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BACTERIAL NITROGEN CYCLES IN AGRICULTURAL SETTINGS

Dissertation

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Kurzfassung

Mikrobielle Gemeinschaften im Boden spielen eine bedeutende Rolle bei der Umsetzung von Bodennährstoffen, so auch Stickstoff (N) und damit verbundenen N-Verlusten in landwirtschaftlichen Böden. Wie eine Vielzahl oft eng miteinander verknüpfter Parameter, die diese Organismengruppen beinflussen und zu Änderungen in Anzahl und Zusammensetzung führen, zusammenspielen, ist großteils noch ungeklärt.

Um tiefere Einblicke in diese `biotic-black box` zu erlangen, wurden Ammonium-oxidierende und denitrifizierende mikrobielle Gemeinschaften in verschiedenen Bodentypen, unter verschiedenen Stickstoff-Düngevarianten und Bepflanzung mit unterschiedlichen Kultivaren von Gerste, teils in Kombination mit wachstumsfördernden Bakterien, untersucht. Mittels kultivierungs-unabhängiger molekularer Methoden wurden Struktur- und Abundanzänderungen verfolgt und mit diversen Bodenparametern korreliert. Bodentextur und Unterschiede in Kohlenstoff und Stickstoffpools bestimmen die Unterschiede in denitrifizierenden und nitrifizierenden Gemeinschaftsstrukturen und Abundanzen. Ammonium Beigabe induzierte Veränderungen der Zusammensetzung und Erhöhung der Quantität der Ammuniumoxidiererenden Bakterien (AOB). Diese Effekte zeigte sich verstärkt in Bulk Boden, aber auch in geringerem Ausmaß im Rhizosphären-Boden. Inokulation mit PGPB führte zu einer Vermehrung von AOBs. Ammonium oxidierende Archaea (AOA) hingegen varierten in Struktur und Abundanz in Ammonium- als auch in Nitrat-behandelten Böden, mit verstärkten Effekten bei Nitrat-Düngung. In den untersuchten Böden spielten daher vermutlich AOBs die größere Rolle bei der Umwandlung von Ammonium. Die genaue Rolle der AOAs ist noch unklar und erfordert weiterführende Studien. Denitrifizierer unterschieden sich in mit Gerste bepflanzten Böden im Vergleich zu brachliegenden Kontrollen abhängig vom Bodentyp. Das Alter der Pflanze spielte eine größere Rolle als die N Form mit der gedüngt wurde, da mit Zunahme der Pflanzenbiomasse auch die Abundanz der Denitrifizierer anstieg.

Abstract

Even though soil microbial communities play an essential role in nitrogen (N) conversion processes in arable soils, parameters and drivers influencing the diversity, activity and quantity of these communities are still a ´biotic-black-box´. Thus, in a first approach, from five different arable soils an extensive biochemical dataset was correlated to abundance and structure of functional bacterial and archaeal marker genes in order to get a deeper insight in microbial N cycling. In two follow up greenhouse pot experiments more specifically the effects on ammonia oxidizing and denitrifying communities upon application of different forms of mineral fertilizer, growth of different barley genotypes combined with inoculation with a plant growth promoting bacterium (PGPB) were tested on bulk and rhizospheric soils. Community structures and abundance of ammonium oxidizing bacteria (AOB) and archaea (AOA) as well as denitrifying bacteria were determined using a culture-independent approach targeting molecular marker genes.

Soil texture and differences in C and N pool sizes mainly account for differences in denitrifier and nitrifier community structure and abundance. Nitrifier and denitrifier diversity and abundance in agricultural soils are differentially influenced by specific soil characteristics.

Ammonium amendment induced changes in ammonia oxidizing bacterial (AOB) community composition and an increase in abundance in bulk soil and rhizosphere, with changes in AOB numbers lagging behind relative to changes in soil ammonium. More pronounced increases in AOB abundance were found in bulk soil but AOB numbers were also enhanced in the rhizosphere upon plant inoculation with a PGPB. AOA numbers varied in abundance in both ammonium and nitrate amended soils, also each treatment shifted the AOA composition. Alltogether AOA underwent shifts in correspondence with soil nitrate rather than ammonium. AOB were thus considered as the main agents responsible for fertilizer ammonium oxidation, while the roles of AOA in soil N cycling still need to be clarified. Community structures of denitrifiers were found to differ in soils planted with barley relative to unfertilized and unplanted control soils depending on soil type. Plant age rather than N amendment showed to be the decisive factor introducing an increase in denitrifying communities.

CONTENTS

OUTLINE OF THE THESIS

Microbial communities are important players in the global biogeochemical cycles in soil environments. Especially in agricultural systems where the anthropogenic influence has increased since industrialisation, bacterial community structures, diversities and abundances are strongly affected. Intensive fertilization application, plant species and tillage change biochemical parameters and soil structure. The focus of this thesis is on nitrogen fertilizer effects on nitrifying and denitrifying bacterial communities since these organisms play a major role in N transformations and plant N availability as well as in nitrogen losses due to either emission of gaseous nitrogen forms or nitrate leaching. Various environmental hazards are the consequences since the emission of NO and N_2O boosts the greenhouse gas effect and nitrate leaching pollutes the groundwater. This thesis describes functional communities and their relations to biochemical data and gas fluxes in different soil types (**Chapter1**) in a small scale laboratory system. N dynamics upon N amendment and the effect on ammonium oxidizing bacteria and archaea (**Chapter 2**) and plant cultivar and bacterial inoculation effects on denitrifiying bacterial guilds were objected (**Chapter 3**) in greenhouse pot experiments. A culture independent approach was applied using T-RFLP analysis based on functional marker genes, encoding for key enzymes in nitrogen cycling, to determine community structures of bacteria and archaea harbouring these enzymes. To explore potential activities, the functional groups were quantified via SYBRGreen real time assays. Nitrogen turnover was followed by gas measurements, ammonium and nitrate determination in the soil as well as nitrate concentrations in the leaching water.

Comparing various different soil types, both nitrifier and denitrifier community composition and abundance appeared to differ significantly between the soil types and were most strongly affected by various C and N pool sizes, while correlations were rather poor with gas fluxes or specific microbial activities that were measured along with molecular analyses (Chapter 1).

Studying short term effects of N fertilizer in form of KNO_3, NH_4NO_3 or $[NH_4]_2SO_4$, testing different barley cultivars and one bacterial growth promoting inoculant revealed to affect ammonium oxidizers and denitrifiers differentially. Short time effects of the fertilizer treatments were seen on bacterial ammonium oxidizing (AOB) communities, which were reflected in structural and quantitative changes depending on ammonium concentration. Bacterial *amoA* copy numbers showed a rapid and continuous increase from day seven upon ammonium sulfate or ammonium nitrate amendment followed by a decrease upon substrate depletion. Ammonium oxidizing archaea (AOA) were more abundant than AOB from the beginning, and showed some shifts in community composition and changes in abundance but primary upon nitrate application rather than ammonium indicating a major role of AOB in ammonium oxidation in the subjected soils. Plant genotype had no influence on the quantity of AOB before and after N amendment but bacterial inoculation strongly influenced the functional communities. No clear effects of fertilizer application were detected on the structure and abundance of denitrifier communities based on *nirS* and *nosZ* gene analysis. Conclusively, nitrate addition was not a modulating factor for the denitrifying communities. With one soil type, denitrifying (*nirS* and *nosZ*) communities differed significantly between fertilized and unplanted/unfertilized control soils, indicating a plant associated effect probably due to root exudation. Objecting different cultivars of barley over an extended time period, showed an increase in denitrifier abundance associated with plant biomass independent of genotype proposing a plant age effect. Also a ratio change *nirS/nosZ* across time indicates a shift in denitrification end products. A delayed nitrate supply due to nitrification was favorable for plant biomass yield but led to increased nitrate leaching.

This study explored the influence of N fertilization on denitrifying and ammonia oxidizing communities and revealed pronounced insights into the dynamics of these communities, but to unravel the `biotic-black-box` there are still many open questions to be asked and answered in future experiments. For example, the role of archaea in the N cycling processes is still unclear

and needs further observation. The difficult cultivation of archaea is limiting the information about the lifestyle and thus the interpretation of environmental influences in ecological studies. Furthermore there is always the question: Who is out there? Are there still unknown organisms that may have more impact than the known ones? Also the matter of which part of the population is active at what time is a major challenge for microbial ecology in the future. Gene expression and metagenomic studies are under development to solve these issues but are facing still methodological limitations.

INTRODUCTION

1. N fertilization efficiencies arable soils

Nitrogen (N) is a limiting factor in agricultural soils, it limits plant growth and thus the net primary production. Only simple, soluble forms of N as simple organic compounds (e.g. amino acids, amino sugars), NO_3^- and NH_4^+ can be used by plants. In natural ecosystems these N sources are supplied to plants through the microbial processes as products of N fixation and mineralization (Schimel & Bennett, 2004), i.e. the activity of soil microbes determines the amount of N that is transformed to plant-usable forms. Modern industrial agriculture used 2007 worldwide 110 Mio tons N fertilizer to increase crop yield (FAO, 2009). But N uptake and conversion into protein and thus biomass is limited differentially in different crops. Depending on the plant species only 40 to 60% of N applied can be recovered from crops with a global average value of 50% (Fig.1) (Mosier, 2002). Volatilization of NH_3, denitrification and nitrate leaching are the consequences, as well as a decline in crop production through depletion of macro- and micronutrients (Aulakh & Malhi, 2005).

Consequential damages are nitrate leaching to the ground water and associated effects on human and environmental health e.g. asphyxiation (blue baby syndrome) or eutrophication of groundwater on a local scale. From a global viewpoint, NO and N_2O emissions resulting from denitrification and ammonification affect the weather and environment. N_2O acts as a greenhouse gas, with a GWP (Global Warming Potential) of 295 in the next 100 years. It can survive 114 years in the atmosphere and makes up 9% of total greenhouse gas emissions. From this up to 70% of N_2O emissions originate from agriculture (IPCC, 2007). Furthermore, NO either leads together with volatile organic compounds to formation of ozone (O_3) in the troposphere or can act as a precursor for nitric acid deposition via atmospheric transport e.g (Jackson *et al.*, 2008).

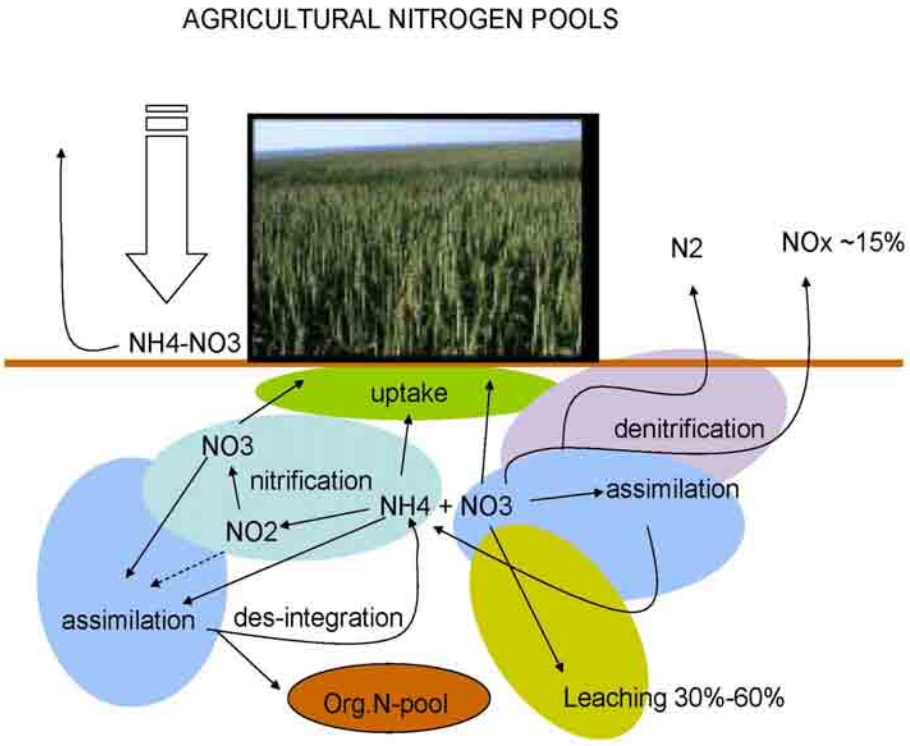


Figure 1 Input of nitrogen fertilizer (NH_4-NO_3) triggers a number of transformation processes such as uptake (assimilation) of both nitrogen forms by plant roots, bacteria and fungi as well as selective steps performed only by certain soil microbial communities, such as nitrification or denitrification. Both latter processes release green house gases from soil. (NITROGENOM proposal)

2. Forms of Nitrogen in soil

N in soil occurs organically bound or mineral, in nine different oxidative states (+ V to –III) (Robertson & Groffman, 2007). The main steps in conversion from one form to another are: N_2 fixation (N_2→ organic N), N mineralization (organic N→ NH_4^+), N immobilization or assimilation (uptake of inorganic forms of N by microbes or other soil heterotrophs), nitrification (NH_4^+→ NO_2^-) and denitrification (NO_3^-→ NO_2^- → NO→ N_2O → N_2).

The oxidation state plays a role in the availability for plants and microorganisms, which take up mainly the mineral N forms NH_4^+ and NO_3^-. Additionally, depending on their charge, the compounds show different migration behaviour in the soil. While ammonium is relatively immobile in soil due to adherence of the cation-exchange site to soil organic matter and

negative clay minerals, nitrate is susceptible to leaching out of the rooting zone into deeper soil layers and into the ground water when precipitation exceeds evapotranspiration. By transforming ammonium into nitrate, nitrifying microorganisms may trigger leaching losses, which in agricultural soils commonly comprise 30 to 60% of the fertilizer N applied. But on the other hand NO_3^- has the advantage to be easier accessible for roots and thus for plant supply.

Intermediaries and endproducts in these conversion processes are often gases that can escape to the environment as nitrogen dioxide (NO_2), nitric oxide (NO), nitrous oxide (N_2O), dinitrogen (N_2) and ammonium (NH_3) (Robertson & Groffman, 2007).

3. Nitrogen transformation and the main players

N mineralization (organic N→ NH_4^+) entitles the conversion of organic N into inorganic form. N immobilization or assimilation on the other hand is the uptake of inorganic forms of N by microbes or other soil heterotrophs. Both are very fundamental processes and therefore widely distributed in nature and regulated very straightforward. Almost all heterotrophic soil organisms (e.g. bacteria, fungi) can mineralize and immobilize N, increasing activity with temperature and intermediate water content (Robertson & Groffman, 2007).

On the opposite, more or less specialized guilds formed by bacteria and archaea have been evidenced as the dominant nitrifying and denitrifying organisms in soils (Hayatsu et al., 2008; Leininger et al, 2006; Treusch et al., 2005). Autotrophic nitrification, which is considered the dominant nitrification process in soils is a two step process, carried out by two specific and phylogenetically restricted groups of microorganisms: ammonia oxidizers and nitrite oxidizers. Ammonia oxidizers possess the enzymes ammonia monooxygenase and NH_2OH oxidoreductase, which catalyze the conversion of NH_4^+ to NO_2^-. The latter is transformed into

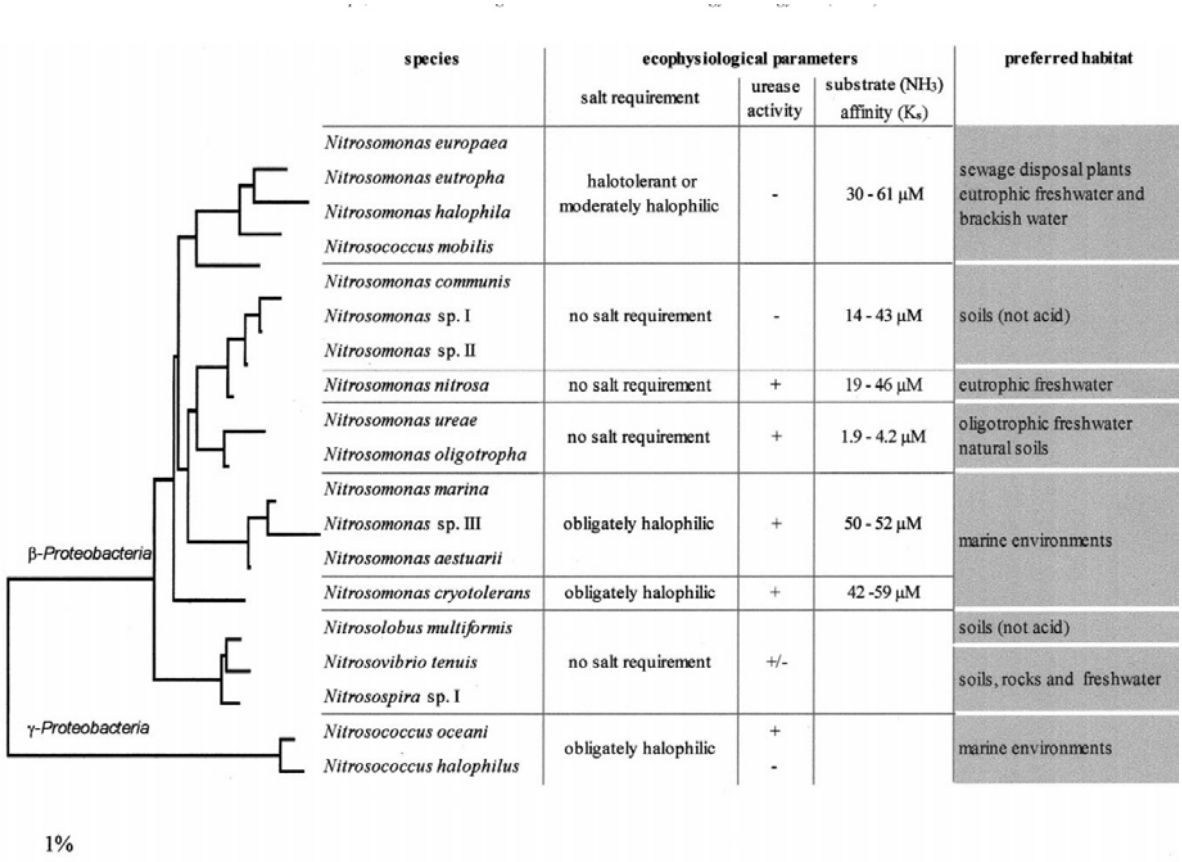
NO_3^- by nitrite oxidizers with the help of the enzyme nitrite oxidoreductase. Ammonia oxidizers can also switch to nitrite reduction resulting in N_2O production when O_2 is limiting. Denitrification is the stepwise reduction of nitrate to NO, N_2O or N_2, whereby nitrate instead of O_2 is used as a terminal electron acceptor during respiration. Denitrifiers are found in many different genera which can either fully or partially reduce NO_3^- depending on whether they possess genes for the whole pathway or not. Nitrate reductase (Nar) and Nitrite reductase (Nir), which catalyse transformation of NO_3^- into NO_2^- and NO_2^- to NO, respectively, are found in all denitrifiers, whereas nitrous oxide reductase (Nos) is not ubiquitously distributed. Nitrous oxide reductase is needed for the conversion of N_2O into N_2, e.g lack of this enzyme indicates greenhouse gas production. Mostly heterotrophic bacteria are denitrifying under oxygen limited conditions, i.e. when soil pores become water-saturated and oxygen diffusion is drastically slowed.

4. Ammonia oxidizers

Diverstiy

Mobilized N in form of NH_4^+ can be oxidised by nitrifiers in two steps into nitrate. Transformation of NH_4^+ to NO_2^- is catalyzed by Ammonia Oxidising Bacteria (AOB) or archaea (AOA). These are specialized groups of bacteria and lately also archaea that were found to possess an enzyme called ammonia monooxygenase (amo) catalyzing the first and rate limiting step of nitrification (Treusch *et al.*, 2005, Leininger *et al.*, 2006, Hayatsu *et al.*, 2008). Ammonia oxidizing bacteria (AOB) are aerobic, chemoautotrophic organisms affiliated with Beta- or Gammaproteobacteria (Kowalchuk & Stephen, 2001, Koops *et al.*, 2003). The major groups in these two phlyogenetically distinct groups based on 16SrRNA contain the genera *Nitrosomonas, Nitrosospira, Nitrosovibrio* and *Nitrosolobus* considering the Betaproteobacteria. The second group affiliated with the Gammaproteobacteria is

represented by two species of *Nitrosomonas* (Koops *et al.*, 2003). In soils different representatives of AOB, mainly depending on soil pH, were found showing low diversity. Whereas acidic soils, lacking free ammonia, were dominated by urease-positive species of the genera *Nitrosospira* and *Nitrosovibrio* (Stephen *et al.*, 1996, Smith *et al.*, 2001), also found in moderately acidic oligotrophic soils, neutral soils were rich in *Nitrosomonas communis* lineages and strains of *Nitrosolobus multiformis* (Fig.2) (Koops & Pommerening-Roser, 2001, Koops *et al.*, 2003).



species	ecophysiological parameters			preferred habitat
	salt requirement	urease activity	substrate (NH_3) affinity (K_s)	
Nitrosomonas europaea *Nitrosomonas eutropha* *Nitrosomonas halophila* *Nitrosococcus mobilis*	halotolerant or moderately halophilic	-	30 - 61 µM	sewage disposal plants eutrophic freshwater and brackish water
Nitrosomonas communis *Nitrosomonas* sp. I *Nitrosomonas* sp. II	no salt requirement	-	14 - 43 µM	soils (not acid)
Nitrosomonas nitrosa	no salt requirement	+	19 - 46 µM	eutrophic freshwater
Nitrosomonas ureae *Nitrosomonas oligotropha*	no salt requirement	+	1.9 - 4.2 µM	oligotrophic freshwater natural soils
Nitrosomonas marina *Nitrosomonas* sp. III *Nitrosomonas aestuarii*	obligately halophilic	+	50 - 52 µM	marine environments
Nitrosomonas cryotolerans	obligately halophilic	+	42 -59 µM	
Nitrosolobus multiformis	no salt requirement	+/-		soils (not acid)
Nitrosovibrio tenuis *Nitrosospira* sp. I				soils, rocks and freshwater
Nitrosococcus oceani	obligately halophilic	+		marine environments
Nitrosococcus halophilus		-		

Figure 2 16S rRNA gene based dendrogram of cultured ammonia-oxidizing bacteria including ecophysiological parameters and preferred habitats (Koops & Pommerening-Roser, 2001)

The lifestyle, the diversity and the phylogeny of ammonia oxidizing archaea (AOA) assigned right now to the group of mesophilic Crenarchaeota is still unknown (Treusch *et al.*, 2005, Francis *et al.*, 2007). First traces for archaea possessing ammonia oxidizing activities were found by Venter and collegues (2004) in their Sargasso sea shot gun sequence project where *amoA* gene homologues were found in a contig with genes of archaeal origin (Venter *et al.*,

2004). Similarly, almost at the same time a soil metagenome study revealed an archaeal role in global energy cycles (Schleper *et al.*, 2005). The final proof was an isolated fosmid that contained 16S rRNA sequences as well as *amoA* and *amoB* subunits (Treusch *et al.*, 2005). Since then AOA were found in diverse environments and are so far separated based on *amoA* gene comparison into a marine and terrestrial group mostly belonging to Crenarchaeota Group 1.1a and 1.1b (Fig. 3) (Prosser & Nicol, 2008).

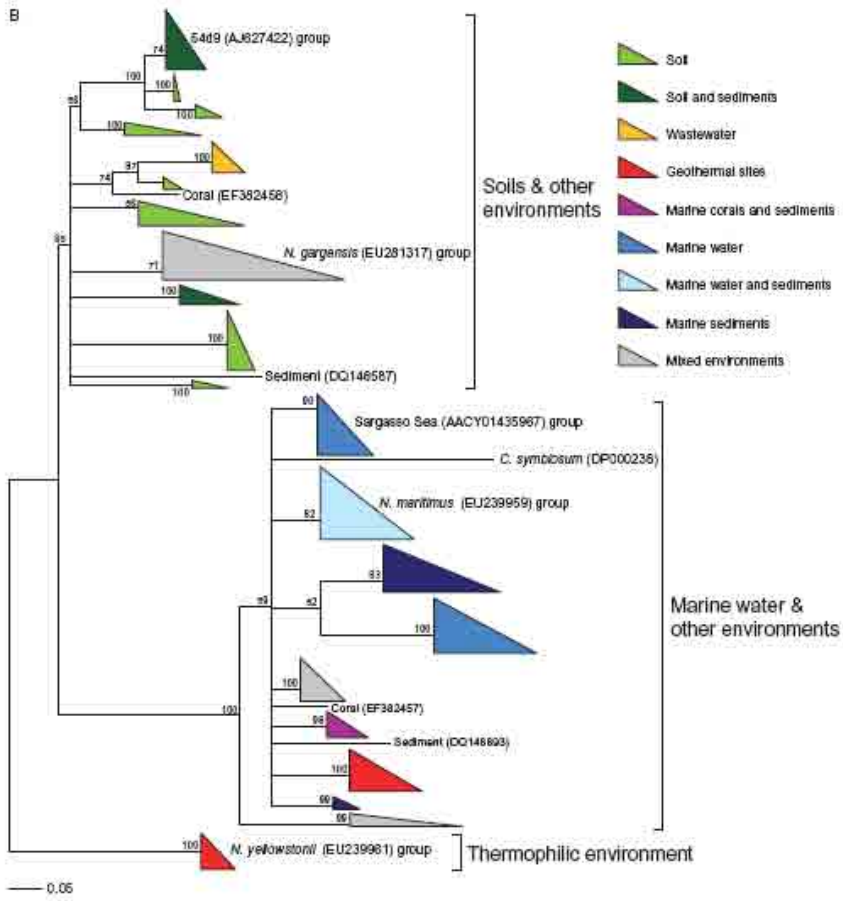


Figure 3 *amoA* gene-defined lineages within the kingdom *Crenarchaeota*. The height and length of triangular blocks are proportional to the number of sequences and maximum branch length within lineages respectively (Prosser & Nicol, 2008)

Key enzymes in ammonia oxidation

All bacterial ammonia oxidizers affiliated to Gamma- and Betaproteobacteria possess the enzyme ammonia monooxygenase, which catalyzes the conversion of NH_4^+ to NH_2OH.

NH_2OH oxidoreductase helps to transform the latter into NO_2^-. Ammonia monooxygenase is the key enzyme in this process since it catalyzes the rate limiting step. It consists of several subunits, is membrane-bound and occurs in 2 to 3 copies per cell in the so far characterised bacteria. Recently Crenarchaea were found to habour distantly related *amoA* genes in a metagenome approach as well as in marine isolates (Koenneke *et al.*, 2005, Treusch *et al.*, 2005).

Parameters affecting ammonia oxidizers in agricultural soils

A variety of studies deal with the subject of fertilization effect on AOB in arable soils, mainly comparing different combinations of mineral and organic fertilizers and reporting a significant effect of NH_4^+ on the abundance and structures of ammonia oxidizing bacterial communities (Hermansson & Lindgren, 2001, Okano *et al.*, 2004, Enwall *et al.*, 2005, Chu *et al.*, 2007, Enwall *et al.*, 2007, He *et al.*, 2007, Cavagnaro *et al.*, 2008, Wang *et al.*, 2009). Also temperature (Avrahami & Conrad, 2003, Avrahami *et al.*, 2003, Avrahami & Conrad, 2005, Avrahami & Bohannan, 2007), soil moisture (Avrahami & Bohannan, 2007) and soil pH (Prosser & Embley, 2002, Koops *et al.*, 2003, Enwall *et al.*, 2007, He *et al.*, 2007) play important roles in the ecology of AOBs. On the other hand effects of fertilization on the archaeal counterparts are not so well studied since knowledge of their existence is still young. AOA outnumbered AOB up to 232 times in diverse soil habitats and thus seems to play a major role in nitrification in soil (Fig. 4) (Leininger *et al.*, 2006). Nitrogen fertilizer application induced minor (Shen *et al.*, 2008) or no changes (Wang *et al.*, 2009) in structure or abundance of AOA, whereas growth stimulation was reported under organic manure treatment (He *et al.*, 2007, Schauss *et al.*, 2009) as well as root exudate influence in rice rhizosphere (Chen *et al.*, 2008). Recently a functional redundancy for AOB and AOA in soil environments was postulated (Schauss *et al.*, 2009). But information about modulating factors is rather low and the role of AOA in nitrification still unclear.

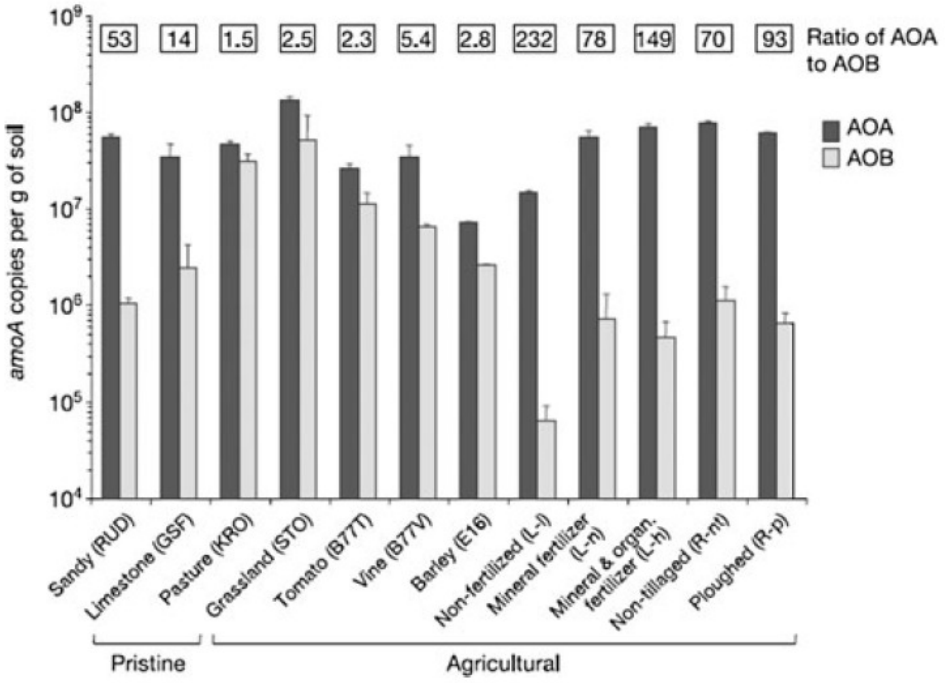


Figure 4 Abundances and ratios of archaeal (AOA) and bacterial (AOB) amoA genes in various soil environments (Leininger *et al.*, 2006)

5. Denitrifiers

Diversity

Conversion of NO_3^- to N_2O or N_2 is a process carried out by denitrifiers. Bacteria, fungi and archaea were found to perform denitrification (Zumft, 1997, Takaya, 2002, Cabello *et al.*, 2004, Philippot *et al.*, 2007) (Table 1) but bacteria seem to be the main drivers in soil. With members found in more than 50 different genera (Zumft et al., 1997), gram-positive and gram-negative, heterotrophic bacteria capable of denitrification arose to be a very diverse group of organisms. Depending on whether or not they have the enzyme nitrous oxide reductase, NO_3^- can be completely reduced to N_2 or partial denitrification to N_2O occurs. The enzymatic modification of soluble NO_2^- into gaseous form is an important factor in this process. It has been suggested that each soil hosts a nearly unique denitrifier community, as a consequence of inherent soil properties and specific environmental conditions (Hallin *et al.*, 2006).

Table 1 Archaeal, bacterial and fungal genera which contain at least one denitrifying strain (Philippot *et al.*, 2007)

Genus	Example of species	Source
Archaea		
Haloarcula	*marismortui*	(Yoshimatsu *et al.*, 2000)
Halobacterium	*denitrificans*	(Tomlinson *et al.*, 1986)
Pyrobaculum	*aerophilum*	(Völkl *et al.*, 1993)
Bacteria		
Firmicutes		
Bacillus	*azotoformans, stearotermophilus*	(Ho *et al.*, 1993; Pichinoty *et al.*, 1976b)
Paenibacillus	*terrae*	(Horn *et al.*, 2005)
Actinomycetes		
Corynebacterium	*nephridii*	(Har *et al.*, 1965)
Streptomyces	*thioluteus*, sp.	(Chèneby *et al.*, 2000; Shoun *et al.*, 1998)
Bacteroides		
Flavobacterium	sp., *denitrificans*	(Horn *et al.*, 2005; Pichinoty *et al.*, 1976a)
Flexibacter	*canadiensis*	(Jones *et al.*, 1992)
Aquifaceae		
Hydrogenobacter	*thermophilus*	(Suzuki *et al.*, 2006)
Proteobacteria Alphaproteobacteria		
Agrobacterium	sp.	(Chèneby *et al.*, 2000)
Azospirillum	*lipoferum*	(Neyra *et al.*, 1977)
Bradyrhizobium	sp., *japonicum*	(Monza *et al.*, 2006; van Berkum and Keyser, 1985)
Brucella	*melitensis*	(Baek *et al.*, 2004)
Hyphomicrobium	sp.	(Sperl and Hoare, 1971)
Mesorhizobium	*loti*	(Monza *et al.*, 2006)
Ochrobactrum	*anthropi*	(Kim *et al.*, 2006)
Paracoccus	*pantotrophus*	(Robertson and Kuenen, 1983)
Pseudovibrio	*denitrificans*	(Shieh *et al.*, 2004)
Rhizobium	sp.	(Arrese-Igor *et al.*, 1992)
Rhodobacter	*sphaeroides*	(Sabaty *et al.*, 1994)
Rhodopseudomonas	*salustris*	(Kim *et al.*, 1999)
Sinorhizobium	*meliloti*	(Daniel *et al.*, 1982)
Betaproteobacteria		
Acidovorax	sp.	(Heylen *et al.*, 2006; Schloe *et al.*, 2000)
Alcaligenes	*faecalis*	(Vanniel *et al.*, 1992)
Achromobacter	sp.	(Youatt, 1957)
Aquaspirillum	*magnetotacticum*	(Bazylinski and Blakemore, 1983)
Azoarcus	*tolulyticus, anaerobius*	(Fries *et al.*, 1994; Springer *et al.*, 1998)

(*continued*)

Table 1 (*continued*)

Genus	Example of species	Source
Azonexus	*caeni*	(Quan *et al.*, 2006)
Azospira	sp.	(Heylen *et al.*, 2006)
Azovibrio	sp.	(Heylen *et al.*, 2006)
Burkholderia	sp.	(Chèneby *et al.*, 2000)
Chromobacterium	sp.	(Grant and Payne, 1981)
Comamonas	sp., *denitrificans*	(Gumaelius *et al.*, 2001; Patureau *et al.*, 1994)
Cupriavidus	*necator*	(Pfitzner and Schegel, 1973)
Dechloromonas	*denitrificans*	(Horn *et al.*, 2005)
Denitratisoma	*oestradiolicum*	(Fahrbach *et al.*, 2006)
Kingella	*denitrificans*, sp.	(Grant and Payne, 1981; Snell and Lepage, 1976)
Microvirgula	*aerodenitrificans*	(Patureau *et al.*, 1998)
Neisseria	sp.	(Grant and Payne, 1981)
Nitrosomonas	*europaea, eutropha*	(Poth and Focht, 1985; Zart and Bock, 1998)
Ottowia	*thiooxydans*	(Springs *et al.*, 2004)
Ralstonia	*basilensis*	(Stamper *et al.*, 2002)
Rubrivivax	sp.	(Magnusson *et al.*, 1998)
Sterolibacterium	*denitrificans*	(Tarlera and Denner, 2003)
Thauera	*aromatica, mechernichensis*	(Schloten *et al.*, 1999; Song *et al.*, 1998)
Thibacillus	*denitrificans*	(Hole *et al.*, 1996)
Gammaproteobacteria		
Halomonas	*desiderata, campisalis*	(Berendes *et al.*, 1996; Mormile *et al.*, 1999)
Luteimonas	*mephitis*	(Finkmann *et al.*, 2000)
Pseudomonas	*fluorescens*, sp.	(Gamble *et al.*, 1977; Philippot *et al.*, 2001)
Pseudoxanthomonas	*taiwanensis*	(Chen *et al.*, 2002)
Sheuanella	*putrefaciens, denitrificans*	(Brettar and Hofle, 1993)
Stenotrophomonas	*nitritireducens*	(Finkmann *et al.*, 2000)
Thioalkalivibrio	*denitrificans*	(Sorokin *et al.*, 2001)
Zobellella	*denitrificans, taiwanensis*	(Lin and Shieh, 2006)
Epsilonproteobacteria		
Nitratifractor	*salsuginis*	(Nakagawa *et al.*, 2005)
Nitratiruptor	*tergarcus*	(Nakagawa *et al.*, 2005)
Thiomicrospira	*denitrificans*	(Brettar *et al.*, 2006)
Eukaryota		
Fungi		
Fusarium	*oxysporum*	(Tanimoto *et al.*, 1992)

Key enzymes in the denitrification pathway

Denitrification can be considered a four-step process driven by seven different enzymes that respond differently to external and internal signals. Low oxygen tension and the presence of nitrate and nitrite are the major ones (Zumft, 1997).

A key step in denitrification since soluble compounds are reduced to gaseous molecules is nitrite reductase, which occurs in two functional equivalents, copper containing (NirK) and cytochrome cd1 nitrite reductase (NirS) (Zumft, 1997, Braker *et al.*, 1998, Braker *et al.*, 2001, Wolsing & Prieme, 2004, Kandeler *et al.*, 2006, Bremer *et al.*, 2007). Both enzymes are periplasmic, occur in 1 or 2 gene copies per cell (Etchebehere & Tiedje, 2005) but have so far never been found together in one organism. They can be used as functional markers but their phylogenetic information is rather poor since members are taxonomically wide-spread and sequences derived from one habitat are often more related than *nir* genes derived from closely related genera (Heylen *et al*., 2006).

Another key enzyme is the nitrous oxide reductase (Nos) which converts N_2O to N_2 and defines whether the N is fully reduced to N_2 or the potent greenhouse gas N_2O is emitted (Zumft, 1997, Henry *et al.*, 2006, Henry *et al.*, 2008). It is a periplasmic, homodimeric enzyme with the catalytic subunit NosZ.

Parameters affecting denitrifiers in agricultural soils

Denitrification, which is regulated primarily by available organic carbon, soil NO_3^- content and anoxic conditions (Bothe *et al.*, 2000), shows generally higher rates in grassland than in agricultural soils and also varies in dependence of soil texture. Denitrifier communities appear to be strongly determined in their structural composition by C availability, pH, moisture and temperature e.g. (Wallenstein *et al.*, 2006, Philippot *et al.*, 2007), and hence fertilizer application involving changes in soil pH and C and N contents has shown major effects on

denitrifier diversity and composition (Enwall et al., 2005). Furthermore, plant species selectively affect the composition of denitrifying guilds in soils due to specific root exudation (Philippot et al., 2002; Sharma et al., 2005).

Looking at NO_3^- reduction, fertilization influence on denitrifiers revealed a major role of root exudates and plant species or functional groups (Sharma *et al.*, 2005, Bremer *et al.*, 2007, Dandie *et al.*, 2008, Henry *et al.*, 2008, Ruiz-Rueda *et al.*, 2009), whereas fluctuations to N supply revealed contrasting results indicating changes in *nirK* upon NH_4-N application (Avrahami *et al.*, 2002), but high resistence of *narG* nitrate reducing bacteria upon short-term nitrate fluctuations (Deiglmayr *et al.*, 2006). Relations of community pattern changes to soil pH were found in a long-term fertilization experiment with lower pH values upon $(NH_4)_2SO_4$ and sewage sludge treatment (Enwall *et al.*, 2005).

Denitrifiers, however, form a diverse group of microbes performing a complex multi-stepwise process (Fig.5), and it remains still unclear which are the main environmental drivers determining their composition, abundances, and specific activities.

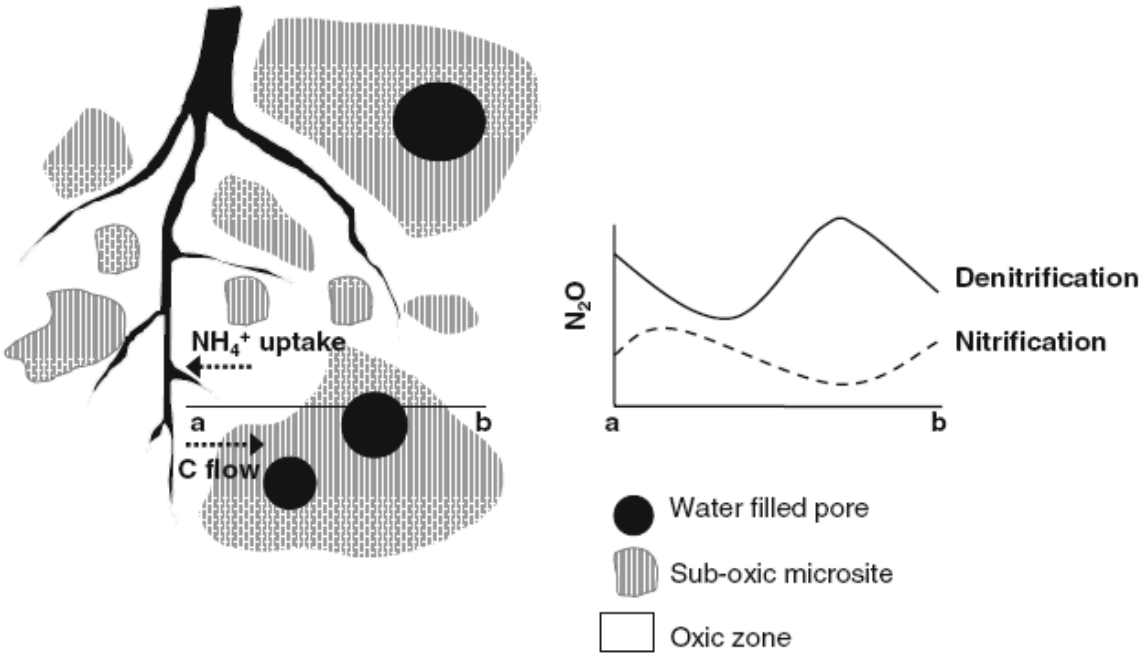


Figure 5 Nitrification and denitrification depending N_2O release influenced by carbon, oxygen and NH_4^+ supply in the rhizosphere (Philippot *et al.*, 2009)

6. Cultivation-independent approaches

Since only 1% of bacteria can be cultivated from soil, cultivation-independent methods are applied to characterize functional bacterial communities. Thus PCR based methods analyzing fragments of functional marker genes for the fore cited bacterial key players in the nitrogen cycle have been used in several studies (Bothe et al., 2000). Usually housekeeping genes as rRNA, RNA polymerase (e.g. *rpoB*), topoisomerase (e.g. *gyrB*)(Watanabe *et al.*, 2001), elongation factors G (EF-G) and Tu (EF-Tu), F1F0 ATP synthase β-subunit (*atpD*), the RecA protein (*recA*) and the HSP60 heat shock protein (Sigler *et al.*, 1998) genes are used to target the bacteria of interest. Most commonly the genes encoding for the small subunit rRNA are used to address complex microbial communities in soil and other environments. These genes are used because they are ubiquitär, conserved in some region and variable in other parts and phylogenetic relationships as well as chronometric development can be concluded (Woese, 1987). General molecular markers are useful to study total bacterial communities and closely related groups e.g. ammonia oxidizing bacteria. Since denitrifiers build a functional guild and share specific functions but show no or minor phylogenetic relationship general markers are not able to discriminate. Instead, enzymes encoding for genes involved in denitrification are being targeted. PCR primer sets have been developed and used in various studies for almost all functional genes involved in both, denitrification and ammonia oxidation (Braker *et al.*, 1998, Braker *et al.*, 2000, Purkhold *et al.*, 2000, Kowalchuk & Stephen, 2001, Braker & Tiedje, 2003, Koops *et al.*, 2003, Purkhold *et al.*, 2003, Throbäck *et al.*, 2004, Philippot, 2005).

Functional marker genes

Focusing on nitrification processes, genes encoding for the enzyme Ammonia monooxygenase, a multisubunit membrane bound enzyme, were used in former studies e.g. (Purkhold *et al.*, 2000, Koops *et al.*, 2003, Purkhold *et al.*, 2003). Fragments of the *amoA* gene encoding for the catalytic α-subunit can be used as functional and phylogenetic markers for autotrophic ammonia-oxidizing bacteria (AOB) affiliated with the Beta- and Gammaproteobacteria. Usually 2 to 3 copies of *amoA* genes are found in one cell.

AOBs have been supposed to be the most important bacterial contributors to aerobic ammonia oxidation in soils (Prosser, 1989, Purkhold *et al.*, 2000, Prosser & Embley, 2002). But a homolog of this ammonia monooxygenase gene has been found only recently related with mesophilic crenarchaeota in soil and marine environments (Francis *et al.*, 2005, Treusch *et al.*, 2005). Since then archael *amoA* copy numbers where proven to numerically dominate over AOB in several soil ecosystems (Leininger *et al.*, 2006, Nicol *et al.*, 2008, Prosser & Nicol, 2008, Tourna *et al.*, 2008).

For denitrification steps the enzyme nitrite reductase plays a key role since soluble nitrogen dioxide is reduced to gaseous molecules. Two equivalents of nitrite reductases, copper containing (NirK) and cytochrom cd1 (NirS) nitrite reductase, are so far known and have been applied to analyse denitrifying communities ((Braker *et al.*, 2000, Braker *et al.*, 2001, Prieme *et al.*, 2002, Metz *et al.*, 2003, Henry *et al.*, 2004, Throbäck *et al.*, 2004, Wolsing & Prieme, 2004, Sharma *et al.*, 2005, Kandeler *et al.*, 2006).

The reduction of N_2O to N_2 is the last step in denitrification and driven by a nitrous oxide reductase (Nos). The catalytic subunit *nosZ* was subjected by several environmental studies (Scala & Kerkhof, 1998, Scala & Kerkhof, 1999, Delorme *et al.*, 2003, Stres *et al.*, 2004, Throbäck *et al.*, 2004, Henry *et al.*, 2006, Horn *et al.*, 2006, Kandeler *et al.*, 2006). Since it is

easily inhibited by disturbances as low oxygene levels or heavy metals, denitrification processes result in N_2O as an end-product.

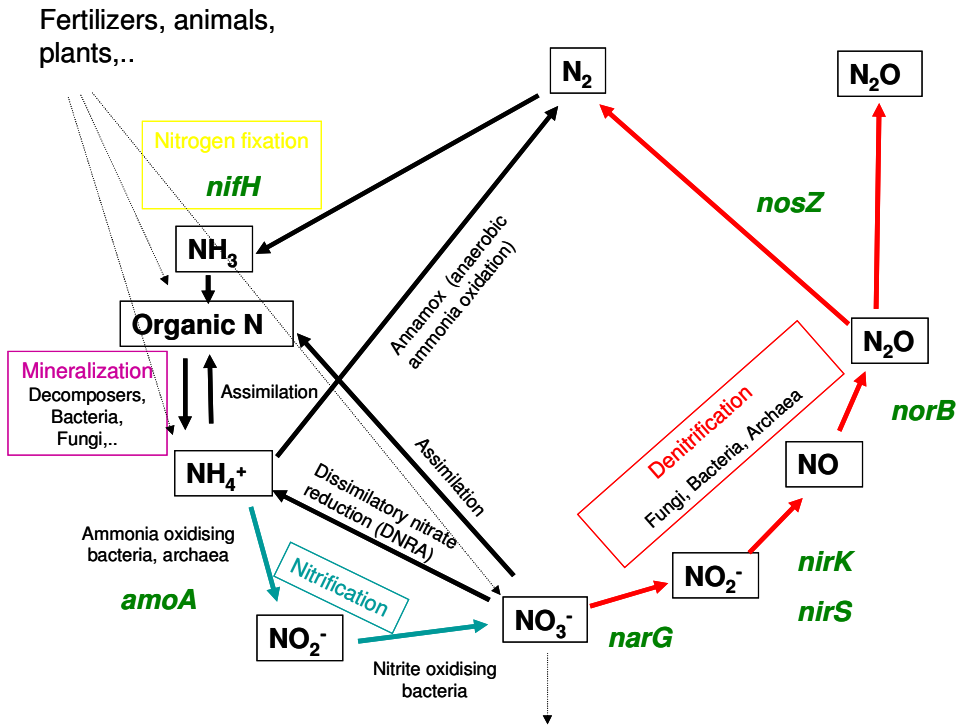


Figure 6 Functional marker genes in the N cycle

Molecular fingerprinting of microbial community structures

PCR derived from environmental samples run on an agarose gel results in a single band but consists of a mixture of different sequences. This mixture of amplicons should reflect the composition of the functional community. To resolve the pooled mix either a combination of cloning and sequencing techniques or fingerprinting methods are used.

For resolution of the polymorphism most commonly the following methods are applied: Terminal Restriction Length Polymorphism (T-RFLP), DGGE (Denaturing Gradient Gel Electrophoresis) or TGGE (Temperature Gradient Gel Electrophoresis), molecular cloning methods or microarrays.

Comparing the above mentioned fingerprinting methods T-RFLP is the one with the highest resolution, the output signal is digital and it is easier to handle than acrylamide gels. PCR is done with one of the primers carrying a fluorescent label followed by digest with a restriction enzyme (4-6 bp cutter) and size separation on a polymer matrix. Fragments harboring the fluorescent label are separated according to length and the migration behavior is detected at different time points by a fluorescence detector. The fluorescence units measured over time result in a chromatogram. With each sample an internal standard labeled with another fluorescent dye has to be run. Different concentrations lead to different peak heights and areas considering this method as semi-quantitative.

Abundance measurements - Quantitative real-time PCR

To gain more knowledge of the *in situ* microorganisms and get an idea of potential activities in the soil, it is often necessary to quantify the functional communities. Again cultivation dependent approaches like the most probable number method (MPN) (Volz, 1977), where serious dilutions of soil suspensions are plated and colonies counted, lead to an underestimation since as mentioned above approximately only 1% of soil microorganisms can be cultivated. The same principle was applied for a PCR technique, called MPN-PCR, stopping PCR reactions at different cycle numbers but this is rather labor intensive and applicable to only a restricted number of samples. Thus, to measure abundances a molecular approach again PCR based to watch amplicon accumulation in `real time` has been applied in several studies using either fluorescent dyes or oligo probes (Ginzinger, 2002, Zhang & Fang, 2006, Sharma *et al.*, 2007). Whereas for bacterial ammonia oxidizers both kind of assays were applied (e.g. Okano *et al.*, 2004, He *et al.*, 2007, Jia & Conrad, 2009), for denitrification genes SYBRgreen assays are most common since primers and probes are more difficult to design due to the heterogeneity of this functional group (Philippot, 2006). SYBRgreen is a fluorescent dye that binds to double-stranded DNA and only the DNA-dye complex shows fluorescence activity. Thus after every PCR cycle the accumulation of double-strand products

can be measured. After setting a threshold and determing the cycle number at the threshold line, the so called Ct values, the starting quantities can be calculated when compared to a standard curve consisting of serial dilutions of gene copies with known abundance. PCR efficiencies between 80-115% and a slope between -3.0 to -3.9 are good criteria for the quality of your run and standard curve, respectively (Zhang & Fang, 2006).

To assure the specificity following each PCR run a melting curve analysis is performed, where temperature is continuously increased which leads to a decrease of DNA-dye complexes due to melting of double strands causing a decrease in fluorescence. The melting depends on the G+C content, thus if products are specific all melt at the same temperature and when plotting temperature against the decrease of fluorescence per change in degrees temperature only one peak occurs.

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CHAPTER 1

COMPARISON OF ABUNDANCES AND COMMUNITY CHARACTERISTICS OF AMMONIA OXIDIZERS AND DENITRIFIERS IN FIVE AGRICULTURAL SOILS BY USE OF A SMALL-SCALE MICROCOSM SYSTEM

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Abstract

A small-scale laboratory format microcosm system specifically designed for an integrated approach to studying soil nitrogen (N) cycling was evaluated regarding its suitability for the molecular based study of nitrifying and denitrifying communities from five agricultural sites. Ammonia oxidizing bacterial (AOB) and archaeal (AOA) as well as nitrite reducing bacterial communities were compared regarding their structural composition and abundances, and these data were set in relation to their physical and chemical soil environments and to microbial process and gas flux rates. *nirS* and *nirK* gene based terminal restriction fragment length polymorphism (T-RFLP) patterns of denitrifying communities were distinct in the various soils, and they were considerably more diverse than *amoA* gene based AOB and AOA community profiles. Both *nirS* and *nirK* denitrifier abundances (as determined via quantitative PCR) were strongly related to readily available dissolved soil carbon, whereas variability in denitrifier community composition was associated with varying total and microbial C and N pool sizes. AOBs were more abundant in sandy, well aerated soils than in clay loam soils high in organic matter and microbial biomass. Archaeal *amoA* gene copies outnumbered bacterial ones 3 to 136 times in the various soils, and AOAs showed contrasting environmental dependences to AOBs. From these results it may be concluded that bacterial and archaeal nitrifiers as well as denitrifiers will respond specifically to measures of agricultural soil management, because they involve manipulations of the physical and chemical soil environment. The microcosm system used proved suitable for the high-throughput analysis of soils regarding molecular microbiological characteristics concomitantly with soil chemical and process measurements performed under defined experimental conditions.

Keywords: Microbial communities, denitrifiers, ammonium oxidizers, qPCR, T-RFLP, microcosm

1. Introduction

Nitrifying and denitrifying microorganisms take a decisive influence on the fate of nitrogen (N) in soil (Francis *et al.*, 2007), which has environmental implications such as pollution of ground waters and alteration of the atmospheric chemistry and climate change. In agricultural soils, up to 50% of the mineral N applied as fertilizers evade uptake by plants for conversion into biomass and hence are prone to losses to the environment (FAO, 1995). N losses may occur via leaching of nitrate into deeper soil layers and ground waters and during stepwise reduction of nitrate to gaseous N forms via microbial denitrification. NO and N_2O gases that are released in denitrification and also during nitrification enhance the greenhouse effect and deplete stratospheric ozone (Conrad, 1996). Nitrifying microorganisms, by transforming ammonium into mobile nitrate, thus may act as linking agents that potentially trigger N losses and cause environmental hazard.

While the biogeochemical and physical controls on soil N cycling are generally understood, studying the microbial players involved has been difficult until the advent of environmental molecular biology techniques (Braker *et al.*, 1998, Kowalchuk & Stephen, 2001, Prosser & Embley, 2002, Philippot, 2005, Wallenstein *et al.*, 2006). Studying soil microbial community dynamics in situ is also hampered by the enormous complexity and spatial and temporal heterogeneity of the soil system. These issues may be partly dealt with by using an appropriate sampling resolution in time and space (Ranjard *et al.*, 2003). However, as it is not easily possible to manipulate microbial communities in a natural system, it may in some cases be useful to conduct additional experiments in a laboratory format microcosm system (Copley, 2000). Ideally, such a microcosm system will allow the parallel, combined analysis of multiple factors influencing microbial N cycling in specific environmental settings. By using a combined molecular and biochemical approach, microbial communities performing

key steps in soil N cycling may be studied under defined conditions for exploring effects of specific experimental manipulations.

As the key performers in soil nitrification and denitrification more or less specialized guilds of bacteria and archaea have been identified (Treusch *et al.*, 2005, Leininger *et al.*, 2006, Hayatsu *et al.*, 2008), which show incongruent characteristics regarding physiology and phylogeny. Bacterial ammonia oxidizers (AOBs) comprise obligatory aerobic, chemoautotrophic organisms restricted to just a few groups within the proteobacteria (Kowalchuk & Stephen, 2001). The lifestyle of ammonia oxidizing archaea (AOAs) assigned within the mesophilic Crenarchaeota subphylum (Treusch *et al.*, 2005), by contrast, is widely unknown (Francis *et al.*, 2007). Both autotrophic or heterotrophic carbon incorporation has been found in isolates (Hallam *et al.*, 2006, Nicol & Schleper, 2006), suggesting a mixotrophic lifestyle dominated by autotrophy (Francis *et al.*, 2007). Denitrifying bacteria are widespread among over 50 different genera as primarily heterotrophic, facultative performers under low-oxygen conditions (Zumft, 1997).

The present study uses a functional gene based approach to target nitrifying and denitrifying communities represented by the ammonia oxidizing and nitrite reducing functional microbial groups. AOBs affiliated with the beta- and gamma-proteobacteria were studied via analysis of *amoA* genes, encoding the α subunit of ammonia monooxygenase (Kowalchuk & Stephen, 2001). For the study of AOAs a homologue of the bacterial *amoA* gene (Leininger *et al.*, 2006, Francis *et al.*, 2007, He *et al.*, 2007) was targeted. In analyses of denitrifiers, genes encoding nitrite reductases (Braker *et al.*, 2001, Wolsing & Prieme, 2004) were analyzed. The two functionally equivalent nitrite reductase enzymes contain copper (NirK) or cytochrom cd1 (NirS), respectively, and catalyze the first step in denitrification that produces N gas and at the same time discriminate denitrifiers against other nitrate reducing bacteria (Zumft, 1997, Philippot, 2005).

Microcosms were set up which allowed performing molecular analyses concomitantly with process and biochemical measurements in both the soil and atmosphere compartments within specifically developed test tubes (Inselsbacher *et al.*, 2009). Nitrifying and denitrifying microbial communities were compared in five soils that were typical of agricultural sites in Lower Austria and represented different bedrocks, soil textures, pH values, and water and humus contents. Thus, relationships were explored between the community characteristics as well as the abundances of ammonia oxidizing and nitrite reducing microorganisms and (i) general soil physical and chemical characteristics, (ii) the sizes of various soil C and N pools, and (iii) microbial process and gas flux data. Community structures of AOBs, AOAs and nitrite reducing bacteria were studied by using terminal restriction fragment length polymorphism (T-RFLP) analysis of the respective functional markers, whereas real-time PCR assays were performed on the same marker genes to measure the abundances of these groups. The overall aim of the study was to evaluate the suitability of the microcosm experimental system for an integrated, molecular and biochemical based approach to studying soil N cycling.

2. Materials and Methods

2.1. Soil sampling and microcosm set-up

Agricultural soils were collected at five different locations in Lower Austria: Maissau, Purkersdorf, Niederschleinz, Riederberg and Tulln. They were selected based on the following criteria: coverage of a range of different bedrocks, textures, water conditions and humus contents; suitability for barley cultivation; location at various, typical agricultural sites within Lower Austria; and pH range within 5 and 7, which is typical of agricultural soils of that region (Table 1).

Soils were taken from 0 to 20 cm depth and were transported into the laboratory in cooling boxes, where they were stored at 4 °C for one week. Prior to analysis, soils were sieved to 2 mm, homogenized by mixing and adjusted to 70% field capacity.

Experiments were set up in test tubes specifically designed for concomitant chemical and gas flux measurements (Inselsbacher et al. 2009). Triplicate samples of the various soils (30 ml each) were incubated in the test tubes for seven days, and during that time were kept in a climate chamber with a 15 h to 9 h day-night cycle at 21 and 18 °C, respectively, and with a relative air moisture content of 55%. During incubation, the test tube atmospheres were repeatedly sampled for CO_2, methane, and nitrogen gas analyses. Following final headspace sampling at day seven, aliquots of the soils were taken from the tubes for biochemical and molecular analyses.

2.2. Chemical and biochemical analyses and gas flux measurements

Biochemical and gas flux analyses were done as described in (Inselsbacher *et al.*, 2009). In brief, the soil water content and pH were measured, and NH_4^+-N, NO_3-N and total dissolved C (TDC) and N (TDN) were determined in KCl extracts. Microbial biomass C and N were analyzed by using a chloroform fumigation-extraction method, and total N and C as well as natural ^{15}N and ^{13}C abundances were measured by applying isotope ratio mass spectrometry (IRMS). Gross mineralization and gross nitrification rates were measured in ^{15}N pool dilution assays, in addition to the measurement of nitrate reductase and dehydrogenase activities. N_2O, CO_2 and CH_4 sampled from the test tube atmospheres were analyzed via automated headspace chromatography.

2.3. Molecular analyses

DNA was extracted from 0.5 g of soil each taken from three replicate test tubes by using the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, Ohio, USA) as described by the

manufacturer, and was quantified photometrically (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA). Amplification of genes encoding the ammonium monooxygenase catalytic subunit A (*amoA*) was accomplished by using the primers amoA1F /amoA2R (Rotthauwe *et al.*, 1997) for bacteria and Arch-amoAF/Arch-amoAR (Francis *et al.*, 2005) for archaea, yielding fragments of 491 bp and 635 bp length, respectively. Genes coding for copper and cytochrome cd1 nitrite reductases *(nirK* and *nirS*) were PCR amplified using the primer pairs nirK1F and nirK5R (Braker *et al.*, 1998) and Cd3aF and R3cd (Michotey *et al.*, 2000, Throbäck *et al.*, 2004), yielding gene fragments of 515 bp (*nirK*) and 425 bp (*nirS*) length. All forward primers were 6-carboxyfluorescein (FAM) labeled at the 5´ end.

Amplifications were performed in 25 µl reactions containing 1x buffer, 3 mM $MgCL_2$, 0.2 mM dNTPs, 4%(v/v) DMSO, 1% (w/v) Bovine Serum Albumine (BSA), 1 U of FIREpol Polymerase (Solis BIODYNE, Estonia), and 0.2 µM of each primer for amplification of archaeal *amoA*, *nirK* and *nirS* and 0.24 µM of each primer for bacterial *amoA* amplification, respectively. DNA amounts of 25 to 100 ng were applied to each batch. Amplifications were performed in a Whatman T1 thermocycler using the following programs: an initial denaturing step at 95°C for 5 min, followed by 38 cycles of 30s at 95°C, 40s at 55°C (*nirK*) or 58°C (*nirS*), 30s at 72°C and a final extension step at 72°C for 10 min. For bacterial/ archaeal *amoA* reactions 35/ 30 cycles at an annealing temperature of 60°C / 53°C (each step 1 min) were performed.

PCR amplified *amoA* and Arch-*amoA* were subjected to T-RFLP analysis using the *Alu*I restriction enzyme, whereas *Rsa*I and *Hae*III were used for restriction digestion of *nirK* and *nirS* gene fragments, respectively. Choice of restriction enzymes was based on pre-experiments in which three different enzymes (*Rsa*I, *Alu*I, *Hae*III) were used in in silico analyses of 20 sequences of clones obtained from Niederschleinz soil. To reduce bias two PCR products were pooled for each digestion essay. 20 µl restriction mixtures containing 200

ng PCR product, 1x buffer and 0.5 µl *Rsa*I/*Hae*III (10 U/µl, Promega) were prepared for *nirK*/ *nirS* analysis. 10 µl reaction mixtures consisting of 7 µl PCR product, 1x buffer and 0.5 µl *Alu*I (10U/µl, Promega) were used for bacterial/ archaeal *amoA* analysis. Following incubation for 4 h at 37°C, digestion batches were purified by passage through DNA grade Sephadex G50 (GE Healthcare) columns. Five µl of purified product were mixed with 15 µl HiDi-Formamide (Applied Biosystems) and 0.3 µl 500 ROX™ Size Standard (Applied Biosystems) and denatured at 95°C for 2 min. Detection of FAM-labeled terminal restriction fragments was done by capillary electrophoresis using an ABI 3100 automatic DNA sequencer.

SYBR-Green Assays were performed in an iCycler iQ Multicolor Real Time PCR Detection System (BIO-Rad Laboratories) using the same but unlabeled primers as for T-RFLP analysis. Twenty-five µl reactions were composed of 12.5 µl 2x IQ ™ SYBR®-Green Supermix (BIO-Rad Laboratories), 0.5 µM of each primer for *amoA*/ Arch-*amoA* and *nirS*, 0.2 µM of each primer for *nirK*, 2.5% (v/v) DMSO and 2.4% (w/v) BSA. Standards and samples were processed in triplicates. The thermocycler program for *nirS* and *nirK* was set on 95°C for 3 min, 45 cycles of 95°C for 30s, 58°C (*nirS*) or 55°C (*nirK*) for 35s, 72°C for 45s and data collection at 78°C for 45s. *amoA*/Arch-*amoA* PCR was carried out using the following protocol: 95°C for 3 min, 45 cycles of 95°C for 1 min, 57°C/53°C for 1 min, 72°C for 1 min and data collection at 78°C for 1 min. Melting curve analysis was done in order to confirm the specificity of the PCR product. To check for inhibition a two times dilution series (1:2 to 1:32) was done using one representative sample of each soil. No inhibiting effects were found for any of the samples.

PCR products of each targeted gene were cloned (Strataclone PCR cloning Kit, Stratagene) and sequenced to proof specificity. Plasmids were isolated using the Quantum Prep Plasmid Miniprep Kit (BIO-Rad) and DNA concentrations were determined by spectroscopy to calculate copy numbers. Standard curves from serial dilutions of known amounts of the target

genes were generated for each run, showing correlation coefficients (R^2) of 0.996 to 0.999 and PCR efficiencies of 92 to 102%.

2.4. Statistical analysis

T-RFLPs were transformed into numerical data using GenoScan 3.7 software. To reduce background noise peaks with intensities higher than three times standard deviation were binned and normalized as described in (Abdo *et al.*, 2006) using the statistical program R together with the filtering and binning macro provided at the IBEST homepage (http://www.ibest.uidaho.edu/tools/trflp_stats/instructions.php). Peaks that occurred in at least two replicates and with a percentage higher than 2% were considered as major ones. Non-metric multidimensional scaling (MDS) using the Primer 5 statistical program (Primer E, Plymouth, UK) was performed for each T-RFLP data matrix. Correlations between the two functions for 2- D MDS plots of T-RFLPs from each functional community and soil chemical and microbial activity parameters were calculated using Spearman`s correlations. To screen for differences and to analyze major distribution patterns, Analysis of similarity (ANOSIM) and species contributions to similarity (SIMPER) calculations were performed using Primer 5 software (Primer E, Plymouth, UK) as described in (Wolsing & Prieme, 2004).

3. Results

3.1. Chemical data and gas flux rates

Chemical soil data as well as microbial process and gas emission rates are presented in Inselsbacher et al. (2009). Available phosphate and various C and N pool sizes in the five soils are shown in Table 2.

3.2. Microbial community structures

T-RFLP analysis of bacterial *amoA* genes yielded seven different peaks for all soils. Community profiles from Riederberg and Tulln soils contained five major peaks, followed by Niederschleinz soils with four and Maissau and Purkersdorf soils with only two major peaks. The T-RFs clustered according to the sampling sites in MDS score plots. However, while T-RFs from Riederberg and Niederschleinz soils were clearly differentiated from each other (R = 0.778 in ANOSIM pair-wise comparison) and from the other soils, the T-RF-profiles of the other soils were more similar, resulting in a global R value of 0.216 (P= 0.05). The profiles from Riederberg and Niederschleinz soils missed a fragment of 388 bp length which was present in the other soils. The Riederberg profiles additionally were lacking a T-RF of 201 bp length and instead showed fragments of 56, 62, 66, 68 and 76 bp length, which were absent in profiles from Maissau and Purkersdorf soils.

Archaeal *amoA* community profiles contained nine T-RFs, which consistently were present in the profiles of all samples. However, variation in individual peak heights resulted in separate clusters being formed by T-RFs from Niederschleinz and Riederberg soils in MDS plots (Fig.1b). Fragments of 260 bp were at least four times more abundant in N and R soils, whereas peak heights of 139 and 170 bp fragments were 1.2 times lower here than in the other profiles. Correspondingly, a high R value (R=1.000) was obtained for either of the

Niederschleinz and Riederberg soils when they were compared to Purkersdorf, Maissau or Tulln soils. T-RFs of 260, 139 and 170 bp length together with T-RFs of 131, 166 and 169 bp explained 60% of the total dissimilarity between the soils.

In T-RFLP profiles of *nirS* amplicons, 34 different peaks were detected across all samples. Highest peak numbers were observed in Niederschleinz and Tulln soils, reflecting 18 different OTUs each. Purkersdorf and Riederberg soils gave both 15 major peaks in the community profiles. Maissau soil with 10 major T-RF peaks showed the lowest OTU numbers. In the score plot of MDS T-RFs from the individual sites formed distinct clusters, with T-RFs from Purkersdorf and Riederberg soils being most strongly separated from the other T-RFs (Fig.1c). Accordingly, T-RFs from the various soils displayed high dissimilarities in ANOSIM pair wise comparison with a global R value of 0.998 (P=0.000). Based on SIMPER analysis, T-RFs of 67, 75, 101, 102, 115, 134, 135, 137, 253, and 259 bp length explained at least 60% of dissimilarity and thus primarily discriminated between sample sites.

T-RFLP profiles of *nirK* amplicons contained 25 major peaks across all soils. T-RFs derived from Niederschleinz soil reflected 11 different OTUs, followed by Tulln, Riederberg, and Purkersdorf soils with 8, 8, 7 and 6 major peaks, respectively. ANOSIM pair-wise comparison revealed significant dissimilarities among the various soils in T-RFs. T-RFs from the individual sites were separated in the score plot of MDS (Fig. 1d). The global R value of 1.000 (P=0.000) confirms the differences between the soils observed in MDS. Based on SIMPER analysis, *nirK* T-RFs of 96,102, 157, 158, 168, 207, 291, 338, and 339 bp length proved to be most important for discriminating between the soils.

3.3. Functional gene abundances

Bacterial *amoA* copy numbers ranged between 4.0 x 10^6 and 9.7 x 10^6 copies g^{-1} dry soil in the various soils, with 2.4 times more *amoA* copies prevailing in Maissau than in Riederberg

soil. Purkersdorf, Niederschleinz and Tulln soils had similar *amoA* copy numbers, while higher and lower numbers were found in Maissau and Riederberg soils, respectively (Fig. 2a). Archaeal *amoA* gene abundances were highest in Riederberg soil with 5.5 x 10^8 copies, followed by Niederschleinz soil with 1.3 x 10^8 copies and Maissau, Purkersdorf and Tulln soils with 2.9, 2.0 and 1.5 x 10^7 copies, respectively (Fig. 2b). Archaeal *amoA* genes outnumbered bacterial *amoA* copies 136-fold in Riederberg soil. The ratio of archaeal to bacterial *amoA* gene copies was 31 in Niederschleinz soil, while Maissau, Purkersdorf and Tulln soils had ratios between 3 and 4.

nirS abundances were highest in Riederberg and Maissau soils, amounting 6.7 and 8.3 x 10^8 copies g^{-1} dry soil, whereas Purkersdorf, Niederschleinz and Tulln soils contained significantly lower *nirS* copy numbers of 2.0 to 3.6 x 10^8 copies g^{-1} dry soil (Fig.2c).

All soils studied contained at least 175-fold higher *nirS* than *nirK* gene copy numbers. By far highest *nirK* abundances of 4.7 x 10^6 copies g^{-1} dry soil were found in Riederberg soil, exceeding values in the other soils by 3 to 11 fold. In Maissau and Purkersdorf soils *nirK* abundances comprised 1.2 and 1.6 x 10^6 copies g^{-1} dry soil, while in Tulln and Niederschleinz soils lowest *nirK* abundances of 4.3 and 5.7 x 10^5 copies g^{-1} dry soil were measured (Fig.2d).

3.4. Correlations of microbial data with biochemical and process data

Correlations of 2-D MDS functions calculated from T-RF-community data with soil physical, chemical and microbiological characteristics are shown in Table 3, reflecting the major environmental determinants of functional community composition. In addition, correlations of functional gene abundances with environmental variables and process data are given in Table 3.

MDS functions of bacterial *amoA* T-RFs showed highest correlations with total C and N concentrations and biomass C and N content. Bacterial *amoA* gene abundances were negatively correlated with total and biomass C and N (Table 3).

The first function of the MDS plot of archaeal *amoA* T-RFs correlated negatively with microbial biomass N, total C and N, and dehydrogenase activity, while a positive correlation was found with gross-mineralization and biomass C to N ratio. Function 2 gave negative correlations with PO_4^-. Archaeal *amoA* copy numbers showed positive correlations with pH, C to N ratio and dehydrogenase activity, signifying the overall metabolic activity of the soils, and were negatively correlated with PO_4^- and biomass C to N.

MDS function 1 of *nirS* T-RFLP profiles showed negative correlations with soil pH, total C and N, biomass N and dehydrogenase activity as well as positive correlations with microbial biomass C to N ratio and Gross mineralization (Table 3). *nirS* abundance data showed positive correlations with TDC, TDN, NH_4^+-concentrations and negative correlations with $\delta^{15}N$ (Table 3). MDS functions of *nirK* based T-RFs correlated highly with the C to N ratio, with total C and N, microbial biomass C and N. Like *nirS* abundance, *nirK* copy numbers correlated positively with TDC, TDN and NH_4^+ concentrations and negatively with $\delta^{15}N$. Additionally, *nirK* copy numbers correlated positively with CO_2 emission rates and the soil water content and negatively with $\delta^{13}C$, PO_4^- and nitrate reductase activity (Table 3). In addition, *nirS* and *nirK* copy numbers were positively correlated ($R= 0.91$; $P< 0.001$).

4. Discussion

Using a lab-scale microcosm system enabled functional gene based analysis of the community characteristics and abundances of ammonia oxidizing and nitrite reducing soil microbial communities while at the same time a large set of chemical and microbiological data was collected from both the soil and atmosphere compartments. Advantages of the test tube system used include that it is small-scale and high-throughput, and that defined environmental and soil conditions (e.g. homogeneous and constant values of soil moisture and bulk density,

defined temperature conditions) may be maintained during specific experimental manipulations (Inselsbacher *et al.*, 2009). Previously, a high potential of the system for elucidating N pathways at the soil-plant-microbe interface was implicated. Furthermore, bacterial and fungal community patterns were found unchanging during soil incubation within a test period of four weeks, suggesting that the system was stable over time and thus suitable also for molecular based microbial community analyses (Inselsbacher *et al.*, 2009). In the present study, specific functional microbial communities from five different soils were characterized in relation to a range of soil and process related factors by using this microcosm system.

Under defined moisture and temperature conditions maintained within the test tube system, the nitrite reducing and ammonia oxidizing microbial groups from the various soils showed specific characteristics regarding diversities and community structures. Communities of nitrite reducing bacteria appeared to be considerably more diverse than communities of bacterial (AOB) and archaeal (AOA) ammonia oxidizers based on the numbers of peaks contained in T-RFLP community profiles. In addition, nitrite reducers harboring either of the *nirS* and *nirK* encoded nitrite reductase homologues formed highly distinct communities in the various soils. This complies to the notion that denitrifying communities are genetically heterogeneous, forming an almost unique pattern in each soil due to effects of specific soil conditions (Hallin *et al.*, 2006). Structural differentiation was also apparent among the bacterial ammonia oxidizing communities in that they showed varying levels of structural complexity in the different soils. Archaeal ammonia oxidizers, however, formed widely uniform communities in the different soils.

Low AOB diversity in agricultural soils may be a consequence of repeated ploughing or fertilization with ammonium rich fertilizers (Bruns *et al.*, 1999, Mendum & Hirsch, 2002, Enwall *et al.*, 2007, He *et al.*, 2007). In compliance with this, highest phylotype numbers were seen in the AOB community profiles from Riederberg soil, which in contrast to the other soils

had been covered with grassland in the previous season. Still, it is uncertain if agricultural soils indeed host such a narrow group of nitrifiers or if available PCR primers are too selective to cover the full range of nitrifying bacteria present. Among the five soils studied, dissimilarities in AOB community composition were most pronounced in Niederschleinz and Riederberg soils based on the presence or absence of defined phylotypes. Contrasting to the other profiles, in the community profiles of these two soils a T-RF of 392 bp length was absent, which supposedly represents *Nitrosospira* sp. cluster 4 organisms (Mintie *et al.*, 2003). Profiles of the Riederberg grassland soil in addition were lacking a T-RF of 201 bp length, which consistently had high abundances in the profiles of the other soils. This restriction fragment was possibly derived from *Nitrosospira* sp. cluster 3 organisms, which have been shown to dominate in ammonium fertilized agricultural plots (Mendum & Hirsch, 2002, He *et al.*, 2007).

Across soils from a wide range of ecosystem types, mean annual temperature appeared to be most important for shaping AOB community composition (Fierer *et al.*, 2009). The soils used in the present study were collected within a geographically and climatically narrow range, and they were incubated under defined temperature conditions. Here, differences in AOB community composition corresponded mainly with differences in total and microbial C and N contents. In addition to diverging vegetation cover, the significantly higher C and N status of the Riederberg soil may account for its distinct and more complex AOB community as compared to the other four soils. In analogy to this, AOB community changes were seen in maize field soils following stubble retention treatment involving C and N input (Steven *et al.*, 2007).

Clear differences existed in AOB abundance among the various soils. Sandy Maissau soil, originating from silicate material and being comparably poor in organic matter and microbial biomass, contained by far highest bacterial *amoA* copy numbers, while lowest *amoA* abundance was found in Riederberg soil of silty clay loam texture, which was high in organic

C and microbial biomass. Soil texture is likely to be critical for AOB abundance, since soil aeration is supportive to the growth of aerobic AOBs. In addition, the chemolithoautotrophic nature of AOBs may account for an inverse relationship of AOB abundance with soil C and microbial biomass content. Being slow growing and poor competitors for ammonium-N against plants and heterotrophs (Paul, 2007), AOBs might be surpassed in growth by heterotrophic bacteria more easily in high-biomass soil that is rich in organic matter. Previous studies have reported effects of soil pH on the growth and activity as well as the community structure of AOBs in arable and grassland soils (Prosser & Embley, 2002, Koops *et al.*, 2003, Enwall *et al.*, 2007, He *et al.*, 2007). However, neither abundances nor community structures of AOBs showed relationships with soil pH in the present study. While AOBs have been found responsive to soil ammonia addition regarding their community structure and abundance in some soils (Mendum *et al.*, 1999, Hermansson & Lindgren, 2001, Okano *et al.*, 2004, Enwall *et al.*, 2007, He *et al.*, 2007, Cavagnaro *et al.*, 2008, Wang *et al.*, 2009), in the present study they were unaffected by the variability in ammonia concentration among the soils.

Although community patterns of archaeal ammonia oxidizers (AOAs) from the different soils were composed of consistent phylotypes, profiles from Riederberg and Niederschleinz together formed a separate cluster in MDS plots based on different relative abundances of T-RFs. Overall, our results reflect high conformity in AOA community composition in the various soils. This may be a consequence of the fact that so far a narrow phylogenetic range has been seen among archaeal *amoA* sequences retrieved from soils, all being assigned within the Crenarchaeota group 1 b.b (Nicol & Schleper, 2006).

AOA abundance, by contrast, differed considerably among the soils. Moreover, the relative numbers of bacterial and archaeal *amoA* copies varied significantly among the various soils. While archaeal *amoA* genes were always more abundant than their bacterial counterparts, this dominance varied between 3 and 136 times. Archaeal *amoA* copies dominated most strongly

over bacterial ones in Riederberg grassland soil. Also Niederschleinz soil contained markedly higher archaeal *amoA* numbers than the other agricultural soils, and at the same time AOA community structure was similar to that in Riederberg soils.

The factors underlying observed ratios of AOA to AOB abundance, which have proven highly variable in various grassland and cropped soils, still remain unknown (Leininger *et al.*, 2006). Numbers of bacterial and archaeal *amoA* copies measured in the present study were in a similar range as in other pristine and agricultural soils (Leininger *et al.*, 2006, He *et al.*, 2007, Adair & Schwartz, 2008, Shen *et al.*, 2008). Based on their numerical dominance, archaea were presumed to be the predominating ammonia-oxidizing prokaryotes in soils (Leininger *et al.*, 2006). Ammonia oxidizing activity, however, has recently been shown to be predominantly of bacterial origin even in soils where archaeal *amoA* copies were more abundant than bacterial ones (Jia & Conrad, 2009). Lately, AOAs have been suggested to serve as a "backup" to maintain ammonium oxidation in disturbed soils rather than being the main drivers (Schauss *et al.*, 2009). Exploring the environmental constraints of AOAs may help understand the metabolic and bioenergetic pathways used by AOAs (Francis *et al.*, 2007). In the five soils studied archaeal *amoA* abundance appeared to be related to overall soil metabolic activity. In addition, high archaeal copy numbers seemed to be associated with slightly higher pH values and lower soil phosphate content. While the specific demands of AOAs remain unknown, this may indicate a more versatile metabolism and adaptation to a wider range of growth conditions as compared to AOBs (Leininger et al., 2006). As compared to AOBs, different environmental requirements are suggested for AOAs, indicating that these groups may occupy different niches in spite of apparent functional redundancy (Schauss *et al.*, 2009).

Both *nirS* and *nirK* gene abundances were strongly related to the content of dissolved carbon in the soils, reflecting dependence of heterotrophic denitrifiers on carbon availability (Sharma *et al.*, 2005, Bremer *et al.*, 2007, Dandie *et al.*, 2008, Henry *et al.*, 2008). In addition, highest

nir copy numbers occurring in Riederberg soil together with a distinct *nirS* denitrifier community pattern may be consequences of high rhizospheric deposition of specific quality in the grassland soil. Differences in ammonium concentration also seemed to partly account for differences among the soils in *nirS* and *nirK* gene abundances. In concordance with findings in other soil environments nitrate supply seemed to control neither denitrifier abundance nor structure (Mergel *et al.*, 2001, Wallenstein *et al.*, 2006). Among the soil factors studied, total and microbial C and N contents appeared to be most important in structuring the communities of nitrite reducers.

Both nitrifier and denitrifier community composition and abundance appeared to be most strongly affected by various soil C and N pool sizes, while correlations were rather poor with gas fluxes or specific microbial activities that were measured alongside with molecular analyses. This is probably due to the fact that molecular analyses were based on DNA and hence reflect potentials and not activities. In addition, our comprehension of the organisms involved in soil nitrogen cycling is incomplete, so that some important players may still evade molecular analysis. It is well evident, for instance, that nitrite reduction is not exclusively accomplished by bacterial heterotrophs targeted with commonly available primer sets (Jason *et al.*, 2007), since nitrite reductase genes have been found among AOBs, AOAs, anammox bacteria and even eukaryotes (Treusch *et al.*, 2005, Risgaard-Petersen *et al.*, 2006, Cantera & Stein, 2007, Francis *et al.*, 2007, Kartal *et al.*, 2007).

The comparative analyses performed within the present study demonstrate that nitrifying and denitrifying microbial groups are differentially influenced by specific soil characteristics in various environmental settings. Data obtained via molecular analyses were related with those derived from chemical and process measurements with the aim of testing the microcosm system used for its suitability for future manipulative studies on soil N cycling. Together with previous findings by Inselsbacher et al. (2009) evidencing that the system was stable during an extended time period, the present results implicate that the microcosm system is an

appropriate tool for the high-throughput testing of large sample numbers in studies on soil N cycling that use an integrated molecular and process-oriented approach. Future applications may consider selecting a comprehensive set of soils that vary in major microbiological characteristics such as AOA to AOB ratio or functional gene diversities and abundances for the parallel and high-throughput testing regarding the effects of multiple influencing factors under specific experimental treatments or incubation conditions. Such studies may prove valuable for complementing field studies on soil N cycling because they allow specific environmental manipulations under defined conditions.

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Table 1 Site information and physicochemical properties of the five soils used in this study (Inselsbacher *et al.*, 2009).

Site	Geographic location	Land use type	pH	Soil type and geology	Soil texture	Clay (%)	Silt (%)	Sand (%)	Soil water regime	Humus (%)
R[a)]	48°15'0'' N 16°04'0'' E	Grassland	6.6	Calcaric Cambisol from Clay Flysch Material	Silty clay loam	29.1	53.8	17.2	Changing water conditions	5.8
M	48°34'0'' N 15°49'0'' E	Arable field (varying crops)	7.0	Cambisol from silicate material	Sandy clay loam	0.0	70.4	29.6	Dry	1.6
P	48°12'25'' N 16°10'37'' E	Arable field (Winter barley)	5.7	(Gleyic) Cambisol from Sandy Loamy Flysch Material	Sandy clay loam	1.7	64.9	33.4	Moist	1.6
N	48°35'59'' N 15°10'24'' E	Arable field (varying crops)	7.2	Chernozem from Loess	Silt loam	17.7	74.2	8.0	Moderately dry	2.6
T	48°20'0'' N 16°03'0'' E	Arable field (varying crops)	6.2	Pseudogley, Planosol	Silty clay	43.2	48.3	8.6	Moderately moist	3.2

[a)] R = Riederberg, M = Maissau, P = Purkersdorf, N = Niederschleinz, T = Tulln

Table 2 Available phosphate and C and N pool sizes in the five agricultural soils, 0-20 cm top soil layer (Inselsbacher *et al.*, 2009). Different letters in columns indicate significant differences between sites at $p<0.05$.

Site	available PO_4^-	Total N	Total C	C to N	Biomass N	Biomass C	TDN	TDC	NH_4-N	NO_3-N
	(mg g^{-1} dw)				(µg N g^{-1} dw)	(mg C g^{-1} dw)	(µg g^{-1} dw)		(µg g^{-1} dw)	
R[a)]	0.07±0.02^{d}	4.78±0.07^{a}	47.7±0.9^{a}	10.0±0.1^{b}	179.7±12.2^{a}	2.267±0.05^{a}	35.6±1.2^{ab}	61.3±0.8^{a}	2.15±0.17^{a}	17.59±2.41^{b}
M	3.07±0.10^{b}	1.45±0.05^{d}	13.7±0.3^{d}	9.4±0.1^{c}	10.1± 1.1^{c}	0.153±0.01^{c}	48.7±3.2^{a}	41.0±1.5^{b}	1.18±0.21^{b}	42.30±1.25^{a}
P	0.66±0.03^{d}	1.63±0.05^{cd}	16.2±0.5^{c}	10.0±0.1^{b}	14.6± 0.8^{c}	0.371±0.03^{c}	23.4±0.8^{ab}	40.2±1.4^{bc}	1.31±0.23^{ab}	18.61±1.17^{b}
N	1.33±0.07^{c}	1.86±0.02^{c}	26.4±0.2^{b}	14.2±0.1^{a}	22.9± 2.5^{c}	0.302±0.03^{c}	20.4±0.4^{b}	31.2±1.3^{d}	1.35±0.18^{b}	19.25±0.97^{b}
T	5.83±0.29^{a}	3.27±0.05^{b}	29.3±0.5^{b}	9.0±0.0^{d}	52.9± 2.5^{b}	1.289±0.11^{b}	15.1±1.5^{b}	33.1±2.4^{cd}	1.28±0.15^{b}	15.94±1.39^{b}

[a)] R = Riederberg, M = Maissau, P = Purkersdorf, N = Niederschleinz, T = Tulln

Table 3 Spearman's correlations between the scores of the first two functions of MDS of bacterial and archaeal *amoA,* and *nirS* and *nirK* T-RFs and soil environmental data as well as between bacterial and archaeal *amoA,* and *nirS*, and *nirK* copy numbers derived from RT-PCR and soil environmental data.

		T-RFLP Community analysis								RT-PCR			
		Bact. *amoA*		Arch. *amoA*		*nirS*		*nirK*		Bact. *amoA*	Arch. *amoA*	*nirS*	*nirK*
		MDS 1	MDS 2	MDS 1	MDS 2	MDS 1	MDS 2	MDS 1	MDS 2				
Soil chemistry	Soil pH			-0.579[b]		-0.535[b]			-0.701[a]		0.578[b]		
	PO_4^-				-0.699[a]		0.817[a]				-0.614[b]		-0.650[a]
	Cl^-		-0593[b]										
C and N pools	Ntot	0.741[a]	-0.620[b]	-0.656[a]		-0.744[a]		0.861[a]		-0.604[b]			
	Ctot	0.756[a]	-0.620[b]	-0.643[a]		-0.721[a]		0.886[a]		-0.657[a]			
	C to N ratio	0.658[a]											
	NO_3-N												
	NH_4-N						-0.581[b]					0.586[b]	0.696[a]
	Biomass N	0.818[a]	-0.603[b]	-0.670[a]		-0.714[a]		0.893[a]		-0.657[a]			
	Biomass C	0.637[b]	-0.612[b]					0.693[a]	0.581[b]	-0.521[b]			
	Biomass C to N	-0.575[b]		0.531[b]		0.550[b]		-0.614[b]			-0.614[b]		
	TDN						-0.599[b]					0.711[a]	0.571[b]
	TDC						-0.806[a]					0.857[a]	0.889[a]
	DON												
	$\delta^{15}N$						0.861[a]					-0.789[a]	-0.804[a]
	$\delta^{13}C$												-0.664[a]
Gas emission	CO_2-C						-0.620[b]		0.637[b]				0.679[a]
	N_2O-N								0.556[b]				
	CH_4-C												
Microbial processes	Gross-N-Min			0.696[a]		0.693[a]		-0.639[b]					
	Gross-Nitrification												
	Dehydrogenase			-0.674[a]		-0.643[a]		0.607[b]			0.907[a]		
	Nitrate reductase							-0.552[b]					-0.531[b]

[a] significant at $p<0.01$, [b] significant at $p<0.05$.

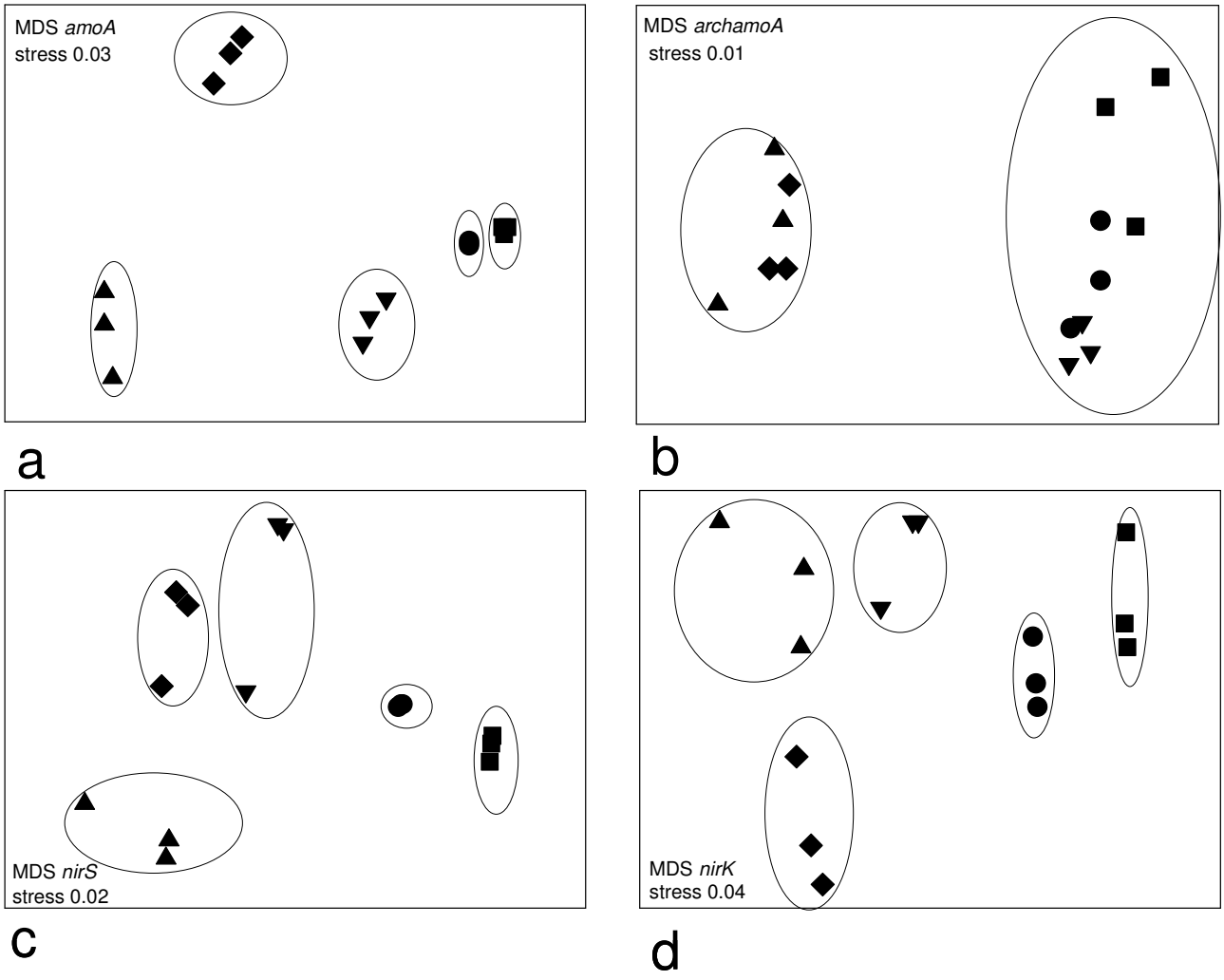
MDS *amoA*
stress 0.03
a
MDS *archamoA*
stress 0.01
b
MDS *nirS*
stress 0.02
c
MDS *nirK*
stress 0.04
d

Figure 1

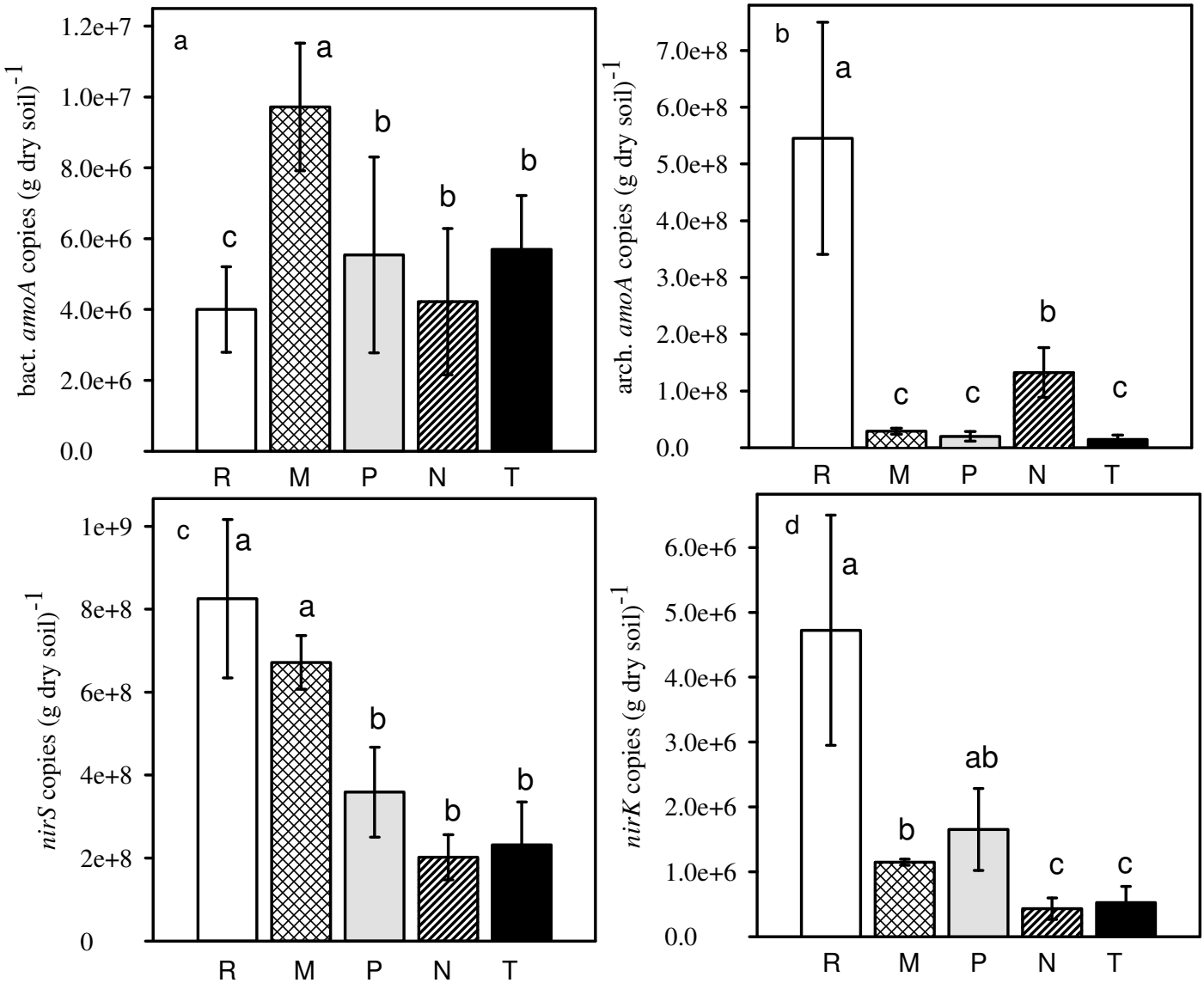
bact. amoA copies (g dry soil)-1
1.2e+7
1.0e+7
8.0e+6
6.0e+6
4.0e+6
2.0e+6
0.0
a
a
b
b
b
c
R
M
P
N
T
arch. amoA copies (g dry soil)-1
7.0e+8
6.0e+8
5.0e+8
4.0e+8
3.0e+8
2.0e+8
1.0e+8
0.0
b
a
c
c
b
c
R
M
P
N
T
nirS copies (g dry soil)-1
1e+9
8e+8
6e+8
4e+8
2e+8
0
c
a
a
b
b
b
R
M
P
N
T
nirK copies (g dry soil)-1
6.0e+6
5.0e+6
4.0e+6
3.0e+6
2.0e+6
1.0e+6
0.0
d
a
b
ab
c
c
R
M
P
N
T

Figure 2

Figure legends

Figure 1. Plots of MDS analysis performed on bacterial *amoA* (a), archaeal *amoA* (b), *nirS* (c) and *nirK* (d) T-RFLPs from Riederberg ▲, Maissau ●, Purkersdorf ■, Niederschleinz ♦ and Tulln ▼ soils (n=3).

Figure 2. Results of real time-PCR analysis of bacterial *amoA* (a), archaeal *amoA* (b), *nirS* (c) and *nirK* (d) genes in Riederberg (R), Maissau (M), Purkersdorf (P), Niederschleinz (N) and Tulln (T) soils (n=3); different letters indicate significant differences between the soils ($p < 0.05$).

CHAPTER 2

SHORT-TERM DYNAMICS OF AMMONIA OXIDIZING COMMUNITIES IN BARLEY PLANTED BULK SOIL AND RHIZOSPHERE FOLLOWING NITRATE AND AMMONIUM FERTILIZER AMENDMENT

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Abstract

In two experiments set up in the greenhouse the community dynamics of ammonia oxidizing bacteria (AOB) and archaea (AOA) upon N fertilizer amendment was studied via *amoA* gene-based T-RFLP profiling and real-time PCR quantification. First, ammonia oxidizers in two contrasting barley-planted soils were monitored following addition of either $[NH_4]_2SO_4$, NH_4NO_3 or KNO_3. Subsequently, the dynamics of ammonia oxidizers was studied specifically in the rhizospheres of two different barley genotypes upon $[NH_4]_2SO_4$ versus KNO_3 addition and effects of plant inoculation with the plant growth promoting *B. phytofirmans* PsJN strain were explored. AOB changed in community composition and increased in abundance upon ammonium amendment in bulk soil and rhizosphere, with changes in bacterial *amoA* copies lagging behind relative to changes in soil ammonium. Increases in AOB abundance were generally stronger in bulk soil but were enhanced in the rhizosphere upon plant inoculation with *B. phytofirmans* PsJN. AOA underwent shifts in community composition and abundance in both ammonium and nitrate amended soils; and archaeal *amoA* copy numbers varied in correspondence with soil nitrate rather than ammonium. AOB were thus considered as the main agents responsible for fertilizer ammonium oxidation, while the roles of AOA in soil N cycling still need to be clarified.

1. Introduction

Nitrifying microbial communities have been shown to respond in their abundances and activities to the nitrogen (N) fertilization regime in arable soils (Chu *et al.*, 2007, Enwall *et al.*, 2007, Cavagnaro *et al.*, 2008, Hallin *et al.*, 2009). This has agronomic and environmental implications, since nitrate set free by nitrifiers as a mobile N form may easily leach out of soils, causing N losses and polluting groundwater. In addition to emission of NO_x during nitrification, the nitrate produced serves as a substrate in denitrification, and may hence be reduced to radiatively active NO_x and N_2O gases, causing volatile N losses and environmental hazards (Conrad, 1996).

While it is generally acknowledged that up to 50% of the fertilizer N applied to agricultural soils is not taken up by the plants (FAO, 1995, FAO, 2009), the pathways that the various fertilizer forms actually take upon field application are not fully understood. Until recently chemoautotrophic bacteria affiliated with the *Beta-* or *Gammaproteobacteria* were believed to be exclusively responsible for oxidizing ammonia-N in soil, since they use ammonia-N as their sole energy source (Kowalchuk & Stephen, 2001). However, among the microorganisms possessing ammonia monooxygenase encoding *amoA* genes are also archaea assigned to the mesophilic Crenarchaeota group, which are hence capable of catalyzing the first and rate limiting step of nitrification. The lifestyle of ammonia oxidizing archaea as well as their environmental constraints and their importance in various soil systems are still unknown (Treusch *et al.*, 2005, Francis *et al.*, 2007).

Ammonium fertilization has been shown to significantly affect the abundance and composition of ammonia oxidizing bacterial communities (AOB;(Chu *et al.*, 2007, Enwall *et al.*, 2007, Cavagnaro *et al.*, 2008). Fertilizer effects on archaeal ammonia oxidizing communities (AOA), by contrast, are less well studied and seem to be incongruent. Mineral

nitrogen fertilizer application resulted in minor (Shen *et al.*, 2008) or no changes (Di *et al.*, 2009, Wang *et al.*, 2009) in the structure or abundance of AOA communities, whereas growth stimulation was reported under organic manure treatment (He *et al.*, 2007, Schauss *et al.*, 2009). Functional redundancy of AOB and AOA in soil environments has been postulated (Schauss *et al.*, 2009). However, a minor role of archaeal nitrification in agricultural soils has recently been suggested due to an oligotrophic lifestyle (Erguder *et al.*, 2009, Martens-Habbena *et al.*, 2009)and based on the observation that archaeal *amoA* gene patterns were uncoupled from ammonia oxidation activity (Di *et al.*, 2009, Jia & Conrad, 2009).

The present study aims at exploring the dynamics of ammonia oxidizing communities upon performing commonly used agricultural practices, i.e. the application of mineral N fertilizer and plant inoculation with plant growth promoting bacteria (PGPB). The main objective was to determine if, in what respect and how rapidly mineral N applied to agricultural soils influences the AOB and AOA microbial groups. A first experiment was directed towards exploring the effects of KNO_3, NH_4NO_3 and $[NH_4]_2SO_4$ fertilizer amendment on AOB and AOA community composition and abundance alongside with effects of the soil type. In a second experiment, the combined effects of N fertilization, the use of different barley genotypes and inoculation with the PGPB *Burkholderia phytofirmans* strain PsJN (Sessitsch *et al*., 2005) on the *in situ* ammonia oxidizing communities in the rhizosphere were explored until depletion of the added fertilizer N. In both experiments, bacterial and archaeal *amoA* genes were targeted in terminal restriction fragment length polymorphism (T-RFLP) and quantitative real-time PCR analysis.

2. Material and Methods

2.1. Soil sampling

In February 2008, soil was sampled from the top layer of two arable fields situated in Purkersdorf (48°15`0``N, 16°0`0``E) and Niederschleinz (48°35`59``N) in Lower Austria.

Purkersdorf soil was classified as cambisol from Sandy Loamy Flysch Material with sandy clay loam texture and had a pH of 5.7, while Niederschleinz soil was a chernozem from Loess with a pH of 7.2. The soils were kept at 4 °C during transportation to the lab, where they were sieved to 2 mm and then stored at 4 °C until analysis. Site characteristics of the sampling locations are given in Table 1.

2.2. Experimental set-up

Two experiments were performed, which focused on (1) N fertilizer effects on ammonia oxidizing microbial communities in two different soils planted with barley and (2) N fertilizer effects on ammonia oxidizers in the rhizospheres of two different barley genotypes. These experiments are in the following referred to as experiment 1 and experiment 2, respectively.

Experiment 1 was set up in the greenhouse in April 2008. Ninety 1 l-pots were filled with 300 g of sieved and homogenized soil from the Purkersdorf and Niederschleinz agricultural sites, respectively. One barley var. Morex (BCC906) plant was grown from seed in each pot. After 10 days of plant growth 40 mg N per pot was applied with a syringe at multiple spots as either KNO_3, NH_4NO_3 or $[NH_4]_2SO_4$ to allow for uniform distribution in the soil (Inselsbacher *et al.*, 2009). To prevent nutrient insufficiencies soils were additionally fertilized with 10 mL of a 37 mM K_2HPO_4 solution. Soils from three replicate pots per treatment and soil type were destructively sampled at five different time points: directly after fertilization (0 d), after two days (2 d), one (7 d), two (14 d) and three weeks (21 d) following fertilization. At the first sampling event (0 d) unamended and unplanted soils from three replicate pots were sampled as controls in addition to the samples given above. Prior to soil sampling, the barley plants were carefully removed. The soils from the individual pots were thoroughly mixed, comprising both bulk and rhizosphere soil but being strongly dominated by bulk soil. Therefore, soils in experiment 1 are subsequently referred to as “bulk” soils. Aliquots of 9 g

and 0.5 g of homogenized soil were used for chemical and molecular microbiological analyses, respectively.

Experiment 2 was set up in March 2009 to analyze nitrifying communities in the rhizosphere and to study plant genotype effects. As seeds of the variety Morex used in experiment 1 were no longer available, seeds of barley var. MR 3/51 (HOR 11371) and barley var. Arupo (BCC812) were sown in little (4 cm diameter) turf pots containing 20 g of Purkersdorf soil. An additional set of genotype 1 barley seeds was used that had been inoculated with a GFP-labeled, kanamycin resistant culture of *B. phytofirmans* PsJN (PsJN::gfp2x). This was done by surface sterilizing the seeds (by washing for 2 minutes with 5% NaOCl, for 2 minutes with 70% ethanol and two washings with sterile water) and then leaving them for five minutes in a two times diluted overnight culture of the inoculant at a final concentration of 6.75 x 10^7 CFU mL^{-1}. After five days, 30 plantlets per variant were transferred together with the turf pots into 1 l pots containing 300 g of Purkersdorf soil. After another five days, mineral N fertilizer was applied with a syringe in the form of either $[NH_4]_2SO_4$ or KNO_3 at a final concentration of 40 mg N per pot. To avoid growth deficiencies 35 mg of K_2O were added to each pot. Three replicate pots each were destructively sampled at four time points within four weeks: immediately after N amendment (0 d), after two weeks (14 d), three weeks (21 d) and four weeks (28 d), resulting in 72 samples in total from four sampling occasions. Here, soil contained in the turf pots that was adhering to the barley roots was sampled. Plant stems and roots were carefully detached from the soil in the turf pots and soil attached to barley roots was collected by use of a spatula. In pots of inoculated plants, approximately 0.5 g of soil was collected from the spermosphere, i.e. soil adhering to the seed, and incubated in 1 mL 0.9% NaCl at room temperature for 2 h with agitation. Hundred µL of serial dilutions (10^{-1} to 10^{-3}) of the suspension were then plated on LB plates with kanamycin [50 mg mL^{-1}], which after three days incubation at 28 °C were screened for GFP-labelled PsJN colonies under UV light. In addition, plant fresh and dry weight were determined in shoots and roots.

2.3. Soil chemical analyses

The soil water content was determined after drying at 105 °C, and pH was measured by glass electrode in 0.0125 M $CaCl_2$. NH_4-N was measured in extracts prepared from 2 g aliquots of homogenized soil in 15 mL KCl by a modified indophenol reaction (Kandeler & Gerber, 1988). NO_3-N was measured in soil extracts prepared from 5 g soil in 0.0125 M $CaCl_2$ by ion chromatography (Inselsbacher *et al.*, 2009).

2.4. Molecular analyses

Nucleic acid extraction

DNA was extracted from 0.5 g of soil taken from three replicate pots per treatment and sampling occasion by using the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, Ohio, USA) as described by the manufacturer, and was quantified photometrically (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA).

Bacterial and archaeal ammonia oxidizer and total bacterial community structures

Amplification of genes encoding the ammonia monooxygenase catalytic subunit A (*amoA*) was accomplished using primers *amoA*1F/*amoA*2R (Rotthauwe *et al.*, 1997) for bacteria and Arch-*amoA*F/Arch-*amoA*R (Francis *et al.*, 2005) for archaea, yielding fragments of 491 bp and 635 bp length, respectively. For amplification of bacterial 16S rRNA genes the primers 8F (Edwards *et al.*, 1989) and 1520R (Massol-Deya *et al.*, 1995) were used. All forward primers were 6-carboxyfluorescein (FAM) labeled at the 5´ end.

Amplifications were performed in 25 µL reactions containing 1x buffer, 3 mM $MgCL_2$, 0.2 mM dNTPs, 4%(v/v) DMSO, 1% (w/v) Bovine Serum Albumine (BSA), 1 U of FIREpol polymerase (Solis BIODYNE, Estonia), and 0.2 µM of each primer for amplification of

archaeal *amoA* and *16 S rRNA* genes but 0.24 µM of each primer for bacterial *amoA* amplification. 10 to 100 ng DNA was added to the reactions. Amplifications were performed in a Whatman T1 thermocycler using the following programs for bacterial / archaeal *amoA* reactions: an initial denaturing step at 95°C for 5 min, followed by 35/ 30 cycles of 1 min at 95°C, 1 min at 60°C / 53°C, 1 min at 72°C and a final extension step at 72°C for 10 min. The program for 16S rRNA gene amplification was set as follows: 95°C for 5 min, 30 cycles of 95°C 1min, 53°C 1 min and 72°C 2 min, with a final extension step at 72°C for 10 min.

PCR amplified 16S rRNA, *amoA* and Arch-*amoA* gene fragments were subjected to T-RFLP analysis using the *Alu*I restriction enzyme. The choice of restriction enzyme was based on *in silico* testing of the resolution in T-RFLP profiles when using three different enzymes (*Rsa*I, *Alu*I, *Hae*III) for 20 cloned sequences of each gene. To reduce bias two PCR products were pooled for each digestion assay. Ten µL mixtures for bacterial and archaeal *amoA* analysis contained 7 µL PCR product, 1x buffer and 0.5 µL *Alu*I (10U μL^{-1}, Promega), and were incubated for 3 h at 37°C. For 16S rRNA gene analysis the amount of restriction enzyme was doubled.

Digestion assays were purified by passage through DNA grade Sephadex G50 (GE Healthcare) columns. Ten µL of purified product were mixed with 10 µL HiDi-Formamide (Applied Biosystems) and 0.3 µL 500 ROX™ Size Standard (Applied Biosystems) and denatured at 95°C for 2 min. Detection of FAM-labeled terminal restriction fragments was done by capillary electrophoresis using an ABI 3100 automatic DNA sequencer.

Quantification of bacterial and archaeal *amoA* genes

SYBR-Green Assays were performed in an iCycler iQ Multicolor Real Time PCR Detection System (BIO-Rad Laboratories) using the same but unlabeled primers as for T-RFLP analysis. Twenty-five µL reactions were composed of 12.5 µL 2x IQ ™ SYBR®-Green Supermix (BIO-Rad Laboratories), 0.5 µM of each primer for *amoA* / Arch-*amoA* , 2.5% (v/v)

DMSO and 2.4% (w/v) BSA. Standards and samples were processed in triplicates. The thermocycler program for *amoA* / Arch-*amoA* PCR was carried out using the following protocol: 95°C for 3 min, 45 cycles of 95°C for 1 min, 57°C/53°C for 1 min, 72°C for 1 min and data collection at 78°C for 1 min. Melting curve analysis was done in order to confirm the specificity of the PCR product. To check for inhibition a two times dilution series of one representative of each soil was done. No inhibiting effects could be seen for any of the samples.

PCR products of each targeted gene were cloned (Strataclone PCR cloning Kit, Stratagene) and sequenced to confirm specificity. Plasmids were isolated using the Quantum Prep Plasmid Miniprep Kit (BIO-Rad) and DNA concentrations were determined by spectroscopy to calculate copy numbers. Standard curves from serial dilutions of known amounts of the target genes were generated for each run, showing correlation coefficients (R^2) of 0.994 to 1.000 and PCR efficiencies of 88 to 102%.

2.5. Plant nitrogen content

Plant Nitrogen contents were determined using an elemental analyzer as described in ÖNORM G 1073 (ÖNORM, 1988).

2.6. Statistical analyses

T-RFLPs were transformed into numerical data using GeneScan 3.7 software (Applied Biosystems). To reduce background noise, peaks with intensities higher than three times the standard deviation were binned and normalized as described by (Abdo *et al.*, 2006) using the statistical program R together with the filtering and binning macro provided at the IBEST homepage (http://www.ibest.uidaho.edu/tools/trflp_stats/instructions.php). Peaks that occurred in at least two replicates and with a percentage equal or higher than 2% were

considered as major ones. Discriminant analysis using the statistical program SPSS (11.0 for Windows) was performed for each T-RFLP data matrix. Functional gene abundance data were subjected to ANOVA (analysis of variance) using the Duncan post-hoc test to test for significant differences between sampling times. Analysis of similarity (ANOSIM) was performed with the statistical program Primer 5 (PRIMER-E, Plymouth) as described in (Clarke, 1993) to test for differences between treatments and time points.

3. Results

3.1. pH and soil mineral N

In experiments 1 and 2 soil pH and mineral N contents were monitored during three and four weeks following the various N amendments, respectively. In experiment 1, pH in Purkersdorf soil changed after KNO_3 addition from 6.5 (0 d) to 6.7 (21 d), while $[NH_4]_2SO_4$ and NH_4NO_3 addition resulted in decreasing pH of finally 5.6 and 6.3. Niederschleinz soil, by contrast, showed only minor variations in pH over time and between treatments. In experiment 2 the initial pH of 6.4 in Purkersdorf rhizosphere decreased upon $[NH_4]_2SO_4$ addition to a minimum of 5.2 after two weeks, followed by an increase up to 5.9 after 4 weeks. Upon KNO_3 amendment pH decreased continuously to a final value of 5.8.

Ammonium concentrations in Purkersdorf and Niederschleinz soils were 7.61 (± 0.34) and 7.32 (± 0.57) µg NH_4-N g^{-1} DW at the beginning of experiment 1, which remained constant upon KNO_3 addition. After $[NH_4]_2SO_4$ and NH_4NO_3 amendment, the added ammonium got continuously depleted within 21 days in Purkersdorf soil, while in Niederschleinz soil ammonium decreased to initial concentrations already on day 14 and 21 in $[NH_4]_2SO_4$ and NH_4NO_3 amended soils, respectively (Fig. 2, Fig. S1). In experiment 2, the ammonium concentration in Purkersdorf rhizosphere soil was initially 15 (±1.06) µg NH_4-N g^{-1} DW. Following $[NH_4]_2SO_4$ amendment, ammonium decreased continuously to minimum 4.58

(±0.11) µg NH_4-N g^{-1} DW on day 28. In the KNO_3 treatments, the initial NH_4-N content decreased over time to minimum 2.89 (±0.64) µg NH_4-N g^{-1} DW.

Upon KNO_3 addition to Purkersdorf soil in experiment 1 the initial nitrate concentration of 9.64 (± 2.82) was raised almost tenfold and then continuously declined to minimum 27.64 (± 9.9) µg NO_3-N g^{-1} DW on day 21. Nitrate in NH_4NO_3 and $[NH_4]_2SO_4$ amended soils amounted to maximum 63.90 (± 5.83) and 86.08 (± 30.37) (day 0 and day 2, respectively) and decreased to 32.05 (± 15.44) and 12.34 (± 4.91) µg NO_3-N g^{-1} DW on day 21. Nitrate in Niederschleinz soil was initially 22.07 (± 7.51) µg NO_3-N g^{-1} DW and decreased almost to initial values following KNO_3 and NH_4NO_3 amendments on day 21. Nitrate in $[NH_4]_2SO_4$ fertilized soil incresased up to 53.93 (± 8.84) µg NO_3-N g^{-1} DW. Low nitrate concentrations were generally measured in Purkersdorf rhizosphere soils in experiment 2. Upon KNO_3 amendment, nitrate in barley rhizospheres was between 37.78 (± 10.96) and 54.84 (±12.95) µg NO_3-N g^{-1} DW soil, and decreased almost beyond the detection limit within two weeks. In $[NH_4]_2SO_4$ amended soils initial nitrate concentrations of 13.42 (±1.83) µg NO_3-N g^{-1} DW soil remained constant during the first two weeks and then decreased below the detection limit.

3.2. 16S rRNA and amoA *community structures*

In experiment 1, T-RFLP community profiles of 16S rRNA and bacterial and archaeal *amoA* genes from soils receiving three different N amendments sampled on day 0 (0 d) and day 21 (21 d) (n=3 per treatment) were compared among each other and with unfertilized unplanted control soils (n=3) (Fig. 1). Peaks accounting for significant differences among treatments and sampling times ($P<0.005$) together with a summary of the statistical analysis results are given in Table 2. Profiles from Purkersdorf versus Niederschleinz soil contained 32 versus 21 T-RFs for 16S rRNA, 9 versus 4 T-RFs for bacterial *amoA* and 11 versus 9 T-RFs for archaeal

amoA communities. 12 and 2 T-RFs in the 16S rRNA and bacterial *amoA* profiles, respectively, were common to both soils, while the remaining T-RFs were unique to either soil. The Purkersdorf archaeal *amoA* T-RFLP profiles contained the same 9 T-RFs that were present also in the Niederschleinz profiles together with two additional unique T-RFs.

In discriminant analyses of T-RFLP data, summed up eigenvalues for functions 1 and 2 explained at least 87 % of the total variance in all cases (Table 2). Most of the functions had highly significant Wilk`s lambda values ($P<0.005$) and high canonical correlation coefficients ($r > 0.934$) except for functions 1 and 2 in the analysis of bacterial *amoA* in Niederschleinz soil, which did not fully explain data variability.

In the 16S rRNA discriminant plot from Purkersdorf soil, T-RFs from $[NH_4]_2SO_4$ and NH_4NO_3 treated samples from day 21 each formed most distinct clusters relative to the other samples. In the discriminant plot of 16S rRNA profiles from Niederschleinz soil, T-RFs from all samples from day 21 formed clusters according to the various N treatments, which were well separated along function 1 from day 0 and untreated control samples (Fig. 1a).

Similar to the 16S rRNA profiles, also the bacterial *amoA* T-RFs from $[NH_4]_2SO_4$ and NH_4NO_3 treated Purkersdorf soils from day 21 were well distinguished against the remaining soils in the discriminant plot. Among Niederschleinz bacterial *amoA* T-RFs, only those from soils amended with $[NH_4]_2SO_4$ and sampled on day 21 formed a separate cluster (Fig. 1b).

Archaeal *amoA* T-RFs from Purkersdorf soil formed separate clusters in the discriminant plot according to the various treatments and sampling times. In the discriminant plot of archaeal *amoA* T-RFs from Niederschleinz soil, NH_4NO_3 amended samples from day 21 were most strongly separated from the other samples, which were also grouped according to treatment but to a lesser extent so than the Purkersdorf samples (Fig. 1c). Archaeal *amoA* profiles from differentially treated Purkersdorf and Niederschleinz samples, respectively, differed regarding the relative abundances of several peaks but consistently contained the same major peaks (Table 2).

3.3. Bacterial and archaeal AmoA *gene abundances*

In experiment 1, $[NH_4]_2SO_4$ amendment resulted in increasing bacterial *amoA* copy numbers at least from day 7 following N addition, with maximum copy numbers occurring on day 21 (Fig. 2, Table S1) in both Purkersdorf and Niederschleinz soils. In the NH_4NO_3 fertilized soils a corresponding but less pronounced increase in bacterial *amoA* copy numbers occurred (Fig. S1, Table S1). Archaeal *amoA* genes were 10 (Purkersdorf) and 21 (Niederschleinz) times more abundant than bacterial *amoA* genes in the initial (untreated) soils. Following $[NH_4]_2SO_4$ amendment, however, archaeal over bacterial *amoA* dominance decreased to 1.3 (Purkersdorf) and 6.1 fold (Niederschleinz). Throughout the experiment, archaeal *amoA* copies varied in abundance in both soils. In contrast to bacterial *amoA* genes, changes in archaeal *amoA* copy numbers were similar in all N treatments of the two soils and corresponded more to changes in nitrate than ammonium (Fig. 3).

In experiment 2, bacterial *amoA* copies increased up to four-fold upon ammonium amendment, amounting to highest numbers on day 21. This increase was strongest in $[NH_4]_2SO_4$ amended rhizospheres of PsJN inoculated MR3/51 plants. A rapid decrease in bacterial *amoA* copies occured after depletion of the added ammonium, so that initial *amoA* copy numbers were reached on day 28 in all soils (Fig. 2). Archaeal *amoA* gene copies were initially equally abundant as the bacterial ones in the barley rhizosphere, but were outnumbered by bacterial *amoA* genes up to 6fold on day 14 following $[NH_4]_2SO_4$ addition. Archaeal *amoA* copies showed similar fluctuations to those seen in experiment 1 (Figure 3, Table S2). Again, variations in archaeal copy numbers were in the same range following both $[NH_4]_2SO_4$ and KNO_3 amendments (Fig. 3).

3.4. Plant biomass and N contents

In experiment 2, $[NH_4]_2SO_4$ fertilization resulted in almost two times more above ground plant biomass of barley var. MR 3/51 as compared to the KNO_3 treatment on day 28. Var. Arupo also showed enhanced growth upon $[NH_4]_2SO_4$ fertilization as compared to KNO_3 fertilization, but less pronounced than MR3/51 barley. MR3/51 plants (70.1 ± 4.5 to 98.4 ±3.0 mg dry weight) generally had higher biomass than Arupo plants (54.1 ± 4.0 to 56.6 ± 9.0 mg dry weight).

N contents in barley var. MR 3/51 ranged from 1.20 (± 0.20) to 3.28 (± 0.24) % and were higher when soils were amended with $[NH_4]_2SO_4$ versus KNO_3. Differences between treatments were not significant with var. Arupo, where the N contents upon $[NH_4]_2SO_4$ and KNO_3 amendment were 2.73 (± 0.96) and 2.39 (±0.96) %, respectively.

Average root biomass was 23 (± 2.14) mg DW in var. Arupo and non-inoculated MR3/51 plants and 27 (± 5.28) mg DW in *B. phytofirmans* strain PSJN inoculated MR3/51 plants on day 28. Upon $[NH_4]_2SO_4$ application, root biomass of PsJN inoculated MR3/51 (37 ± 0.35 mg dry weight) was enhanced relative to var. Arupo and non-inoculated MR3/51 plants (23 ± 0.36 mg dry weight). However, no plant growth promoting effect was shown for *B. phytofirmans* strain PsJN, although the bacterium was recovered from the soils of all inoculated plants with CFUs ranging from $3.83x10^4$ to $3.74x10^5$ per g FW. Plant inoculation with *B. phytofirmans* together with soil $[NH_4]_2SO_4$ amendment was associated with a lower plant N content ($p<0.05$) as compared to non-inoculated plants, whereas KNO_3 treatment resulted in no such differences.

4. Discussion

Experiment 1 - N fertilizer effects in different soil types

Soil type and physico-chemical qualities are well known to affect bacterial communities and their activities including nitrogen (N) transformations (Cheneby *et al.*, 2000, Sessitsch *et al.*, 2001, Girvan *et al.*, 2003). Hence, specific responses to $[NH_4]_2SO_4$, NH_4NO_3 and KNO_3 fertilization regimes were expected in Purkersdorf and Niederschleinz soils, which differed in characteristics such as porosity and clay content, pH and C and N status (Table 1). Distinct total bacterial and ammonia oxidizing bacterial (AOB) communities were hosted by the two soils, while ammonia oxidizing archaeal (AOA) communities were more similar. Niederschleinz versus Purkersdorf soil contained twice and three times more bacterial and archaeal *amoA* copies, but both soils had similar AOA to AOB ratios of 10 and 21, as contrasted to other soil habitats where AOA may dominate over AOB up to several hundred times (Leininger *et al.*, 2006). N amendment, however, resulted in corresponding effects on the nitrifying microbial groups in both soils: AOB changed in abundance and community structure only following ammonium fertilizer application; whereas changes in AOA communities occurred both in ammonium and nitrate amended soils (Figs. 1-3).

The total bacterial communities in Purkersdorf and Niederschleinz soils underwent structural changes in all three N fertilization regimes. Community structures of the AOB subsets changed specifically only upon ammonium amendment, however, to a lesser extent so in Niederschleinz soil (Fig. 1). AOB community shifts may have been promoted by pH effects in Purkersdorf soil (Prosser & Embley, 2002, Koops *et al.*, 2003, Nicol *et al.*, 2008), where pH values decreased upon ammonium amendment, presumably due to the liberation of hydrogen ions during ammonia oxidation. PH was less affected in Niederschleinz soil, which was high in carbonate with a strong buffering capacity (Fig. 2).

AOB abundance in Purkersdorf and Niederschleinz soils increased six and 4.5 times upon $[NH_4]_2SO_4$ addition, respectively (Fig. 2). Fertilizer NH_4-N got immediately diminished in the soil following application together with increasing NO_3-N, but AOB numbers increased only with a delay of two to seven days (Fig. 2). Obviously, ammonia oxidation was brought about initially through enhanced activity of the resident AOB community, involving increased *amoA* transcription. Analysis of *amoA* transcripts may allow insights into the actual ammonia oxidizing activity as compared to *amoA* based quantification of AOB, which reflects the dynamics in AOB population size.

Fertilizer effects on AOB communities in the two soils were seen to depend on the amount of ammonium applied, since NH_4NO_3 amendment containing half the amount of NH_4-N resulted in corresponding but less pronounced alterations in *amoA* copy numbers and community profiles than application of $[NH_4]_2SO_4$ (Fig. S1, Table S1). Increasing AOB abundances following NH_4-N amendment to soils have consistently been reported (Hermansson & Lindgren, 2001, Okano *et al.*, 2004, Chu *et al.*, 2007, Enwall *et al.*, 2007, He *et al.*, 2007, Cavagnaro *et al.*, 2008). Effects on AOB community composition upon addition of mineral N fertilizers, by contrast, varied, including pronounced (Mendum & Hirsch, 2002) or minor changes in AOB community profiles (He *et al.*, 2007) and increased or reduced AOB diversity (Chu *et al.*, 2007).

While fluctuations in AOA abundance were seen throughout the study period (Fig. 3), they were similar in all fertilizer treatments, without specific relatedness to ammonium availability. Notably, AOA changed in abundance in correspondence with changes in soil nitrate, supplied either as KNO_3 or resulting from ammonia oxidation in $[NH_4]_2SO_4$ and NH_4NO_3 amended soils. This suggests a potential role of AOA in nitrate reduction, as has been indicated earlier by the presence of denitrification genes in AOA fosmid clones (Treusch *et al.*, 2005, Hallam *et al.*, 2006). Although important roles of AOA in C and N cycling have been implicated in various molecular studies (Hallam *et al.*, 2006, Leininger *et al.*, 2006,

Chen *et al.*, 2008), further unraveling of their lifestyles and ecological functions is still impeded by the lack of AOA isolates.

In studies of other fertilized soils including rice paddies NH_4-N amendment resulted in increased AOB but unchanged AOA abundances, suggesting involvement of mainly AOB in ammonia oxidation (Shen *et al.*, 2008, Di *et al.*, 2009, Wang *et al.*, 2009). Recently, a greater importance of AOB than AOA for ammonia oxidation in an agricultural soil has been demonstrated through DNA stable isotope probing (Jia & Conrad, 2009). Considering the high ammonia conversion rates displayed by AOB isolates (Prosser, 1989, Okano *et al.*, 2004) and the rapid growth response of AOB following NH_4-N addition to Purkersdorf and Niederschleinz soils, it seems most likely that AOB and not AOA were mainly responsible for consuming and oxidizing the applied ammonium. As ammonia oxidizing rates were low in AOA isolates (Könneke *et al.*, 2005, de la Torre *et al.*, 2008), a backup function of AOAs was suggested under either disturbed (Schauss *et al.*, 2009) or low nutrient conditions (Erguder *et al.*, 2009, Martens-Habbena *et al.*, 2009), which may be supported by our findings.

Experiment 2 – N fertilizer effects in the rhizospheres of different barley genotypes

AOB increased in abundance also in the rhizospheres of two different barley genotypes grown on Purkersdorf soil following $[NH_4]_2SO_4$ addition. In the barley root environment the supplied NH_4-N got continuously and completely depleted within 28 days. In parallel, also the numbers of AOB declined rapidly from day 21, having reached peak abundances, towards initial levels on day 28 (Fig. 2). AOB numbers and NH_4-N concentrations were similar in the rhizospheres of both barley genotypes. As compared with *amoA* copy number increases in Purkersdorf bulk soil, the same fertilizer treatment caused a weaker increase in bacterial *amoA* genes in the barley rhizosphere. NH_4-N concentrations were higher in the rhizosphere than in bulk soil after 14 days but amounted to similar values on day 21. In contrast to experiment 1, where nitrate concentrations fluctuated in a similar way in the various

fertilization regimes, in the barley root environment nitrate concentrations declined to low levels immediately after fertilization (Fig. 2). This indicates rapid uptake of nitrate by plant roots, as has been demonstrated previously in barley especially during early plant growth stages (Inselsbacher *et al.*, 2010), and probably also denitrification activity which is commonly favored by rhizosphere conditions (Cavigelli & Robertson, 2000, Philippot *et al.*, 2007, Philippot *et al.*, 2009).

Competition with plants for NH_4-N was probably a critical factor restricting further growth of AOBs and resulting in rapidly declining AOB numbers concomitantly with decreasing soil ammonium concentrations soon after fertilizer application. Similarly, competition for NH_4-N was shown to negatively affect gross nitrification rates in the rhizosphere of wild oat (Herman *et al.*, 2006). Organic carbon or nitrification inhibitors released from plant roots may additionally inhibit autotrophic AOBs (Verhagen & Laanbroek, 1991, Strauss & Lamberti, 2000, Subbarao *et al.*, 2007).

In concordance with bulk soil AOA dynamics, AOA abundances fluctuated over time also in the barley rhizospheres, but again without specific relatedness to NH_4-N availability but rather in correspondence with soil nitrate concentrations (Fig. 3). That AOA numbers were initially lower after $[NH_4]_2SO_4$ versus KNO_3 addition may reflect limited provision of nitrate through ammonia oxidation together with high plant nitrate uptake during early barley growth stages. While ammonia-based chemolithoautotrophic energy metabolism by AOA has been supported in studies of the so far only isolated free-living AOA strain, '*Candidatus* Nitrosopumilus maritimus' strain SCM1 (Könneke *et al.*, 2005), nitrate reduction by AOA has not been evidenced, stressing the need for further exploring AOA lifestyle traits. Ratios of bacterial to archaeal *amoA* copies changed even more pronounced in favor of AOB following NH_4-N addition in the rhizosphere than in bulk soils, which reinforces the notion that AOB and not AOA presumably were the dominant players in nitrification.

N fertilizer effects following plant inoculation with B. phytofirmans PsJN

In experiment 2, highest AOB numbers were observed in the rhizosphere of barley var. M13/5 inoculated with *B. phytofirmans* strain PsJN three weeks following $[NH_4]_2SO_4$ amendment (Fig. 2). Alongside with higher AOB quantities, lower N contents were found in the barley plants.

While plant growth-promoting bacteria in general may promote plant nutrient acquisition through multiple mechanisms (Adesemoye & Kloepper, 2009), strain PsJN specifically is not able to fix atmospheric nitrogen. This strain has the potential for high 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and IAA production (Sessitsch *et al*., 2005; unpubl. results). Furthermore, it efficiently colonizes the rhizosphere and in particular the endosphere of many different plant species. The strong increase in AOB abundance in the rhizosphere of PSJN inoculated barley plants upon $[NH_4]_2SO_4$ addition may have been brought about via the inhibition of plant N uptake or through the release of plant compounds conducive to AOB ammonia oxidation and growth. In such a scenario, ACC deaminase activity by PSJN in the barley rhizosphere and endosphere causes cleavage of plant derived ACC into ammonia and α-ketobutyrate, thus providing additional ammonia-substrate for AOB and at the same time lowering the plant nitrogen content. The potential transfer pathways of ACC and its degradation products involved, however, remain unclear. While this has not been examined more closely here, effects of the PsJN strain on root architecture may play an indirect role in controlling AOB abundance in the rhizosphere. Increased plant competition for nutrients with endophytic PsJN bacteria may in addition exert some influence on the plant N budget, effecting that plant N concentrations were lowered.

5. Conclusions

Concluding, elevated soil nitrate or ammonium concentrations and altered pH values were short-term effects of N fertilizer amendment to barley-planted soils, since initial levels were reestablished within less than four weeks. Associated dynamics in AOB communities lagged behind approximately one week relative to increased or decreased NH_4-N availability, whereas AOA communities did not respond exclusively to variations in soil NH_4-N status but varied in abundance in correspondence with soil NO_3-N. Retrieving AOA isolates from soil in pure culture would be helpful in future attempts to clarify the importance and roles of AOA in various soil environments, because this would allow exploring lifestyle traits and relationships with environmental factors and other microorganisms. Our results furthermore suggest that plant-growth promoting bacteria may influence N cycling communities in the soil, entailing potential effects on N loss and N gas emission. The processes and mechanisms involved, however, require further investigation.

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Table 1 Site characteristics and physical and chemical properties of soils (0-20 cm soil layer) collected in Purkersdorf and Niederschleinz located in the vicinity of Vienna in Lower Austria

	Purkersdorf	**Niederschleinz**
Soil type	Gleyic Cambisol from sandy loamy flysch	Chernozem from Loess
Geographic site	48°12’25’’ N 16°10’37’’ E	48°35’59’’ N 15°10’24’’ E
Altitude (m. a. sl.)	248	244
Management	Winter barley	Arable field
Water condition	Moist	Moderately dry
Clay (%)	1.7	17.7
Silt (%)	64.9	74.2
Sand (%)	33.4	8
pH (KCl)	5.67	7.15
$CaCO_3$ (%)	0.06	8.5
Exchange capacity (mval%)	11.2	15.4
Base saturation (%EC)	81.4	98.1
Bulk density (g DW mL^{-1})	1.06	0.96

Table 2 Results of comparative statistical analysis of 16S and bacterial and archaeal *amoA* T-RFLP community data.

Soil		16S rRNA	Bacterial *amoA*	Archaeal *amoA*
Purkersdorf	∑ T-RFs [a]	21	4	9
	FC 1 % [b]	80.3	71.0	67.5
	(R /Wilk`s λ)	(0.991/0.000)	(0.859/0.106*)	(0.992/0.000)
	FC 2 % [b]	12.3	17.0	19.4
	(R /Wilk`s λ)	(0.945/0.005)	(0.635/0.404*)	(0.975/0.000)
	Cumulative % [b]	92.6	88.1	87.0
	Sign. T-RFs [c]	153, 196	226	136, 139, 165, 170, 246, 261
Niederschleinz	∑ T-RFs [a]	33	9	11
	FC 1 % [b]	67.0	82.0	51.3
	(R /Wilk`s λ)	(0.979/0.000)	(0.991/0.000)	(0.997/0.000)
	FC 2 % [b]	19.8	9.8	39.2
	(R /Wilk`s λ)	(0.933/0.010)	(0.934/0.007)	(0.996/0.000)
	Cumulative % [b]	86.8	92.2	90.5
	Sign. T-RFs [c]	209, 281, 241, 248, 274	64, 71, 201, 211	137, 138, 139, 165, 169, 170, 208, 209, 246, 260

[a] Number of major peaks

[b] Eigenvalues of Functions (FC) 1 and 2 derived from Canonical Discriminant Analysis and the corresponding correlation coefficients (R) and Wilk`s lambda values. * no statistical significance ($p > 0.05$)

[c] T-RFs that are significantly different among soils receiving various fertilizer treatments based on ANOVA analysis ($p < 0.05$)

7. Figure legends

Figure 1 Score plots of discriminant analysis performed on T-RFLP data of (a) 16S, (b) bacterial *amoA* and (c) archaeal *amoA* gene fragments from Purkersdorf and Niederschleinz soils in experiment 1. Besides analysis of unplanted, unfertilized control soils (n=3) (■), soils (n=3) receiving $[NH_4]_2SO_4$ (Δ, ▲), NH_4NO_3 (◊, ♦) and KNO_3 (○, ●) were analyzed immediately after N amendment (open symbols) and after 21 days (filled symbols). Encircled symbol groups signify that T-RFs were discriminated from other samples in ANOSIM analysis.

Figure 2 Dynamics in AOB community size (measured as bacterial *amoA* copy numbers) in relation to soil NH_4-N and NO_3-N concentrations, and soil pH values, following $[NH_4]_2SO_4$ amendment. Barley-planted bulk soils from (a) Purkersdorf and (b) Niederschleinz, and Purkersdorf rhizospheres beneath (c) barley var. MR3/51, (d) barley var. MR3/51 inoculated with *Burkholderia* strain PsJN and (e) barley var. Apuro were repeatedly analyzed immediately after N application (day 0) and at intervals of about 7 days. Symbols represent means (n=3) ± standard errors. Significant differences in AOB abundance between time points are indicated by * ($p<0.05$).

Figure 3 Dynamics in AOA community size (measured as archaeal *amoA* copy numbers) following various N amendments. Barley-planted (a) Purkersdorf and (b) Niederschleinz bulk soils under $[NH_4]_2SO_4$, NH_4NO_3 and KNO_3 treatments; and Purkersdorf rhizospheres beneath (c) barley var. MR3/51, (d) barley var. MR3/51 inoculated with *Burkholderia* strain PsJN and (e) barley var. Apuro under $[NH_4]_2SO_4$ and KNO_3 treatments. Soils were repeatedly analyzed immediately after N application (day 0) and at intervals of about 7 days. Symbols represent means (n=3) ± standard errors. Significant differences in AOA abundance between time points are indicated by * ($p<0.05$) and ** ($p<0.01$).

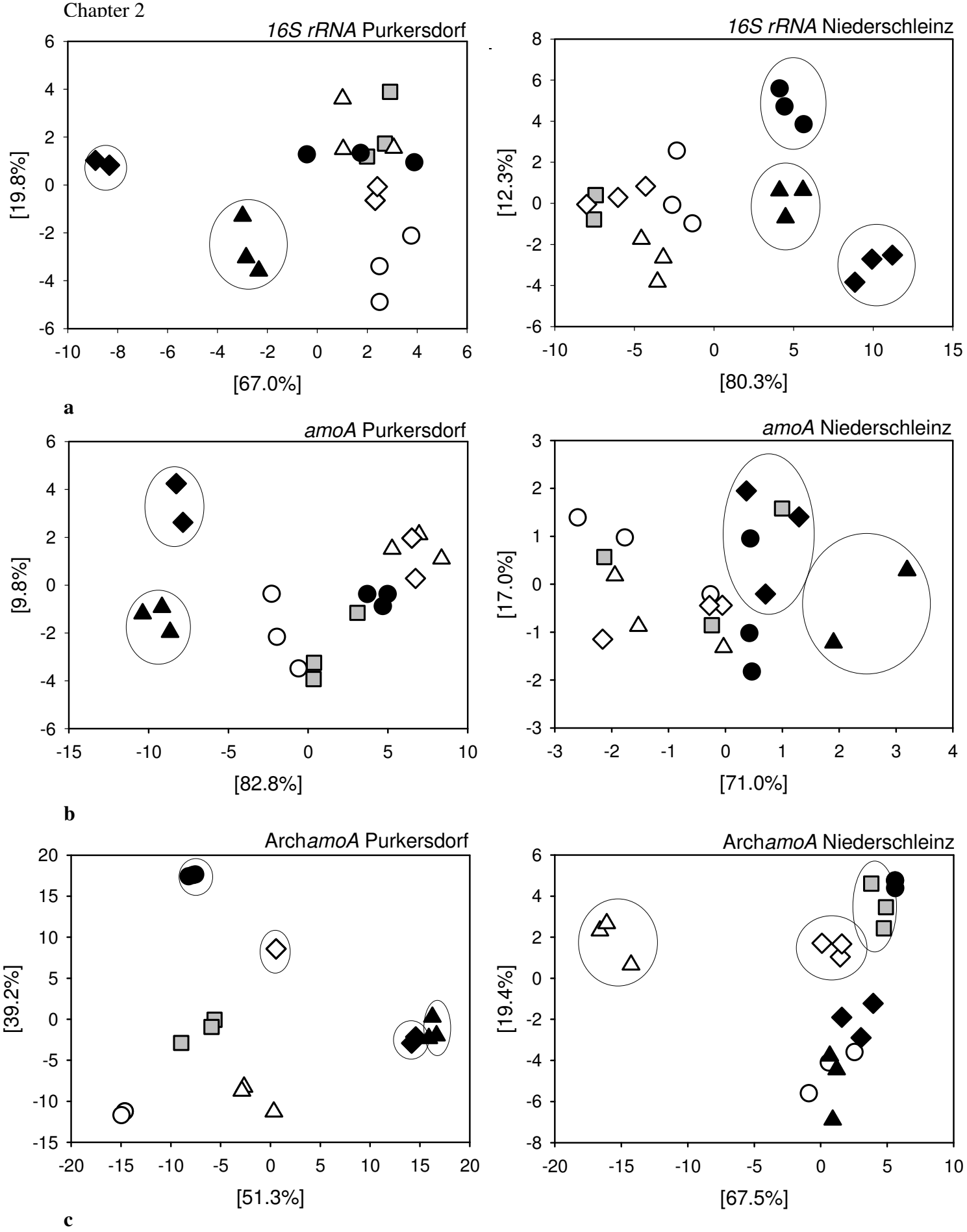

16S rRNA Purkersdorf
[19.8%]
[67.0%]
16S rRNA Niederschleinz
[12.3%]
[80.3%]
a
amoA Purkersdorf
[9.8%]
[82.8%]
amoA Niederschleinz
[17.0%]
[71.0%]
b
ArchamoA Purkersdorf
[39.2%]
[51.3%]
ArchamoA Niederschleinz
[19.4%]
[67.5%]
c

Figure 1

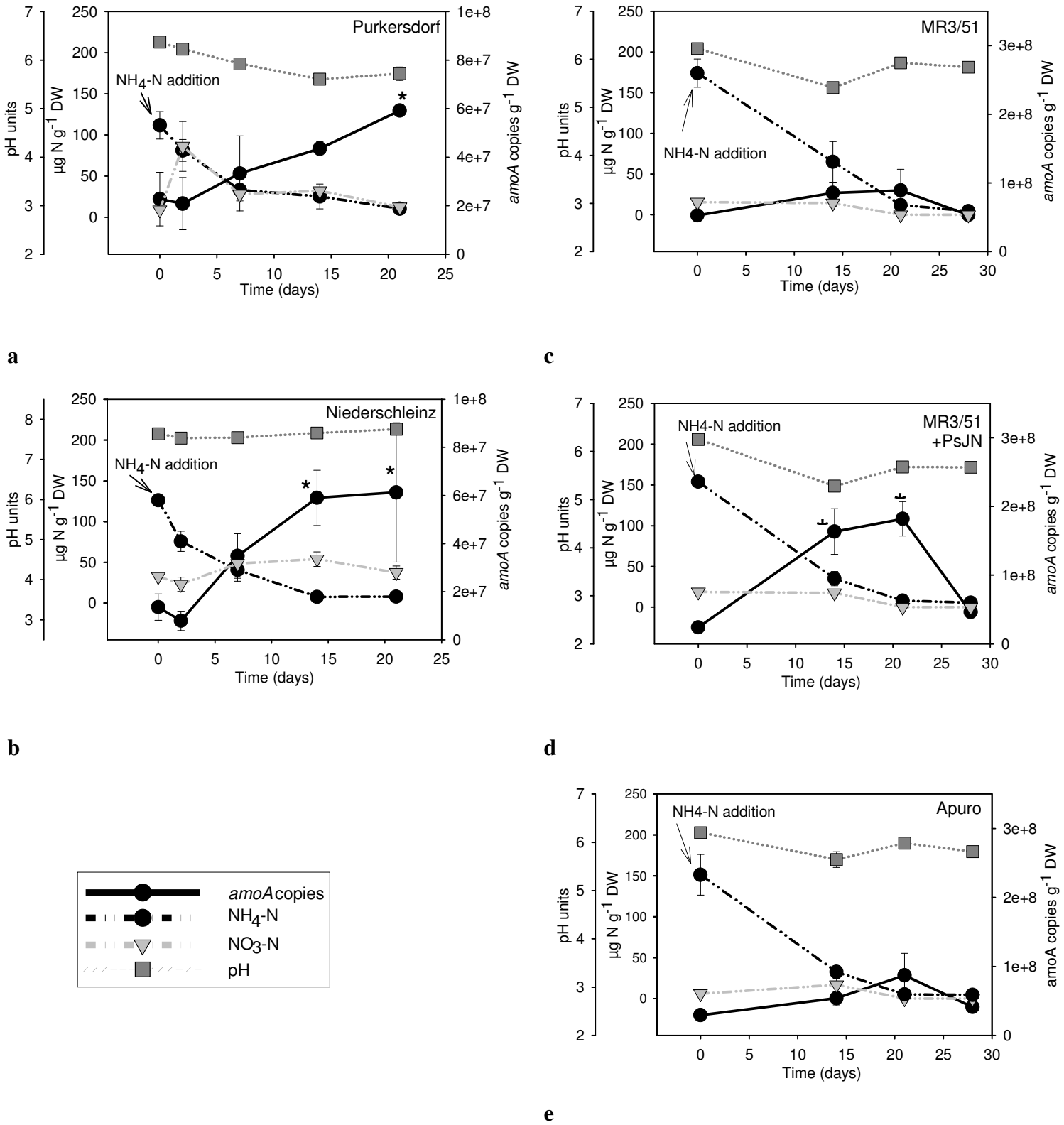
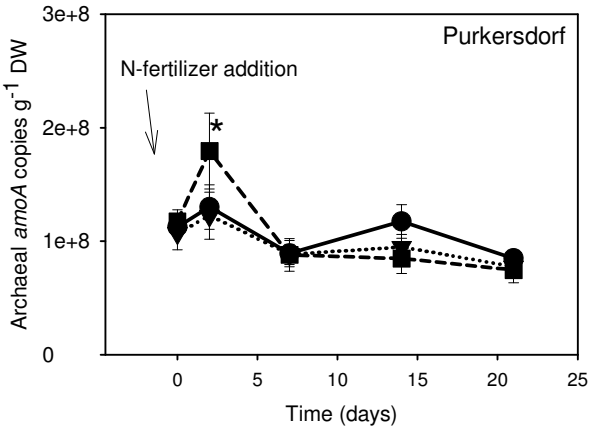
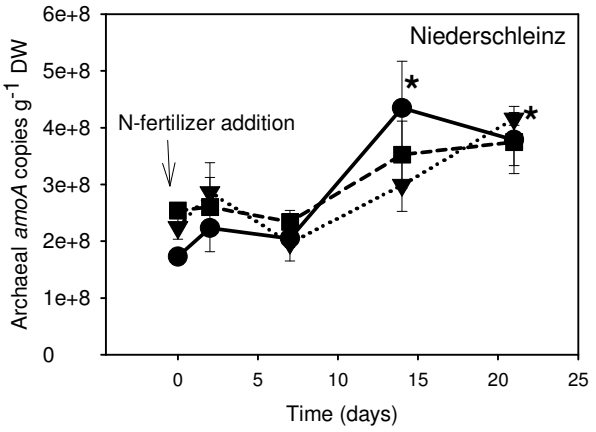

Purkersdorf
NH4-N addition
MR3/51
Niederschleinz
MR3/51 +PsJN
Apuro
pH units
µg N g-1 DW
amoA copies g-1 DW
Time (days)
amoA copies
NH4-N
NO3-N
pH
a
b
c
d
e

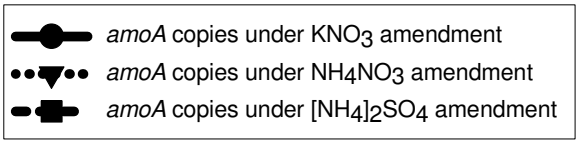
Figure 2

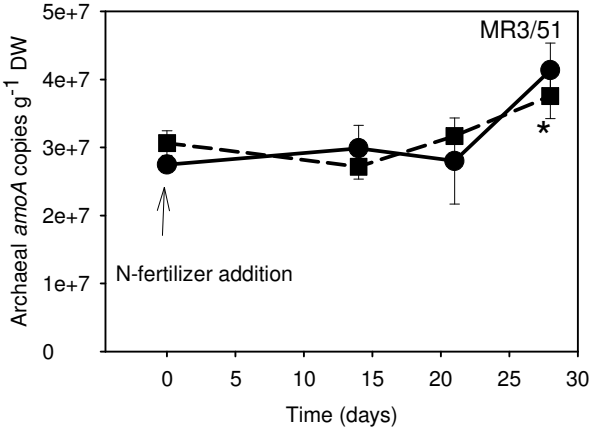


a

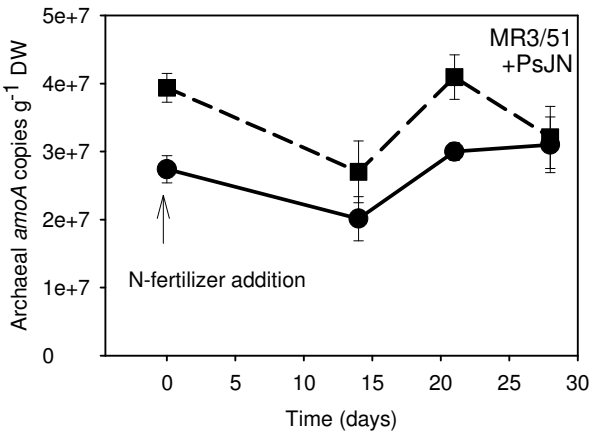


b

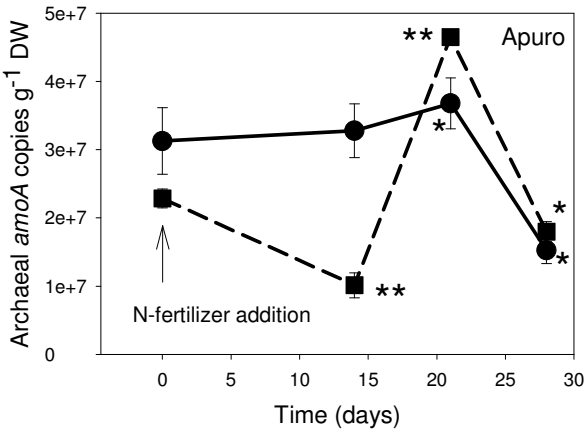




c



d



e

Figure 3

Supporting Information

Table S1. Copy numbers (g DW soil)$^{-1}$ of bacterial (1) and archaeal (2) ammonia monooxygenase subunit A gene fragments (*amoA*) in soils treated with KNO_3, $[NH_4]_2SO_4$ and NH_4NO_3 derived from sites Purkersdorf and Niederschleinz in experiment 1. Different letters indicate differences within one treatment at $p<0.05$ based on Duncan post-hoc test.

		Niederschleinz						*Purkersdorf*					
gene copies g (DW)$^{-1}$	Time[a]	KNO_3		NH_4NO_3		$[NH_4]_2SO_4$		KNO_3		NH_4NO_3		$[NH_4]_2SO_4$	
(1) AOB	no	1.39E+07	(±1.36E+07)^{a}					9.19E+06	(±1.00E+06)^{b}				
	0 d	1.45E+06	(±4.59E+04)^{b}	6.29E+06	(±1.40E+06)^{b}	1.36E+07	(±5.50E+06)^{b}	1.31E+07	(±1.71E+06)^{b}	1.03E+07	(±1.63E+06)^{b}	2.27E+07	(±1.10E+07)^{b}
	2 d	1.74E+06	(±6.57E+05)^{b}	1.10E+07	(±6.26E+06)^{b}	7.91E+06	(±4.02E+06)^{b}	1.74E+07	(±4.10E+06)^{ab}	1.64E+07	(±1.01E+07)^{ab}	2.09E+07	(±1.08E+07)^{b}
	7 d	1.48E+06	(±1.81E+05)^{b}	1.13E+07	(±4.89E+06)^{b}	3.49E+07	(±9.24E+06)^{ab}	1.59E+07	(±2.39E+06)^{ab}	2.24E+07	(±6.87E+06)^{ab}	3.33E+07	(±1.55E+07)^{b}
	14 d	1.75E+06	(±6.16E+04)^{b}	2.54E+07	(±1.39E+07)^{a}	5.90E+07	(±1.15E+07)^{a}	2.05E+07	(±8.88E+06)^{a}	3.29E+07	(±4.37E+06)^{a}	4.35E+07	(±2.85E+06)^{ab}
	21 d	1.93E+06	(±2.72E+05)^{b}	1.59E+07	(±1.29E+06)^{ab}	6.13E+07	(±2.90E+07)^{a}	2.34E+07	(±7.80E+06)^{ab}	3.08E+07	(±1.22E+07)^{ab}	5.92E+07	(±6.18E+05)^{a}
(2) AOA	no	2.91E+08	(±9.76E+07)^{ab}					9.39E+07	(±4.11E+07)^{a}				
	0 d	1.73E+08	(±1.18E+07)^{b}	2.24E+08	(±5.11E+07)^{b}	2.54E+08	(±7.34E+06)^{a}	1.13E+08	(±1.90E+07)^{a}	1.07E+08	(±3.59E+07)^{a}	1.18E+08	(±2.47E+07)^{ab}
	2 d	2.23E+08	(±2.73E+07)^{ab}	2.86E+08	(±6.49E+07)^{ab}	2.60E+08	(±1.92E+08)^{a}	1.30E+08	(±4.78E+07)^{a}	1.23E+08	(±5.09E+07)^{a}	1.79E+08	(±8.17E+07)^{a}
	7 d	2.05E+08	(±3.93E+07)^{b}	1.97E+08	(±7.73E+07)^{b}	2.34E+08	(±4.91E+07)^{a}	8.92E+07	(±2.83E+07)^{a}	8.84E+07	(±2.17E+07)^{a}	8.79E+07	(±3.54E+07)^{b}
	14 d	4.35E+08	(±2.02E+08)^{a}	2.99E+08	(±1.15E+08)^{ab}	3.52E+08	(±1.45E+08)^{a}	1.17E+08	(±3.65E+07)^{a}	9.48E+07	(±2.71E+07)^{a}	8.47E+07	(±3.21E+07)^{b}
	21 d	3.78E+08	(±1.45E+08)^{ab}	4.16E+08	(±2.75E+07)^{a}	3.75E+08	(±1.02E+08)^{a}	8.50E+07	(±1.56E+07)^{a}	7.76E+07	(±1.28E+07)^{a}	7.46E+07	(±2.75E+07)^{b}

[a] Soils were analyzed immediately after N amendment (0 d) and after 2, 7, 14 and 21 days. no= untreated, unplanted control soil.

Supporting Information

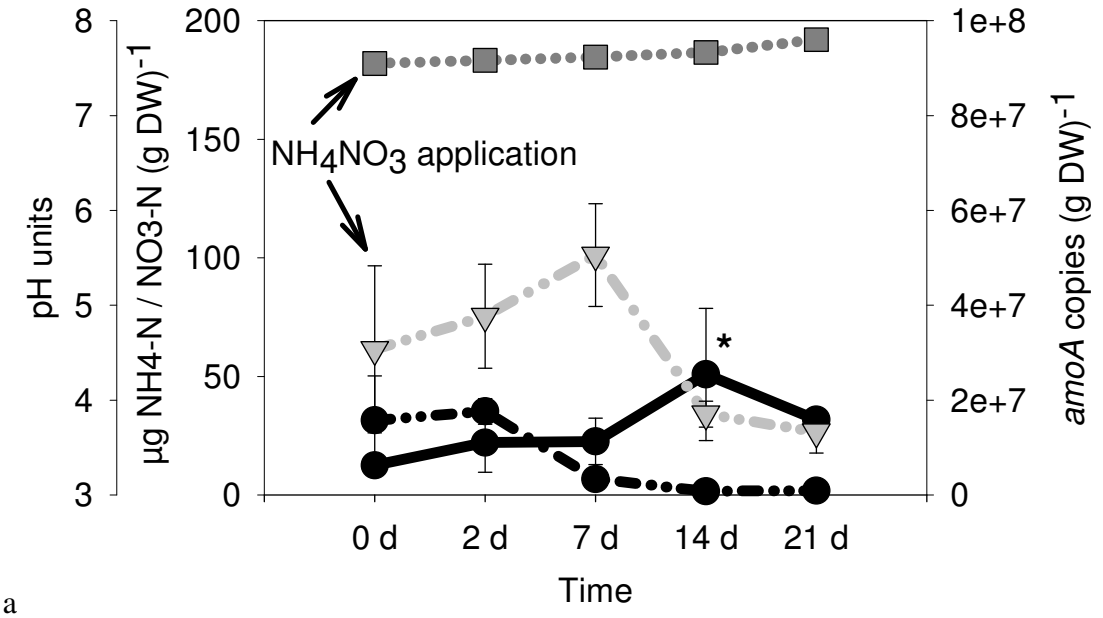
Table S2. Copy numbers (g DW soil)$^{-1}$ of bacterial (AOB) and archaeal (AOA) ammonia monooxygenase subunit A gene fragments (*amoA*) in Purkersdorf rhizospheres beneath barley var. MR3/51 (G1), barley var. MR3/51 inoculated with Burkholderia strain PsJN (G1 PSJN) and barley var. Apuro (G2) under [NH4]2SO4 and KNO3 treatments in experiment 2. Different letters indicate differences within one treatment at p<0.05 based on Duncan post-hoc test.

	Barley Genotype	day 0[a]		14 days		21 days		28 days	
AOB									
$[NH_4]_2SO_4$	G1	5.25E+07	(±2.01E+07)^{bc}	8.54E+07	(±3.80E+07)^{b}	8.90E+07	(±7.50E+07)^{b}	5.32E+07	(±1.27E+07)^{bc}
	G1 PSJN	2.41E+07	(±8.89E+06)^{bc}	1.63E+08	(±8.14E+07)^{a}	1.82E+08	(±6.09E+07)^{a}	4.64E+07	(±6.75E+06)^{bc}
	G2	2.94E+07	(±1.18E+07)^{bc}	5.40E+07	(±2.41E+07)^{bc}	8.71E+07	(±7.83E+07)^{b}	4.13E+07	(±1.10E+07)^{bc}
KNO_3	G1	2.09E+07	(±5.32E+06)^{bc}	2.42E+07	(±3.00E+06)^{bc}	2.94E+07	(±1.58E+07)^{bc}	2.90E+07	(±8.06E+06)^{bc}
	G1 PSJN	1.22E+07	(±7.25E+06)^{c}	5.37E+07	(±2.21E+07)^{bc}	2.70E+07	(±9.35E+06)^{bc}	3.11E+07	(±1.10E+07)^{bc}
	G2	5.18E+07	(±2.71E+07)^{bc}	3.72E+07	(±1.66E+07)^{bc}	4.81E+07	(±9.48E+06)^{bc}	2.58E+07	(±2.00E+06)^{bc}
AOA									
$[NH_4]_2SO_4$	G1	3.07E+07	(±4.41E+06)^{bcde}	2.72E+07	(±4.55E+06)^{bcde}	3.17E+07	(±4.25E+05)^{abcd}	3.76E+07	(±8.15E+06)^{abc}
	G1 PSJN	3.94E+07	(±5.21E+06)^{ab}	2.70E+07	(±1.11E+07)^{bcde}	4.10E+07	(±7.96E+06)^{ab}	3.21E+07	(±1.12E+07)^{abcd}
	G2	2.29E+07	(±3.35E+06)^{cdef}	1.01E+07	(±4.46E+06)^{f}	4.65E+07	(±2.18E+06)^{a}	1.80E+07	(±3.60E+06)^{def}
KNO_3	G1	2.75E+07	(±2.39E+06)^{bcde}	2.99E+07	(±8.21E+06)^{bcde}	2.80E+07	(±1.55E+07)^{bcde}	4.14E+07	(±9.83E+06)^{ab}
	G1 PSJN	2.74E+07	(±4.88E+06)^{bcde}	2.01E+07	(±7.98E+06)^{def}	3.00E+07	(±3.21E+06)^{bcde}	3.10E+07	(±1.00E+07)^{abcde}
	G2	3.13E+07	(±1.19E+07)^{abcd}	3.28E+07	(±9.70E+06)^{abcd}	3.68E+07	(±9.13E+06)^{abc}	1.53E+07	(±4.81E+06)^{ef}

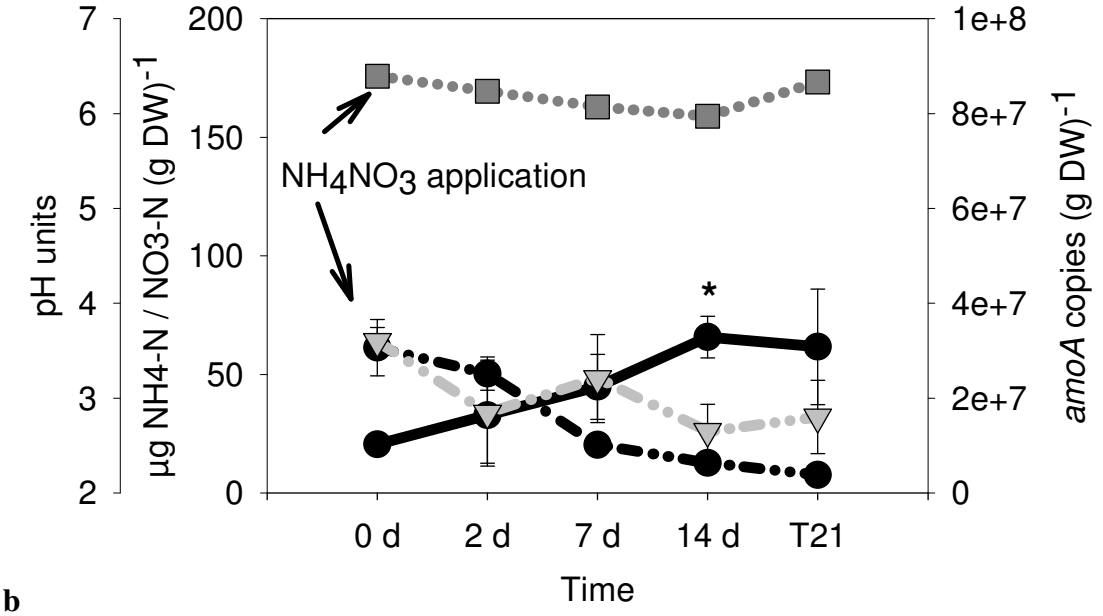
[a] Soils were analyzed immediately after N amendment (day 0) and after 14, 21 and 28 days.

Supporting Information

Figure S1 Dynamics in AOB community size (measured as bacterial *amoA* copy numbers) in relation to soil NH_4-N and NO_3-N concentrations, and soil pH values, following NH_4NO_3 amendment. Barley-planted bulk soils from (a) Purkersdorf and (b) Niederschleinz were repeatedly analyzed immediately after N application (day 0) and at intervals of about 7 days. Symbols represent means (n=3) ± standard errors. Significant differences in AOB abundance between time points are indicated by * ($p<0.05$).


pH units
µg NH4-N / NO3-N (g DW)-1
amoA copies (g DW)-1
NH4NO3 application
*
0 d
2 d
7 d
14 d
21 d
Time

a


pH units
µg NH4-N / NO3-N (g DW)-1
amoA copies (g DW)-1
NH4NO3 application
*
0 d
2 d
7 d
14 d
T21
Time

b

Figure S1

CHAPTER 3

SHORT-TERM CHANGES IN BACTERIAL DENITRIFIER COMMUNITY STRUCTURE AND ABUNDANCE IN SOILS PLANTED WITH BARLEY ARE AFFECTED BY PLANT GROWTH STAGE AND SOIL TYPE BUT NOT BY N FERTILIZATION

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Abstract

Parameters and interactions which influence N conversion in arable soils are still a biotic-black-box. Thus, application of different forms of mineral fertilizer to barley plantlets grown on two selected soils from Lower Austria were tested for denitrifying community changes in greenhouse pot experiment. For testing the influence of the form of applied mineral fertilizer barley var. Morex was grown on two arable soils in the greenhouse. N fertilizer was applied in form of KNO_3, NH_4NO_3 and $[NH_4]_2SO_4$. Samples were collected over a period of three weeks. Furthermore different genotypes of barley combined with KNO_3 or $[NH_4]_2SO_4$ amendments were screened over an elongated time period of four weeks. pH, NO_3, NH_4 contents, plant biomass and nitrate leaching were determined and Terminal Restriction Fragment Length Polymorphism (T-RFLP) analyses of functional genes, encoding enzymes involved in N cycling processes, were performed. Gene abundances were measured using a SYBR green Real-Time PCR assay.

Community structures of denitrifiers were found to differ in soils planted with barley relative to unfertilized and unplanted control soils depending on soil type. Quantitative real-time PCR measurements showed that *nirS* and *nosZ* abundances changed across time but were unaffected by the different fertilizer treatments. Plant age showed to be the decisive factor introducing an increase in both *nirS* and *nosZ* denitrifying communities. Ratios of *nirS* to *nosZ* copy numbers changed across time indicating a shift in potential denitrification end products. Highest yields of plant biomass but also highest NO_3^- leaching rates were detected with the $[NH_4]SO_4$ treatments.

1. Introduction

While it is generally acknowledged that up to 50% of the fertilizer N applied to agricultural soils is not taken up by the plants (FAO, 1995, FAO, 2009), the pathways that the various forms of fertilizer actually take upon application to the field are not fully understood. Denitrification and nitrate leaching are two major pathways of N loss which both have a negative environmental impact, the former enhancing the greenhouse effect and depleting stratospheric ozone, the later leading to the pollution of ground waters. Microbial communities take a significant influence on the fate of N in soils through their activities. Denitrifying bacteria use nitrate as a substrate for the sequential reduction via NO_2 and NO to N_2O and N_2, causing potential environmental hazards by liberating radiatively active gases (Conrad, 1996).

Denitrifiers form a phylogenetically broad group, and not all of them possess the full set of denitrification enzymes along the reductive pathway. Hence, organisms involved in individual denitrification steps may be affected differentially by various fertilization regimes, which have an impact on the amount and species of denitrification product released. Denitrification in soil is influenced by plants through the release of root exudates as carbon sources for denitrifiers, which vary depending on plant species, growth stage, root zone and nutrition, and through root architecture and migration due to effects on soil aeration (Philippot, *et al.*, 2007, Philippot, *et al.*, 2009). Influences of agricultural crops on denitrification rates and N_2O emission via effects on carbon, oxygen and nitrate availability are well documented (Cavigelli & Robertson, 2000, Philippot, *et al.*, 2007, Philippot, *et al.*, 2009), but few studies have so far addressed plant species effects on denitrifying microbial communities (Patra, *et al.*, 2006, Bremer, *et al.*, 2007, Bremer, *et al.*, 2009, Hai, *et al.*, 2009, Ruiz-Rueda, *et al.*, 2009). When analyzing the denitrifying communities in the rhizosphere of different grasses and forbs a change upon plant species due to e.g. root exudates, as well as a time effect connected with

environmental conditions was revealed in a stagnic gleysol (Bremer, *et al.*, 2007). However, the addition of artificial maize root exudates to unplanted soil resulted in changes in potential nitrate reductase and denitrification activities but had no effects on denitrifier community composition or abundance (Henry, *et al.*, 2008). It has been suggested that plant effects on the structural composition of denitrifier communities are brought about primarily through the impact of plant roots on the soil structure during root migration and elongation (Philippot, *et al.*, 2007). In addition, plant effects on bacterial communities seem to strongly depend on the soil type (Clays-Josserand, *et al.*, 1999, Wieland, *et al.*, 2001, Marschner, *et al.*, 2004). Nitrate reducing communities appeared to differ in composition in planted versus unplanted soil in dependence of the soil type (Cheneby, *et al.*, 2003).

The objectives in this study were to determine (1) how different mineral N fertilizers, applied as KNO_3, NH_4NO_3 and $[NH_4]_2\ SO_4$, affect the denitrifying communities in barley planted soil and (2) if plant genotype plays a major role in structuring denitrifier communities. Two successive pot experiments were set up in the greenhouse where barley was grown for three and four weeks, respectively. The first experiment examined N fertilizer effects in two different soils, while the second experiment used two different barley genotypes to study plant effects in the rhizosphere. Abundances and community structures of denitrifying microbial communities was studied by using quantitative real-time PCR and terminal restriction fragment length polymorphism (T-RFLP) analysis of *nirS* and *nosZ* genes. Alongside with molecular microbial analyses, ammonia and nitrate contents were monitored in the soils throughout the study period. Thereby we aimed at describing the temporal dynamics of the soil mineral N pools following fertilizer application together with effects on denitrifier abundance and community composition.

2. Material and Methods

2.1. Sampling sites

The top layers of two arable soils from Purkersdorf (48°15`0``N, 16°0`0``E) and Niederschleinz (48°35`59``N), both situated in Lower Austria, were collected in February 2008 and were transported to the lab on ice, where they were sieved to 2 mm and stored at 4°C until usage. Purkersdorf soil was a cambisol of sandy loamy Flysch material with a pH of 5.7, whereas Niederschleinz soil was a chernozem from loess of silt loam structure with a pH of 7.2. Other soil characteristics are given in Table 1.

2.2. N amendment experiment (experiment 1)

Experiments were set up in the greenhouse in April 2008. For sieved and homogenized Purkersdorf and Niederschleinz soil 300 g were filled into each of 45 one liter-pots per soil type. One barley plant var. Morex was grown per pot, and the pots were uniformly watered when necessary. After 10 days of plant growth 40 mg N per pot were applied with a syringe as either KNO_3, NH_4NO_3 or $[NH_4]_2\ SO_4$ to 15 pots each for each soil type. To prevent nutrient insufficiencies the soils were additionally fertilized with 10 ml of a 37 mM K_2HPO_4 solution. Soils were destructively sampled from three replicate pots per treatment and soil type at five successive time points: directly after fertilization (0d), and two days (2d), one (7d), two (14d) and three weeks (21d) following fertilization. At the first sampling event (0d) unamended and unplanted soils from three replicate pots (no) were sampled as controls in addition to the samples given above. Prior to collecting the soils, the barley plants were carefully removed. The soils from the individual pots were thoroughly mixed and aliquots of 9 and 0.5 g were taken for chemical and molecular microbiological analyses, respectively.

2.3. Genotype experiment (experiment 2)

The second experiment was set up in March 2009, when barley genotypes MR 3/51 (HOR 11371; G1) and Apuro (BCC812; G2) were grown on Purkersdorf soil in the greenhouse. Variety Morex used in experiment 1 was not longer available because of shortage in seeds.

Other than in experiment 1, barley was grown from seeds in little turf pots (5 cm height, 4 cm diameter) containing 20 g of soil; and five days after seed germination 30 plantlets of each barley genotype were transferred together with the turf pots into 1 l pots filled with 300 g of soil each. On the bottom of each pot a polyester fibre bag (app. 12 cm diameter) filled with ion exchange resin (6g of Amberlite, IR 120 and 6g of Dowex 1x8) was placed for measurement of nitrate translocation. After another five days, mineral N fertilizer was applied as either $[NH_4]_2SO_4$ or KNO_3 at a total amount of 40 mg N per pot. To avoid growth deficiencies 10 ml of 37 mM K_2HPO_4 solution (35 mg of K_2O per pot) was added to each pot. Plants were watered uniformly when necessary.

Soil sampling was done destructively from three replicate pots per plant genotype directly after N amendment (0d), and after two weeks (14d), three weeks (21d) and four weeks (28d). Soil was sampled from the rhizosphere contained in the turf pots and the fresh weight of plant shoots and roots was determined for plant biomass measurements.

2.4. Chemical analyses

The soil water content was determined after drying at 105 °C and soil pH was measured by glass electrode in 0.0125M $CaCl_2$. Extracts for NH_4-N and NO_3-N analyses were prepared from fresh soil and were stored at -20°C until further analysis. Extracts for NH_4^+-N analysis were prepared from 2 g aliquots of homogenized soil in 15 ml KCl and analysis of NH_4^+-N was done by using a modified indophenol reaction (Kandeler & Gerber, 1988). NO_3^-N was measured in soil extracts prepared from 5 g soil in 0.0125 M $CaCl_2$ by ion chromatography (Inselsbacher*, et al.*, 2009).

Each ion exchange pad was extracted two times with 100ml 1molar NaCl solution with agitation for 30 min. The filtered extracts were analysed for nitrate as mentioned above.

2.5. Molecular Analyses

DNA extraction

DNA was extracted from 0.5 g of soil by using the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, Ohio, USA) as described by the manufacturer, and was quantified photometrically (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA).

T-RFLP analysis of 16S rRNA, nirS and nosZ gene fragments

Bacterial 16S rRNA genes were PCR amplified using the universal primers 8F (Edwards*, et al.*, 1989) and 1520R (Massol-Deya*, et al.*, 1995). For genes coding for cytochrome cd1 nitrite reductase *(nirS*) the primer pair Cd3aF and R3cd was used (Michotey*, et al.*, 2000, Throbäck*, et al.*, 2004), yielding gene fragments of 425 bp length. Nitrous oxide reductase genes were amplified using the primers *nosZ*F2002 and *nosZ*R2002 (Rösch et al., 2002), yielding 705 bp fragments. All forward primers were 6-carboxyfluorescein (FAM) labeled at the 5´ end.

Amplifications were performed in 25 µl reactions containing 1x buffer, 3 mM $MgCL_2$, 0.2 mM dNTPs, 4%(v/v) DMSO, 1% (w/v) Bovine Serum Albumine (BSA), 1 U of FIREpol Polymerase (Solis BIODYNE, Estonia), and 0.2 µM of each primer for amplification of 16S *rRNA* or *nirS* and 1µM of each primer for *nosZ* amplification. 10 to 100 ng DNA was applied to the individual reactions. Amplifications were performed in a Whatman T1 thermocycler using the following program for *nirS* genes: An initial denaturing step at 95°C for 5 min was followed by 38 cycles of 30s at 95°C, 40s at 58°C, 30s at 72°C and a final extension step at 72°C for 10 min. For amplification of *nirS* genes denaturation at 95°C for 5 min was followed by 35 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C and a final extension step at 72°C for 10 min. The program for 16S *rRNA* gene amplification included 95°C for 5 min

followed by 30 cycles of 95°C for 1min, 53°C for 1 min and 72°C for 2 min, with a final extension step at 72°C for 10 min.

PCR amplified 16S *rRNA* and *nosZ* gene fragments were subjected to T-RFLP analysis using the *Alu*I restriction enzyme, while *Hae*III was used for restriction digestion of *nirS* gene fragments. Choice of restriction enzymes was based on pre-experiments testing three different enzymes (*Rsa*I, *Alu*I, *Hae*III) for 20 cloned sequences of each gene *in silico*. Restriction enzymes giving the greatest resolution were used for further analysis. To reduce bias two PCR products were pooled for each digestion essay. Twenty µl restriction mixtures containing 5 µl PCR product corresponding to app. 200 ng, 1x buffer and 0.5 µl *Hae*III (10 U/µl, Promega) for *nirS* analysis were incubated for 3 h at 37°C.

NosZ PCR products were eluted from gel using the QIAQUICK gel elution kit, eluted in 15µl dH2O and restricted with 1x buffer and 0.5µl *Alu*I (10U/µl, Promega) for 3 h at 37°C. For 16S *rRNA* PCR products the amount of enzyme was doubled.

Digestion batches were purified by passage through DNA grade Sephadex G50 (GE Healthcare) columns. Ten µl of purified product were mixed with 10 µl HiDi-Formamide (Applied Biosystems) and 0.3 µl 500 ROX™ Size Standard (Applied Biosystems) and denatured at 95°C for 2 min. Detection of FAM-labeled terminal restriction fragments was done by capillary electrophoresis using an ABI 3100 automatic DNA sequencer.

Quantification of functional marker genes with qPCR

Q-PCR was performed with SYBR-Green based detection using an iCycler iQ Multicolor Real Time PCR Detection System (BIO-Rad Laboratories) with the same but unlabeled primers as for T-RFLP analysis exept *nosZ* for which primers *nosZ*2F and *nosZ*2R (Henry*, et al.*, 2006) were used. Total reactions of 25 µl were composed of 12.5 µl 2x IQ ™ SYBR®-Green Supermix (BIO-Rad Laboratories), 0.5 µM of each primer for *nirS*, 0.4 µM of each primer for *nosZ*, 2.5% (v/v) DMSO and 2.4% (w/v) BSA. Standards and samples were

processed in triplicates. The thermocycler program for *nirS* was set on 95°C for 3 min, 45 cycles of 95°C for 30s, 58°C (*nirS*) for 35s, 72°C for 45s and data collection at 78°C for 45s. *NosZ* was amplified with the following PCR program: 95°C for 5 min, 6 cycles of 94°C for 30s, 65°C for 45s with decreased setpoint temperature after cycle 2 by 1°C, 72°C for 30s, 40 cycles of 94°C for 30s, 60°C for 45 s and 72°C for 30s (data collection).

Melting curve analysis was done in order to confirm the specificity of the PCR product. To check for inhibition a two times dilution (1:2 to 1:32) series of one representative of each soil was done. No inhibiting effects could be seen for any of the samples. PCR products of each targeted gene were cloned (Strataclone PCR cloning Kit, Stratagene) and sequenced to proof specificity. Plasmids were isolated using the Quantum Prep Plasmid Miniprep Kit (BIO-Rad) and DNA concentrations were determined by spectroscopy to calculate copy numbers. Standard curves from serial dilutions of known amounts of the target genes were generated for each run, showing correlation coefficients (R^2) of 0.994 to 0.999 and PCR efficiencies of 87 to 99%.

Cloning of functional markers

A *nirS* clone library was constructed from the Purkersdorf unplanted unfertilized soil in experiment 2 using StrataClone™ PCR Cloning Kit as described by the manufacturer (Stratagene, Canada). A total of 60 clones were picked, PCR amplified with M13 primers and sequenced by AGOWA (http://www.agowa.de/). Sequences were compared to NCBI database using nucleotide BLAST. Restriction sites with *HaeIII* were determined *in silico* with the CLC Sequence Viewer 5.1.2 (http://www.clcbio.com/).Through alignment and data base comparison repetitive sequences were detected. Clones were related to T-RFLP profiles and one representative clone for each peak that was found to differ significantly when compared with ANOVA is shown in table 2.

2.6. Statistical Analysis

T-RFLPs were transformed into numerical data using GenoScan 3.7 software. To reduce background noise peaks with intensities higher than three times standard deviation were binned and normalized as described by (Abdo*, et al.*, 2006) using the statistical program R together with the filtering and binning macro provided at the IBEST homepage (http://www.ibest.uidaho.edu/tools/trflp_stats/instructions.php). Non-metric multidimensional scaling (MDS) and Analysis of Similarity (ANOSIM) using the Primer 5 statistical program (Primer E, Plymouth, UK) was performed for each T-RFLP data matrix. To check for significant differences in abundances the data set was subjected to ANOVA (Analysis of Variance). Spearman`s correlations were calculated for qPCR data and biochemical parameters.

3. Results

3.1. pH values

Following N fertilization the pH values changed over time in both soils, but changes were more pronounced in Purkersdorf than in Niederschleinz soil. In experiment 1 pH changed after nitrate addition from 6.5 (0 d) to 6.8 (14 d) and 6.7 (21 d) in Purkersdorf soil. Ammonium application resulted in decreasing pH values of 5.6 (21d), while in NH_4NO_3 amended soils the pH first decreased to 6.0 (14 d) and then amounted 6.3 (21d). Soil Niederschleinz showed less variation in pH over time in the various treatments, with most pronounced changes occurring in KNO_3 amended soils from pH 7.7 (0 d) to 7.9 (21d).

In Purkersdorf rhizosphere soil in experiment 2 the pH decreased from 6.4 (0 d) to 5.2 (14 d) followed by an increase to 5.6 (21 d) and 5.9 (28 d) following $[NH_4]_2SO_4$ addition. Upon KNO_3 amendment pH continuously decreased from 6.4 (0 d) to 5.8 (28 d).

3.2. Soil nitrate

In experiment 1 NO_3^--N contents varied considerably over time in both soils. Initial nitrate concentrations in Purkersdorf soil were 9.64 (± 2.82) µg NO_3-N (g DW)$^{-1}$. Following KNO_3 amendment, concentrations were 93.02 (± 12.41) µg NO_3-N (g DW)$^{-1}$, which continuously declined over time to 27.64 (± 9.9) µg NO_3-N (g DW)$^{-1}$ at 21 d. Amendment of NH_4NO_3 resulted in nitrate concentrations of 63.90 µg NO_3-N (g DW)$^{-1}$ at 0 d and 32.05 µg NO_3-N (g DW)$^{-1}$ at 21 d. $[NH_4]_2SO_4$ addition led to increasing nitrate concentrations of 86.08 (± 30.37) µg NO_3-N (g DW)$^{-1}$ two days after the fertilizer had been applied, followed by decreasing values of 27.86 (± 7.92) µg NO_3-N (g DW)$^{-1}$ at 7 d and 12.34 (± 4.91) µg NO_3-N (g DW)$^{-1}$ at 21d.

In Niederschleinz soil the initial nitrate concentration was 22.07 (± 7.51) µg NO_3-N (g DW)$^{-1}$, which increased upon fertilization with KNO_3 and NH_4NO_3 to 116.52 (± 8.38) and 61.39 (± 35.22) µg NO_3-N (g DW)$^{-1}$, respectively. Following KNO_3 amendment soil nitrate decreased from day seven to 44.37 (± 13.35) µg NO_3-N (g DW)$^{-1}$ at 21 d. In NH_4NO_3 amended soil nitrate increased until day seven to 101.21 (± 21.65) µg NO_3-N (g DW)$^{-1}$, followed by decreasing values of 26.61 (± 8.83) µg NO_3-N (g DW)$^{-1}$ at 21 d. In NH_4NO_3 fertilized soil nitrate concentrations increased to 53.93 (± 8.84) µg NO_3-N (g DW)$^{-1}$ at 14 d and were 37.47 (± 8.10) µg NO_3-N (g DW)$^{-1}$at 21 d.

Lower nitrate concentrations were measured in Purkersdorf rhizosphere soil in experiment 2 as compared to experiment 1. Highest levels were detected with Genotype MR 3/51 and Genotype Apuro with 42.07 (±18.12) and 54.84 (±12.95) µg NO_3-N/ g DW soil two hours after KNO_3 amendment. Thus total NO_3-N corresponded to 11.33 and 13.16 mg per pot, respectively, which is not even half of the applied concentration. After two weeks the NO_3-N contents in the KNO_3 treatments decreased to almost zero. In $[NH_4]_2SO_4$ treated soils nitrate concentrations remained stable during the first two weeks and then decreased below the detection limit at days 21 and 28 (Figure 4).

3.3. Soil ammonium

In the beginning of experiment 1, the ammonium concentration in Niederschleinz soil was 7.32 (± 0.57) µg NH_4-N (g DW)$^{-1}$, which remained constant upon KNO_3 addition during the study period. Immediately after $[NH_4]_2SO_4$ and NH_4NO_3 fertilizer application, 126.12 (± 5.62) and 37.82 (± 6.95) µg NH_4-N (g DW)-1 were measured, respectively. Initial NH_4 concentrations were reached at day 14 in the ammonium fertilized pots, whereas in NH_4NO_3 fertilized pots the initial NH_4^+ concentrations were reached already at day 7.

In Purkersdorf soil initial ammonium contents of 7.61 (± 0.34) µg NH_4-N (g DW)$^{-1}$ increased to 111.66 (± 16.72) µg NH_4-N (g DW)$^{-1}$ and 61.29 (± 11.88) µg NH_4-N (g DW)$^{-1}$ immediately

after $[NH_4]_2SO_4$ and NH_4NO_3 application, respectively. Initial NH_4-N concentrations were reached after 3 weeks.

In experiment 2, $[NH_4]_2SO_4$ amended soils contained 151.35 (±60.86) to 173.95 (±42.46) µg NH_4-N g^{-1} DW soil app. two hours after amendment, corresponding to a total of 45 to 52 mg NH_4-N per pot. The original soil contained 15 (±1.06) µg NH_4-N g^{-1} DW before fertilization. NH_4-N decreased continuously to reach values of 4.58 to 5.53 µg NH_4-N/ gDW at 28 d. In the KNO_3 treatment, the initial amount of ammonium of 13.57 (±0.62) to 15.70 (±2.96) µg NH_4-N/ g DW soil decreased over time to final levels of 2.89 (±0.64) to 4.57 (±0.36) µg NH_4-N/ g DW soil.

3.4. Nitrate translocation

Translocation of NO_3-N with the soil solution to ion exchange resins measured in experiment 2 was higher following $[NH_4]_2SO_4$ amendment as compared to KNO_3 amendment. Additionally the barley genotype played a role, since higher concentrations were found with Genotype Apuro than with barley var. MR3/51. Four weeks after $[NH_4]_2SO_4$ addition in total 10.8 and 20.9 mg NO_3-N were translocated onto ion exchange resins with Genotype MR3/15 and Genotype Apuro, respectively; while after KNO_3 amendment 5.5 and 9.9 mg NO_3-N were translocated for each genotype. (Table 3)

3.5. Total bacterial and denitrifier community composition

In experiment 1 and 2 gene fragments of 16S *rRNA*, *nirS* and *nosZ* genes were analyzed in terms of community structure with Terminal Restriction fragment length polymorphism (T-RFLP). In experiment 1 in general for each gene the composition of the community from unfertilized pots (n=3) was compared to profiles derived from the three different treatments KNO_3, NH_4NO_3 and $[NH_4]_2SO_4$ at 0d and 21d (n=3). In experiment 2 four different

treatments, namely (1) Genotype MR3/51 + $[NH_4]_2SO_4$, (2) Genotype MR3/51+ KNO_3, (3) Genotype Apuro + $[NH_4]2SO_4$, and (4) Genotype Apuro + KNO_3, were compared directly after fertilization (0d) and 28d days upon N amendment. Binned and normalized data matrixes were subjected to ANOVA and ANOSIM tests as well as MDS plots were calculated.

In experiment 1 T-RFLP profiles of Niederschleinz and Purkersdorf soil showed a total of 21 and 31 major T-RFs for 16S *rRNA*, respectively. In both soils MDS plots revealed a separation across time supported by low stress values (0.08 and 0.04) but gave low global R values (0.165 and 0.231, respectively) using ANOSIM test.

The time effect was also present in 16 S *rRNA* communities from rhizosphere soil of Purkersdorf in experiment 2 consisting of a total of 30 T-RFs. Even though T-RFs were not so well clustered in the MDS plots (stress 0.12) ANOSIM analysis showed highly significant global R values (0.709; p=0.000) indicating a community change across time (Fig.3).

In experiment 1 T-RFLP profils derived from *HaeIII* restricted *nirS* gene fragments contained 17 and 10 T-RFs for Niederschleinz and Purkersdorf soil, respectively. Samples of Niederschleinz soil clustered in the MDS plot (stress 0.06) according to time but gave a low global R value of 0.034 using ANOSIM test. In MDS plot (stress 0.04) from Purkersdorf soil derived from *nirS* T-RF data, unplanted/unfertilized samples clustered separately from the other samples. Eventhough global R values were low (0.195) subjecting the data to ANOVA Duncan posthoc test significant differences were found with several OTUs. Figure 2 shows relative peak areas of *nirS* –T-RFs (%) with different letters indicating differences at $p < 0.01$.

When focusing on the rhizosphere in experiment 2 again a time dependent change was detected for *nirS* communities. The overall data matrix consisted of 14 major T-RFs. Samples clustered according to time when subjected to MDS analysis (stress 0.05) supported by highly significant global R values (R=0.635; p=0.000).

NosZ community profiles detected 15 T-RFs for Niederschleinz soil and 22 T-RFs for Purkersdorf soil in experiment 1. Data derived from *nosZ* T-RFLPs with both soils scattered

randomly in the MDS plots showing also higher stress values (0.14 and 0.12) and low global R values with ANOSIM tests (0.168 and 0.310). Thus neither treatment effects nor significant changes across time were determined for *nosZ* denitrifying community patterns (Fig.1).

In experiment 2 on the other hand, a significant change across time was detected for *nosZ* communities in Purkersdorf soil. Data derived from *NosZ* T-RFs were well separated across time in the MDS plot supported by stress values of 0.05 and highly significant global R values (0.825; p=0.000).

3.6. NirS clone libraries

NirS clone libraries were constructed for experiment 1 Purkersdorf soil unplanted /unfertilized sample (Pno) as well as for a pool of 0d samples from each KNO_3, $[NH_4]_2SO_4$ and NH_4NO_3 amendment (P0d). From each clone library 30 clones were randomly picked and sequenced. All 60 sequenced clone were found to show high score similarities to *nirS* genes when compared to NCBI database. Sequences that could be related to changes in the *nirS* community profiles (Fig.2) between planted and unplanted soils e.i. T-RFs 75, 137, 259, and 363 are shown in table 2. No matching sequence was found for T-RF 274. Up to six base pair difference between length of T-RF in profil and T-RF in the corresponding sequence where considered the same phylotype. Selected sequences related to T-RFs showed 83 to 99 % similarties with uncultured *nirS* genes (Table 2). When different sequences showed same terminal restriction site, more than one sequence is listed, e.g. three different sequences were found to possess T-RF 137 but showed different matches with database derived sequences.

3.5. Functional gene abundances

In experiment 1 copy numbers in Purkersdorf soil ranged from 8.78×10^7 ($\pm 2.72 \times 10^7$) to 2.46×10^8 ($\pm 1.60 \times 10^8$) *nirS* copies $(g\ DW)^{-1}$ and 1.54×10^8 ($\pm 1.44 \times 10^7$) to 7.99×10^8 ($\pm$

2.97 x 10^8) *nosZ* copies (g DW)$^{-1}$. In samples from Niederschleinz soil 5.65 x 10^7 (1.21 x 10^7) to 1.26 x 10^8 (± 7.91 x 10^7) *nirS* copies (g DW)$^{-1}$ and 5.80 x 10^7 (± 1.05 x 10^7) to 1.29 x 10^8 (± 2.94 x 10^7) *nosZ* copies (g DW)$^{-1}$ were determined. Significant ($P<0.05$) lower *nirS* copy numbers were only seen for NH_4NO_3 treatment in Niederschleinz soil at 7d compared to the other time points.

In experiment 2 subjecting rhizosphere soil from site Purkersdorf, *nirS* gene copy numbers reached from 3.55x10^7 (±7.92x10^6) to 1.14x10^8 (±2.96x10^7) per g DW. In most cases, samples taken at 21d and 28d had significantly higher abundances than at 0d and 14d independent which treatment. That corresponds to up to 3.21 times more copies of *nirS* that were detected at the third and fourth sampling (Fig. 4).

Abundance of *nosZ* gene copy numbers ranged from 1.48x10^7 (±6.23x10^5) to 2.80x10^8 (±1.76x10^7) showing up to 18.92 –fold higher copy numbers at the 21d and 28d sampling occasions (Figure 4). Ratios between the *nirS* and *nosZ* genes changed arcoss time. At 0d and 14d *nirS* genes were up to 1.8 times more abundant in all treatments, whereas ratios of *nosZ:nirS* increased at 21d and 28d to finally *nosZ* overtake *nirS* up to 2.6-fold.

NirS and *nosZ* copy numbers correlated positively to plant biomass (R= 0.661 and 0.630; $p\leq0.01$). Negative correlations were found to NO_3-N (R= -0.560 and -0.633; $p\leq0.01$), NH_4-N (R= -0.576 and -0.631; $p\leq0.01$) and soil pH (R= -0.370 and -0.290; $p\leq0.01$)

3.6. Plant biomass and plant N content

Shoot biomass of the barley plants was higher following $[NH_4]_2SO_4$ than KNO_3 fertilizer application. Barley var. MR 5/31 yielded almost two times more shoot biomass in the $[NH_4]_2SO_4$ versus KNO_3 treatment, while differences among fertilizer treatments were less pronounced in barley var. Apuro. Barley var. MR 5/31 yielded more shoot biomass than var. Apuro in both fertilization regimes (Fig. 4).

Similar to shoot biomass, barley var. MR 3/51 treated with $[NH_4]_2SO_4$ had with 3.28 (± 0.24) % significantly ($p>0.01$) more N in the plant, than the samples amended with KNO_3 (1.49 ± 0.18 %). This effect again was not significant with Var. Arupo (G2), where the N contents upon $[NH_4]_2SO_4$ and KNO_3 were 2.73 (± 0.96) and 2.39 (±0.96) %, respectively.

4. Discussion

Mineral N fertilizer effects on bulk soil denitrifiers

Effects of mineral nitrogen (N) fertilizer application on denitrifier community composition and abundance were studied in two successive pot experiments using barley-planted soils. In the first experiment, barley var. Morex was grown on two different agricultural soils, Purkersdorf and Niederschleinz. Total bacterial communities as well as their denitrifying subsets differed in composition and abundances in these two soils, which were contrasted in clay content, soil C and N, C to N ratios and pH. Following application of various forms of mineral N fertilizer, neither abundances nor community structures of denitrifiers were significantly changed in spite of changing mineral N pool sizes that were indicative of soil N processes. Copy numbers of *nirS* and *nosZ* genes were six and three times higher in Purkersdorf versus Niederschleinz soil but remained constant in both soils during three weeks following either KNO_3, $[NH_4]_2SO_4$ or NH_4NO_3 fertilization; and also *nirS* and *nosZ* denitrifier community composition remained unaffected. Previously, a high resistance of *narG* denitrifying communities towards short–term fluctuations in soil nitrate content was seen in glacier foreland grassland soils (Deiglmayr*, et al.*, 2006). *NirK* structures in a silt loam soil were changed only after four weeks upon NH_4-N addition (Avrahami*, et al.*, 2002), suggesting that in the present study *nirS*-based community changes might have been visible during an extended study period, although *nirS* and *nirK* homologues may show diverging reactions to N amendments.

However, in long term experiments involving the combined use of organic and mineral N fertilizers for seven to 50 years, changes in the structures and activities of *narG*, *nirK, nirS* and *nosZ* denitrifier communities occurred (Dambreville*, et al.*, 2006, Hallin*, et al.*, 2009). These changes were largely attributed to pH effects alongside with changes in organic matter and nutrient pools, and to the introduction of external microbial communities with the pig

manure (Dambreville*, et al.*, 2006). PH effects were also held responsible for changes in *narG* and *nosZ* gene diversity after $(NH_4)_2SO_4$ and sewage sludge treatment of a clay loam soil (Enwall*, et al.*, 2005). Addition of mineral N fertilizer alone apparently had no major impact on the *nirS* and *nosZ* denitrifier communities in the present experiment, even though soil pH decreased by 0.9 units in Purkersdorf soil following NH_4 application. However, it seems that bacterial community members other than denitrifiers were affected by the treatments or changed over time because 16S rRNA gene profiles were shifted at day 21 following N amendments as compared with the profiles before fertilizer addition (Fig.1).

The only community that was distinct among the various treatments was that of *nirS* denitrifiers in unplanted and unfertilized control soil from Purkersdorf, supposedly reflecting an influence of the presence or absence of plants on denitrifier community composition (Fig.2, Table 2). *NosZ* communities, by contrast, showed no plant-related effects. No differential effects were seen on denitrifier communities in unplanted control versus fertilizer treated barley-planted soils from Niederschleinz. Since the analyses in this experiment were performed on aliquots of the homogenized bulk soils contained in the pots, effects such as root exudation that take place in close proximity to plant roots could not be adequately addressed. In a similar way, no differences in *nosZ* and *narG* community patterns were found between cropped and uncropped plots in a long term fertilization experiment where bulk soil samples were analyzed (Enwall*, et al.*, 2005).

Plant-associated effects on denitrifier communities during mineral N fertilization

In a second experiment *nirS* and *nosZ* denitrifier communities in the barley rhizosphere were specifically targeted by analyzing soil from the root environment that was enclosed in small turf “pre-growth” pots. Two different barley cultivars, MR 3/51 and Apuro, were grown on Purkersdorf soil that was amended with mineral N in the form of either KNO_3 or $[NH_4]_2SO_4$. As compared with the first experiment, almost one order of magnitude lower numbers of *nirS*

and *nosZ* gene copies were contained in the rhizospheres prior to fertilization. Longer storage of the soil until the start of the experiment may play a role in decreased copy numbers.

However, in contrast to the measurements in the bulk soil samples, significant increases in *nirS* and *nosZ* denitrifier abundances were seen in the barley rhizospheres on day 21 after both fertilizer treatments, with unchanging or slightly decreasing copy numbers on day 28 (Fig.4). Higher bacterial numbers and denitrifying activities in the rhizosphere versus bulk soil have commonly been observed as a consequence of nutrient provision through plant root exudation (Cheneby*, et al.*, 2004, Philippot*, et al.*, 2009).

During the four week-study period barley biomass increased from an overall average of 0.3 to 4.4 g in parallel with increasing *nirS* and *nosZ* copy numbers (Fig. 4). Together with the observations from the first experiment this suggests that the plant developmental stage was critical for denitrifier abundance in the rhizosphere, probably through effects such as the growth-dependent release of C rich microbial substrates from plant roots and root effects on soil structure. Alongside with increasing denitrifier abundance, soil nitrate in the rhizosphere got continuously and rapidly depleted following N application, indicating denitrification activity as well as efficient N plant uptake (Fig.4). In the bulk-dominated soils of the first experiment, by contrast, no changes in denitrifier abundance occurred and nitrate concentrations were highly variable and eventually increased in ammonia amended soils, most likely due to nitrification (Table I supplement).

In addition to denitrification gene abundances, also the structures of the *nirS* and *nosZ* communities were changed in the barley rhizospheres following both N fertilizer treatments (Fig. 3), while no such effects were seen in the bulk soils of the first experiment. Thus, it seems that plant-associated effects such as the release of exudates and root effects on the soil structure were decisive for shaping the denitrifier communities in response to increased N availability. Root exudates have previously shown to exert a selective influence on bacterial community composition (Hartmann*, et al.*, 2009) and effects of plant age on microbial

communities via carbon release into the soil have often been reported (Nguyen, 2003, Jones*, et al.*, 2004, Jones*, et al.*, 2009). The plant developmental stage had a major influence on the size of N cycling communities in an inoculation study involving leguminous plants (Babic*, et al.*, 2008). Similar to our findings an increase of *nirS* and *nosZ* copy numbers was found with time due to supposingly changes in root exudation, when the early trifoliate stage was compared to early bud development and late flower stage.

In the rhizosphere multiple interwoven factors exert an influence on denitrifier composition and activity. These include not only nitrate and carbon supply and oxygen partial pressure but also chemical and physical soil qualities and root migration, which modifies soil compaction and creates microsites for denitrification (Philippot*, et al.*, 2007). In addition, rhizosphere effects on microbial community composition were found to strongly depend on the soil type (Clays-Josserand*, et al.*, 1999, Wieland*, et al.*, 2001, Marschner*, et al.*, 2004, Costa*, et al.*, 2006). This may explain why the presence of plants seemed to affect *nirS* communities in Purkersdorf but not in Niederschleinz soil in the first experiment.

Prior to N amendment, *nirS* and *nosZ* gene abundances did not differ in the rhizospheres of the two barley cultivars MR 3/51 and Apuro, and no difference was seen in the structural composition of the denitrifier communities. Plant species and cultivar effects on denitrification activities have previously been described (e.g. Philippot et al, 2007, Sharma et al, 2005), but no such effects were seen on denitrifier diversity and community composition (Deiglmayr*, et al.*, 2004, Sharma*, et al.*, 2005). The two barley cultivars used in the present study exhibited a similar root architecture, and hence as the role of root structure mentioned above a corresponding performance regarding soil N cycling may be expected (Philippot*, et al.*, 2007). Nevertheless, divergent responses to the different fertilization regimes were seen in that peak *nosZ* copy numbers were measured with cultivar MR 3/51 following KNO_3 and with cultivar Apuro following $[NH_4]_2SO_4$ amendment, respectively. Communities seem to react

fast upon changes in substrate concentrations and peaks may have been missed, thus a higher sampling resolution covering more time points should be considered for further experiments. While the rhizospheres of both barley cultivars contained three times more *nirS* than *nosZ* copies at the beginning of the experiment, this ratio was inverted when *nosZ* copies outnumbered *nirS* copies up to 2.1 times on day 21 following KNO_3 and $[NH_4]_2SO_4$ amendment. It is a common belief that most denitrifiers do not reduce nitrous oxide to N_2 in complete denitrification as long as other N substrates are available for reduction that yield more energy and induction of *nosZ* has been observed in denitrifier isolates upon accumulation of nitric oxide (Arai*, et al.*, 2003). Hence, peak *nosZ* numbers in the present study may reflect continuous denitrification activity, resulting in the depletion of nitrate and nitrite as substrates for denitrifiers together with NO accumulation. It is noteworthy that N_2 was previously identified as the predominant denitrification product close to the roots in soils planted with barley, and that denitrification rates correlated positively with root biomass (Klemedtsson*, et al.*, 1986). However, other studies have reported an increase in the *nirS*+*nirK* to *nosZ* ratio with increasing plant growth (Sharma*, et al.*, 2004, Babic*, et al.*, 2008).

Plant biomass and denitrifier abundance

Higher plant biomass was yielded in consequence of $[NH_4]_2SO_4$ versus KNO_3 fertilizer application to barley var. MR 3/51 plants (Fig. 4). With Genotype Apuro, which in general produced less shoot biomass than MR 3/51, this N amendment effect was also seen with but to a minor extend. Together with enhanced biomass, also the N contents were elevated upon $[NH_4]_2SO_4$ treatment. Since preferential uptake of NO_3 over NH_4 was previously seen in the barley cultivar Morex used in the first experiment of this study (Inselsbacher*, et al.*, 2009), it may be concluded that nitrate provided through nitrification of the added ammonia was more conducive to plant growth than a one-time application of nitrate fertilizer. In support of this,

enhanced growth of wheat and higher microbial activities in the rhizosphere were observed following NH_4-N application when nitrification was taking place transforming NH_4-N into nitrate compared to nitrification inhibited conditions or direct nitrate supply (Mahmood*, et al.*, 2005).

While $[NH_4]_2SO_4$ versus KNO_3 fertilizer application yielded higher plant biomass, it also resulted in higher availability of nitrate that is prone to denitrification and leaching from the soil. Although denitrification processes were not measured in the present study, numbers of *nirS* and *nosZ* gene copies did not differ due to the fertilization regimes applied suggesting similar denitrification rates. However, following ammonia versus nitrate amendment higher amounts of nitrate were translocated through the soil onto ion exchange resins (Table 3), indicating higher potential leaching losses into deeper soil layers and to ground waters.

Concluding, structure and abundance of denitrifier communities based on *nirS* and *nosZ* gene analysis was mainly affected by plant presents and plant age depending on soil type, thus nitrate addition was not a modulating factor for the denitrifying communities. Denitrifying (*nirS*) bacterial guilds in fertilized Purkersdorf soils differed significantly from those in unplanted and unfertilized control soils, indicating a plant associated effect probably due to root exudation. Objecting different cultivars of barley in rhizosphere soil an increase in denitrifier abundance associated with plant biomass independent of genotype was discovered proposing a plant age effect rather than a genotype related community change. Also a ratio change *nirS/nosZ* across time indicates a shift in denitrification end products. A delayed nitrate supply due to nitrification was favorable for plant biomass yield but led to increased nitrate leaching stretching the importance of the time point of fertilizer application.

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Table 1 Site characteristics and physical and chemical properties of two soils (0-20 cm soil layer) collected in Purkersdorf and Niederschleinz located in the vicinity of Vienna in Lower Austria

	Purkersdorf	**Niederschleinz**
Soil type	Gleyic Cambisol from sandy loamy flysch	Chernozem from Loess
Geographic site	48°12'25'' N 16°10'37'' E	48°35'59'' N 15°10'24'' E
Altitude (m. a. sl.)	248	244
Management	Winter barley	Arable field
Water condition	Moist	Moderately dry
Clay (%)	1.7	17.7
Silt (%)	64.9	74.2
Sand (%)	33.4	8
Total C (mg C g^{-1} DW)	16.2	26.4
Total N (mg N g^{-1} DW)	1.63	1.86
C/N	9.96	14.17
pH (KCl)	5.67	7.15
$CaCO_3$ (%)	0.06	8.5
Exchange capacity (mval%)	11.2	15.4
Base saturation (%EC)	81.4	98.1
Bulk density (g DW ml^{-1})	1.06	0.96

Table 2 Results of selected sequences compared with NCBI database of clones derived from soil Purkersdorf *nirS* unplanted/ unfertilized (Pno) corresponding to T-RFs that differed significantly to planted samples (P0d)

Clone no (length bp)	T-RF in sequence	T-RF in profile	Closest match to NCBI database	Similarity (query coverage) [%]	Reference
Cl 5 P0d (410)	79	75	Uncultured bacterium clone S1m_nirS-22 NirS (nirS) gene, partial cds DQ337883.1	85 (99)	Kim,O.-S., Junier,P., Imhoff,J. and Witzel,K.-P. Comparison of denitrifying bacterial communities in the water column and sediment of two lakes and the Baltic Sea. Unpublished
Cl 38 P0d (413)	79	75	Uncultured bacterium clone S9-N-40 putative dissimilatory reductase (nirS) gene, partial cds EU235898.1	83 (84)	Dang,H. and Wang,J. Diversity and spatial distribution of sediment NirS-type denitrifying communities in response to environmental gradients in Changjiang Estuary and East China Sea. Unpublished
Cl 11 P0d (409)	136	137	Uncultured bacterium clone DGGE band AS7K nitrite reductase (nirS) gene, partial cds AY583421.1	99 (99)	Throback,I.N., Enwall,K., Jarvis,A. and Hallin,S. Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol. Ecol. 49 (3), 401-417 (2004)
Cl 32 P0d (409)	136	137	Uncultured bacterium clone M-nirS-1 nitrite reductase-like (nirS) gene, partial sequence. EU650277.1	91 (97)	Orlando,J., Espinosa,F., Braker,G. and Caru,M. Composition of the bacterial community, nitrifiers and denitrifiers from the semiarid soil of the Chilean sclerophyllous matorral. Unpublished
Cl 34 P0d (410)	136	137	Uncultured bacterium clone DGGE band BS3J nitrite reductase (nirS) gene, partial cds. AY583430.1	91 (99)	Throback,I.N., Enwall,K., Jarvis,A. and Hallin,S. Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol. Ecol. 49 (3), 401-417 (2004)
Cl 3 P0d (410)	253	259	Uncultured bacterium 2303 genomic sequence EU910857.1	99 (99)	Demaneche,S., Philippot,L., David,M.M., Navarro,E., Vogel,T.M. and Simonet,P. Characterization of denitrification gene clusters of soil bacteria via a metagenomic approach. Appl. Environ. Microbiol. 75 (2), 534-537 (2009)
Cl 3 Pno (409)	368	363	Uncultured bacterium clone S12m_nirS-13 nitrite reductase (nirS) gene, partial cds EF615479.1	88 (98)	Kim,O.-S., Junier,P., Imhoff,J.F. and Witzel,K.-P. Widely and differently distributed nitrite reductase (nirK and nirS) genes in the water column and sediment-water interface of two lakes and the Baltic Sea. Unpublished

Table 3 Translocation of NO_3^- Ions

Genotype	Treatment	day 0	14 days	21 days	28 days
HOR 11371	NO_3	3.7 (±0.9)^{d}	3.6 (±0.8)^{d}	4.0 (±0.7)^{d}	5.5 (±0.7)^{cd}
	NH_4	3.3 (±0.5)^{cd}	5.2 (±0.3)^{d}	11.1 (±4.5)^{bcd}	10.8 (±0.7)^{b}
BCC812	NO_3	5.0 (±0.8)^{d}	7.5 (±3.2)^{bcd}	5.8 (±2.0)^{cd}	9.9 (±6.0)^{bcd}
	NH_4	3.6 (±1.3)^{d}	13.8 (±2.7)^{abc}	9.0 (±1.8)^{bcd}	20.9 (±1.7)^{a}

Different letters indicate significant differences at 0.001 level with One-way ANOVA using Duncan similarity test; NO_3 (=KNO_3) and NH_4 (=$[NH_4]_2SO_4$) indicate the fertilization regime, whereas + stands for inoculation with *B. phytofirmans* strain PsJN. Day 0 = day of fertilization

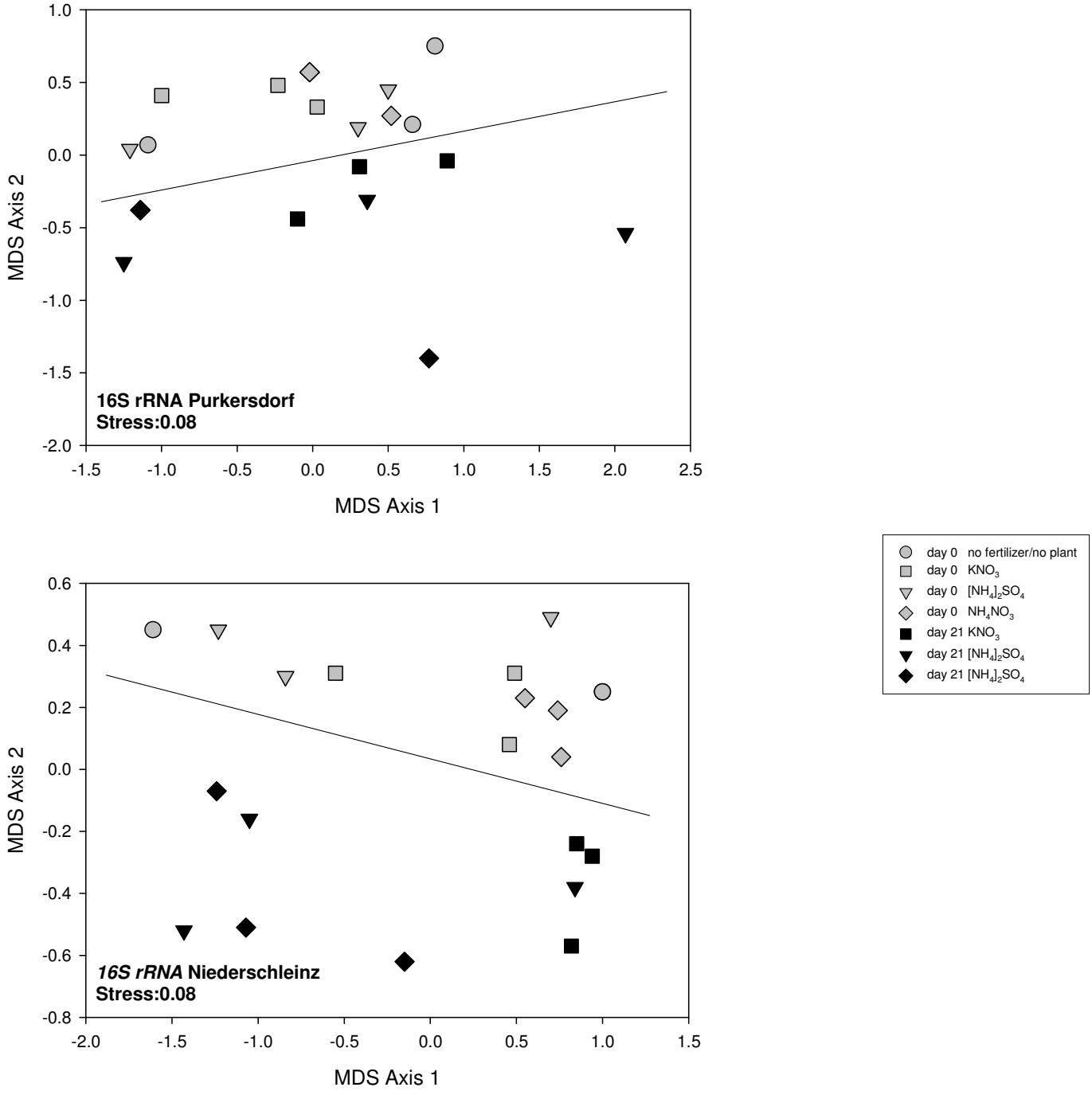
6. Figure legends

Figure 1 *Experiment 1*: MDS plots derived from T-RFLP data of *AluI* restricted 16S *rRNA* gene fragments of Purkersdorf and Niederschleinz soil. Samples without plant and no fertilizer (●); with $[NH_4]_2SO_4$ application (▼); NH_4NO_3 application (♦) or KNO_3 application (■) were analyzed at day 0 (grey symbols) and day 21 (black symbols) (n=3).

Figure 2 *Experiment 1*: Relative abundances (%) of T-RFs peak areas derived from restriction with Hae*III* of *nirS* gene fragments from Purkersdorf soil. Means ± standard errors of triplicate T-RF peak areas are shown from treatments without plant and fertilizer (no), directly after either $[NH_4]_2SO_4$, NH_4NO_3 or KNO_3 amendment (0d) and after 21 (21d) days (n=3);different letters indicating significant differences at $P<0.01$.

Figure 3 *Experiment 2*: MDS plots derived from normalized and binned data sets of (a)16S *rRNA*, (b) *nirS* and (c) *nosZ* T-RFLP analysis comparing samples from treatments Genotype MR 3/51 KNO_3 (♦) and $[NH_4]_2SO_4$ (■); Genotype Apuro KNO_3 (▼) and $[NH_4]_2SO_4$ (▲) directly after fertilization (day 0; symbols in grey) with samples from the last sampling point (day 28; symbols in black) in Purkersdorf soil (n=3).

Figure 4 *Experiment 2*: Dynamics of plant biomass, NO_3-N and *nirS/nosZ* copy numbers in the rhizosphere of Purkersdorf soil **(a)** Genotype MR3/51 KNO_3; **(b)** Genotype MR3/51 $[NH_4]_2SO_4$ **(c)** Genotype Apuro KNO_3 **(d)** Genotype Apuro $[NH_4]_2SO_4$; directly after application (0d), 14 (14d), 21 (21d) and 28days (28d) . Plots show means ± standard errors of triplicate samples (n=3). Significant differences at the 0.05 and 0.01 level are labeled with * and **, respectively


MDS Axis 2
MDS Axis 1
16S rRNA Purkersdorf
Stress:0.08
16S rRNA Niederschleinz
Stress:0.08
day 0 no fertilizer/no plant
day 0 KNO3
day 0 [NH4]2SO4
day 0 NH4NO3
day 21 KNO3
day 21 [NH4]2SO4
day 21 [NH4]2SO4

(a)

Figure 1

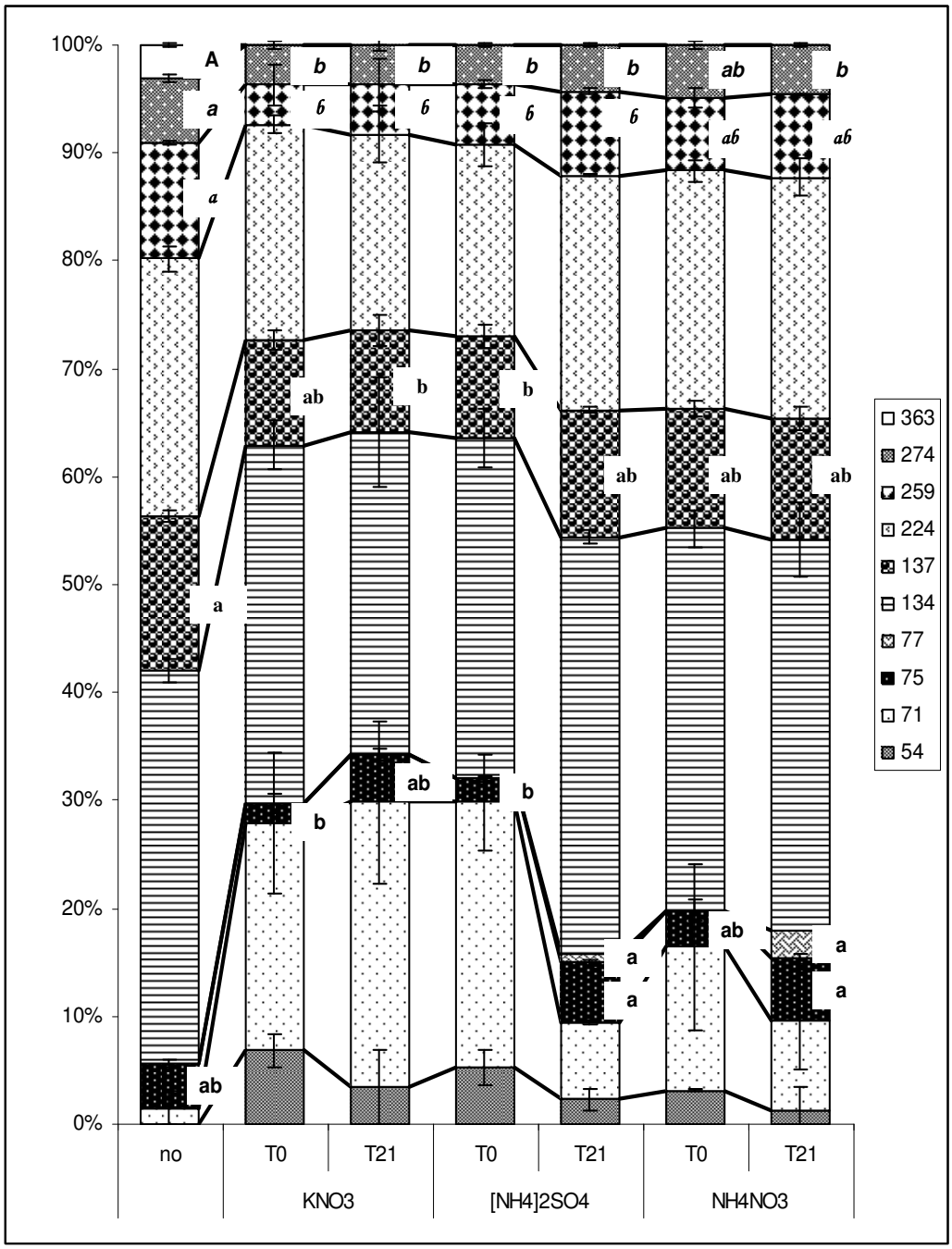
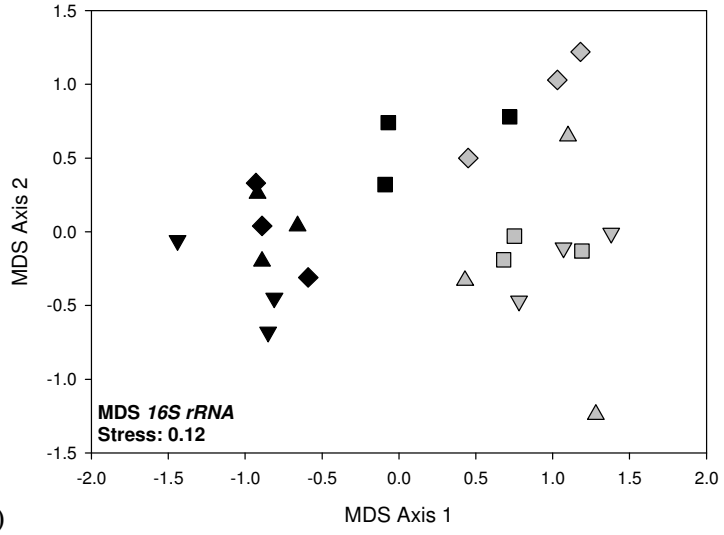
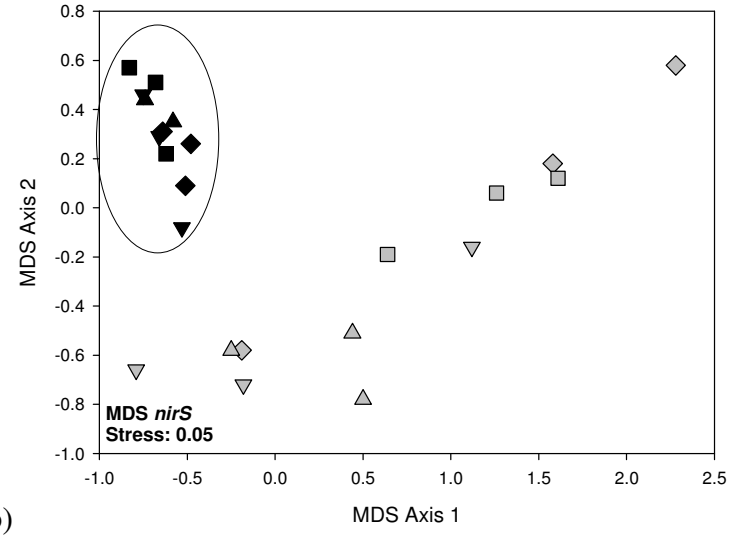

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
363
274
259
224
137
134
77
75
71
54
no
T0
T21
T0
T21
T0
T21
KNO3
[NH4]2SO4
NH4NO3

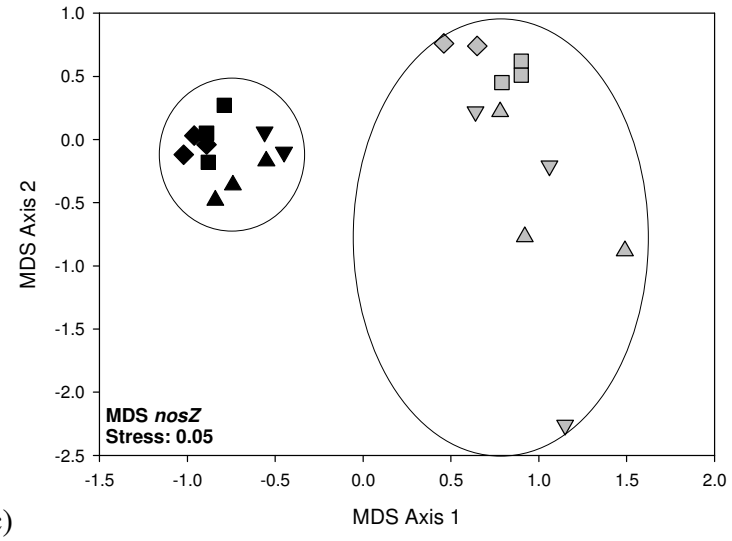
Figure 2


MDS Axis 2
MDS Axis 1
MDS *16S rRNA*
Stress: 0.12

(a)


MDS Axis 2
MDS Axis 1
MDS *nirS*
Stress: 0.05

(b)


MDS Axis 2
MDS Axis 1
MDS *nosZ*
Stress: 0.05

(c)

Figure 3

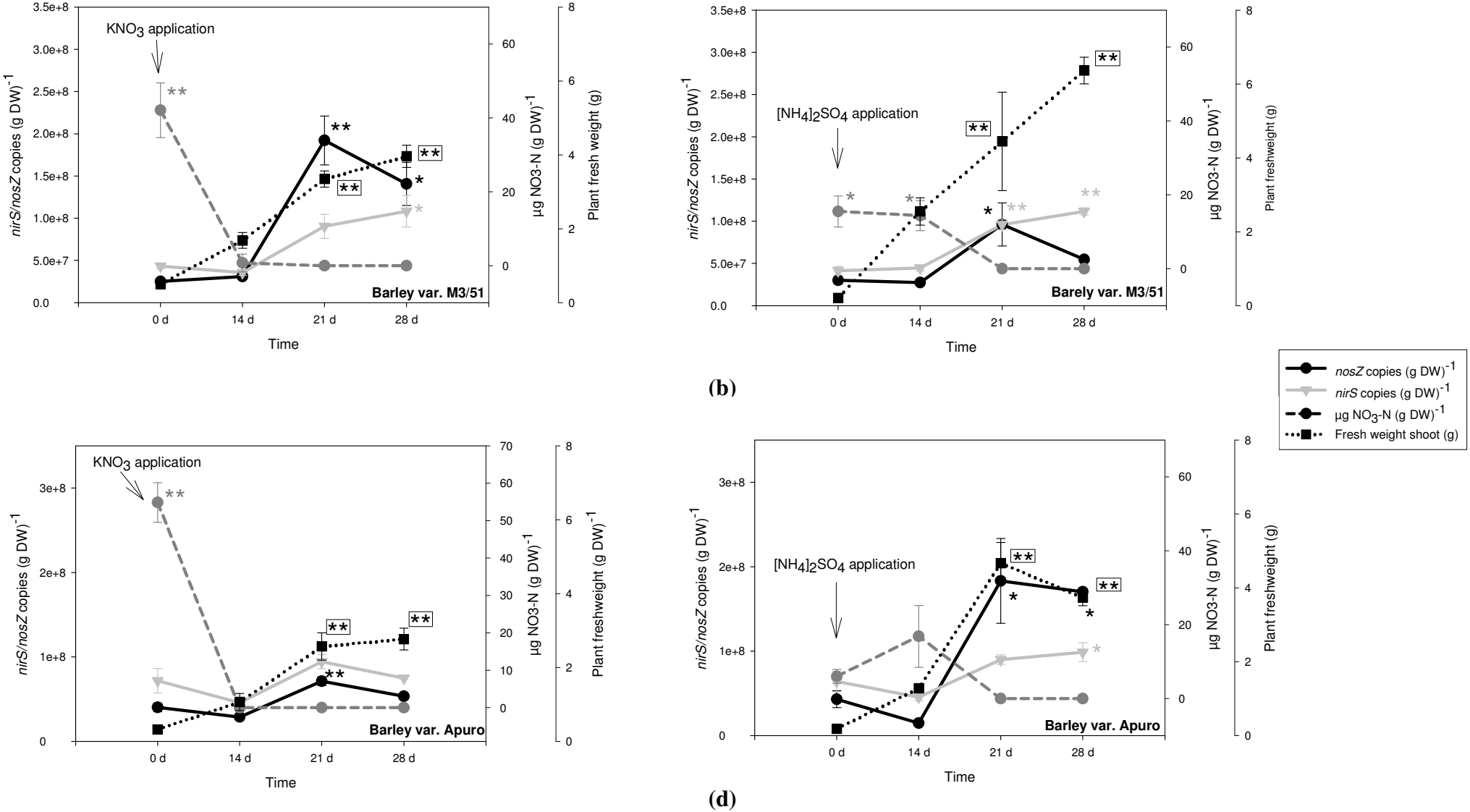

KNO3 application
nirS/nosZ copies (g DW)-1
µg NO3-N (g DW)-1
Plant fresh weight (g)
Barley var. M3/51
Time
0 d
14 d
21 d
28 d
(a)
[NH4]2SO4 application
Barely var. M3/51
(b)
Barley var. Apuro
(c)
(d)
nosZ copies (g DW)-1
nirS copies (g DW)-1
µg NO3-N (g DW)-1
Fresh weight shoot (g)

Figure 4

Supporting Information

Supplement Table I Experiment 1: Copy numbers of functional gene fragements in (g DW soil)$^{-1}$of Nitrous oxide reductase (nosZ) (1) and Chytochrome cd1 containing Nitrite reducatase (nirS) (2) in soils treated with KNO_3, $[NH_4]2SO_4$ and NH_4NO_3 derived from sites Purkersdorf and Niederschleinz. Different letters indicate differences inbetween one treatment at p<0.05.with DUNCAN post-hoc test

		Niederschleinz						*Purkersdorf*					
gene copies g (DW)$^{-1}$	time	**KNO_3**		**NH_4NO_3**		**$[NH_4]_2SO_4$**		**KNO_3**		**NH_4NO_3**		**$[NH_4]_2SO_4$**	
(1) nosZ	no	9.09E+07	(±2.15E+07)^{a}					5.79E+08	(±1.27E+08)^{a}				
	0d	5.93E+07	(±2.78E+06)^{a}	1.12E+08	(±1.91E+07)^{a}	1.03E+08	(±6.61E+07)^{a}	6.33E+08	(±1.73E+08)^{a}	7.72E+08	(±3.58E+08)^{a}	3.28E+08	(±5.22E+07)^{a}
	2d	7.06E+07	(±8.92E+06)^{a}	1.01E+08	(±2.84E+07)^{a}	7.50E+07	(±4.54E+07)^{a}	6.51E+08	(±1.60E+08)^{a}	6.81E+08	(±3.06E+08)^{a}	5.62E+08	(±1.99E+08)^{a}
	7d	6.24E+07	(±9.11E+06)^{a}	8.56E+07	(±2.63E+07)^{a}	5.80E+07	(±1.05E+07)^{a}	7.99E+08	(±2.97E+08)^{a}	5.98E+08	(±7.03E+07)^{a}	1.54E+08	(±1.44E+07)^{a}
	14d	1.29E+08	(±2.94E+07)^{a}	1.28E+08	(±4.33E+07)^{a}	1.11E+08	(±3.65E+07)^{a}	6.55E+08	(±2.81E+08)^{a}	6.41E+08	(±7.27E+07)^{a}	4.95E+08	(±3.25E+08)^{a}
	21d	1.19E+08	(±4.53E+07)^{a}	1.33E+08	(±3.59E+07)^{a}	9.04E+07	(±3.64E+07)^{a}	5.42E+08	(±1.84E+07)^{a}	5.38E+08	(±1.10E+08)^{a}	2.93E+08	(±1.13E+08)^{a}
(2) nirS	no	6.46E+07	(±4.89E+06)^{a}					1.63E+08	(±9.29E+06)^{a}				
	0d	5.66E+07	(±8.84E+06)^{a}	7.19E+07	(±1.67E+07)^{ab}	1.26E+08	(±7.91E+07)^{a}	1.51E+08	(±3.02E+07)^{a}	2.28E+08	(±1.21E+08)^{a}	1.45E+08	(±1.05E+07)^{a}
	2d	8.37E+07	(±2.18E+07)^{a}	8.89E+07	(±2.74E+07)^{a}	9.33E+07	(±5.58E+07)^{a}	1.61E+08	(±3.02E+07)^{a}	1.81E+08	(±7.11E+07)^{a}	2.28E+08	(±3.40E+07)^{a}
	7d	7.41E+07	(±1.69E+07)^{a}	5.65E+07	(±1.21E+07)^{b}	6.22E+07	(±1.63E+07)^{a}	1.39E+08	(±1.52E+07)^{a}	1.72E+08	(±3.54E+07)^{a}	2.46E+08	(±1.60E+08)^{a}
	14d	9.62E+07	(±2.21E+07)^{a}	6.92E+07	(±2.08E+07)^{ab}	1.25E+08	(±3.38E+07)^{a}	2.20E+08	(±2.42E+07)^{a}	1.72E+08	(±1.88E+07)^{a}	1.92E+08	(±1.88E+08)^{a}
	21d	8.63E+07	(±3.35E+07)^{a}	9.06E+07	(±2.01E+06)^{a}	9.39E+07	(±3.87E+07)^{a}	1.24E+08	(±1.94E+07)^{a}	1.29E+08	(±1.83E+07)^{a}	8.78E+07	(±2.72E+07)^{a}

Supporting Information

Supplement Table II Experiment 2: Copy numbers of functional gene fragements in (g DW soil)$^{-1}$ of (1) *nirS* and (2) *nosZ* copy in soils treated with KNO_3, $[NH_4]_2SO_4$ derived from sites Purkersdorf. NO_3 (=KNO_3) and NH_4 (=$[NH_4]_2SO_4$) indicate the fertilization regime, whereas PsJN stands for inoculation with B. phytofirmans strain PsJN. Day 0 = day of fertilization. Different letters indicate differences at p<0.05 with Duncan post-hoc test numbers

		day 0		14 days		21 days		28 days	
(1) *nirS*	G1 no3	4.33E+07	(±7.38E+06)^{fg}	3.55E+07	(±7.92E+06)^{g}	9.05E+07	(±3.49E+07)^{abcd}	1.08E+08	(±4.55E+07)^{ab}
	G1 nh4	4.14E+07	(±1.24E+07)^{fg}	4.47E+07	(±8.72E+06)^{fg}	9.61E+07	(±1.50E+07)^{ab}	1.12E+08	(±1.05E+07)^{a}
	G2 no3	7.17E+07	(±3.56E+07)^{abcdefg}	4.57E+07	(±1.25E+07)^{efg}	9.43E+07	(±2.04E+07)^{abc}	7.48E+07	(±2.59E+06)^{abcdefg}
	G2 nh4	6.41E+07	(±2.97E+07)^{bcdefg}	4.56E+07	(±1.01E+07)^{efg}	9.00E+07	(±1.36E+07)^{abcde}	9.87E+07	(±2.73E+07)^{ab}
(2) *nosZ*	G1 no3	2.51E+07	(±3.72E+06)^{fg}	3.10E+07	(±5.29E+06)^{fg}	1.92E+08	(±7.11E+07)^{b}	1.41E+08	(±6.25E+07)^{abcd}
	G1 nh4	3.00E+07	(±1.05E+07)^{fg}	2.74E+07	(±4.25E+06)^{fg}	9.62E+07	(±6.28E+07)^{defg}	5.46E+07	(±4.37E+06)^{fg}
	G2 no3	4.03E+07	(±1.29E+07)^{fg}	2.88E+07	(±6.63E+06)^{fg}	7.12E+07	(±1.26E+07)^{efg}	5.33E+07	(±9.00E+06)^{efg}
	G2 nh4	4.31E+07	(±2.45E+07)^{efg}	1.48E+07	(±6.23E+05)^{g}	1.83E+08	(±1.23E+08)^{ab}	1.70E+08	(±8.18E+06)^{abc}

FINAL CONCLUSIONS AND FUTURE PROSPECTIVES

Even though there are numerous and extensive studies on N cycling bacterial groups in arable soils and N uptake efficiency, the environmental and chemical variations across time affecting the community structures, as well as the distribution of Nitrogen between microorganisms, plant and N losses are still unclear. The parameters influencing activity and composition of bacterial guilds in soil ecosystems are strongly interwoven. Thus it is difficult to determine and interpret environmental impacts that lead to changes in community structure and function. Therefore, the aim of this thesis was to contribute to the understanding of key players in denitrification and ammonia oxidation concerning community structure and potential activities in bulk soil and in the rhizosphere of a model plant (barley) modulated by mineral nitrogen applications and different plant genotypes. Furthermore, the impact of inoculation with a plant-growth promoting bacterium was assessed.

Plenty of baseline information was gained correlating nitrifier and denitrifier community composition in various different soil types to a vast number of soil biochemical parameters, gas fluxes and enzyme activities. Communities were most strongly affected by soil types and various C and N pool sizes. Based on this information two contrasting soils were selected for the N fertilization experiments which showed that ammonia oxidizing bacteria (AOB) in short time dynamics are mainly influenced by the amount of NH_4^+ applied, whereas ammonia oxidizing archaea (AOA) were influenced by parameters other than NH_4^+ content and thus seem to inhabit different environmental niches.

As the AOBs reacted directly to the applied ammonium amounts, it can be concluded that the amount of ammonium is the primary factor modeling these communities in the performed experiments and soils. Changes were mainly seen in abundance, whereas structural changes were less pronounced, which may indicate that ammonium supply enhanced these AOBs, which were also most abundant. Thus with the combination of quantitative PCR and

fingerprinting methods a higher resolution of the community changes in soils was accomplished. Nevertheless, a delayed reaction upon ammonium supply was recognized supposing a lag phase between usage of NH_4^+ and cell proliferation, which enhances the importance of measurements of actual activity rather than potential activity. DNA-based methods allow to reveal insight on abundance (irrespective of cell vitality), community structure and shifts and, as mentioned above, to draw conclusions on potential activities. In the future the development of RNA-based methods will be a major challenge to explore the role of AOB in nitrogen dynamics in soils including the following challenges: quantitative and clean RNA extractions from soil samples, reducing problems related to low mRNA stability, and adaptation of sampling times including detailed resolution of mRNA production across time. Since mRNA is fast produced and also degraded depending of *in situ* soil microbial community and soil composition, it would be desirable to analyze in each soil observed detailed mRNA kinetics to adjust the choice of time scale to the planned experimental setup.

Inoculation with the plant growth-promoting bacterial strain (PGPB) *B. phytofirmans* PsJN had a significant influence on the abundance of AOB dependent on the plant genotype. The inoculated bacterial strain had no obvious tendency to interfere with N cycling in soil, since neither N-fixing nor denitrifying functions are known for *B. phytofirmans* strain PsJN. Thus interactions with the barely plant seem to introduce changes in nutrient availability, followed by effects on bacterial communities and plant N contents. The combination PGPB and mineral N fertilization did not result in enhanced biomass yield but a decrease in plant N in the observed barley cultivars indicating that inoculation in this case had an undesireable effect. This experiment resulted in important baseline information, since the application of PGPB in agriculture are practice and may influence N cycling and N uptake efficiency. Further experiments including different barely variations and PGP-bacterial strains should clarify these mechanisms.

In the present study important knowledge was gained for archaeal ammonia oxidizing communities. Since this group is suggested a major role in soil NH_4 oxidation because of high numbers in most arable soils but was detected only recently, there are limited data available. Thus the short-time dynamics observed in this study, as well as the reaction upon different N forms have provided important information on the potential role of this group of organisms.

Even though the AOA have the possibility to use NH_4^+ as an energy source, this functional group did not react, in contrary to their bacterial homologues, primary on the amount of NH_4^+ applied. Concluding, in the observed soils the AOB were considered the major agents of NH_4^+ oxidation. There are even some traces that AOA are inhibited by high levels of NH_4^+. Literature on the importance of archaeal ammonium oxidation is contradictory. Even though they were found to be more abundant than AOB in most arable soils, the ammonium conversion rates seem to be low and the view changed from considering them as main players to attach this group a back-up function in ammonia oxidation in arable soils. In the present study, rather than to ammonium a connection to nitrate was observed indicating denitrifying potential within this group. Archaeal denitrification genes were found on fosmids in a recent metagenome study but denitrification activity has still to be proven, e.g. with cultivated organisms.

The actual role of archaea as ammonia oxidizers and denitrifiers still keeps thrilling and will be an exiting topic for further explorations. Apart from gene expression studies as mentioned above, the minimal knowledge about the AOAs` mode of life makes interpretation of environmental data difficult. Thus, the isolation of strains from soil to explore the lifestyle in more detail is of utmost importance. Cultivation of these slow-growing organisms and further the isolation of single strains will be one of the major challenges for the next years.

Objecting the dynamics of denitrifiers in barley planted soils, plant age together with soil type were the factors introducing changes into bacterial denitrifying communities. In one of the studied soil types increased plant biomass resulted in enhanced denitrifier abundance as well

as in ratio changes between *nirS* and *nosZ* harboring denitrifiers indicating a shift in denitrification end products. Nitrate addition was not a modulating factor for the denitrifying communities in the objected soil types supposing that the soils were rather carbon-limited than short in nitrate. Thus this suggests that in terms of denitrification the time point of fertilization rather than the N form applied plays an essential role to the fate of nitrogen.

Altogether this study explored the influence of N fertilization on denitrifying and ammonia oxidizing communities and revealed insight into the dynamics of these communities, but to unravel the `biotic-black-box` there are still, as mentioned above, many open questions to be asked and answered in future experiments. Furthermore there are always the questions: Who is out there? Are there still unknown organisms that may have more impact than the known ones? Also the matter of which part of the population is active at what time is a major challenge for microbial ecology in the future. Gene expression and metagenomic studies are developed to solve these issues but are facing still methodological limitations.

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