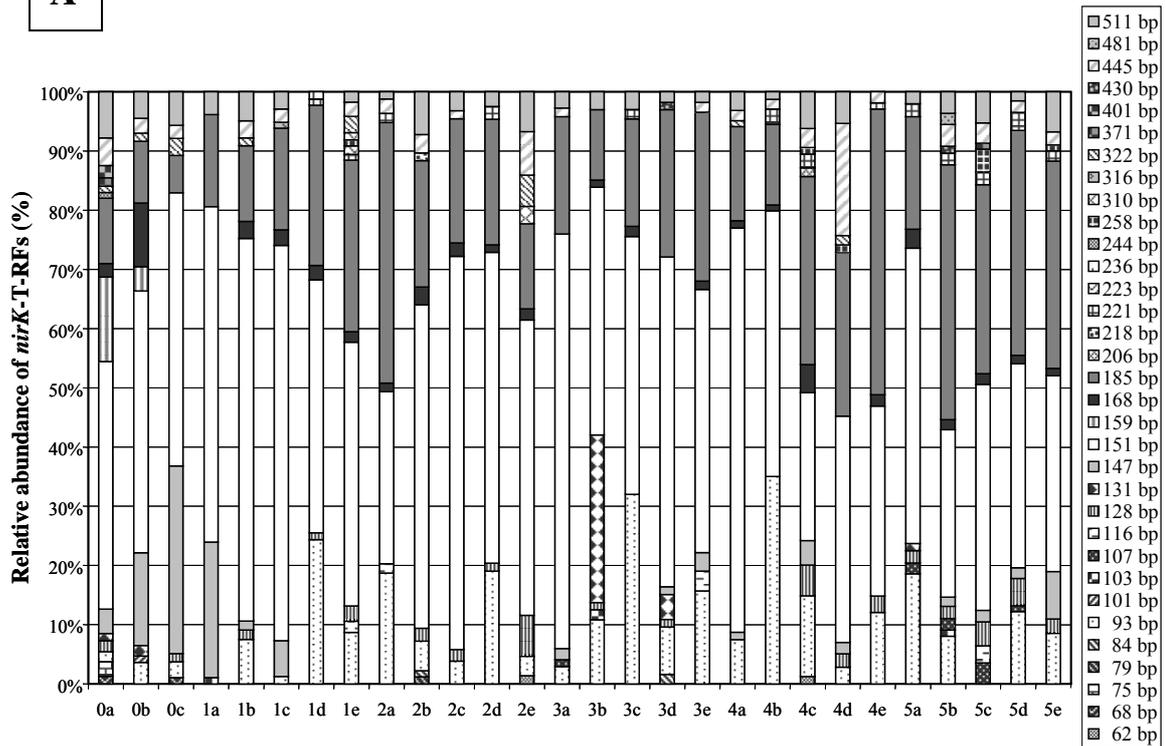
Fig. 9. MPNs of mesocosm soil samples in summer 2004. Data are means ($n=5$, plot 0 $n=3$), bars indicate standard errors of the mean. Different letters indicate significant differences (Tukey's test, $P < 0.05$). (0, unplanted; 2a, *Holcus lanatus* and *Arrhenatherum elatius*; 2b, *Holcus lanatus* and *Geranium pratense*; 2c, *Holcus lanatus* and *Plantago lanceolata*; 4, *Holcus lanatus*, *Arrhenatherum elatius*, *Geranium pratense*, *Plantago lanceolata*; 8, like 4, and *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Taraxacum officinale*, *Ranunculus acris*).

The most probable number (MPN) method was used to determine the number of nitrate reducing microorganisms in the mesocosm soils in summer 2004. No significant different MPNs were detected in the mesocosm soils (Fig. 9), and plant diversity and composition were unrelated to the most probable number of nitrate reducing microorganisms (F-values 2.36 and 0.77 for the effect of plant diversity and for the effect of plant combination, respectively).

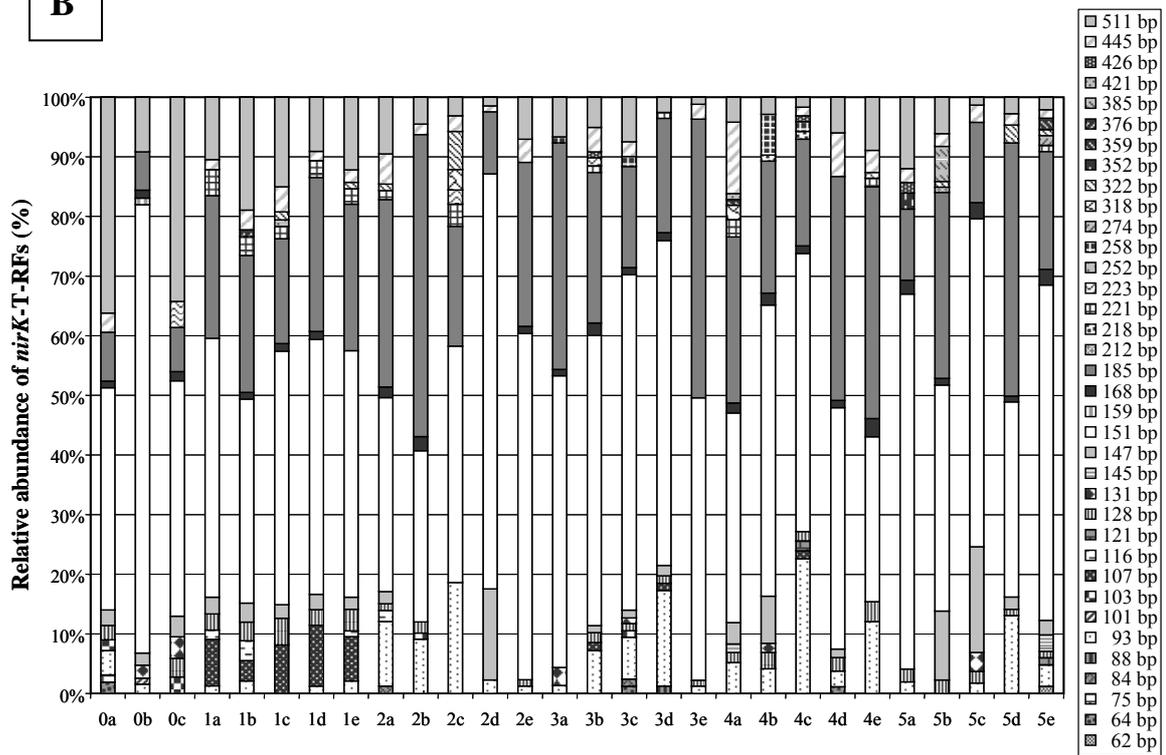
4.2.4 Influence of plant diversity, plant combination, and sampling time on the *nirK*-type denitrifier communities

NirK genes were successfully amplified from the soil of all plant mesocosms, and the *nirK*-type denitrifier community was subsequently resolved by T-RFLP. The T-RF-profiles of soil from replicate mesocosms planted with the same species were similar indicating that the results were reproducible and representative for the denitrifier community in the soil of these mesocosms. T-RFLP analysis showed marked differences in the structure of the denitrifier community in the soil of mesocosms planted with different plants (Fig. 10).

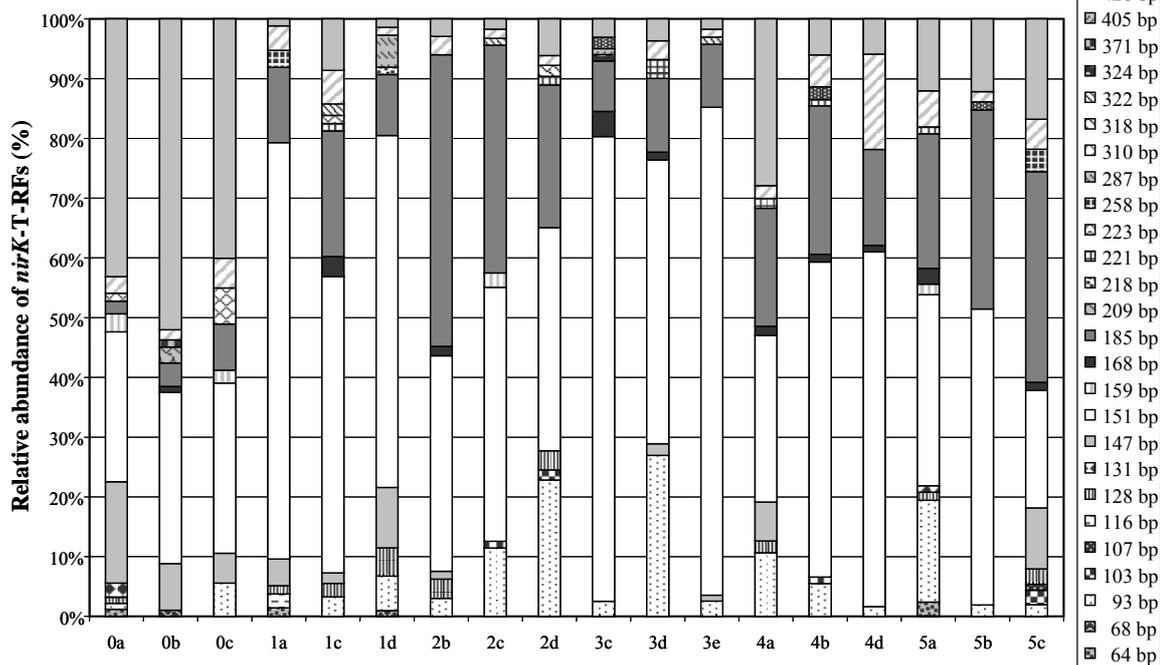
A



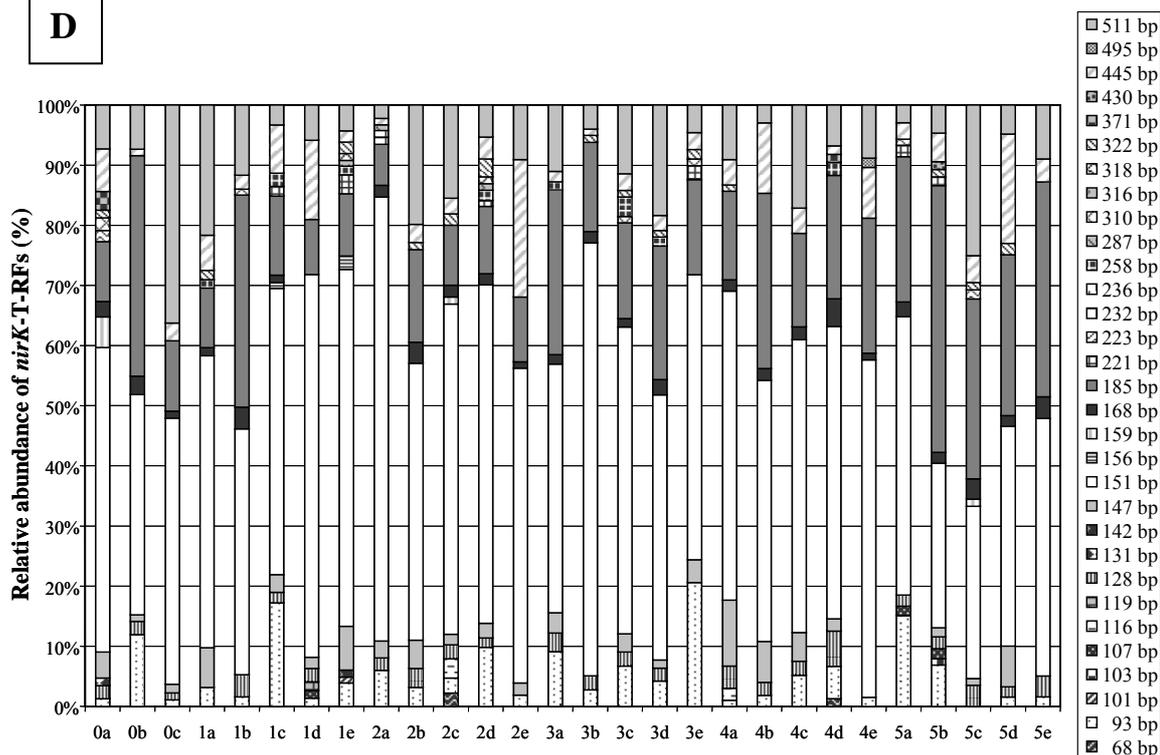
B



C



D



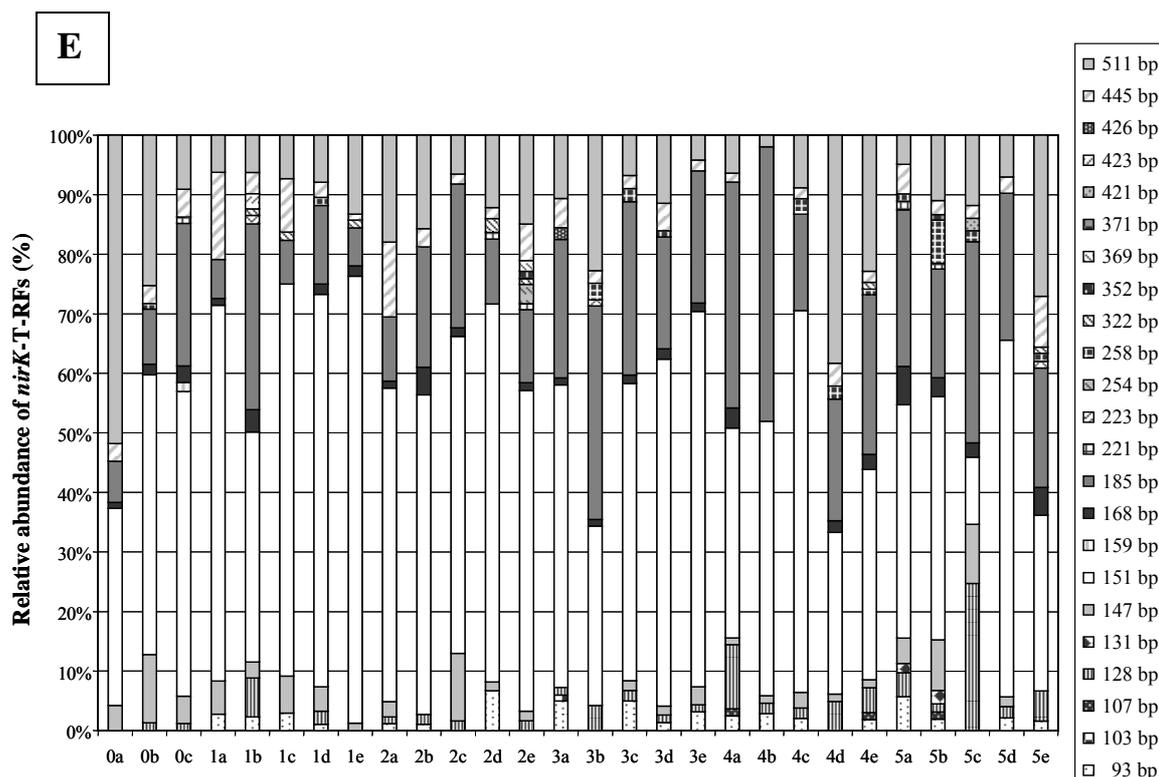
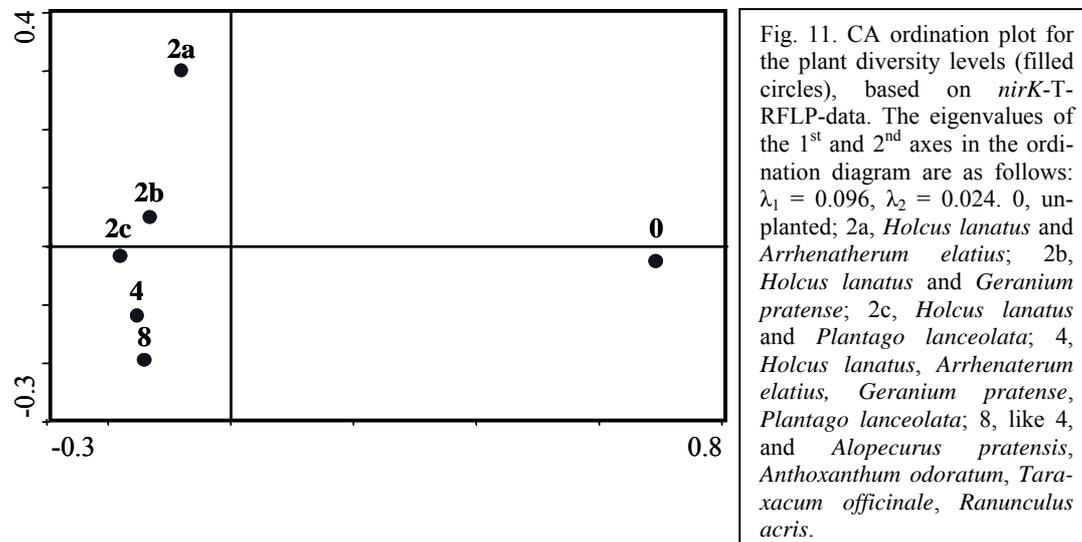


Fig. 10. Relative abundance of *nirK* terminal restriction fragments (T-RFs) from soil of plant mesocosms in summer 2003 (A), autumn 2003 (B), winter 2003 (C), summer 2004 (D), and autumn 2004 (E). Peak size is given in base pairs, the relative abundance of T-RFs in percentage of total peak height. 0, unplanted; 1a-e, *Holcus lanatus* and *Arrhenatherum elatius*; 2a-e, *Holcus lanatus* and *Geranium pratense*; 3a-e, *Holcus lanatus* and *Plantago lanceolata*; 4a-e *Holcus lanatus*, *Arrhenatherum elatius*, *Geranium pratense*, *Plantago lanceolata*; 5a-e, like 4, and *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Taraxacum officinale*, *Ranunculus acris*.

Like in the soil of plant microcosms, some fragments of comparably high relative abundance (e.g. T-RFs of 93, 151, and 185 bp) were detected in soil of all mesocosms at all sampling times (Fig. 10), but varied in their relative abundances depending on the plant combination. In contrast, less abundant fragments occurred both in relation to several plant combinations at all sampling times (e.g. the 322 bp T-RF was found most often in the combination of *Holcus* and *Geranium*, but also in other combinations) and in relation to a single plant combination at one sampling time (e.g. the 401 bp T-RF of unplanted soil in summer 2003).

The T-RFLP-dataset was analysed by ordination techniques (CA and CCA) that reflected the nested design of the experiment (CA ordination diagrams corresponding to the single T-RFLP-histograms (Fig. 10) are shown in the appendix (Fig. 5, 6, 7, 8, 9). Plant diversity affected the relative abundance of *nirK*-type denitrifiers in the mesocosm soils ($P = 0.0334$; Monte Carlo permutation test within CCA) (Fig. 11). Only four levels of diversity were used in the experiment (0, 2, 4, and 8 plant species). The denitrifier community of unplanted soil was clearly separated from the communities

of planted soil. Soil planted with *Holcus lanatus* and *Arrhenatherum elatius* (2a) was also different from the other planted soils, whereas soil planted with 4 and 8 plant species had a similar denitrifier community.



Plant combination also influenced the relative abundance of *nirK*-type denitrifiers ($P = 0.0002$; Monte Carlo permutation test) (Fig. 12). In the ordination diagram, T-RFs scattering around plant combination points indicate *nirK*-OTUs that typically occurred with a given plant combination. Unplanted soil had a very dissimilar denitrifier community composition compared to planted soil. Soil from the plant combinations 4 and 8 had a quite similar denitrifier community, whereas that from 2a was very different from those of all other plant combinations. T-RFs placed in the center of the diagram either represent *nirK* operational taxonomic units (OTUs) occurring in the soil of all plant combinations or they represent those that occur mainly in soil of plant combinations 2b and 2c.

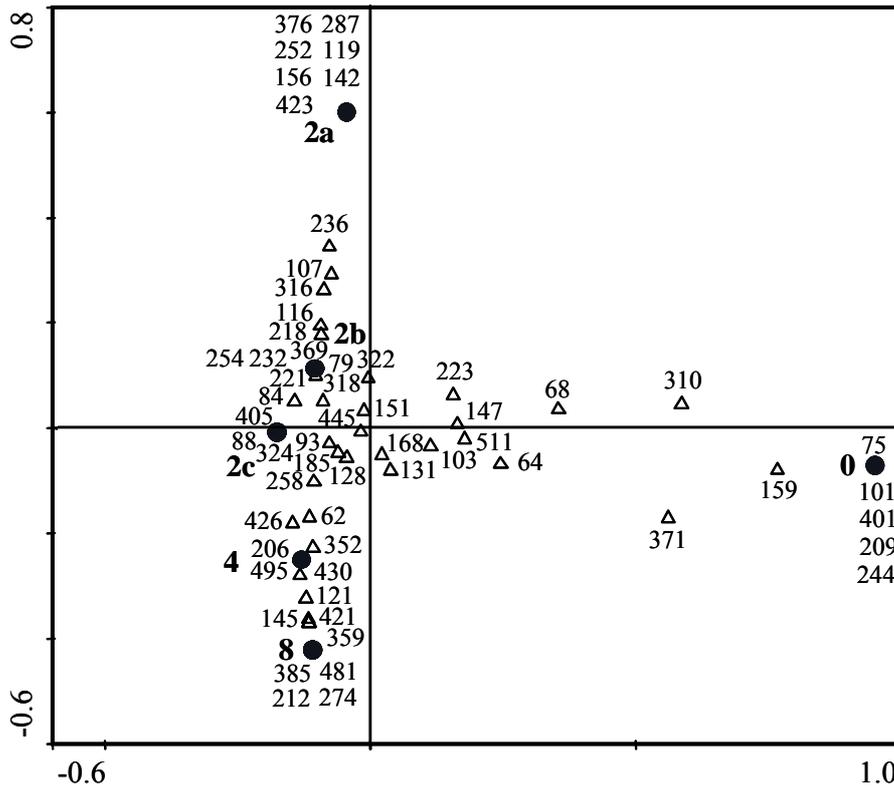


Fig. 12. CCA ordination plot for the plant combinations (filled circles) and T-RFs (triangles, labelled according to fragment size [bp]), based on *nirK*-T-RFLP-data. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.078$, $\lambda_2 = 0.025$. 0, unplanted; 2a, *Holcus lanatus* and *Arrhenatherum elatius*; 2b, *Holcus lanatus* and *Geranium pratense*; 2c, *Holcus lanatus* and *Plantago lanceolata*; 4, *Holcus lanatus*, *Arrhenatherum elatius*, *Geranium pratense*, *Plantago lanceolata*; 8, like 4, and *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Taraxacum officinale*, *Ranunculus acris*.

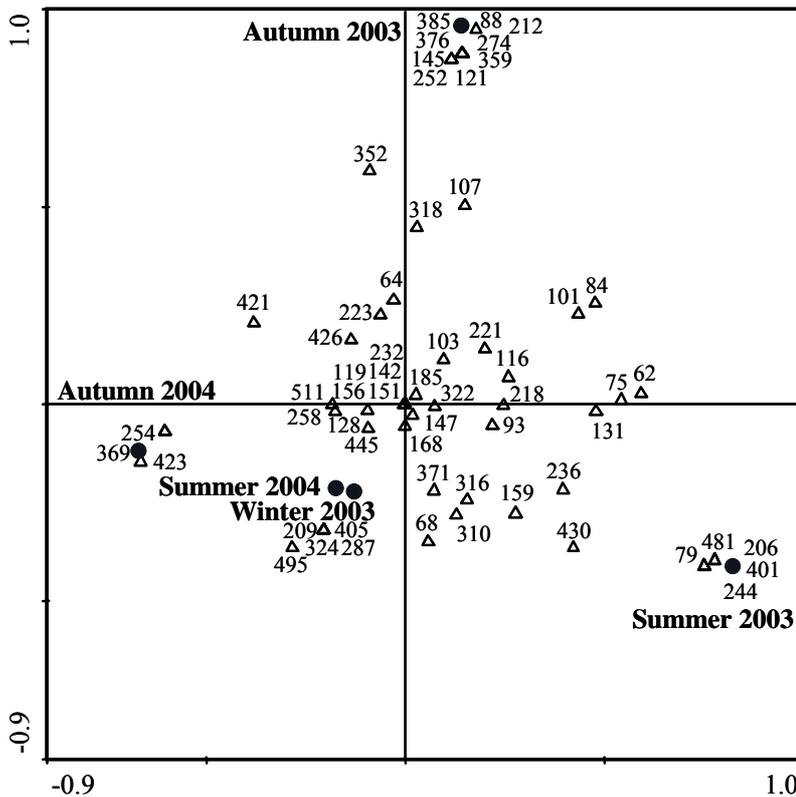


Fig. 13. CCA ordination plot for the sampling times (filled circles) and T-RFs (triangles, labelled according to fragment size [bp]), based on *nirK*-T-RFLP-data. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.042$, $\lambda_2 = 0.018$.

A general effect of sampling time on the composition of the *nirK*-type soil denitrifier community was detected ($P = 0.0002$; Monte Carlo permutation test) (Fig. 13). Interestingly, the community compositions of *nirK*-type denitrifiers in the soil sampled in summer, autumn and winter 2003 were as different from each other as they were from the samples taken in autumn 2004, but the community compositions of *nirK*-type denitrifiers in the soil sampled in winter 2003 and summer 2004 were similar indicating that effects of mesocosm development were at least as important as seasonal effects.

Many *nirK*-OTUs were placed at intermediate positions demonstrating their association with two or more sampling times. For instance, the 430 bp fragment was found in the summers 2003 and 2004 in soil planted with 8 plant species. I also observed that *nirK*-OTUs which were unrelated to sampling time matched with *nirK*-OTUs unrelated to plant combinations (e.g. T-RFs of 151, 168, and 185 bp) indicating the general occurrence of the respective denitrifiers in the given soil.

In addition to the effect of sampling time, time as a linear factor (to analyse whether there was a continuous development of the community over time) also influenced the composition of *nirK*-type denitrifiers in mesocosm soil (the linear trend component was $P = 0.0002$; Monte Carlo permutation test; ordination diagram not shown, because of high complexity), but the interactions of time as a linear factor with plant diversity and with plant combination were both not significant ($P = 0.52$ and $P = 0.34$; Monte Carlo permutation tests).

However, the general interactions of time with plant diversity and plant combination were both significant ($P = 0.0022$ and $P = 0.001$; Monte Carlo permutation tests) indicating that the effects of plant diversity and combination varied with sampling time (see appendix Fig. 10 for the ordination diagram of the interaction of plant diversity with time; the ordination diagram of the interaction of plant combination with time is not shown due to high complexity).

The two summers and autumns of 2003 and 2004 were chosen to further scrutinize the effects of sampling time on the community composition of *nirK*-type denitrifiers. The denitrifier community composition differed significantly between 2003 and 2004, independent of the season ($P = 0.0002$; Monte Carlo permutation test; ordination diagram not shown). Furthermore, the denitrifier community composition in both summers was found to be significantly different from that in both autumns ($P = 0.0006$; Monte Carlo permutation test; ordination diagram not shown), but the inter-

action of season with year was not significant ($P = 0.70$; Monte Carlo permutation test). The last finding indicates that the differences between summer and autumn in one year were quite similar to the differences between summer and autumn in the second year.

4.2.5 Richness, Shannon diversity, and Shannon evenness of the *nirK*-type denitrifier communities

Richness (i.e. the number of *nirK*-OTUs), Shannon diversity and Shannon evenness indices were used as additional characters to describe the structure of the *nirK*-type denitrifier communities. The impact of the experimental factors (i.e. plant diversity, plant combination, sampling time, and interactions between these factors) on denitrifier diversity indices was tested by ANOVA according to the hierarchical experimental design (Table 12).

Table 12. Effects of experimental factors (plant diversity etc.) on diversity indices according to the nested experimental design. The effects of plant diversity were tested against the variation among plant combinations, that of plant combinations against the variation among the individual mesocosms, and the effect of time against the residual. The effect of the interaction between plant diversity and time was tested against the interaction between plant combination and time. ANOVA, (+) $P < 0.1$; n.s. not significant.

	F-values		
	Richness (i.e. number of <i>nirK</i> -OTUs)	Shannon diversity index	Shannon evenness index
log Plant diversity	2.46 n.s.	3.44 n.s.	3.43 n.s.
Plant combination	0.94 n.s.	2.35 n.s.	3.23 (+)
Mesocosm	1.06 n.s.	0.60 n.s.	0.38 n.s.
Sampling time	2.17 (+)	1.00 n.s.	0.26 n.s.
Plant div. x time	1.46 n.s.	0.73 n.s.	0.77 n.s.
Plant comb. x time	0.81 n.s.	1.43 n.s.	1.49 n.s.

Richness was only affected by sampling time, being highest in autumn 2003 (mean 9.9) and lowest in autumn 2004 (mean 8.6). Shannon diversity was unrelated to the

factors tested. Shannon evenness was only affected by plant combination. It was highest in soil planted with 8 plant species (mean 0.69) and lowest in soil planted with *Holcus lanatus* and *Plantago lanceolata* (mean 0.61).

4.2.6 Interactions of denitrifier diversity and functioning

To detect possible interactions between the denitrifier enzyme activity and N₂O production rates, DEA values were included in the nested ANOVA (Table 13).

Table 13. Effects of the factors (DEA etc.) on N₂O production rates according to the nested experimental design. Plant diversity and DEA were used as covariables. ANCOVA, (+) P < 0.1; P (*) < 0.05; P (***) < 0.001; n.s. not significant.

	F-values	
	Net N ₂ O production	Gross N ₂ O production
DEA	173 (***)	80.6 (***)
(log) Plant diversity	5.4 (+)	11.0 (*)
Plant combination	1.6 n.s.	1.2 n.s.
Mesocosm	5.1 (***)	2 (*)
Sampling time	219 (***)	72.8 (***)
(log) Div. x time	2.7 n.s.	4.3 (+)
Plant comb. x time	7.9 (***)	1.9 (*)

Denitrifier enzyme activity influenced net and gross N₂O production rates (see appendix Fig. 11 and 12 for scatter plots of net and gross rates with DEA), but the effects of the other factors on the rates did not change substantially (see Table 10 and 11 for comparison). Only the effect of plant combination disappeared.

Sample scores along the first and second axis (generated by canonical correspondence analysis) of *nirK*-OTUs were also included in the nested ANOVA to check for relationships between *nirK*-type denitrifier composition and function (Table 14, 15 and 16).

Table 14. Effects of factors (sample scores along axis 1 etc.) on N₂O production rates and denitrifier enzyme activity according to the nested experimental design. Plant diversity and sample scores along axis 1 were used as covariables. ANCOVA, (+) P < 0.1; P (*) < 0.05; P (***) < 0.001; n.s. not significant.

	F-values		
	Net N ₂ O production	Gross N ₂ O production	DEA
Axis 1	40.6 (***)	25.5 (***)	24.4 (***)
(log) Plant diversity	7.2 (+)	9.9 (*)	5.1 (+)
Plant combination	3.7 (*)	3.7 (*)	10.3 (***)
Mesocosm	3.9 (***)	1.6 n.s.	1.0 n.s.
Sampling time	225.4 (***)	83.9 (***)	4.1 (*)
(log) Div. x time	2.0 n.s.	3.1 n.s.	10.3 (***)
Plant comb. x time	5.9 (***)	2.0 (+)	5.7 (***)

Sample scores along axis 1 had a significant effect on net and gross N₂O production rates and on the denitrifier enzyme activity. The effects of plant combination remained, even when the effects of denitrifier community composition were partialled out, indicating that plant combination had additional effects on N₂O production rates and denitrifier enzyme activity that could not be explained by differences in denitrifier community composition.

Table 15. Effects of factors (sample scores along axis 2 etc.) on N₂O production rates and denitrifier enzyme activity according to the nested experimental design. Plant diversity and sample scores along axis 2 were used as covariables. ANCOVA, (+) P < 0.1; P (*) < 0.05; P (**) < 0.01; P (***) < 0.001; n.s. not significant.

	F-values		
	Net N ₂ O production	Gross N ₂ O production	DEA
Axis 2	60.2 (***)	16.1 (***)	7.0 (*)
(log) Plant diversity	11.5 (*)	13.4 (*)	7.2 (+)
Plant combination	2.7 (+)	4.3 (**)	8.2 (***)
Mesocosm	4.0 (***)	1.3 n.s.	1.2 n.s.
Sampling time	206.1 (***)	81.9 (***)	4.0 (*)
(log) Div. x time	3.8 (+)	4.2 (+)	2.3 n.s.
Plant comb. x time	6.2 (***)	2.0 (+)	5.7 (***)

Sample scores along axis 2 explained more of the variance in net production rates than sample scores along axis 1. In contrast, sample scores along axis 2 explained less variance in gross production rates and denitrifier enzyme activity than sample scores along axis 1.

Table 16. Effects of factors (sample scores along axis 1 etc.) on N₂O production rates and denitrifier enzyme activity according to the nested experimental design. Plant diversity and sample scores along axis 1 and 2 were used as covariables. ANCOVA, (+) P < 0.1; P (*) < 0.05; P (**) < 0.01; P (***) < 0.001; n.s. not significant.

	F-values		
	Net N ₂ O production	Gross N ₂ O production	DEA
Axis 1	40.3 (***)	25.5 (***)	24.4 (***)
Axis 2	60.1 (***)	16.4 (***)	7.4 (**)
(log) Plant diversity	6.7 (+)	9.1 (*)	4.8 (+)
Plant combination	3.3 (*)	4.2 (*)	8.0 (***)
Mesocosm	3.7 (***)	1.3 n.s.	1.2 n.s.
Sampling time	210.2 (***)	83.2 (***)	3.9 (*)
(log) Div. x time	2.2 n.s.	3.0 n.s.	2.6 n.s.
Plant comb. x time	5.7 (***)	2.0 (+)	5.6 (***)

In summary, sample scores along both axes and denitrifier enzyme activity explained some of the variance in net and gross N₂O production and denitrifier enzyme activity. However, the effects of the plants on both rates remained.

The relationships between diversity indices, N₂O production rates and denitrifier enzyme activity were tested by nested ANOVAs (Tables 17, 18 and 19).

Table 17. Effects of factors (richness of *nirK*-T-RFs etc.) on N₂O production rates and denitrifier enzyme activity according to the nested experimental design. Plant diversity and richness were used as covariables. ANCOVA, (+) P < 0.1; P (*) < 0.05; P (**) < 0.01; P (***) < 0.001; n.s. not significant.

	F-values		
	Net N ₂ O production	Gross N ₂ O production	DEA
Richness	12.4 (***)	10.0 (**)	0.4 n.s.
(log) Plant diversity	10.9 (*)	11.4 (*)	7.0 (+)
Plant combination	2.6 (+)	3.3 (*)	10.7 (***)
Mesocosm	4.8 (***)	2.0 (*)	1.0 n.s.
Sampling time	201.2 (***)	71.9 (***)	3.1 (+)
(log) Div. x time	3.3 (+)	4.4 (+)	2.9 n.s.
Plant comb. x time	6.6 (***)	1.8 n.s.	5.6 (***)

Table 18. Effects of factors (Shannon index of *nirK*-T-RFs etc.) on N₂O production rates and denitrifier enzyme activity according to the nested experimental design. Plant diversity and Shannon were used as covariables. ANCOVA, (+) P < 0.1; P (*) < 0.05; P (**) < 0.01; P (***) < 0.001; n.s. not significant.

	F-values		
	Net N ₂ O production	Gross N ₂ O production	DEA
Shannon index	22.0 (***)	16.6 (**)	2.0 n.s.
(log) Plant diversity	8.3 (*)	9.8 (*)	7.2 (+)
Plant combination	3.2 (*)	4.0 (*)	10.1 (***)
Mesocosm	4.6 (***)	1.7 (+)	0.9 n.s.
Sampling time	198.2 (***)	71.7 (***)	3.4 (*)
(log) Div. x time	3.5 (+)	4.7 (*)	2.9 n.s.
Plant comb. x time	6.4 (***)	1.7 n.s.	5.4 (***)

Richness influenced net and gross N₂O production rates, but not denitrifier enzyme activity. The relationship between richness and net and gross production rates is shown in the appendix (Fig. 13 and 14).

The Shannon diversity and evenness affected net and gross N₂O production rates, but not denitrifier enzyme activity. Denitrifier enzyme activity was only influenced by plant combination (and diversity), sampling time and the interaction of plant combination with sampling time.

Table 19. Effects of factors (evenness of *nirK*-T-RFs etc.) on N₂O production rates and denitrifier enzyme activity according to the nested experimental design. Plant diversity and evenness were used as covariables. ANCOVA, (+) P < 0.1; P (*) < 0.05; P (**) < 0.01; P (***) < 0.001; n.s. not significant.

	F-values		
	Net N ₂ O production	Gross N ₂ O production	DEA
Evenness	14.1 (**)	10.7 (**)	1.6 n.s.
(log) Plant diversity	8.9 (*)	11.2 (*)	7.3 (+)
Plant combination	3.5 (*)	4.4 (**)	10.3 (***)
Mesocosm	4.1 (***)	1.5 n.s.	0.9 n.s.
Sampling time	206.2 (***)	77.9 (***)	3.8 (*)
(log) Div. x time	3.5 (+)	4.4 (+)	2.8 n.s.
Plant comb. x time	6.4 (***)	1.8 n.s.	5.2 (***)

The relationships between Shannon diversity and evenness with net and gross production rates are shown in the appendix (Fig. 15, 16, 17, 18).

4.2.7 Phylogenetic analyses of amplified *nirK* genes from microcosm and mesocosm soil

Representative *nirK*-PCR products from the soil of plant microcosms and mesocosms were cloned and partial *nirK* gene fragments (516 bp) of clones were sequenced. All sequenced clones were *nirK*-like. A dendrogram was calculated using clone sequences obtained in my study as well as additional *nirK* sequences of the EMBL database, including taxonomically characterized isolates and *nirK* sequences

of unknown affiliation retrieved from soil and other habitats. The dendrogram for *nirK* from uncultured and cultured denitrifiers showed six major clusters of *nirK* sequences (Fig. 14, cluster I to VI) and several subclusters in cluster I, II and III. The overall topology was supported by neighbour joining and parsimony analyses and the clusters and subclusters were defined if sequences were consistently grouping together. To avoid crowding of the tree, I then manually removed the sequences from organisms of unknown phylogenetic affiliation except those obtained in this study. The *nirK* sequences were distributed within the six branches of the *nirK* tree. The majority of my clones (155) from soil of both habitats (plant microcosms and mesocosms) 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. TA-921 i-NH₄ also grouping in this cluster is the most closely related sequence from a cultured representative. However, the *nirK* sequences obtained in this study were not closely related to this sequence. Another abundant group of clones (21) grouped in cluster II. Part of these sequences (14) grouped in the vicinity to *nirK* from *Rhodopseudomonas palustris* (cluster IIa), while the remainder of the sequences in this cluster was related to *nirK* from *Bradyrhizobium japonicum* (cluster IIb). Cluster III contained the second most abundant group of clones (28). Within this cluster the genes from the nearest related cultured representatives are those from *Rhizobium sllae* (cluster IIIa) and *Pseudomonas* sp (cluster IIIb), while the other sequences in this cluster formed three distinct subclusters (cluster IIIc, d and e). In cluster IV two sequences from the soil of *Holcus lanatus* grouped in the vicinity, but not closely, to *nirK* from *Rhodobacter spaeroides*. Five sequences were grouped in cluster V and were affiliated with *nirK* from *Mesorhizobium* sp. In cluster VI one sequence was affiliated with *nirK* from *Alcaligenes* sp.

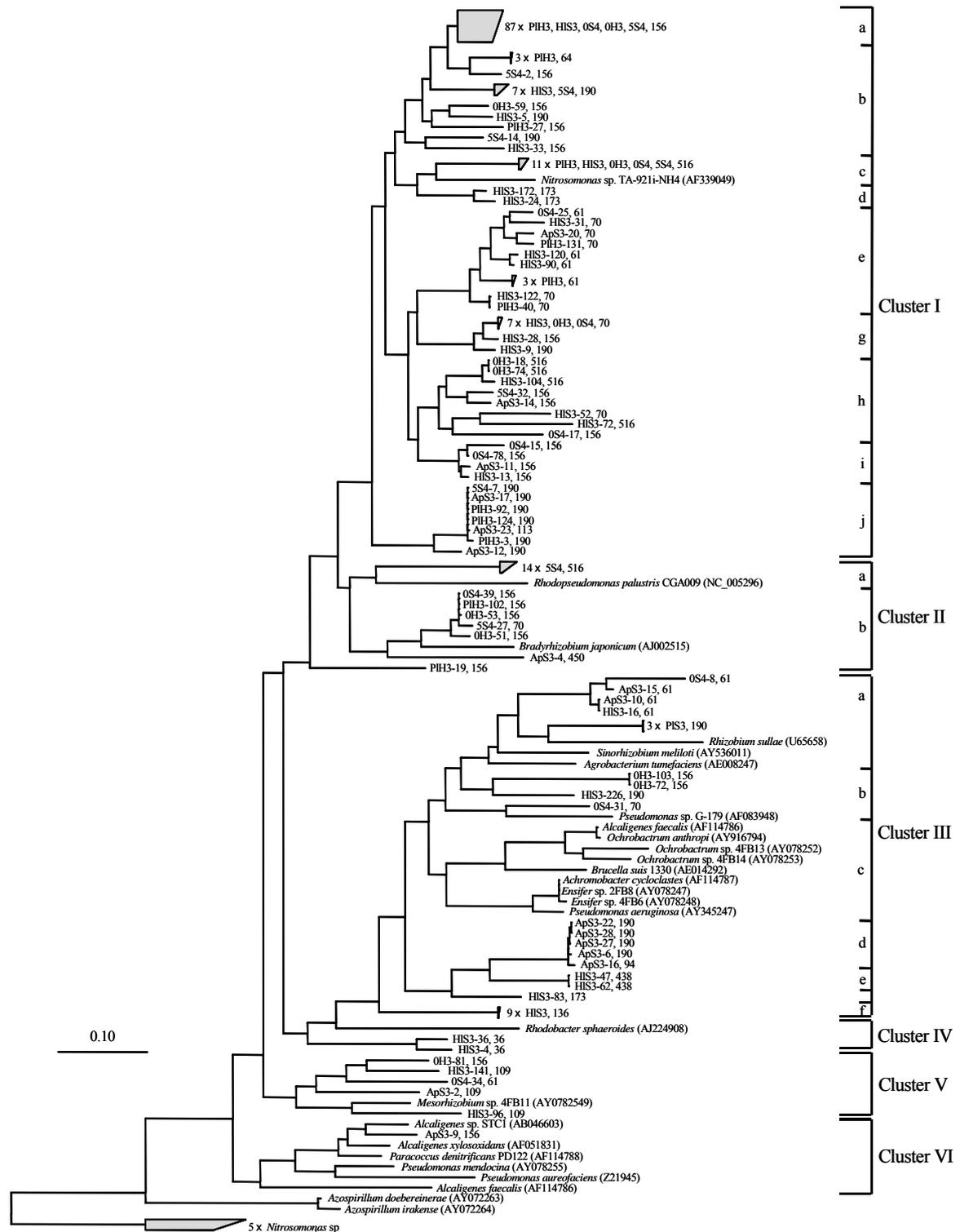


Fig. 14. Maximum likelihood tree based on partial *nirK* sequences (432 nucleotide positions) from cultured *nirK*-type denitrifiers and sequences from microcosm and mesocosm soil. Tree topology is supported by parsimony and neighbour joining method. Hl, clones obtained from soil of *Holcus lanatus*, Pl, clones obtained from soil of *Plantago lanceolata*, Ap, clones obtained from soil of *Alopecurus pratensis*, 0, unplanted soil, 5, soil with 8 plant species. S3, summer 2003, H3, autumn 2003, S4, summer 2004. Numbers after the name of the clone indicate the respective size of T-RFs after *in silico* analysis with the restriction enzyme *Hae*III. Accession numbers are in parentheses.

NirK gene sequences were cleaved *in silico* with the restriction enzyme *HaeIII* using the program TRF-CUT and were successfully assigned to experimentally derived T-RFs (Table 20).

Table 20. Assignment of theoretical T-RFs to experimentally derived T-RFs and the occurrence of these T-RFs in the phylogenetic clusters (Fig. 14).

Theoretical T-RFs	Experimental T-RFs	Occurrence in phylogenetic clusters					
36	-- ^a					IV	
61	-- ^a	I		III			V
64	-- ^a	I					
70	-- ^a	I	II	III			
94	86 ^b			III			
109	101						V
113	103	I					
136	131			III			
156	151	I	II	III		V	VI
173	168	I		III			
190	185	I		III			
438	430 ^b			III			
450	445		II				
516	511	I	II				

^a experimental detection not accurate, fragments <60 bp lacking from the internal standard

^b excluded from the T-RFLP analysis due to its minor abundance (<1%)

T-RFs from *in silico* analysis differed by 5 to 10 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. Calculation of theoretical T-RFs demonstrated that *nirK* sequences with T-RFs of 61, 64, 70, 113 (experimental 103 bp), 156 (151), 173 (168), 190 (185), and 516 (511) bp clustered in cluster I (Fig. 13, Table 20). In cluster I, subclusters Ia, Ic, Id, and Ii consisted of sequences with specific T-RFs, e.g. 87 *nirK* sequences with the 156 bp T-RF formed the distinct subcluster Ia. However, sequences with different T-RFs clustered in subclusters Ib, Ie, Ig, Ih, Ij. Sequences with a theoretical T-RF of

156, 173, 190 and 516 bp which corresponded to the dominant T-RFs in the soil of all microcosms and mesocosms, were found in cluster I, II, III, V, and VI. In contrast to these abundant T-RFs, less abundant T-RFs referred to sequences in specific sub-clusters (subclusters IIIe, IIIf, and cluster IV consisted of sequences with the theoretical 438, 136 and 36 bp T-RFs, respectively). The theoretical 136 bp T-RF (experimental 131 bp T-RF) originated from soil of *Holcus lanatus* in summer 2003, where it was most frequently detected. This subcluster did not contain sequences from cultivated relatives. In cluster V, *nirK* sequences with the unique theoretical T-RF of 109 bp were affiliated with *nirK* of *Mesorhizobium* sp.. The corresponding experimental T-RF of 101 bp was most frequently found in T-RFLP-profiles from soil of *Holcus lanatus*. The majority of *nirK* sequences derived from mesocosm soil were affiliated with sequences from microcosm soil in cluster I.

4.2.8 Quantification of *nirK*-type denitrifying microorganisms by MPN-PCR

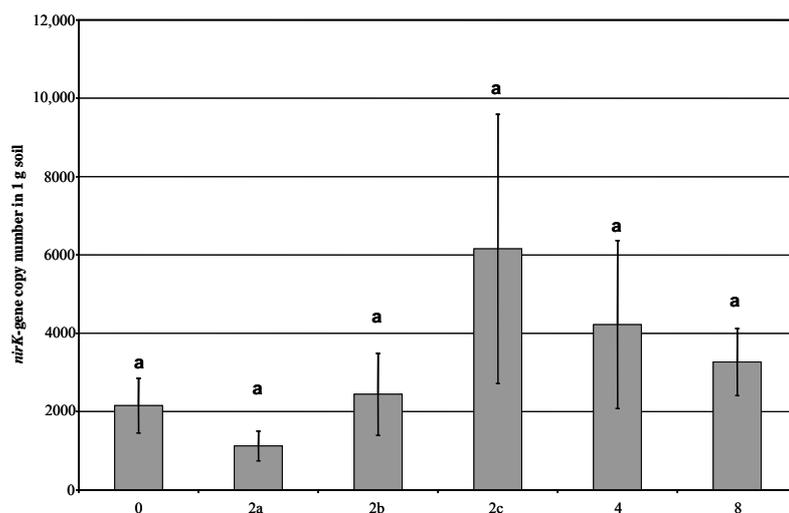


Fig. 15. *NirK*-gene copy number in 1g of mesocosm soil samples in summer 2004. Data are means (n=5, for 0 n=3), bars indicate standard errors of the mean. Different letters indicate significant differences (Tukey's test, $P < 0.05$). (0, unplanted; 2a, *Holcus lanatus* and *Arrhenatherum elatius*; 2b, *Holcus lanatus* and *Geranium pratense*; 2c, *Holcus lanatus* and *Plantago lanceolata*; 4, *Holcus lanatus*, *Arrhenatherum elatius*, *Geranium pratense*, *Plantago lanceolata*; 8, like 4, and *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Taraxacum officinale*, *Ranunculus acris*).

MPN-PCR was used to quantify the *nirK*-type denitrifying microorganisms in mesocosm soils of summer 2004. No significant different *nirK*-gene copy numbers were detected in the soil of the mesocosms (Fig. 15). Neither plant diversity nor plant combination affected the *nirK*-gene copy number in soil (F values 0.08 and 2.02, respectively).

5. DISCUSSION

The aim of my study was to test the hypotheses that non-leguminous grassland plant species and their diversity and composition exert a specific impact on the diversity, composition, and function of *nirK*-type denitrifiers, and that the denitrifier community composition differs over time or over seasons. Since I focussed on *nirK* as the only functional marker gene for denitrifiers, an important part of the denitrifier community in soils, the *nirS*-type denitrifiers, remains to be explored by other studies.

DNA extraction, PCR amplification and T-RFLP as a community fingerprinting approach can suffer from bias, but I assume that these effects can be minimized, since a standardized DNA extraction procedure for pooled mixed samples from replicate microcosms and mesocosms was used, the PCR products were pooled, and T-RF peak heights were standardized finally. Therefore the samples could be compared on a relative basis as it was done in previous studies (Castro-González et al. 2005, Lüders and Friedrich 2003, Priemé et al. 2002). Correspondence analyses have proven to be effective tools for evaluating data of fingerprinting analyses like DGGE (Salles et al. 2004, Yang and Crowley 2000) and T-RFLP (Ayala-del-Rio et al. 2004, Castro-González et al. 2005, Kennedy et al. 2005, Noll et al. 2005). Furthermore, by plotting species and environmental variables, the identification of T-RFs that respond to certain experimental factors (plant functional group, plant identity, plant diversity and combination, sampling time and interactions of interest) was possible.

5.1 Plant microcosms

In the first experiment eight plant monocultures were set up to investigate the effect of individual plant species representing two plant functional groups (grasses vs. forbs) on the diversity and composition of *nirK*-type denitrifiers.

5.1.1 Plant-specific impacts on the *nirK*-type denitrifier community composition

Many different environmental factors shape the species composition of a functional group of soil microorganisms, but I assumed that the functional group of a plant or the species identity of a plant exerts a highly selective effect on *nirK*-type denitrifiers that is at least as strong as that of the soil. The successful amplification of *nirK* genes revealed the presence of *nirK*-type denitrifiers in the soil of all plant microcosms. Given that precondition I found that the factor plant functional group (grasses

vs. forbs) did not affect the community composition of *nirK*-type denitrifiers. There were also no differences in root biomass, and carbon and nitrogen content between soil planted with grasses and soil planted with forbs at the end of the experiment (Bremer et al. 2007, in prep., see appendix p.87). However, the pH was higher in soil planted with grasses than in soil planted with forbs (Bremer et al. 2007, in prep., see appendix p.87). According to Hinsinger et al. (2003) 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. The authors also suggest that plant species may have the ability to react to bulk soil pH values by either reducing or increasing their rhizosphere pH. The four grasses used in my experimental setup belong to the same family, the *Poaceae* (class: *Mono-cotyledonae*), whereas each forb belongs to a different family (the *Asteraceae*, the *Geraniaceae*, the *Plantaginaceae*, and the *Ranunculaceae*; class: *Dicotyledonae*) (Sitte et al. 1999). It is well known that forbs have a less intensive root system than grasses but these differences in taxonomy, root systems and pH did not lead to the development of plant group-specific denitrifier communities in the soil.

In contrast to the plant functional group, plant species identity influenced the community composition of *nirK*-type denitrifiers. Plant species identity also influenced the total amount of N and C in the soil, the pH and the root biomass (Bremer et al. 2007, in prep., see appendix p.87). The most distinct denitrifier community composition was found in the soil from *Holcus lanatus*. This soil had the highest C content and the highest soil pH together with the lowest root biomass, probably due to a high root turnover in these microcosms (Bremer et al. 2007, in prep., see appendix p.87), so the observed plant-mediated effects on soil characteristics may be responsible for the differences in denitrifier community compositions. *Alopecurus pratensis* had the highest root biomass per plant in June 2004 (Bremer et al. 2007, in prep., see appendix p.87) and its denitrifier community composition was also quite dissimilar from that of the other plants. Plant species like *Anthoxanthum odoratum* and *Plantago lanceolata* harbored similar *nirK*-type denitrifier community compositions and did not differ in their soil characteristics. In contrast, soil from *Geranium pratense* had the lowest pH (Bremer et al. 2007, in prep., see appendix p.87), but did not have a very distinct denitrifier community composition.

Generally, non-symbiotic plant-specific microbial communities or differences in the microbial community composition in associations with plants may be attributed to

differences in the amount and composition of root exudates. Exudation is a dynamic process which varies depending on plant species, physiological status of the plant (Smalla et al. 2001), root zone (Jaeger et al. 1999), and nutritional conditions (Grayston et al. 1998). 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 mineralisation). Specific exudates may also selectively favor some microbial strains over others. Interestingly, microorganisms and plants of the family *Poaceae* excrete siderophores to solubilize and transport iron (Crowley and Gries 1994). Iron can be replaced by bivalent copper in phytosiderophores (Chaignon et al. 2002). Both, plants and microorganisms, can compete for these phytosiderophores and alter iron and copper availability (Marschner and Crowley 1998, Yang and Crowley 2000). Sequestration of copper and iron by plants may impact denitrification since within this process iron and copper are required for enzyme activity (Zumft 1997).

CCA allowed the identification of plant-specific *nirK*-OTUs probably representing *nirK*-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 weather conditions were identical for all microcosms, therefore this factor was not responsible for the patterns observed. In a recent study, Costa et al. (2006) observed effects of plant species identity (i.e. oilseed rape and strawberry) and year to year variation on the community composition of *Pseudomonas* in rhizosphere soils, based on 16S rRNA gene fragments.

5.1.2 Seasonal variations of the *nirK*-type denitrifier community composition

The community composition of *nirK*-type denitrifiers generally varied between sampling dates, and also in a directional way. But to assume a consistent trend in microcosm development based on three sampling times is rather speculative. The general effect of time was not unexpected, because many studies have reported seasonal effects on soil microbial communities in the past (Grayston et al. 2001, Griffiths et al. 2003, Kennedy et al. 2005). Seasonal climatic changes govern physical soil characteristics such as moisture and temperature. My results are in accordance with those of Wolsing and Priemé (2004), who reported a significant seasonal shift in the community structure of *nirK*-type denitrifiers in spring, summer and autumn of a single year. Surprisingly, I observed different *nirK*-type denitrifier community compositions for the same season (summer) in different years (2003 and 2004) emphasiz-

ing the variation of environmental conditions and their influence on certain soil characteristics. Apart from differences in denitrifier community composition due to seasonal impacts, the general effect of time varied for individual plant species. The interaction of time effects with plant identity hints on a development of the plant microcosms with age, either due to aging of the plant itself or progressing root development in the microcosms. The observation that the denitrifier community composition was different in two consecutive summers may support this notion.

I assume that the most abundant *nirK*-OTUs that were ubiquitous in the soil from all plant species at any sampling time originate from denitrifiers that are well adapted to the intrinsic characteristics of the microcosm soil substrate and to the surrounding climatic conditions. In contrast, the occurrence of less abundant but rather specific *nirK*-OTUs may be linked to special environmental factors like plant identity, sampling time or the development of individual plant microcosms.

The phylogenetic analysis of *nirK* sequences derived from plant microcosm soil is discussed in chapter 5.2.3.

5.1.3 Description of the *nirK*-type denitrifier community structure by diversity indices

Richness (i.e. the number of different *nirK*-OTUs), Shannon diversity and Shannon evenness indices were used as additional characters to describe possible differences in the structure of the *nirK*-type denitrifier communities. The number of *nirK*-OTUs was higher in soil of forbs than in soil of grasses, and it was higher in autumn 2003 than in the two summers. Shannon diversity of *nirK*-OTUs was unrelated to the factors tested, but Shannon evenness was influenced by plant identity, and this effect varied with sampling time. In soil of *Geranium pratense* the *nirK*-type denitrifier community had the highest evenness, whereas in soil of *Holcus lanatus* the community had the lowest evenness. Evenness decreased in the soil communities sampled in summer 2003 to autumn 2003 to summer 2004.

However, the use of diversity indices to describe community diversity and evenness in ecological studies is discussed controversially (Hartmann and Widmer 2006, Hill et al. 2003, Martin 2002, Purvis and Hector 2000; but see also Wilsey and Polley 2002, Polley et al. 2003). First, unlike CCA, the calculation of Shannon indices comprises a weighting of species, i.e. more weight per individual is given to rare than common species. Consequently, it is more sensitive to absolute than relative changes

in the abundance of species. Second, the index can be an underestimate of its true value due to incomplete coverage. Since the Shannon evenness index is derived from the Shannon index, it suffers from the same biases. In contrast to CCA, both indices also do not incorporate species identity.

5.2 Planted mesocosms (lysimeters)

Planted mesocosms were set up to examine the effects of plant diversity, plant combination, time and the interactions between these factors on the *nirK*-type denitrifier community and on their functional traits.

5.2.1 Impacts of plant diversity, plant combination, and time on the *nirK*-type denitrifier community

Many different environmental factors shape the composition of a functional group of soil microorganisms, but I assumed that not only single plant species excreting root exudates, but also plant diversity and combination exert a highly selective effect on *nirK*-type denitrifiers. The successful amplification of *nirK* genes revealed the presence of *nirK*-type denitrifiers in the soil of all plant mesocosms. Given that precondition I found that plant diversity and plant combination affected the community composition of *nirK*-type denitrifiers. Plant combination also affected the Shannon evenness of the denitrifier communities (the usefulness and significance of diversity indices was already discussed in chapter 5.1.3). I assume that the ubiquitous *nirK*-OTUs that were most abundant in the soil from all plant combinations at any sampling time originate from denitrifiers that are well adapted to the intrinsic characteristics of the mesocosm soil substrate and to the surrounding climatic conditions. In contrast, the occurrence of less abundant but rather specific *nirK*-OTUs may be linked to special environmental factors like plant diversity, plant combination, sampling time or the development of individual plant mesocosms.

Non-symbiotic plant-specific microbial communities or differences in the microbial community in associations with plants may be attributed to differences in the amount, spectra and composition of root exudates (see also chapter 5.1.1). An increasing number of plants or a varying plant combination seems to enhance or broaden the amount, spectra and composition of exudates resulting in plant diversity or plant combination specific *nirK*-type denitrifier communities.

Zak et al. (2003) used phospholipid fatty acid (PLFA) profiles to demonstrate that soil microbial communities differed with plant species richness (1-16 species) in a long-term experimental grassland system; plant diversity increased microbial biomass and modified the microbial composition, but this effect was attributed to increases in plant production. These findings are supported by Carney and Matson (2006). They found that tropical plant diversity (1, 3, 5, <25 species), plant identity and combination, and sampling time in particular influenced microbial community composition (based on PLFA profiles). The authors also observed an increase in soil carbon concentrations with plant diversity, which may be an index of plant productivity.

Apart from the single effect of sampling time on the *nirK*-type denitrifier community composition and richness, a linear effect of time could be demonstrated with this experiment. Generally, a linear, i.e. directional effect of time, can be explained by succession. Moore (2001) described ecosystem succession as a passage through a series of stages that eventually stabilizes at a predetermined endpoint, the climax or maturity state. This state is supposed to have the greatest biomass, equal nutrient imports and exports, the highest species richness and complexity. In the case of denitrifiers, Sharma et al. (2006) described for the first time succession of *napA*- and *nirS*-type denitrifiers in soil after freeze-thaw stress. In my study, the *nirK*-type denitrifier community composition in mesocosms soils in summer and autumn 2003 was different from that in summer and autumn 2004 indicating a development of the mesocosms from year to year. This development can be due to aging of the plant mesocosms, but it can also be caused by the climatic anomaly in summer 2003. This year was particularly hot and dry in Europe, with July temperatures up to 6°C above long-term means, and annual precipitation deficits up to 300 mm, 50% below the average (Ciais et al. 2005). The *nirK*-type denitrifier community composition also differed between summer and autumn in both years indicating a seasonal succession, but the differences between summer and autumn in both years were similar, therefore contradicting the hypothesis of a linear succession over two years. Moore (2001) pointed out that the time scale is the major problem in studies of succession, since the stages may simply be artefacts of the time dimension. Long-term experiments instead of short-term experiments are required to test the hypothesis of microbial succession in soil. Malý et al. (2000) noted that most studies about plant diversity and composition effects refer to a relatively short period of time (less than 5 years) so that the prevailing

soil microbial conditions may stronger influence the plant community than vice versa. However, the last argument does not refer to the planted mesocosms.

The effects of plant diversity and composition also interacted with sampling time indicating differences in the *nirK*-type denitrifier community composition as a consequence of a development of the plant mesocosms. Aging of the mesocosms comprises different or increasing amounts of organic carbon, which can be released by mature plants, a more advanced root system (higher root density), or other factors like mycorrhizal colonization providing additional carbon.

Growing plants develop a root system which gets more advanced with increasing plant age. Root systems and root decomposability are important factors of carbon dynamics in soil of temperate grasslands. Especially root life spans are supposed to be plant specific and highly variable, e.g. average root life spans of herbaceous plants range from two weeks to more than a year (Eissenstat and Yanai 1997). Van der Krift and Berendse (2002) observed that root life spans of plant species from fertile habitats were significantly shorter than the root life spans of plant species from low fertility habitats. This means, that plants from nutrient-rich habitats add more carbon to the soil through dead root tissues than plants from nutrient-poor habitats. Unfortunately, root life spans of many plant species have not been determined yet, only the average root life span in *Arrhenatherum elatius* is known to be 40 weeks (van der Krift and Berendse 2002). *Arrhenatherum elatius*, *Alopecurus pratensis*, *Taraxacum officinale* and *Geranium pratense* are plant species of nutrient-rich habitats (according to Ellenbergs indicator values (EIV), <http://www.boku.ac.at/statediv/edv/botanik/zeigerwerte>). *Holcus lanatus* is a species of habitats with intermediate nitrogen contents, whereas the other plant species are indifferent to nitrogen.

Reuter (2005) investigated the root characteristics of the plants in the mesocosm set up, which I also used for my work. He reported lower mean root diameters and higher root turnover rates in soil of the plant combinations *Holcus lanatus* with *Arrhenatherum elatius* (2a) and *Holcus lanatus* with *Geranium pratense* (2b) in the years 2003 and 2004. The carbon input by dead roots was also higher in the first ten centimeters of the soil of these two plant combinations (referring to the years 2001 to 2004), but the accumulation of carbon was higher in soil of the other plant combinations (*Holcus lanatus* with *Plantago lanceolata* (2c), the four (4) and the eight plant species combination (8)). Reuter (2005) assumed that this can be explained by the higher degree of mycorrhizal root infection or the higher proportion of forbs growing

in these mesocosms. Taken together, Reuter (2005) found an influence of plant species combination but not of plant diversity on the input and accumulation of carbon in the mesocosm soils.

The development of the plants and their root systems in mesocosm soil with time can be accompanied by mycorrhizal colonization providing additional carbon. It is known that arbuscular mycorrhizal (AM) fungi can colonize the roots of *Holcus lanatus* (Gonzalez-Chavez et al. 2002), *Arrhenatherum elatius* (Wijesinghe et al. 2001) and *Plantago lanceolata* (Olsson and Johnson 2005). AM fungi are important regulators of the carbon flux from plants to the soil (Zhu and Miller 2003). Olsson and Johnson (2005) showed a rapid transfer of plant assimilates to AM fungi and a gradual release of C from roots to rhizosphere bacteria by ^{13}C enrichment of signature fatty acids. Reuter (2005) found that the proportion of mycorrhized root length was higher in the mesocosms planted with *Plantago lanceolata* than in mesocosms without this plant indicating an effect of plant species identity. For all planted mesocosms he observed that the proportion of mycorrhized root length was 40-60%, measured in the first ten centimeters of the mesocosm soils.

5.2.2 Interdependencies between soil nitrate concentrations, N_2O production rates, denitrifier enzyme activity, MPN, plants, and denitrifier communities

In soil, the process of denitrification requires nitrate as a substrate. Mineral nitrate was added to the plant mesocosms with the NPK-fertilizer “Blaukorn” twice a year. In winter 2003 and autumn 2004, but not in summer 2004, nitrate concentrations in the upper soil layer of unplanted mesocosms were higher than in the upper soil layer of planted mesocosms. Nitrate concentrations in soil of the planted mesocosms were low and did not differ from each other at the three sampling times. But there were significant differences between unplanted and planted mesocosms in autumn and winter.

Generally, the fate of nitrate in soil is plant uptake, microbial denitrification or leaching. In the unplanted mesocosms the uptake by plants is missing causing the relatively higher concentrations of nitrate in these soils. Here, nitrate can only be taken up by microorganisms or leached with soil water. As I will show later the nitrate uptake by denitrifying microorganisms is not the limiting factor influencing denitrifier functioning, so that leaching seems to be the important factor regulating nitrate concentrations in unplanted soil. In the planted mesocosms nitrate concentrations were surprisingly not influenced by plant diversity, plant combination and sampling time,

either because of similar demands of the different plant species or of different demands of the different plant species complementing one another.

Despite high nitrate concentrations in soil of unplanted mesocosms, the net and gross N_2O production rates were low in these soils and therefore not depending on nitrate concentrations. This result is in accordance with other studies (Dendooven and Anderson 1994, Mounier et al. 2004, Müller et al. 2004) which found that high nitrate concentrations in grassland soil are not a prerequisite for high N_2O production, but contradictory results have been published, too (Arah and Smith 1989). Since the low net and gross N_2O production rates of unplanted soil were similar to the rates of planted soil in winter, but different to the rates of planted soil in summer and autumn, denitrification in these soils seems to be limited by carbon supply of growing plants, an assumption, which also explains why both rates were generally lowest in winter, intermediate in autumn and highest in summer. Many studies have stressed the dependency of N_2O production rates on the amount, type and composition of carbon substrates (Azam et al. 2002, Dendooven et al. 1996, Hume et al. 2002, Lin et al. 2002, Mounier et al. 2004). The N_2O producing denitrifier community probably competes for carbon with other aerobic heterotrophs, since denitrifiers seldom exercise their denitrification capacity (Tiedje 1994). Hence, the input of organic carbon by plants into soils may indirectly affect N_2O emissions. In the present study, plant diversity, plant combination, time, the interaction of plant diversity with time, and the denitrifier enzyme activity influenced net and gross N_2O production rates. Niklaus et al. (2006) studied the effects of plant species diversity and composition on N_2O emission by nitrification. They found that N_2O emissions from soil decreased with plant diversity and increased in the presence of legumes, and also differed under varying plant community compositions, especially when they contained legumes. In my study, the effect of plant diversity was mainly a result of differences between unplanted and planted soil and of differences between soil planted with two and four plant species. Varying plant combinations may increase the amount, composition and spectra of organic carbon available for heterotrophic microorganisms. The influence of sampling time, i.e. season or temperature, on N_2O production rates was reported in other studies (Flessa et al. 1995, Groffman and Tiedje 1989, Holtan-Hartwig et al. 2002). However, in my study the effect of sampling time was only indirectly caused by temperature effects, since all rates were measured under the same anoxic and standardized lab conditions, so that the effect of time is caused by different denitrifier enzyme activi-

ties or differences in the availability of carbon substances. Bakken (1988) compared denitrification rates in soil under growing barley, wheat and oats and concluded that variations in plant growth rates controlled the differences in denitrification, because rates of photosynthesis could be used to predict denitrification rates.

Net and gross N_2O production rates were linearly related in mesocosm soil, but this relationship cannot be assumed a priori, because the denitrifier community composition and the environmental conditions may be different in other soils. In two of the 18 samples (soil of the plots 2a and 2b in autumn) net production rates surprisingly exceeded gross production rates. Several explanations for this phenomenon are possible but not equally likely. First, chemodenitrification may have taken place, but pH-values were determined for all slurries and were found to be too high (6.1-6.4) to favor chemodenitrification (Laughlin and Stevens 2002). Second, small amounts of oxygen can lead to an underestimation of (gross) N_2O production rates, because oxygen can oxidize the intermediate nitric oxide in the presence of acetylene (Bollmann and Conrad 1997, Dunfield and Knowles 1997). Consequently, nitric oxide is depleted and cannot be reduced to N_2O leading to an underestimation of gross N_2O production rates (Bollmann and Conrad 1997, Dunfield and Knowles 1997). The degree of underestimation can vary with different soils, as Murray and Knowles (2004) pointed out. These authors suggested in an earlier study that with an adequate amount of carbon and nitrate the loss of nitric oxide caused by acetylene-catalysed oxidation would not be large enough to substantially reduce N_2O production (Murray and Knowles 2003). However, it is still unknown what happens if the amount of carbon is not adequate but rather the limiting factor and if varying supplies of carbon substances (i.e. plant root material) increase or decrease the scavenging of nitric oxide. In my study, much care was taken to avoid oxygen input into the slurries (e.g. flushing the syringes with N_2 before gas samples were taken, slowly withdrawing the needle from the stopper to allow closing of the gap), but the possibility of underestimating N_2O production rates due to oxygen input can not be fully excluded. A third reason for lower gross than net N_2O rates may be that the pool of nitric oxide is depleted because it can react with humic materials, organic free radicals, transition metals and disrupted cell contents or metabolites in the presence of acetylene in the soil slurries (Conrad 1996, Dunfield and Knowles 1997).

Fourth, denitrifying fungi could have grown invisibly in the soil slurries, because no fungal inhibitor was used. Filamentous fungi (Shoun et al. 1992), yeasts (Tsuruta

et al. 1998), and some actinomycetes (Shoun et al. 1998) have the capability to denitrify. They are facultative anaerobes and produce N_2O as an end product (Laughlin and Stevens, 2002), probably due to the lack of N_2O reductase (Shoun et al. 1992). Therefore their denitrification pathway can not be influenced by acetylene and that may explain relatively similar net- and gross production rates in the slurries of some mesocosm soils. Laughlin and Stevens (2002) found that fungi were responsible for most of the N_2O production in a temperate grassland soil. However, in a coculture of a fungus (*Fusarium oxysporum*) and a bacterium (*Pseudomonas stutzeri*), N_2O produced by the fungus was rapidly consumed by the bacterium (Liu et al. 2006).

In this study, both, *nirS*- and *nirK*-type denitrifiers are assumed to contribute to the N_2O production rates measured. Although *nirS*-type denitrifiers were not investigated with molecular techniques, the emission rates depended on richness, Shannon diversity and Shannon evenness indices calculated on the basis of *nirK*-T-RFs and on the T-RF-scores along the first and second axis after ordination, thus providing hints on a relationship between *nirK*-type denitrifier diversity and function. Recently, Wertz et al. (2006) reported that decreasing the richness of *nirK*-type denitrifiers did not affect their associated soil functions, probably because the number of species remaining after diversity loss was still sufficiently high to allow the maintenance of functioning. Hence, a vast diversity or redundancy of soil microorganisms can make ecosystem functioning largely insensitive to richness losses, even in the case of functions performed by specialized groups. However, in my study the effects of the plants on N_2O production rates remained even after elimination of the influence of the other factors like *nirK*-type denitrifier community composition (sample scores along the ordination axes), denitrifier richness, diversity, and evenness. Thus, plant diversity and combination had additional effects on N_2O production rates which could not be explained by differences in the characteristics of the *nirK*-type denitrifier community. It also remains unknown to what extent nitrate respirers, nitrifier denitrification or fungi contribute to the measured N_2O production rates in the mesocosm soils.

The denitrifier enzyme activity (DEA) (Tiedje 1994) was determined as a way of assessing the potential optimum activity of existing denitrifying enzymes in soil. Generally, the DEA in the mesocosm soils ranged from 0.2 to 50 ng N_2O -N $g^{-1} h^{-1}$. Rich et al. (2003) determined values of 10 to 100 ng N_2O -N $g^{-1} h^{-1}$ in a meadow soil from the Western Cascade Mountains of Oregon and classified them as low to intermediate values. My results match those of Barnard et al. (2005) and Boyle et al. (2006) show-

ing no significant correlation between soil nitrate concentrations and DEA. DEA has also not to be well correlated with in situ denitrification rates or N₂O fluxes (Wrage et al. 2001), but in my study DEA was related with net and gross N₂O production rates. Despite the addition of glucose as a carbon source in the DEA assay, plant diversity, plant composition and time also affected the DEA emphasizing the importance of the amount, composition or spectra of organic carbon supplied by the plants. In soil, DEA was significantly increased by the presence of plants, especially when they were actively growing during summer and autumn. Low DEA in the soil of unplanted mesocosms and in the soil of planted mesocosms in winter contrasted with higher DEA in soil with different plant combinations in summer. In former studies, plant species influenced DEA measured in the rhizosphere (soil adhering to the roots) of potted tree seedlings (Priha et al. 1999) and in soil below grass tufts dominated by *Holcus lanatus*, *Arrhenatherum elatius* and *Dactylis glomerata* (Patra et al. 2006). In another study (Le Roux et al. 2003) five different plant species (including *Holcus lanatus* and *Arrhenatherum elatius*) and different plant combinations did not affect soil DEA.

Effects of time on DEA were already reported in the past (Barnard et al. 2005, Boyle et al. 2006), but in the present study the effect of time also varied with plant combination indicating a development of the plant mesocosms with age, either due to aging of the plants themselves or a progressing root development in the mesocosms. Richness, Shannon diversity and Shannon evenness indices of *nirK*-OTUs did not influence denitrifier enzyme activity, maybe because only *nirK*-type and not *nirS*-type denitrifiers were taken into account. Sample scores along the first and second axes after ordination had a significant effect on DEA pointing at a relationship between *nirK*-type denitrifier community composition and denitrifier enzyme activity.

Since differences in the activity of a community may be partially explained by differences in its size, the most probable number method (MPN) was applied. MPN of nitrate reducing microorganisms in the mesocosm soils of summer 2004 did not depend on plant diversity or composition, but on individual mesocosms indicating a bad replication, -even when the effect of individual mesocosms was partialled out-, or indicating an insensitive method. The MPN of the nitrate reducing microorganisms in the mesocosm soils was low but it corresponds to results obtained by Schwarz (2002) for an agricultural soil. The MPN methods do not provide a “real” value but rather give a range of values which contains the “true” value with 95% probability (confi-

dence intervals). Thus the huge ranges can easily overlap and hide possible differences confounding statistical evaluations. Furthermore, MPN is known to be time consuming and selective, since its efficiency relies on the culture medium used and on the physiological state of a cell. Unculturable organisms are not targeted. Therefore it underestimates the number of organisms of interest, like protozoa (Ekelund and Rønn 1994), nitrifying bacteria (Degrange and Bardin 1995, Féray et al. 1999), and denitrifying bacteria (Michotey et al. 2000). An extrapolation from the culturable fraction of denitrifying bacteria to the total fraction of denitrifiers is not possible, since the culturable fraction of denitrifiers is unknown (Michotey et al. 2000) and it contains both, *nirK*-type and *nirS*-type denitrifiers in unknown proportions.

5.2.3 Phylogenetic analyses

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. Ten different *nirK*-OTUs detected in the soil of plant microcosms and planted mesocosms could be assigned to *nirK* sequences in my clone libraries by T-RFLP-analysis of clones and using TRF-CUT. The majority of *nirK* sequences (155 of 212) obtained in my study were related to *nirK* sequences of uncultured organisms from soil and activated sludge and to *Nitrosomonas* sp. TA-921 i-NH₄ as the nearest cultivated representative. Four theoretical T-RFs of the *nirK* sequences in cluster I (156, 173, 190, and 516 bp) corresponded to the experimental T-RFs (151, 168, 185, and 511 bp), which were found to be ubiquitous in the soil of all plant microcosms and planted mesocosms at any sampling time, but which also demonstrated differences in their relative abundances depending on plant identity, diversity, composition, and sampling time. However, closely related *nirK* sequences sharing the same T-RF can represent the same organism. In clusters II, III and V several of our *nirK* sequences were affiliated with *nirK* genes of denitrifiers from the order *Rhizobiales*, namely *Bradyrhizobium*, *Rhizobium*, and *Mesorhizobium*. The results of the present study accord with other studies that found numerous functional marker genes for denitrification in soils grouping with denitrification genes of these representatives of the *Rhizobiales* (Philippot et al. 2002, Prieme et al. 2002, Rich et al. 2003). *Rhizobiales* usually live in symbiosis with legumes, but can also be found free-living in soil when leguminous plants are absent. In cluster III, *nirK* sequences with a specific T-RF (theoretical, 136 bp, experimental, 131 bp) from soil planted with

Holcus lanatus form a distinct subcluster (III_f) without a close relationship to *nirK* from any cultivated organism indicating a group of denitrifiers with yet unknown *nirK*-genotype. In cluster V, *nirK* sequences with the theoretical T-RFs of 36 and 109 bp (experimental 101 bp) from soil planted with *Holcus lanatus* were grouped with *nirK* of *Mesorhizobium*.

In a recent study, Heylen et al. (2006) outlined the constraints of phylogenetic analyses based on the *nirK* gene. They found that phylogenetic *nirK* sequence analysis was incongruent with the 16S rRNA phylogeny at the family and genus level. *NirK* gene sequences were also found to be more similar to *nirK* sequences from the same habitat than to *nirK* sequences retrieved from highly related taxa, because of horizontal gene transfer. Only *nirK* sequences of the *Rhizobiaceae* and *Nitrosomonas* clustered together according to their taxonomic classification. The authors further noted that the fallibility of the primers should be kept in mind when community analyses are conducted and that it is not known if all groups of microorganisms experience horizontal gene transfer to the same extent and under which environmental circumstances.

5.2.4 MPN-PCR as a quantification method

The *nirK*-gene copy number in 1 g soil of summer 2004, as determined by MPN-PCR, did not depend on plant diversity and composition, but on individual mesocosms due to bad replication. Ferre (1992) and Rongsen and Liren (1997) argued that MPN-PCR is only a semiquantitative method, since it provides estimates and not absolute quantification. MPN-PCR as well as the MPN method do not provide a “real” value but rather give a range of values which contains the “true” value with 95% probability. Thus the huge ranges can easily overlap and hide possible differences exacerbating statistical evaluations.

MPN-PCR assays for prokaryote nucleic acid detection in soil and sediment samples have been successfully applied (Chern et al. 2004, Degrange and Bardin 1995, Féray et al. 1999). In the case of denitrifiers, *nirS*-type denitrifiers were quantified by MPN-PCR in marine samples (Michotey et al. 2000) and by real-time PCR in soil, groundwater, and sediment samples (Grüntzig et al. 2001, Henry et al. 2004). In the study of Henry et al. the *nirK*-gene copy number in 1 g soil ranged from 9.7×10^4 to 3.9×10^6 , being higher than the *nirK*-gene copy number in the mesocosm soils (mean

values ranged from 1.1×10^3 to 6.1×10^3). This discrepancy in *nirK*-gene copy numbers can be explained by the generally low MPN of nitrate-reducing microorganisms in the mesocosm soils, by the loss of target DNA during extraction and sample dilution and by the extent of optimized PCR conditions (Fredslund et al. 2001). I also observed two other problems, which may occur during dilutions of environmental DNA. As a consequence of dilution, any environmental contaminant present in a sample will be minimized together with the target DNA (Chandler 1998, Wilson 1997). Either the contaminants may affect PCR amplification when only a low copy number of DNA is present (Miller et al. 1999) or the low contaminant concentration enables the reappearance of a positive amplification signal after a negative one. Furthermore, the use of degenerated primers can increase the risk of non-specific annealing onto non-target sequences, leading to a putative loss of specificity and sensitivity (Henry et al. 2004).

Since the proportion of *nirK*-type and *nirS*-type denitrifiers among the total denitrifying community in nature is still unknown (Henry et al. 2004, Michotey et al. 2000), one cannot determine the portion of one type of denitrifiers from the other.

5.3 Aspects of diversity-function relationships

When diversity-ecosystem function relationships are investigated, some considerations have to be taken into account. In a study of Hooper and Vitousek (1998) the effects of plant composition and diversity on nutrient cycling in soil were investigated over one year. They found that some soil characteristics differed stronger among seasons than among treatments within season and therefore raised the question under which conditions one will most likely see ecosystem-level effects of diversity in a given ecosystem.

Another aspect is the “sampling effect” or “selection probability effect”. This effect states that the communities which have the highest diversity have a greater probability of being dominated by the most productive species of the entire species pool (Wardle 1999). If the traits of dominant species are taken into account, species compositional effects may be found to be more pronounced than species diversity effects. Conversely, strong diversity effects tend to be detected only in those studies in which the sampling effect may play an important role (Wardle 1999). The sampling effect can be avoided by comparing the performance of single species in monoculture with multiple species mixtures (Wardle 1999), as it was done in recent studies (Car-

ney and Matson 2006, Niklaus et al. 2006). Interestingly, these and other studies (Hooper and Vitousek 1997, 1998) found compositional effects to be quite as important or more important than diversity effects influencing soil functions or other characteristics. In my study, plant microcosms cannot be directly compared to plant mesocosms, since the plants of the microcosms were grown in pots and the plants of the mesocosms were grown in concrete lysimeters. Nevertheless, I want to speculate about some aspects of diversity-function relationships observed in this study. When *Holcus lanatus* was cultivated in monoculture, it showed a very dissimilar *nirK*-type denitrifier community composition in the soil compared to soil of the other plant species. In addition, a few partial *nirK* sequences retrieved from soil of *Holcus lanatus* grown in monoculture indicated a group of denitrifiers with yet unknown *nirK*-genotype. Taken together, *Holcus lanatus* seems to be a species with special traits. Considering that *Holcus lanatus* was the only plant species occurring in all plant combinations, one might expect that the soil denitrifier community composition under the different plant combinations is quite similar (due to the special traits of *Holcus*). But the denitrifier community compositions in soil with different plant combinations were not similar, maybe because the special traits of *Holcus lanatus* were more or less impaired by the traits of the other plant species.

N₂O production rates and denitrifier enzyme activity of soil with different plant combinations also varied. Unfortunately, N₂O production rates and denitrifier enzyme activity were not measured in plant microcosms and cannot be compared with each other.

6. CONCLUSIONS AND OUTLOOK

This study provides for the first time a detailed insight into the relationships between typical non-leguminous grassland plant species, the respective *nirK*-type denitrifier communities in soil and their functioning. Plant species identity affected the evenness and composition of *nirK*-type denitrifiers in soil, and plant functional group (grasses vs. forbs) affected their richness. Plant diversity and combination influenced the composition of *nirK*-type denitrifiers, and plant combination also influenced their evenness. Thus, the first hypothesis pointing at a linkage between ecosystem and microbial diversity was supported. Yet, it is impossible to infer the genetic diversity

of denitrifiers from phylogenetic analyses, since the phylogeny of *nirK* gene sequences is incongruent with the 16S rRNA gene phylogeny and a distribution of denitrification genes via horizontal gene transfer is likely to have occurred. However, T-RFLP-analyses in combination with ordination techniques like CA or CCA are effective tools to reveal factors influencing microbial community compositions. Differences in microbial communities are rarely driven by single environmental factors and are often linked to interactions between two or more factors.

The second hypothesis stating that the diversity and composition of denitrifier communities affects their functioning, i.e. the reduction of N₂O emission from soil, was also supported. Richness, Shannon diversity, Shannon evenness and the composition of the *nirK*-type denitrifiers influenced net and gross N₂O production rates, and denitrifier composition influenced denitrifier enzyme activity. The question if highly diverse denitrifier soil communities produce less N₂O (complementarity effect) is difficult to answer. In my study, the impact of plant species and sampling time on N₂O emission rates remained after the elimination of the influence of *nirK*-type denitrifier community characteristics. N₂O emission rates did not show a linear or exponential relationship with *nirK*-type denitrifier community traits.

I conclude that non-leguminous plant species of temperate grassland exert a species-specific effect on a functional group of soil microorganisms, the *nirK*-type denitrifiers. I also observed different *nirK* communities at different sampling times and interactions between the effects of time and plant species suggesting differences due to seasonal conditions or due to the development of the individual plant microcosms and mesocosms.

Future research should specify the definition of community diversity (richness, evenness or composition) to provide instructive and detailed insights into the complex linkages between above- and belowground communities and their functioning. Furthermore, a better knowledge about root exudation of individual plant species is required.

APPENDIX

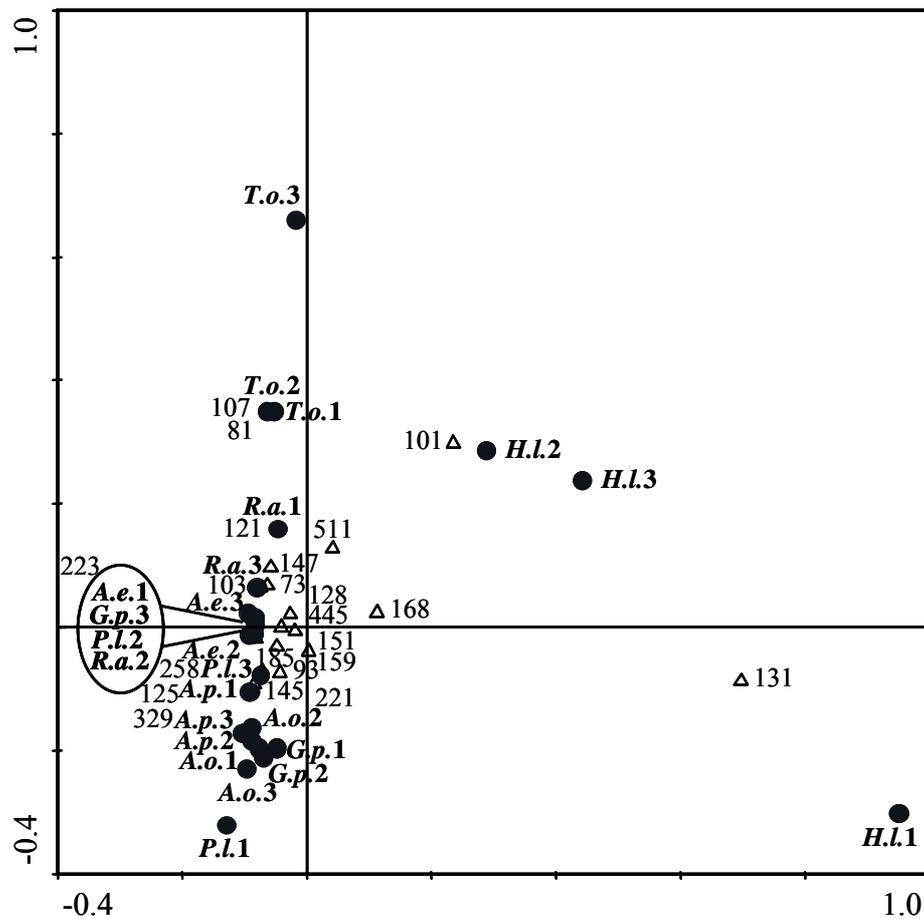


Fig. 1. CA ordination plot for the soil samples of individual plant species (filled circles) and T-RFs (triangles, labelled according to fragment size [bp]) based on *nirK*-T-RFLP-data obtained for microcosms in summer 2003. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.283$, $\lambda_2 = 0.069$. *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*. 1-3, number of replicates.

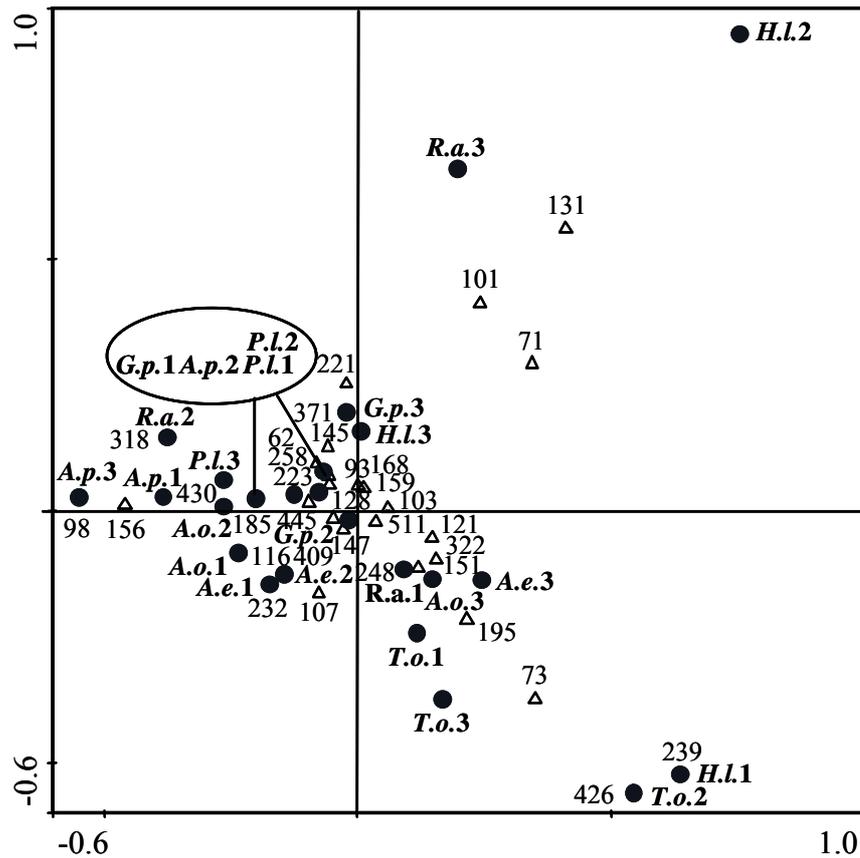


Fig. 2. CA ordination plot for the soil samples of individual plant species (filled circles) and T-RFs (triangles, labelled according to fragment size [bp]) based on *nirK*-T-RFLP-data obtained for microcosms in autumn 2003. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.132$, $\lambda_2 = 0.122$. *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*. 1-3, number of replicates.

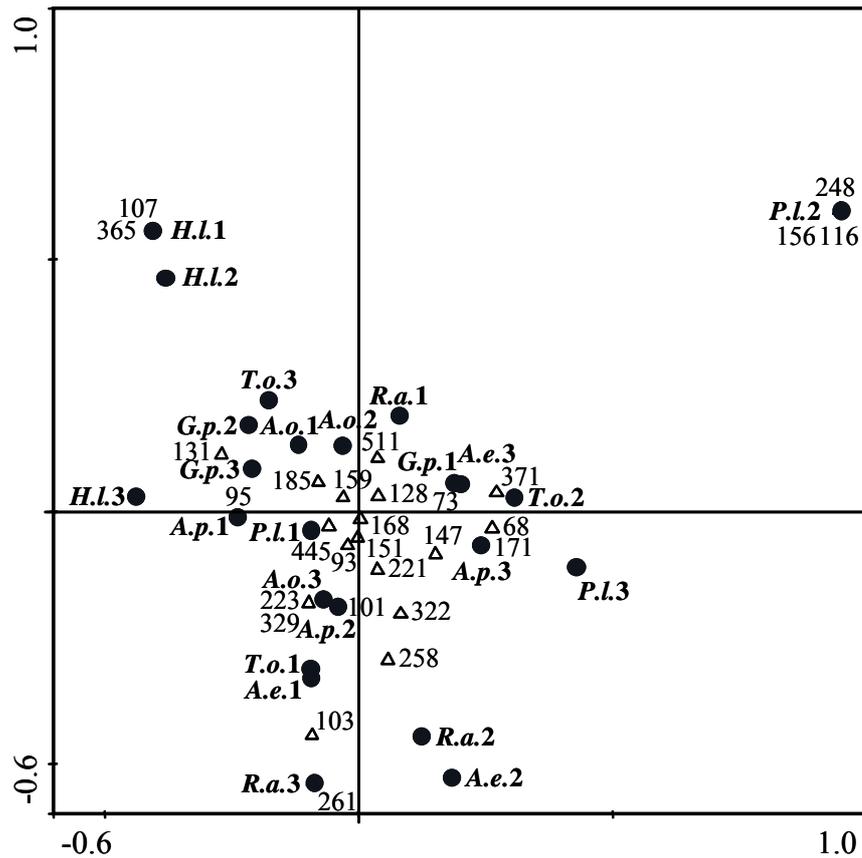


Fig. 3. CA ordination plot for the soil samples of individual plant species (filled circles) and T-RFs (triangles, labelled according to fragment size [bp]) based on *nirK*-T-RFLP-data obtained for microcosms in summer 2004. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.101$, $\lambda_2 = 0.087$. *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*. 1-3, number of replicates.

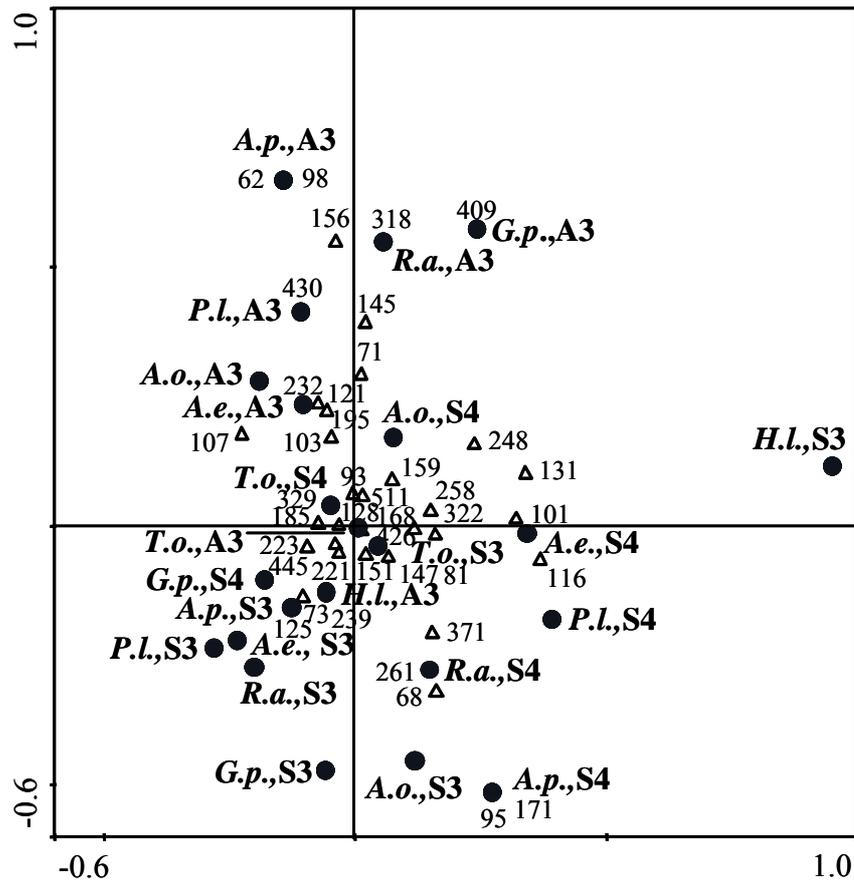


Fig. 4. CCA ordination plot for the effect of plant species identity and sampling time on the *nirK*-type denitrifier community. The different microcosms are denoted by filled circles and T-RFs by triangles, labelled according to fragment size [bp]) based on *nirK*-T-RFLP-data. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.058$, $\lambda_2 = 0.042$. *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*; S3, summer 2003; A3, autumn 2003; S4, summer 2004.

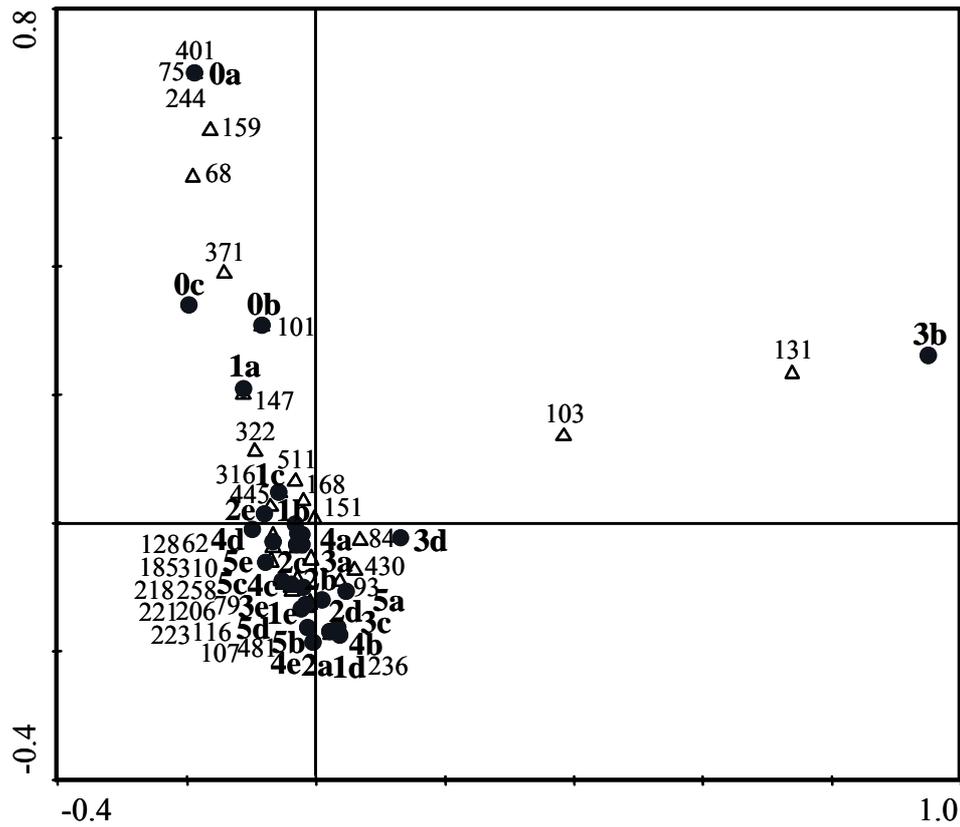


Fig. 5. CA ordination plot for the soil samples of the plant mesocosms (filled circles) and T-RFs (triangles, labelled according to fragment size [bp]) based on *nirK*-T-RFLP-data obtained for summer 2003. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.228$, $\lambda_2 = 0.218$. 0a-c, unplanted mesocosms; 1a-e *Holcus lanatus* and *Arrhenatherum elatius*; 2a-e, *Holcus lanatus* and *Geranium pratense*; 3a-e, *Holcus lanatus* and *Plantago lanceolata*; 4a-e *Holcus lanatus*, *Arrhenatherum elatius*, *Geranium pratense*, *Plantago lanceolata*; 5a-e, like 4, and *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Taraxacum officinale*, *Ranunculus acris*.

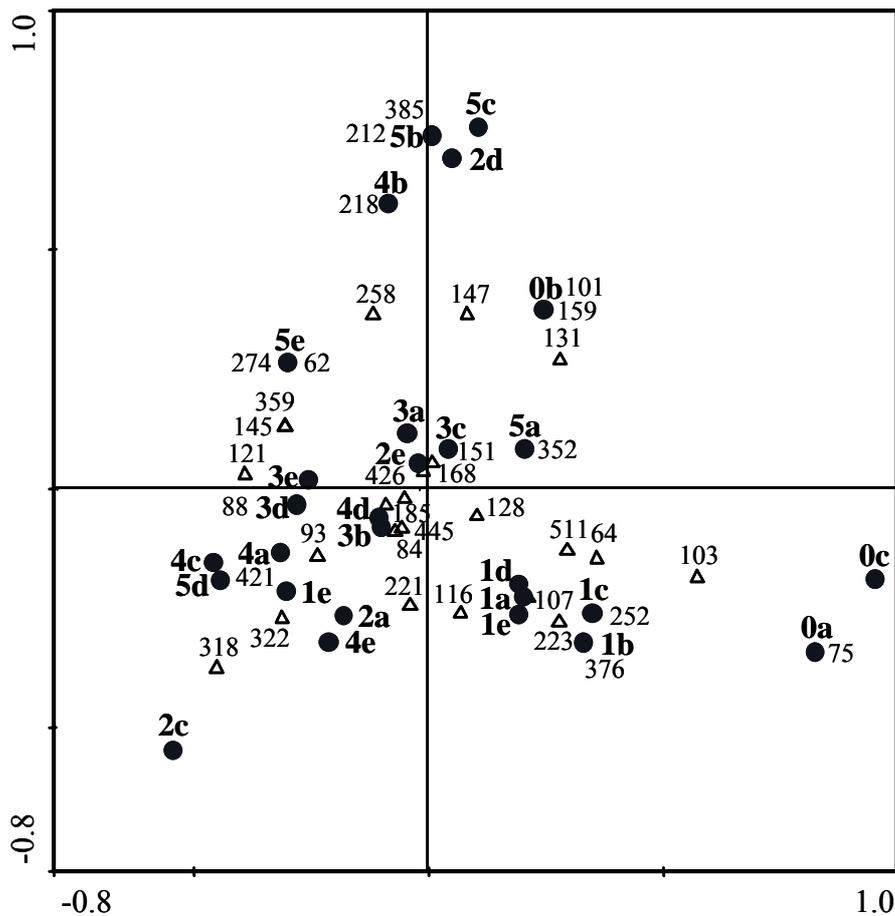


Fig. 6. CA ordination plot for the soil samples of the plant mesocosms (filled circles) and T-RFs (triangles, labelled according to fragment size [bp]) based on *nirK*-T-RFLP-data obtained for autumn 2003. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.149$, $\lambda_2 = 0.119$. 0a-c, unplanted mesocosms; 1a-e *Holcus lanatus* and *Arrhenatherum elatius*; 2a-e, *Holcus lanatus* and *Geranium pratense*; 3a-e, *Holcus lanatus* and *Plantago lanceolata*; 4a-e *Holcus lanatus*, *Arrhenatherum elatius*, *Geranium pratense*, *Plantago lanceolata*; 5a-e, like 4, and *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Taraxacum officinale*, *Ranunculus acris*.

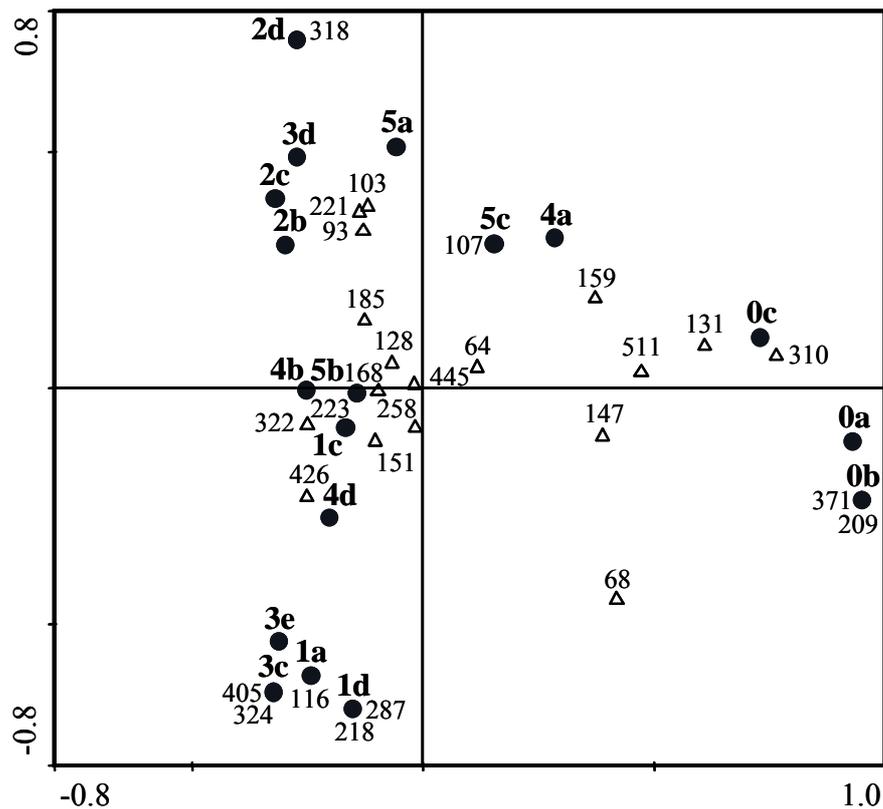


Fig. 7. CA ordination plot for the soil samples of the plant mesocosms (filled circles) and T-RFs (triangles, labelled according to fragment size [bp]) based on *nirK*-T-RFLP-data obtained for winter 2003. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.296$, $\lambda_2 = 0.131$. 0a-c, unplanted mesocosms; 1a-e *Holcus lanatus* and *Arrhenatherum elatius*; 2a-e, *Holcus lanatus* and *Geranium pratense*; 3a-e, *Holcus lanatus* and *Plantago lanceolata*; 4a-e *Holcus lanatus*, *Arrhenatherum elatius*, *Geranium pratense*, *Plantago lanceolata*; 5a-e, like 4, and *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Taraxacum officinale*, *Ranunculus acris*.

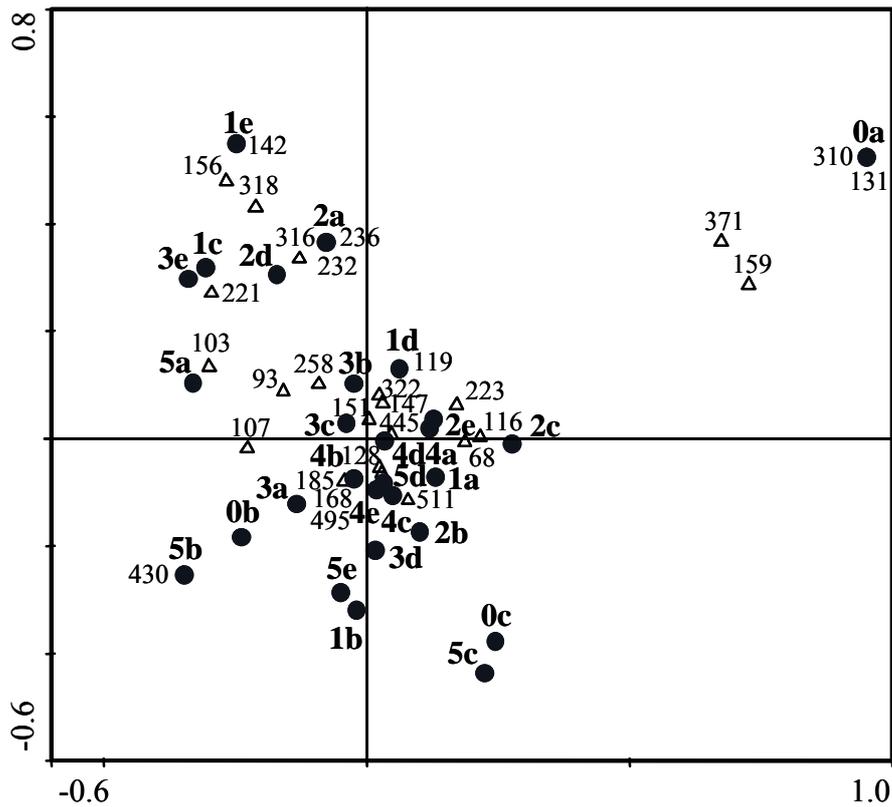


Fig. 8. CA ordination plot for the soil samples of the plant mesocosms (filled circles) and T-RFs (triangles, labelled according to fragment size [bp]) based on *nirK*-T-RFLP-data obtained for summer 2004. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.106$, $\lambda_2 = 0.093$. 0a-c, unplanted mesocosms; 1a-e *Holcus lanatus* and *Arrhenatherum elatius*; 2a-e, *Holcus lanatus* and *Geranium pratense*; 3a-e, *Holcus lanatus* and *Plantago lanceolata*; 4a-e *Holcus lanatus*, *Arrhenatherum elatius*, *Geranium pratense*, *Plantago lanceolata*; 5a-e, like 4, and *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Taraxacum officinale*, *Ranunculus acris*.

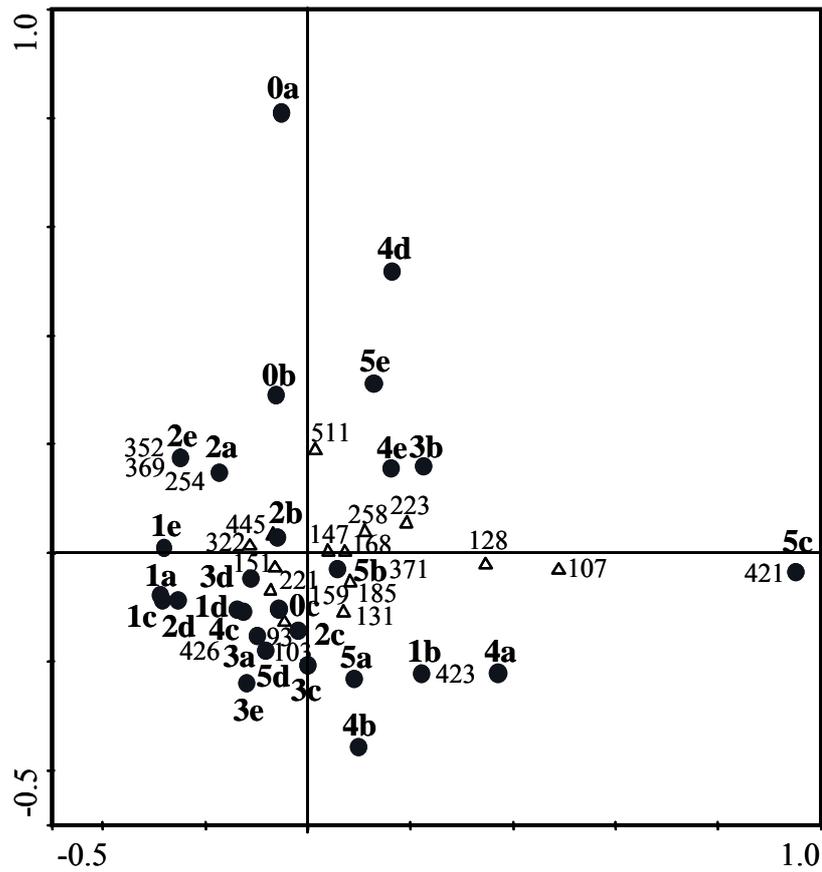


Fig. 9. CA ordination plot for the soil samples of the plant mesocosms (filled circles) and T-RFs (triangles, labelled according to fragment size [bp]) based on *nirK*-T-RFLP-data obtained for autumn 2004. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.146$, $\lambda_2 = 0.106$. 0a-c, unplanted mesocosms; 1a-e *Holcus lanatus* and *Arrhenatherum elatius*; 2a-e, *Holcus lanatus* and *Geranium pratense*; 3a-e, *Holcus lanatus* and *Plantago lanceolata*; 4a-e *Holcus lanatus*, *Arrhenatherum elatius*, *Geranium pratense*, *Plantago lanceolata*; 5a-e, like 4, and *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Taraxacum officinale*, *Ranunculus acris*.

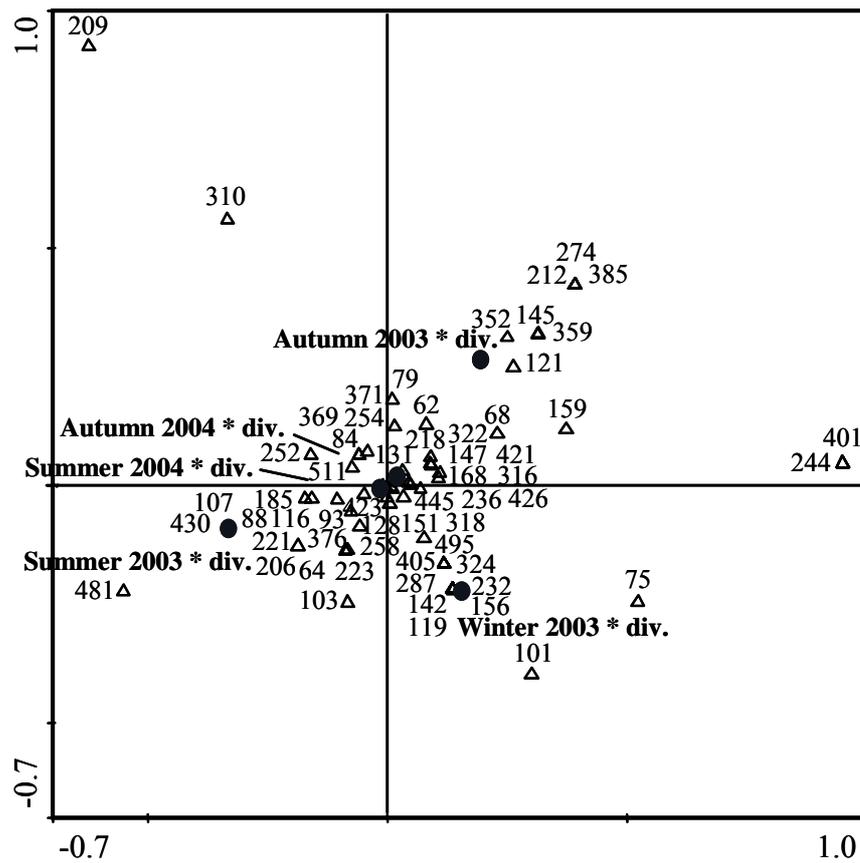


Fig. 10. CCA ordination plot for the effect of plant species number (diversity) and sampling time on the *nirK*-type denitrifier community. The different sampling times are denoted by filled circles and T-RFs by triangles, labelled according to fragment size [bp]) based on *nirK*-T-RFLP-data. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.039$, $\lambda_2 = 0.023$.

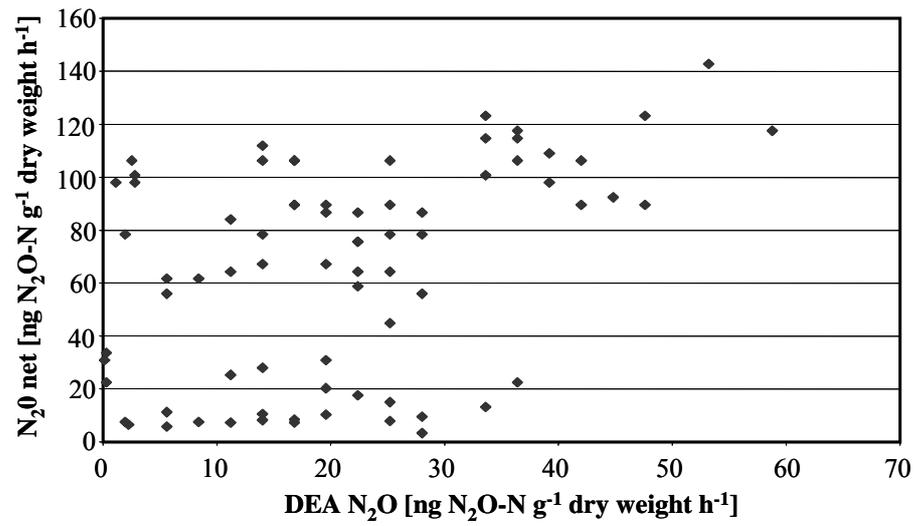


Fig. 11. The relationship between net N₂O production rates and denitrifier enzyme activity (DEA) in mesocosm soil.

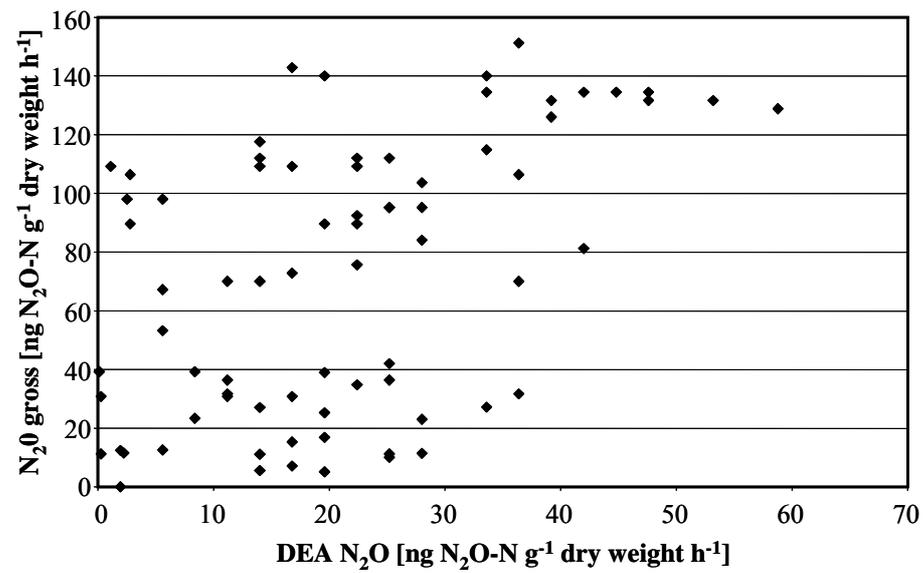


Fig. 12. The relationship between gross N₂O production rates and denitrifier enzyme activity (DEA) in mesocosm soil.

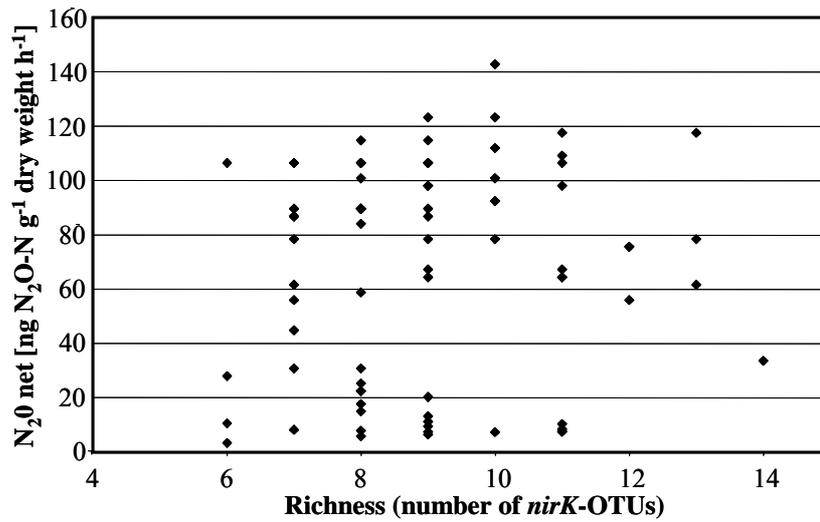


Fig. 13. The relationship between net N₂O production rates and richness of *nirK*-OTUs in mesocosm soil.

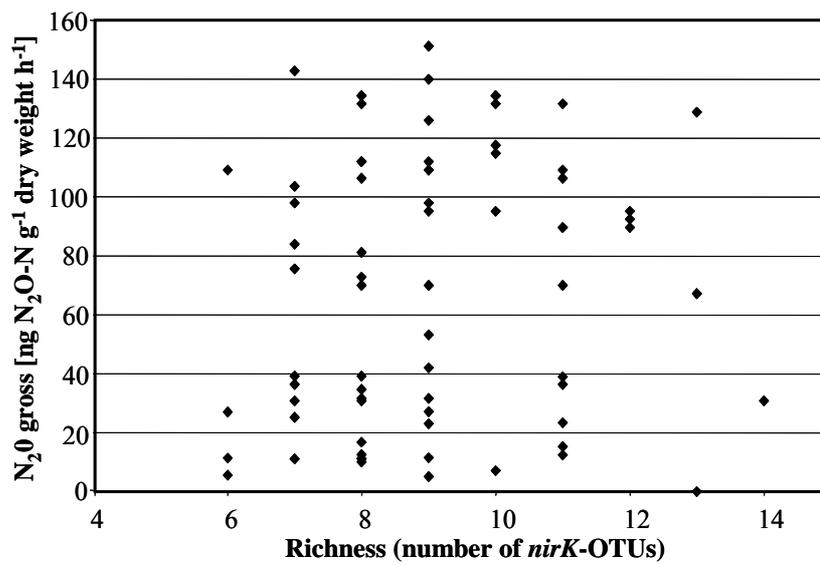


Fig. 14. The relationship between gross N₂O production rates and richness of *nirK*-OTUs in mesocosm soil.

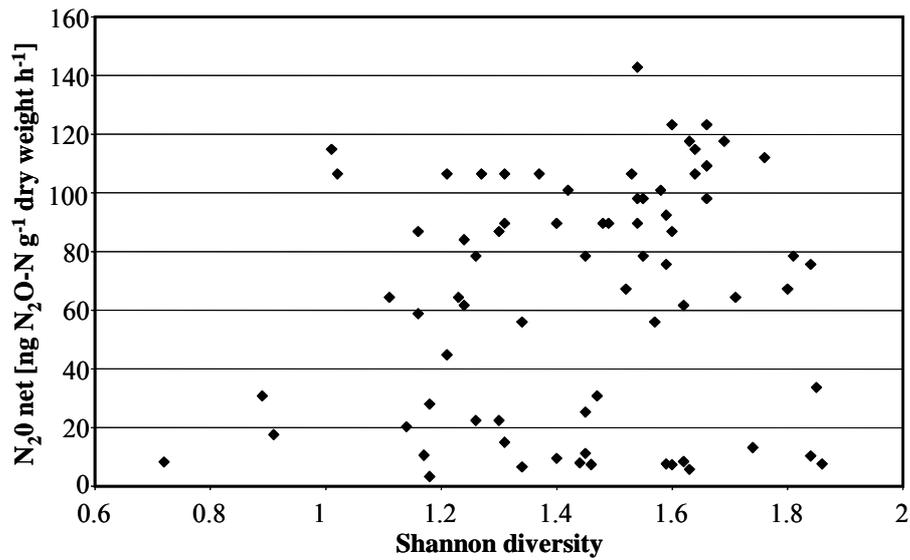


Fig. 15. The relationship between net N₂O production rates and Shannon diversity indices of *nirK*-OTUs in mesocosm soil.

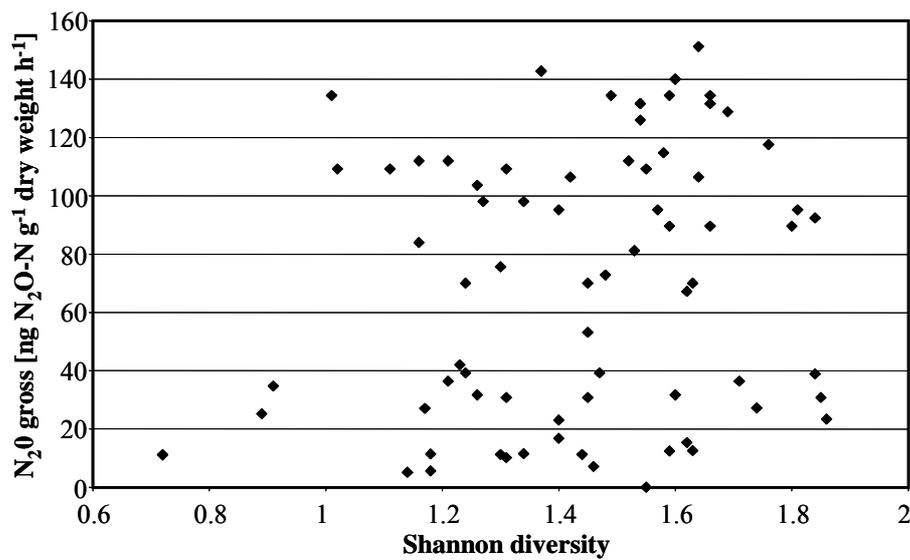


Fig. 16. The relationship between gross N₂O production rates and Shannon diversity indices of *nirK*-OTUs in mesocosm soil.

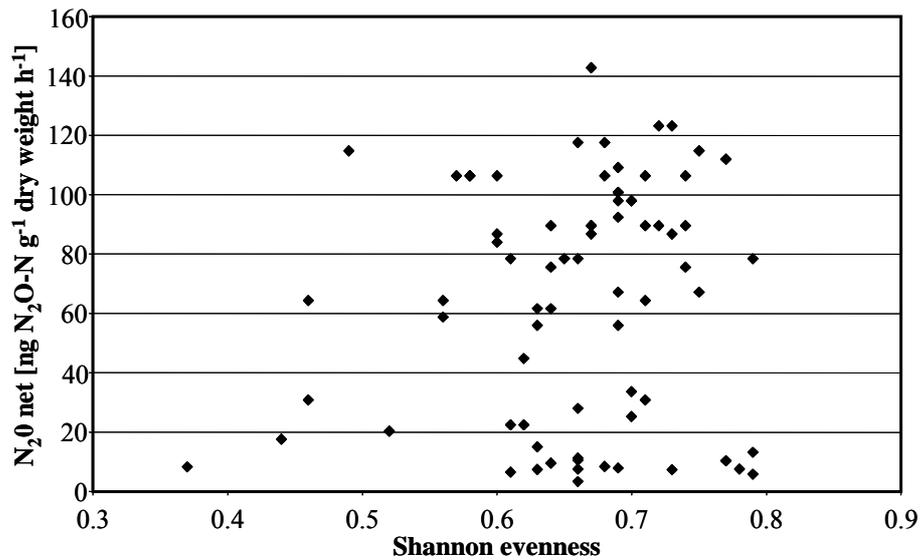


Fig. 17. The relationship between net N₂O production rates and Shannon evenness indices of *nirK*-OTUs in mesocosm soil.

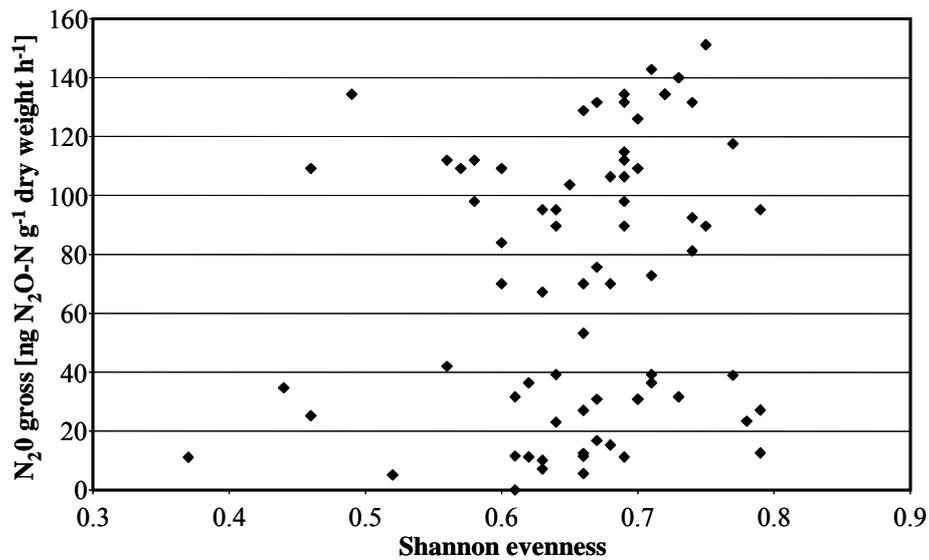


Fig. 18. The relationship between gross N₂O production rates and Shannon evenness indices of *nirK*-OTUs in mesocosm soil.



Fig. 19. Picture of the plant microcosms (monocultures) in the Ecological Botanical Garden of the University of Bayreuth, summer 2003.



Fig. 20. Picture of a single mesocosm (lysimeter) in the Ecological Botanical Garden of the University of Bayreuth, summer 2003.



Fig. 21. Picture of the mesocosm (lysimeter) arrangement in the Ecological Botanical Garden of the University of Bayreuth, summer 2003.

**Impact of plant functional group, plant species and sampling time on diversity
and composition of *nirK*-type denitrifier communities in soil**

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Running title: Impact of plant species on denitrifier communities

Abstract

We studied the influence of eight non-leguminous 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 analysed 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 analysed by canonical correspondence analysis (CCA). Whereas 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 growth of plants in the microcosms. The effect of plant species identity on denitrifier community composition could be due to observed plant-induced differences in total soil nitrogen and carbon, soil pH and root biomasses or due to the amount and composition of 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 non-leguminous plants and their development in the microcosms over time influenced the composition of denitrifier communities in soil, but environmental conditions had additional significant effects.

Introduction

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 (10, 13, 21, 31, 46, 49, 51). 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 (26, 32). However, the root exudation of an individual plant may also depend on its growth conditions and developmental stage thereby potentially masking species-specific effects (12, 45). Apart from the plants, soil type, soil structure and specific characteristics also affect the microbial community (6, 7, 19, 30), and these soil effects have often been found to be more important than the identity of the plant species and its root exudates (16). In addition, seasonal changes of environmental conditions may also influence the amount, activity and composition of soil microorganisms (15, 22, 28, 41, 43, 46).

Most studies of plant-microbe relationships have focused on the overall microbial community composition based on the analysis of the 16S rRNA genes. However, there is also evidence that individual functional groups of soil microorganisms like methanotrophs (23), ammonia-oxidising bacteria (25) and denitrifying bacteria (9) 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 of unplanted soil (35). 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 USA (39). 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 (34). *NirK*-type denitrifiers were also found in the rhizosphere of three legume crops, and the diversity and composition of *nirK* transcripts was influenced by plant species identity (44). Apart from effects induced by plants, the community composition of denitrifying bacteria has also been shown to be influenced by the seasonal variation of environmental conditions (52).

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 mainly driven by facultative anaerobic bacteria, which use oxidized nitrogen compounds as alternative electron acceptors for energy production (55). The key enzyme in dissimilatory denitrification is nitrite reductase, since the ability to reduce nitrite to nitric oxide separates nitrate respirers from denitrifiers (55). Two structurally different nitrite reductases are found among denitrifiers. The copper-containing nitrite reductase is encoded by *nirK*, the heme *cd*₁-containing enzyme by *nirS*. Both genes have been effectively appointed as functional markers genes to detect communities of denitrifiers in the environment, e.g. in soils (17, 37), activated sludge (48), and marine environments (5, 8). However, in a number of studies *nirK* could be more readily amplified from soils than *nirS* (37, 44, 52).

The objectives of our study were to analyse the *nirK*-type denitrifier community in soil associated with different non-leguminous plant species, representing two functional groups of plants (grasses and forbs), from unimproved temperate grasslands. Grasses have a more intensive root system than forbs (45). Hence we hypothesized

that plant functional group and plant species identity influence denitrifier diversity and composition. We further hypothesized that denitrifier community composition differs between seasons. Eight typical grassland plant species from Western Europe were cultivated in individual microcosms containing the same soil. *NirK* genes were extracted from the soil, PCR amplified from environmental DNA and analysed by terminal restriction fragment length polymorphism (T-RFLP). We evaluated the impact of different experimental factors (plant functional group, plant species, sampling time, and interactions between these factors) on denitrifier diversity and community composition by nested analysis of variance (ANOVA) and by canonical correspondence analysis (CCA). In addition, we tested the influence of plant functional group and plant species identity on soil properties (total nitrogen and carbon content, and pH) at the end of the experiment. Amplicons of *nirK* from two soil samples were cloned and sequenced to identify important members of the denitrifier community.

MATERIALS AND METHODS

Experimental setup and soil sampling. In autumn 2001, monocultures of eight non-leguminous grassland plants representing two functional plant groups were grown in microcosms (Ø 20 cm, depth 100 cm) under ambient environmental conditions in the Ecological Botanical Garden of the University of Bayreuth. Functional plant groups had been defined in the past, in many cases according to morphological traits (27, 36, 50). In the present study we assigned two functional plant groups, grasses and forbs, 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*. Three replicate microcosms were set up for

each species. 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: $\text{pH}_{(\text{CaCl}_2)}$ 4.9, $\text{NH}_4^+\text{-N}_{(1\text{ M KCl})}$ 19.6 mg kg^{-1} , $\text{NO}_3^-\text{-N}_{(1\text{ M KCl})}$ 27.0 mg kg^{-1} , and it consisted of 7% sand, 78% silt and 16% clay (24). The soil was thoroughly mixed and steamed (12 h at 100° C) to kill weed seeds. The plants were grown for three years and watered if necessary. The experimental set up is described in detail in the study of Reuter (38). In summer (June) and autumn (September) 2003 and in summer (June) 2004 two soil samples were taken from each microcosm to a depth of 6 cm (about 10 g soil), pooled, thoroughly 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 microcosms (38).

DNA extraction. Soil (0.5 g), 0.5 g glass-beads (\O 0.17-0.18 mm, B. Braun Biotech Int. GmbH, Melsungen, Germany), 800 μl sterile sodium phosphate buffer and 260 μl sterile SDS buffer were mixed. Cell lysis was done with a bead-beater (BIO 101 Mini Bead Beater, Savant, New York, USA) for 45 s with 6.5 ms^{-1} . Samples were centrifuged for 15 minutes at 14,000 rpm 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-isoamylalcohol (25:24:1) and 600 μl chloroform-isoamylalcohol (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 volumes of 100% isopropanol at room temperature.

After centrifugation (60 min, 14,000 rpm at room temperature) the DNA pellet was washed with ice-cold 70% (vol/vol) ethanol and centrifuged again (10 min, 14,000 rpm at 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 recommendations. Subsequently, the purity and the quantity of the DNA was 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 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 PCR reactions were done and pooled afterwards to minimize PCR artefacts. All PCR reactions were done in the cycler Primus 96^{plus} (MWG Biotech, Ebersberg, Germany). 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 for 40 s. A last step was done at 72°C for 7 min. The quality and the quantity of PCR products was determined by electro-

phoresis of an aliquot of each PCR reaction 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 l^{-1}).

T-RFLP analysis of *nirK*-type denitrifiers. For T-RFLP analysis, the reverse PCR primer nirK5R was 5'-end labelled with 6-carboxyfluorescein. PCR products of the correct size were excised from the agarose gel and purified with the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany), because in some cases PCR by-products of unexpected sizes were amplified, too. The purified PCR products (100 ng) were hydrolysed with 5 U of the restriction endonuclease *Hae*III (New England Biolabs, Frankfurt, Germany) overnight at 37°C (1). Digestions were cleaned up with Autoseq G-50 columns (Amersham Biosciences, Piscataway, NJ, USA) 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). Restriction fragments were separated on an ABI 310 automated sequencer (Applied Biosystems, Darmstadt, Germany). Since T-RFs can vary slightly in size, T-RFLP patterns were inspected visually and peak size differences of one or two bp were confirmed by comparing the respective peaks of all patterns. Afterwards, the lengths of the fluorescently labeled terminal restriction fragments (T-RFs) were defined by comparison to the internal length standard with GeneScan 3.71 software (Applied Biosystems). Peaks with a fluorescence of 50 U over the background fluorescence and larger than 60 bp were analysed by peak height. The relative abundances of T-RFs in a sample, given in percent, were calculated and normalized in an iterative standardization procedure according to Dunbar et al. (11).

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. These samples were selected for cloning because T-RFLP patterns obtained from *Plantago lanceolata* microcosms included most of the T-RFs also present in the profiles for all other plants. In contrast, profiles of *Holcus lanatus* indicated a 131-bp fragment of high relative abundance that was specific for microcosms of only some of the plants. 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 sequenced 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 reactions were purified with Autoseq G-50 columns (Amersham Biosciences) and analysed on an ABI 377 DNA sequencer (Applied Biosystems).

Phylogenetic analysis. Phylogenetic analyses were done with ARB (<http://www.arb-home.de>). *NirK* sequences 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

neighbour 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 analysed *in silico* for terminal restriction fragments obtained by cleavage with the restriction endonuclease *HaeIII* using TRF-CUT (40) 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, time and the interactions on T-RFLP-profiles were explored by ordination techniques. After an initial detrended correspondence analysis had indicated that a unimodal response model was more appropriate than a linear model, the data were analysed by correspondence analysis (CA) and canonical correspondence analysis (CCA). A CA is a method to describe the structure of a dataset. In addition, a CCA allows to relate community variation to environmental variation. It uses the individual T-RF obtained from each replicate microcosm and their relative abundance as input variables and calculates the position of all the T-RFs in a two-dimensional ordination. Samples with a similar community composition are placed closer together, samples with a more dissimilar community are positioned further apart. Because of the nested design of the experiment, covariables and dummy variables were included in the analyses. Covariables are concomitant variables whose effect is partialled out when analysing the effects of the variables of

interest. Dummy variables are nominal variables defined as 1 or 0, that code for the levels of a factor.

First, single CAs were performed with the T-RFLP-datasets for the three sampling times to view the structure of the data and the quality of replication. Second, several CCAs were performed with different numbers of replicates for the effect of the factors of interest and to reflect the nested design of the experiment (54). The effect of plant group (grasses vs. forbs) was tested using a dataset consisting of average OTU abundances for the individual plant species. The effect of plant species on *nirK*-OTUs was tested using a dataset consisting of average OTU abundances for the individual microcosms and with the effect of plant group partialled out by using plant group as a dummy-coded covariable. The effect of time on *nirK*-OTU abundance was tested using the dataset containing the measurements taken for the individual microcosms at the three sampling times and with the variation due to microcosms partialled out. The effect of interactions on *nirK*-OTU abundance was tested using the dataset containing the measurements taken for the individual microcosms at the three sampling times and with the variation due to microcosms and time partialled out. Monte Carlo permutation tests (based on 5000 random unrestricted permutations with blocks defined by the covariable microcosm) as available in CANOCO 4.5 (47) were used to test the hypothesis that relative abundances of *nirK*-OTUs were unrelated to the factors plant functional group, plant species, sampling time and the interactions between these factors. The effects of the experimental factors plant functional group and plant species on the soil parameters were explored by analysis of variance (ANOVA) according to the nested experimental design. Thus, the effects of plant functional group were tested against the variation among plant species, and those of plant species identity against

the variation among the microcosms. Values for total nitrogen content were log-transformed prior to the analysis.

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. *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 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 supplementary material Fig.1 for T-RFLP-profiles of the replicates at the three sampling times and Fig. 2 for the corresponding ordination diagrams). When the T-RFLP-dataset of one sampling time was averaged, the histograms showed marked differences among the structure of the denitrifier community in the soil of microcosms planted with different plants (as an example the results of 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 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 some of the plant species (e.g. the 131-bp T-RF with *Holcus lanatus*, *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 dataset was further analysed by canonical correspondence analysis (CCA). With a CCA the variation in the abundance data can be explained via the

ordination axes by environmental variables (plant functional group, plant species identity, sampling time, and interactions). CCAs were performed with different numbers of replicates for the effect of the factors of interest. The functional group of a plant (grasses vs. forbs) did not significantly affect the *nirK*-type denitrifier community in the soil ($P = 0.60$; Monte Carlo permutation test within CCA) (data not shown), but plant species identity significantly influenced the relative abundance of *nirK*-T-RFs ($P = 0.0002$; Monte Carlo permutation test) (Fig. 2). In the ordination diagram T-RFs scattering around plant species indicate *nirK*-OTUs that typically occurred with a given plant species. Moreover, plant species with a similar soil denitrifier community are positioned closely together. For instance, soil from the plants *Plantago lanceolata*, *Anthoxanthum odoratum* and *Ranunculus acris* had a similar denitrifier community whereas that from *Holcus lanatus* was very different from those of all other plant species. T-RFs placed in the center of the diagram either represent *nirK* operational taxonomic units (OTUs) occurring in the soil from all plant species or they represent those that occur only in the soil from *Ranunculus acris*.

There was also a general significant effect of sampling time on the composition of the *nirK*-type soil denitrifier community ($P = 0.0002$; Monte Carlo permutation test) (Fig. 3). Interestingly, the communities of *nirK*-type denitrifiers in the soil sampled in the two summers 2003 and 2004 were as different from each other as they were from 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 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*. We also observed that *nirK*-

OTUs which were unrelated to sampling time matched with *nirK*-OTUs unrelated to plant species (e.g. T-RFs of 128, 151, and 185 bp) indicating the general occurrence of the respective denitrifiers in the given soil substrate. The effect of time as a linear factor was also found to be significant ($P = 0.0002$; Monte Carlo permutation test), pointing at a trend of microcosm development (data not shown).

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 ($P = 0.003$; Monte Carlo permutation test) indicating that the effect of plant species identity varied with sampling time (see supplementary material Fig. 3 for the ordination diagram). However, the interactions of time as a linear factor with plant functional group and with plant identity were not significant (data not shown)

Soil characteristics. At the end of the experiment in summer 2004 total carbon and nitrogen content, pH, and root biomass per plant were determined for the individual microcosms. 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 pH and the lowest root biomass (Table 1). *Alopecurus pratensis* produced the highest root biomass per plant.

Plant functional group only affected soil pH ($P < 0.05$). It was significantly higher in soil planted with grasses than in soil planted with forbs. Plant species identity influenced total nitrogen ($P < 0.01$) and carbon content ($P < 0.05$), soil pH ($P < 0.001$) and root biomass ($P < 0.01$).

***NirK* phylogeny.** 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 of the EMBL database, including taxonomically characterized isolates and *nirK* sequences of unknown 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, cluster I to V) and several subclusters in cluster I and II. The overall topology was supported by neighbour 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 our clones (86) from soil from 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. 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. Part of these sequences grouped in the vicinity, but not closely, to *nirK* from *Rhizobium sullae* (cluster IIa), one clone was related to *nirK* from *Pseudomonas* sp. reclassified as a member of the *Rhizobiales* within the α -Proteobacteria (cluster IIb), while the remainder of the sequences in this cluster formed two distinct subclusters (cluster IId and e). Two sequences from soil from *Plantago lanceolata* were grouped in cluster III and were related to *nirK* from *Blastobacter de-*

nitrificans 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. *NirK* sequences were cleaved *in silico* with the restriction 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 our 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 <60 bp were lacking from the internal standard.

Calculation of theoretical T-RFs demonstrated that *nirK* sequences with T-RFs of 61 bp, 64, 70, 156 (experimental 151 bp), 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 one referring to an abundant T-RF in the soil from all plant microcosms, were either grouped in single subclusters (e.g. Ib, Ih) or in a single cluster (I). However, sequences with different T-RFs clustered in subcluster Ic, Id, and Ie. Se-

quences with a theoretical T-RF 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 cluster 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. *NirK* sequences with the unique theoretical T-RF of 109 bp, which were most frequently found in T-RFLP profiles from soil from *Holcus lanatus*, but also in soil of *Alopecurus pratensis*, *Taraxacum officinale*, and *Ranunculus acris* (Fig. 1, 3, and 4) were affiliated with *nirK* of *Mesorhizobium* sp. in cluster IV.

DISCUSSION

The aim of our study was to test the hypotheses that non-leguminous grassland plant species representing two functional groups (grasses and forbs) with different root systems exert a species-specific impact on a functional group of soil microorganisms, the *nirK*-type denitrifiers.

We are aware that using *nirK* genes as the only functional marker for denitrifiers neglects an important part of the denitrifier community in soils, the *nirS* containing denitrifiers. Furthermore, DNA extraction, PCR amplification and T-RFLP as a community fingerprinting approach can suffer from bias, but we assume that these effects can be minimized, since a standardized DNA extraction procedure for pooled mixed samples from replicate microcosms was used, the PCR products were pooled, and T-

RF peak heights were standardized finally. Therefore the samples could be compared on a relative basis as was done in previous studies (8, 29, 33). Correspondence analyses have proven to be effective tools for evaluating data of fingerprinting analyses like DGGE (42, 53) and T-RFLP (2, 8, 22, 33). Furthermore, by plotting species and environmental variables, the identification of T-RFs that respond to certain experimental factors (plant functional group, plant species identity, sampling time) is possible.

Many different environmental factors shape the species composition of a functional group of soil microorganisms, but we hypothesized that plant functional group and plant species identity exert a highly selective effect on *nirK*-type denitrifiers that is at least as strong as that of the soil. The successful amplification of *nirK* genes revealed the presence of *nirK*-type denitrifiers in the soil from all plant microcosms. Given that precondition we found that the factor plant functional group (grasses vs. forbs) did not affect the community composition of *nirK*-type denitrifiers. There were also no differences in root biomass, and carbon and nitrogen content between soil planted with grasses and soil planted with forbs at the end of the experiment. However, the pH was higher in soil planted with grasses than in soil planted with forbs. According to Hinsinger et al. (18) 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. The authors also suggest that plant species may have the ability to react to bulk soil pH values by either reducing or increasing their rhizosphere pH. The four grasses used in our experimental setup belong to the same family, the *Poaceae* (class: *Monocotyledonae*), whereas each forb belongs to a different family (the *Asteraceae*, the *Geraniaceae*, the *Plantaginaceae*, and the *Ranunculaceae*; class: *Dicotyledonae*) (45). It is well known that forbs have a less intensive root system than grasses but these differences in taxonomy,

root systems and pH did not lead to the development of plant group-specific denitrifier communities in the soil.

In contrast to the functional group of a plant, the plant species identity influenced the community composition of *nirK*-type denitrifiers. Plant species identity also influenced the total amount of N and C in the soil, the pH and the root biomass. The most distinct denitrifier community composition was found in the soil from *Holcus lanatus*. This soil had the highest C content and the highest soil pH together with the lowest root biomass, probably due to a high root turnover in these microcosms. We assume that the observed plant-mediated effects on soil characteristics may cause the differences in denitrifier community compositions. *Alopecurus pratensis* had the highest root biomass per plant in June 2004 and its denitrifier community composition was also quite dissimilar from that of the other plants. Plant species like *Anthoxanthum odoratum* and *Plantago lanceolata* harbored similar *nirK*-type denitrifier community compositions and did not differ in their soil characteristics. In contrast, soil from *Geranium pratense* had the lowest pH, but did not have a very distinct denitrifier community composition. Although we observed some correlations between the plant species, their soil characteristics and the respective denitrifier community composition, we can not draw conclusions about causes and effects, since the processes involved are complex, too few plant species were involved and the soil characteristics were not measured throughout the whole experiment.

CCA allowed the identification of plant-specific *nirK*-OTUs probably representing *nirK*-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. To our knowledge this study provides for the first time insight into the relationships between typical non-leguminous grassland plant species and the respective *nirK*-type denitri-

fier communities in soil. The weather conditions were identical for all microcosms, therefore this factor was not responsible for the patterns observed.

Generally, non-symbiotic plant-specific microbial communities or differences in the microbial community composition in association with plants may be attributed to differences in the amount and composition of root exudates. Exudation is a dynamic process which varies depending on plant species, physiological status of the plant (45), root zone (20), and nutritional conditions (13). 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 mineralisation). Specific exudates may also selectively favor some microbial strains over others.

The community composition of *nirK*-type denitrifiers varied among sampling dates, and also in a directional way. But to assume a consistent trend in microcosm development based on three sampling times is rather speculative. The general effect of time was not unexpected, because many studies have reported seasonal effects on soil microbial communities in the past (3, 14, 15, 22, 28, 43). Seasonal climatic changes govern physical soil characteristics such as moisture and temperature. Our results are in accordance with those of Wolsing and Priemé (52), who reported a significant seasonal shift in the community structure of *nirK*-type denitrifiers in spring, summer and autumn of a single year. Surprisingly, we observed different *nirK*-type denitrifier community composition for the same season (summer) in different years (2003 and 2004) emphasizing the variation of environmental conditions and their influence on certain soil characteristics. Apart from differences in denitrifier community composition due to seasonal impacts, the effect of time varied for individual plant species. The variation of time effects with plant identity hints on a development of the plant microcosms with age, either due to aging of the plant itself or a progressing root

development in the microcosms. The observation that the denitrifier community composition was different in two consecutive summers may support this notion.

We assume that the most abundant *nirK*-OTUs that were ubiquitous in the soil from all plant species at any sampling time originate from denitrifiers that are well adapted to the characteristics of the microcosm soil substrate and to the surrounding climatic conditions. In contrast, the occurrence of less abundant but rather specific *nirK*-OTUs may be linked to special environmental factors like 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. Six of 16 different *nirK*-OTUs detected in the soil from *Holcus lanatus* and *Plantago lanceolata* could be assigned to *nirK* sequences in our clone libraries by T-RFLP-analysis of clones and using TRF-CUT. The majority of our *nirK* sequences (86 of 109) were related to *nirK* sequences of uncultured organisms from soil and activated sludge and to *Nitrosomonas* sp. TA-921 i-NH₄ as the nearest cultivated representative. Four theoretical T-RFs of the *nirK* sequences in cluster I (156, 173, 190, and 516 bp) corresponded to the experimental T-RFs (151, 168, 185, and 511 bp), which were found to be ubiquitous in the soil from all plant microcosms at any sampling time, but which also demonstrated differences in their relative abundances depending on plant identity and sampling time. However, our results showed that closely related *nirK* sequences sharing the same T-RF can represent the same organism. In clusters II, III and IV several of our *nirK* sequences were affiliated with *nirK* genes of denitrifiers from the order *Rhizobiales*, namely *Rhizobium*, *Bradyrhizobium*, *Blastobacter*, and *Mesorhizobium*. The results of the present study accord with other studies that found numerous functional marker genes for denitrifi-

cation in soils grouping with denitrification genes of these representatives of the *Rhizobiales* (35, 37, 39). *Rhizobiales* usually live in symbiosis with legumes, but can also be found free-living in soil when leguminous plants are absent.

In cluster II, *nirK* sequences with a specific T-RF (theoretical, 136 bp, experimental, 131 bp) from soil planted with *Holcus lanatus* form a distinct subcluster (IIe) without a close relationship to *nirK* from any cultivated organism indicating a group of denitrifiers with yet unknown *nirK*-genotype. In cluster IV, *nirK* sequences with the theoretical T-RFs of 36 and 109 bp (experimental 101 bp) from soil planted with *Holcus lanatus* were grouped with *nirK* of *Mesorhizobium*.

We conclude that non-leguminous plant species of temperate grassland exert a species-specific effect on a functional group of soil microorganisms, the *nirK*-type denitrifiers. This effect is mainly due to differences in the abundance of individual *nirK*-OTUs, and it may also be the result of complex interactions between plants, microorganisms and soil properties. The functional group of a plant (grasses vs. forbs) did not show an effect on the *nirK*-type denitrifier community composition. We also observed different *nirK* communities at different sampling times and an interaction between the effects of time and plant species identity suggesting changes due to seasonal conditions or due to the development of the individual plant microcosms. However, it is impossible to infer a phylogenetic relationship of denitrifiers without cultivation studies since a distribution of denitrification genes via horizontal gene transfer is likely to have occurred. Further research on root exudation of individual plant species could provide instructive insights into the black box of non-leguminous plant-microbe relationships.

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TABLE 1. Characteristics of the soil in the microcosms at the end of the experiment. Mean values of the three replicates are shown together with the standard errors of the mean. Different letters indicate significant differences between the values ($P < 0.05$; one way ANOVA with Tukey's HSD test).

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

FIG. 1. Averaged relative abundance of *nirK* terminal restriction fragments (T-RFs) from soil of replicate plant microcosms in summer 2003. Peak size is given in base pairs, the relative abundance of T-RFs in percentage of total peak height. (For data on individual microcosms see supplementary material Fig.1 and 2.) Fragment sizes within the graph indicate the size [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 plant species (filled circles) and T-RFs (triangles, labelled according to fragment size [bp]) based on *nirK*-T-RFLP-data obtained for summer and autumn 2003 and for summer 2004. The eigenvalues of the 1st and 2nd 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*.

FIG. 3. CCA ordination plot for the sampling times (filled circles) and T-RFs (triangles, labelled according to fragment size [bp]) based on *nirK*-T-RFLP-data from microcosms of plant replicates. The eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.076$, $\lambda_2 = 0.023$.

FIG. 4. Maximum likelihood tree based on partial *nirK* sequences (432 nucleotide positions) from cultured *nirK*-type denitrifiers and sequences from microcosm soil. Tree topology is supported by parsimony and neighbour joining method. Dashed lines indi-

cate 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 size of T-RFs after *in silico* analysis with the restriction enzyme *Hae*III. Accession numbers are in parentheses.

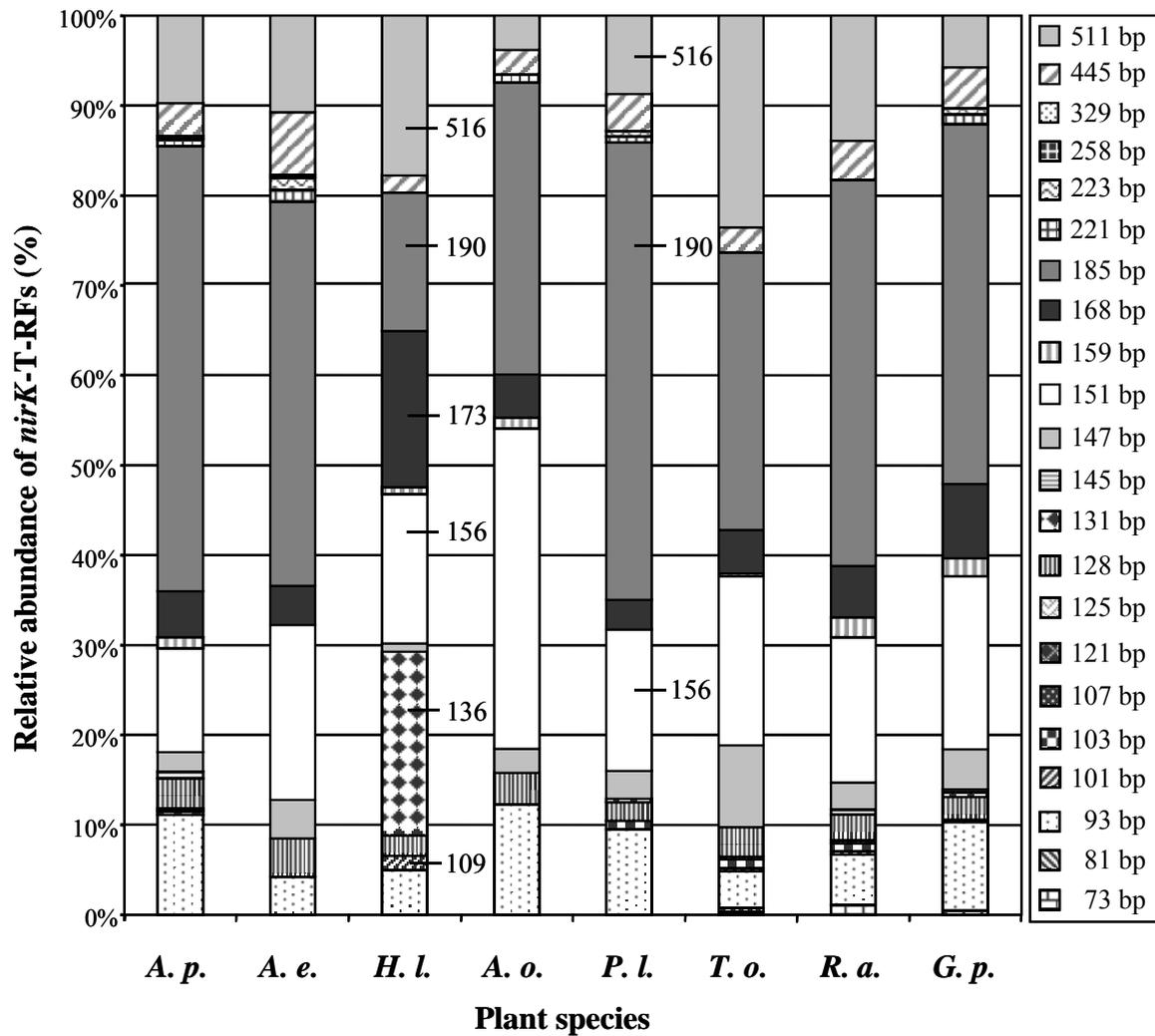


FIG. 1

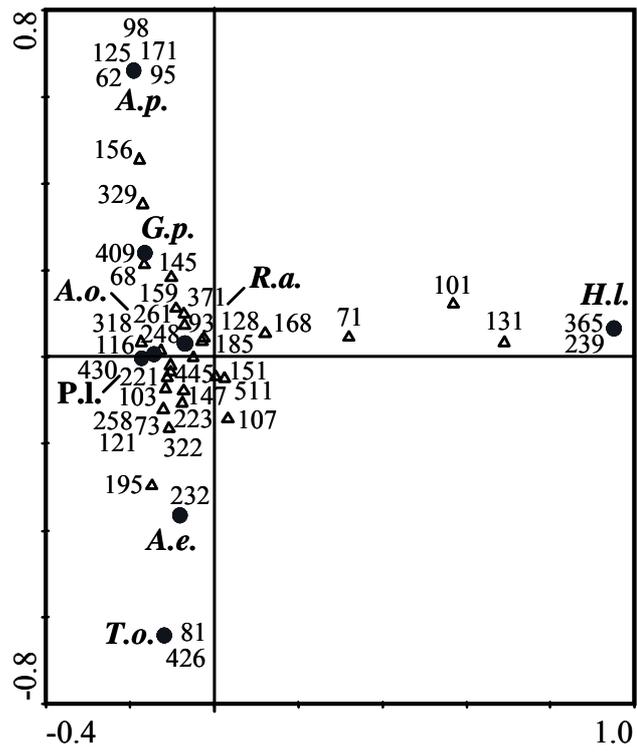


FIG. 2

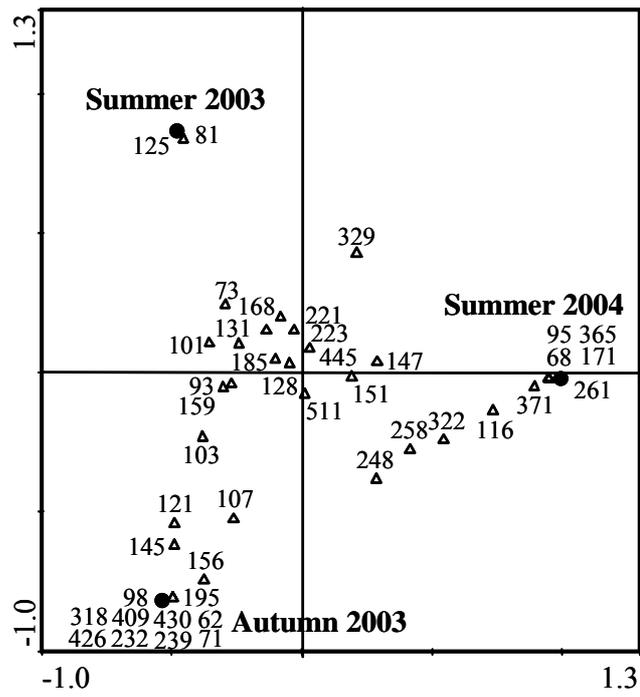


FIG. 3



FIG. 4

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